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Analysis of Mitochondrial DNA Structuring Between Colonies of the World's Smallest Penguin (Eudyptula minor) in New South Wales, Australia

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SIT Study Abroad

Analysis of mitochondrial DNA structuring between colonies of the world's smallest penguin (*Eudyptula minor*) in New South Wales, Australia

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ABSTRACT

The Little Penguin, *Eudyptula minor*, is a flightless seabird that is endemic to Australia and New Zealand. It can be found nesting on both on and offshore colonies along the coasts of both countries and it is the only penguin currently found breeding on mainland Australia. The IUCN Red List of Threatened Species lists *E. minor* as "Least Concerned," but numbers have noticeably dropped in recorded history due to a number of direct and indirect anthropogenic influences. One particular location of decline is Manly, New South Wales that contains the last onshore breeding colony of *E. minor* in NSW, Australia. In order to determine the most appropriate management strategy for the Manly colony as well as other New South Wales colonies, the mitochondrial genetic structuring was evaluated for the nine colonies that *E. minor* is known to breed on in New South Wales.

Statistically significant phylogenetic structuring was not observed in this study, but due to the low sample size these results cannot be definitively stated. There was evidence of genotypic similarities all along the coast of New South Wales, including the northernmost colony of Broughton Island and the southernmost colony of Montague Island. Theories surrounding the genetic homogeneity among the majority of the colonies include past or present gene flow or a recent founders event. The data analyzed in this study points towards the need to focus conservation efforts on all colonies in New South Wales and not just the Manly colony. By maintaining the health of offshore colonies, particularly those in close proximity to Manly, the chances of rebuilding the Manly population will increase.

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1. INTRODUCTION

1.1 Background Information

The Little Penguin (*Eudyptula minor*) is an endemic of Australia and New Zealand, the smallest of 18 penguin species (Sergent et. al 2004), and the only species of penguin currently found breeding on mainland Australia (Rogers 1995). They are found predominantly in temperate seas (Banks et. al 2008) of offshore islands along the coasts of New Zealand (Peucker et. al 2009) and extending along the Australian coastline from Port Stephens in New South Wales, south to Victoria, South Australia, and as far north as Fremantle in Western Australia (Sergent et. al 2004). Like all penguins, *E. minor* is a flightless seabird (Overeem et. al 2008) that uses land predominantly for breeding and its yearly molt (Peucker et. al 2009). Breeding colonies can occur in a variety of coastal habitats including beaches and rocky shores with anywhere from a few pairs to 15,000 individuals (ie Gabo and Tullaberga Islands; BirdLife International 2013). Individuals mature between 2-3 years of age and often maintain the same breeding partner throughout life (Stahel and Gales 1987), though extra-pair copulations and mate switching do occur (Billing et. al 2007). During breeding season, partners will take turns incubating their eggs/ protecting their chicks during the day while their partner forages at sea. Then, at dusk, the pair will exchange roles and the parent that was just at sea will incubate his/her eggs/ feed his/her chicks. This cycle continues for approximately 8 weeks until the chicks have fledged and leave the nests (Stahel and Gales 1987). The breeding seasons vary geographically and interannually, but usually takes place during the winter and spring with 1-2 clutches of 2 eggs laid per breeding season (Overeem et. al 2008). The low fecundity of the species, coupled with a high mortality rate of fledglings

(Overeem et. al 2008) leads to low numbers of successful offspring produced each season.

1.2 Population Genetics

Population genetics, the study of allele frequency distributions and changes due to evolutionary factors, has come to be an integral component in conservation biology (Avise 1995). Genetic subdivisions can lead to subspeciation (genetically and possibly phenotypically different species that can interbreed) and therefore different management strategies (Taylor & Dizon 1996). In sexually reproducing species, molecular markers provide an insight into the pedigree of the species by providing evidence of heterozygosity, gene flow, and genetic distinctiveness (Avise 1995). In contrast to the traditional use of banding to evaluate intercolony movements, molecular approaches have been found to be more effective for the following reasons: (1) data can be obtained from a greater number of colonies, (2) movement patterns can be approximated based on long term time scales and are therefore less likely to be biased by rare observations, and (3) genetic surveys have been found to be less debilitating than banding (Overeem et. al 2008).

1.3 Mitochondrial DNA

Within higher animal species, mitochondrial DNA (mtDNA) has come to be a heavily utilized source for phylogeographic structuring because of its relatively rapid evolutionary rate, about 10 times higher than that estimated for single-copy nuclear DNA (Brown et. al 1979), and its non-recombining mode of inheritance (Banks et. al 2008; Avise 1995) from the maternal lineage (Overeem et. al 2008). As a highly conserved and neutral marker (Avise et. al 1987), the control region of the mtDNA is ideal for

evaluating the past and present evolutionary lineage of individuals within a population and can form a bridge between systemics and population genetics (Avise et. al 1987).

