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Using Molecular Genetic and Demographic Tools to Improve Management of Ex Situ Avian Populations

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USING MOLECULAR GENETIC AND DEMOGRAPHIC TOOLS TO IMPROVE MANAGEMENT OF *EX SITU* AVIAN POPULATIONS

by

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ABSTRACT

Small populations, specifically those that are isolated from others, are more prone to extinction than larger inter-connected populations. The risks that these small isolated populations face include loss of genetic diversity due to founder effects and inbreeding due to population bottlenecks, as well as demographic uncertainty due to fluctuating fecundity and mortality rates and impacts of external environmental factors. *Ex situ* populations, including those managed as conservation breeding programs with species recovery aims, as well as those that do not have reintroduction goals but are managed for long term population sustainability, suffer from the same extinction risks as small and isolated natural populations. Using three separate avian species which have different life histories and population structures, I investigated impacts of multiple genetic and demographic management strategies on these *ex situ* populations. I examined the use of molecular genetic datasets including microsatellites and single nucleotide polymorphisms (SNPs) to determine their utility for reconstructing pedigrees, examining individual relatedness within populations, and compared results of measuring genetic diversity through theoretical methods verses those obtained from a molecular dataset. These methods can then ultimately be applied to improve future management including improving studbook datasets and to measure actual loss of genetic diversity. I also used analytical strategies including population viability analysis to determine how management practices influence demographic parameters and determine the future probability of population extinction. The genetic and demographic analyses of both the

historic management of an *ex situ* population, and its current status, are a first step in hypothesizing the potential directions for future management and understanding the likelihood of survival of an *ex situ* population.

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CHAPTER ONE: INTRODUCTION

Genetic diversity of small and isolated populations is greatly reduced by founder effects and inbreeding due to population bottlenecks. The amount of diversity lost depends on many factors, including the severity of the bottleneck, the size of the founding population, the population growth, and mutation rates (Bouzat 2010, Wright 1931). As wildlife populations become smaller, a number of interacting stochastic processes can destabilize populations, including genetic effects (inbreeding and loss of adaptability) and volatility of the breeding structure (sex ratio imbalances, unstable age distribution, and disrupted social systems) (Lacy 2000). In small populations, inbreeding can greatly reduce the average individual fitness, and loss of genetic variability or genetic erosion from random genetic drift can diminish future adaptability to a changing environment (Bijlsma and Loeschcke 2012, Lande 1988). Maintenance of genetic diversity is of particular concern for bottlenecked species, since population bottlenecks may reduce the amount of genetic variation and consequently result in lowered fitness, reduced potential for future adaptation, as well as elevated extinction rates (Jamieson 2015, Zhang et al. 2006).

Small populations, specifically those that are isolated from others such as island populations, are more prone to extinction than larger inter-connected, or mainland populations. For example, island endemic species typically have higher extinction rates than non-endemic species (Frankham 1998, Loehle and Eschenbach 2011, Rybicki and Hanski 2013). When immigration rates into isolated or insular populations are sufficiently high, extinction rates are reduced (Brown and Kodric-Brown 1977). This is

due to the demographic and genetic contributions of immigrants which increase the size and fitness of these populations, therefore reducing their probability of extinction (Brown and Kodric-Brown 1977). Small populations, including insular species, typically suffer increased inbreeding relative to large populations or meta-populations due to bottlenecks at foundation and lower subsequent population sizes (Frankham 1998). Evidence indicates that the majority of threatened species, including island endemics, have lower genetic diversity than taxonomically related non-threatened species, implying that factors such as inbreeding can increase the probability of extinction before extinction events occur (Jamieson 2007). There is compelling evidence that inbreeding depression and loss of genetic variation lead to increased extinction risk in laboratory populations, and that inbreeding depression in wild populations can affect population growth rates which ultimately contributes to population extinction risk (Frankham 2005, Frankham et al. 2014, Hedrick and Garcia-Dorado 2016).

Despite having the ability to fly and seeming ease of dispersal, avian species are at great risk for extinction, with more than 13% of all species considered threatened (IUCN 2015). Those species predicted to be at highest risk of extinction are those found in the geographic regions with high species richness, including most tropical regions, as well as those species that are non-migratory or insular (Lee and Jetz 2011, Loehle and Eschenbach 2011). Additionally, species-specific life history characteristics may impact extinction risk in birds, including those with large body size, reduced fecundity, and/or slow development (Bennett and Owens 1997; Lee and Jetz 2011). Most avian extinctions within the past 200 years have been of insular species, with the causes of extinction for these species including introduced predators, competitors, or disease

(Savidge 1987; Freed et al. 2008; Loehle and Eschenbach 2011). Avian species on islands and those that exist in small populations continue to decline and lose suitable habitat and therefore many species may become reliant on *ex situ* reproduction, supplementation, or reintroduction to sustain them (Collar and Butchart 2014).

Ex situ populations, including those managed as conservation breeding programs with species recovery aims, as well as those that do not have reintroduction goals but are managed for long term population sustainability, suffer from the same extinction risks as small, isolated natural populations. Small populations brought into *ex situ* environments often experience a secondary bottleneck due to limited number of founders and the inevitable inbreeding and genetic drift that occurs in populations with extremely low or no migration. While theoretical models of loss of gene diversity have long been used to document the loss of hypothetical alleles in *ex situ* managed populations, measuring the direct loss through time of genetic diversity at the molecular level has not yet been examined. The most commonly used models used to manage *ex situ* populations and their loss or maintenance of gene diversity require a known pedigree, or the ancestral linkages between individuals, to calculate genetic characteristics of the population (Attard et al. 2016, Hammerly et al. 2013, Lacy 1995, Lacy et al. 1995). There is also a lack of empirical evidence to address the assumptions present in the theoretical models used to calculate loss of gene diversity. In most cases, *ex situ* conservation projects do not incorporate genetic analyses prior to initiation, and can thus suffer from inbreeding and/or outbreeding depression (Witzenberger and Hochkirch 2011). Future *ex situ* management programs should aim to incorporate measures of genetic diversity including founder relationships, observed and expected

heterozygosities, and inbreeding levels prior to establishing the population (Witzenberger and Hochkirch 2011). Furthermore, detailed long-term viability analyses of *ex situ* managed populations are few, and rarely do these studies look closely at the genetic impacts of viability by incorporating measures of inbreeding depression and projecting future loss of genetic diversity at the molecular or allelic level. Understanding the processes contributing to loss of population viability and developing methods to maintain the current levels of genetic diversity in small populations of both *ex situ* and declining wild populations takes on immediate importance (Jamieson 2015, Haig and Ballou 1995).

In this dissertation, I aimed to investigate impacts of genetic and demographic management strategies on small *ex situ* populations of avian species. I first examined the impacts that incorrect pedigree information can have on understanding relationships and studbook data in a small flock of marabou stork (*Leptoptilus crumeniferus*; Ferrie et al. 2013). Next, I examined the use of microsatellites to understand relationships in a flock of carmine bee-eater (*Merops n. nubicus*). Because of species-specific behaviors such as extra-pair fertilizations, mating multiply, and intraspecific nest parasitism, this population has a completely unknown pedigree. I examined how behavior and genetic relatedness information can help elucidate demographic and genetic characteristics of this flock. Next, I examined the use of a genome-wide scan of molecular markers (single nucleotide polymorphisms or SNPs) in a known pedigree population for a species that is extinct in the wild and managed for eventual release or reintroduction, the Guam kingfisher (*Todiramphus cinnamominus*). I examined the congruence between the known pedigree and parentage assignment software using SNPs, as well as compared

the rate of loss of gene diversity when using the theoretical pedigree methods compared to the measures of gene diversity obtained from the SNP information. As current management strategies for *ex situ* populations are based on theoretical models, there is often a demonstrated need to incorporate molecular genetic studies in *ex situ* management and to validate those theoretical models with molecular data (Witzenberger and Hochkirk 2011). Finally, I combined demographic factors such as fecundity and mortality, husbandry factors such as management of clutch size, and genetic factors including inbreeding depression and loss of gene diversity to develop a model of population viability for the Guam kingfisher. Most current PVA-based risk assessments ignore or do not adequately model genetic factors such as inbreeding depression and genetic impacts on evolutionary potential (Frankham et al. 2014). This work is paramount, as proper models of population viability which incorporate multiple aspects of management including genetic data, impacts of inbreeding depression, and husbandry factors, are rare and underutilized in studying small, closed populations, such as those maintained in an *ex situ* setting or those populations which will serve as the sole source for a future reintroduction. With the widespread evidence for increasing extinction rates of taxa and the increasing impacts of climate change, new *ex situ* approaches which are effectively integrated with *in situ* strategies becoming even more necessary to conserve species (Pritchard et al. 2012). Overall, these studies show how genetic information can and should be used to inform genetic decisions for reproductive management as well as to evaluate loss of gene diversity which translates to eventual loss of adaptive potential in *ex situ* managed programs. In addition, genetic and demographic parameters should be incorporated into PVAs to provide insights into the

persistence of species and how that persistence might change if individuals are removed to re-found a natural population.

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CHAPTER TWO: IDENTIFYING PARENTAGE USING MOLECULAR MARKERS: IMPROVING ACCURACY OF STUDBOOK RECORDS FOR A CAPTIVE FLOCK OF MARABOU STORKS (*LEPTOPTILUS CRUMENIFERUS*)

Abstract

Extra-pair copulations (EPCs) leading to extra-pair fertilization (EPF) are common in avian mating systems, despite the prevalence of observed social monogamy in many species. Colonially-breeding birds are interesting species to investigate the prevalence of EPCs and EPF because they show nesting habits including close proximity of nest sites and sexual partners, which are proposed to promote alternative reproductive tactics. Endemic to Africa, the colonial marabou stork (*Leptoptilos crumeniferus*) is one of the most commonly held avian species in North American zoos. The aims of this study were to use genetic information to verify parentage in a population of marabou stork housed at Disney's Animal Kingdom® based on five microsatellite loci and to investigate reproductive behavior. We compared genetic analyses of parents and offspring to studbook data collected through behavioral observations of parental behavior at the nest. Using genetic analyses to reconstruct the pedigree of the marabou stork flock using the program COLONY led to improvement of studbook records by determining parentage of an individual that had previously unknown parentage, and identified one individual that had a sire that differed genetically

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from studbook records. An important contribution of our analyses was the identification and verification of the most likely parents for offspring hatched in this colony and improving incorrect or undocumented parentage in the studbook. Additionally, the colonial nature of this species makes it difficult to observe and understand reproductive behavior. Gaining better understanding of the mating system of a species is essential for successful breeding and captive management.

Key Words: captive breeding, extra-pair paternity, microsatellite, parentage, pedigree, studbook

Introduction

Avian mating systems vary widely, with many species practicing extra-pair copulations (EPCs) and extra-pair fertilizations (EPFs) at much higher rates than assumed, particularly those species previously thought to be both socially and reproductively monogamous [Avise, 1996; Miño et al., 2011]. Parentage studies using molecular techniques have improved understanding of the evolution and behavioral ecology of birds, revealing unsolved aspects of their reproductive biology and natural history [Avise, 2004]. With the application of various molecular genetic techniques, extra-pair paternity has been found in 90% of all avian species studied, with over 11% of offspring from socially monogamous species being the result of extra-pair paternity and true genetic monogamy in less than 25% of socially monogamous species [Griffith et al., 2002]. Using molecular techniques to determine genetic relatedness among

individuals and assess kinship is a powerful tool for investigating issues such as mating systems, parental care, dispersal and other biological parameters in natural and captive populations of birds [Frankham et al., 2002; Avise, 2004].

Colonially-breeding avian species are interesting species in which to investigate the prevalence of EPCs and EPFs because they show nesting habits proposed to promote alternative reproductive tactics such as close proximity of nest sites and sexual partners [Miño et al., 2011]. Indeed, Møller and Birkhead [1992, 1993] reported that extra-pair copulations are found to occur more frequently in colonial than dispersed nesting birds. They interpreted this finding as the result of either increased proximity of individuals or lower intensity of mate-guarding in colonial species. Species in the order Ciconiiformes include examples of those that nest colonially and therefore have high competition for nest sites [Burger, 1981]. Their proximity when nesting could easily lead to EPCs, EPFs, and extra-pair paternity (EPP). Studies have documented non-monogamous mating systems in Ciconiiformes such as the roseate spoonbill (*Platalea ajaja*) and the wood stork [*Mycteria americana*; Miño et al., 2009].

One of the hypotheses proposed as to why females accept EPCs is to avoid the potentially larger cost of rejecting a persistent or aggressive male [Kempenaers and Dhondt, 1993]. In this case, females do not necessarily benefit directly from the EPC, but instead avoid the costs associated with refusing the male. In colonial species with a high level of nest-site competition or where nests can easily be destroyed, nest guarding may prevent continuous mate guarding, and females may be left alone and subsequently attacked and forced into extra-pair copulations by males [e.g. white ibis (*Eudocimus albus*; Frederick, 1987b)]. It has also been suggested that aggressive male

behaviors and forced copulations are more common among colonial than solitary species [Gowaty and Buschhaus, 1998]. Frederick [1987a] reported that white ibis (*Eudocimus albus*) males were frequently observed biting and beating the heads of females, while female were observed to “protest” and “lack cooperation” in copulation attempts.

Native to tropical Africa, marabou storks (*Leptoptilos crumeniferus*) are the largest storks of the Ciconiidae family, with an average height of 120 cm, wing-span of 2.9 m and pronounced sexual dimorphism [Hancock et al., 1992]. These birds are colonial and have been observed to nest with a few to hundreds of other pairs. In breeding season, male marabou storks establish and aggressively protect a nest-site. Females in breeding condition will repeatedly visit the nest site despite overly aggressive displays from the male that include threat displays and bodily assaults on the female [Kahl, 1966]. Females respond with a submissive display and the male eventually allows her to approach and remain at the nest-site [Kahl, 1966]. Once the pair is formed, they begin work on nest construction or repair. It is during this nest building phase that most copulations occur and eggs are laid soon after the nest is completed [Kahl, 1966]. While many descriptions exist about the behavioral courtship and pairing rituals between males and females in the marabou stork [Kahl, 1966; Pomeroy, 1978] as well as demographic parameters of specific colonies including fecundity [Monadjem, 2005] and juvenile dispersal [Monadjem et al., 2008], little information is available about the interactions between pairs and non-paired individuals at the colony site.

Marabou storks are one of the most commonly held birds in North American zoos, yet many zoos are rarely successful in breeding this species [Terkel, 1994]. For example, between 1978 and 2002, only eight North American institutions successfully bred marabou storks out of approximately 25 institutions attempting to breed [Hejna, 2002]. Despite the challenges around successfully breeding this species, little research exists on the causes of these reproductive impediments [Kuhar et al., 2004]. Furthermore, the captive population faces a significant challenge related to trauma-related mortalities. Between 2006 and 2007, within the captive population held in European institutions, 32% of deaths were attributed to intraspecific aggression, 16% to interspecific aggression (from hoofed stock), and 18% to aggression of an unknown source. Numerous other deaths from conspecifics at zoological institutions throughout the world have been reported [Terkel, 1994]. There have been at least seven deaths from intraspecific aggression in the North American population (Schutz, personal communication) in addition to multiple conflicts resulting in injury requiring veterinary intervention since 2007. It has been observed that females are most often the recipients of this intraspecific aggression leading to a captive population that is heavily skewed towards males [Schutz, 2011]. This skewed sex ratio further contributes to the reproductive challenges facing the captive population and it is possible that the presence of female-targeted aggression in this species could lead to forced copulations, and thus extra-pair fertilizations in this species.

Breeding programs of many species managed by zoos and aquaria rely on a studbook, which is a database comprised of the pedigree information and major life history events for every individual in a defined population [Earnhardt et al., 2005]. This

studbook provides data for pedigree analyses, which are the foundation to analyzing and managing the demographic and genetic health of captive populations [Ballou and Foose, 1996]. Accurate pedigrees provide information on inbreeding, kinships among individuals, and the distribution of individual founder contributions to a population [Ivy and Lacy, 2010]. However, for pedigree analyses to be effective, the pedigree must be accurate and complete. In the case of pedigrees with missing or incorrect information, molecular genetic tools have the ability to improve breeding programs [see Ivy and Lacy, 2010 for a detailed discussion]. The North American regional marabou stork studbook was first published in 2002 [Hejna, 2002] and the data from the pedigree have been used to analyze the population and publish two breeding plans for this species in North American zoos [Schutz and Christman, 2009; Schutz and Ferrie, 2012].

The aim of this study was to use genetic information to verify parentage in an *ex situ* population of marabou storks based on five microsatellite loci. Using molecular genetic techniques, we reconstructed the pedigree of a colony of marabou storks to determine if the genetic pedigree supports the presumed pedigree constructed using parental behaviors observed over multiple breeding seasons. We also used the genetic pedigree to interpret behavior during the breeding season in this colony. Our analyses constitute the first attempt to clarify aspects of the genetic mating system in the marabou stork, which is under-studied both in the wild and in captive populations. Investigating these factors may improve the understanding of this species' reproductive behavior and the management of a colonial species in an *ex situ* population.

Materials and Methods

Study Subjects

Disney's Animal Kingdom® was one of the most successful breeding institutions for marabou stork in North America with the largest flock at one time [Schutz, 2011], and as in other zoological institutions displaying this species, this flock had a history of intraspecific aggression. From 1998 through 2012, Disney's Animal Kingdom® exhibited a flock of marabou storks that varied in number from six to 15 birds in an enclosure approximately three acres in size.

Sample Collection and DNA Extraction

Blood samples were collected from 11 marabou storks as part of their routine physical examinations. Twelve blood samples were also collected from four additional institutions in the state of Florida to increase sample size as well as to include birds that previously lived in the Disney's Animal Kingdom® flock. Approximately 0.25g of liver tissue preserved in 95% ETOH were also included from seven samples from deceased birds. Blood samples were preserved by placing three to four drops of blood (approximately 0.25 mL) into 1.5 mL tubes containing 1.0 mL of Longmire Buffer (100 mM Tris HCL pH 8.0, 100 mM EDTA, 10 mM NaCl, 0.5% SDS; Longmire et al., 1988). Two samples were obtained from each bird. In total, we collected samples from 30

individual birds. Genomic DNA was extracted using a standard phenol-chloroform extraction protocol [Sambrook and Russell, 2001] followed by an ethanol precipitation. Samples were then re-suspended in double-distilled water and the DNA concentration was determined using a Nano-Drop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Samples were then visualized on a 1% agarose gel to confirm that genomic DNA had not fragmented during extraction. All samples were diluted with double-distilled water to a final concentration of 20ng/µl.

Microsatellite Development and Genotyping

To isolate microsatellite loci, we generated random DNA fragments (~200-2500 bp) using degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR), using the K6-MW primer and protocol [Macas et al., 1996]. Microsatellite enrichment of the PCR-amplified genomic fragments employed a 3'-biotinylated $(GATA)_8$ repeat motif bound to streptavidin-coated particles (Promega Corporation, Madison, WI) enriched via magnetic separation. Enriched DOP-PCR products were made double stranded by a subsequent DOP-PCR and cloned using TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Clones positive for $(CA)_n$ or $(GATA)_n$ microsatellites were identified using the screening procedure of Cabe and Marshall [2001]. In brief, we conducted two PCRs per colony: one PCR included T3 and T7 primers while the second included the $(GATA)_8$ primer in addition to the T3 and T7 primers. We visualized the product of the PCR reactions on a 2.0% agarose gel and positive clones (those containing microsatellites) were identified by a distinctive smear in the $(GATA)_8$ reaction. We then

sequenced positive clones and developed PCR primers from flanking regions of DNA surrounding the microsatellite repeats. In total, we developed microsatellite primer sets for 10 loci and genotyped 30 individuals at these loci.

PCRs for all loci were performed in a 20 µl reaction containing 1 µl of template DNA diluted to 20 ng/µl, 2 µl of 10X PCR buffer, 1.25 µl of 25 mM of $MgCl_2$, 200 µM of each dNTP, 0.5 µl of 10 µM M13 labeled (Schuelke 2000) forward primer and 1 µl of 10 µM reverse primer, 1 µl of 10 µM fluorescently-labeled M13 primer, 0.2 µl dimethyl sulfoxide, and 1 unit of Taq polymerase. The fluorescently labeled dyes were ABI DS-30 (6-FAM, HEX, NED; Applied Biosystems, Life Technologies Corporation, Carlsbad, CA). We performed PCRs using a BioRad MyCycler thermalcycler (Bio-Rad Laboratories, Hercules, CA). The initial denaturing step was 94 °C for 4 mins, followed by 35 cycles of 30 secs at 94 °C, 30 secs at the annealing temperature, and 45 secs at 72 °C, then a final extension cycle at 72 °C for 7 mins, and a hold at 4 °C. Annealing temperatures for all loci are listed in Table 1. We visualized PCR products on a 2% agarose gel prior to genotyping.

PCR products were sized using an ABI PRISM® 3730 DNA Analyzer (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA) in 5 µl multi-plexed reactions at the University of Arizona Genetics Core (Tucson, AZ). Alleles were sized with respect to size standard ROX (DS-30, Applied Biosystems, Life Technologies Corporation, Carlsbad, CA) using the Peak-Scanner Software (v1.0, Applied Biosystems, Life Technologies Corporation, Carlsbad, CA).

Basic Genetic Parameters

Five of the 10 loci were found to be polymorphic and were used for further analyses (Table 1). We used the program Micro-checker (van Oosterhaut et al., 2004) to check for the presence of null alleles and scoring errors such as peak stuttering or allelic dropout. The software program FSTAT2.9.3 [Goudet, 1995] was used to test for deviation from Hardy-Weinberg Equilibrium and for evidence of linkage disequilibrium between pairs of loci, as well as to calculate number of alleles and allelic richness of each locus.

We also estimated probability of identity (PID) in the five polymorphic loci using the program Gimlet [v1.33, Valière, 2002]. PID estimates the likelihood of sampling the same genotype by chance given the diversity of the loci used in the analysis, and in general, should be less than 0.001 for a randomly sampled population and less than 0.05 in a population comprised of siblings [Schwartz and Monfort, 2008]. Gimlet was also used to calculate the observed and expected heterozygosities for the microsatellite loci used in this study. Basic genetic parameters were calculated with all individual birds (N = 30).

Parentage Analysis

Of the 30 samples collected, 18 were used specifically for parentage analyses, as these were birds that had lived in the Disney's Animal Kingdom® flock and were either offspring hatched in this flock or were potential parents. The remaining 12

individuals were excluded from parentage analyses as they were never a part of the study flock, or lived in the flock after the study period. Between August 2002 to March 2007, 12 males and 15 females were present in this population of marabou storks. An individual was determined to be a potential sire or dam if they were sexually mature (greater than five years for males, and four years for females), and were present in the flock during a portion of the timeline of this study and if chicks hatched during that time. Institutional animal records were examined to determine each bird's timeline of presence in the flock. Comparing the timeline of each individual's presence in the flock, as well as when they became sexually mature, to the dates that the egg of each offspring was laid allowed us to reduce the number of candidate sires and candidate dams in the flock to eight for both sexes. We genetically sampled nine offspring (individuals hatched at Disney's Animal Kingdom®), 6 candidate sires and four candidate dams. Therefore, the probabilities that the sire and dam were included in the sampled dataset were 75% and 50% respectively. We used these a priori probabilities in both CERVUS [v3.0.3; Kalinowski et al., 2007] and COLONY [v2.0.3.0; Wang and Santure, 2009] for parentage screening. By examining dates of hatch and each individual's timeline of presence in the flock, we were able to exclude some candidate males from paternity analysis for three offspring, and exclude some candidate females from maternity analysis for all nine offspring.

Using the program CERVUS [v3.0.3; Kalinowski et al., 2007], which uses a pair-wise likelihood comparison based approach to assigning parentage, we first ran an allele frequency analysis on the five loci discussed above, which we used to calculate multilocus parental exclusion probabilities [Selvin, 1980]. We then ran a parentage

analysis simulation to determine the parent pair with known sexes. We included the probabilities that the candidate parents were sampled. This simulation calculates the critical delta of each assignment, which is a derivative of the likelihood score used in the parentage analysis and provides a threshold for assigning parentage with varying levels of confidence [Kalinowski et al., 2007]. Finally, we conducted a parentage analysis of the parent pair with known sexes including a separate file for each list of candidate sires and dams.

We also used COLONY [v2.0.3.0; Wang and Santure, 2009], which implements a full-pedigree maximum likelihood method to assign parentage and sibship among individuals with multi-locus genotypes, to run a parentage analysis and examine family relationships. For the COLONY analysis, we chose a polygamous mating system which allows for maternal-only and paternal-only sib-ships (half sibs) and no inbreeding. We ran a full likelihood analysis with a long run length and no sib-ship prior. We input the five microsatellite marker types, and included an error rate of 0.02, an error rate for sibship reconstruction suggested by Wang [2004]. The genotypes of the nine offspring, six candidate sires, and four candidate dams were input as separate files, as well as the sire and dam exclusion for each offspring.

Finally, we ran a kinship identification test to assign the parent pair in Gimlet [v1.33; Valière, 2002], which uses a pair-wise comparison of the parent-offspring genotypes using the alleles at each locus and a threshold for number of allelic incompatibilities. We accepted kinship with no incompatibilities per locus and input demographic information including sex, dates of birth and death, and age at first

reproduction of four years for females and five years for males. We also limited kinship to the potential sires and dams by timeline of presence in the flock as described above. To compare parentage recorded from the studbook to the genetic analyses, we first recorded each sire and dam listed in the North American regional marabou stork studbook [Schutz, 2011] for each offspring hatched in population. These putative parents were recorded in the studbook based on animal keepers' observations of nesting, incubation and chick-rearing behaviors. We then used the results of the CERVUS, COLONY, and Gimlet parentage analysis to assign sires and dams based on strict confidence (95%) and relaxed confidence (80%). Last, we built a pedigree of the flock based on the parentage results, as well as the best configuration of families provided by COLONY.

Results

Using FSTAT [v2.9.3; Goudet, 1995] the five polymorphic loci were found to be in Hardy-Weinberg equilibrium using the Bonferroni corrected significance value of $\alpha = 0.01$, and there was no evidence of linkage disequilibrium between the loci based on the Bonferroni corrected significance value of $\alpha = 0.005$. Locus Lcru101 shows signs of null alleles when analyzing with Micro-checker; however, there was no evidence of scoring error due to stuttering or of allelic dropout. Population mean expected heterozygosity was 0.74 and mean observed heterozygosity was 0.69 (Table 1).

Table 1. Characterization of five microsatellite loci used in marabou stork (*Leptoptilus crumeniferus*) identity and parentage analysis.

Locus	Primer Sequence (5'-3')	T_a (°C)	Repeat motif	Size (bp)	Number of Alleles	Allelic Richness	H_{exp}	H_{obs}	$PI_{unbiased}$	PI_{SIB}
Lcru105	F: *TTATAAAACGTGGCGGGAAG R: CCATTTAGCACAAACAAATTCC	54	$(CA)_{14}$	164-192	11	10.583	0.80	0.67	0.04870	0.3673
Lcru108	F: *CTTGGTGGGCACCTAGCAG R: CATGGCACCATGAAAAAGAG	54	$^{+}(GATA)_{13}$	311-331	7	6.862	0.75	0.77	0.004070	0.1470
Lcru101	F: *CCCAAAAGGCAAATGCATAC R: GTTGAGGGGGAAGAAAATGC	52	$^{+}(CA)_{41}$	184-207	9	9.000	0.72	0.50	0.0003558	0.02482
Lcru109	F: *TGCAGGAGCACAAGTAGATG R: AGGGGTAAAAAGCGAAGCTG	54	$^{+}(GATA)_{10}$	214-234	6	5.900	0.74	0.90	0.00003207	0.05972
Lcru107	F: *ACTGAGAACGGGATTTGTCC R: TTCTGGAAAAACCGAAGACC	54	$^{+}(CATA)_4(GATA)_{15}(CATA)_4$	246-266	5	4.999	0.70	0.60	0.000003977	0.01086
Mean							0.74	0.69		

Table 1 footnote: Loci were amplified using PCR conditions described in the text. Loci are sorted by rank of unbiased probability of identity (PI), which was calculated using Gimlet (v1.33; Valière 2002). Number of alleles and allelic richness (based on minimum sample size of 27 diploid individuals) were calculated with FSTAT (v2.9.3; Goudet, 1995). Expected and observed heterozygosity were calculated in Gimlet. The asterisk (*) in the primer sequence denotes a 5' tail (TGTAAAACGACGGCCAGT) attached to the primer sequence; + in the repeat motif represents an imperfect repeat in the sequence; T_a, annealing temperature; H_{exp}, expected heterozygosity; H_{obs}, observed heterozygosity; $PI_{unbiased}$, unbiased probability of identity; PI_{SIB}, probability of identity in a population of all siblings. Note that values for $PI_{unbiased}$ and PI_{SIB} are cumulative, such that the value listed for Lcru107 is the cumulative effect of all loci.

The cumulative unbiased probability of identity (over all loci) was 0.000003977, suggesting that approximately one in 250,000 genotypes will match by chance alone. The cumulative probability of identity in a population comprised of full siblings was 0.01086, suggesting that approximately one in 100 genotypes would match by chance if all individuals were full siblings. These five loci therefore show sufficient discrimination ability for this study and were used in parentage screening of the captive-hatched individuals in the marabou stork population.

The parentage of each offspring hatched in the flock obtained from the studbook and the three methods of assignment based on molecular markers (CERVUS, COLONY and Gimlet) are shown in Table 2.

