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Population genetics and mating system of Antarctic fur seals, Arctocephalus gazella, at Livingston Island, Antarctica

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Population genetics and mating system of Antarctic fur seals, *Arctocephalus gazella*, at Livingston Island, Antarctica

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Marine Biology

by

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2012

The Dissertation of Carolina Aimoré Bonin is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

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2012

DEDICATION

This thesis is dedicated to my family.

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ACKNOWLEDGEMENTS

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Chapter 3, in full, is being prepared for submission: Bonin, C., M. Goebel, G. O'Corry-Crowe and R. Burton. Rematings are rare among Antarctic fur seals (*Arctocephalus gazella*) despite high levels of site fidelity and polygyny. The dissertation author was the primary investigator and author of this paper.

Chapter 4, in full, was published in the Journal of Experimental Marine Biology and Ecology: Bonin, C., M. Goebel, G. O'Corry-Crowe and R. Burton. 2012. Twins or not? Genetic analysis of putative twins in Antarctic fur seals, *Arctocephalus gazella*, on the South Shetland Islands. Journal of Experimental Marine Biology and Ecology 412:13-19. The dissertation author was the primary investigator and author of this paper.

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Population genetics
Vertebrate mating systems
Animal Behavior
Molecular ecology

ABSTRACT OF THE DISSERTATION

Population genetics and mating system of Antarctic fur seals, *Arctocephalus gazella*, at Livingston Island, Antarctica

by

Carolina Aimoré Bonin

Doctor of Philosophy in Marine Biology

University of California, San Diego, 2012

Professor Ronald Burton, Chair

Antarctic fur seals, *Arctocephalus gazella*, were hunted to near-extinction in the early 1800's, but have recovered during the past 70 years to re-colonize most of their historical range. The large South Georgia (SG) fur seal population has been considered the main source of immigrants that re-colonized other areas, including Livingston Island (LI). Despite being one of the most exploited marine mammal species, clear evidence for a genetic bottleneck is lacking and instead, exceptionally high genetic diversity has been detected. Nevertheless, little is known about population-level patterns of genetic structure, or how this species' polygynous mating system may influence such patterns. This thesis fills some of this knowledge gap via extensive efforts in the field and in the laboratory, where over 1,000 individual samples were processed to obtain data on 17 highly polymorphic microsatellite markers; of these, 365 were also sequenced for mtDNA hypervariable region 1. The results of this work uncovered: (i) unexpected genetic differentiation between SG and LI indicating that LI was re-colonized by

immigrants from one or more sources in addition to SG, (ii) remarkably high male reproductive success at a low-density LI colony during four breeding seasons, suggesting reduced competition among males at LI relative to the high-density colony of Bird Island (SG) (iii) a low percentage of rematings among individuals over the course of a decade, which was surprising considering the high level of breeding site fidelity and male reproductive skew found in this species, and (iv) a case of multiple-paternity in Antarctic fur seals among twins, showing that females may often escape control of territorial males within a breeding period. Not only do these findings provide unique insights into the remarkable re-colonization of Antarctic fur seals, but they also emphasize the importance of satellite populations for harboring genetic diversity through a period of profound anthropogenic disturbance. Additionally, by revealing complexities within male and female breeding behavior, this work advances our overall understanding of polygyny, providing insight into how it might function under different population densities and how individuals may interact over the course of their lives within this mating system.

INTRODUCTION

INTRODUCTION

The Antarctic fur seal

The Antarctic fur seal (*Arctocephalus gazella*, Peters, 1875), also formerly known as Kerguelen fur seal, is a southern member of the eared seal family (Otariidae), and is circumpolar in distribution. Breeding populations are found on islands typically south of the Antarctic Polar Front, near South America (South Georgia, South Sandwich, South Orkney and the South Shetland Islands), Africa (Bouvet, Prince Edward, Crozet, Kerguelen, Amsterdam and Heard Islands) and Australia (Macquarie Islands; Hofmeyr et al., 2006; Figure 0-1). South Georgia possibly holds over 4 million seals, corresponding to 95% of the global *A. gazella* population, and Bouvet is the second largest with 66,000 animals (Hofmeyr et al., 2005). The South Shetland Islands comprise the third most populous region, with nearly 21,000 seals (Hucke-Gaete et al., 2004). Breeding colonies are much smaller at all other areas (Hofmeyr et al., 2005).

Thanks to the establishment of research stations near some of the largest breeding colonies, the diet and life history of Antarctic fur seals have been fairly well studied. Fur seals feed primarily on Antarctic krill, *Euphausia superba*, within the Atlantic sector of the Southern ocean (e.g. South Georgia and South Shetland Islands; Reid and Arnould, 1996; Osman et al., 2004), while fishes are predominant in the diet for seal populations breeding in the southern Indian Ocean (e.g. Heard Island; Green et al., 1989). At the South Shetlands, myctophid fishes (*Gymnoscopelus nicholsi*, *Electrona antarctica* and *Electrona carlsbergi*) are of secondary dietary importance to krill, followed by squids and penguins (Osman et al., 2004). Antarctic fur seals are extremely sexually dimorphic with bulls being four times heavier than females (Forcada and Staniland, 2009; Figure 0-2). Their reproductive cycle

typically lasts one year; males and females meet to breed at the beginning of the austral summer. Male fur seals arrive on traditional breeding beaches in early November and fight to establish their territories. Females arrive a few weeks after males and usually give birth to a single pup within a few days of arrival, becoming receptive to breeding with males after the perinatal period, which lasts 5-8 days (Doidge et al., 1986; Lunn & Boyd, 1991). After this period, Antarctic fur mothers typically alternate 2-7 days foraging at sea with 1-2 days of nursing ashore during the 4-month lactation period (Doidge et al., 1986). Female fur seals first give birth between 3 and 6 years of age, and reproduction reaches a peak at 7-9 years. Female reproductive performance increases with age and experience until 11 years, and subsequently declines with increasing age (Lunn et al., 1994). Males reach sexual maturity between 3-4 years of age, but usually do not breed until 7-8 years old. Males have much shorter life expectancy than females, which can live up to 20 years of age (Forcada & Staniland, 2009).

The exploitation and recovery of Antarctic fur seals

Although the Antarctic seems remote to humans relative to the rest of the world, it has been the stage of intense exploitation for the past 200 years. Seals were targeted in the 18th and 19th centuries, large whales at the beginning of the 20th century, and several fisheries were subsequently established, including the Antarctic krill fisheries in the late 1960's (Mori and Butterworth, 2006). Two species of fur seals (*Arctocephalus gazella* and *A. tropicalis*), and the southern elephant seal (*Mirounga leonina*) were specifically targeted for exploitation. Fur seal pelts were used to produce valuable clothing and hats that were sold mostly in British and Chinese markets (Hucke-Gaete et al., 2004). From elephant seal blubber, high-quality oil was extracted and used in industry (Kock, 2007). Nearly one million elephant seals where

taken at South Georgia (Laws, 1960); yet, their harvesting was ancillary to Antarctic fur seals (Kock, 2007).

Sealing activities consistently occurred at several Antarctic and sub-Antarctic islands from approximately 1786 until 1825 (Figure 0-3), when Antarctic fur seals were considered near-extinct throughout most of their historical distribution (Bonner, 1968; Laws, 1973; McCann and Doidge, 1987). At South Georgia alone 1.2 million pelts had been taken by 1822 and at the South Shetland Islands, towards the southern limit of the species distribution, another 250,000 seals had been harvested only three years after the Islands were first discovered (McCann and Doidge, 1987).

Despite intense exploitation, Antarctic fur seals re-colonized most of their range. These recoveries were well documented at South Georgia and South Shetland Islands, two of the best studied breeding colonies of this species. At South Georgia, the population recovery did not begin until the 1940's (Bonner, 1968) and at South Shetland Islands, fur seals were only again sighted in small numbers during the summer of 1958-59 (O'Gorman, 1961). During the past 70 years, fur seal populations have continued to grow, reaching a global population of several million (Forcada and Staniland, 2009). This recovery constitutes one of the most dramatic recovery events ever documented for a marine mammal (Laws, 1985) and has been loosely associated with an excess biomass of Antarctic krill that resulted from the predatory release of killing nearly 2 million large whales some decades earlier (Laws, 1977; Laws, 1985). Whaling released *ca*. 150 million tones of krill from annual large whale consumption, which is thought to have benefited other species, a hypothesis known as the "Krill Surplus Hypothesis" (see Laws, 1977). This hypothesis has faced recent controversy because pre- and post-whaling population sizes for the relatively small minke whale (*Balaenoptera bonaerensis*), which were thought to have benefitted from the "krill surplus", were actually found to be similar (Ruegg et al., 2010). Regardless of this controversy, it is

undeniable that the re-colonization success of Antarctic fur seals was largely due to conservation actions initiated in the mid- 1960's (Hucke-Gaete et al., 2004), including the establishment of the Antarctic Treaty (AT) and listing of Antarctic fur seals as a "Specially Protected Species" under the AT in 1964.

Successive actions and treaties involving multiple signatory nations mark the contemporary history of Antarctic living resources conservation and management. With the goal of regulating marine mammal harvests, the International Whaling Convention (1948) and the Convention for the Conservation of Antarctic Seals (CCAS; 1978) were established. These initial steps gave way to a more holistic conservation-management paradigm, coined by the Convention for the Conservation of Antarctic Marine Living Resources (CCAMLR) in 1982 (reviewed by Kock, 2007). CCAMLR has 25 signatory nation-members and establishes a body (commission and scientific committee) for the management of Antarctic living resources using an ecosystem-based approach (ccamlr.org). The United States has commercial fisheries in Antarctic waters, and must therefore comply with CCAMLR mandates and participate in fisheries regulation and management. In 1982 a public law created the US Antarctic Marine Living Resources Program (managed by NOAA Fisheries' Antarctic Ecosystem Research Division) as a response to the US obligation to engage in CCAMLR (swfsc.noaa.gov). Since 1986 the US AMLR monitors krill biomass and its dependent populations of seabirds and seals off the Antarctic Peninsula region. At Cape Shirreff, Livingston Island, US AMLR has monitored Antarctic fur seals since 1997 by conducting systematic studies of female attendance behavior, recruitment of juveniles, annual pup production and foraging behavior. As a result, the US AMLR Program has archived a large amount of observational data and numerous tissue samples, which have allowed for the unique opportunity to conduct an in-depth investigation of Antarctic fur seal behavior as it relates to their puzzling re-colonization history.

In this thesis, genetic data and cutting-edge analyses techniques were applied to more than 1,000 individual Antarctic fur seals, mostly sampled at Livingston Island, in order to address lingering questions about the re-colonization process and mating system of the species. In particular, Chapter 1 addresses the hypothesis that Livingston Island was mostly re-colonized by immigrant seals from South Georgia and estimates current levels of gene flow between these populations. In Chapter 2, male reproductive success is investigated at a low-density breeding site, with the goal of understanding how population density may affect individual breeding success. Chapter 3 explores the breeding behavior of females monitored over a decade to estimate levels of remating among individuals over time. Finally, Chapter 4 tests for the presence of biological twins among putative cases that were observed in the field, and considers the consequences of twinning on female breeding behavior. The results of each chapter were interpreted from within the framework of intra-specific genetic diversity, and in particular, were compared to a similar suite of studies conducted by the British Antarctic Survey Program at the high-density colony of Bird Island, South Georgia.