1.4 Conservation/ Declining Population

While the IUCN Red List of Threatened Species currently lists *E. minor* as "Least Concerned" ("IUCN Red List" 2013), numbers have noticeably declined in recorded history (Sergent et. al 2004). Remains found in Aboriginal middens signify that *E. minor* colonies were far more extensive on the Australian mainland prior to European settlement (Rogers et. al 1995; Sergent et. al 2004). While the global population size has not been calculated, the Australian population is estimated as under 1,000,000 individuals ("IUCN Red List" 2013), with an estimated 25,000 pairs nesting on and off the coast of New South Wales (New South Wales Government 2011A). Both direct and indirect anthropogenic influences, namely the introduction of carnivores (e.g. foxes, dogs, rats, and cats) (Dann 1992) and habitat degradation (Sergent et. al 2004), have been acknowledged as major causes of low breeding success rate and high mortality rate for the species, leading to population decline (Overeem et. al 2008). Habitat degradation is specifically destructive to *E. minor* because due to the already fragmented habitats, further destruction can potentially drive a colony to extinction (Leidner and Haddad 2011) because it limits the number of immigrants entering the population. Genetic drift and inbreeding are major concerns in a small and isolated population because they often times lead to a loss of genetic variability. With a loss of genetic variability often comes a decline in fitness and adaptability, which affects the reproductive success rate and increases mortality within the population, thereby leading to an even smaller population where the cycle begins anew. This cycle of decreasing population size is known as the

"extinction vortex" (Frankham et. al 2004). Furthermore, overfishing of important prey species (e.g. schooling fish and krill, Dann 1992; Sergent et. al 2004), oil spills (e.g. Baron oil, Sergent et. al 2004; Dann 1992; Overeem et. al 2008), toxins in the water (Sergent et. al 2004), and plastic waste (Dann 1992) have negatively affected *E. minor* numbers.

While *E. minor* has very high dispersal potential (Peucker et. al 2009), particularly within the first year of fledging when the bird may travel hundreds of km from its natal colony (Stahel and Gales 1987), they are also generally philopatric (Overeem et. al 2008; Billing et. al 2007). A lack of migration amongst colonies is potentially alarming because without enough gene flow to continue bringing new alleles into the population, the colony may lack genetic variance to maintain heterozygous structuring (Overeem et. al 2008), which will stunt the evolutionary potential of the population.

1.5 Climate Change

It has been widely recognized that climatic changes is one of the biggest threats to biodiversity (Thomas et al. 2004). Changes in the geographical distribution and abundance have been observed in a wide variety of species since the dawn of the 20th century, and many more are expected in the near future (Fordham et al. 2013). *E. minor* is no exception to this worldwide trepidation as they are known to be unable to withstand temperatures above 35°C due to their heavy layers of insulated feathers used for spending extended periods of time in the water (Stahel and Gales 1987). New South Wales' *E. minor* is particularly at risk because it contains the northernmost colony of Broughton

Island. The penguins may have no choice but to move south to cooler waters due to the heat and the potential migration of their prey species.

1.6 Manly Colony

A secluded cove in Manly of Sydney's North Harbor is home to the only known remaining breeding colony of *E. minor* in New South Wales. Over a five-year monitoring program, this population that once numbered in the hundreds has decreased to an average of 54 breeding pairs in recent years due to a loss of suitable habitat from urbanization and expansion, attacks by introduced predators such as dogs and foxes, and disturbance of nesting sites. *E. minor* numbers in Manly are so low that this colony has been listed as an endangered population according to the Threatened Species Conservation Act and areas of the harbor have been declared "critical habitat" for the population (New South Wales Government 2011B). In 2000, a Recovery Plan was put into effect with the goal of saving the Manly colony and thereby removing its endangered listing. These plans have included mapping and annual monitoring of the population, educating the public about management threats to the colony, and ending commercial fishing in North Sydney Harbor, to name a few (NSW National Parks 2007). However, the conservation efforts being undertaken at Manly have been primarily focused on Manly, without much attention being given to the offshore colonies, including Lion Island approximately 30km away.

1.7 Aims

The aim of this study is to determine the mitochondrial genetic structuring and variation of individuals from nine colonies in New South Wales where *E. minor* is known to breed. In doing so, the null hypothesis that there is no genetic structuring between

colonies will be evaluated. This knowledge and understanding of mtDNA control region structuring will be able to assist researchers and conservation management experts in assessing the degree of genetic structuring/ mixing between colonies. Thereby, further evidence will indicate whether each individual colony of penguins should be given different management strategies, if all *E. minor* in New South Wales should be treated as a metapopulation (spatially separated populations of the same species that still can interact and interbreed), or a combination of the two. This is particularly important because as numbers are declining in NSW's last remaining onshore colony of Manly, conservation efforts may unwisely be exclusively focused there instead of spreading the efforts to local New South Wales offshore colonies. This publication will shed further light on the genetic variance of New South Wales colonies of *E. minor* through the sampling and mtDNA analysis of individuals from each of the 9 known NSW colonies.