Table 2. Sires and dams of offspring hatched in the Disney's Animal Kingdom® marabou stork (*Leptoptilus crumeniferus*) flock determined from the studbook data [Schutz, 2011], and three methods of parentage assignment.

Offspring Sampled	Hatch Year	Sex	Sire				Dam			
			Studbook	CERVUS	COLONY	GIMLET	Studbook	CERVUS	COLONY	GIMLET
406	2002	F	296‡		296*	296/ 375	369§			
415	2003	F	296‡	296*	296**	296	369§			
427	2004	M	296‡	296*	296**	296/ 368+	369§			
428	2004	M	375‡		375	383	371§			406
431	2005	F	296‡	296**	296**	296/ 368+	369§			
432	2005	M	296‡	368**	368**	368+	369§			
433	2007	M	368‡	352**	368**	368	364‡	364	364**	364
410	2002	U	368‡		368		364‡	364*	364**	364
n242	2002	U	UNK	375*	368**	368+/ 375	UNK	381	364**	364

‡: Putative parent included in genetic sampling.
§: Putative parent NOT included in genetic sampling.
**: Assignment confidence >95%.
*: Assignment confidence >80%.
+: Missing allele in Gimlet analysis.

Table 2 footnote. Methods of parentage assignment include CERVUS v3.0.3 [Kalinowski et al., 2007], COLONY v2.0.3.0 [Wang and Santure, 2009], and Gimlet v1.22 [Valière, 2002]. Light gray shaded boxes are assigned parents that match the putative sire and dam with greater than 80% confidence. Dark gray shaded boxes reflect an individual with assigned parents in genetic analyses that do not match to putative parents from studbook. No allelic mismatches were present in assignments made with >95% or >80% confidence from CERVUS and COLONY results.

Of the three programs, analyses from COLONY resulted in more assignments (sires: 6 with strict, and 1 with relaxed confidence; dams: 3 with strict confidence), which were made with higher confidence than CERVUS (sires: 3 with strict, and 3 with relaxed confidence; dams: 1 with relaxed, and 2 with low confidence) and Gimlet (Table 2). The results of assignment tests showed that five offspring had sires match the studbook parentage and that two offspring had dams match the studbook with strict or relaxed confidence. Two additional offspring were assigned sires that matched the studbook with low confidence (<80%); studbook (SB) 428 was assigned sire SB 375, with low confidence (77%, COLONY) but there were no mismatching alleles between sire and offspring genotypes. SB 410 was assigned sire SB 368 with almost no confidence (4%, COLONY). There was one mismatching allele between these two individuals, and a missing genotype in SB 368.

Reconstructing the pedigree based on COLONY best family configuration results, paternity, maternity, full sib-ship, and half sib-ship assignments resulted in three discrepancies from the recorded studbook data (Fig. 1).

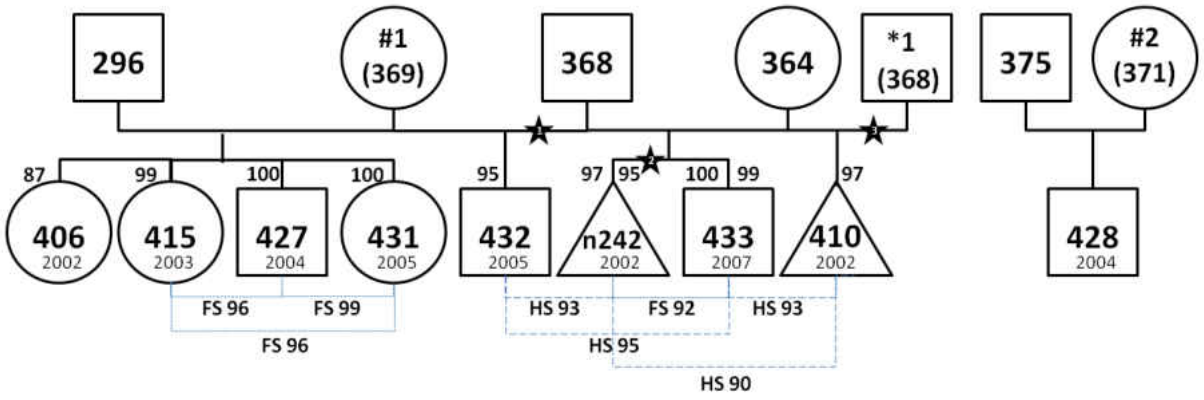

Figure 1. Pedigree of Disney's Animal Kingdom® marabou stork (*Leptoptilus crumeniferus*) flock based on COLONY [v2.0.3.0; Wang and Santure, 2009] best configuration results, paternity, maternity, full sib-ship and half sib-ship assignments. Individuals sampled for genetic analyses are labeled within squares (males), circles (females), and triangles (unknown sex). Year of hatch is listed below individual number. Probabilities of assignment for sires are to the left of the offspring node, and for dams to the right of the offspring node. Probability of assignment for full-siblings (FS) and half-siblings (HS) are below pedigree. Stars (★) represent relationships in the genetic pedigree that do not match the studbook records. These differences are numbered, and are discussed in the text in numerical order. * represents either unsampled putative sire or relationship unsubstantiated by genetic analyses (see text), # represents unsampled putative dams or relationship unsubstantiated by genetic analyses. In the case where a putative sire or dam is listed in the studbook, these are included under the * or #.

First, we found one case of incorrect assignment of a sire that was assigned differently from the studbook (Star 1, Fig.1). Studbook (SB) 296 was listed as the sire of offspring SB 432 with dam SB 369. The daily observation notes of reproductive behavior list one clutch of four eggs for this pair (SB 296 and SB 369) in 2005. In all three analyses (CERVUS, COLONY, and Gimlet), SB 368 was assigned as the sire to SB 432, with greater than 95% confidence (CERVUS and COLONY; Table 2). Offspring SB 432 was laid as an egg in early May 2005, and at this time male SB 368 had just had a clutch fail (April 2005) and was observed in breeding color and copulating with his mate (SB 364).

Thus it appears that offspring SB 432 is a result of an extra-pair copulation between male SB 368, and female SB 369, who was not reproductively monogamous in 2005 (Fig. 1). Second, we were also able to determine parentage of an individual that had previously unknown parentage (no parents listed in the studbook; Table 2). Following assignment tests, offspring SB n242 assigned to sire SB 368 (97%, COLONY; Table 2) and dam SB 364 (95%, COLONY; Table 2; Star 2; Fig. 1). Third, offspring SB 410, which according to the studbook would have been a clutch-mate to SB n242 with the recorded sire SB 368 and dam SB 364 did not assign to any sire in the other assignment tests (Table 1; Star 3; Fig. 1). SB 410 did assign to dam SB 364 with strict confidence in both CERVUS and COLONY (95%). However, the presence of a null allele in the putative sire (SB 368), could explain this discrepancy. SB 368 was scored as a homozygote at locus Lcru105, but if it was scored such that it was a heterozygote exhibiting a null allele, it would yield a result that is consistent with a single family group; therefore, the putative sire (SB 368) could have given the null allele to offspring SB 410.

Discussion

This study compared studbook data to genetic analyses of parents and offspring from a captive population of marabou storks. Reconstructing the pedigree from genetic analyses has shown that one pair in the flock housed at Disney's Animal Kingdom® was not reproductively monogamous and, like many other avian species, this species may make use of a mating system other than strict monogamy. Pairs change mates between

years, and our evidence suggests that even within one clutch there is evidence for extra-pair fertilization and multiple-paternity. Despite the small sample size in this study, the identification of extra-pair fertilization in one captive flock of marabou storks suggests that the rates of this behavior may be quite high in this species in the appropriate environment. Evaluation of the studbook records suggest that data in the studbook may be missing, with no known parents recorded for some offspring, or may be incorrect, with a sire or dam listed that is not the genetic parent. While we did find one individual that did not assign with any confidence to a sire (SB 410, Star 3; Fig. 1), it is likely due to presence of a null allele, and provides a cautionary tale of adhering strictly to genetic data, particularly in that the genetic data can have scoring or sequencing errors. More microsatellite loci may have helped in resolving the parentage of this individual by reducing effects of potential error. Overall, these genetic analyses led us to two major findings in our pedigree that differed from studbook records. Both inaccuracies in the studbook can have implications on future genetic and demographic analyses or management of the population.

A common challenge in genetic parentage studies is that not all potential parents are sampled. In our study only 75% of potential sires and 50% of potential dams were included. In this study, we were not able to compare studbook data to genetic data for most dam assignments, and two individuals assigned to sires with little to no confidence (Table 2). SB 428 did not assign to a sire with confidence, and it is possible in this case that the studbook could be incorrect and we did not sample the true genetic parent. Alternatively, this may be a case that our data did not have the ability to discern the parent. Using genetic data for parentage assignment, there is always the potential to

assign a parent incorrectly, particularly based on a limited sampling of the potential parents, as in this study. However, there is also the ability to exclude a parent if molecular analyses do not support that the parent listed in the studbook is the genetic parent. We believe that we avoided potential errors of incorrect parent assignments by carefully reviewing animal records and timelines to determine which exact individuals were potential parents, and by adhering to assignments with high confidence and no allelic mismatches. Future studies can avoid these challenges by including more molecular markers (ie more microsatellites) to strengthen the parentage assignment analysis methods. Opportunistic sampling and storage of genetic material of more individuals and species in general can also help future studies reduce issues of missing samples.

One of the most important contributions of our genetic analyses to pedigree building was the identification and verification of the most likely parents for offspring hatched in this colony. This study revealed that one female reproduced with a male who was not her behavioral mate, resulting in one clutch in 2005 with multiple-paternity and observations demonstrating that the chick resulting from this copulation was reared by an individual other than its genetic parent. In this colony of marabou storks, sex ratio was male biased leading to the presence of unpaired, potentially aggressive males, and nest sites were all constructed less than a meter from each other simulating a situation of high density nesting. Using the pedigree and observations of parental behavior that led to determination of parents for the studbook allows us to examine the factors that support an environment that promotes EPCs and the reproductive behavior observed in this colony.

Both paired as well as unpaired males in colonial species have been observed attempting to aggressively copulate with females besides their social mates [Gowaty and Buschhausf, 1998]. Unpaired males direct aggressive "copulations" at unmated or unguarded females or females moving unescorted in the environment [Gowaty and Buschhausf, 1998]. Males may themselves create the dangerous environment for the advantage they will accrue from completing copulation, especially those who might otherwise have little or no opportunity to mate due to subordinate position or inability to gain a mate [Gowaty and Buschhausf, 1998]. We have frequently observed increased aggression between males and females at the nest site, even between birds assumed to be monogamous and paired for multiple breeding seasons (not just in newly established pairings). This female-targeted aggression often leads to visible injury in the females (small lacerations to head and gular sac, and limping) and has at times escalated to death of the female. At this point, we do not understand males' motives for this aggression, but it could be due to two situations. First, males may require females to "trade" copulations for protection [Gowaty and Buschhausf, 1998] from other males and the aggression could be a result of the female's refusal. Second, the aggression could be retribution for the female's solicitation of an EPC. In social animals, there is evidence that retaliatory aggression is common, and individuals often punish group members to maintain dominance relationships, discourage cheats, and discipline sexual partners [Clutton-Brock and Parker, 1995]. More information is needed to examine these hypotheses.

High densities of breeding adults and nest sites can increase the chances of encounters between fertile males and females, reducing the energetic costs for

individuals searching for extra-pair mates and favoring increased rates of extra-pair copulations [Miño et al., 2011; Thusius et al., 2001; Westneat and Sherman, 1997]. Although extra-pair encounters might increase with increasing density of individuals in the colony, at higher densities social mates are forced into greater proximity, which should make it easier for males to see and repel intruders, to control female movements, or to witness an EPC and perform compensatory within-pair copulation [Westneat and Sherman, 1997]. Alternatively, increasing density may severely impair a male's abilities to successfully guard his social mate, because either there are too many males to chase away or nests are clustered at sites distant from other resources important to males. In addition, habitat complexity allows females more opportunity to escape from male surveillance and increasing density makes it more valuable for them to do so [Westneat and Sherman, 1997]. In this colony of marabou storks, we observe that nests are, in fact, clustered closely together, males are observed interacting aggressively at the nest site, and the exhibit size and configuration of the environment may allow females to remove themselves from being guarded by their mate. Additionally, as a part of husbandry management in this colony, birds are often required to enter a feeding pen to receive daily feedings, leading to a situation in which males may choose to leave their females unguarded. Under these conditions, the skewed sex ratio and unguarded females leads to a favorable environment for EPCs to occur.

This study further supports the fact that molecular data can be useful in verifying recorded parentage and missing data in studbooks of captive populations [Ivy et al., 2009; Jones et al., 2002] which are essential for pedigree analyses of the population’s genetic composition and demographic history. Accurate studbook information can

provide information on mating system and behavior. Understanding the mating system of a species is essential for successful breeding, captive management, and conservation programs [Miño et al., 2009]. This study contributes to better understanding of the mating system of a zoo-living colony of marabou storks by helping to disentangle the relationships among the chicks hatched in this breeding colony and supplementing or correcting records in this species' studbook. At this point, little information exists regarding the actual mating systems of wild marabou stork beyond behavior of individuals during breeding season [Kahl, 1966]. Future work should investigate the female's role in accepting EPCs and EPFs and how these behaviors may increase the genetic quality of offspring [Neff and Pitcher, 2005]. Other work may examine the complex social and reproductive behaviors between individuals in a colony of wild marabou stork, as well as to seek a greater understanding of the social structure and aggressive interactions in these birds both in zoos and in the wild in order to assist in management of captive populations of marabou storks and other colonial nesting birds [Miño et al., 2009]. Finally, understanding how frequently extra-pair paternity occurs in colonially nesting species will have implications for the genetic management of captive avian populations.

Conclusions

1. Using genetic analyses to reconstruct the pedigree of the marabou stork flock led to improvement of studbook records by determining parentage of an individual

that had previously unknown parentage, and identified one individual that had a sire that differed genetically from studbook records, confirming one case of extra-pair fertilization and extra-pair paternity in this species.

2. Our analyses contributed to the identification and verification of the most likely parents for offspring hatched in one *ex situ* marabou stork colony and improved some studbook records.
3. Using molecular genetic analyses can lead to a better understanding of the mating system of species with cryptic or difficult to study reproductive behavior. Understanding these behaviors can be essential for developing a successful breeding and captive management program.

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CHAPTER THREE: USING BEHAVIORAL OBSERVATIONS AND GENETIC MARKERS TO CHARACTERIZE THE FLEXIBLE REPRODUCTIVE SYSTEM IN AN *EX SITU* POPULATION OF CARMINE BEE-EATERS (*MEROPS N. NUBICUS*)

Abstract

Combining behavioral observations with population genetic analyses in *ex situ* populations can be used to evaluate factors that determine the success of a breeding program. Avian species living in a colony accrue reproductive benefits by taking advantage of multiple reproductive strategies. Growing evidence suggests that both extra pair fertilizations (EPFs) and intra-specific nest parasitism (ISNP) are sufficiently frequent in colonial birds to produce patterns of relatedness that differ from those inferred from behavioral observations. The aim of this study was to use behavioral and genetic information to examine relatedness and reproductive behaviors in an *ex situ* colony of Northern carmine bee-eaters (*Merops n. nubicus*). We conducted behavioral observations to determine social parents (i.e. the males and females that entered/exited each nest with the highest frequency). We also used microsatellite marker to conduct parentage analysis and calculate relatedness between individuals in the colony. Using two methods of parentage analysis, we were able to determine one or both parents of all offspring. Males and females differ in their parental behavior during the different reproductive phases. Finally, both males and females were found to use reproductive strategies other than monogamy. In general, EPFs and ISNP are two of the

consequences of social living, with multiple individuals utilizing these various strategies for reproduction. In summary, our study found that Northern carmine bee-eaters use a flexible social system that enables individuals within populations to take advantage of suitable nest conditions and mating strategies as they arise, which allows for some individuals in this *ex situ* colony to have improved reproductive success.

Keywords: behavior, colony, parentage, population, relatedness, reproduction

Introduction

Conducting research on *ex situ* avian populations not only provides information that can be applied to the improvement of the daily management of that species, but also presents the opportunity to gather comparative data to natural populations and those species which are difficult to observe in the wild (Bouchard and Anderson 2011; Leighton 2014). Studying reproductive behavior strategies with behavioral observations combined with population genetic analyses can be used to evaluate factors that help determine the success of *ex situ* breeding programs. For example, reproductive behaviors, pedigree, and microsatellites have been used to compare effective number of breeders in the *in situ* and *ex situ* populations of bearded vultures (*Gypaetus barbatus*; Guatshi et al. 2003). Effects of inbreeding and levels of mitochondrial diversity were compared to survival and reproduction in the pink pigeon (*Columba mayeri*; Swinnerton et al. 2004) and in lesser kestrels (*Falco naumanni*), microsatellites were

used to examine individual reproductive success and overall genetic health of individuals produced in *ex situ* breeding programs destined for reintroduction to the wild (Alcaide et al. 2010). Molecular genetic analyses are particularly useful in evaluating the breeding programs of avian populations of those species that live in large flocks or colonies, as well as those that have more complex reproductive systems that are difficult to track using basic pedigree information.

Colonial living in birds has been found to have both positive reproductive benefits as well as costs to both sexes (Rolland et al. 1998; Covas and Griesser 2007). Individuals living in a colony accrue reproductive benefits by taking advantage of the density of individuals and the potential to utilize multiple reproductive strategies. Pairs may have increased clutch size and higher offspring survival (Stacey and Koenig 1990; Covas et al. 2006). From the perspective of a reproductive female, colonial nesting provides opportunities for intra-specific nest parasitism (ISNP), or having others incubate and care for her offspring (Emlen and Wrege 1986;Yom-Tov 2001; Covas et al. 2006). Females may have increased production of offspring due to having multiple male social and/or reproductive partners through extra-pair copulations (EPC; Li and Brown 2002). Male-specific reproductive benefits include the opportunity for multiple matings through extra-pair fertilizations (EPF) and the avoidance of some of the expenses of parental investment (Covas et al. 2006). Although group living offers some individuals the opportunity of benefitting at the expense of others (Emlen and Wrege 1986), there can also be costs to breeding in high densities. These include uncertain parentage due to EPF, intra-specific cannibalism (Wittenberger and Hunt 1985), and infanticide of chicks (Wittenberger and Hunt 1985; Danchin and Wagner 1997), as well

as inter-specific depredation of chicks (Rolland et al. 1998). When ISNP is present, it can lead to reduced fitness in birds that are raising the chicks of non-relatives. Thus, while offering reproductive benefits, colonial living also comes with potential costs.

The family of bee-eaters, *Meropidae*, consists of 26 species with considerable diversity in social and breeding behaviors (Burt 2002; Boland 2004). They may be sedentary or migratory, and pair-breeding and/or cooperative with some species having extremely complex social organizations at nest sites (Burt 2002; Boland 2004). Because of the variation in nesting behaviors, this group has been used to examine questions related to costs and benefits of coloniality, sociality, and the presence of various reproductive strategies such as cooperative breeding (Burt 2002). Wrege and Emlen (1991) studied several colonies of white-fronted bee-eaters (*Merops bullockoides*) to examine ISNP, finding that parasitizing females tend to remove host eggs from the nest, and host females frequently discard eggs that are not their own (Elston et al. 2007). In some species, nests are rarely left unguarded due to threats of predation and ISNP (Burt 2002). In other species of bee-eaters, males guard their mates against EPCs, as in the blue-tailed bee-eater (*M. philippinus*; Burt 2002). However, males frequently make use of EPCs if provided an opportunity as a means to sire additional offspring without the cost of parental investment (del Hoyo et al. 2001; Elston et al. 2007).

Growing evidence suggests that both EPCs and ISNP occur frequently enough in birds to produce patterns of relatedness that differ appreciably from those inferred from observational studies. The presence of these reproductive strategies could have major effects when examining relatedness and the identities of birds performing behaviors such as ISNP and multiple mating (Jones et al. 1991). Early studies examining

relatedness in bee-eaters did not account for EPC or ISNP due to the lack of genetic data. The authors recognized these behaviors were likely occurring, but assumed they were at low rates. However, they acknowledged that their estimates of relatedness may be overestimated due to the presence of these behaviors (Emlen and Wrege 1988; Lessells 1990).

Northern carmine bee-eaters (*M. nubicus nubicus*) are a subspecies native to the savannah woodlands, rivers, and grass plains of Africa (Fry 1972). They are opportunistic, insectivorous birds that forage aerially, consuming honeybees, grasshoppers, and flying ants (Nickerson 1958; Fry 1984; Fry et al. 1992). While it has been previously determined that carmine bee-eaters are colonial breeders (Nickerson 1958; Fry 1972), little research has been done on the reproductive strategies used in their colonies and very few observations have been conducted on wild colonies (Fry 1972). The aim of this study therefore, was to use genetic and behavioral information to test hypotheses related to life history parameters, relatedness, and reproductive behaviors of individuals in an *ex situ* colony of Northern carmine bee-eaters. We hypothesized that alternative reproductive strategies are utilized in this colonial species and specifically predicted that ISNP and EPFs are present. To test our predictions, we used genetic data and behavioral observations. For the birds' behavior, we predict that males and females will differ in their parental behaviors throughout breeding season. We determined the family relationships between individuals in an *ex situ* colony of Northern carmine bee-eaters using parentage analyses and by comparing relatedness values between individuals. We then used the parentage and relatedness to interpret reproductive and parental behaviors observed during five breeding seasons. Our

analyses allow us to clarify aspects of the behavioral and genetic mating system in the Northern carmine bee-eater in an *ex situ* colony. Investigating these factors may improve the understanding of this relatively under-studied species' social and reproductive behavior and yield improvements in the management of this and other colonial species in an *ex situ* population by understanding the complex social structure and balance in a breeding colony.

Materials and Methods

Study Species

The carmine bee-eater colony examined in this study is held in the behind-the-scenes Avian Research Center (ARC) at Disney's Animal Kingdom, Lake Buena Vista, Florida, USA. The population increased in number from 9 individuals in 2006 to 27 individuals in 2012 through both reproduction and transfer into the colony from other zoological collections. See Elston et al. (2007) for full description of enclosure, diet, and husbandry protocols. In 2005, a new design to the artificial nest box was implemented that gave animal keepers better access to nests and chicks, enhanced hygiene, and provided both better airflow and space, in order to promote parental rearing of young (Elston et al. 2007). We increased the number of nest tunnels and nest boxes in the enclosure from 5 in 2005, to 9 in 2006-2010, to 21 in 2011. Additional husbandry practices were also altered over time which improved fledging success, including

replacing viable eggs with dummy eggs and artificially incubating eggs until they were externally pipped before returning them to the nest boxes to prevent breakage and potential expulsion from the nest.

Behavioral Data Collection

Behavioral observations were performed during the excavation, nesting, and chick-rearing phases, following the methods in Elston et al. (2007) from April through July in the years 2005-2007, 2009, and 2011. This species' breeding season is compact and highly synchronized with the entire season from reproduction through fledging lasting less than 10 weeks each year. The colony was observed for 15 minutes each in the morning and in the afternoon, approximately 5 days per week for a total of 113.25 observer hours over five years. Birds were individually identified by colored bands. We recorded all occurrences of birds visiting the nest platform (i.e. landing on the numbered block outside the nest tunnel; see Fig. 2), entering, and exiting the nest cavity along with the identification of each individual.

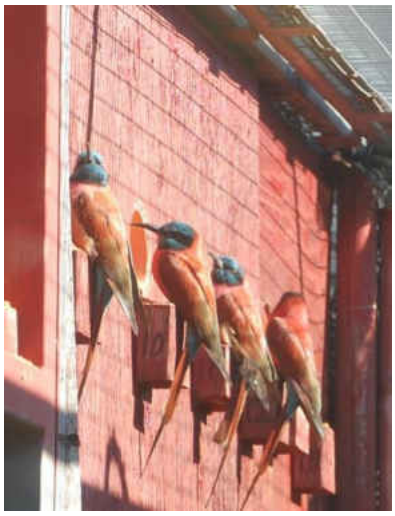

Figure 2. Photo of adult carmine bee-eaters (*Merops n. nubicus*) visiting a nest cavity.

Duration in the nest box was recorded when possible. We also recorded if the individual was carrying a food item into the nest. A single data collector was utilized for each year of data collection.

Behavioral Analyses

We segmented each breeding season into three periods: excavation of nest tunnels, incubation of eggs, and rearing of chicks. Nests were considered active during the excavation phase if an adult was observed entering and exiting during the observations and if eggs/chicks were present in later phases. We counted the total number of times all individuals visited each nest platform during observations each year, which may be a behavior of males who are mate or nest-guarding, or of birds seeking EPC or ISNP. All of the following comparisons between males and females were done

using mixed models with outcome variables log transformed before analysis. To determine which birds were most likely to be guarding nests, we evaluated whether the total number of visits per year between males and females, and between resident and non-resident males were significantly different. To investigate which birds more frequently performed parental behaviors (incubation, brooding, and chick provisioning), we counted the total number of times each individual entered or exited a nest cavity each year. The male and female with the highest frequency of entering and exiting throughout each breeding season were determined to be the nest residents and the social parents of the eggs laid in each nest cavity. All other birds that entered or exited at lower frequencies were considered non-residents. Additionally, we tested for differences between males and females in average time spent in nest during the three stages of the breeding season to further examine parental investment. Finally, we tested for differences in rates of food provisioning to the nest between male and female parents. All statistics were calculated with JMP Pro 12.1.0 (2015 SAS Institute, Cary, North Carolina, USA). Values reported in the Results are mean ± SE.

Genetic Data Collection

Blood samples were collected in 2009 from 15 bee-eaters during routine physical examinations. Three to 4 drops of blood (~15 µL per drop) were placed into 1.5 mL tubes containing 1.0 mL of Longmire Buffer (100 mM Tris HCL pH 8.0, 100 mM EDTA, 10 mM NaCl, 0.5% SDS; Longmire et al. 1988). Tissue (liver or muscle) was collected

from 32 deceased embryos or chicks between 2009 and 2012 and preserved in ethanol. Contour feathers were also collected opportunistically from 14 juvenile bee-eaters in 2011 and 2012. In total, DNA samples from 61 individual birds (adults, embryos, or chicks) were collected for this study. Sampling for this study began in 2010, so prior to this year only living birds were sampled; however all fertile eggs, chicks and adults were sampled in 2010 and 2011.

Genomic DNA from tissue and blood was extracted using a standard phenol-chloroform extraction protocol (Sambrook and Russell 2001). Genomic DNA from feathers was extracted using a DNeasy Blood & Tissue Kit (Quiagen, Valencia, California, USA) with modifications as described in Bush et al. (2005). The concentration of DNA in each sample was determined with a Nano-Drop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) and was visualized on a 1% agarose gel. All samples were diluted with double-distilled water to a final concentration of 20 ng μL-1.

We generated eight species-specific microsatellite loci and tested an additional six loci originally isolated in Merops apiaster which also amplified in Merops ornatus (Dasmahapatra et al. 2004; Adcock et al. 2006) for this study. We genotyped 61 individuals at these 14 loci. See Supplement 1 for microsatellite loci generation, PCR conditions and genotyping methods.

Genetic Analyses

Eight of the 14 loci were found to be polymorphic and were therefore used for further analyses (Table 3).

Table 3. Characterization of eight microsatellite loci used in carmine bee-eater (*Merops n. nubicus*) identity and parentage analysis. Loci were amplified using PCR conditions described in the text. Three loci (identified by the symbol ‡) were originally identified in Dasmahapatra *et al*. 2004. Loci are sorted by rank of cumulative unbiased probability of identity (PI), which was calculated using Gimlet v1.33 (Valière 2002). Number of alleles and allelic richness (based on minimum sample size of 47 diploid individuals) were calculated with FSTAT 2.9.3 (Goudet 1995). Expected and observed heterozygosity were calculated in Gimlet v1.33. The asterisk (*) in the primer sequence denotes a 5' tail (TGTAAAACGACGGCCAGT) attached to the primer sequence; + in the repeat motif represents an imperfect repeat in the sequence; T_a, annealing temperature; H_{exp}, expected heterozygosity; H_{obs}, observed heterozygosity; $PI_{unbiased}$, unbiased probability of identity; PI_{SIB}, probability of identity in a population of all siblings. Note that values for $PI_{unbiased}$ and PI_{SIB} are cumulative, such that the value listed for Mnub104 is the cumulative effect of all loci. NE-PP is the non-exclusion probability of the parent pair from CERVUS (Kalinowski et al. 2007) results.