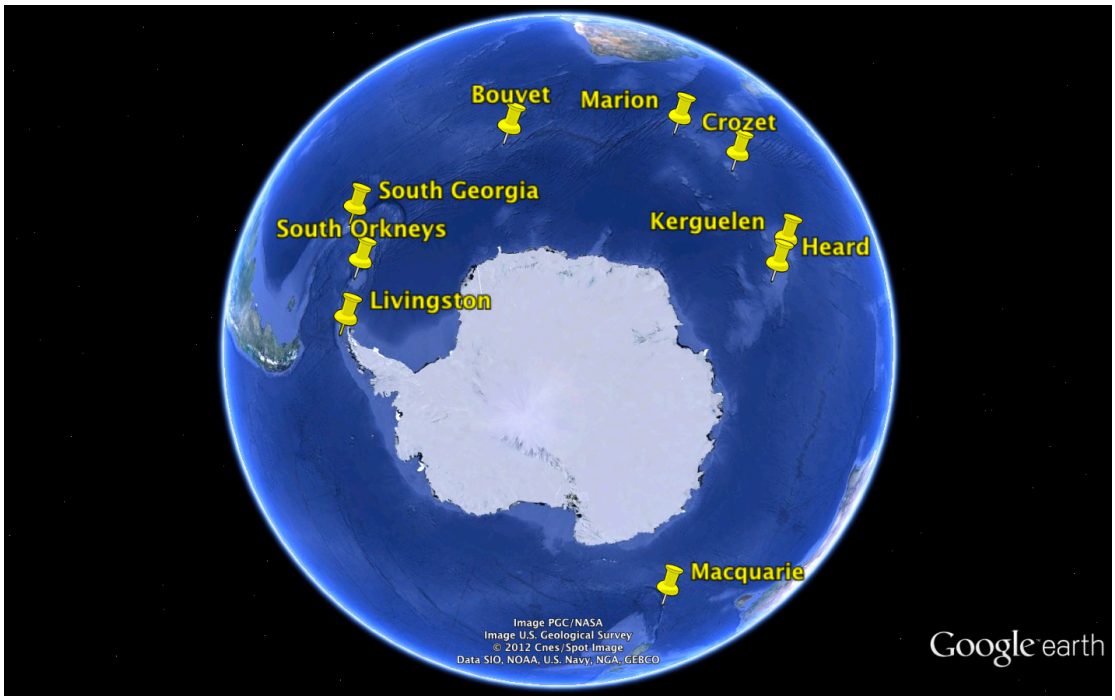

Fig. 0-1: Antarctic fur seal, *Arctocephalus gazella*, breeding colonies around the Antarctic continent.

Fig. 0-2. Antarctic fur seals, *Arctocephalus gazella*, at Livingston Island, Antarctica: male on the left, females are on the right and upper left. Photo Credit: Carolina Bonin.

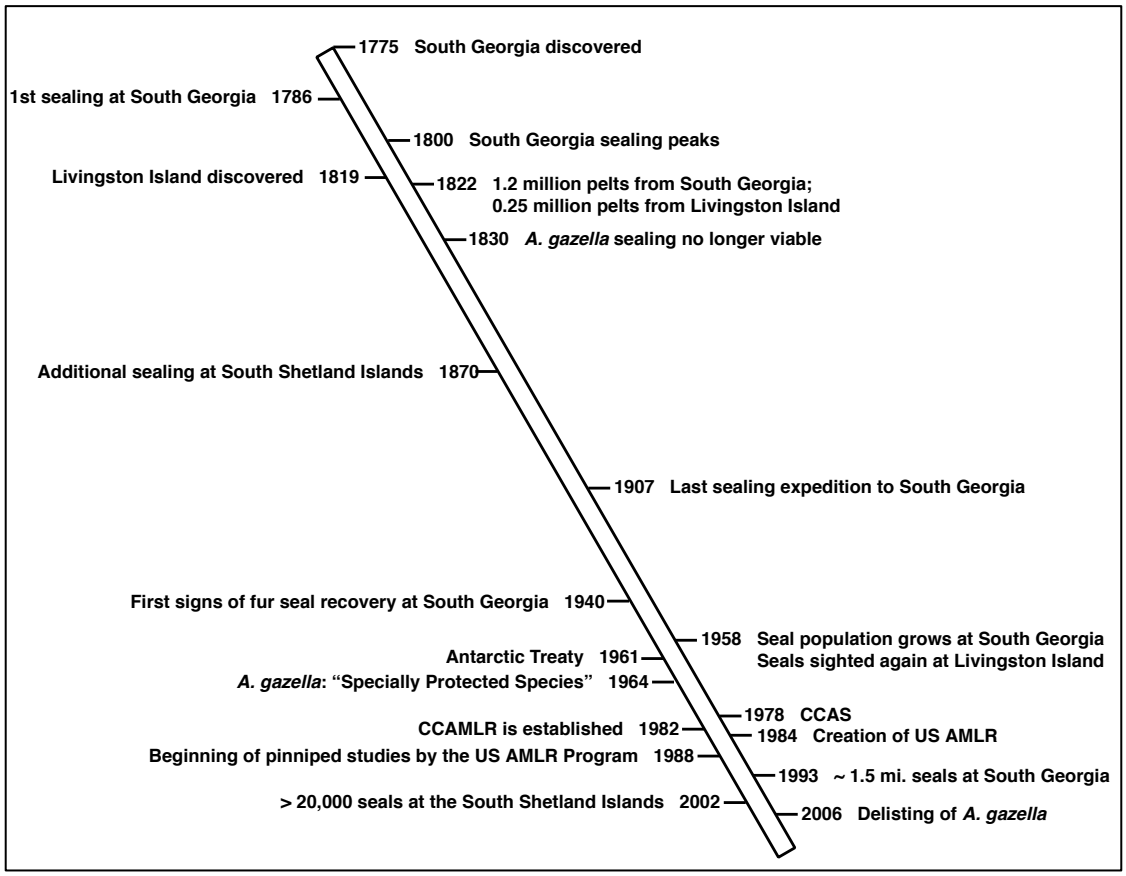

Fig. 0-3. South Georgia (SG) and Livingston Island (LI) Antarctic fur seal exploitation timeline. Legend: SSI= South Shetlands; CCAS= Convention for the Conservation of Antarctic Seals; CCAMLR= Convention for the Conservation of Marine Living Resources; AMLR= Antarctic Marine Living Resources.

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CHAPTER 1: Unexpected genetic differences between recently re-colonized Antarctic fur seal (*Arctocephalus gazella*) populations

ABSTRACT

Many species have been heavily exploited by man leading to local extirpations, yet few studies have attempted to unravel subsequent re-colonization histories. This has led to a significant gap in our knowledge of the long-term effects of exploitation on the amount and structure of contemporary genetic variation, with important implications for conservation. The Antarctic fur seal provides a particularly interesting case in point, having been virtually exterminated in the 1800's but subsequently staged a dramatic recovery to re-colonize much of its range. South Georgia (SG), where a few million seals currently breed, is thought to have been the main source of immigrants to other locations including Livingston Island (LI). To evaluate this hypothesis, we genotyped 366 individuals from these two populations at 17 microsatellite loci and sequenced a 263bp fragment of the mitochondrial hypervariable region 1. Contrary to expectations, we found weak but highly significant genetic differences at both types of marker, with 51% of LI individuals carrying haplotypes that were not observed in 246 animals from SG. Moreover, the youngest of three sequentially founded colonies at Livingston Island showed similarity to South Georgia at mitochondrial DNA but not microsatellites, implying temporal and sex-specific variation in re-colonization. Our findings emphasize the importance of relict populations and provide insights into the mechanisms by which severely depleted populations can recover while maintaining surprisingly high levels of genetic diversity.

INTRODUCTION

Colonization is a fundamental process in the dynamics of metapopulations (Gaggiotti et al., 2004). The birth and death of demes and patterns of migration among them (e.g. migrational variance) can have direct consequences for the maintenance of genetic diversity (Gaggiotti and Smouse, 1996; Pannell and Charlesworth, 2000). Founder effects can be especially important, with both the number and genetic composition of founding individuals exerting a profound influence on the way in which genetic variation becomes partitioned among populations (Pannel and Charlesworth, 1999; Gaggiotti et al., 2004). For example, populations established by small numbers of founders can rapidly develop striking genetic differences relative to the source. Such events can be considered as agents of "rapid genetic change" (Leblois and Slatkin, 2007).

In pinnipeds, colonization is viewed as a density dependent process. For example, both geographic distance and demography were found to be important factors in the establishment of new grey seal (*Halichoerus grypus*) colonies (Gaggiotti et al., 2002). Similarly, research on expanding populations of South American sea lions (*Otaria flavescens*) suggests that competition for space and harassment from conspecifics may cause a "spill over" effect to nearby breeding sites (Grandi et al., 2008). Although several otariid populations have been dramatically reduced by exploitation, most have staged a rapid recovery (Gerber and Hilborn, 2001), thereby providing particularly interesting case studies of re-colonization. Here, the use of the term "re-colonization" applies to the process by which a population is extirpated from a given area to subsequently reoccupy it, establishing a breeding colony.

The Antarctic fur seal (*Arctocephalus gazella*) is one of the most heavily exploited of all pinniped species. Breeding colonies on Antarctic and sub-Antarctic islands were subject to indiscriminate commercial sealing from around 1790 until 1825, when the species was

virtually extirpated from all of its distribution range. Antarctic fur seals have a broad circumpolar distribution, but South Georgia (SG) currently supports the largest breeding population of possibly over 4 million seals (Hofmeyer et al., 2005). In other regions, Antarctic fur seals breed in much smaller numbers. For example, the South Shetland Islands, at the southern limit of the species' breeding distribution, has a population size somewhere in the order of 20,000 animals (Hucke-Gaete et al., 2004).

SG was the stage of the most intense sealing activities in the Southern Ocean. James Weddell (1825) estimated that over 1.2 million fur seal pelts had been taken by 1822, and for a long time it was thought that the species had been extirpated from the area. The last sealing expedition to SG took place in 1907, but fur seal numbers showed no sign of recovery until around four decades later (reviewed by Trathan and Reid, 2009). By the early 1990's, the total number of fur seals was estimated at over a million individuals (Boyd, 1993).

The South Shetland Islands were discovered in 1819 and thereafter sealing quickly reached its peak, with 250,000 fur seal pelts taken between 1820 and 1822 (McCann and Doidge, 1987). By 1825, the population was so depleted that sealing became no longer commercially viable, leading to a hiatus up to the 1870's when another round of hunting further reduced population sizes throughout much of the archipelago. From 1925 to 1951, three ships often visited the islands and reported no signs of fur seals until the austral summer of 1958-59, when two groups of 27 and 15 non-breeding individuals respectively were observed ashore at Cape Shirreff, Livingston Island (O'Gorman, 1961). Subsequently, the population rapidly recovered, particularly during the mid 1960's and 1970's when the growth rate was estimated to be in excess of 50% (Hucke-Gaete et al., 2004). This rapid growth was largely attributed to immigration from the disproportionally large and rapidly expanding SG population (Hucke-Gaete et al., 2004), which was considered to be the source population for all other regions (Bonner, 1968; Laws, 1973). Sporadic re-sightings of flipper-tagged

individuals together with satellite-tracking information from other localities (e.g. South Orkney Islands; Waluda et al., 2010) are consistent with SG being the main source of seals dispersing to other regions, but these data are largely anecdotal and genetic data to test this hypothesis are so far lacking.

Despite the Antarctic fur seal providing an interesting model for understanding the impact of historical exploitation on a highly vagile, widely distributed marine mammal, only a single study of population structure has so far been conducted (Wynen et al. 2000). Using data from 145 mtDNA control region sequences from 13 breeding sites including SG and the South Shetland Islands, low levels of genetic differentiation were reported. Based on haplotype frequency data, three broad geographical regions were proposed: a "western region" comprising SG, Bouvet, Marion and South Shetland Islands, an "eastern region" including Kerguelen and Macquire, and an "intermediary group" comprising Crozet and Heard Islands. Within the western region, SG and Bouvet were the hypothesized sources of immigrants that re-colonized Marion, Heard and the South Shetland Islands.

Wynen et al. (2000) also documented several haplotypes that were unique to some of the smaller fur seal populations, notably those from the eastern region. Although the sample sizes used for this study were too small to draw firm conclusions ($n \leq 20$ per population), the authors interpreted the absence of these haplotypes from SG as meaning that contemporary fur seal populations may have been founded from more than one source. This merits further exploration since excluding SG as the main source of fur seal immigrants would have important implications for understanding how this species was able to maintain high levels of genetic diversity despite intense range-wide exploitation.

Here, we use a larger sample, of 366 fur seal individuals, to document genetic relationships between Livingston Island (LI) on the South Shetland Islands and it's main putative source population within the western region, SG. To provide both matrilineal and bi-

parental perspectives, all individuals were sequenced at a 316bp fragment of the mitochondrial hypervariable region 1 (HVR1) and genotyped at 17 highly polymorphic microsatellite loci. We also added a fine-scale perspective by including individuals from three populations at LI that were successively established during the late 20th Century. Our aims were to (i) evaluate support for the hypothesis that fur seal colonies at LI were established by individuals from SG; (ii) compare levels of genetic diversity between SG and LI; (iii) estimate the number of recent migrants between SG and LI; and (iv) examine the probable origin of individuals sampled at the youngest re-colonized site at LI.