2. MATERIALS AND METHODS

2.1 Sampling and DNA extraction

Genetic data was collected from 9 colonies in New South Wales, Australia. These colonies, from north to south, are Broughton Island (32.6158° S, 152.3172° E), Cabbage Tree Island (32.6817° S, 152.2344° E), Lion Island (33.5569° S, 151.3177° E), Manly (33.7962° S, 151.2827° E), Five Islands (34.4832°S 150.9330°E), Bowen Island (49.3833° N, 123.3833° W), Brush Island (35.52917°S 150.41667° E), Tollgate Islands (35.7485°S, 150.2679°E), and Montague Island (36.2500° S, 150.2167° E) (Figure 1; Table 1). Blood samples were taken from between 11 and 50 individuals per colony during the breeding seasons (early October through late December) of 2012 and 2013.

The blood was drawn using a standard method (Overeem et. al 2008; Ellegren 1996; Radford and Blakey 2000) in which between 50 and 100µL was taken from the foot. This sample was added to 1mL of Longmire's buffer and stored at room temperature until DNA extraction was performed.

Figure 1: Location of nine known *Eudyptula minor* colonies in New South Wales, Australia. Photo courtesy of Google Earth.

Table 1: Approximate distances (km) between nine known *Eudyptula minor* colonies in New South Wales, Australia

	Broughton Island	Cabbage Tree Island	Lion Island	Manly	Five Islands	Bowen Island	Brush Island	Tollgate Island	Montague Island
Broughton Island	0	11.6	143.56	169.06	249.06	318.86	362.22	399.53	457.26
Cabbage Tree Island	11.6	0	127.29	151.74	233.83	303.73	347.55	383.66	432.51
Lion Island	143.56	127.29	0	28.4	110.35	180.73	234.15	262.35	314.54
Manly	169.06	151.74	28.4	0	82.71	152.67	206.73	234.19	288.03
Five Islands	249.06	233.83	110.35	82.71	0	72.64	125.82	151.98	207.12
Bowen Island	318.86	303.73	180.73	152.67	72.64	0	55.06	82.8	136.84
Brush Island	362.22	347.55	234.15	206.73	125.82	55.06	0	28.47	85.43
Tollgate Island	399.53	383.66	262.35	234.19	151.98	82.8	28.47	0	56.05
Montague Island	457.26	432.51	314.54	288.03	207.12	136.84	85.43	56.05	0

2.2 Mitochondrial DNA

In order to test the phylogeographic structuring of the colonies sampled, a ~600bp fragments of the mtDNA control region (Roeder 2002) was sequenced from between 2 and 4 individuals per colony.

2.2A Extraction

The mtDNA from *E. minor* was extracted using the standard procedure for the Qiagen DNEasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) with two modifications. The first was that in the initial step of the procedure, 100µL of blood and Longmire's buffer were mixed with 20µL of proteinase K and 100µL of PBS instead of utilizing pure blood because the blood had already been stored in the buffer from sampling. The second was that 100µL of buffer AE was used for the elution steps instead of 200µL in order to increase the final DNA concentration in the eluate.

2.2B Nanodrop

2μL of the extracted DNA was placed on a NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific, Scoresby Vic) to test the purity and concentration of the DNA. Those containing concentrations above 20 ng/μL were selected for amplification in order to limit the amount of dilution necessary prior to the amplification step. Additionally, the 260/280 value (absorbance at 260 and 280nm) was taken into consideration to assess DNA purity, with an ideal ratio being approximately 1.8nm. The 260/230 value was also used as a second measure of DNA purity, with an idea ration being between 2.0 and 2.2nm.

2.2C Amplification

Mitochondrial DNA was amplified through a Polymerase Chain Reaction (PCR) using the Qiagen *Taq* PCR core kit with the following ratio of reagents: 2μL of Q solution; 1μL of Qiagen 10x buffer CL (containing gel loading buffer); 0.2μL of dNTP; 1μL of the forward primer 'L-tRNAglu,' 1μL of the reverse primer 'H-Dbox' (Roeder 2002), 2μM each; 0.1μL of Taq polymerase; 3.7μL of water; and 1μl of DNA per individual. This mixture was then placed in the Eppendorf Thermocycler for 4 minutes at 94°C, before beginning 40 cycles of 10 seconds at 94°C, 10 seconds at 55°C, and 35 seconds at 72°C. After the 40 cycles were completed, the mixture stood at 72°C for 5 minutes before cooling to 4°C.

2.2D Gel Electrophoresis

In order to verify the length of the mtDNA control region that was amplified in the PCR, a 1.5% agarose gel was prepared using 0.3g of agarose, 20mL of 1x TBE, and 2μL of Gel-Red staining solution. 3μL of PCR product containing Gel Loading buffer

was loaded into each well and run against the GelPilot 100bp ladder (Qiagen, Valencia, CA, USA) for approximately 25 minutes at 100V.

2.2E Product Cleanup

In order to prepare the amplified product for sequencing by removing excess primers and nucleotides, ExoSAP-IT reagent and the procedure outlined USB ExoSAP-IT PCR Product Cleanup was utilized (Affymetrix, Santa Clara, CA, USA).