Locus	Primer Sequence (5'-3')	T_a (°C)	Repeat motif	Size (bp)	Number of Alleles	Allelic Richness	H_{exp}	H_{obs}	$PI_{unbiased}$	PI_{SIB}	NE-PP
Be19.2‡	*GTCAAGTGGGCTGTTGGAG AAGAGGGGCTACTTCCAAGC	59	$(AC)_9$	189-211	9	8.796	0.82	0.82	0.04408	0.3517	0.16
Be2.31‡	*CTTCAGGCAAGTGACCACAG CAGAGGGACACCAGAGCTTC	61	$(AC)_{13}$	183-211	11	10.719	0.79	0.61	0.002470	0.1315	0.18
Mnub103	*TGTGATGCAAAAAGCCAAGAGG CAGGCTGGCTGGTTTTGTCC	62	$(GATA)_5(GACA)_3$	231-263	6	5.965	0.70	0.64	0.0003068	0.05731	0.13
Mnub102	*GAATGATATGTCATGGGGGAAT CAAAAAGGTTCGCCATCACT	62	$(GATA)_8$	163-179	5	4.783	0.58	0.59	0.00006349	0.01500	0.47
Mnub107	*CATCAGCCCATTCACAAAAGACTG CATCCAGCTTGCAGAACACGA	51	$(CTAT)_5$	231-247	5	4.887	0.58	0.46	0.00001394	0.007761	0.44
Be2.46‡	*AATGGCTGTAAGTGGTCATGG TGATTTCATCCCAGATGTGC	59	$(AC)_{5n3}(AC)_{3n8}(AC)_8$	198-207	4	4.000	0.55	0.59	0.000003120	0.002197	0.49
Mnub105	*ACACATTGCCATGAGGACAGC TGCACATGGATGCTTTTTGC	60	$^{+}(GATA)_{12}$	237-269	7	6.726	0.61	0.80	0.0000007052	0.02899	0.49
Mnub104	*ACATTGCCATGGGGACAGC TGATTTGAATGCTGCTGTTTGC	60	$(GATA)_{13}$	190-222	7	7.000	0.57	0.61	0.0000001874	0.004124	0.48
Mean							0.65	0.64			

The software program FSTAT2.9.3 (Goudet 1995) was used to test for deviation from Hardy-Weinberg Equilibrium and for evidence of linkage disequilibrium. The program Micro-checker (van Oosterhaut et al. 2004) was used to check for the presence of null alleles and scoring errors such as peak stuttering or allelic dropout. We also estimated probability of identity (PI) of the eight polymorphic loci using the program Gimlet (v1.33; Valière 2002). Gimlet was also used to calculate the observed and expected heterozygosities for the microsatellite loci used in this population.

From 2006 to 2011, 14 potential sires and 12 potential dams were present in the colony. A bird was considered a potential parent if it was present when eggs were laid (May and June of each year). Eleven candidate sires and 10 candidate dams were genetically sampled, leading to a probability that the sire and dam were included in the sampled dataset of 78.6% and 83.3% respectively. These *a priori* probabilities were used in both CERVUS (v3.0.3; Kalinowski et al. 2007) and COLONY (v2.0; Jones and Wang 2009) for parentage screening (see Supplement 1 for program settings). The remaining potential parents (three potential sires and two potential dams) were not sampled because they died before our samples were collected. During this time period, we collected genetic samples from a total of 50 offspring (chicks or fertile eggs/embryos). By examining dates of hatch and each potential parent individual's timeline of presence in the colony, we were able to exclude some candidate males from paternity analysis for 29 of the offspring, and exclude some candidate females from maternity analysis for all 50 offspring with varying number of potential sires and dams for each offspring. In both programs, parentage is assigned with confidence levels of 95% (strict) and 80% (relaxed) confidence. We used both programs (CERVUS and

COLONY) for parentage screening because they use different methods to assign parents, and are known to differ in levels of confidence in assignment, with COLONY typically producing more assignments at a higher confidence level (Ferrie et al. 2013). We also completed a visual comparison between the genotypes of the offspring and those individuals identified as their social parents and determined how many loci were mismatched between the offspring and social parents. Finally, to further examine the relationships between all individuals in the colony, and to determine relationships that were not assigned in parentage analyses, we estimated relatedness between all individuals using Coancenstry (Wang 2011). All three programs assume a genotyping error rate of 0.02 as suggested by Wang (2004).

Application of Genetic Analyses to Investigate Behavior

We examined life history parameters and occurrence of various reproductive strategies for this species, including age at first and last reproduction, clutch size, multiple paternity in clutches, ISNP, and males with multiple mates. In these analyses, we only considered genetic parentage assignments that were assigned with >95% confidence in both programs unless specifically noted. To investigate if inbreeding is occurring, we also compared the relatedness of behavioral parents at each nest and, when both parents of an egg were assigned with >95% confidence in both COLONY and CERVUS, we examined relatedness of the genetic pair.

Results

Behavioral Analyses

In five years of observations, we determined 30 of the 50 offspring's social parents (individual male and female) through observations of birds entering/exiting the nests (Fig. 3; Table 13 in Supplement).

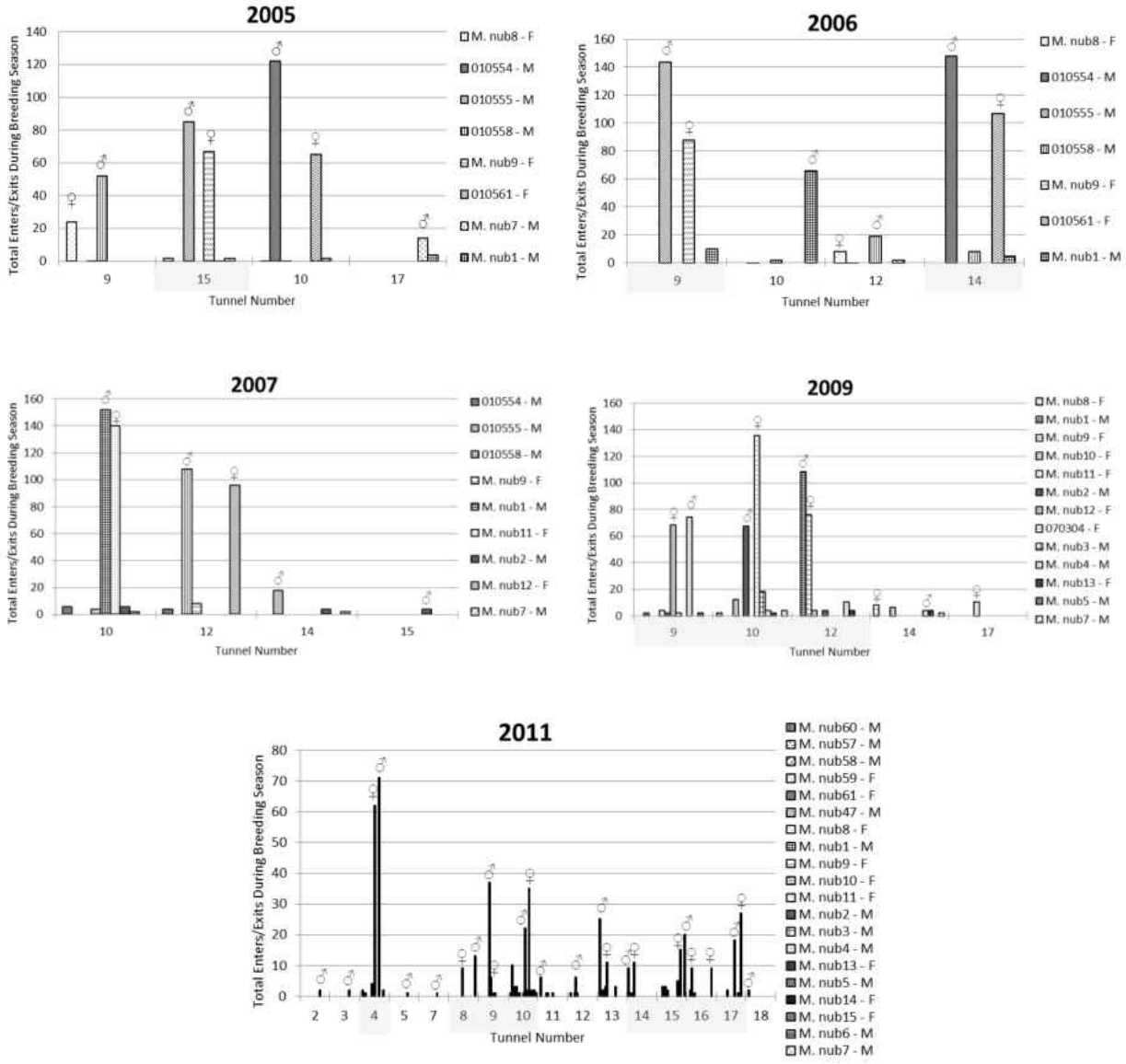

Figure 3. Total enters to and exits of each nest tunnel per individual bird by year. The birds that had the highest number of enters/exits combined per year were considered the social parent of the eggs/chicks in the nest. Gray highlighted nest tunnels are those that had eggs/chicks present. The male and female with the most enters/exits to each nest are identified by ♂ and ♀ symbols.

The social parents were considered the resident of the nest tunnel, and all birds that visited at lower rates were considered non-residents of that nest tunnel. Throughout all

stages of the breeding season (excavation, incubation, chick rearing), birds other than the social parents were also observed visiting the nest tunnel. Total visits per year were lognormally distributed, so we transformed to natural log of visits per year, and considered year and bird ID as random effects in our mixed models. Males were observed visiting the nest tunnels more often than females, although this was not significant (t = -1.77, DFDen = 26.4 p = 0.09) and there was no evidence that males that were the social parent or resident at the nest tunnel visited more than non-resident males (t = -0.73, DFDen = 37.0, p = 0.47).

Prior to chicks hatching, multiple birds in addition to the social parents were observed entering and exiting nest tunnels. Among all active nest tunnels and prior to chicks hatching, 66.67% were entered/exited by non-social parents during excavation and incubation. When examining differences during the three periods of the breeding season and the two sexes, the duration of time spent in the nest tunnel was significantly different between sex and period (Fig. 4; $F_{5, 58}$ = 8.047, p < 0.0001) with females spending longer time in the nest than males, and incubation period having the longest entrances by females.

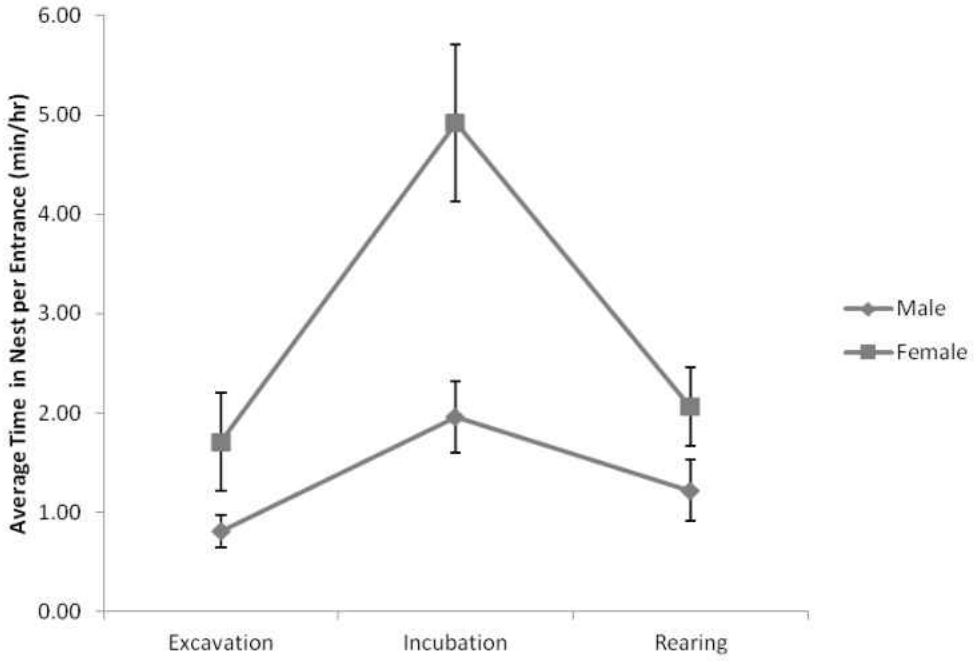

Figure 4. Average time in nest per entrance/exit (min hr^{-1}) of all males and females during three nesting phases: excavation (M = 10, F = 10), incubation (M = 13, F = 11), and chick rearing (M = 10, F = 10). Mean ± SE for each phase.

When examining food provisioning (number of food items brought to cavity per hour) by each parent, males brought food 0.67 ± 0.21, *n* = 10, times per hour, and females brought food 0.51 ± 0.16, *n* = 10, times per hour. These data were also lognormally distributed and after transforming and using a mixed model which examined difference in sex with year and bird ID as random factors, there was no difference in the rate that males and females provisioned nests (*t* = -0.78, DFDen = 25.1, *p* = 0.44). Food provisioning by non-social parents was only observed twice throughout the study.

Genetic Analyses

The eight polymorphic loci were found to be in Hardy-Weinberg equilibrium using the Bonferroni corrected significance value of $\alpha = 0.00625$, and there was no evidence of linkage disequilibrium between loci based on the Bonferroni corrected significance value of $\alpha = 0.001786$. Locus Be2.31 showed signs of null alleles when analyzed with Micro-checker; however, there was no evidence of scoring error due to stuttering or of allelic dropout. The cumulative unbiased probability of identity (PI; over all loci) was 1.874×10^{-7}, suggesting that approximately one in 10 million genotypes will match by chance alone and the PI in a population comprised of full siblings was 2.197×10^{-3}, suggesting that approximately one in 450 genotypes would match by chance if all individuals were full siblings. Therefore, these eight loci show sufficient discrimination ability and were used in parentage screening and relatedness analyses.

The parentage of each offspring hatched in the population obtained from the behavioral observations and the two methods of assignment based on molecular markers (COLONY and CERVUS) are shown in Table 13 in the supplemental materials. We analyzed parentage in 50 offspring (embryos and chicks) and thus there were 50 potential pairings. Of the two assignment programs, analyses from COLONY resulted in more assignments with higher confidence (sires: 35 with strict, 4 with relaxed confidence, 7 with low confidence, and four offspring not assigned a sire; dams: 31 with strict, 3 with relaxed confidence, 13 with low confidence, and two offspring not assigned a dam) than the assignments from CERVUS (sires: 20 with strict, and 16 with relaxed confidence, 13 with low confidence, and 1 offspring not assigned a sire; dams: 19 with

strict, 20 with relaxed confidence, 10 with low confidence, and one offspring not assigned a dam; Table 13). In comparing the congruence of assignments between the two programs, 34 assignments of sires were the same in both programs, and 35 assignments of dams were the same. Non matching assignments are also displayed in Table 13.

Application of Genetic Analyses to Investigate Behavior

Basic life history parameters for this species were determined from the results of parentage analyses. Both males and females were reproductively viable before they reached one year (eggs are laid or fertilized at slightly older than 11 months). The oldest male to have fertilized an egg was at least 17 years, and the oldest female to lay a fertile egg was at least 13 years. Both of these birds were wild caught and have estimated hatch years, so these ages may be underestimates. One female was assigned as the dam to four eggs in 2010 with >95% confidence and an additional three eggs with >80% confidence, suggesting females' fecundity may be 1-7 eggs in one breeding season. These eggs were spread out across three nests, such that total fecundity is larger than clutch size, which was observed to be 1-5 eggs per nest with an average of 2.8±1.08 in 2010 and 2011 (years for which complete nests were sampled, see supplemental results, Table 13). Our observed clutch size is slightly greater than previous estimates by Nickerson (1958), who determined that *M. n. nubicus* had clutch size ranging from 1-3 in natural colonies.

When comparing the social parents to the genetic parents, we found that 15 sires and 15 dams were both socially and genetically identified as parents, with both assignment programs giving the same parentage assignment, although the confidence ranged from no confidence to high confidence in the assignments (Table 13). In all of these cases, where both programs assigned the same individual as the social parent, the visual comparison of genotypes matched completely or in four cases there was just one loci mismatched. Also, an additional 6 sires and 4 dams matched as social parent and genetic parent in one of the assignment programs, and in these cases there were two loci mismatches identified. However, there were 4 sires and 5 dams that the genetic assignments were matching in both programs, yet the social parent assignment did not match, suggesting the presence of EPF and/or ISNP. The number of loci mismatched in these cases ranged from 0-7. We found clutches with multiple paternity in both 2010 and 2011 (Table 13). In 2010, tunnel 17 had two eggs laid by the same female that were sired by different males (>95% sire assignments COLONY; Table 13). Females were also found to parasitize the nest of other pairs; specifically, four instances of ISNP were found in 2011(>95% dam assignments COLONY; Table 13). Males were found to mate multiply and use a strategy of EPF. In 2011, in two cases we confirmed with >95% confidence in parentage assignments males that had offspring with their social mate, but also sired offspring with other females. In these cases where we suggest ISNP or EPF, there are almost always mismatches in the genotypes between offspring and social parents (Table 13). In total, when both methods assigned parentage with high confidence, we found that 28.6% of all nests had ISNP by females (4 of 14) and 14.3%

of the nests had EPF by males (2 of 14) across the four years where both genetic and behavior data were collected.

The average relatedness in the colony was 0.11 ± 0.004. Social pairs were related to each other with an average relatedness of 0.14 ± 0.05, and genetic pairs that were assigned to an offspring with >80% confidence had an average relatedness of 0.09 ± 0.05. Comparing the parentage results and relatedness values, we found that most pairs, both social and genetic, were unrelated (11 of 16, and 10 of 12 respectively). However, we did observe both social and genetic pairings of parent-offspring, full or half siblings, and grand-parent to grand-offspring. Only two of these genetic pairings (one father-daughter, one half-sibling) resulted in living, inbred offspring.

After chicks began hatching, 24.24% of tunnels had individuals that were not the social parents enter or exit. The total time non-social parent individuals were observed spending time in the tunnels was low (98.12 s ± 48.79) across the four weeks post hatching. In just two cases, a non-social parent bird was observed entering with food. Both observations were of male birds. We could not assess the relationship of the first bird to the parents of this nest, as it was not included in our genetic sampling. The second individual was likely unrelated to the male parent at this nest (r = 0.07) but was the offspring of the female parent at this nest-box location from the previous year (r = 0.62, >95% confidence dam assignment in both COLONY and CERVUS).

Discussion

In this study, we observed behaviors during the breeding season related to parental investment and reproductive output, and used genetic analyses to determine parentage and relatedness between individual Northern carmine bee-eaters in an *ex situ* colony. We examined behavioral differences in males and females parents, finding differing levels of contribution in behaviors from each sex depending on phase of the breeding season. Using two methods of parentage analysis, we were able to determine one or both parents of all 50 offspring from the colony with at least 80% confidence, and supplement the parentage assignments by performing a visual comparison of number of loci mismatching between social parents and genetic assignments, as well as calculating relatedness values between all individuals. Finally, we found that birds made use of alternative reproductive strategies including ISNP and EPFs.

When examining behaviors that may play a role in reproduction and levels of parental investment, males were observed visiting the nests more than females, although this result was not significantly different. These results are similar to the nest-visiting observed at the beginning of the breeding season in wild colonies of carmine bee-eaters in which visiting nests, but not entering, may serve as a reproductive strategy for either resident or non-resident males (Fry 1972). For the former, it may be advantageous to guard exiting females or nest contents. For the latter, nest visiting can provide EPF or nest-guarding opportunities (Burt 2002). In our study, females were observed spending more time than males in the nest during the incubation period (Fig. 4) suggesting that females invest time incubating and tending to eggs, whereas males

may play a greater role in guarding the nest against intra- and inter-specific predation. Indeed males were often observed perched nearby the holding box during the incubation period (Elston et al. 2007). However, we found no difference in duration spent in the nest between males and females during excavation and chick rearing (Elston et al. 2007; Fig. 4). Females may reduce their maternal investment during these phases due to the high energy requirements in laying and incubating eggs, and as a measure against ISNP (Emlen and Wrege 1986) or they may be the sex more biologically equipped to do the majority of incubation as obvious brood patches have been described in the females of black-headed bee-eater (*M. breweri*; Schmidt and Branch 2005). More research into sex differences in parental care and investment may uncover why these differences exist.

When observing rates of food provisioning to chicks, we found no difference between male and females, also agreeing with the findings of Elston et al. (2007), suggesting that both parents provide equal investment during rearing, and males are either confident in their paternity or cannot distinguish between chicks that are not genetically their offspring. In fact, as part of the husbandry in this colony to reduce egg breakage, eggs are incubated, and then not necessarily returned to the tunnel in which they were laid. While this may be a confounding factor in studying parental behavior, we do not have any evidence that suggests that the birds provide less care to chicks that are not their own; indeed the rate of food provisioning to chicks was similar across nest boxes (Elston et al. 2007, G. M. Ferrie personal observation) and males of many avian species do not have the ability to discriminate kin (Kempenaers and Sheldon 1996). These results suggest that Northern carmine bee-eaters provide equal investment in

feeding offspring regardless of parentage and future studies that specifically manipulate which nests chicks are returned to could test kin recognition (Komdeur and Hatchwell 1999) and parental investment in this *ex situ* environment.

With regards to cooperative breeding in this species, we cannot confirm that helpers are present during chick rearing. Provisioning by non-parental birds was only observed twice in five years; in 2007 we observed an unpaired adult male enter one nest with food three times, and in 2011 we observed a juvenile male that was paired and successfully fledged chicks at a nearby nest provision the nest that was located ~0.5 m directly below his own nest three times as well. We could not assess the relationship of the first bird to the parents of this nest and the second individual was only related to the female parent. This one observation of helping at a related female's nest may have just been a random occurrence. While males are more commonly observed as helpers in bee-eaters (Brooke 1994) and having grown offspring remain to assist in rearing young is common (Emlen and Wrege 1992), more regular observation of provisioning by non-parents will be necessary to confirm that helpers are present at the chick rearing stage of breeding season in this *ex situ* colony (Boland 2004). We may not have observed typical helping behavior in this colony for a few reasons. First, food resources are provided to the birds, and while they are not unlimited, there is also not much food competition, thus more birds can dedicate energy to reproductive attempts at their own nest rather than needing to forage. At this point nest sites are also not limited, as every year there are nests that do not get utilized. Second, the average relatedness in the colony was ~0.11, or at a level less than half-siblings. Perhaps colony relatedness needs to be higher before the benefits of helping kin outweigh the costs of attempting

reproduction. Finally, it is also possible that Northern Carmine Bee-eaters are not cooperative at all, as in their sister taxon (Emlen 1990).

As predicted, both males and females were found to use reproductive strategies other than monogamy. From the female's perspective, we found clutches with multiple paternity and they were also found to parasitize the nest of other pairs. Both fecundity and clutch size were larger than wild populations, which may be an artefact of being in a resource-rich *ex situ* environment, which may enhance the levels of ISNP leading to larger clutches. Males were found to mate multiply and use a strategy of EPF. EPF may occur more frequently in this population, as we only considered cases of high confidence assignment which were supplemented with visual comparisons of number of mismatches of loci in genotypes (supplemental information, Table 13). These behaviors are all common methods that both males and females use to increase their reproductive output, and reduce parental care. Paired males make use of a mixed mating strategy in which the male, while remaining monogamously paired, takes advantage of extra pair opportunities, made possible by synchronized colonial breeding (Trivers 1972; Emlen and Wrege 1986; Rohwer and Freeman 1989). White-fronted bee-eater females were found to parasitize the nests of parents or close relatives, suggesting that some hosts will tolerate ISNP by close kin (Emlen and Wrege 1986). However, white-fronted bee-eaters will toss eggs that have been dumped in their nest by parasitizing females and they are highly territorial, ejecting intruders from their nests with physical contact (Emlen and Wrege 1986; Boland 2004). In this population of Northern carmine bee-eaters, we have observed eggs thrown out of nests and breakage within the nest, which may be the results of females removing eggs that are not her own. We have also observed long

lasting aggressive interactions between individuals in the nest tunnels. ISNP may pose the primary threat to certainty of parentage in bee-eaters, even more so than forced copulations resulting in EPFs (Emlen and Wrege 1986). Future studies should compare the rates of these behaviors to determine their frequency.

In general, forced or voluntary EPCs and ISNP are two of the consequences and costs of social living (Emlen and Wrege 1986; Yom-Tov 2001), having fitness benefits for some individuals, but not all. Birds remain in colonies and sustain these potential costs, suggesting that the selective advantage of colonial living outweighs the costs (Emlen and Wrege 1986). In some species, breeding pairs exhibit moderately high levels of social monogamy, with pairs of European and white-fronted bee-eaters renesting together at 88% and 87% of nests respectively (Lessells and Krebs 1989; Emlen 1990) with both sedentary and migratory bee-eaters having similar rates of pair fidelity (Boland 2004). However, in our colony, we found some pairings with the same mate in following seasons (as in 3 pairs in 2004 and 2005 as in Elston et al. 2007), whereas others paired with different individuals year after year. With more years of observation on a greater number of pairs, we should be able to have a better understanding of how often pairs choose the same mate the next year, and how often they find a new mate; however the re-pairing rate in our colony may not be comparable to large, migratory, natural populations, as our population is relatively small and does not have a natural ability to migrate or disperse. In bee-eater species, inbreeding is avoided by using a strategy in which females disperse to join new colonies and the social organization is patrilocal extended family groups or clans (Emlen and Wrege 1992). At this time, our colony is not managed this way with no emigration from the

colony; however there is occasional immigration in the form of newly introduced birds. There have been examples of inbreeding in the colony and we may be underestimating inbreeding, as we have been conservative in accepting only parentage assignments with >80% probability. Future colony management will need to include a strategy that allows for dispersal of females to avoid inbreeding when choosing a mate.

In summary, our study found that bee-eaters in an *ex situ* setting use a flexible social system that enables individuals to take advantage of suitable nest conditions and mating strategies as they arise, including EPFs and ISNP (Boland 2004). These strategies allow the birds to improve individual reproductive output while taking advantage of the high density of individuals and nest sites and greater foraging efficiency which are characteristic of colonial living (Beauchamp 1999). Future studies or experiments could examine these reproductive behaviors to determine if individuals maintain one or multiple strategies, or if they choose different strategies each year. Also, as colony size increases and resources such as nest tunnels or living space become more limited, will birds be more likely to choose a strategy based on their age or experience or some other factor? We have seen our colony grow from nine to 27 individuals and the number of nest tunnels provided was increased from five to 21. While nest tunnels have not yet been limiting (the maximum number excavated in 2011 was 16 and only eight of these tunnels were used by a pair throughout the entire season) this is obviously a finite resource which has the potential to influence pair success, mate choice, and other factors in reproduction (Yom-Tov 2001). This study gave some insight into the various reproductive strategies present in an *ex situ* colony of Northern carmine bee-eaters. Due to the small size of our colony compared to natural

settings, the specific frequency of these behaviors may not be generalizable to an *in situ* colony, however little information currently exists from observational field studies on this species. Therefore this study may serve as a comparative baseline for future studies on the reproductive behavior of this species in their natural environment. Furthermore, this study offers zoological managers concerned with reproduction of birds in an *ex situ* setting a better understanding of the complexity of colonial managed species. Our methods can be used to evaluate management consequences including effective number of breeders and the impact on loss of genetic variability (Gautshi et al. 2003) in a colonial setting.

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CHAPTER FOUR: USING A MOLECULAR GENETIC DATASET TO UNDERSTAND POPULATION GENETIC HISTORY IN AN *EX SITU* MANAGED POPULATION OF GUAM KINGFISHERS (*TODIRAMPHUS CINNAMOMINUS*)

Introduction

Biologists are becoming increasingly interested in using molecular genetic approaches and understanding the transfer of genetic material from one generation to the next in order to answer questions of ecological, evolutionary, and conservation relevance (Manel et al. 2005). Contemporary events in population history can be studied using assignment methods, which use genetic information to determine population membership of individuals and answers questions related to classification and clustering of individuals and populations (Manel et al. 2005). Traditional assignment tests have been used to assign individuals to their population or location of origin and have been applied to areas such as identifying the source of stranded common bottlenose dolphins (*Tursiops truncatus*) in the Gulf of Mexico after the 2010 Deepwater Horizon oil spill (Thomas et al. 2017), and in wildlife forensics to identify movement of trafficked plant and animal materials, such as in corals (Ledoux et al. 2016). Genetic mixture analysis aims to uncover genetic composition of a population and how those populations change in space and time and is used extensively in fisheries management to identify composition of specific stocks or commercial lineages (Liu et al. 2016). Finally, parentage analysis, which involves assigning the parents of specific individuals,

has been used to understand mating structure and behavioral ecology of species (Kaiser et al. 2016), detecting family groups within populations and tracing individuals, such as captive-bred fishery-hatched fish when they escape or mix with wild populations (Bylemans et al. 2016). Controlled breeding programs, for both commercial and conservation purposes, extensively apply the assignment methods discussed above to improve management and attain goals such as increased economic gain through production goals in livestock (Raoul et al. 2016), selection of traits in agriculture or aquaculture (Chavanne et al. 2016), or maintenance of gene diversity and adaptive potential in endangered species (Attard et al. 2016). Although molecular genetic techniques have been used extensively to study aspects of population genetics in natural populations, more recently, these same techniques have been incorporated into the study *ex situ* populations as a means to better manage these assurance populations.

The goals of *ex situ* conservation breeding programs include maintaining populations so that they are both demographically self-sustaining and genetically healthy (Lacy 1994). From a genetic perspective, the specific goals of *ex situ* management are to minimize the loss of genetic diversity in order to maintain future adaptive potential in species that are candidates for eventual reintroduction to natural habitats (Frankham 2008, Haig et al. 1990, Williams and Hoffman 2009). The objective of genetic management is the preservation of the genetic variation of the population from which the founders were drawn, as well as to improve on the rate of loss of genetic variation that would be expected in random mating situations (Caballero and Toro 2000). Numerous studies have shown that genetic management based on pedigree

analysis produces the most genetically diverse populations, particularly through the strategy of equalizing founder contribution through minimizing mean kinship (Ballou and Lacy 1995, Haig et al. 1990).