MATERIALS AND METHODS

Study sites and sample collection

SG (35°47'-38°01'W and 53°58'-54°53'S) is a sub Antarctic island situated approximately 1000km southeast of the Falkland Islands (Figure A1-1- Appendix 1). Antarctic fur seal pups were tissue sampled by Hoffman et al. (2011) at seven sampling sites during the austral summer of 2003-2004 (Table 1-1). LI is the southernmost Antarctic fur seal breeding area and is one of the South Shetland Islands, a 500km-long archipelago towards the north of the Antarctic Peninsula (Figure A1-1 Appendix 1). Sampling was conducted at Cape Shirreff (62°27’S; 60°47’W), an ice-free peninsula approximately 3km long and located at the western end of LI’s north coast.

Cape Shirreff fur seal pups were sampled at three sites (West, East and North; hereafter designated LI-W, LI-E and LI-N respectively; Figure A1-1 Appendix 1, Table 1-1). LI-W is the oldest breeding site where the first records of fur seals were collected in the late 1950’s (O’Gorman, 1961). LI-N was re-colonized in the 1980’s, whereas LI-E is the most

recently established breeding area, dating to 2001-2002. Samples were collected during the austral summers of 2008-09 at LI- E, and 2009-10 at LI- W and LI- N. Tissue samples were preserved in either 20% dimethylsulphoxide (DMSO) saturated with salt (NaCl) or 95% ethanol (ETOH) stored at -20°C. Total genomic DNA was subsequently extracted from LI tissue samples using a NaCl precipitation method (adapted from Miller et al., 1988). SG samples were extracted using either a Chelex 100 protocol (for DNA used in sequencing) or a Dneasy blood and tissue extraction kit, Qiagen, USA (for DNA used in genotyping).

Mitochondrial DNA sequencing

A 316bp HVR1 fragment was PCR amplified using the primers Thr/Pro (5'-TCCCTAAGACTCAAGGAAGAG-3') and Cent (5'- GAGCGAGAAGAGGTACACTTT-3') as detailed by Wynen et al. (2000) and Hoffman et al. (2011). Sequencing was initially carried out using the forward primer, but whenever sequences had < 100% quality scores (as was the case for 24 out of the 119 LI samples), the reverse strand was also sequenced. In addition, 24 samples were independently replicated for quality control purposes, but no errors were detected. The sequences were edited using SEQUENCHER v. 4.8 for Windows (GeneCodes Corporation©, Ann Arbor, MI). The sequences were then trimmed to the final length of 263bp following Hoffman et al. (2011) to eliminate insertions and deletions, including the highly repetitive "TC landmark" previously described by Wynen et al. (2000). Alignment was conducted using BIOEDIT v. 5.0.6 (Hall, 1999).

Microsatellite genotyping

Tissue samples previously genotyped by Hoffman et al. (2011) were transported to La Jolla, CA (USA) where they were re-extracted and genotyped in the same laboratory where the LI samples were processed (Southwest Fisheries Science Center, NOAA Fisheries). This measure was undertaken to assure that the genotype data for the two regions would be directly comparable.

All samples were genotyped at 17 microsatellite markers: Ag10 (Hoffman et al., 2008), Agaz8, Agaz9 (Hoffman, 2009); Hl4, Hl16, Lc28 (Davis et al., 2002); Hg3.7 (Gemmell et al., 1997); M11A, M2B (Hoelzel, 1999); Pvc29, Pvc78 (Coltman et al., 1996); ZcCgDh1.8, ZcCgDh4.7, ZcCgDh48, ZcCgDh5.8, ZcCgDh7tg, ZcCgDhB.14 (Hernandez-Velasquez et al., 2005) using the annealing temperatures shown in Table A1-1 Appendix 1. PCR amplification and fragment analysis protocols are described in detail by Bonin et al. (2012). Following Hoffman and Amos (2005), we also independently re-genotyped eight samples (2.2% of the samples) at all 17 loci. The resulting genotyping error rate was low at 0.02 per reaction, consistent with a previously published rate for a similar marker panel in the same laboratory (Bonin et al., 2012).

Mitochondrial sequence analysis

Molecular diversity indices for the dataset, including haplotype (gene) diversity, the number of polymorphic sites (S), nucleotide diversity (π) and the average number of nucleotide differences (k) were assessed using DNAsp v. 5.10.01 (Librado and Rozas, 2009). Genetic differentiation was estimated using Φ statistics within a hierarchical Analysis of Molecular Variance (AMOVA; Excoffier et al., 1992) framework in the program ARLEQUIN v. 3.5.1.2 (Excoffier and Lischer, 2010). The hierarchical levels corresponded to tests at the individual level (within sites), among the ten sampling sites and between the two regions: LI (3 sites) and SG (10 sites). Significance (p values) was obtained using 1,000 permutations. A median joining network (MJ) of the mtDNA haplotypes was constructed using NETWORK v. 4.6.1 (Bandelt et al., 1999).

Microsatellite data analysis

The microsatellite dataset was tested for deviations from Hardy-Weinberg equilibrium (heterozygote deficit) and linkage disequilibrium using 100,000 dememorizations and 10,000 iterations per batch within GENEPOP v. 4.0 (Raymond & Rousset, 1995). Null allele frequencies were estimated using MICROCHECKER v. 2.2.3 (Van Oosterhout et al., 2004). FSTAT v.2.9.3.2 (Goudet, 2001) was used to calculate F-statistics, estimate variance components within individuals, among individuals within sampling sites and among sampling sites. Genetic differentiation was estimated via the calculation of global and pairwise F_{ST} (θ; Weir and Cockerham, 1984). Allelic richness, and expected and observed heterozygosities

(Hs, Ho) were compared among populations using two-tailed, sample size weighted statistical tests within FSTAT based on 10,000 permutations of the dataset.

For comparison, we also analized our data within STRUCTURE v. 2.3.4 (Pritchard et al., 2000), a program that is conservative in the sense that it does not rely on a priori stratification of individuals by location. STRUCTURE uses genotype data to probabilistically assign individuals to clusters based on the assumption that deviations from Hardy-Weinberg and linkage equilibrium should be minimized within the resulting clusters. Detection of the true number of clusters (K) based solely on the log probability of data (Ln[Pr(x|K)] is not always straightforward, particularly where population structure is weak or follows an isolation-by-distance pattern. Thus, after the true value of K is reached, the log probability of the data often reaches a plateau or increases at very small increments. Consequently, we applied the ad hoc statistical method of Evanno et al. (2005), which focuses on the rate of change in the log probability of data between successive K values. A conspicuous "jump" or increase in the log probability of data (equivalent to the highest ΔK) indicates the uppermost hierarchical number of clusters present in the dataset. We initially ran STRUCTURE without a priori sampling location information, but later repeated the same analyses incorporating location information and setting the parameter LOCPRIOR to 1. All analyses were conducted using the following parameters: admixture, allele frequencies correlated, 10,000 burn-in period and 100,000 MCMC repetitions. We conducted five independent runs for K= 1-10 and used STRUCTURE HARVESTER web core (Earl et al., 2012) to interpret the resulting outputs.

Detection of recent migrants

Maximum likelihood methods as implemented in the program MIGRATE can be powerful tools for exploring migration rates among populations or sub-populations. However, these approaches can be strongly affected by un-sampled or "ghost" populations (Slatkin, 2005). Having only sampled two of several globally distributed Antarctic fur seal populations we therefore detected individuals with recent migrant ancestry (i.e. to a maximum of two generations back) using the approach of Rannala and Mountain (1997). This derives the probability distribution of allele frequencies in each population using a Bayesian approach and then calculates assignment probabilities for each individual via comparison against those distributions. This tends to work well even when populations are only weakly differentiated, although power decreases as migrant ancestry goes back in time across generations. We implemented this analysis within GENECLASS2 (Piry et al., 2004) using Rannala and Mountain's (1997) Bayesian criterion and the simulation algorithm proposed by Paetkau et al. (2004). MCMC re-sampling was performed with 10,000 simulated individuals and a p value threshold of 0.01. In order to verify the robustness of GENECLASS2 results, we also used STRUCTURE to identify individuals with recent migrant ancestry. We set up migrant detection runs in STRUCTURE with the same parameters and run-lengths described earlier. Three independent runs were performed to detect migrant descendants only within two generations (GENSBACK= 2) for each of three alternative migration model priors (MIGPRIOR= 0.01, 0.03 and 0.05).

RESULTS

Mitochondrial DNA sequences

A total of 52 polymorphic sites and 41 haplotypes were observed among the 365 HVR1 mtDNA sequences. Thirteen haplotypes were only observed in SG (n = 246 individuals), five of which were represented by more than one individual. Fifteen haplotypes were unique to LI (n = 119 individuals), ten of which were sampled more than once. Remarkably, these unique regional haplotypes were found in 51% of the individuals sampled at LI, with the highest of incidence being observed at the oldest colony (54%, LI-W), the lowest at the youngest colony (38%, LI-E) and an intermediate proportion at the colony of intermediary age (46%, LI- N).

Approximately 95% of the variation in the sequence data was observed among individuals within sampling locations (AMOVA, $\Phi ST = 0.04838$, $p = 0.00098 \pm 0.0098$), while the remaining 5% was largely partitioned between SG and LI ($\Phi CT = 0.05008$, $p = 0.00880 \pm 0.00288$). A negligible proportion of the total variance could be attributed to sampling sites within these two regions ($\Phi SC = -0.00179$, $p = 0.53177 \pm 0.01354$). Consistent with this pattern, most of the significant pairwise ΦST values (9 out of 11 significant values, $p < 0.05$; Table A1-2 Appendix 1) were observed in comparisons between SG and LI. Sequence diversity indices were comparable between SG and LI (Table 2) despite the former having a much larger population size.

A median joining network constructed using all of the samples contained 12 hypothetical median vectors (un-sampled sequences) and three unresolved links (loops) despite attempts to reduce its complexity using post-processing calculations within the program NETWORK. Nevertheless, many of the most common haplotypes were present in

both SG and LI, whereas haplotypes unique to LI tended to be in peripheral positions, although they were represented by > 50% of the individuals sampled at LI (Figure A1-2 Appendix 1).

Microsatellites

Our microsatellite panel was highly informative (average number of alleles per locus= 13.76 ± 6.95; HE= 0.81) and the proportion of missing data was low at 1.6%. There was no clear indication of null alleles, allelic dropout or linkage disequilibrium (Table A1-1 Appendix 1). Four loci deviated significantly from Hardy-Weinberg equilibrium, although only two of these values remained significant following Bonferroni correction for multiple statistical tests. Moreover, these loci were not found to be consistently out of equilibrium when the data were analiz*ed* separately for SG and LI, suggesting that these deviations could be due to a Wahlund effect (heterozygosity reduction due to population sub-structuring).

The global F_{ST} (θ) for the microsatellite dataset was 0.014 (95% CI= 0.010- 0.018; 99% CI= 0.009-0.019). Pairwise F_{ST} values among sampling sites were mostly significant in comparisons involving SG and LI (23 out of 24 inter-region comparisons (Table A1-3 Appendix 1). Non-significant, low pairwise F_{ST} values were indicative of a lack of genetic structuring within South Georgia (overall F_{ST} = 0.0008 ± 0.006; range = -0.009 - 0.018). At Livingston Island a similar result was obtained (overall F_{ST} = 0.008 ± 0.003; range = 0.005 - 0.010) and only comparisons involving the youngest colony (LI-E) reached statistical significance (LI-E vs. LI-W, FST = 0.009; LI-E vs. LI-N, F_{ST} = 0.0127). Allelic richness and mean observed (Ho) and expected (Hs) heterozygosity did not differ significantly between SG and LI (p = 0.196, 0.803 and 0.170 respectively in two-tailed comparisons).