2.2F Sanger Sequencing, Ethanol Cleanup, and Sequencing

The mtDNA was sequenced by dye termination method Sanger Sequencing based on BigDye Terminator v3.1 chemistry (Life Technologies Corporation, Grand Island, NY, USA), in which each of the four dideoxynucleotide (ddNTPs) chain terminators is labeled with a different fluorescent dye, which emits different wavelengths of light to stop the sequencing when it is incorporated into the DNA during the sequencing reaction. The procedure was in concordance with *The Ramaciotti Centre for Gene Function Analysis*'s 1.5 mL tube Clean Up Procedure. In doing this, the product was ready for sequencing, which also took place at *The Ramaciotti Centre*. Only 'H-Dbox' reverse primer was utilized for the sequencing due to the length heteroplasmy at both ends of the fragment (Overeem et al 2008).

2.2G Sequence Verification

The program *Geneious* (Geneious® 6.1.6, Biomatters development team) was used to analyze and edit the sequences returned from *The Ramaciotti Centre*. The BLAST feature was utilized to ensure that the sequences did indeed code for the control region of the mtDNA.

2.2H Sequence analysis

To analyze the obtained sequences, the raw data loaded into the Geneious® software were aligned using the ClustalW function for multiple sequence alignment. To illustrate which mitochondrial haplotypes occur in which population, the software DNASP v. 5.10.01 (Librado & Rozas 2009) was used to generate a haplotype data file. This file was then used to identify the population affiliation of the individual haplotypes that were displayed in a haplotype network, which was generated using the software NETWORK version 4.6.0.0 (Polzin & Daneshmand 2011). Additionally, population differentiation was tested using the generated haplotypes for analysis in Arlequin version 3.1.5.2 (Excoffier & Schneider 2005).

2.2I Statistical testing

An F_{ST} statistical test was run to assess the variance of genetic markers for the colonies with 2 or more individuals' DNA sequenced and analyzed. The product of this test is an F_{ST} value with a corresponding p-value. An F_{ST} value of 0 shows no dissimilarity, which points towards individuals from different colonies interbreeding freely. An F_{ST} value of 1 shows complete dissimilarity, which points towards a lack of genetic mixing between colonies. Negative F_{ST} values have been obtained due to the small sample size, of which the calculations correct for sampling bias. All negative values will be assumed to be 0 for analysis purposes. The p-values indicate whether the F_{ST} values are significant, with values <0.05 being significant.

3. RESULTS

Among the 16 individuals analyzed from 6 New South Wales colonies, 7 haplotypes and 10 polymorphic nucleotides were found within 288bp of the

mitochondrial control region sequence. The 288bp were used instead of 410bp in order to include all 16 samples, because some of the viable regions of the individual sequences were shorter than others. The most common haplotype was found in individuals from Broughton, Cabbage Tree, Lion, Bowen, and Montague Islands, which extend all along the coast of New South Wales (Figure 2). The greatest divergences amongst individuals of the same colony were seen in Cabbage Tree Island (3-4 nucleotides), Brush Island (6 nucleotides) and Bowen Island (7 nucleotides), with all remaining colonies having no more than 1 nucleotide difference amongst individuals. No DNA from Manly, Five Islands, or Tollgate Islands could be sequenced successfully.

The F_{ST} statistical testing (Table 2) showed no statistically significant results, likely due to the small sample size. Despite the lack of statistically significance, the comparison of the genetic structuring of Montague and Brush Islands yielded the highest F_{ST} (0.512) and lowest p-values (0.063). Additionally, the comparison of genetic structuring of Montague and Cabbage Tree Islands yielded a notably high F_{ST} value (0.244) and low p-value (0.108).