Gene diversity (*GD*), also termed expected heterozygosity, is a common measure of genetic variation within a population (Wright 1969, Nei 1973). In the absence of new variation introduced to a population by mutation or immigration, in the absence of natural selection, and in a randomly mating population of constant size, gene diversity decays due to genetic drift according to

$$GD_t = GD_0 \times \left[1 - \frac{1}{2\mathrm{N}}\right]^{\mathrm{t}} \qquad (1)$$

where *t* denotes number of generations, N is the number of individuals in each generation, and GD_0 is initial gene diversity (Lacy 1995). Projections of the value of *GD* at time *t* will differ based on the starting values used in the above equation, specifically in the value of GD_0 and in N. In pedigree management of *ex situ* populations, GD_0 is assumed to be one, given that the GD_0 of the baseline population is not known and the founders are assumed to be a representative sampling of the wild population (Lacy 1995). The value for N is the number of founders, which are also assumed to be randomly sampled and unrelated.

In order to calculate gene diversity in a population as described above, an accurate pedigree of the population in question is essential. Pedigree analysis, or parentage testing, is a powerful tool in studies of population demographics (Launhardt

et al. 2001), ecology and behavior (Mitani et al. 2002) and estimation and minimization of individual inbreeding (Bulmer 1973; Tzika et al. 2009). It has also been used in conservation management for estimation of effective population size (Morin et al. 2004). In cases where animals have an unknown pedigree, molecular markers can be used to create a relative ranking of degrees of relatedness (Haig et al. 1995; Henkel et al. 2011) and kinship (Blouin 2003), or given complete sampling, recreate a complete pedigree. However, in an open system, where not all potential parents have been sampled, and with limited genetic information and high genotyping error, exclusion methods may fail to assign parentage or assign false parentage (Hauser et al. 2011). Additionally, estimates of diversity based on pedigree are limited when parentage information is missing (Henkel et al. 2011). Increasingly, managers of *ex situ* populations are combining molecular genetic analyses with pedigree analyses to allow for calculations of gene diversity in those populations where pedigree information is missing or questionable, and if founders of the *ex situ* population were related to another (Henkel et al. 2011, Russello and Amato 2004, Witzenberger and Hochkirch 2011). This is done to provide a more accurate picture of the current genetic structure of the population (Henkel et al. 2011) and to fill in gaps in pedigree information (Russello and Amato 2004). Depending on methods of genetic management, pedigree information and/or an understanding of genetic structure is instrumental in understanding the exchange of genetic material from parents to offspring and subsequent loss over time.

However, the calculations of loss of gene diversity in pedigree analysis are theoretical and based on expected loss according to the pedigree. Two common methods to estimate loss of diversity in a pedigree include gene-drop simulations, in

which hypothetical alleles are assigned to founders and Monte Carlo methods are used to assign a random genotype for each descendant (MacCluer et al. 1986) and through the use of mean kinship values, which calculate the probability that two alleles sampled at random from homologous loci will be homozygous by descent from a common ancestor (Lacy 1995). Both methods are based on two main assumptions 1) that the founders of the pedigreed population are randomly sampled, unrelated, and thus possess unique alleles (Lacy 1995; MacCluer et al. 1986), and 2) that the variance effective population size (N_e) is representative of the reproduction in the population, such that the genes transmitted to each generation are a random sample of the genes of the previous generation (Lacy 1995). Calculating gene diversity using these methods is the first step to understanding rate of loss of genetic diversity in *ex situ* managed populations. However, these measurements of gene diversity have never been validated by calculating direct loss of genetic diversity at the molecular level by knowing the true relationships among the founders and how alleles are being passed on relative to the individuals breeding in the population. Identifying the congruence of pedigree gene diversity and gene diversity calculations from genetic markers is critical for future conservation management goals. *Ex situ* assurance populations typically only use pedigree data and natural populations typically only utilize molecular genetic data and as the need for a continuum of management develops, or comparison between wild and *ex situ* populations becomes more evident, the tools used to understand transfer of genetic material between individuals and populations should be comparable.

Various genetic tools have been developed to help evaluate and quantify loss of genetic diversity within populations. These tools can be used to empirically monitor and

test how well *ex situ* populations are being managed to effectively limit loss of diversity in these populations (Lacy 2009). Previously, neutral microsatellite loci have been used extensively to examine founder effects, bottlenecks (Hawley et al. 2006), population fragmentation, genetic drift (Taylor et al. 2007), and effective population size (Johnson et al. 2004). Theoretical models predict that small, bottlenecked populations will show reduced levels of genetic variation at these neutral loci compared to pre-bottleneck levels (e.g. Groombridge et al. 2000, Wisely et al. 2002). Single nucleotide polymorphisms (SNPs) represent a more recently developed class of polymorphic genetic marker common in most genomes now being used in population based studies (Vignal et al. 2002). SNPs can be used to survey both neutral variation as well as genes under selection in natural populations (Tokarska et al. 2009). SNPs offer the potential for genome wide scans of selectively neutral or adaptive variation, with simple mutation models, powerful analytical methods, and application to noninvasive and historical DNA (Morin et al. 2009). It has been shown that using as few as 40 - 100 SNPs, depending on level of heterozygosity, can provide high probabilities of parentage exclusions and has the power to identify individuals (Morin et al. 2009), particularly when studying historical demography, in which many unlinked nuclear loci may be needed to estimate population genetic parameters with statistical confidence (Brumfield et al. 2003). However, more recent studies have demonstrated that using reduced representation libraries have enabled parentage analysis and other population specific questions with more than one thousand SNPs in any non-model species (Ekblom and Galindo 2011, Peterson et al. 2012). Overall, SNPs have been shown to be an effective tool to study bottlenecked species in which heterozygosity of other markers such as microsatellites

may be low (Tokarska et al. 2009) and for reconstructing groups of kin, or assigning parentage (Hauser et al. 2011). It is also possible to use this information to assess population structure and size, follow population reductions and expansions, and evaluate effective population size (Morin et al. 2004).

In this study, using a dataset of SNPs and the known pedigree of the Guam kingfisher, I first test the ability of SNPs to assign parentage. I predict that given complete sampling of parents, the SNP dataset will have high confidence of parentage assignment. I also examine the loss of gene diversity in the same population by examining the loss of heterozygosity over time as calculated with pedigree analyses verses with the molecular data. I predict that the SNP dataset will have lower gene diversity than in the current evaluations of levels of gene diversity with the pedigree results due to the assumptions that are present in the pedigree based analysis that are not in calculations of gene diversity using molecular data. I also use a SNP dataset from deep within the pedigree (generations one and two) to examine the assumptions of unrelated founders by analyzing the family clustering of these. I predict that these individuals deep within the pedigree will have cluster together due to closer relationships between founders than is assumed in the pedigree dataset, as found in Haig et al. (1995). Finally, I aimed to use museum specimens as a means to estimate levels of gene diversity in the wild population of Guam kingfishers prior to the drastic bottleneck event which impacted the taxon and the subsequent founding of the *ex situ* assurance population. I discuss these results with how they may impact future consequences of genetic management in this conservation breeding population and other *ex situ* management programs.

Materials and Methods

Study Species

The Guam kingfisher (*Todiramphus cinnamominus*) is an ideal species for examining genetic questions in an *ex situ* managed population because this species is an island endemic that has experienced severe population decline, habitat loss, and introduction of an invasive predator. Inadvertent introduction of the brown tree snake (*Boiga irregularis*) to Guam caused a precipitous decline or extinction for all of Guam's forest bird species (Savidge 1987). This kingfisher now exists solely in a small zoo-managed population as the last of the Guam kingfishers in the wild were observed in 1988. From 1984 through 1986, 29 birds were captured to found the zoo population (Haig and Ballou 1995); however, only 16 founders contributed genetically to the current population.

In 1995, Haig and Ballou found no genetic diversity in Guam kingfishers using allozyme analyses (29 enzymes screened). However, DNA profile analyses of founders suggested that six to seven founders were close relatives from one family group (some founders were likely siblings while others were likely more distant relatives; Haig et al. 1995). This was important information incorporated into the early breeding plans, as most *ex situ* populations are managed with the goal of reducing the loss of gene diversity by equalizing the contribution of founders under the assumption of unrelated

founders. The DNA profile analysis allowed for designating two sets of founders as siblings (Haig and Ballou 1995). Also, early pedigree analyses suggested that as much as 6% heterozygosity was lost in the first 10 years of *ex situ* management (1984-1993; Bahner 1993), indicating that the severity of the bottleneck had increased since Guam kingfishers were brought into zoos (Haig and Ballou 1995). In 2015, following pedigree analyses, gene diversity was reported as 87.69% of the founding population and inbreeding (F) was 0.0727 (Bahner and Ray 2015). Genetic diversity measured in the kingfishers may reflect both a founder effect and the subsequent bottleneck of captive populations (Haig and Ballou 1995). Monitoring actual genetic loss using molecular data is rarely carried out for *ex situ* populations, and the unique opportunity is available to examine loss of genetic diversity in this long-term managed conservation breeding population.

Researchers on Guam have developed, tested and implemented numerous control tools to reduce or eliminate brown tree snakes from key areas of the island in order to prepare habitat for the reintroduction of the Guam kingfisher (Engeman and Vice 2001; Johnston et al. 2002). In addition to predator-controlled release areas on Guam, islands outside the kingfishers' natural home range are being considered for reintroduction efforts using the birds from the *ex situ* population. Island selection models have analyzed various attributes to find suitable additional introduction sites in the Pacific (Laws and Kesler 2015). With a plan for reintroduction to the wild on the horizon, there is the unique opportunity to assess genetic diversity in a population that has been through an intense bottleneck and subsequent *ex situ* management. Population growth was low from initial founding in 1984 until 2000, due to the typical lag phase common in

managing a new species in human care (Fig. 1). As pairing success and hand-rearing methods improved, the population has experienced a steady increase in size since 2000 (Fig. 1). Holding space for more individuals is being sought as the population will soon need to increase even more in order to meet the demands for the reintroduction. By analyzing single nucleotide polymorphisms found throughout the genome of *T. cinnamominus*, levels of genetic diversity in this population from founding to its current state can be compared, the extent of the bottleneck can be determined, and the influence of genetic drift and loss of diversity can be estimated. Also, the strategy of genetic management using gene drop analysis (Haig et al. 1990) and mean kinship can be evaluated to determine if it was successful at reducing the loss of genetic diversity by directly comparing pedigree analyses to calculations based on molecular analyses.

Sample Collection and DNA Extraction

DNA samples were collected from 208 individual Guam kingfishers from birds that were currently living at time of sample collection, as well as historical samples of birds from throughout the history of the *ex situ* population and from museum samples of birds that were living on Guam prior to the population decline (see Appendix C for sample details). From 86 living birds, blood samples were taken and preserved by placing three to four drops (approximately 0.25 mL) into 1.5 mL tubes containing 1.0 mL of Longmire Buffer (100 mM Tris HCL pH 8.0, 100 mM EDTA, 10 mM NaCl, 0.5% SDS; Longmire et al., 1988). Approximately 0.25g of tissue (liver, heart, muscle) was

collected and stored in 95% ETOH from 44 deceased birds. I also obtained samples from 38 museum skins (28 toe pads and 10 samples cut from the apterium). I collected approximately 6-8 contour feathers from 13 living birds, and drops of blood from toe clips on filter paper (3mm FTA card) from seven living birds. Finally, twenty pre-extracted samples were obtained from the San Diego Zoo Institute for Conservation Research Genetics Department (San Diego, CA, USA). A total of 85 birds living in 2015 were included in the sampling, with the remaining 123 individuals sampled from earlier generations and birds that had lived on Guam.

Genomic DNA was extracted using Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, California, USA) for blood and tissues or QIAamp DNA Micro Kit (Qiagen, Valencia, California, USA) without the carrier RNA for feathers, blood cards, toe clips and skin. RNase A (4 µl, 100 mg/ml) was added to each sample according to kit instructions. Extractions were done in a laboratory that had not ever had avian DNA present. Following extraction, the DNA concentration was determined using a Qubit® 3.0 Fluorometer (Invitrogen, ThermoFisher Scientific Inc., Waltham, MA, USA). Samples were then visualized on a 1% agarose gel to confirm that genomic DNA had not fragmented during extraction. All samples that had a final concentration greater than 50 ng/µl were diluted with double-distilled water to a final concentration of 50 ng/µl.

SNP Genotyping and Analysis

Samples were genotyped for single nucleotide polymorphisms (SNPs) using the nextRAD (Nextera – tagmented reductively amplified DNA) genotyping-by-sequencing

(GBS) approach as implemented by SNPsaurus (Institute of Molecular Biology, Eugene, OR, USA). Briefly, nextRAD uses selective primers to amplify fragments across the genome, as opposed to using restriction enzymes followed by size selection as in similar RADseq approaches (Etter & Johnson, 2012). Genomic DNA was fragmented using the Nextera reagent (Illumina, Inc.) which also ligates short adapter sequences to the ends of each fragment. Fragmented DNA was then amplified, with one of the primers matching the adapter and extending nine arbitrary nucleotides into the genomic DNA with the selective sequence (Russello et al. 2015; Siliceo-Cantero et al. 2016). Each reaction utilized 20 ng of genomic DNA and GBS was completed with a NextSeq 500 Desktop Sequencer (Illumina, Inc.) at the Institute of Molecular Biology, Eugene, OR to generate sequencing reads 75 bp in length. The genotyping analysis used customs scripts (SNPsaurus, LLC) that created a de novo reference from abundant reads across the combined set of samples (Russello et al. 2015).

In order to examine which samples may have had bacterial contamination prior to sequencing, two hundred sequences from each kingfisher sample were blasted against sequences from 158 other species with data obtained from GenBank. This information was also used to test how closely the Guam kingfisher sample sequences aligned to other species. All samples that had greater than 10% matches to the blasted sequences were examined closely at the genotype level to determine if data were low quality or if they were missing a significant proportion of the genotypes and thus should be removed from analyses.

Parentage Assignment

SNP genotypes were used to run simulations of paternity analysis using CERVUS and COLONY to verify the genetic relationships obtained with SNPs with the historic recorded pedigree. These programs differ in their approach to parentage assignment. CERVUS uses simulated parents and offspring to determine a cut-off point of log-likelihood (LOD) scores for true parents, which are then used to identify parent-offspring pairs in empirical data (Kalinowski et al. 2007). Additionally, CERVUS only considers parent-offspring relationships, therefore ignoring information intrinsic in larger family groups (Hauser et al. 2011). COLONY uses a group-wise method to find the most likely configuration of full-sib and half-sib families in the data, as well as provides estimation of probabilities of parent-offspring assignments (Jones and Wang 2009, Wang and Santure 2009). Because offspring sharing the same parents must necessarily be full-sibs, COLONY uses more information in the data and is more powerful under most circumstances (Walling et al. 2010).

Using CERVUS 3.0, which uses a pair-wise likelihood comparison based approach to assign parentage, I first ran an allele frequency analysis on the 1361 SNPs, which are used to calculate multilocus parental exclusion probabilities (Selvin 1980). I then ran a parentage analysis simulation of the parent pair with known sexes. A total of 73 candidate dams and 90 candidate sires were sampled. These values were entered under the “Simulated Genotypes”. There have been 98 total males in the population that have reproduced, and of these, 45 were sampled. Thus, the probability that the sire was sampled and included in the sampling was entered as 46%. There have been 104 total

females that produced chicks or fertile eggs, and of these, 42 were sampled. Thus, the probability that the dam is included was 40%. Default values calculated by CERVUS were used for proportion of loci typed and proportion of loci mistyped (0.01) as well as minimum loci typed (680 loci). This parentage analysis simulation calculates the critical delta of each assignment, which is a derivative of the likelihood score used in parentage analysis and provides a threshold for assigning varying levels of confidence in the parentage analysis (Kalinowski et al. 2007). Finally, I conducted a parentage analysis of the parent pair with known sexes including all sampled individuals as candidate sires and dams for each offspring. This analysis assigns the most likely candidate parent pair using the likelihood score, and the predetermined confidence levels of 95% (strict confidence) and 80% (relaxed confidence). Analyses returned the two most likely sires and dams for each offspring.

I also used COLONY (v2.0; Jones and Wang 2009), which implements a full-pedigree maximum likelihood method to assign parentage and sib-ship among individuals with multi-locus genotypes, to run a parentage analysis and examine family relationships. For the COLONY analysis, I chose a polygamous mating system for both sexes due to the management history of the population in which both sexes of birds may have multiple mates in their lifetime and based on the software constraints which allows for maternal-only and paternal-only sib-ships (half-sibs). I also chose to include inbreeding, with the *a priori* information that there is some level of inbreeding in the pedigree, particularly in recent generations. I specified that the species is diploid, and ran a full likelihood analysis with a long run length with medium likelihood precision, which considers more configurations in the simulated annealing process (Jones and

Wang 2009). For the run specifications, I used program defaults (no updating of allele frequencies, sibship scaling, a single run), and a weak sib-ship prior. I input the SNP markers, and included an error rate of 0.01 (Anderson 2010). The genotypes of the 169 potential offspring, which is the complete dataset as all individuals included were zoo-hatched, as well as the 90 candidate sires, and 73 candidate dams were input as separate files, the same probabilities of sampling the candidate parents as above (46% for sires and 40% for dams). All males and females were included in the lists of potential parents in order to test the ability of the SNPs to determine parentage in a larger sample. Known paternal/maternal sibships and excluded paternity/maternity were both entered as "0" to again test the ability of the SNP data to determine true parentage out of the entire sample of individuals.

Finally, parentage assignment results were compared against studbook data to examine rates of assignment with SNP data that matched the recorded parents in the studbook. In both programs CERVUS and COLONY, parentage is assigned with confidence levels of 95% and 80%.

Family Cluster Analysis

I performed a second parentage analyses using the same input parameters as described above for the COLONY analysis (Jones and Wang 2009), this time with the 20 individuals from my sample dataset that were in the first or second generation of the managed breeding population. Generation was followed from the dam's lineage, as generations are not discrete in this population. When both males and females are

specified as polygamous, as in our analysis, some offspring who do not share parents may still be linked in the pedigree (Wang 2016). This analysis can also be used to examine those relationships of the first generation birds, whose founder parents are assumed unrelated. If there is some relatedness present, these birds should cluster together as a family. I also examined the pedigree to determine from which founders each of these birds was descended.

Persistence of Gene Diversity

To examine loss of gene diversity, or rate of loss of heterozygosity in the *ex situ* population, I first calculated the population's gene diversity by year using the program PMx 1.4 (Ballou et al. 2016). I exported the pedigree file from Poplink 2.4 (Faust et al. 2012) using a filter of 1980 – present (October 2016) and all animals living in North America (which includes Guam) for the demographic filter, and for the genetics filter, all animals living in North America (including Guam) from 1980 – present (October 2016). In PMx, the project was created using the .ped file and the genetics and demographics .csv moves files. PMx calculates gene diversity based on the kinship matrix (GD = 1 – MK; Lacy 1995). For each year, this value was calculated for all birds living on 1 January. The pedigree of the current, managed population has two pairs of founder birds that were identified as siblings by Haig et al. (1995). I compared the loss of gene diversity from the pedigree with these relationships included verses the original assumption such that all founder birds were unrelated.

Then, to examine loss of heterozygosity as measured in the genotyped portion of the population, I grouped individuals living on 1 January for every five years from 1985-2015, and calculated observed and unbiased expected heterozygosity for this subset of birds using the program GenAlEx 6.503 (Peakall and Smouse 2006, 2012). I then calculated the linear regression using JMP Pro 12 for each of these three measures of gene diversity over from 1990 through 2015 to examine whether diversity is lost, gained, or remains the same over this time period.

Results

A total of 1361 SNPs were generated through nextRAD GBS and a total of 180 samples were genotyped by sequencing. Of the original 208 samples collected, 28 samples were not genotyped due to the low quality of the DNA after extraction. This included all museum skins of birds collected from the wild. Therefore, I was unable to complete my aim to quantify genetic diversity of the wild Guam kingfisher population prior to the bottleneck. After examining the genotyped individuals that had 10% or greater of their sequences match to the blasted sequences of other species from GenBank, as well as examining the genotypes of these individuals to determine what percentage of the SNP genotyping data was missing, 11 additional individuals were removed from further downstream analysis. These samples were missing most (greater than 50%) or all of their genotypes. Nine of these samples came from museum skins, and thus had degraded DNA after extraction, although not all museum samples were

excluded. An additional two samples were from very low concentration DNA from the samples sent by San Diego Zoo. After removing the 39 individuals listed above, the 169 remaining individuals were used for population wide descriptive analyses as described below.

According to CERVUS, 182 of the 1361 loci (13.4%) showed evidence of null alleles or F(null) value greater than 0.05. A locus with a large positive estimate of null allele frequency indicates an excess of homozygotes but does not necessarily imply that a null allele is present and in the absence of known parent-offspring relationships it is difficult to identify a null allele with certainty and thus parentage results from CERVUS should consider this in the results (Pemberton et al. 1995). Because the population is known to have inbreeding present, non-random mating (Bahner and Ray 2015), and an excess of homozygotes at many loci (average expected heterozygosity across all individuals at all loci = 0.35 from CERVUS and GenALEx analyses), all SNPs were used for further downstream parentage analysis.

Parentage Assignment

A total of 168 birds were assigned sires and dams with CERVUS providing the two most likely parents for both sire and dam (see Table 4 for overall parentage results).

Table 4. Summary of parentage assignments from CERVUS 3.0 (Kalinowski et al. 2007).

		Confidence (%)	Critical Delta	Assignments		Assignment Rate	
				Observed	Expected	Observed	Expected
Dam alone	Strict	95.0	0.00	120	19	71%	11%
	Relaxed	80.0	0.00	120	19	71%	11%
	Unassigned			48	149	29%	89%
Sire alone	Strict	95.0	0.00	126	22	75%	13%
	Relaxed	80.0	0.00	126	22	75%	13%
	Unassigned			42	146	25%	87%
Parent pair	Strict	95.0	0.00	42	5	25%	3%
(sexes known)	Relaxed	80.0	0.00	42	5	25%	3%
	Unassigned			126	163	75%	97%

A total of 41 individuals had their sire assigned the same as the studbook at the strict confidence level and a total of 45 individuals had their dam assigned the same as the studbook with strict confidence. An additional 29 males and 29 females had parentage which matched the studbook assigned at a lower confidence level with a different individual assigned either at the strict or same lower confidence level. In cases where the matching studbook parent was not assigned with strict confidence but another individual was assigned with strict confidence most of these assignments (106 of 109 for sires and 100 of 101 for dams) were of other high order relationships such as parent, full sibling, or offspring (as recorded in the studbook).

From the COLONY parentage results, a total of 23 sires were assigned, 21 at 100% probability and two at 73% probability. When comparing the recorded sires from the studbook, the 21 individuals that were returned as a sire with 100% probability matched in both the recorded sire and the sire determined by SNPs. For the two individuals that were assigned sires with 73%, one of the assigned sires was a full sibling of that individual from the same clutch, and the other assigned the offspring as sire rather than the sire itself (which was not included in the genetic sampling). For the maternity assignments, a total of 27 dams were assigned with 100% probability. When

comparing the recorded dams from the studbook 26 of 27 assigned dams matched the dam from the studbook. For the one individual that did not match, the assigned dam was an offspring of this individual.

Family Cluster Analysis

Using the birds from the first and second generation, I examined the best family cluster for these birds to estimate relationships of founders. Ten clusters were differentiated by COLONY. Table 5 shows these clusters, the probability of the cluster, the inferred father and mother identified from the software, as well as the sire and dam recorded in the studbook and the list of founders from which they descended.

Table 5. Best family cluster analysis of the birds sampled from the first and second generation from COLONY analyses (v 2.0; Jones and Wang 2009). Prob = probability of cluster, Father and Mother inferred from parentage analysis, SB# = studbook number of individual, Sire and Dam from studbook, Gen = generation from wild based on dam's lineage, Founder = list of founders each individual is descended from based on pedigree analysis.

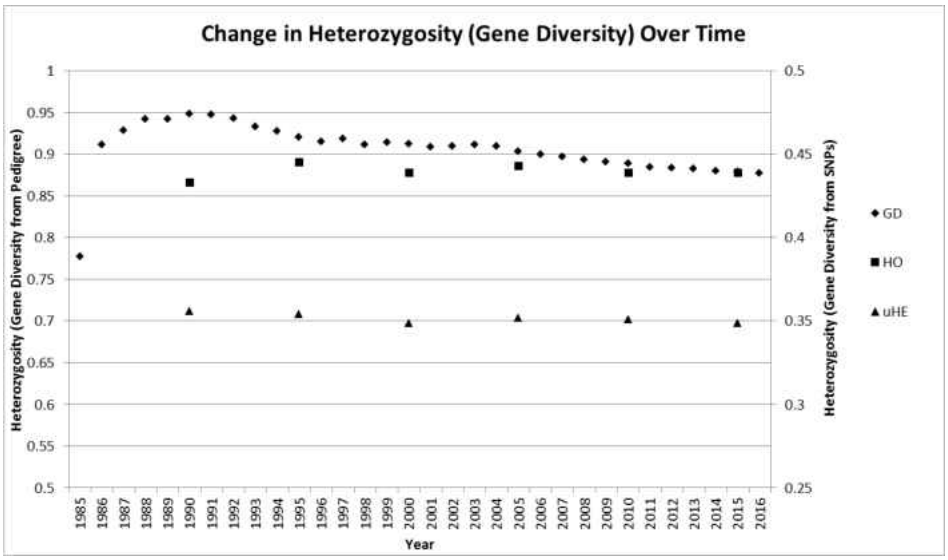
Cluster	Prob	Father	Mother	SB#	Sire	Dam	Gen	Founder													
1	1.000	*1	#1	36	9	10	1	10	WILD1	WILD2											
1	1.000	*2	#2	38	3	4	1		WILD1	WILD2	4										
1	1.000	*2	#1	139	3	44	2	10	WILD1	WILD2											
1	1.000	*2	#1	145	3	44	2	10	WILD1	WILD2											
2	0.809	*3	#3	112	19	30	2				11	12	19								
3	1.000	*4	#4	122	26	27	1							26	27						
4	1.000	*5	#5	130	1	2	1									1	2				
5	0.808	*6	#6	148	19	74	2											15	16	19	
6	1.000	*7	#7	170	111	61	2				11	12									17	WILD3	WILD4
7	1.000	*8	#8	176	88	92	2														17	WILD3	WILD4
7	1.000	*8	#8	209	88	92	2														17	WILD3	WILD4
8	0.968	*9	#9	186	96	99	2				11	12						15	16		17	WILD3	WILD4
8	0.968	*9	#12	142	96	99	2				11	12						15	16		17	WILD3	WILD4
9	0.991	*10	#10	189	135	91	2				11	12									17	WILD3	WILD4
9	0.991	*10	#10	199	135	91	2				11	12									17	WILD3	WILD4
9	0.991	*10	#10	225	135	91	2				11	12									17	WILD3	WILD4
9	0.991	*10	#10	246	135	91	2				11	12									17	WILD3	WILD4
10	0.970	*11	#11	216	24	99	2														17	WILD3	WILD4		24
10	0.970	*12	#11	261	24	99	2														17	WILD3	WILD4		24
10	0.970	*13	#11	263	24	99	2														17	WILD3	WILD4		24

Most birds clustered based on first order families, with those with the same parents and same founders clustering together. One bird, SB#170 clustered separately from the birds that had the same founder lineages, but this bird also had different parents (clusters 6 and 9; Table 11). Another bird, SB# 38 is the only descendant of Founder SB#4. This bird clustered with three other birds that shared two of the same founders (WILD1/WILD2), although it was half siblings with two other birds in its cluster and a first cousin to the third bird according to the pedigree relationships.

Persistence of Gene Diversity

Loss of gene diversity in the Guam kingfisher population from founding in 1985 to present (i.e. 2016) as determined by pedigree analysis and mean kinship of the population, as well as the observed and unbiased expected heterozygosity is shown in Figure 5.

A)



B)

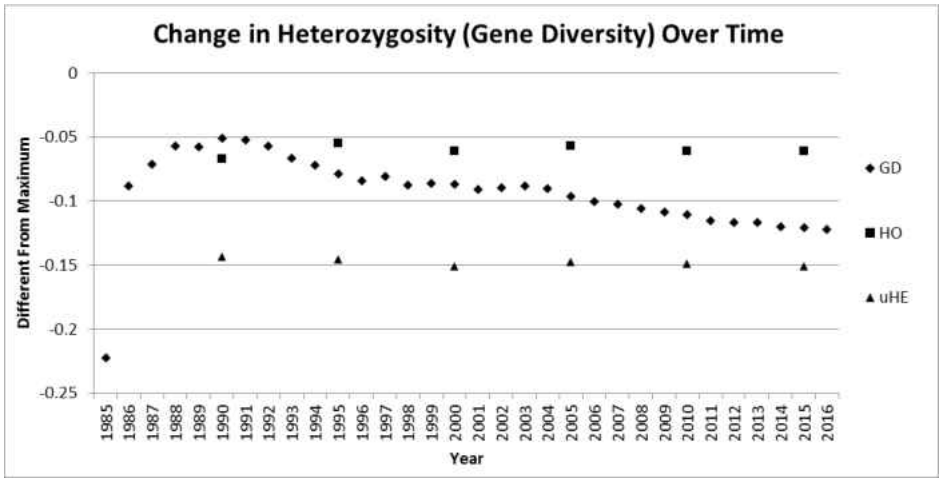


Figure 5. A) Change in heterozygosity over time in the Guam kingfisher SSP from founding (1985) to current (2016) as determined by pedigree analysis from the studbook (left y-axis) and molecular analysis using SNPs (right y-axis). GD = gene diversity calculated as GD = 1 – MK where MK is the mean kinship of the population (Lacy 1995) calculated from the studbook of the birds living on 1 January of each year. HO = observed heterozygosity. uHE = unbiased expected heterozygosity. HO and uHE calculated from birds in the SNP dataset living on 1 January of each year. SNPs have a maximum of 0.5 as a biallelic marker. B) Change in heterozygosity over time as measures as a difference from the maximum value.