Consistent with the above analyses, STRUCTURE identified two clusters (K = 2) based on the approach of Evanno et al. (Figure 1B). These coincided perfectly with SG and LI

(Figure 1-2 A), with the majority of individuals having a high posterior probability of assignment to their respective cluster (minimum of 90% for SG and 79% for LI individuals). Additional STRUCTURE runs conducted separately for LI and SG found no clear evidence of further subdivision within these two regions (results not shown). Similar results were obtained using the LOCPRIOR setting, which takes into account the sampling locations of each individual (Figure 1-2 B), although the clustering appears marginally improved in that less admixture was observed within SG.

Detection of individuals with migrant ancestry

The program GENECLASS2 detected three pups with migrant ancestry via exclusion tests within LI-N ($p = 0.0007$, 0.0077 and 0.0041 respectively). Two of these were assigned to SG with > 99.5% probability, while the third individual was not confidently assigned to either SG or LI, suggesting that it could have originated from another, un-sampled location. The program STRUCTURE identified one of the same migrants using a migration prior of 0.01 and confirmed the second migrant with a higher migration prior of 0.05, while assignment probabilities to the population of origin (LI) were 0.004 ($p < 0.0001$) and 0.391 ($p < 0.01$) respectively.

DISCUSSION

Relatively few studies have attempted to unravel the re-colonization histories of heavily hunted species such as marine mammals, leading to a significant gap in our knowledge of the long-term effects of exploitation. Consequently, we conducted a genetic analysis of Antarctic fur seal pups from LI and its putative source population SG. We detected highly significant population differentiation and identified numerous unique mitochondrial haplotypes within LI, allowing us to reject a simple scenario of re-colonization from SG. Our study thereby provides insights into the mechanisms by which severely depleted populations can recover while maintaining surprisingly high levels of genetic diversity.

The finding of genetic differentiation between SG and LI was unexpected given our original working hypothesis that LI was re-colonized by immigrants from the rapidly expanding population of SG. It is also in contrast to previous studies of Australian and Northern fur seals that found no population structure despite these species having also been heavily exploited (Lancaster 2010, Dickerson 2010). Nevertheless, genetic structuring was broadly consistent between the nuclear and mitochondrial genomes, suggesting that it is mediated via both males and females.

Our data clearly suggest that LI was not re-colonized solely from SG, but the number and identity of source populations remains open to question. One possibility is that Antarctic fur seals may have survived sealing at isolated locations within the South Shetland Islands archipelago, allowing them to rapidly re-colonize the nearby vacant rookeries at LI. This explanation is supported by a recent study (Hoffman et al. 2011), which suggests that the South Georgia population may not have been as heavily reduced by sealing as previously thought. The same could conceivably apply to the South Shetland population because, although the remarkably rapid growth rate was attributed to immigration (Hucke-Gaete et al.,

2004), systematic censuses incorporating all breeding areas in the South Shetlands did not commence until 1987 (Bengtson et al., 1990), and there was no direct evidence of exchange of individuals from studied populations. Thus, relict populations in areas such as the San Telmo Islets could well have been overlooked.

It is also possible that LI was re-colonized by immigrants from multiple source populations from further afield. The best candidate for a source population within the "western region" proposed by Wynen et al (2000) is Bouvet Island. Mainly due to its inaccessible location, seals were not completely exterminated at Bouvet, which currently holds the second largest fur seal population (Hofmeyr et al., 2005). Other islands within the western region are less likely to have been significant sources of immigrants as their pup production is much lower, in most cases less than 400 and not more than 1,000 pups per year (Hofmeyr et al., 1997; Page et al., 2003; Hofmeyer et al., 2005; Waluda et al., 2010).

One line of evidence that is consistent with multiple founder populations is the high genetic diversity and large number of unique haplotypes found in LI, despite this population being orders of magnitude smaller than SG. However, to determine the relative contributions, if any, of populations such as Bouvet Island would require allele frequency data from other colonies. Nevertheless, such an explanation would be consistent with the recent observation that northern fur seals (*Callorhinus ursinus*) also maintained high levels of genetic diversity in the face of heavy historical exploitation; a fact attributed to the existence of multiple refugia coupled with historically high dispersal rates (Pinsky et al., 2010).

As initially reported for SG (Hoffman et al., 2011), we found little evidence for genetic structuring within LI, although contrasting results were obtained for mtDNA and microsatellites regarding the newest colony, LI-E. Individuals from this locality were found to cluster together with those from LI based on the microsatellite data, but showed greater similarity to SG than the other two LI colonies based on mtDNA. Such a finding may offer a

novel insight into the colonization process, as it implies that many of the females that founded LI-E may have originated from SG, whereas the males they mated with were probably of local origin.

We found evidence for at least two pups from LI having immigrant ancestry from SG within the last two generations. Although we were not able to formally estimate migration rates within a maximum likelihood framework due to incomplete population sampling, this provides evidence in support of some level of contemporary gene flow between SG and LI. It also raises the possibility that, if LI population growth resulted mostly from local recruitment, population structure may actually have been even more pronounced than it is today. This points towards a complex re-colonization history, although to disentangle this further will require large sample sizes of individuals from all of the main fur seal colonies. The inclusion of pre-exploitation samples, if these could be obtained, would also provide valuable insights into temporal variation in the amount and geographic partitioning of genetic variation with respect to exploitation history.

Our findings strongly support the hypothesis that LI was re-colonized by one or more unsampled source populations in addition to SG. This highlights the importance of satellite populations, which although demographically less significant, can harbor high levels of genetic diversity. Such populations could become increasingly important for maintaining the genetic diversity of polar species that are facing mounting threats from rapid environmental change.

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Table 1-1: Number of Antarctic fur seals, *Arctocephalus gazella*, sampled at South Georgia and Livingston Island.

Region	Sampling site	Samples sequenced	Samples genotyped
Livingston Island	East	26	28
	West	46	43
	North	47	49
Subtotal		119	120
South Georgia	Willis Islands	16	15
	Bird Island	167	171
	Prince Olav	12	12
	Leith Harbor, Husvik	13	11
	Cooper Bay	14	14
	AnnenKov Island	15	14
	Wilson Harbor	9	9
Subtotal		246*	246
Total		365	366

* Sequences from South Georgia previously published by Hoffman et al. (2011).

Table 1-2: Molecular diversity indices for Antarctic fur seals, *Arctocephalus gazella*, sampled in two regions (South Georgia and Livingston Island) sequenced for 263 bp fragment (HVR1) of the mtDNA and genotyped using 17 microsatellite markers.

Molecular diversity indices	**South Georgia**	**Livingston Island**
Number of individuals sequenced	246	119
Number of unique haplotypes	13	15
Average number of nucleotide differences	9.02	9.019
Nucleotide diversity	0.034	0.034
Number of individuals genotyped	246	120
Mean number of alleles	11.824 ± 4.94	12.588 ± 5.26
Allelic richness	6.021	6.343
Mean heterozygotes proportion	0.799 ± 0.115	0.802 ± 0.086
Mean Nei's genetic diversity	0.807 ± 0.104	0.822 ± 0.08

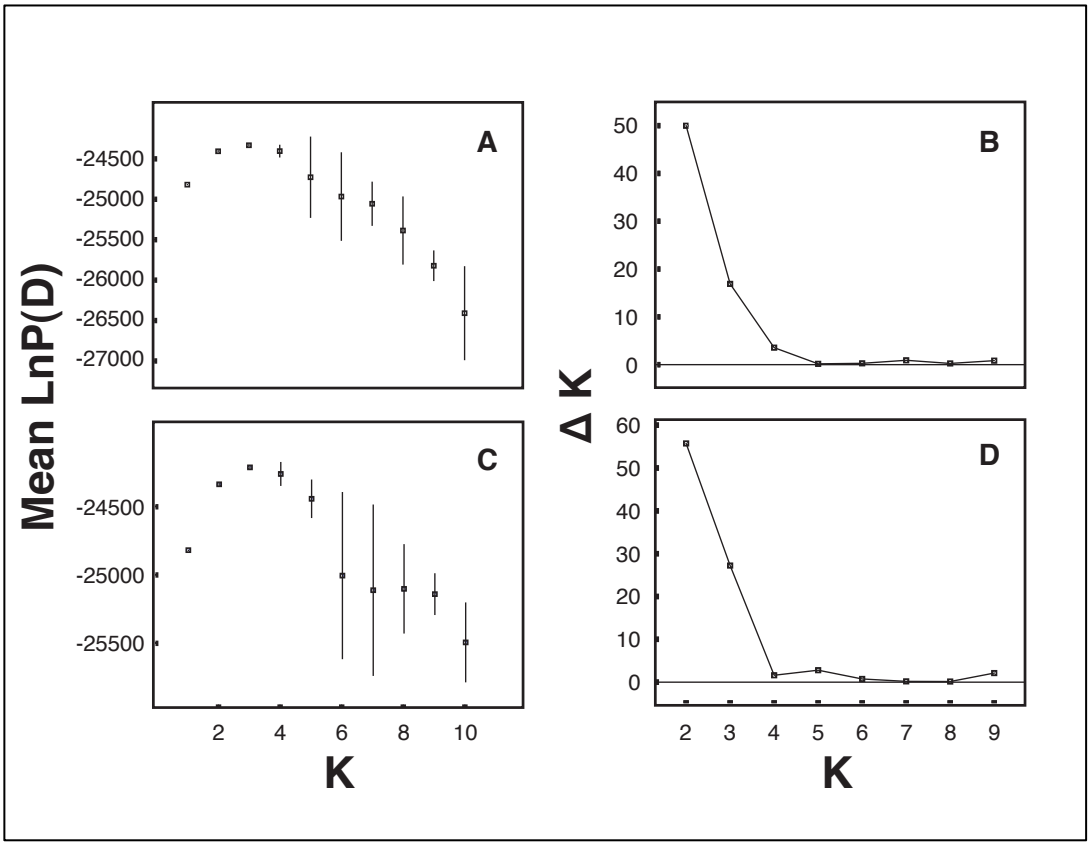


Fig. 1-1: Results of Bayesian cluster analyses within the program STRUCTURE v.2.3.3 (Pritchard et al., 2000) based on 366 Antarctic fur seals genotyped for 17 microsatellite loci. Shown are plots of mean and standard deviation of the posterior probabilities of K (LnP(D)) plus variation in the rate of increase of LnP(D) with successive K values (ΔK). Five simulations were conducted for each value of K between one and ten. A, B) Results of runs without a priori population information. C, D) Results of runs with population information (sampling locations).

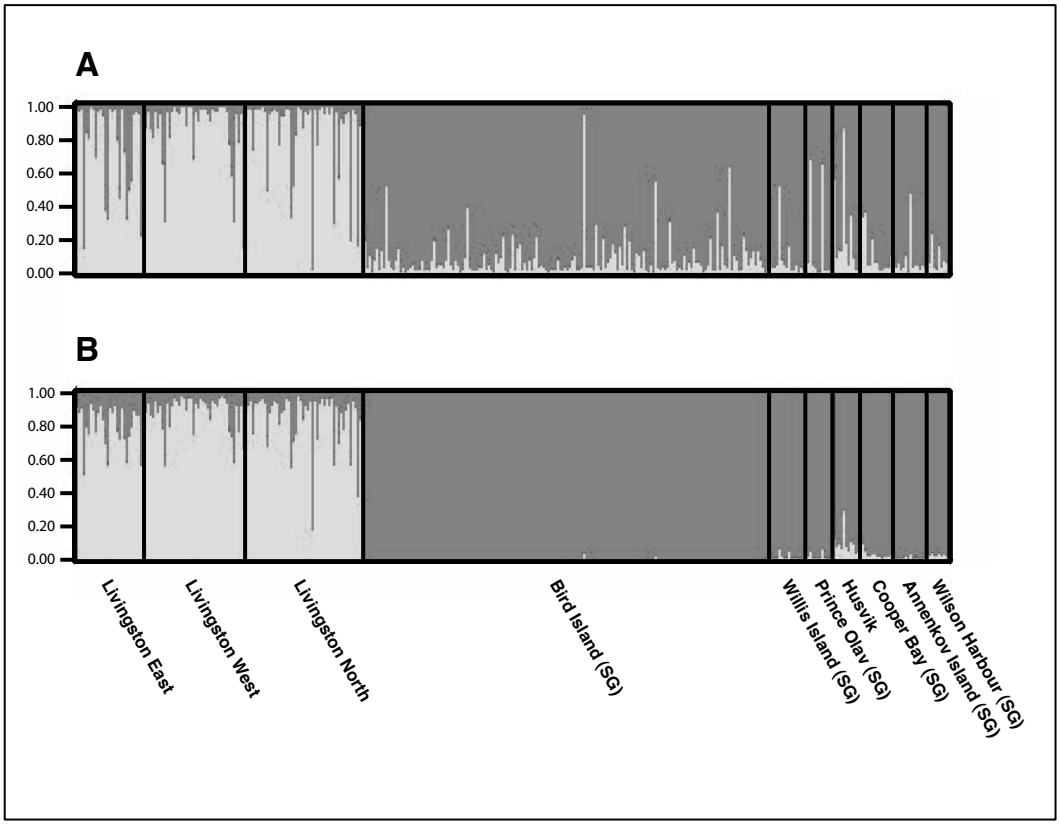


Fig. 1-2: Posterior probability of assignment for Antarctic fur seal individuals (vertical bars) into clusters according to Bayesian analyses in STRUCTURE v.2.3.3 (Pritchard et al., 2000). Clusters corresponding to South Georgia and Livingston Island regions are denoted by dark and light grey respectively. A) Results without a priori population information. B) Results of analyses incorporating the sampling locations of individuals.