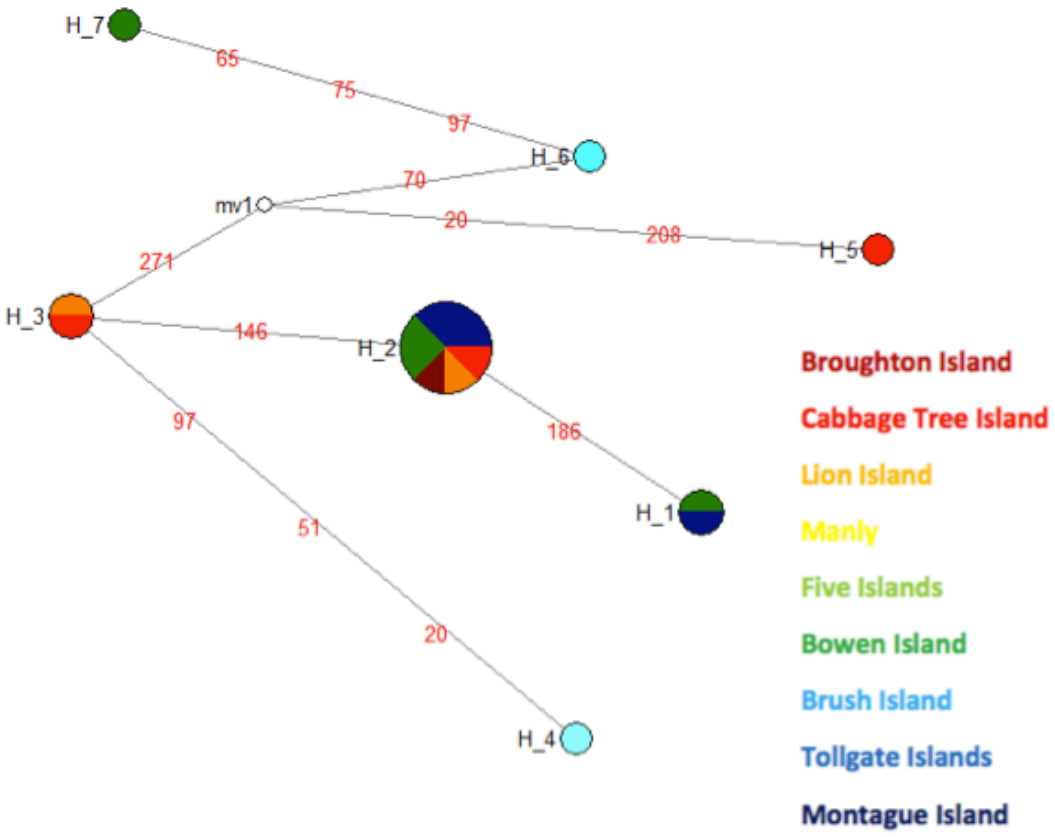

Figure 2: Haplotype map of the 16 *Eudyptula minor* individuals from 6 colonies (Manly, Five Islands, and Tollgate Islands not included).

Table 2: F_{ST} and p-values comparing the 5 colonies with 2 or more individuals' mtDNA sequenced and analyzed. F_{ST} values are on the lower diagonal and corresponding p-values are on the upper diagonal.

	Cabbage Tree Island	**Lion Island**	**Bowen Island**	**Brush Island**	**Montague Island**
Cabbage Tree Island	*	0.991	0.991	0.793	0.108
Lion Island	-0.326	*	0.991	0.991	0.640
Bowen Island	-0.1	-0.274	*	0.703	0.486
Brush Island	-0.134	0	-0.152	*	0.063
Montague Island	0.244	0.111	0.019	0.512	*

4. DISCUSSION

4.1 Genotype Mapping

Because none of the F_{ST} values calculated showed statistically significant structuring, the connectivity of the individual colonies cannot be determined. However, based on the genotype map it is evident that there are genotypic similarities all along the coast of New South Wales. The most commonly seen haplotype is evident in 5 of the 6 colonies sampled, including the most northern colony of Broughton Island and the most southern of Montague Island. This evidence combined with all p values being greater than 0.05 and the prominence of F_{ST} values <0.15 (8 out of the 10 comparisons) points towards panmixis between the colonies.

Conversely, the relatively high F_{ST} value comparing Cabbage Tree Island and Montague Island indicates the likelihood that distance may play a role in genetic structuring between colonies. These two islands are 432.51km apart, making it unlikely that individuals between the two colonies would frequently travel that great distance to breed because *E. minor* species has been found to be highly philopatric (Overeem et. al 2008). However, based on the limited data presented above, there is no evidence of genetic structuring between Broughton and Montague Islands, which are 457.26km apart.

It should be noted that the F_{ST} value comparing Brush and Lion Islands was the only one calculated to be 0. This should point towards no dissimilarities in the genetic structuring between the colonies, but by looking at the haplotype map (Figure 2) one can see that Brush Island represents haplotypes 4 and 6 while Lion Island represents haplotypes 2 and 3. It is possible that Brush Island and Lion Island individuals have a similar evolutionary lineage and that the Brush Island divergences can be linked to those

of Lion Island. However, with such a small sample size definitive results are difficult to conclude.

Tentatively and based on the data presented above, there does not appear to be genetic structuring between the individual colonies of *E. minor* in New South Wales, based on the mtDNA analysis. This evidence indicates either past or present gene flow between the colonies, which though not commonly seen, has been observed in fledglings moving to non-natal colonies to breed (Overeem et. al 2008). Previous research based on the mtDNA control region and microsatellite genes (Overeem et. al 2008; Banks et. al 2008) has found two deep and well-supported lineages of *E. minor*: one consisting of Australia and Otago, New Zealand and the other consisting of the rest of New Zealand. The lack of phylogeographic structuring of eastern Australian colonies is consistent with the previous research that identifies the Australian clade as having similar ancestry (Overeem et. al 2008; Banks et. al 2008; Peucker et. al 2009). However, further data with larger sample sizes that also incorporate Manly, Five Islands, and Tollgate Islands would be needed to state definitively the link between populations.