Each of the six time segments used to calculate these population statistics with the molecular dataset included a varying number of individuals, which was based on the individuals living on 1 January of the specified year (Table 6).

Table 6. Sampling numbers and percentage of population sampled from the Guam kingfisher SSP from birds living on 1 January of six different time periods. Overall summary statistics from population genetic analyses of the molecular dataset are also presented.

Year	Number of Individual Sample	Total Number of Birds Living	Percentage of Population Sampled	Ho	uHe	F	Ne
1990	5	59	8.47%	0.43	0.32	-0.31	1.56
1995	13	51	25.49%	0.45	0.35	-0.24	1.58
2000	19	59	32.20%	0.44	0.35	-0.23	1.58
2005	34	72	47.22%	0.44	0.35	-0.21	1.59
2010	65	117	55.56%	0.44	0.35	-0.20	1.59
2015	85	157	54.14%	0.44	0.35	-0.20	1.59

Ho = observed heterozygosity = number of heterozygotes / N
uHe = unbiased heterozygosity = (2N/(2N-1))*He
F = fixation index = (He – Ho)/He = 1 – (Ho/He)
Ne = number of effective alleles = 1/(sum pi^2)
Where pi is the frequency of the ith allele for the population and the sum pi^2 is the sum of the squared population allele frequencies.

Using pedigree analysis from the studbook, the maximum population gene diversity was reached in 1990 (92.42%), and declined to 87.80% in 2016 ($y = 6.11 - 0.0026x$, $R^2 = 0.95$, $p < 0.0001$; Figure 6, Table 5). For heterozygosity calculated from the molecular data, observed heterozygosity has remained consistent (43.30% in 1990 and 43.90% in 2015; $y = 0.26 + 0.00009x$, $R^2 = 0.04$, $p = 0.69$; Figure 5) and unbiased expected heterozygosity has declined slightly although not significantly (35.60% in 1990 to 34.90% in 2015; $y = 0.82 - 0.0002x$, $R^2 = 0.62$, $p = 0.06$; Figure 5). Additionally, comparing the current pedigree analyses which includes the relationship between two sets of founders as full siblings to a pedigree where all founders are assumed unrelated

demonstrates that less gene diversity is lost in the pedigree with unrelated founders (Figure 6).

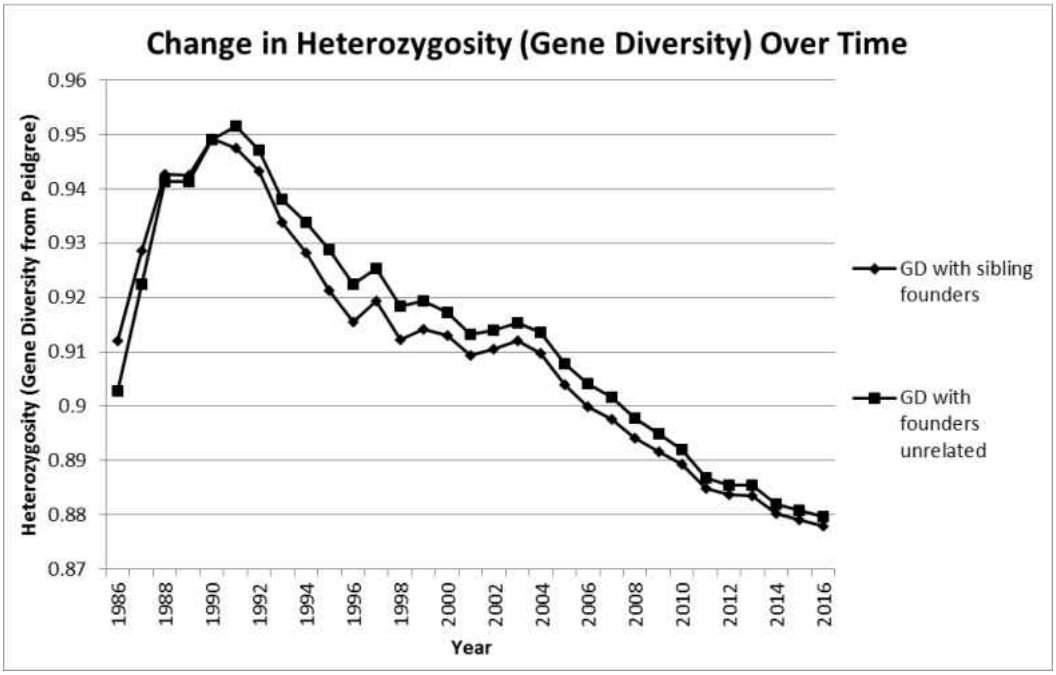


Figure 6. Change in heterozygosity over time in the Guam kingfisher SSP from founding to current (2016) as determined by pedigree analysis from the studbook. The bottom line represents the pedigree as it is used in current analyses and projections, which considers two sets of founders related as determined by Haig et al. (2015). The bottom line represents the loss of gene diversity if all founders were considered unrelated.

Discussion

In this study, I used a dataset of SNPs to test the ability of these genetic markers to assign parentage in the Guam kingfisher SSP® and to compare loss of genetic diversity as predicted from a pedigree versus estimated from molecular markers. I found that the molecular dataset had high confidence in parentage assignment, although

sometimes these assignments were mis-identified for other first-order relatives. I also found that the SNP dataset had lower level of gene diversity than what is calculated with the pedigree methods, however more gene diversity has been maintained over time than assumed. These differences are likely due to the assumptions that are present in the pedigree based analysis that are not in calculations of gene diversity using molecular data. I also examined the relationships of birds from the first and second generation, early in the populations' history, to examine the assumptions of unrelated founders by analyzing the family clustering of these individual. My results support the original results of Haig et al. (1995) for the relatedness of early founders. I discuss the implications for future use of these methods in understanding genetic structure and loss of diversity and how they may impact future consequences of genetic management in a conservation breeding population and other *ex situ* managed programs.

Parentage results from both CERVUS and COLONY provided high confidence assignments for many individuals. While the CERVUS results provided more parentage assignments, the number of assignments that did not match the assigned parentage in the studbook yet returned a first order relative was high (63% of total sire assignments and 60% of dam assignments). Labuschagne et al. (2015) used a set of N = 15 SNPs to complete parentage assignment to compare to studbook records in the *ex situ* African penguin (*Spheniscus demersus*) population in South Africa and also found high success rate in assignment in CERVUS. They did however, also encounter incorrect assignments made with high confidence, although they do not discuss which birds were assigned in lieu of the parent recorded in the studbook (Labuschagne et al. 2015). A similar analysis was undertaken within the Tasmanian devil *ex situ* population

(*Sarcophilus harrisii*) in which 267 SNPs were used to conduct parentage assignment and 68% of individuals without pedigree information failed to have parents assigned (Wright et al. 2015). Results from both studies, as well as this current analysis on the Guam kingfisher, suggest that when the "true" parent is not included in the candidate parent list, there is a higher likelihood of incorrect assignment. Ultimately, this is a consequence of incomplete sampling that must be considered when attempting to reconstruct a pedigree, or could be uncovered in cases where studbook data is wrong, either through the recording of incorrect parents in animal records, or if biology of the species is not considered (e.g. presence of extra-pair copulations, Ferrie et al. 2013). In the Guam kingfisher population, the birds are maintained as monogamous pairs. While it is possible there are errors in the studbook, they would be as a result of human error in data entry, and likely found during studbook data validation since both parents would need to be at the same location at the time a chick was hatched. Therefore, in our parentage assignments in this study, it is more likely that incorrect assignments were due to incomplete sampling.

Based on the parentage results described above, it appears that if a goal of applying a SNP dataset to population genetic analyses is to reconstruct a pedigree that has missing parentage information, it is likely that first-order relationships may be confused, assigning offspring or siblings as parents and vice versa, and incorrect assignments are likely to occur even at high likelihood when there are gaps in the sampling effort through the depth of the pedigree. However, if the goal of doing parentage assignments using a SNP dataset is to evaluate kinship or relatedness for population level analyses, these incorrect parentage assignments can still be useful in

evaluating and defining a kinship value between individuals, particularly in that full siblings and parent-offspring relationships are the same first-order kinship relationships (Hauser et al. 2011; Ivy et al. 2016).

With the major assumption in pedigree management of unrelated founders, there is always a concern that *ex situ* populations may have higher inbreeding than assumed, and may have lower gene diversity than projected. Numerous studies have therefore utilized molecular genetic data to uncover these founder relationships, clarify studbook records and improve genetic management, such as in the whooping crane (*Grus americana*, Jones et al. 2002), St. Vincent parrot (*Amazona guildingii*, Russello and Amato 2004) and Guam rail (*Rallus owstoni*, Haig et al. 1994). In an attempt to determine if the founders were related in order to provide more information to the baseline of the pedigree analysis, I performed a family cluster parentage analysis with the first and second generation birds. The cluster analysis separated all birds into clusters based on their founder lineages, and did not provide any additional information into founder relationships. One founder, SB# 4 did cluster with three other founders, but that cluster likely occurred more based on the relationship of the individuals descended from founders WILD1/WILD2 rather than SB# 4 being related to those founders (Table 5). Because this founder line died out after the second generation, and only produced three descendants, we cannot look at this founder line in more detail, nor does it impact the current pedigree analysis. The traditional methods of assuming unrelated founders in *ex situ* populations will lead to inaccurate evaluations of mean kinship, inbreeding, and levels of gene diversity (da Silva et al. 2010).

The loss of gene diversity in a closed population is inevitable. Furthermore, calculations of loss of gene diversity done with a pedigree include assumptions that assume there are no prior relationships between the founders. The goal of maintaining gene diversity through management focuses on a strategy that leads to retention of founders' gene diversity (Lacy et al. 1995). The loss of gene diversity in the Guam kingfisher SSP as measured by the pedigree is shown in Figures 5 and 5. The rate of loss shown here is susceptible to those assumed parameters discussed above, namely that founders are completely unrelated. More likely, and as discovered by Haig et al. (1995), the founders in this population had some prior level of relationships. Haig et al. (1995) used allozymes to examine parentage in the Guam kingfishers by visually comparing bands on a gel of putative parents and offspring. Putative parents were assumed to be true genetic parents when offspring DNA profiles contained fewer mismatched bands than could be attributed to mutation (Haig et al. 1995). All parents were identified correctly using this method, which is expected, given that pairs are housed in monogamous pairs (Haig et al. 1995). The authors used UPGMA clustering of genetic distances and found that three clusters of founders had higher similarity than expected for unrelated birds. Their results suggested linking two sets of founders as siblings (Haig et al. 1995). In calculating the loss of gene diversity with all founders assumed to be unrelated as well as with the relationships determined by Haig et al. (1995) which are the founder relationships used in management today (since these results became available), the loss of gene diversity occurs at different levels (Figure 6). As expected, the pedigree which assumes all founders are unrelated demonstrates improved levels of gene diversity (Figure 6), and when founder relationships are

incorporated, gene diversity measures are lower. These results reinforce the importance of uncovering founder relationships to best model loss of gene diversity, and for future planning of conversation breeding programs, to aim to sample unrelated founders from multiple populations or sites and to uncover any relationships in these founders prior to intensive breeding management (Witzenberger and Hochkirch 2011).

When examining the comparisons in loss of gene diversity between the theoretical calculation (i.e. pedigree) verses actual loss, the molecular data, both in observed and expected heterozygosity, shows little to no decline (Fig. 5). The absolute difference from the maximum (Fig. 5B) shows that unbiased expected heterozygosity (uHe) is lower than both observed heterozygosity and pedigree calculations. This value may be the more appropriate representation of the gene diversity as measured from the SNP dataset as uHe accounts for the related individuals and presence of inbreeding in the sampled population. There may be little to no decline at the molecular level due to the success of the management strategy which aims to minimize mean kinship applied by this program (Montgomery et al. 1997). Ito et al. (2016) recently compared actual genetic diversity as calculated by two mitochondrial genes to pedigree analyses in two species of zebra and found similar results with little correlation in diversity measures. They suggest that this pattern is being driving by the assumptions related to founder relationships (Ito et al. 2016).

For the broader perspective of conservation breeding programs and *ex situ* populations and their relationships to conserving species, the results from this paper suggest that for most breeding programs up until this point in time, managers have relied exclusively on theoretical analyses as derived from a pedigree to understand the

loss of genetic diversity (Ito et al. 2016). I have also demonstrated that even in a species with a known pedigree, there are limitations to the theoretical methods that should caution managers in making decisions and interpreting the genetic analyses, and comparisons between managed programs, even closely related species, should not be made (Ballou et al. 2010, Ito et al. 2016). With the molecular methods demonstrated in this paper becoming more widely available and economical, greater efforts should be made to understand the actual genetic diversity in managed populations and in comparing them to their wild counterparts (Forstmeier et al. 2007, Ito et al. 2016, Witzenberger and Hochkirch 2011). Beyond understanding loss of diversity, these markers are also useful in undertaking analyses on inbreeding levels, non-neutral markers such as MHC or other genes that may demonstrate adaptations to having lived in an *ex situ* environment (Pelletier et al. 2009, Witzenberger and Hochkirch 2011). Having a better comprehension of genetic diversity and composition of all populations, whether under human care, or in the wild, will provide quantitative points of reference for which conservation goals can be measured and achieved.

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CHAPTER FIVE: POPULATION VIABILITY ANALYSIS FOR A SPECIES THAT IS EXTINCT IN THE WILD: CONSIDERATIONS FOR MAINTAINING THE *EX SITU* POULATION OF GUAM KINGFISHER (*TODIRAMPHUS CINNAMOMINUS*)

Introduction

Demographic stochasticity is the random variation in the numbers of births, deaths, and the sex ratio in a population that results from the outcomes of probabilistic events of reproduction, mortality, and sex determination (Shaffer 1981). Recent analyses have shown that some populations can be quite sensitive to these stochastic processes, often in unexpected ways (Lacy 2000, Lee et al. 2017) and the impact of demographic stochasticity is magnified by small sample size (Sim et al. 2011). Demographic stochasticity arises because, at any time, individuals of a given age or developmental stage have differing probabilities of survival and reproduction (Lande 1988). Additionally, inbreeding depression acting on demographic rates can become a significant contributor to population decline in populations smaller than several hundred individuals, even if genetic problems are not the primary threat (Lacy 2000). When the increases in demographic fluctuations of small populations are taken into consideration, rates of loss of genetic variation and accumulation of inbreeding can be much faster than has been previously suggested (Lacy 2000). Demographic models can be used as an objective tool to evaluate the risk of various management scenarios, to identify the demographic parameters to which the populations are most sensitive, and to indicate

where research is more urgently needed to provide the information necessary for management of the population (Bustamante 1996, Converse et al. 2013, Di Minin and Griffiths 2011). In addition, demographic modeling allows for a quantitative assessment of population status, diagnosing potential causes of population decline and comparing strategies that might change the population's trajectory (Faust et al. 2006).

Population viability analysis (PVA) is a quantitative analysis of population dynamics, used to evaluate data and model various scenarios in order to anticipate the likelihood that a population will persist into the future (Gilpin and Soulé 1986). PVA incorporates the demographic parameters specific to a population and their sensitivity to potential changes in these values, as well as genetic processes that may affect persistence. Quantitative and mathematical complexity vary among models and can include aspects of spatially explicit models, sensitivity analyses, and genetic data, and the use of packaged viability computer programs has become common (Lethbridge and Strauss 2015, Reed et al. 2002). Recent advances in PVA have led to the incorporation of new types of information, such as molecular genetic data for understanding molecular ecology and processes (Hoban 2014) and improved methods for evaluating and incorporating parameter uncertainty (Heard et al. 2013, McGowan et al. 2011) and individual uncertainty (Kendall and Fox 2002, Moore et al. 2012). Therefore, it is important to consider carefully which PVA model is most appropriate for a particular analysis. An individual-based simulation program that models the stochastic process of small populations can account for factors such as fluctuations in sex ratio, mate availability, and inbreeding (Lacy 2000). Individual-based PVA models best approximate the dynamics of small populations, particularly those with low intrinsic growth rates and

stable social systems such as mammalian and avian systems (Lacy 2000). Individual-based modeling is a powerful alternative approach to matrix-based modeling that allows for the incorporation of characteristics of individuals that may impact demography. A model that uses these individual data can more realistically simulate the potential for growth of a closed population in short- and long-term time frames (Faust et al. 2006).

While individual-based PVA has been frequently applied to conservation and management strategies of wild populations (Carroll et al. 2014, Moore et al. 2012), detailed demographic and genetic analyses examining the viability of *ex situ* populations are much less common and usually focused on long-lived, charismatic species. For example, Bustamante et al. (1996) examined the *ex situ* population of the bearded vulture (*Gypaetus barbatus*) and the viability of extraction rates for release and determined the need to improve hatchling survival to support expanded releases. Faust et al. (2006) evaluated the declining *ex situ* population of Asian elephants (*Elephas maximus*) in the United States and found that multiple management changes, particularly related to increasing the number of births per year, improving reproductive rates, and altering the birth sex ratio towards females, were all necessary to reverse the negative population trajectory. To evaluate the success of *ex situ*-born, reintroduced bighorn sheep (*Ovis Canadensis*), Ostermann et al. (2001) compared the survival and recruitment ability of the reintroduced individuals compared to wild sheep, and found similar survival and low recruit recruitment rates in both groups, and determined that the supplemented population was neither growing nor viable for an extended period of time. These studies, and others, have examined such factors as the probability of persistence of the species in *ex situ* settings (Faust et al. 2006, Sukumar et al. 1997, Wiese 2000),

the sensitivity of a population to change in demographic parameters (Bustamante 1996, Earnhardt et al. 2009, Faust et al. 2006, Rodriguez-Clark and Sanchez-Mercado 2006, Wiese 2000, Zeoli et al. 2008), and the integration of the *ex situ* population into *in situ* management of the species (Bustamante 1996, Osterman et al. 2001, Rodriguez-Clark and Sanchez-Mercado 2006, Zeoli et al. 2008).

In this paper, I implement a PVA to study the *ex situ* population of Guam kingfishers. Conducting a PVA is an important management tool at this point in the population's history due to the availability of extensive information on the individuals in the population, its management history, as well as the existence of specific goals for the short and long term management. There are few, if any, taxa that have gone extinct in the wild, and for which a scientific PVA was published prior to reintroduction, with the aim of maintaining the sole source population. Even in such well known recovery programs as the California condor (*Gymnogyps californianus*), no published PVA exists from the early history of the reintroduction program. Only simplistic models have been completed for *G. californianus*, and these have focused on basic demography of the reintroduced birds (Meretsky et al. 2000), but no PVAs have focused on maintenance of the *ex situ* source population. With the development of the field of reintroduction, there is a need to improve and inform future reintroductions through the application of a hypothetico-deductive method with models derived from observation and data collection and hypotheses subject to testing (Seddon et al. 2007). In order to optimize effective *ex situ* management strategies prior to and during initial phases of reintroduction, I conducted individual-based stochastic simulations that incorporate demographic and genetic information, as well as factors related to the management of this species in zoos

to address a number of questions relative to the future management of this species. Specifically, I developed and tested scenarios related to future changes in carrying capacity, evaluated impacts of inbreeding depression, changes in genetic management methods, tested the sensitivity in various demographic parameters, and examined the impacts of harvesting birds for a reintroduction.

The baseline scenario was constructed with data obtained from both the Guam kingfisher studbook (Bahner 2015) and the most recent breeding and transfer plan and population analysis (Bahner and Bryan 2016) in order to represent the most recent history of management in this population. The first altered scenario examined changes in carrying capacity. Based on population simulations that find larger population sizes retain higher levels of heterozygosity (Lacy 1987), I predict that an increase in carrying capacity will lead to improved maintenance of gene diversity and maintain a low probability of population extinction.

The fact that inbreeding depression plays a role in the extinction of populations is not a controversy in itself. However, the magnitude of the effect and the role it plays in relationship to other factors is debated (Charlesworth and Charlesworth 1987, Lynch 1991). In a meta-analysis by O'Grady et al. (2006), it was determined that disregarding the influence of inbreeding depression on extinction risk will lead to serious overestimates of the survival prospects of threatened mammalian and avian taxa. While the number of lethal alleles and effects of inbreeding depression have not previously been studied in the Guam kingfisher, inbreeding depression should be considered as inbreeding is likely to continue increasing in this population, and inbreeding depression effects may become prominent. I predict than an increase in lethal alleles in the Guam

kingfisher population will have negative impacts on the probability of survival in this population.

In addition to inbreeding depression, other genetic scenarios are important to consider. The population is currently managed with a strict set of guidelines typical of *ex situ* populations including creating pairs that are well matched in mean kinship, and avoiding pairings that create inbred offspring (Ballou and Lacy 1995). These strategies are incorporated in the baseline, but I test scenarios of altering these guidelines. I predict that changing genetic management strategies will have impacts on the percentage of gene diversity maintained after 100 years.

Sensitivity analysis allows for the exploration of the effect of alterations of different parameters and to investigate the consequences of measurement errors or alteration related to management or threats (Boyce 1992, Penn et al. 2000). Sensitivity analysis is conducted by varying a parameter by a small amount around its estimated value and calculating the change in population growth rate given an absolute change in a single demographic element (Crooks et al. 1998). Sensitivity testing is essential to document the uncertainty in the model projections that result from uncertainty in input parameter values (Lacy 1993). A sensitivity analysis of the baseline scenario was conducted to determine which vital rates are most critical to the population's dynamics. If the model's results are highly sensitive to a parameter, there are two interpretations: 1) that the parameter is a good target for management actions if it can be altered in practice, or 2) that if there is any uncertainty in the value of the parameter, it is important to estimate correctly and effort should be put into collecting more data or studies focused on this parameter (Hosack et al. 2002). I expect that alterations in demographic

parameters such as mortality rates and reproductive rates will influence the persistence of this population.

This population of Guam kingfishers will serve as the sole source for reintroduction of this species back to the wild. Therefore, it is imperative to examine the effect on various scenarios related to removal of individuals for reintroduction while maintaining the population at a specified population size and maximizing gene diversity. Many projects focus on the rate of extraction needed to support the supplementation or augmentation to create a viable wild populations (e.g. Ostermann et al. 2001), but rarely do these PVAs consider the health of the *ex situ* source populations. However, Bustamante (1996) did examine the rate of extraction and the number of hatchlings needed to support that reintroduction. Because the Guam kingfisher is extinct in the wild, there is little room for error when harvesting birds for a future reintroduction, and I predict that age of harvest, number of birds harvested, and the population size needed to maintain the *ex situ* population will impact population persistence.

While the information in these analyses is specific to Guam kingfishers, these questions and methods apply to a range of species managed in *ex situ* populations, particularly for those conservation breeding programs that would like to determine long term impacts on population viability when making specific management changes, or in those programs that have a reintroduction component.

Methods

Demographic Parameters and Parameterization of Life Table

Realistic population modeling relies on estimates of vital rates for the species in question (Doak et al. 1994), particularly quantification of life history such as mortality and reproductive rates. Age specific vital rates, or the differences in mortality and fecundity in each age class, are known to vary among the stages observed in managed populations (Ballou and Foose 1996). Those rates will differ during the founding stage of a population compared to when the population is managed at its space capacity. In order to calculate parameters for the PVA that are representative of the biology of the species as well as those that are representative of the recent management strategies, I examined the census of the population (Figure 7), and calculated multiple life tables to understand how different time periods of management have influenced demographic parameters.

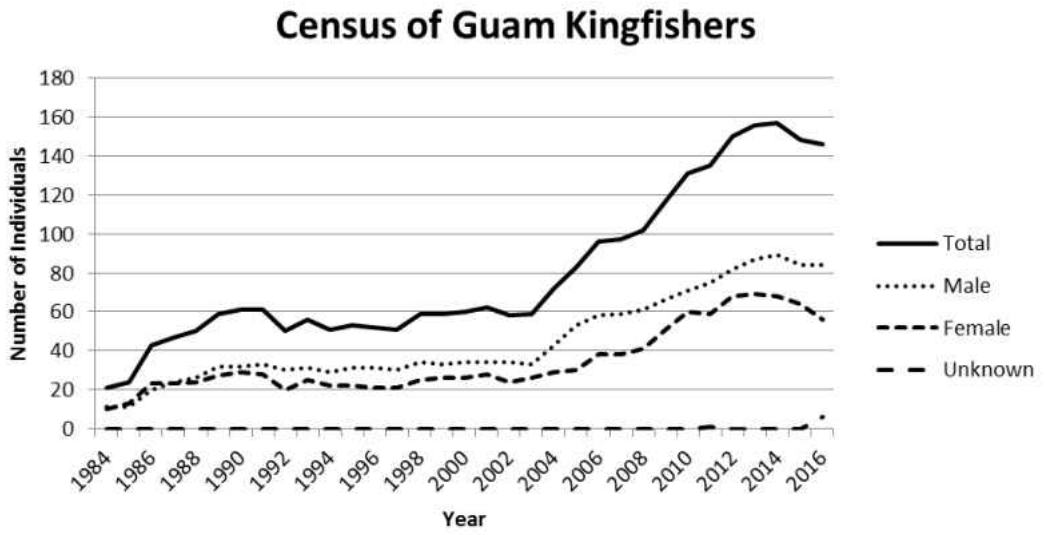


Figure 7. Census of Guam kingfisher Species Survival Plan® population from 1984-1986. Census was taken on the last day of every year.

The current SSP management plan uses demographic data from 1980-2016 (Bahner and Bryan 2016) but I also examined the time period of rapid growth from 2004-2016 to determine if fecundity and/or mortality rates differed between these datasets. The demographic life tables were calculated using the program PMx (Ballou et al. 2016) using the most recently published studbook data (Bahner 2015) and demographic vital rates extracted from the studbook database were used for viability analysis of the *ex situ* population under various projected scenarios described below. Male and female life tables incorporate Kaplan-Meier estimates of survivorship (l_x), annual age-specific survival and mortality rates (p_x and q_x), life expectancy (E_x), fecundity (M_x), and the sample size used to calculate those rates. Therefore, a range of possible rates for mortality and fecundity were investigated based on different stages of management of

the *ex situ* population to ensure the rates are most representative of the current population. Life tables from PMx (Ballou et al. 2016) are included as Tables 7 and 8.

Table 7. Guam kingfisher life table with demographic data of *ex situ* population from 1980-2016.

	Males						Females					
Age (years)	Qx	Risk Qx	Lx	Mx	Risk Mx	Ex	Qx	Risk Qx	Lx	Mx	Risk Mx	Ex
0	0.41	209.60	1.00	0.00	209.60	6.65	0.40	195.70	1.00	0.00	195.70	4.85
1	0.07	184.50	0.59	0.05	184.50	9.56	0.05	176.60	0.60	0.08	176.60	6.39
2	0.07	170.80	0.55	0.18	170.80	9.24	0.10	165.40	0.57	0.27	165.40	5.67
3	0.07	159.50	0.51	0.25	159.50	8.84	0.17	146.00	0.51	0.44	146.00	5.21
4	0.08	137.30	0.47	0.34	137.30	8.45	0.13	115.30	0.42	0.70	115.30	5.09
5	0.08	111.30	0.43	0.43	111.30	8.13	0.15	94.70	0.37	0.48	94.70	4.69
6	0.11	94.40	0.40	0.41	94.40	7.78	0.10	77.00	0.31	0.46	77.00	4.34
7	0.12	77.00	0.35	0.55	77.00	7.60	0.23	60.20	0.28	0.38	60.20	3.70
8	0.09	62.70	0.31	0.45	62.70	7.53	0.16	41.70	0.22	0.18	41.70	3.49
9	0.09	54.60	0.28	0.17	54.60	7.19	0.44	26.50	0.19	0.11	26.50	2.95
10	0.12	47.00	0.26	0.12	47.00	6.77	0.17	14.50	0.10	0.11	14.50	3.49
11	0.12	38.10	0.23	0.12	38.10	6.57	0.31	8.60	0.09	0.00	8.60	3.00
12	0.09	28.20	0.20	0.12	28.20	6.37	0.01	5.70	0.06	0.08	5.70	2.88
13	0.14	21.60	0.18	0.14	21.60	5.93	0.40	3.70	0.06	0.00	3.70	1.90
14	0.15	18.50	0.16	0.06	18.50	5.71	0.50	1.20	0.04	0.00	1.20	1.50
15	0.06	15.50	0.13	0.20	15.50	5.54	1.00	0.60	0.02	0.00	0.60	1.00
16	0.00	15.00	0.12	0.23	15.00	4.84	1.00	0.00	0.00	0.00	0.00	0.00
17	0.15	12.30	0.12	0.29	12.30	3.84	1.00	0.00	0.00	0.00	0.00	0.00
18	0.25	8.90	0.10	0.00	8.90	3.36	1.00	0.00	0.00	0.00	0.00	0.00
19	0.15	6.60	0.08	0.08	6.60	3.13	1.00	0.00	0.00	0.00	0.00	0.00
20	0.26	4.90	0.07	0.00	4.90	2.52	1.00	0.00	0.00	0.00	0.00	0.00
21	0.34	3.30	0.05	0.00	3.30	2.05	1.00	0.00	0.00	0.00	0.00	0.00
22	0.41	2.00	0.03	0.00	2.00	1.59	1.00	0.00	0.00	0.00	0.00	0.00
23	1.00	0.00	0.02	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00
24	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00

Table 8. Guam kingfisher life table with demographic data of *ex situ* population from 2004-2016.