APPENDIX 1-1

Table A1-1: Microsatellite loci used to genotype 366 Antarctic fur seal samples (*n*= 246 from South Georgia, *n*= 120 from Livingston Island). The table includes details of PCR annealing temperatures (Tm), number of alleles (k), observed and expected heterozygosities (Ho and H_E), and Hardy-Weinberg equilibrium test p values for the total dataset (*p* global), for Livingston Island (*p* LI) and South Georgia samples (*p* SG). Significant deviations for Hardy-Weinberg equilibrium are highlighted in bold.

Locus	Source	Species	Tm	K	Ho	He	p Global	p LI	p SG
Ag10 t	Hoffman et al. 2007	*Arctocephalus gazella*	50	8	0.751	0.761	0.568	0.754	0.508
Agaz8t	Hoffman et al. 2009	*Arctocephalus gazella*	52	19	0.838	0.864	**0**	0.103	**0.001**
Agaz9t	Hoffman et al. 2009	*Arctocephalus gazella*	50	10	0.775	0.807	0.098	0.072	0.244
Hg3.7 t	Gemmell et al. 1997	*Halichoerus grypus*	50	13	0.833	0.853	0.004	0.007	0.479
Hl-16 t	Davis et al. 2002	*Hydrurga leptonyx*	56	35	0.864	0.887	0.322	0.021	0.837
HI-4 t	Davis et al. 2002	*Hydrurga leptonyx*	52	5	0.529	0.601	0.008	0.051	0.003
Lc-28 t	Davis et al. 2002	*Lobodon carcinophaga*	58	14	0.845	0.855	**0**	0.003	0.06
M2B t	Hoelzel 1999	*Mirounga angustirostris*	56	13	0.832	0.845	0.027	0.74	0.014
M11C t	Russ Hoelzel unpubl.	*Mirounga angustirostris*	55	20	0.882	0.899	0.148	0.006	0.892
Pvc29	Coltman et al. 1996	*Phoca vitulina*	52	15	0.869	0.865	0.358	0.613	0.426
Pvc78	Coltman et al. 1996	*Phoca vitulina*	55	10	0.804	0.814	0.283	0.551	0.269
ZcCgDh1.8 t	Hernandez-Velazquez et al. 2005	*Zalophus californianus*	60	9	0.757	0.77	0.125	0.327	0.39
ZcCgDh4.7 t	Hernandez-Velazquez et al. 2005	*Zalophus californianus*	60	14	0.849	0.88	0.257	0.073	0.688
ZcCgDh48 t	Hernandez-Velazquez et al. 2005	*Zalophus californianus*	55	10	0.624	0.602	0.87	0.657	0.885
ZcCgDh5.8	Hernandez-Velazquez et al. 2005	*Zalophus californianus*	60	15	0.886	0.881	0.288	0.671	0.546
ZcCgDh7tg t	Hernandez-Velazquez et al. 2005	*Zalophus californianus*	55	18	0.907	0.889	0.694	0.656	0.747
ZcCgDhB.14 t	Hernandez-Velazquez et al. 2005	*Zalophus californianus*	60	6	0.762	0.771	0.022	0.285	0.046

Table A1-2: Pairwise Φ_{ST}s (above diagonal) and corresponding p values (below diagonal) estimated for 365 Antarctic fur seals, *Arctocephalus gazella*, sampled at 10 sites across two regions (South Georgia and Livingston Island) and sequenced for 316 bp of the mtDNA HVR1. Statistically significant comparisons ($p < 0.05$) are highlighted in bold. Sample sizes are given in Table 1.

	South Georgia							Livingston Island		
	Husvik	**Wilson Harbor**	**Prince Olav**	**Cooper Bay**	**Annenkov**	**Willis Island**	**Bird Island**	**LI- East**	**LI- West**	**LI- North**
Husvik		-0.027	0.069	0.015	0.011	0.084	0	-0.006	0.003	0.028
Wilson Harbor	0.636±0.004		-0.009	-0.044	-0.061	0.045	-0.019	-0.027	0.018	0.016
Prince Olav	**0.046±0.002**	0.505±0.004		0.015	0.002	-0.042	0.001	0.012	0.072	0.055
Cooper Bay	0.329±0.004	0.795±0.004	0.282±0.004		-0.027	0.036	-0.009	0.029	0.065	0.076
Annenkov	0.316±0.004	0.961±0.001	0.372±0.004	0.746±0.004		0.054	0.008	-0.008	0.041	0.022
Willis Island	**0.040±0.002**	0.168±0.003	0.606±0.004	0.155±0.003	0.097±0.002		0.013	0.05	0.103	0.099
Bird Island	0.396±0.005	0.662±0.004	0.392±0.004	0.590±0.004	0.263±0.004	0.206±0.003		0.017	0.052	0.057
LI- East	0.526±0.004	0.799±0.003	0.263±0.004	0.117±0.003	0.599±0.004	0.054±0.002	0.102±0.002		-0.007	-0.016
LI- West	0.371±0.005	0.218±0.004	**0.015±0.001**	**0.012±0.001**	**0.037±0.001**	**0.000±0.000**	**0.000±0.000**	0.654±0.004		-0.004
LI- North	0.108±0.003	0.236±0.004	**0.033±0.001**	**0.004±0.000**	0.124±0.002	**0.001±0.000**	**0.000±0.000**	0.932±0.002	0.587±0.004	

Table A1-3: Pairwise F_{ST}s (θ, above diagonal) and corresponding p values (below diagonal) estimated for 366 Antarctic fur seals, *Arctocephalus gazella*, sampled at 10 sites across two regions (South Georgia and Livingston Island) and genotyped at 17 microsatellite loci. Significant comparisons ($p < 0.05$) are highlighted in bold. Sample sizes are given in Table 1.

	South Georgia							Livingston Island		
	Husvik	**Wilson Harbor**	**Prince Olav**	**Cooper Bay**	**Annenkov**	**Willis Island**	**Bird Island**	**LI- East**	**LI- West**	**LI- North**
Husvik		0.003	0.007	-0.001	0.019	0.005	0.006	0.023	0.019	0.014
Wilson Harbor	0.353		-0.002	-0.001	0.007	-0.003	0.001	0.016	0.014	0.017
Prince Olav	0.184	0.716		-0.004	0	-0.009	0.003	0.014	0.021	0.018
Cooper Bay	0.507	0.817	0.834		0	-0.007	-0.003	0.013	0.018	0.016
Annenkov	**0.023**	0.201	0.469	0.569		-0.003	0.004	0.03	0.029	0.024
Willis Island	0.266	0.35	0.904	0.918	0.41		-0.005	0.021	0.019	0.021
Bird Island	0.097	0.528	0.177	0.501	0.167	0.571		0.024	0.021	0.02
LI- East	**<0.001**	**0.032**	**0.002**	**0.006**	**<0.001**	**<0.001**	**<0.001**		0.009	0.013
LI- West	**<0.001**	**0.003**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**		0.005
LI- North	**0.006**	**0.003**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.193	

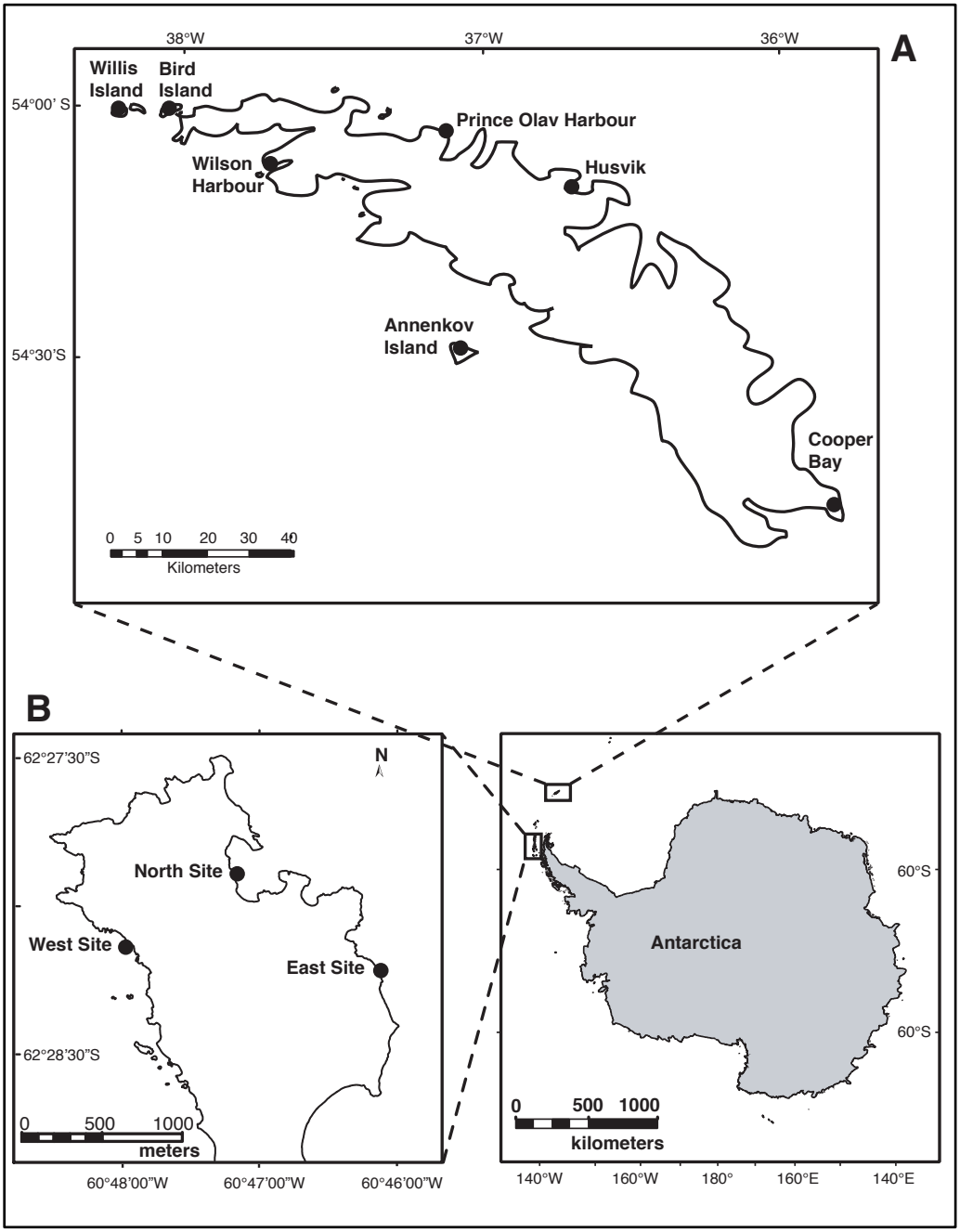
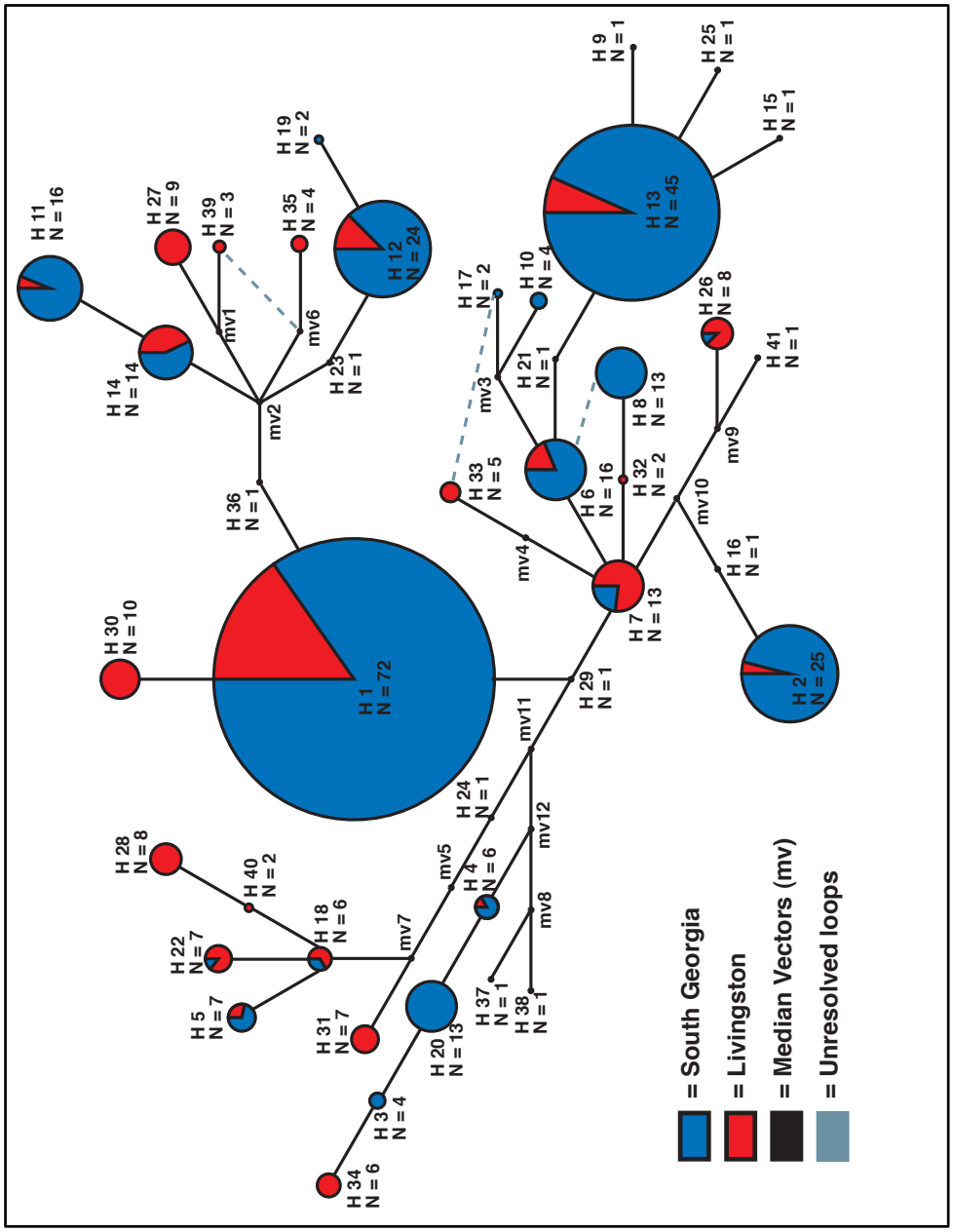