4.2 Methods Validity

Genetic research into New South Wales colonies of *E. minor* is still in a relatively early stage of development, as compared to the more extensive research that has been done in Western Australia, South Australia, and Victoria. In order to truly be able to understand the structuring and viability of the population, the already well established methods will need to become more widely applied in order to have a set system to compare data overtime.

4.2A Banding vs. Invasive and Noninvasive Genetics

Traditionally, flipper banding was utilized to monitor inter-colony movements (Overeem et al. 2008). However, in the field of penguin research this technique has been largely disbanded because of the concern that banded birds may have reduced survival rate as compared to genetically analyzed individuals (NSW National Parks 2007). Since penguins need to use their flippers to propel through the water, banding has the potential to interfere with locomotion and hence foraging, with data suggesting that banded pengins expend 24% more energy than nonbanded individuals. Another possibility is that the bands attract predators because they act as "flashers" (Froget et al. 1998). A study performed on 383 breeding and banded king penguins in 1998 found 67.5% of banded birds that should have started breeding by late November, did not do so until January possibly due to the bands slowing their progress in returning to their colonies. Additionally, 15% less banded birds returned to their colony at Possession Island, Crozet Archipelago than nonbanded birds (Froget et al. 1998).

Conversely, while noninvasive genetic analysis does not require researchers to handle their observed species by utilizing feathers, feces, or hairs for genetic sampling, this often yields low DNA quantity or quality (Taberlet et al. 1999). Additionally, the laboratory cost of avoiding genotyping errors from non-invasive sampling can be 10-20 times higher than if the samples were extracted from blood or tissue (Taberlet and Waits 1998). However, in the field of penguin research is as become the common practice to draw a relatively small amount of blood before readily releasing the animal. The DNA from the nucleated red blood cells can be readily extracted, allowing researchers to understand the past and present reproductive relationships among individuals and populations. This is particularly useful for small or geographically isolated populations,

where gene flow is necessary to prevent inbreeding depression, phenotypic variability, or genetic viability (Beissinger and McCullough 2002).

4.2B Mitochondrial DNA analysis

Experts argue for both the favorability of using mtDNA to assess phylogeographics (Avise 1995) and the limitations of utilizing such a small part of the genome that may not reflect the overall evolutionary development and diversification of the taxa (Cronin 1993). However, mtDNA's rapid pace of nucleotide substitution, its nonrecombining mode of maternal inheritance, and the fact that it is readily accessible has come to bridge a gap between taxonomy and population genetics (Avise et al. 1987). mtDNA has been used to evaluate a great number of species' genetic structures, including the leatherback turtle (Dutton et al. 2013), Mytilus coruscus Gould (Li et al. 2013), and even humans (Martínez-Cortés et al. 2013), to name a few.

In a larger study into the genetic structuring of *E. minor*, other genetic markers such as MHC genes, single-nucleotide polymorphisms (SNPs), and microsatellite markers would have been utilized to paint a complete picture of the individuals' genetic makeups. However, this information is not covered in the realms of this study.

4.3 Linking Genetics and Demography

The necessity to utilize both genetic and demographic methods (Avise 1995) such as mark-recapture and burrow occupancy for conservation cannot be overstated, as both are vital pieces of a puzzle to determine the best possible management strategy for *E. minor*. If two subpopulations are geographically and genetically separated, it is logical and in the best interest of the species to evaluate and treat each as separate entities, including employing different conservation strategies if necessary, in order to potentially

improve conservation outcomes. However, it is unsuitable to combine assumed populations based exclusively on genetic variability or geographic isolation (Taylor and Dizon 1996). In this delicate balance that needs to be established, pooling subpopulations can lead to under protection and splitting subpopulations can lead to over protection (Taylor & Dizon 1996), the resources of which could be utilized elsewhere.

4.4 *E. minor* Worldwide

While the data collected and analyzed in this study suggest a lack of genetic structuring throughout New South Wales, previous research has found two deep and well-supported lineages within the *E. minor* species (Overeem et. al 2008; Banks et. al 2008; Peucker et. al 2009). The first consists of Australia, including Western Australia, and Otago in southern New Zealand (Overeem et. al 2008; Banks et. al 2008; Peucker et. al 2009). Until recently, there was no the genetic structuring between individuals in Western Australia and Victoria (Overeem et. al 2008) was unknown, however a recent study conducted by Sinclair et. al (Unpublished) found significant population structuring in Western Australia. The Perth metropolitan population, which is found at the edge of *E. minor*'s distribution, was found to be genetically divergent from populations located near the center of *E. minor*'s distribution in Western Australia (Sinclair et. al Unpublished).

Based on Sinclair et. al's (Unpublished) findings of genetic divergence in Western Australia as well as the previous studies indicating that there are two clades in Australia and New Zealand (Overeem et. al 2008; Banks et. al 2008; Peucker et. al 2009), it is obvious that the entire population of *E. minor* cannot be classified as one large metapopulation. Therefore, each individual state/country will need o devise management

strategies while keeping in mid that they may be dealing with a separate entity than their neighboring state/country.