	Males						Females					
Age (years)	Qx	Risk Qx	Lx	Mx	Risk Mx	Ex	Qx	Risk Qx	Lx	Mx	Risk Mx	Ex
0	0.32	123.40	1.00	0.00	123.40	8.36	0.37	110.20	1.00	0.00	110.20	5.52
1	0.06	117.30	0.68	0.05	117.30	10.85	0.03	103.40	0.63	0.08	103.40	7.12
2	0.07	108.10	0.64	0.18	108.10	10.46	0.12	96.10	0.62	0.28	96.10	6.30
3	0.06	95.50	0.59	0.19	95.50	10.18	0.15	82.90	0.54	0.48	82.90	6.01
4	0.06	81.80	0.56	0.28	81.80	9.77	0.10	66.60	0.46	0.56	66.60	5.88
5	0.10	65.50	0.52	0.44	65.50	9.32	0.10	58.90	0.42	0.45	58.90	5.43
6	0.14	52.50	0.47	0.43	52.50	9.23	0.06	50.60	0.38	0.32	50.60	4.91
7	0.05	42.50	0.41	0.56	42.50	9.52	0.19	41.60	0.35	0.28	41.60	4.14
8	0.05	35.60	0.39	0.49	35.60	8.95	0.17	26.90	0.29	0.16	26.90	3.87
9	0.03	33.60	0.37	0.12	33.60	8.40	0.43	16.60	0.24	0.08	16.60	3.47
10	0.12	30.90	0.36	0.13	30.90	7.62	0.09	8.80	0.14	0.00	8.80	4.33
11	0.08	25.80	0.31	0.08	25.80	7.55	0.00	4.60	0.12	0.00	4.60	3.67
12	0.05	20.10	0.29	0.00	20.10	7.09	0.00	4.00	0.12	0.13	4.00	2.67
13	0.06	16.40	0.28	0.09	16.40	6.38	0.50	2.70	0.12	0.00	2.70	1.67
14	0.13	15.00	0.26	0.07	15.00	5.74	0.67	1.00	0.06	0.00	1.00	1.33
15	0.08	12.50	0.23	0.13	12.50	5.41	1.00	0.90	0.02	0.00	0.90	1.00
16	0.00	12.30	0.21	0.00	12.30	4.78	1.00	0.00	0.00	0.00	0.00	0.00
17	0.18	10.80	0.21	0.00	10.80	3.78	1.00	0.00	0.00	0.00	0.00	0.00
18	0.21	7.20	0.17	0.00	7.20	3.40	1.00	0.00	0.00	0.00	0.00	0.00
19	0.15	5.70	0.14	0.00	5.70	3.04	1.00	0.00	0.00	0.00	0.00	0.00
20	0.32	3.90	0.11	0.00	3.90	2.40	1.00	0.00	0.00	0.00	0.00	0.00
21	0.34	3.00	0.08	0.00	3.00	2.05	1.00	0.00	0.00	0.00	0.00	0.00
22	0.41	2.00	0.05	0.00	2.00	1.59	1.00	0.00	0.00	0.00	0.00	0.00
23	1.00	0.00	0.03	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00
24	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00

Viability Analysis of Ex Situ Population

This study enables the predictive estimates of how well the current management strategies will foster persistence of the *ex situ* population of the Guam kingfisher. Specifically, the models investigated here allowed me to test altered management strategies to determine how these strategies impact long-term persistence. Comparisons of these models enabled me to address the following questions:

(1) Under current practices, what is the risk of population decline or extinction?

(2) If the space available to the *ex situ* population was increased, how does this increase impact the risk of extinction?

(3) What change in demographic rates would lead to a population decline or increase in extinction risk?

(4) What is the impact of current genetic management practices on the loss in genetic diversity?

(5) What role may inbreeding depression play on the potential for extinction?

(6) How many individuals and what age class can be removed for a reintroduction program without leading to risk of population decline?

Under all scenarios, I summarized probability of extinction in 100 years, as well as resulting levels of gene diversity to determine success of the specific strategy. The following scenarios and inputs were used to examine these questions:

Scenario 1 – Baseline:

The baseline scenario used parameters that most closely represent the current (i.e. 2016) management strategy of the Guam Kingfisher Species Survival Plan (SSP®). In the baseline scenario, an individual-based simulation was run using Vortex 10.0.7.9 (released 28 January 2015; Lacy and Pollack 2014) in which each individual survives with the probability determined by its age and sex, and number of offspring produced by each female is sampled from a Poisson distribution with the mean set to the age-specific fecundity, under the constraint that there must be sufficient adult males available for the breeding females (Lacy et al. 2012). Stochastic projections are more

realistic for smaller population size, as skewed sex ratio and other chance events will typically depress and cause large variation in population growth (Lacy 2000).

The scenario settings were input with one population (the current *ex situ* population), with extinction defined as when only one sex remains. I ran 500 iterations for 100 years each. The reproductive system for this species is long-term monogamy, with the age of first offspring being five years for males and four years for females (median age at first hatch calculated from studbook data). The maximum age of reproduction was entered as 19 for males and 12 for females. These data are from the maximum ages at which reproduction has occurred in the studbook dataset in the history of *ex situ* management. The maximum lifespan was entered as 24, as the oldest male in the population lived to be slightly older than 23 years old.

While biologically possible for females to produce seven clutches in a year by removing eggs and artificially incubating so that females re-clutch, management strategies in recent years have limited females to six clutches per year to reduce the physical demand on the female to produce eggs. The model was therefore adjusted to allow for no more than 6 clutches in a year and the maximum number of progeny per clutch is three. The scenario included a 1:1 sex ratio at hatch (total hatches of known sex individuals from 1980-2016 = 446, $\chi^2 = 1.085$, df = 1, $p = 0.30$). I used Poplink 2.4's (Faust et al. 2012) to estimate the average proportion of females breeding in a given year as the proportion of females of reproductive age that produced a fertile egg, which averaged 52.7% of females over the past 10 years. The distribution of clutches per year from 1984-2016 laid by a female in a year is shown Figure 8.

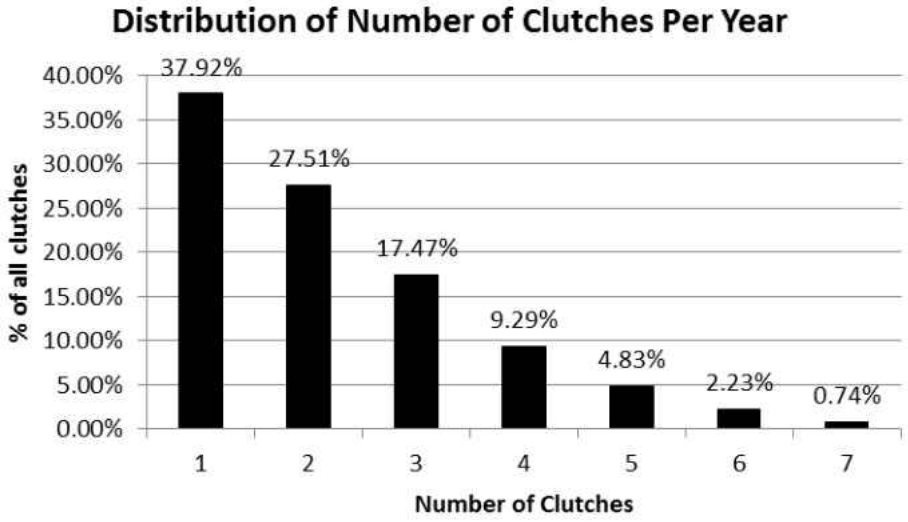


Figure 8. Distribution of number of clutches per female per year from 1984-2016.

A total of 269 clutches were laid during this time with a clutch defined as eggs laid by a single female within a period of seven days. The distribution of number of offspring per female per clutch is one offspring = 65.19%, two offspring = 33.33%, three offspring = 1.48%. These values were determined from reproductive reports in Poplink 2.4 and are determined by the predetermined hatch date range for clutchmates of seven days.

Mortality (q_x) rates are extracted from the life table for both males and females (from Table 7) for the age classes of 0-1, 1-2, 2-3, 3-4, and after 4 (for females), and 4-5 and after 5 (for males) are listed in Table 9.

Table 9. Juvenile and adult mortality rates by sex. First year mortality rates are calculated from chicks that have hatched.

Age class	Females (% age class)	Age class	Males (% age class)
0-1	40.0	0-1	41.0
1-2	5.0	1-2	7.0
2-3	10.0	2-3	7.0
3-4	17.0	3-4	7.0
After 4	24.0	4-5	8.0
		After 5	15.0

For mate monopolization values, because the reproductive system is long-term monogamy, all males in the population are available to breed (100%). Carrying capacity was set at K = 150, which is the approximate size of the population at the start of 2016. The initial population was input from the studbook with living birds as of 1 January 2016 and the pedigree of the population. The population starting size on this date was N = 148 and the starting age structure of the population was defined by this input file. The scenario included genetic management setting to pair according to mean kinship with a static mean kinship list, to as closely match the history of genetic management of the *ex situ* population as possible. This scenario was also set to calculate the number of pairings required each year to bring or maintain the population at its carrying capacity. Finally, this scenario was set to prevent pairings between birds with kinships greater than 0.25 since at this point in the population's genetic management pairings between siblings have been purposefully avoided.

Scenario 2 – Carrying Capacity:

There is a proposal to build an additional 1-2 breeding centers in the next 5-10 years. If these breeding centers can hold five breeding pairs or approximately 10 offspring for a total of 10 additional birds, this would increase the carrying capacity to 160 or 170. Also, a population target size of 250 was previously set as the goal for the managed population to reach prior to conducting releases or removing birds for supplementing a release population. Therefore, I examined the probability of extinction under models of expanded carrying capacity. Given the plans for growth of the Guam kingfisher *ex situ* population, I tested three additional carrying capacity scenarios of 160, 170, and 250. All remaining model parameters matched those of the baseline model.

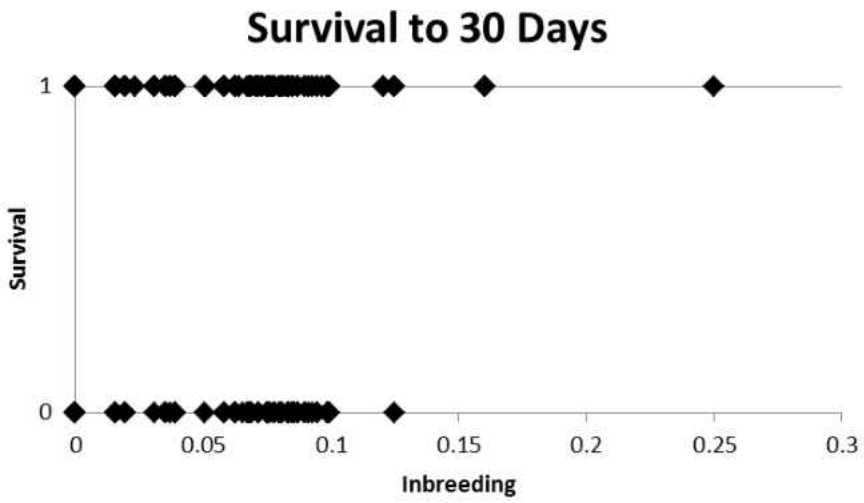
Scenario 3 – Inbreeding Depression:

Previously, 3.14 lethal equivalents were used in most Vortex PVAs in cases where number of lethal equivalents was unknown. This value, obtained from Ralls (1988) study of captive mammals, had been the only comprehensive reference examining lethal equivalents in multiple species. However, in O'Grady et al.'s (2006) meta-analysis, they presented diploid lethal equivalents in avian species affecting fecundity and survival varying from 0.74 to >13.44. Based on recent arguments that *ex situ* populations are likely to have lower lethal alleles than wild populations due to their sheltered environment in which they are impacted by fewer stressors (Lacy et al. 2015),

the mid-point of O'Grady's suggested values of 6.29 is now a more commonly assumed value.

Vortex models inbreeding depression as a reduction in the vital rate for first-year survival among inbred individuals. I first examined this assumption in Guam kingfisher population by conducting a logistic regression comparing the inbreeding value for each individual hatched in the population to survival to 30 days and to one year. There is no evidence at this point that there is inbreeding depression occurring in first year survival for 30 days ($N = 637$, $p>0.32$, Figure 9A) or to one year ($N = 637$, $p>0.74$, Figure 9B).

A)



B)

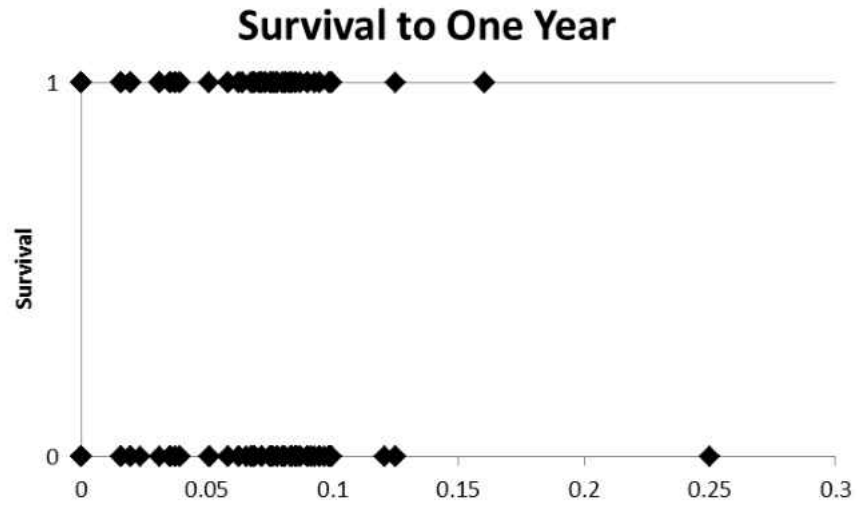


Figure 9. Comparison of individual inbreeding level and survival to 30 days (A) or to one year (B) in all hatches in the Guam kingfisher population from 1984-2016.

However, even though there is not evidence for inbreeding depression in first year survival at this time, it may be present in another aspect of this population such as reduced fecundity or reduced adult survival, or as inbreeding levels increase, may have

impacts on the population into the future. Since no inbreeding depression was included in the baseline scenario, in this scenario I tested the impacts of including both the values of 3.14 and 6.29 lethal equivalents, with the default of 50 percent due to recessive lethal alleles.

Scenario 4 – Genetic Management:

Most *ex situ* populations are managed such that when breeding recommendations and pairings are made, a static mean kinship list is used where the kinship of each individual is left unchanged within each year as pairs are selected. The Guam kingfisher population is managed with a static mean kinship list, which is the more appropriate strategy if many pairings fail. The dynamic mean kinship list is projected to preserve genetic diversity if most males and females that are paired do produce the expected offspring (i.e. the % females breeding is high, Lacy et al. 2015). Because the percent of females breeding in the baseline scenario is above 50%, I tested a scenario in which a dynamic mean kinship was used to determine the predicted improvement in maintenance of gene diversity.

In addition to changing the way pairs are made, another genetic management strategy related to inbreeding avoidance is also used. In the baseline, I chose to avoid pairings that would result in offspring with inbreeding levels of 0.25, or of that of full siblings. In the history of genetic management of this population, just one individual with this level of inbreeding was hatched. Therefore, I chose to test a scenario where the

restrictions on inbreeding would be even greater with no individuals paired that would produce offspring with an inbreeding level of 0.125, or half-siblings.

Scenario 5 – Sensitivity Analysis of Mortality and Reproduction:

For the sensitivity testing scenario, I examined changes in first year mortality of both sexes, adult mortality of both sexes, and a change in the percentage of females breeding. The minimum and maximum as well as the range of the parameter space tested are listed in Table 10.

Table 10. Sensitivity test variables with the minimum, maximum and increments tested.

Test Description	Base Value	Minimum	Maximum	Increment
First Year Mortality	40	15	65	5
Female Adult Mortality	25	5	50	5
Male Adult Mortality	15	5	50	5
Percent of Females Breeding	53	25	75	2

I used 100 samples of each parameter with the single-factor testing option. After completing the sensitivity analysis, I completed a standard least square ANOVA to examine the impact of the four parameters and their relationship to probability of extinction.

Scenario 6 – Removal for Reintroduction (Harvest):

In the final scenario, I varied the proportion of various age classes to determine an appropriate number of available birds (i.e. harvested individuals) for reintroduction

throughout the next 100 years. All scenarios started with first harvest occurring in five years, and with a frequency of recurrence of every five years after for the next 100 years. I examined extraction of juveniles in age classes two and three, as well as scenarios with adult birds only and tested different numbers of birds in these scenarios (Table 11). I also tested these simulations under the current carrying capacity of 150, and the higher carrying capacity of 250 individuals (Table 11).

Table 11. Harvest scenarios of the Guam kingfisher population to determine availability of birds for reintroduction. All harvest scenarios start at year 5 and end at year 100. When the population size condition is included, the total population size must be greater than 90% of carrying capacity in order for harvest to occur.

Harvest Scenarios	Sex Ratio	Age Class	Carrying Capacity
Adults	5 males, 5 females	After 5 males, After 4 females	150
Adults	5 males, 5 females	After 5 males, After 4 females	250
Adults	3 males, 3 females	After 5 males, After 4 females	150
Adults	3 males, 3 females	After 5 males, After 4 females	250
Juveniles	4 males, 4 females	2 individuals from age 2-3, and 2 from age 3-4 from both sexes	150
Juveniles	4 males, 4 females	2 individuals from age 2-3, and 2 from age 3-4 from both sexes	250

Results

The results of the baseline scenario predict that there is a 0.00% probability of extinction in 100 years with 83.64% of gene diversity projected to remain after the 100 years. Summary statistics of all scenarios, probability of extinction after 100 years, and projected gene diversity in 100 years are in Table 12.

Table 12. Results from population viability analyses for the Guam Kingfisher (*Todiramphus cinnamominus*) Species Survival Plan® including baseline scenario and multiple alternate scenarios. All scenarios run for 500 iterations.

Scenario	Det-R	Stoch-R	SD (Stoch-R)	PE	N-extant	SD (N-extant)	GeneDiv	SD (GD)
Baseline	0.044	0.010	0.061	0.00	144.49	7.19	0.8364	0.0402
Carrying Capacity – N = 160	0.044	0.011	0.060	0.00	154.19	8.74	0.8412	0.0384
Carrying Capacity – N = 170	0.044	0.011	0.059	0.00	164.94	8.93	0.8384	0.0426
Carrying Capacity – N = 250	0.044	0.013	0.055	0.00	244.52	7.24	0.8551	0.0356
Inbreeding Depression - 3.14	0.044	0.000	0.067	0.02	128.00	31.33	0.8230	0.0507
Inbreeding Depression - 6.29	0.044	-0.022	0.093	0.35	58.05	42.60	0.7638	0.1036
Dynamic Mean Kinship	0.044	0.010	0.061	0.00	144.33	8.32	0.8335	0.0471
Prevent Inbreeding 0.125	0.044	-0.031	0.107	0.94	6.48	3.88	0.7516	0.0930
Harvest Adults – 5.5, K = 150	0.044	-0.005	0.097	0.20	125.81	26.79	0.8187	0.0524
Harvest Adults – 5.5, K = 250	0.044	0.001	0.088	0.17	228.67	34.48	0.8385	0.0430
Harvest Adults – 3.3, K = 150	0.044	0.004	0.073	0.04	135.38	17.93	0.8257	0.0434
Harvest Adults – 3.3, K = 250	0.044	0.009	0.065	0.02	238.38	21.47	0.8459	0.0403
Harvest Juveniles – 4.4, K = 150	0.044	0.004	0.073	0.02	132.75	20.38	0.8278	0.0488
Harvest Juveniles – 4.4, K = 250	0.044	0.008	0.065	0.03	234.94	25.23	0.8464	0.0404

Under all strategies in the increased carrying capacity scenarios, the populations reach their target carrying capacity (Figure 10) and the risk of extinction is 0.00%.

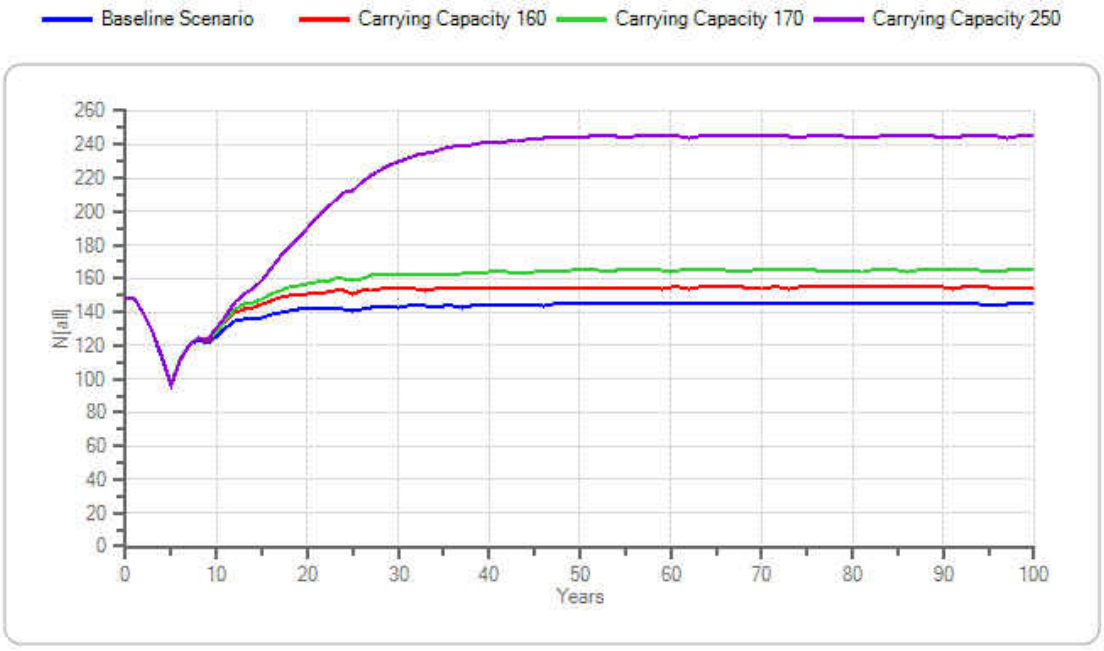


Figure 10. Baseline and Carrying Capacity Scenarios demonstrating predicted population size at the end of 100 years for the Guam kingfisher SSP®.

This model also found that the population with 250 spaces is predicted to retain the highest level of gene diversity at 85.51%, although the standard deviations between this projection and the baseline projection do overlap (Table 12).

When including inbreeding depression in the baseline model, the probability of extinction increases and depends on the number of lethal equivalents that are included. If the population has 3.14 lethal equivalents per bird, probability of extinction is 2%, but if the population has 6.29 lethal equivalents, that probability increases to 35.0% (Table 12).

In the genetic management scenarios, using the dynamic mean kinship list when creating breeding pairs each year did not lead to increased gene diversity at 100 years compared to the baseline. However, changing the inbreeding avoidance to preventing pairings that would result in an inbreeding level of 0.125 had a detrimental impact on probability of extinction, leading to a 94% chance of extinction in 100 years (Table 12).

The sensitivity testing demonstrated impacts on probability of extinction of three of the four parameters tested (complete model: $F = 26.98$, $DF = 4$, $p < 0.0001$), including infant mortality ($F = 14.92$, $p = 0.0003$), adult female mortality ($F = 31.60$, $p < 0.0001$), and percent of females breeding ($F = 62.26$, $p < 0.0001$). Male adult mortality did not contribute significantly to the model. For infant mortality, the values between 15% - 50% maintained a low probability of extinction (0.00% – 0.04%) and infant mortality above 55% had the greatest impact (Figure 11).

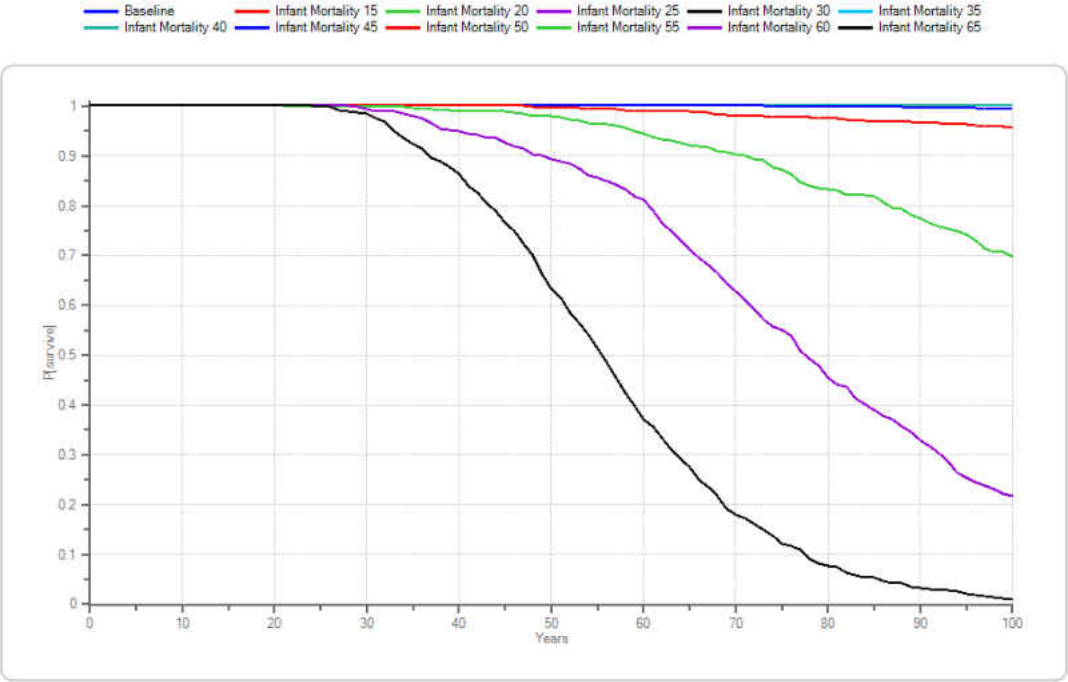


Figure 11. Sensitivity analysis of infant mortality on population probability of extinction in Guam kingfishers.

With female adult mortality, the results were extremely variable. The values from 5% - 30% adult female mortality predicted a low probability of extinction (0.00 – 4%) but increasing mortality to 35% - 50% predicted a 41% - 100% probability of extinction (Figure 12).

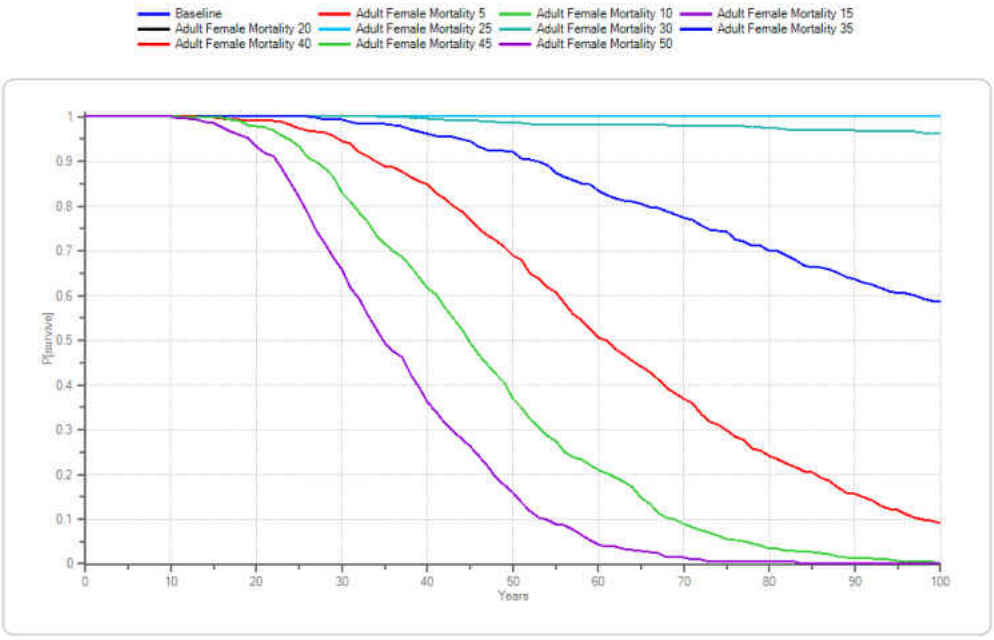


Figure 12. Sensitivity analysis of adult female mortality on population probability of extinction in Guam kingfishers.

However, across most values tested for male adult mortality, probability of extinction remained between 0.00% - 4%, with only male mortality of 45% and 50% predicting a probability of extinction of 19% and 56% respectively (Figure 13).