Fig. A1-1. The sub-Antarctic and Antarctic islands of South Georgia and Livingston where Antarctic fur seals were sampled. **A)** South Georgia sampling sites; **B)** Livingston Island sampling sites.

Fig. A1-2. Medium joining network of 41 haplotypes observed among 365 Antarctic fur seals sampled at South Georgia and Livingston Island and sequenced for 263 bp fragment of the mtDNA control region (HVR1). Dashed lines represent unresolved links among haplotypes.


H 11
N = 16
H 27
N = 9
H 39
N = 3
H 35
N = 4
H 19
N = 2
H 12
N = 24
H 14
N = 14
mv1
mv6
H 23
N = 1
mv2
H 36
N = 1
H 30
N = 10
H 1
N = 72
H 28
N = 8
H 40
N = 2
H 22
N = 7
H 18
N = 6
H 5
N = 7
mv7
H 31
N = 7
H 3
N = 4
H 34
N = 6
H 20
N = 13
mv5
H 24
N = 1
H 4
N = 6
mv11
mv12
mv8
H 37
N = 1
H 38
N = 1
H 29
N = 1
H 7
N = 13
mv4
H 33
N = 5
H 17
N = 2
mv3
H 21
N = 1
H 10
N = 4
H 6
N = 16
H 32
N = 2
H 8
N = 13
H 13
N = 45
H 9
N = 1
H 25
N = 1
H 15
N = 1
H 26
N = 8
H 41
N = 1
mv9
mv10
H 16
N = 1
H 2
N = 25
= South Georgia
= Livingston
= Median Vectors (mv)
= Unresolved loops

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CHAPTER 2: High male reproductive success in a low-density Antarctic fur seal (*Arctocephalus gazella*) breeding colony.

ABSTRACT

Understanding how population density influences mating systems may lead to important insights into the plasticity of breeding behavior, but few natural systems allow for such investigations. Antarctic fur seals (*Arctocephalus gazella*) are an interesting case in point because they breed in colonies of varying densities but have so far only been studied at a high-density site at Bird Island, South Georgia. Consequently, we used 13 highly polymorphic microsatellite loci to conduct a genetic analysis of a low density breeding colony of this species at Livingston Island, where the majority of adults seen ashore (n = 54) were opportunistically sampled together with every pup born (n = 97) over four consecutive seasons. We found unexpectedly high male reproductive skew, with two males accounting for a total of 28% of all pups sampled (n= 97) and 82% of all paternities assigned (n = 34). Moreover, a full likelihood pedigree inference method assigned a further eight paternities to an unsampled male who is inferred to have held a territory in the year before the study began. We also identified eleven pairs of full siblings, including three triplets, implying that their parents re-mated over three consecutive seasons. These findings suggest that territorial male fur seals may achieve greater success in monopolizing access to breeding females when population density is relatively low.

INTRODUCTION

Population density can strongly influence mating strategies (Emlen and Oring 1977; Kokko and Rankin, 2006) because the energetic cost for the monopolization of mates changes with the number of potential competitors (Emlen and Oring 1977). For example, in grey-sided voles, *Myodes rufocanus*, the occurrence of multiple paternity is positively correlated with male density because dominant males no longer effectively monopolize access to their mates in the presence of a relatively high number of contenders (Ishibashi and Saitoh, 2008). Similarly, at higher densities, courtship rates decrease in the rose bitterling fish, *Rhodeus ocellatus*, as individuals seem to spend more time defending resources from rivals (Casalini et al., 2010). Although these dedicated studies provide empirical evidence for enhanced male competition at increased population densities, they were conducted in enclosed settings, and little is known about how these findings can be generally applied to populations in the wild.

Pinnipeds, especially otariids, are a particularly interesting case in point to study the plasticity of mating systems. First, they are highly polygynous, so males typically engage in either defending their mates (female-defense polygyny) or an attractive resource (resource-defense polygyny) from rivals (Emlen and Oring, 1977, Clutton- Brock, 1989). Second, pinniped reproductive strategies have evolved in intimate association with their amphibious existence (offshore marine feeding and terrestrial partuition), which is tightly linked to their typical reproductive synchronicity and gregariousness (Batholomew, 1970). Third, expectations based on mating reproductive behavior are not always matched by results obtained from genetic analysis. In some instances, these analyses corroborated highly polygynous behavior (e.g. elephant seals, *Mirounga leonina*, Hoelzel et al., 1999; Fabiani et al., 2004), while others have surprisingly shown the prevalence of alternative mating strategies, such as aquatic mating (e.g. gray seals, *Halichoerus grypus*; Wilmer et al., 1999).

Finally, pinnipeds breed in colonies with varied densities, allowing for a unique assessment of how breeding behavior may vary accordingly. For example, a shift from territorial-defense to female-defense polygyny has been observed in Northern fur seals (*Callorhinus ursinus*) as populations of this species experienced considerable decline (Kiyota et al., 2008).

The Antarctic fur seal, *Arctocephalus gazella*, provides a unique opportunity to study the effects of population density on polygyny in a colonially breeding pinniped. This species has been studied by the British Antarctic Survey for many decades at the Sub-Antarctic island of South Georgia (SG), where it breeds in extraordinary numbers (possibly > 4 million seals; Hofmeyr et al., 2005). Here, density-dependent pup mortality (e.g. traumatic injury) has been frequently observed (Doidge et al., 1984), and bottom-up processes related to resource availability (particularly for lactating mothers) appear to be important in regulating the population (Reid & Forcada, 2005).

Making use of an especially amenable study colony at Bird Island, where a scaffold walkway is in place allowing virtually every animal ashore to be observed and tissue-sampled, a genetic study now spans almost twenty years. This has shown that territorial males father the majority of pups, and that most matings that take place on land (i.e. terrestrial polygyny; Hoffman et al 2003). Adult males also show extreme site fidelity, both across and within seasons. For example, over half of all individuals return to within a body length of where they held territories in previous seasons, and at the peak of the season when densities are the highest, any male movements are so small as to be indistinguishable from measurement error (Hoffman et al 2006). In contrast, females are considerably more mobile, with only around 40% conceiving with males within a radius of two body lengths. Moreover, females appear to move further to conceive with males who offer a balance of high heterozygosity and low relatedness to themselves, implying mate choice (Hoffman et al 2007). This work is interesting in that it both supports and challenges the traditional view of male-dominated

polygyny, but it also raises an important question: to what extent are these findings, obtained at the crowded Bird Island colony, applicable to lower-density Antarctic fur seal populations that occur elsewhere?

Livingston Island (LI), situated within the South Shetlands archipelago (approximately 1600 km south of SG), holds the southernmost population of Antarctic fur seals. At this remote island, fur seals breed in relatively small numbers (> 21,000 individuals; Hucke-Gaete et al., 2004) and colony densities are far lower than observed at SG. At LI, seal numbers have been limited by the top-down influence of leopard seal predation (Boveng et al., 1998) and there is no clear evidence that density-dependent processes control population growth. For example, traumatic injury due to space limitation has not been observed, nor has the influence of resource competition on lactating females been assessed. Antarctic fur seals breed at several beaches along Cape Shirreff, which is an ice-free peninsula on the north coast of LI. In the austral summer of 2001–2002, seals began occupying an additional area on the Cape's East side (designated LI-E), where annual pup productions above forty have never been recorded. We chose this location for our genetic study because individuals can be relatively easily tracked and tissue-sampled.

Newly occupied areas at LI-E, where seals breed in very low numbers, provide us with an ideal opportunity to elucidate the genetic mating system of a low-density population of Antarctic fur seals for comparison with previous studies of a high-density colony at SG (Hoffman et al 2003, Hoffman et al 2006, Hoffman et al 2007). We therefore conducted a genetic analysis of 172 samples collected over four consecutive breeding seasons. Our primary objective was to evaluate reproductive success of territory-holding males, and to relate this to the number of seasons over which each individual held tenure. In addition, we employed a full pedigree inference method to infer the reproductive success of unsampled males and to estimate the frequency of remating within this small colony.

MATERIALS AND METHODS

Study site

Cape Shirreff (62°27'S, 60°47'W) is an ice-free peninsula located on the north coast of Livingston Island, Antarctica. This study was conducted at a small breeding site, covering an area of approximately 200m^2 on the East side of the Cape, where fur seals started to breed in 2001-02 for the first time after having been extirpated by sealers around a century ago. The study site, hereafter referred to as "LI-E", was subdivided into three sub-units (A, B, C), which were naturally delimited by rock outcrops.

Observational data and tissue sampling

The study colony was monitored on a daily basis during December, when the majority of breeding females were present. Soon after birth, pups were given a small bleach mark on their lower back for identification. These pups were then sampled by taking a small skin plug from the inter-digital membrane of their rear flipper using a 2mm sterile biopsy punch. During the same period, the study area was monitored for the presence of territorial males. Whenever possible, these were identified using natural markings (e.g. scars, coloration) or a bleach mark was applied to the pelage. Because bleach marks are not retained across seasons, we then used genetic recaptures (*sensu* Hoffman et al 2003, Hoffman et al 2006) to identify males returning over multiple seasons. The sampling of adult males was not systematic, but was instead carried out opportunistically to in order to maximize safety to the researchers and minimize stress to the animals. Nevertheless, we made special efforts to attempt to sample all of the males observed during the last two seasons of the study.