4.5 *E. minor* in eastern and southern Australia

One common theory to explain the lack of phylogeographic structuring of *E. minor* in southern and eastern Australia (Overeem et. al 2008; Banks et. al 2008; Peucker et. al 2009) can be paralleled to research done on the ecologically similar Short-Tailed Shearwaters (*Ardenna tenuirostris*). *A. tenuirostris* is a burrowing, colonial-nesting seabird in which both parents care for their young and it is often found living sympatrically with *E. minor* (Peucker et. al 2009). The lack of genetic structuring seen in the highly philopatric *A. tenuirostris* has been explained by a bottleneck event that took place relatively recently (10,000 years ago), followed by founder events involving large numbers of individuals that expanded their range (Overeem et. al 2008; Peucker et. al 2009). A similar scenario could account for the lack of geographic structuring of *E. minor* throughout eastern and southern Australia (Overeem et. al 2008; Banks et. al 2008).

4.6 Future Research

A great deal of research is still required to gain a full understanding of the population dynamics of *E. minor*, particularly in New South Wales. Mitochondrial DNA analysis needs to be coupled with the analysis of different genetic markers such as MHC and microsatellite loci to identify polymorphisms. These analyses need to be compared with those in Western Australia, South Australia, Victoria, and New Zealand, as well as analyses of demographics between colonies. In doing so, a more complete picture of E.

minor population structuring and genetics can be painted in order to definitively determine the best management strategies for the species as a whole.

4.5 Conservation Implications

The genetic data acquired from this study tentatively suggests that there is no statistically significant genetic divergence between New South Wales *E. minor* colonies. However, without a much larger sample size, this cannot be definitively stated. If this is the case, then management experts in New South Wales will be able treat the metapopulation as a single entity and focus management resources on all individuals within New South Wales, extending from Broughton Island to Montague Island. While this does not necessarily mean the even distribution of funds between each individual island, it does take into consideration that maintaining the health of one colony will also help maintain the health of surrounding colonies. This is particularly important when taking into consideration the Endangered Colony of Manly, which is threatened with low numbers due to anthropogenic influences. It is necessary to monitor the health of local offshore colonies such as Lion Island and Five Islands to make sure that these populations don't subsequently decline and hopefully some individuals migrate to the Manly colony. With the knowledge that there are at least 2 genetically different clades of *E. minor* that currently exist (Australia and Otago, and New Zealand), in addition to the sub-structuring found in Western Australia (Sinclair et. al Unpublished), then each individual clade needs to be treated with separate management strategies. The final goal will be to rebuild the colony of Manly and maintain the population size of *E. minor* throughout the remaining NSW colonies, as well as worldwide.

REFERENCES

Avise, J. C. (1995). Mitochondrial DNA Polymorphism and a Connection Between Genetics and Demography Relevance to Conservation. *Conservation Biology*, *9*(3), 686–690.

Avisel, J. C., Arnold, J., Ball, R. M., Lamb, T., Neigell, J. E., Carol, A., & Saunders, N. C. (1987). Intraspecific Phylogeography: The MitochondrialDNA Bridge Between Population Genetics and Systematic. *Annual Review of Ecology and Systematics*, *18*, 489–522.

Banks, J., Cruickshank, R. H., Drayton, G. M., & Paterson, A. M. (2008). Few genetic differences between Victorian and Western Australian blue penguins , Eudyptula minor. *New Zealand Journal of Zoology*, *35*, 265–270.

Beissinger, S., & McCullough, D. (2002). *Population Viability Analysis*. Chicago, IL.

Billing, T. M., Guay, P.-J., Peucker, a. J., & Mulder, R. a. (2007). Isolation and characterization of polymorphic microsatellite loci for the study of paternity and population structure in the little penguin Eudyptula minor. *Molecular Ecology Notes*, *7*(3), 425–427.

BirdLife International (2013) Important Bird Areas factsheet: Gabo and Tullaberga Islands. Downloaded from http://www.birdlife.org on 02/12/2013

Brown, W. M., George, M., & Wilson, a C. (1979). Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Sciences of the United States of America*, *76*(4), 1967–71.

Cronin, M. A. (1993). In My Experience: Mitochondrial DNA in Wildlife Taxonomy and Conservation Biology: Cautionary Notes. *Wildlife Society Bulletin*.

Dann, P. (1992). Distribution , Population Trends and Factors Influencing the Population Size of Little Penguins Eudyptula minor on Phillip Island , Victoria. *EMU*, *91*, 263–272.

Dutton, P. H., Roden, S. E., Stewart, K. R., LaCasella, E., Tiwari, M., Formia, A., … Allman, P. (2013). Population stock structure of leatherback turtles (Dermochelys coriacea) in the Atlantic revealed using mtDNA and microsatellite markers. *Conservation Genetics*, *14*(3), 625–636.

Ellegren H (1996) First gene on avian W chromosome (CHD) provides a tag for universal sexing of non-ratite birds. P Roy Soc B-Biol Sci 263:1635–1641.