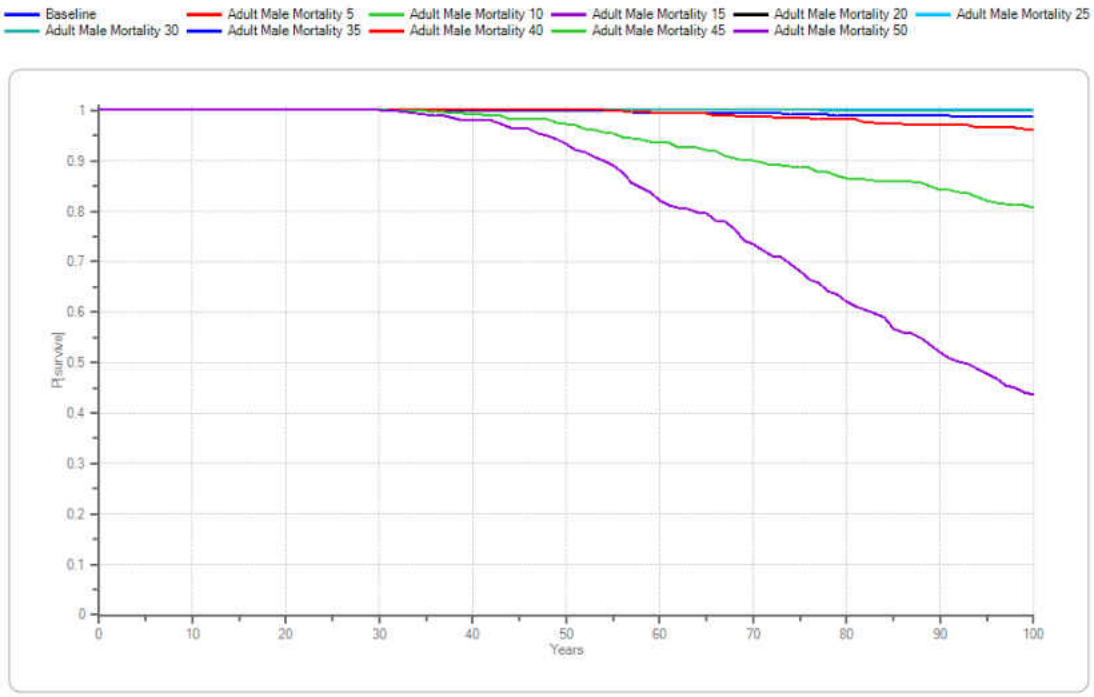


Figure 13. Sensitivity analysis of adult male mortality on population probability of extinction in Guam kingfishers.

Finally, altering the percentage of females breeding had the most variable impact. When 45% - 75% of females reproduced, probability of extinction was between 0.00 – 4%. However, once the value dropped from 31% - 43%, probability of extinction ranged from 12% - 97%, and for values of 25% - 29%, extinction probability was 100% (Figure 14).

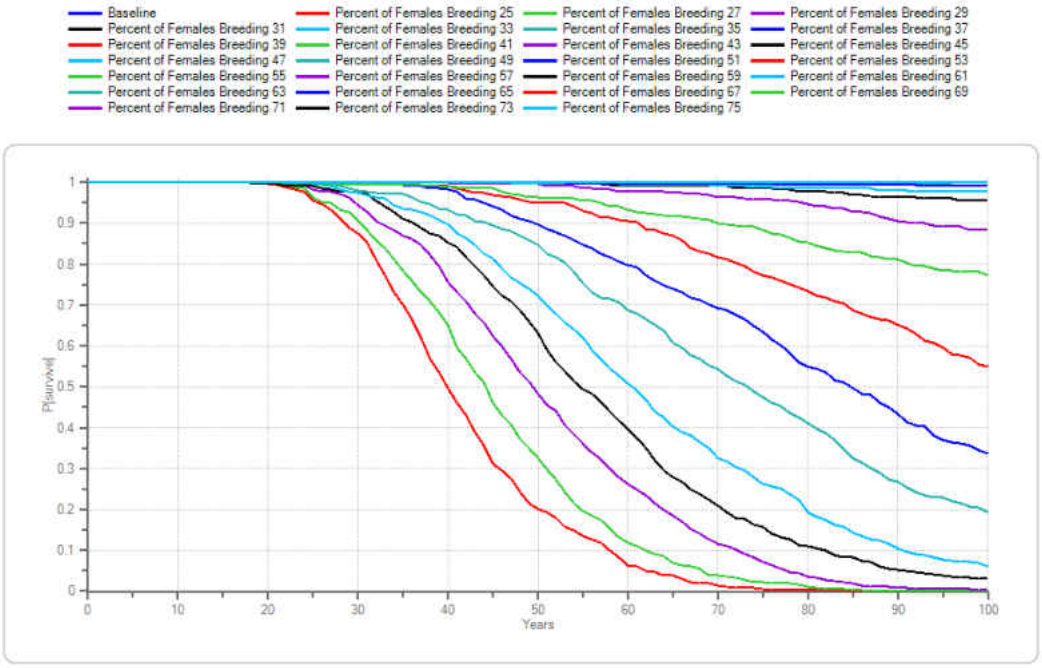


Figure 14. Sensitivity analysis of percent of females breeding on population probability of extinction in Guam kingfishers.

Finally, in the scenarios which examined harvesting birds for reintroduction, the PVA models estimated that population size had little impact on probability of extinction and maintenance of gene diversity than expected, but number and age class of birds had large impacts on probability of survival (Table 12). Extracting six adults (three males, three females) every five years had a low probability of extinction (2% - 4%) and I found similar results for extracting eight juveniles (four males, four females, two from each of the 2-3 and 3-4 age classes) with probability of extinction of 2% - 3%.

Discussion

In this paper, I use a PVA to test both the current management strategies and alternate scenarios in the Guam kingfisher assurance population. The baseline scenario suggests that under current strategies, there will be 0% probability of extinction in 100 years with greater than 83% genetic diversity retained (Table 12). However, as this population is extinct in the wild, and serves as an assurance population which will serve as the single source for future reintroductions, it is necessary to consider how potential management changes will impact this population's future trajectory. Therefore, I tested numerous scenarios and considered the implications of the scenario results in terms of future management of this species.

As expected, our analyses of carrying capacity indicated that an increase in space above the baseline capacity maintained a 0% probability of extinction (Table 12). An increase in carrying capacity also led to the maintenance of increased levels of gene diversity (Table 12). The small increases did not have a noticeable impact, particularly when considering the standard deviation of the results, although the highest carrying capacity of N = 250 did result in the highest projections of future gene diversity (Table 12). Baker (2007) recommends a population size of 200 to be considered a minimally viable *ex situ* population. However, for populations to be self-sustaining with no net loss of genetic diversity, where drift and mutation are thought to be in balance, the minimum population size is predicted to be $N_e = 500$ where N_e is the effective population size (Frankham et al. 2002). Increased population size is directly linked to maintaining high levels of gene diversity, with smaller populations at risk for impacts of genetic drift, inbreeding depression, and stochastic events (Bouzat 2010, Soulé and Wilcox 1980).

However, carrying capacity becomes an important management variable when considering future harvests for reintroduction, particularly as projections are likely to change as birds are extracted (Dimond and Armstrong 2007), particularly if the goal is to maintain the population at a specific size.

The results from the inbreeding depression and genetic management scenarios demonstrated the potential impacts of genetic composition that may soon affect this population. With the incorporation of inbreeding depression at conservative (3.14 lethal equivalents) to moderate (6.29 lethal equivalents) levels, probability of extinction was higher (2% and 35%) than baseline. Because of the resulting broad variation from a small change in lethal equivalents used in this model, more research, specifically on the genetic load that is present in the Guam kingfisher, is warranted to create more accurate predictions. The meta-analysis by O'Grady et al. (2006) found that disregarding the influence of inbreeding depression can lead to serious overestimates of survival prospects of threatened taxa and can lead to the development of inappropriate recovery plans. While it does not appear that inbreeding depression is yet impacting this population, it is likely to become a concern as the presence of inbred individuals in the population increases and the impacts of inbreeding depression may accumulate across multiple life history stages (Grueber et al. 2010). The genetic management scenario indicated that the population would have a very high probability of extinction (94%) if matings that would lead to individuals with inbreeding levels of 0.125 were avoided (Table 12). These results are likely driven by the increase in inbreeding that is already occurring, and the inability to create pairs under these somewhat strict genetic management guidelines. In order to decrease probability of extinction, higher levels of

inbreeding will need to be tolerated, which in turn will likely lead to increased impacts of inbreeding depression and therefore a greater loss of genetic diversity (Lacy 1993). While not always considered as impactful as demographic factors, inbreeding depression and reduced genetic diversity can in some cases be the primary factors that threaten population viability (del Castillo et al. 2011, Menges and Dolan 1998).

The interpretation and application of the results of sensitivity testing should be considered within their ability and capacity to change in a real world situation, i.e. either there is historic evidence of variation in the key vital rate, there is ability to make the suggested alterations based on the results, or more data are required to have a better estimate of the parameter in question (Reed et al. 2002). Of the three vital rates that showed to be impactful in my model (infant mortality, adult female mortality, and percent of females breeding), percentage of females breeding had the greatest impact, and also the greatest variation on the probability of extinction at 100 years (Figure 14). Managers should therefore consider this variable in future efforts to grow this population and determine if there are ways to increase from the baseline value of 53% of females breeding per year. More research may be needed around this variable and understanding of what is driving this result. For example, are some females not being given the opportunity for breeding based on their kinship value and the need to maintain the population at its current carrying capacity, or are many females paired and not successfully reproducing? The other important variables in this sensitivity analysis related to infant and adult female mortality may have less of a capacity for change. Attempts should be made to uncover causes of infant and female mortality to determine if there are strategies to mitigate this, and therefore reduce mortality in these age

classes. Many studies focused on understanding the viability of avian populations have also found similar results demonstrating that either juvenile or adult mortality may be the most sensitive vital rate (Sæther and Bakke 2000), although these results may also be driven by other biological aspects of demography such as life-span, or by type of model implemented (Mortenson and Reed 2016).

The final scenarios tested were related to rates of extraction or harvesting for eventual reintroduction of Guam kingfishers back to the wild. I predicted that an increased carrying capacity would better support the harvesting of more birds for reintroduction, however I found that both scenarios with 10 adult birds extracted had the highest probability of extinction (Table 12). Based on the results of the additional scenarios, it appears that age class and/or a reduced number of birds are better indicators of population survival when harvest is included. A harvest of six adults or eight juveniles both had low probabilities of extinction. Because different strategies and different factors appear to have similar probabilities of success in maintaining the population, other avian reintroductions have made use of an adaptive approach to harvesting source populations for reintroductions, re-evaluating their viability analysis and harvest model after each extraction (Dimond and Armstrong 2007). Finally, while the extraction scenarios completed here considered age class and number, there was not a consideration of which birds, from a genetic perspective, would best to extract and yet maintain the genetic health of the *ex situ* population. The genetic trade-offs of which individuals should be harvested relative to the goals of the reintroduction and maintenance of the source population will need to be considered in the future (Earnhardt 1999).

While the results of the PVA provide a baseline of projections for the future trajectory of the Guam kingfisher population, one of the limitations of this current model (as well as many others, see Conner and White 1999) is the lack of detailed understanding and incorporation of individual variation and its impact on the estimation of extinction risk. Individual variation may be attributed to genotypic or phenotypic variation (Conner and White 1999). Overall, demographic stochasticity results from the differences among individuals and higher variance in demographic rates can lead to greater extinction risks (Kendall and Fox 2002). Populations comprised of uniform individuals are more extinction-prone than populations comprised of variable individuals (Jager 2001). However, most PVAs, including this one, assume that the fates of all individuals are identical such that the stochasticity from each vital rate is modeled from a pre-determined, often over-simplified distribution (Conner and White 1999, Fox and Kendall 2002, Kendall and Fox 2002). Recent analyses have determined that if there is more variation among individuals in a population than is included in the models, existing PVA's may overestimate variance and thus the overall extinction risk may be lower than expected (Conner and White 1999, Fox and Kendall 2002, Jager 2001, Kendall and Fox 2002). Individual variation can be further investigated in this Guam kingfisher PVA by evaluating specific functions of demographic rates. For example, by specifying that demographic rates are functions of the alleles carried by an individual, it is possible to model more in depth genetic process and consider the genetically based individual variation in demographic rates, such as breeding success or lifetime reproductive succession (Conner and White 1999, Lacy et al. 2015). Additional scenarios may examine the heritability of traits, such as high fitness and high survival, and consider

that the presence of individuals with these extreme traits that can survive and reproduce under adverse conditions may extend the probability of a population's persistence (Conner and White 1999, Jager 2001).

The *ex situ* population of Guam kingfishers is on the verge of major changes in management, with population managers considering testing release strategies for the birds, and preparing to increase the population size and increase reproduction. Because of these potential changes, conducting a PVA for this population prior to implementing these changes can better inform and help prioritize the future recovery strategy. The importance of conducting this PVA goes beyond the management of the Guam kingfisher. While most *ex situ* populations have short term goals of reaching and maintaining a target population size, retaining gene diversity and avoiding inbreeding (Lees and Wilcken 2009), very few use a PVA to understand how best to reach these same goals in a longer timeframe. For the Guam kingfisher, this becomes even more significant as this species is extinct in the wild, and there is less room for error in managing the population to meet its goals. Finally, most research of the impacts of reintroductions has been retrospective and gained from post hoc interpretations of monitoring results (Seddon et al. 2007). There is a need to develop better methods of modeling approaches and experiments related to improving outcomes from the release of *ex situ* animals as well as a better use of simulation modeling to identify factors affecting the viability of the reintroduced population (Seddon et al. 2007). These needs should be extended to not just reintroduced populations, but also to conservation breeding programs and in management of species with conservation significance.

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CHAPTER SIX: CONCLUSION AND SIGNIFICANCE

Management of species in *ex situ* environments for conservation purposes, including eventual reintroduction, should incorporate details of the species ecology, especially its life history and demography as well as its genetic composition (Ballou et al. 2010), which may require larger populations than has been suggested on genetic grounds alone (Frankham et al. 2014, Lande 1988). The immediate practical need in biological conservation for understanding the interaction of demographic and genetic factors in the extinction of small populations, therefore, may provide a focus for fundamental advances at the interface of ecology and evolution (Lande 1988). The analyses and results presented in this dissertation demonstrate the testing and improvement of tools related to genetic, demographic, and husbandry management of *ex situ* breeding programs. The papers presented here should provide guidance for more efficient and comprehensive *ex situ* breeding programs that, in turn, could be used as the starting point of a reintroduction program or a conservation breeding program with the goals to increase the probability of the long-term survival of the species (Canessa et al. 2016, Tzika et al. 2009).

The genetic and demographic analyses of both the historic management of an *ex situ* population, and its current status, are a first step in hypothesizing the potential directions for future population management (Maunder and Byers 2005). In addition, aspects of species biology including behavior, natural mating systems, evolutionary perspectives such as adaptation to the *ex situ* environment, and management factors

should be incorporated into setting specific goals for a conservation breeding program (Schulte-Hostedde and Mastromonaco 2015). Moreover, the testing of various proposed management strategies should be utilized as a means further understand the impacts that these strategies may have on the future trajectory of the population. In this dissertation I demonstrated how molecular genetic data can be used to strengthen pedigrees (Ferrie et al. 2013), as well as discuss the need for better understanding of species biology, particularly in reproductive behaviors, and how this knowledge can be translated to planning future reproduction in a species (Ferrie et al. 2013; and see Chapters Three). I also determined that when evaluating genetic diversity at a molecular level with single nucleotide polymorphisms, more diversity is being maintained than projected through the theoretical methods of pedigree analysis currently used to evaluate loss of genetic diversity in a population. Therefore, the strategy of prioritizing breeding pairs with low and well-matched mean kinship is proving more successful than predicted (see Chapter Four). Finally, I demonstrated through a population viability analysis, the importance of specific biological and management factors in influencing a population's long term sustainability, and the impacts that these factors can have on setting short and long term strategic goals for conserving, and potential reintroduction of a population (see Chapter Five). There is a need to continue to build more advanced population viability analyses which incorporate population genomics into new approaches in order to better understand the influence of evolutionary processes on population persistence (Pierson et al. 2014).

Zoological institutions and *ex situ* conservation breeding centers work cooperatively, sharing best practices in animal husbandry, health and welfare,

population management, and broader conservation goals (Conway 2011, Lees and Wilcken 2009). Analytical tools have been in place to inform *ex situ* population management for quite some time; however as habitat continues to disappear and *in situ* population sizes decline, there is an even greater need to develop an integrated approach to population management for species conservation (Byers et al. 2013). *Ex situ* populations can and do function as sources of genetic variation for reintroduction programs, although typical recommendations include a meta-population management approach where mutual and continuous gene flow between wild populations and the *ex situ* population occurs to ensure long-term survival of the species (Ochoa et al. 2016). However, as discussed for the Guam kingfisher, the species is extinct in the wild, so genetic management for reintroduction becomes even more pertinent.

This dissertation has provided insight into some of the many factors important in managing *ex situ* populations for conservation goals demonstrating the need for ever-evolving tools to promote improved science in conservation breeding. In general, the outcomes of these collective studies, and the methods used to achieve these outcomes, should be incorporated broadly into many *ex situ* management programs. Finally, as the need for more intensely managed wild populations develops, and the continuum that bridges *ex situ* to *in situ* population management becomes more evident (Byers et al. 2013), the application of new and scientifically sound management strategies to inform conservation becomes paramount.

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APPENDIX B: CHAPTER THREE SUPPORTING INFORMATION

Supporting Information – Expanded Methods and Results for Molecular Data Collection and Parentage Analysis

Genetic Data Collection

To isolate species-specific microsatellite loci for genetic analyses, we generated random DNA fragments (~200-2500 bp) using degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR), using the K6-MW primer and protocol (Macas et al. 1996; Degner et al. 2009). Microsatellite enrichment of the PCR-amplified genomic fragments employed a 3'-biotinylated $(GATA)_8$ repeat motif bound to streptavidin-coated particles (Promega Corporation, Madison, Wisconsin, USA) enriched via magnetic separation. Enriched DOP-PCR products were made double stranded by a subsequent DOP-PCR and cloned using TOPO TA cloning kit (Invitrogen, Carlsbad, California, USA). Clones positive for $(CA)_n$ or $(GATA)_n$ microsatellites were identified using the screening procedure of Cabe and Marshall (2001). In brief, we conducted two PCRs per colony: one PCR included T3 and T7 primers while the second included the $(GATA)_8$ primer in addition to the T3 and T7 primers. We visualized the product of the PCR reactions on a 2.0% agarose gel and positive clones (those containing microsatellites) were identified by a distinctive smear in the $(GATA)_8$ reaction. We then sequenced positive clones (Applied Biosystems 3730 DNA Analyzer, Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA) and

developed PCR primers from flanking regions of DNA surrounding the microsatellite repeats. In total, we developed microsatellite primer sets for eight loci.

PCRs for all loci were performed in 20 µL reactions containing 1 µL of template DNA diluted to 20 ng μL^{-1}, 2 µL of 10X PCR buffer, 1.25 µL of 25 mM of $MgCl_2$, 200 µM of each dNTP, 0.5 µL of 10 µM M13 labeled (Schuelke 2000) forward primer and 1 µL of 10 µM reverse primer, 1 µL of 10 µM fluorescently-labeled M13 primer, 0.2 µL dimethyl sulfoxide, and 1 unit of Taq polymerase. The fluorescently labeled dyes were ABI DS-30 dye set (6-FAM, HEX, NED; Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA). PCRs were performed using a BioRad MyCycler thermalcycler (Bio-Rad Laboratories, Hercules, California, USA). The initial denaturing step was 94 °C for 4 minutes, followed by 35 cycles of 30 seconds at 94 °C, 30-35 seconds at the annealing temperature, and 45 seconds at 72 °C, then a final extension cycle at 72 °C for 7 minutes, and a hold at 4 °C. Annealing temperatures for all loci are listed in Table 3. PCR products were visualized on a 2% agarose gel before genotyping. PCR products were sized using an ABI PRISM® 3730 DNA Analyzer (Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA) in 5 µL multi-plexed reactions at the University of Arizona Genetics Core (Tucson, Arizona, USA). Alleles were sized with respect to size standard ROX (DS-30, Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA) using the Peak-Scanner Software (v1.0, Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA). Allele sizes were checked for accuracy by double-genotyping some individuals at each locus.

Parentage and Relatedness Analysis

Using CERVUS, which uses a pair-wise likelihood comparison based approach to assign parentage, we first ran an allele frequency analysis on the eight polymorphic loci discussed above, which we used to calculate multilocus parental exclusion probabilities (Selvin 1980). We then ran a parentage analysis simulation of the parent pair with known sexes, including the *a priori* probabilities of sampling of candidate parents shown in the main text. This simulation calculates the critical delta of each assignment, which is a derivative of the likelihood score used in parentage analysis and provides a threshold for assigning varying levels of confidence in the parentage analysis (Kalinowski et al. 2007). Finally, we conducted a parentage analysis of the parent pair with known sexes including a list of candidate sires and dams for each offspring. To be included in analysis, the minimum number of loci typed per individual was four. This analysis assigns the most likely candidate parent pair using the likelihood score, and the predetermined confidence levels of 95% and 80%.

We also used COLONY (v2.0; Jones and Wang 2009), which implements a full-pedigree maximum likelihood method to assign parentage and sib-ship among individuals with multi-locus genotypes, to run a parentage analysis and examine family relationships. For the COLONY analysis, we chose a polygamous mating system which assumes no inbreeding and allows for maternal-only and paternal-only sib-ships (half-sibs). We ran a full likelihood analysis with a long run length, which considers more configurations in the simulated annealing process (Jones and Wang 2009) and no sib-ship prior. We input the eight microsatellite marker types, and included an error rate of

0.02 (Wang 2004). The genotypes of the 50 offspring, 11 candidate sires, and 10 candidate dams were input as separate files. In both programs, parentage is assigned with confidence levels of 95% and 80% confidence.

The program Coancestry (Wang 2011) implements 7 different methods to estimate pair-wise relatedness between individuals and allows for comparison between estimators using simulated data with predefined relationships. We first conducted an analysis using simulated genotypes with the observed allele frequencies at each locus, the proportion of genotypes missing at each locus, and the genotyping error rate of 0.02 (Wang 2004) to calculate correlation coefficients between estimated and true simulated relatedness values. One hundred dyads each of six known relationships (parent-offspring, full-sibs, half-sibs, first cousins, second cousins, and unrelated) were simulated with genotyping errors accounted for in the likelihood calculations. Ninety-five percent confidence intervals (CIs) were calculated with 1000 bootstraps. To determine which method of calculating relatedness was most representative of our data, we tested correlations between the relatedness values of simulated and known relationships in JMP Pro 9.0.0 (2010 SAS Institute, Cary, North Carolina, USA). To obtain relatedness estimates between individuals in our colony, we then conducted an analysis of the empirical data with observed allele frequencies. We included 100 reference individuals used in the triadic likelihood methods and 1000 bootstrapping samples to calculate 95% CIs. Finally, we accounted for potential error in genotyping with the error rate of 0.02 at all loci (Wang 2004).

Based on the comparisons in Coancestry between simulated genotypes and relatedness values between known relationships, the TrioML method of calculating

relatedness (Wang 2007) yielded the highest correlation between genetic (simulated) and true estimates ($r = 0.76$, $p < 0.001$) and also had the lowest variance (0.03) of the seven methods. When the dataset contains relatively unrelated individuals, the triadic likelihood method of calculating relatedness best accounts for genotype error in the data (Wang 2007) and gives the most prudent method of inferring relationships between pairs of individuals (Doutrelant et al. 2011).

Table 13. Results of behavioral observation determining the social parents in each nest tunnel, as well as results of visual comparisons of the number of loci that are mismatched between the offspring genotypes and their social sire and dam, as well as COLONY and CERVUS parentage assignment tests. UNK refers to unknown social sire or dam. ** strict confidence, >95% assignment probability, *relaxed confidence, >80% assignment probability, no * low confidence, <80% assignment probability, ǂ indicates individual was missing a genotype at one or two loci.

Egg/Chick ID	Nest Tunnel	Year	Social Sire	Genetic Sire			Social Dam	Genetic Dam		
				Visual Comparison # Loci Mismatched	COLONY	CERVUS		Visual Comparison # Loci Mismatched	COLONY	CERVUS
M. nub10	15	2005	010555	N/A		M. nub7	M. nub9	0	M. nub9**	M. nub9**
M. nub2	9	2006	010555	N/A		M. nub7	M. nub9	0	M. nub9	M. nub9*
M. nub12	14	2006	010554	N/A		M. nub7	010561	N/A	M. nub11	M. nub10*
M. nub16	9	2009	M. nub4	0	M. nub4**	M. nub4**	M. nub12	0	M. nub12**	M. nub12**
M. nub14	10	2009	M. nub2	2	M. nub7**	M. nub7*	070304	N/A	M. nub10	M. nub10*
M. nub15	10	2009	M. nub2	0	M. nub2**	M. nub2**	070304	N/A		M. nub8
M. nub17	10	2009	M. nub2	0	M. nub2	M. nub2*	070304	N/A	M. nub11**	M. nub11*
M. nub6	12	2009	M. nub1	0	M. nub1**	M. nub1**	M. nub9	0	M. nub9**	M. nub10
M. nub19	9	2010	UNK	N/A	M. nub6**	M. nub6*	UNK	N/A	M. nub10**	M. nub10*
M. nub20	9	2010	UNK	N/A	M. nub1**	M. nub6*	UNK	N/A	M. nub9**	M. nub10
M. nub26	9	2010	UNK	N/A	M. nub1**	M. nub6*	UNK	N/A	M. nub9**	M. nub10
M. nub27	9	2010	UNK	N/A	M. nub6**	M. nub6*	UNK	N/A	M. nub10**	M. nub10**
M. nub57	10	2010	UNK	N/A	M. nub4**	M. nub4**	UNK	N/A	M. nub11**	M. nub12
M. nub58	10	2010	UNK	N/A	M. nub2	M. nub2	UNK	N/A	M. nub13**	M. nub13**
M. nub60	10	2010	UNK	N/A	M. nub4**	M. nub4*	UNK	N/A	M. nub11**	M. nub11*
M. nub18	12	2010	UNK	N/A		M. nub7*	UNK	N/A	M. nub11*	M. nub12*
M. nub24	12	2010	UNK	N/A	M. nub6**	M. nub6**	UNK	N/A	M. nub10**	M. nub10*
M. nub30	12	2010	UNK	N/A	M. nub5		UNK	N/A	M. nub9**	
M. nub59	12	2010	UNK	N/A	M. nub6**	M. nub6*	UNK	N/A	M. nub10**	M. nub10
M. nub22	14	2010	UNK	N/A	M. nub6**	M. nub6*	UNK	N/A	M. nub10	M. nub10*
M. nub47	14	2010	UNK	N/A	M. nub2*	M. nub2**	UNK	N/A	M. nub13**	M. nub15**
M. nub61	14	2010	UNK	N/A	M. nub2**	M. nub2**	UNK	N/A	M. nub13**	M. nub13**
M. nub21	15	2010	UNK	N/A	M. nub7**	M. nub7**	UNK	N/A	M. nub10	M. nub10*
M. nub28	15	2010	UNK	N/A	M. nub7**	M. nub7*	UNK	N/A	M. nub10	M. nub10*
M. nub23	17	2010	UNK	N/A	M. nub3**	M. nub2**	UNK	N/A	M. nub15**	M. nub15**
M. nub29	17	2010	UNK	N/A	M. nub5**	M. nub2	UNK	N/A	M. nub15**	M. nub15**
M. nub25	19	2010	UNK	N/A	M. nub3**	M. nub3**	UNK	N/A	M. nub15**	M. nub15**
M. nub31	19	2010	UNK	N/A	M. nub4**	M. nub4**	UNK	N/A	M. nub12	M. nub12*
M. nub32	4	2011	M. nub4	1	M. nub4	M. nub4	M. nub11	1	M. nub11	M. nub11
M. nub34	8	2011	M. nub6	0	M. nub6*	M. nub6	M. nub10	0	M. nub10*	M. nub59**
ǂM. nub37	8	2011	M. nub6	0	M. nub6**	M. nub6*	M. nub10	0	M. nub10**	M. nub10**
M. nub43	8	2011	M. nub6	0	M. nub6	M. nub6*	M. nub10	0	M. nub10	M. nub10*
ǂM. nub50	8	2011	M. nub6	5	M. nub4**	M. nub4*	M. nub10	2	M. nub11**	M. nub11*
M. nub33	9	2011	M. nub1	1	M. nub1**	M. nub1**	M. nub9	0	M. nub9**	M. nub59**
M. nub36	9	2011	M. nub1	1	M. nub1**	M. nub6*	M. nub9	0	M. nub9**	M. nub9*

Egg/Chick ID	Nest Tunnel	Year	Social Sire	Genetic Sire			Social Dam	Genetic Dam		
				Visual Comparison # Loci Mismatched	COLONY	CERVUS		Visual Comparison # Loci Mismatched	COLONY	CERVUS
ǂM. nub54	9	2011	M. nub1	5	M. nub2	M. nub47	M. nub9	0	M. nub13**	M. nub13*
ǂM. nub55	10	2011	M. nub2	0	M. nub2**	M. nub47	M. nub13	0	M. nub13**	M. nub13
ǂM. nub56	10	2011	M. nub2	0	M. nub2*	M. nub58	M. nub13	0	M. nub58	M. nub14
M. nub44	14	2011	M. nub60	1	M. nub60**	M. nub58**	ǂM. nub61	1	M. nub13**	M. nub13**
ǂM. nub52	14	2011	M. nub60	0	M. nub60**	M. nub60**	ǂM. nub61	0	M. nub61**	M. nub61*
M. nub35	15	2011	M. nub7	0	M. nub7**	M. nub7**	M. nub14	0	M. nub14	M. nub14**
ǂM. nub39	15	2011	M. nub7	1	M. nub7*	M. nub7	M. nub14	1		M. nub13
M. nub42	15	2011	M. nub7	0	M. nub7**	M. nub7**	M. nub14	0	M. nub14	M. nub14**
M. nub46	15	2011	M. nub7	0	M. nub7**	M. nub7**	M. nub14	0	M. nub10	M. nub14*
ǂM. nub51	15	2011	M. nub7	0	M. nub7**	M. nub7**	M. nub14	0	M. nub14	M. nub14**
M. nub38	16	2011	ǂM. nub58	0	M. nub3**	M. nub58**	M. nub15, ǂM.nub59	3 0	M. nub61*	M. nub61**
M.nub40	16	2011	ǂM. nub58	0	M. nub3	Mnub3	M. nub15, ǂM.nub59	1 0	M. nub59**	M. nub59*
M. nub41	16	2011	ǂM. nub58	0	M. nub6**	M. nub58*	M. nub15, ǂM.nub59	4 0	M. nub13**	M. nub59*
M. nub45	17	2011	M. nub3	7	M. nub4**	M. nub4**	M. nub15	0	M. nub15**	M. nub15**
M. nub53	17	2011	M. nub3	2	M. nub4**	M. nub4	M. nub15	0	M. nub15**	M. nub15**

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APPENDIX C: GUAM KINGFISHER GENETIC SAMPLING INFORMATION

Table 14. Table of all Guam kingfisher (*Todiramphus cinnamominus*) genetic samples collected for molecular genetic analyses including their pedigree information (sire and dam, date of hatch), type, location, and date of sample (if known).