During four consecutive breeding seasons (austral summers of 2006-07 – 2009-10) all pups born at LI-East were sampled, along with an estimated 60% of all territorial males sighted ashore. Although males were the focus of this study, adult females at the study site were also sampled during the last two seasons (2008-09; 2009-10). All adult males and females were tissue sampled using a customized, sterile, stainless steel biopsy tip (3mm diameter) attached to a pole. Seals were approached by foot and the biopsy tip was applied to the rear flipper to obtain a small skin plug. All sampling procedures were fully compliant with Marine Mammal Protection Permit No. 774-1847-03 granted by the Office of Protected Resources, National Marine Fisheries Service, United States.

Laboratory procedures and microsatellite genotyping

All tissue samples were preserved in 95% ethanol at -20°C. Total genomic DNA was extracted from tissues using a NaCl precipitation method adapted from Miller et al. (1988). After extraction, genomic DNA was PCR amplified for 13 microsatellite markers: Aa4, Hg3.7 (Gemmell et al., 1997); Hl4, Lc28 (Davis et al., 2002); M2B, M11C (Hoelzel, 1999; R. Hoelzel unpubl.); Pvc29, Pvc78 (Coltman et al., 1996); ZcCgDh1.8, ZcCgDh4.7, ZcCgDh48, ZcCgDh5.8, ZcCgDh7tg, and ZcCgDhB.14 (Hernandez-Velasquez et al., 2005). Amplification protocols, fragment analyses and raw data editing are described in detail by Bonin et al. (2012). Our genotyping error rate, estimated by repeatedly genotyping 6 samples (3.5 % of the dataset) was 0.025 per reaction. The rate of missing data was 3%.

Genetic data analysis

Deviations from Hardy-Weinberg and linkage equilibrium were evaluated using the program CERVUS (Marshall et al., 1998), which was also used to estimate the probability of parental exclusion based on our marker panel.

To determine which individuals had been re-sampled within and among years, we tested for identical multilocus genotypes using CERVUS. We then investigated relationships among the sampled individuals. To fully exploit the fact that our dataset contained putative fathers, mothers, half sibling pups and full sibling pups, we opted for the likelihood method employed by COLONY2 (Wang and Santure, 2009; Jones and Wang, 2010), which performs parentage and sibship assignments simultaneously. COLONY2 uses multilocus genotypes to partition samples into clusters that contain individual(s) linked by parentage, sibship or both. The method then estimates the likelihood of each data partition based on Mendelian rules, which correspond to the product of the likelihood of clusters within partitions. Because there can be numerous configurations for a given dataset, a simulated annealing algorithm searches for the best configuration, each time making small adjustments (i.e. relationship re-assignments) until the configuration with the highest likelihood is reached. Uncertainty is estimated by calculating how often a true dyad relationship is not excluded at the 95% confidence interval.

As described above, clusters may contain full and half-siblings and COLONY2 infers the presence of any "unsampled parent(s)" that are needed to explain the observed relationships. This allows estimation of the total number of parents, and hence the number of individuals that may have evaded our sampling efforts. Within COLONY2, we opted for the

full likelihood method, with intermediate run lengths, and included a standard 1% marker error rate across all loci.

RESULTS

To determine the mating system of a low-density colony of Antarctic fur seals at Livingston Island, we collected 172 tissue samples during four consecutive breeding seasons and genotyped these at 13 microsatellite loci. After excluding duplicate genotypes (see subsequent section), no significant deviations from Hardy-Weinberg equilibrium were observed (lowest p value = 0.10 for locus Pvc29; the results for remaining loci are not shown). The average number of alleles/locus was 11.69 ± 4.32 (range: 6 - 19) and expected heterozygosity (HE) = 0.82, resulting in high power for parentage and identity analysis (non-exclusion probabilities were 8.1×10^{-5} for the first parent, 1.24×10^{-11} for a pair of parents and 5.12×10^{-18} for individual identity). Similarly, paternity assignment probabilities (estimated by COLONY2) indicated high certainty (100% probability) with only a single exception. Most maternities (93%) were also assigned with 100% probability and only two assignments had probabilities below 97%.

Repeatedly sampled adult males

The program CERVUS detected a total of 21 adult genotype matches, representing genetic recaptures of six males and five females across seasons. Two males were recaptured once, two across three seasons, and one across all four seasons. Sampling locality records indicated strong male site fidelity, with all but a single male being recaptured in the same sub-unit of LI- East. The one exception was male A, who was recaptured at an adjacent sub-unit to where he was initially sampled. This was the only recaptured male who was not assigned any paternities.

Parentage analysis

The number of sampled pups ranged from 22 to 38 for the first three seasons, but was lower at 12 individuals during 2009-10 when the site was largely covered with snow and ice. Paternity was assigned to a total of 34 pups using the program Colony (see Table A2-1 Appendix 2-1). Although the paternity assignment was highest for pups sampled during the last breeding season (2009-10, 63%) and lowest (17%) for pups sampled during 2007-08, the overall rate of paternity assignment did not vary significantly across seasons ($\chi 2 = 7.07$, df= 3, $p = 0.07$; Figure 2-1).

Male reproductive success was highly skewed (Figure 2-2), with only 5 out of a total of 23 unique males being assigned any paternities. Of the successful males, four were genetically recaptured in multiple seasons, implying reproductive longevity. The remaining male, who was sampled only once, was assigned a single paternity (this male was not considered as a "top territorial male"). Two males (“A” and “D” in Figure 2) were disproportionately successful, being assigned 13 and 15 paternities respectively during the course of the study, and a maximum of 8 within any season. Together, these two males accounted for 28% of all sampled pups (n = 97) and 82% of all paternities (n = 34). These individuals both sired pups in at least three consecutive seasons. However, male A's success was disproportionately high during the first season and declined thereafter, whereas male D's success remained relatively high and constant for at least three seasons (Figure 2-2).

Maternity

Of 31 adult females sampled, 29 were assigned as mothers of 59% of the pups. The highest maternity assignment rate was obtained for the last season (2009-10) when all pups had their mothers sampled, and the second highest (78%) was obtained for the previous season (2008-09), when adult female sampling was initiated. Out of the total of 29 mothers, 17 pupped during at least two seasons (58%) and 10 of these (34%) returned to pup for three or four seasons.

Unsampled parents

COLONY2 derived a total of 28 fathers and 18 mothers for the entire progeny (97 pups), implying that our sampling captured 20% of total fathers and 61% of mothers over all seasons. Unsampled fathers were estimated by COLONY2 to have sired an average of 2.2 ± 1.28 pups, with a single male accounting for 8 pups. We found that half of these pups were born in the first and second year of the study, implying the presence of a single highly successful male in the colony during the year before we began sampling.

Full siblings

A total of eleven full-siblings were identified (Table 2-2), corresponding to 11% of all pups in the dataset (n = 97). All but two of the full sibling pairs were identified at high confidence, with assignment probabilities equal to or greater than 96%. Among the cases with

high assignment probabilities, we found two triads of full sibling pups, implying that their parents re-mated consecutively across three seasons.

DISCUSSION

Empirical investigations on the interaction between mating systems and population density are paramount to understanding the mechanisms that influence the plasticity of reproductive behavior. However, few studies allow such investigations in natural populations, particularly for long-lived mammals that require multiple seasons of data to accumulate reasonable measures of reproductive success. Antarctic fur seals provide such an opportunity as this species' breeding behavior has been well described for a colony with extremely high density (SG), and here for the first time, we provide comparative data to those studies. We conducted extensive sampling at a low-density Antarctic fur seal breeding site (LI) during four years with the objective of investigating male reproductive success over time. We found high reproductive skew with only 2 out of 23 sampled males being exceptionally successful. These males held the majority of paternities during four seasons, and their reproductive success significantly surpassed the maximum recorded for the species. Our study demonstrates that at lower population densities, individuals can achieve surprisingly high levels of reproductive success likely due to reduced competition.

At face value, the lifetime reproductive success of the most successful males was significantly higher at LI than at SG. Nevertheless, there are some caveats in comparing the results from the studies conducted in these two populations. A direct comparison is challenged by the differences in how studies at SG and LI were conducted. First and most obvious, are the differences in the sampling schemes adopted at the two locations. At SG, the study colony was sub-sampled because the number of pups there (660 pups born annually; Hoffman et al.,

2003) are too numerous to be accounted in their totality. Therefore, it is quite possible that additional pups fathered by the most successful males at SG were missed. Secondly, the sampling at SG only targeted a portion of the total number of territorial males (Hoffman et al., 2003), thus, other very successful males could have been missed there. Third, the SG study spanned two additional seasons (Hoffman et al., 2003) to this study, allowing for a longer-term view of male turnover there. Although these caveats indicate that careful consideration should be taken when interpreting these inter-population comparisons, the maximum number of pups sired within a season was always eight at the two locations, suggesting that seasonal harem size is fairly conserved across populations of Antarctic fur seals. In support of this idea, is the interesting fact that harem size counts ranged between 6 and 11 females at SG in the late 1950's when seals bred at much lower densities there (Bonner, 1968). This consistency is rather encouraging, implying that the comparisons regarding the lifetime reproductive success of the top territorial males at these two locations is likely valid.

The high lifetime reproductive success achieved by LI males can be linked to low colony density in two ways. First, reduced competition may confer LI males an increased ability to control female movements. This has been observed in Northern fur seals, *Callorhinus ursinus*, where population reduction affected male breeding strategies: at lower densities, a higher number of paternities are now attributed to territorial males (Kiyota et al., 2008). If indeed female movements are under stronger control by males at LI than what is observed at SG, the finding of female choice (Hoffman et al., 2007) for Antarctic fur seals may not be applicable to lower density colonies such as LI, where female movements are fairly restricted. Secondly, at lower densities, LI males might be able to remain successful for more seasons. Extensive data collected at SG suggests that most pups are sired when males are in their first or second year of tenure (Hoffman et al., 2003). The data compiled here show that the top males were actually successful for three and four seasons, which is also corroborated

by the finding of full siblings born across three consecutive seasons. Admittedly, our sample size is fairly small and a number of territorial males were missed. Although every effort was made to sample all adult males at LI, a considerable number of them escaped our attempts. During the peak of breeding, individual male turnover can be fairly high, making it challenging to track and sample all individuals (Hoffman et al., 2003). As well, our data shows reduced female breeding site fidelity, revealing that only a small number actually remained at our breeding site for more than three seasons. This suggests that females breeding at our study site, often conceived from males elsewhere, posing an additional challenge for paternity assignment. A more extensive study encompassing nearby breeding sites at LI would be necessary to boost sample sizes and strengthen our interpretations.

Rematings

Our analyses confidently detected multiple cases of full siblings, and even two cases where the same parents re-mated consecutively for three seasons. This finding highlights an implication of strong breeding site fidelity for some individuals, which may lead to a higher proportion of full siblings within a population than might be expected under a system where individuals distribute themselves and mate randomly. A higher incidence of full siblings than expected by chance has been observed in grey seals, *Halichoerus grypus* (Amos et al., 1995). In this case, it was proposed that a compensating mechanism must exist (e.g. females mating with highly heterozygous males) in order to avoid inbreeding depression (Amos et al., 2001). Given that heterozygosity correlates with virtually every fitness trait so far measured in this species (Hoffman et al 2004, Hoffman et al 2007, Hoffman et al 2010), it would be interesting to explore, using a larger sample of pups from LI, if full siblings indeed occur at a high rate within this population and whether such mechanisms could also be operating at LI. More

generally, the question arises as to whether female behaviors that have evolved to maximize offspring heterozygosity could be density dependent in their expression.

In spite of shortcomings, four years of observational and sampling effort at Livingston Island has provided us with a unique opportunity to investigate the reproductive success of males at a low-density breeding site. We found high reproductive skew with a few individuals achieving remarkable lifetime reproductive success at our study site. Taken with caution, comparisons with the findings from the high-density colony of SG seem to indicate that LI males may enjoy more seasons of successful breeding and perhaps exert stronger control over female movements. Overall, this work points out interesting new avenues for mating system studies in Antarctic fur seals, as they may reveal novel insights into how population density affects polygyny.