Eucker, A. M. J. P., Ann, P. E. D., & Urridge, C. H. P. B. (2009). Range-wide phylogeography of the Little Penguin (Eudyptula minor): Evidence of long-distance dispersal. *The Auk*, *126*(2), 397–408.

Fordham, D. a., Akçakaya, H. R., Araújo, M. B., Keith, D. a., & Brook, B. W. (2013). Tools for integrating range change, extinction risk and climate change information into conservation management. *Ecography*, *36*(9), 956–964.

Frankham, R., Ballou, J. D., & Briscoe, D. A. (2004). *A Primer of Conservation Genetics* (p. 94). Cambridge.

Froget, G., Gautier-Clerc, M., Le Maho, Y., & Handrich, Y. (1998). Is Penguin banding harmless? *Polar Biology*, *20*(6), 409–413.

IUCN Red List of Threatened Species. (2013). Eudyptula minor.

Leidner, A. K., & Haddad, N. M. (2011). Combining measures of dispersal to identify conservation strategies in fragmented landscapes. *Conservation biology : the journal of the Society for Conservation Biology*, *25*(5), 1022–31.

Li, J., Ye, Y., Wu, C., Qi, P., Guo, B., & Chen, Y. (2013). Genetic variation of Mytilus coruscus Gould (Bivalvia: Mytilidae) populations in the East China Sea inferred from mtDNA COI gene Sequence. *Biochemical Systematics and Ecology*, *50*, 30–38.

Martínez-Cortés, G., Salazar-Flores, J., Haro-Guerrero, J., Rubi-Castellanos, R., Velarde-Félix, J., Muñoz-Valle, J., … Rangel-Villalobos, H. (2013). Maternal admixture and population structure in Mexican-Mestizos based on mtDNA haplogroups. *American Journal of Physical Anthropology*, *151*(4), 526–537.

New South Wales Government Environment & Heritage (2011) Little penguin. Downloaded from http://www.environment.nsw.gov.au on 05/12/2013

New South Wales Government Environment & Heritage (2011) Little penguin population in Sydney North Harbor. Downloaded from http://www.environment.nsw.gov.au on 05/12/2013

NSW National Parks and Wildlife Authority. (2007). Status of the Endangered Population of Little Penguins Eudyptula minor at Manly Little Penguin Recovery Team October 2007. *Department of Enviornment & Climate Change NSW*, 1–17.

Overeem, R. L., Peucker (nee Mitchelson), A. J., Austin, C. M., Dann, P., & Burridge, C. P. (2008). Contrasting genetic structuring between colonies of the World's smallest penguin, Eudyptula minor (Aves: Spheniscidae). *Conservation Genetics*, *9*(4), 893–905.

Peucker, A. J., Dann, P., & Burridge, C. P. (2009). Range-wide pylogeography of the Little Penguin (Eudyptula minor): evidence of long-distance dispersal. *The Auk*, *126*(2).

Radford AN, Blakey JK (2000) Is variation in brood sex ratios adaptive in the great tit (Parus major)? Behav Ecol 11:294–298

Rodgers, T., Eldershaw, G., & Walraven, E. (1995). Reproductive Success of Little Penguins , Eudyphla minor , on Lion Island , New South Wales. *Wildlife Restoration*, *22*(709-715).

Roeder, A. D., Ritchie, P. A., & Lambert, D. M. (2002). New DNA markers for penguins. *Conservation Genetics*, *3*, 341–344.

Roth, T., & Amrhein, V. (2010). Estimating individual survival using territory occupancy data on unmarked animals. *Journal of Applied Ecology*, *47*(2), 386–392.

Sergent, N., Rogers, T., & Cunningham, M. (2004). Influence of biological and ecological factors on hematological values in wild Little Penguins, Eudyptula minor. *Comparative biochemistry and physiology*, *138*(3), 333–9.

Sinclair, J., Cannell, B., Bradley, S., Wooller, R., & Sherwin, W. (2013). Unexpected patterns of gene-flow at the range-edge highlight significance of peripheral populations of the world's smallest penguin.

Stahel, C., & Gales, R. (1987). *Little Penguin: Fairy Penguins in Australia* (pp. 7–109). Kensington, NSW.

Taberlet, P., Waits, L., & Luikart, G. (1999). Noninvasive genetic sampling: look before you leap. *Trends in ecology & evolution*, *14*(8), 323–327.

Taberlet, P., & Waits, L. P. (1998). Non-invasive genetic sampling. *Tree*, *13*(1), 26–27.

Taylor, B. L., & Dizon, A. E. (1996). The Need to Estimate Power to Link Genetics and Demography for Conservation. *Conservation Biology*, *10*(2), 661–664.
Thomas CD, Cameron A, Green RE, Bakkenes M, Beaumont LJ, Collingham YC, Erasmus BFN, De Siqueira MF, Grainger A, Hannah L (2004) Extinction risk from climate change. Nature, 427, 145-148.