Studbook ID	Generations From Wild Founder (Based on Dam's Lineage)	Sex	Current Location at Sampling*	Current Local ID	Sire Studbook ID	Dam Studbook ID	Hatch Date	Status at Sampling	Tube Label	Sample Type	Collection Date If Known	Sample ID Post Extraction	Notes
25	0	Female	SDNHM	46633	Wild	Wild	1/1/1983	Dead	46633	skin	4/11/1970	T. cin 171e	SDZ 27673
36	1	Female	ANSP	22026	9	10	7/12/1985	Dead	22026	tissue		T. cin 118e	PZG 29328, silver R 624
38	1	Female	ANSP	29069	3	4	8/12/1985	Dead	29069	tissue		T. cin 116e	PZG 30222
68	1	Male	SDNHM	49787	28	29	3/8/1987	Dead	49787	skin	5/27/1996	T. cin 172e	SDZ 36671
106	1	Female	SDNHM	48117	26	27	11/12/1988	Dead	48117	skin	6/29/1992	T. cin 173e	SDZ 31109
112	2	Male	USNM	621040	19	30	4/21/1989	Dead	621040	tissue	3/25/1994	T. cin 151e	NZP 212968
117	2	Male	SDNHM	46866	19	30	5/22/1989	Dead	46866	skin	8/10/1990	T. cin 174e	SDZ 28234
122	1	Female	LSUMZ	B-23829	26	27	7/18/1989	Dead	B-23829	tissue		T. cin 165e	
130	1	Male	LSUMZ	B-57112	1	2	9/7/1989	Dead	B-57112	tissue		T. cin 167e	
139	2	Male	LSUMZ	B-20871	3	44	5/21/1990	Dead	B-20871	tissue		T. cin 164e	
142	2	Female	LSUMZ	B-37273	96	99	6/13/1990	Dead	B-37273	tissue		T. cin 166e	In bag with other 1996 zoo-necropsied birds, SB# 142 or #202
145	2	Male	USNM	621665	3	44	7/16/1990	Dead	621665	tissue	8/28/1997	T. cin 153e	NZP 211632
148	2	Female	LSUMZ	B-57114	19	74	7/19/1990	Dead	B-57114	tissue		T. cin 169e	
164	2	Male	SDNHM	48510	68	74	6/1/1991	Dead	48510	skin	7/5/1993	T. cin 175e	SDZ 32766
170	2	Male	ANSP	22023	111	61	6/17/1991	Dead	22023	tissue		T. cin 117e	PZG 29933
176	2	Male	USNM	623255	88	92	8/18/1991	Dead	623255	tissue	7/31/2000	T. cin 149e	NZP 213719
179	2	Male	SDNHM	48011	68	74	9/24/1991	Dead	48011	skin	4/2/1992	T. cin 176e	SDZ 30644
186	2	Female	SD-WAP	FHDB	96	99	2/29/1992	Dead	DNA 186	DNA from San Diego		T. cin 102e	
189	2	Male	SD-WAP	AHJX	135	91	4/13/1992	Dead	DNA 189	DNA from San Diego		T. cin 93e	
190	2	Male	SDNHM	48116	120	106	5/13/1992	Dead	48116	skin	7/6/1992	T. cin 177e	SDZ 31138
193	2	Male	SDNHM	50804	88	92	5/17/1992	Dead	50804	skin	7/28/2000	T. cin 178e	SDZ 42001

Studbook ID	Generations From Wild Founder (Based on Dam's Lineage)	Sex	Current Location at Sampling*	Current Local ID	Sire Studbook ID	Dam Studbook ID	Hatch Date	Status at Sampling	Tube Label	Sample Type	Collection Date If Known	Sample ID Post Extraction	Notes
199	2	Female	SANDIEGOZ	NHXX	135	91	4/5/1993	Dead	DNA 199	DNA from San Diego		T. cin 103e	
201	3	Female	USNM	587855	131	137	5/25/1993	Dead	587855	tissue	5/26/1994	T. cin 161e	Phoenix 6993
205	3	Male	SANDIEGOZ	305520	131	137	6/26/1993	Dead	OR5700	tissue	12/1/2014	T. cin 127e	
206	3	Male	DISNEY AK	NHXX	131	137	6/27/1993	Dead	tissue 206	tissue		T. cin 108e	
209	2	Female	USNM	623227	88	92	7/31/1993	Dead	623227	tissue	5/2/2001	T. cin 155e	NZP 213724
216	2	Male	DISNEY AK	80107	24	99	5/31/1994	Living	None	feathers, filter paper	12/14/2012	T. cin 129e	
225	2	Male	AGANA	301	135	91	8/5/1994	Living	9	blood		T. cin 9e	
246	2	Male	USNM	623242	135	91	7/2/1995	Dead	623242	tissue	5/26/2000	T. cin 159e	NZP 214152
259	3	Male	DISNEY AK	110190	68	186	5/4/1996	Living	None	feathers, filter paper	12/14/2012	T. cin 130e	
261	2	Male	ST LOUIS	991873	24	99	5/12/1996	Living	50	blood		T. cin 36e	
263	2	Male	DISNEY AK	FHAB	24	99	6/30/1996	Dead	tissue 263	tissue		T. cin 109e	
330	3	Male	SD-WAP	497202	178	199	12/13/1997	Living	330	feathers in bags		T. cin 143e	
331	3	Female	USNM	638592	178	199	1/9/1998	Dead	638592	tissue	11/14/2007	T. cin 163e	NZP 215616
342	3	Male	USNM	631753	189	209	7/3/1998	Dead	631753	tissue	8/15/2002	T. cin 156e	NZP 214083
343	3	Female	SD-WAP	CHXX	135	186	7/30/1998	Dead	DNA 343	DNA from San Diego		T. cin 105e	
344	3	Male	USNM	646629	135	186	8/1/1998	Dead	646629	tissue	3/6/2010	T. cin 152e	NZP 215769
352	3	Female	DISNEY AK	NHXX	189	209	1/31/1999	Dead	tissue 352	tissue		T. cin 111e	
369	4	Male	USNM	631786	178	327	4/3/2000	Dead	631786	tissue	1/29/2002	T. cin 162e	NZP 214848
377	4	Female	SANDIEGOZ	NHXX	24	239	5/21/2000	Dead	DNA 377	DNA from San Diego		T. cin 101e	
382	3	Male	AGANA	30335	26	260	6/24/2000	Living	11	blood		T. cin 11e	
384	3	Male	AGANA	313	207	339	7/18/2000	Living	3	blood		T. cin 3e	
386	4	Male	USNM	623245	176	251	7/19/2000	Dead	623245	tissue	8/20/2000	T. cin 160e	NZP 214643
388	3	Female	USNM	638606	26	260	7/21/2000	Dead	638606	tissue	10/31/2006	T. cin 154e	NZP 214846
389	3	Male	SDNMH	50805	26	260	7/25/2000	Dead	50805	skin	11/25/2001	T. cin 179e	SDZ 43754
389	3	Male	SANDIEGOZ	NHXX	26	260	7/25/2000	Dead	DNA 389	DNA from San Diego		T. cin 95e	
393	4	Female	CHICAGOLP	20762	205	345	5/31/2001	Living	121	feathers in bags		T. cin 137e	
397	4	Female	SD-WAP	NHXX	263	354	7/31/2001	Dead	DNA 397	DNA from San Diego		T. cin 98e	
403	4	Male	CHICAGOLP	22666	365	251	3/7/2002	Living	122	feathers in bags		T. cin 136e	
406	4	Male	SANDIEGOZ	NHXX	259	381	5/3/2002	Dead	DNA 406	DNA from San Diego		T. cin 104e	
411	4	Male	USNM	641556	26	239	5/24/2002	Dead	641556	tissue	9/16/2007	T. cin 157e	NZP 215615
421	4	Female	AGANA	NONE	259	381	4/24/2003	Living	2	blood		T. cin 2e	
424	4	Female	SD-WAP	NHN	259	381	6/1/2003	Dead	DNA 424	DNA from San Diego		T. cin 91e	

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425	4	Male	AGANA	NONE	259	381	6/6/2003	Living	8	blood		T. cin 8e	
427	4	Female	DISNEY AK	110756	259	381	8/7/2003	Living	None	feathers, filter paper	12/14/2012	T. cin 131e	
434	5	Male	SD-WAP	NHXX	344	377	5/5/2004	Dead	DNA 434	DNA from San Diego		T. cin 106e	
435	5	Male	METROZOO	10B037	330	393	5/8/2004	Living	47	blood		T. cin 33e	
440	5	Female	SANDIEGOZ	NHXX	344	377	6/8/2004	Dead	DNA 440	DNA from San Diego		T. cin 89e	
441	5	Male	HOUSTON	22303	330	393	6/10/2004	Living	104	blood		T. cin 67e	
446	4	Female	NY BRONX	NHXX	360	354	6/30/2004	Dead	N2009-503	tissue		T. cin 124e	
447	5	Female	ST LOUIS	106534	330	393	7/12/2004	Living	52	blood		T. cin 38e	
449	4	Male	DISNEY AK	110076	367	352	7/15/2004	Living	None	feathers, filter paper	12/14/2012	T. cin 132e	
451	5	Female	SANDIEGOZ	404134	344	377	7/16/2004	Dead	OR5599	tissue	12/1/2014	T. cin 128e	
451	5	Female	SANDIEGOZ	404134	344	377	7/16/2004	Living	153	blood		T. cin 76e	
452	4	Female	SANDIEGOZ	308384	373	387	7/17/2004	Dead	OR5587	tissue	12/1/2014	T. cin 126e	
454	4	Male	NY BRONX	FHAL	351	374	8/1/2004	Dead	N2006-844	tissue		T. cin 123e	
463	4	Male	SAN ANTON	Y07020	373	387	10/12/2004	Living	33	blood		T. cin 21e	
464	4	Female	NY BRONX	FHXX	373	387	11/18/2004	Dead	N2007-17	tissue		T. cin 120e	
485	5	Male	CHICAGOLP	21530	330	393	7/9/2005	Living	119	feathers in bags		T. cin 139e	
487	4	Male	AGANA	297	384	340	7/10/2005	Living	7	blood		T. cin 7e	
489	5	Male	NZP-CRC	215731	403	397	8/1/2005	Living	489	feathers in bags		T. cin 147e	
492	4	Male	CHICAGOBR	2447	351	374	8/11/2005	Living	161	blood		T. cin 78e	
493	4	Female	LSUMZ	B-91955	373	387	8/12/2005	Dead	B-91955	tissue		T. cin 170e	
496	4	Male	AGANA	296	384	340	8/25/2005	Living	4	blood		T. cin 4e	
497	4	Female	AGANA	NHN	384	340	8/26/2005	Dead	12	blood		T. cin 12e	
499	5	Male	SAN ANTON	Y07031	403	397	9/11/2005	Living	34	blood		T. cin 22e	
505	5	Male	SANDIEGOZ	NHXX	344	377	4/24/2006	Dead	DNA 505	DNA from San Diego		T. cin 96e	
518	5	Female	USNM	644528	344	377	5/26/2006	Dead	644528	tissue	6/15/2009	T. cin 158e	NZP 215739
520	5	Male	PITTS CA	7711	461	439	5/29/2006	Living	37, 38, pen	blood, blood, liver		T. cin 24e	
522	5	Male	NY BRONX	B08025	330	393	6/2/2006	Living	N2012-1032	tissue		T. cin 119e	
528	6	Male	SANDIEGOZ	FHDX	406	451	6/22/2006	Dead	DNA 528	DNA from San Diego		T. cin 94e	
530	5	Female	USNM	644527	344	377	6/26/2006	Dead	644527	tissue	8/22/2009	T. cin 150e	NZP 215732
535	4	Female	DISNEY AK	60217	351	374	7/12/2006	Living	None	feathers, filter paper	12/14/2012	T. cin 133e	
536	4	Male	NZP-WASH	215737	351	374	7/13/2006	Living	None	feathers	1/16/2013	T. cin 141e	
538	4	Female	HOUSTON	22666	373	387	7/17/2006	Living	46	blood		T. cin 32e	

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540	5	Male	BUSCH TAM	64365	461	439	7/18/2006	Living	79	blood, feathers		T. cin 57e	
543	4	Female	CHICAGOLP	22026	359	420	8/17/2006	Living	178	tissue		T. cin 114e	
545	5	Female	SAN ANTON	F08018	461	439	9/21/2006	Living	545	feathers in bags		T. cin 145e	
549	5	Male	SEA WORLD	MK0002	403	397	3/7/2007	Living	69	blood		T. cin 49e	
555	6	Male	SEA WORLD	MK0001	434	424	4/19/2007	Living	67	blood		T. cin 47e	
557	4	Female	METROZOO	B90044	359	420	5/6/2007	Living	103	blood		T. cin 66e	
558	4	Male	PHILADELP	205739	359	420	5/8/2007	Living	57	blood, feathers		T. cin 43e	
561	5	Female	SANDIEGOZ	309053	330	393	5/17/2007	Living	27	blood		T. cin 15e	
567	6	Male	METROZOO	B90116	174	447	7/19/2007	Living	45	blood		T. cin 31e	
568	6	Male	CHICAGOLP	NHXX	174	447	7/21/2007	Dead	177	tissue		T. cin 115e	
569	5	Female	HOUSTON	24645	461	439	7/30/2007	Living	44	blood		T. cin 30e	
571	6	Male	SDNMH	52932	406	451	8/9/2007	Dead	52932	skin	9/19/2008	T. cin 180e	SDZ 51441
571	6	Male	SANDIEGOZ	NHXX	406	451	8/9/2007	Dead	DNA 571	DNA from San Diego		T. cin 99e	
574	5	Male	BUSCH TAM	65040	403	397	2/13/2008	Living	74	blood, feathers	10/8/2013	T. cin 52e	
575	5	Female	RIO GRAND	B10012	403	397	2/15/2008	Living	39	blood		T. cin 25e	
577	5	Female	NZP-WASH	215852	403	397	4/24/2008	Living	None	feathers	1/16/2013	T. cin 140e	
578	5	Female	SANDIEGOZ	NHAA	403	397	4/24/2008	Dead	DNA 578	DNA from San Diego		T. cin 97e	
582	6	Female	PHILADELP	205741	406	451	4/23/2008	Living	59	blood, feathers		T. cin 45e	
583	6	Female	BUSCH TAM	64398	406	451	6/10/2008	Living	32	blood		T. cin 20e	
592	6	Male	SAN ANTON	N09041	434	424	7/17/2008	Living	31	blood		T. cin 19e	
593	5	Female	SD-WAP	NHXX	403	397	9/13/2008	Dead	DNA 593	DNA from San Diego		T. cin 100e	
594	5	Female	PHILADELP	205740	403	397	9/15/2008	Living	58	blood, feathers		T. cin 44e	
595	6	Male	BUSCH TAM	64780	522	508	12/21/2008	Living	82	blood, feathers	10/10/2013	T. cin 60e	
597	6	Female	SEA WORLD	MK0004	522	508	1/2/2009	Living	68	blood		T. cin 48e	
604	5	Female	AGANA	NONE	487	443	4/6/2009	Living	5	blood		T. cin 5e	
609	5	Female	DISNEY AK	100748	403	397	4/27/2009	Living	None	feathers, filter paper	12/14/2012	T. cin 134e	
610	5	Female	LONG B AQ	UNK	492	439	5/5/2009	Living	110	blood		T. cin 69e	
612	6	Male	DISNEY AK	90195	461	484	5/13/2009	Living	113	blood, feathers	12/29/2012	T. cin 70e	
617	6	Female	BUSCH TAM	65124	485	543	6/2/2009	Living	73	blood, feathers	10/10/2013	T. cin 51e	
623	6	Male	DISNEY AK	90310	461	484	6/14/2009	Living	None	feathers, filter paper	12/14/2012	T. cin 135e	
624	6	Female	DISNEY AK	NHN	461	484	6/15/2009	Dead	tissue 624	tissue		T. cin 110e	
625	6	Unknown	SANDIEGOZ	NHXX	406	451	6/16/2009	Dead	DNA 625	DNA from San Diego		T. cin 90e	

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627	5	Male	PHILADELP	205832	492	439	6/17/2009	Living	60	blood, feathers		T. cin 46e	
630	4	Male	AGANA	NONE	384	421	7/3/2009	Living	10	blood		T. cin 10e	
631	4	Female	AGANA	NONE	384	421	7/5/2009	Living	6	blood		T. cin 6e	
639	7	Female	BUSCH TAM	64772	507	539	8/17/2009	Living	81	blood, feathers	10/10/2013	T. cin 59e	
640	4	Male	AGANA	NONE	384	421	8/23/2009	Living	1	blood		T. cin 1e	
655	6	Male	SAN ANTON	A10015	499	545	4/9/2010	Living	25	blood		T. cin 13e	
655	6	Male	ST LOUIS	111037	499	545	4/9/2010	Living	51	blood		T. cin 37e	
656	6	Male	DISNEY AK	100100	461	484	4/9/2010	Living	114	blood, feathers	12/29/2012	T. cin 71e	
660	6	Female	SANDIEGOZ	410045	563	561	4/19/2010	Dead	OR6061	tissue	12/1/2014	T. cin 125e	
660	6	Female	SANDIEGOZ	410045	563	561	4/19/2010	Living	160	blood		T. cin 77e	
665	6	Male	DISNEY AK	100144	461	484	5/1/2010	Living	115	blood, feathers	12/29/2012	T. cin 72e	
669	7	Male	ST LOUIS	107996	507	539	5/14/2010	Living	53	blood		T. cin 39e	
671	7	Female	BUSCH TAM	64605	540	583	5/18/2010	Living	102	blood		T. cin 65e	
673	6	Male	SANDIEGOZ	410061	563	561	5/19/2010	Dead	OR5114	DNA from San Diego	12/1/2014	T. cin 87e	
675	6	Male	SANDIEGOZ	313847	461	484	5/21/2010	Living	144	blood		T. cin 73e	
679	6	Male	CHICAGOLP	22520	485	543	6/3/2010	Living	109	blood		T. cin 68e	
680	6	Female	CHICAGOLP	22522	485	543	6/5/2010	Living	26	blood		T. cin 14e	
682	6	Male	SANDIEGOZ	NHN	563	561	6/13/2010	Dead	DNA 682	DNA from San Diego		T. cin 92e	
687	6	Unkno wn	DISNEY AK	AHN	461	484	7/27/2010	Dead	tissue 687	tissue		T. cin 107e	
696	6	Female	HOUSTON	25662	441	538	9/11/2010	Living	70	blood		T. cin 50e	
699	6	Unkno wn	NY BRONX	IHXX	448	581	1/13/2011	Dead	N2011-29	tissue		T. cin 122e	
700	6	Unkno wn	NY BRONX	IHXX	448	581	1/13/2011	Dead	N2011-30	tissue		T. cin 121e	
701	6	Female	SAN ANTON	J11023	499	545	1/29/2011	Living	29	blood		T. cin 17e	
701	6	Female	SANDIEGOZ	813036	499	545	1/29/2011	Living	167	blood, feathers		T. cin 84e	
704	7	Male	BUSCH TAM	64876	595	639	4/21/2011	Living	75	blood, feathers	10/10/2013	T. cin 53e	
706	6	Male	HOUSTON	26356	441	538	4/27/2011	Living	40	blood		T. cin 26e	
707	6	Female	HOUSTON	26368	441	538	4/29/2011	Living	41	blood		T. cin 27e	
709	6	Male	SANDIEGOZ	411051	563	561	5/17/2011	Living	162	blood		T. cin 79e	
711	6	Female	HOUSTON	26494	441	538	6/5/2011	Living	43	blood		T. cin 29e	
717	6	Male	SAN ANTON	L11011	499	545	7/6/2011	Living	30	blood		T. cin 18e	
719	6	Male	PHILADELP	205825	558	594	7/21/2011	Living	None	feathers		T. cin 148e	
723	6	Male	HOUSTON	26589	373	569	7/25/2011	Living	42	blood		T. cin 28e	

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724	7	Female	NZP-CRC	216002	622	427	7/25/2011	Living	724	feathers in bags		T. cin 146e	
726	6	Female	SAN ANTON	G11022	499	545	8/16/2011	Living	28	blood		T. cin 16e	
728	7	Male	SANDIEGOZ	313852	622	427	8/20/2011	Living	145	blood		T. cin 74e	
737	7	Female	BUSCH TAM	65180	574	617	4/19/2012	Dead	78	blood, feathers	10/10/2013	T. cin 56e	
740	7	Male	BUSCH TAM	65191	595	639	4/30/2012	Living	77	blood, feathers	10/10/2013	T. cin 55e	
741	7	Male	SANDIEGOZ	812076	492	660	5/19/2012	Living	166	blood		T. cin 83e	
742	7	Female	SD-WAP	812080	492	660	5/21/2012	Living	165	blood		T. cin 82e	
743	7	Female	BUSCH TAM	65212	574	617	5/22/2012	Living	76	blood, feathers	10/10/2013	T. cin 54e	
744	6	Male	CHICAGOLP	22972	485	543	5/23/2012	Living	120	feathers in bags		T. cin 138e	
746	6	Unknown	CHICAGOLP	21962	485	543	5/24/2012	Dead	179, 180	tissue		T. cin 113e	
748	6	Male	NZP-WASH	216096	577	536	5/27/2012	Living	None	feathers in bags	2/18/2013	T. cin 142e	
750	6	Female	METROZOO	12B215	441	557	5/28/2012	Living	99	blood		T. cin 62e	
751	6	Female	METROZOO	12B216	441	557	5/28/2012	Living	101	blood		T. cin 64e	
752	7	Male	SAN ANTON	Y12071	499	680	5/28/2012	Living	35	blood		T. cin 23e	
753	6	Male	NZP-CRC	216085	536	577	5/28/2012	Living	753	feathers in bags		T. cin 144e	
756	6	Male	ST LOUIS	110838	349	447	6/16/2012	Dead	49	blood		T. cin 35e	
759	5	Male	DISNEY AK	120497	449	535	6/26/2012	Dead	tissue 759	tissue		T. cin 112e	
760	7	Male	BUSCH TAM	65273	574	617	6/29/2012	Living	80	blood, feathers	10/10/2013	T. cin 58e	
762	7	Female	BUSCH TAM	65290	574	617	7/14/2012	Living	96	blood, feathers	10/10/2013	T. cin 61e	
765	7	Female	SANDIEGOZ	812194	492	660	7/28/2012	Living	168	blood, feathers		T. cin 85e	
767	6	Male	METROZOO	12B366	441	557	8/13/2012	Living	48	blood		T. cin 34e	
768	6	Male	METROZOO	12B367	441	557	8/14/2012	Living	100	blood		T. cin 63e	
774	7	Female	SD-WAP	813001	492	660	1/9/2013	Living	164	blood		T. cin 81e	
782	7	Male	SD-WAP	813047	492	660	4/8/2013	Living	163	blood		T. cin 80e	
783	7	Female	SD-WAP	813049	492	660	4/9/2013	Living	146	blood		T. cin 75e	
793	7	Male	PHILADELP	205926	627	582	5/31/2013	Living	55	blood, feathers		T. cin 41e	
794	7	Male	PHILADELP	205927	627	582	6/1/2013	Living	54	blood, feathers		T. cin 40e	
802	7	Female	PHILADELP	205933	627	582	7/3/2013	Living	56	blood, feathers		T. cin 42e	
803	7	Female	SANDIEGOZ	813184	492	660	7/3/2013	Living	169	blood		T. cin 86e	
825	7	Female	SD-WAP	814138	709	701	6/29/2014	Dead	OR6203	DNA from SD	12/1/2014	T. cin 88e	

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???	???	Male	LSUMZ	B-57113	Unknown	Unknown	Unknown	Dead	B-57113	tissue		T. cin 168e	Two male birds died at HOUSTON on 12/21/1999 - SB#s 197 or 348
None	WILD	Female	MVZ Bird	123479	Wild	Wild	Unknown	Dead	123479	toe pad	5/4/1905	T. cin 181e	see Ornis search results for more details
None	WILD	Male	MVZ Bird	95154	Wild	Wild	Unknown	Dead	95154	toe pad	4/28/1905	T. cin 182e	see Ornis search results for more details
None	WILD	Male	MVZ Bird	95156	Wild	Wild	Unknown	Dead	95156	toe pad	4/28/1905	T. cin 183e	see Ornis search results for more details
None	WILD	Male	MVZ Bird	95157	Wild	Wild	Unknown	Dead	95157	toe pad	4/28/1905	T. cin 184e	see Ornis search results for more details
None	WILD	Male	MVZ Bird	95158	Wild	Wild	Unknown	Dead	95158	toe pad	4/28/1905	T. cin 185e	see Ornis search results for more details
None	WILD	Male	MVZ Bird	95159	Wild	Wild	Unknown	Dead	95159	toe pad	4/28/1905	T. cin 186e	see Ornis search results for more details
None	WILD	Female	MVZ Bird	95160	Wild	Wild	Unknown	Dead	95160	toe pad	4/28/1905	T. cin 187e	see Ornis search results for more details
None	WILD	Female	MVZ Bird	95161	Wild	Wild	Unknown	Dead	95161	toe pad	4/28/1905	T. cin 188e	see Ornis search results for more details
None	WILD	Female	MVZ Bird	95162	Wild	Wild	Unknown	Dead	95162	toe pad	4/28/1905	T. cin 189e	see Ornis search results for more details
None	WILD	Unknown	MVZ Bird	95155	Wild	Wild	Unknown	Dead	95155	toe pad	4/28/1905	T. cin 190e	see Ornis search results for more details
None	WILD	Male	USNM	377969	Wild	Wild	Unknown	Dead	377969	toe pad	6/6/1945	T. cin 191e	see Smithsonian results for more details on sample collection location
None	WILD	Male	USNM	377973	Wild	Wild	Unknown	Dead	377973	toe pad	3/8/1945	T. cin 192e	see Smithsonian results for more details on sample collection location
None	WILD	Male	USNM	377977	Wild	Wild	Unknown	Dead	377977	toe pad	6/4/1945	T. cin 193e	see Smithsonian results for more details on sample collection location

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None	WILD	Male	USNM	377978	Wild	Wild	Unknown	Dead	377978	toe pad	6/16/1945	T. cin 194e	see Smithsonian results for more details on sample collection location
None	WILD	Male	USNM	377980	Wild	Wild	Unknown	Dead	377980	toe pad	5/25/1945	T. cin 195e	see Smithsonian results for more details on sample collection location
None	WILD	Female	USNM	377981	Wild	Wild	Unknown	Dead	377981	toe pad	6/14/1945	T. cin 196e	see Smithsonian results for more details on sample collection location
None	WILD	Female	USNM	377984	Wild	Wild	Unknown	Dead	377984	toe pad	5/25/1945	T. cin 197e	see Smithsonian results for more details on sample collection location
None	WILD	Female	USNM	377985	Wild	Wild	Unknown	Dead	377985	toe pad	6/19/1945	T. cin 198e	see Smithsonian results for more details on sample collection location
None	WILD	Female	USNM	377987	Wild	Wild	Unknown	Dead	377987	toe pad	5/26/1945	T. cin 199e	see Smithsonian results for more details on sample collection location
None	WILD	Female	USNM	377990	Wild	Wild	Unknown	Dead	377990	toe pad	6/28/1945	T. cin 200e	see Smithsonian results for more details on sample collection location
None	WILD	Male	USNM	384866	Wild	Wild	Unknown	Dead	384866	toe pad	7/6/1945	T. cin 201e	see Smithsonian results for more details on sample collection location
None	WILD	Female	USNM	384870	Wild	Wild	Unknown	Dead	384870	toe pad	7/18/1945	T. cin 202e	see Smithsonian results for more details on sample collection location
None	WILD	Unknown	ANSP	21389	Wild	Wild	Unknown	Dead	21389	toe pad	Unknown	T. cin 203e	
None	WILD	Unknown	ANSP	21390	Wild	Wild	Unknown	Dead	21390	toe pad	Unknown	T. cin 204e	
None	WILD	Unknown	ANSP	21391	Wild	Wild	Unknown	Dead	21391	toe pad	Unknown	T. cin 205e	
None	WILD	Unknown	ANSP	21392	Wild	Wild	Unknown	Dead	21392	toe pad	Unknown	T. cin 206e	
None	WILD	Female	AMNH	332419	Wild	Wild	Unknown	Dead	332419	toe pad	8/11/1931	T. cin 207e	

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None	WILD	Female	AMNH	332420	Wild	Wild	Unknown	Dead	332420	toe pad	8/11/1931	T. cin 208e	