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Table 2-1: Number of Antarctic fur seal, *Arctocephalus gazella*, samples collected at Livingston Island during four austral summers and genotyped at 13 microsatellite loci. 'Unique samples' refers to the numbers of samples remaining after excluding those with identical multilocus genotypes (i.e. duplicates).

	Breeding season				**Samples**	
Sample type	2006-07	2007-08	2008-09	2009-10	Collected	Unique
Pups	38	22	29	12	101	97
Adult males	8	9	6	10	33	23
Adult females	1	0	25	12	38	31
Total - season	47	31	60	34	172	151

Table 2-2: Full sibling dyads (Pup IDs 1 and 2) identified among 97 Antarctic fur seal pups, *Arctocephalus gazella*, sampled at Livingston Island, Antarctica between 2006-07 and 2009-10 inclusive. Samples were genotyped for 13 microsatellite loci and relationships among individuals were determined using a full pedigree inference method (COLONY2; Jones and Wang, 2010). Probabilities of relationship assignment for each dyad are shown as well as whether their parents were included in the analysis. Note that cases 1 and 2 both represent triplets of full siblings born in three consecutive seasons to the same parents.

Case	Pup ID 1	Pup ID 2	Probability	Mother	Father
1	62420 (2006-07)	74459 (2007-08)	100%	N	Y
	62420 (2006-07)	78218 (2008-09)	100%	N	Y
	74459 (2007-08)	78218 (2008-09)	100%	N	Y
2	62440 (2006-07)	74452 (2007-08)	100%	Y	Y
	62440 (2006-07)	78235 (2008-09)	100%	Y	Y
	74452 (2007-08)	78235 (2008-09)	100%	Y	Y
3	62442 (2006-07)	74450 (2007-08)	100%	Y	N
4	62436 (2006-07)	74451 (2007-08)	100%	Y	N
5	78246 (2008-09)	93050 (2009-10)	96%	Y	Y
6	62437 (2006-07)	74457 (2007-08)	27%	Y	N
7	74456 (2007-08)	78245 (2008-09)	91%	Y	N

Fig. 2-1: Number of paternity assignments of Antarctic fur seals, *Arctocephalus gazella*, at Livingston Island, Antarctica. The number of pups sampled each breeding season is also shown.

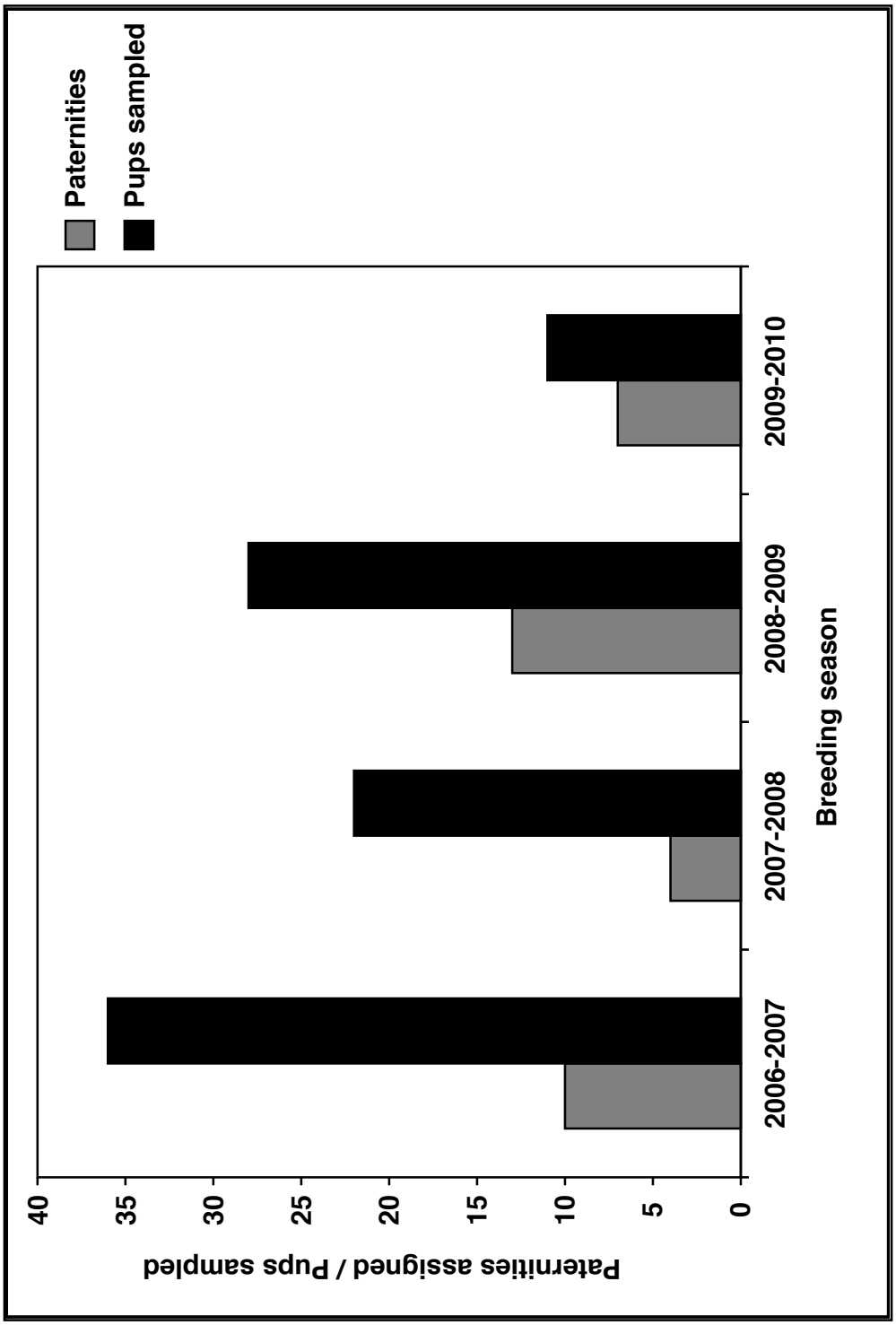
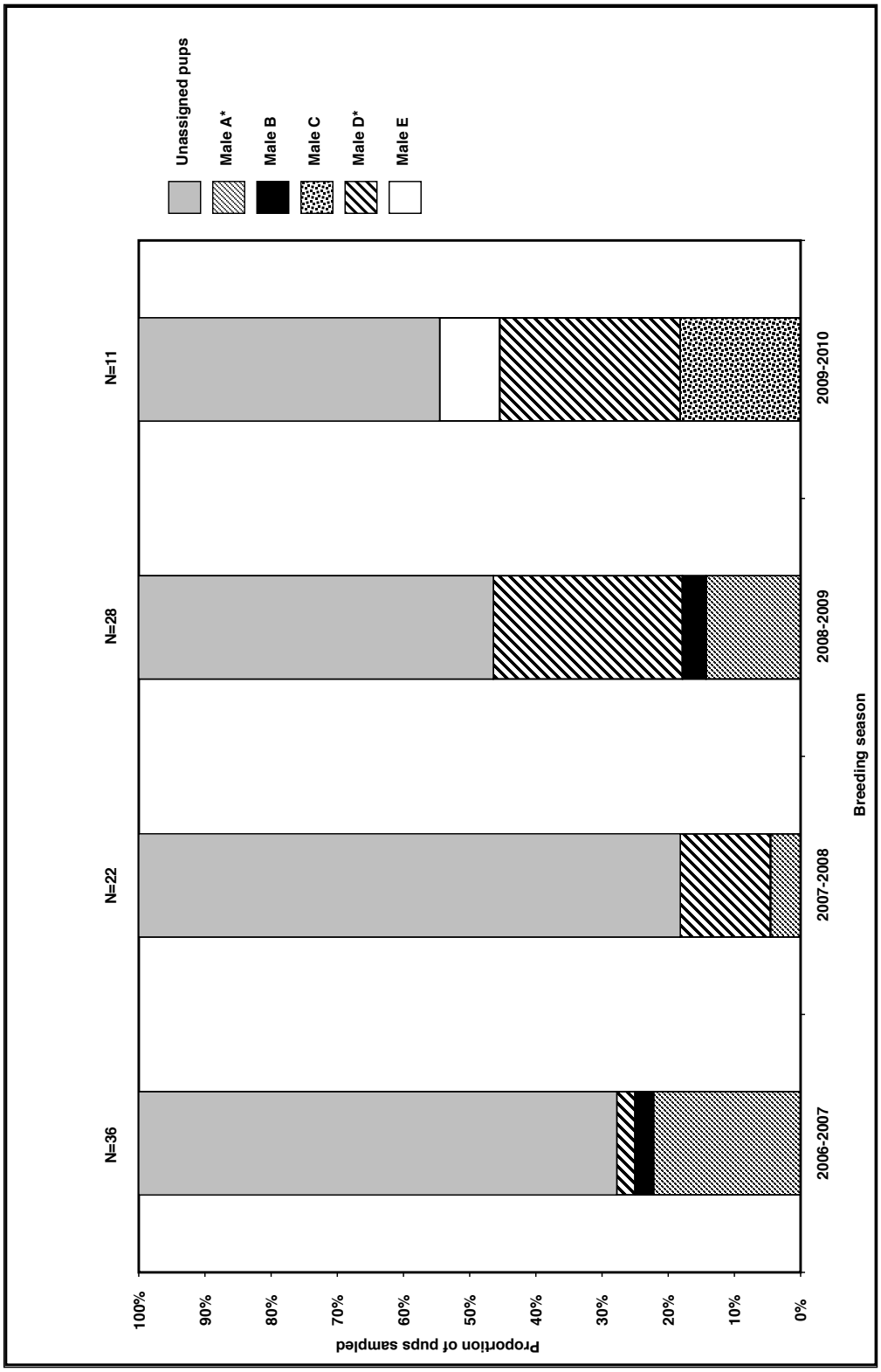

Paternities
Pups sampled
Paternities assigned / Pups sampled
40
35
30
25
20
15
10
5
0
2006-2007
2007-2008
2008-2009
2009-2010
Breeding season

Fig. 2-2: Reproductive success of adult male Antarctic fur seals, *Arctocephalus gazella*, genetically re-captured at Livingston Island, Antarctica, during the austral summers of 2006-07 through 2009-10. Reproductive success is expressed as the proportion of paternities assigned to each male relative to the number of pups sampled per season (shown at the top of the plot columns).


Unassigned pups
Male A*
Male B
Male C
Male D*
Male E
N=36
N=22
N=28
N=11
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Proportion of pups sampled
2006-2007
2007-2008
2008-2009
2009-2010
Breeding season

APPENDIX 2-1

Table A2-1: Paternity and maternity results for Antarctic fur seals, *Arctocephalus gazella*, sampled (n= 97 pups, 23 adult males and 31 adult females) at Livingston Island during four breeding seasons/ austral summers (2006-07 through 2009-10). Results were obtained using the full likelihood method implemented by COLONY2 (Jones and Wang, 2010). The five-digit sample IDs (e.g. 62399) correspond to sampled individuals while one and two-digit numbers (e.g. *1) are unsampled parents inferred by COLONY2. Probabilities of paternity and maternity assignment (0-1) are also shown.

Pup ID*	Pup cohort	Father ID*	Paternity	Mother ID*	Maternity
62399	2006-07	*1		#1	
62400	2006-07	*2		#2	
62401	2006-07	*3		#3	
62402	2006-07	*4		#4	
62403	2006-07	*5		#5	
62408	2006-07	62404M	1	93056F	1
62409	2006-07	62404M	1	#6	
62410	2006-07	*6		93059F	1
62411	2006-07	*6		#7	
62412	2006-07	62404M	1	#8	
62413	2006-07	*7		#9	
62414	2006-07	62404M	1	78233F	1
62415	2006-07	*8		#8	
62416	2006-07	*9		#4	
62417	2006-07	*10		#10	
62418	2006-07	62404M	1	78227F	1
62419	2006-07	*11		#11	
62420	2006-07	62404M	1	#12	
62421	2006-07	62404M	1	#13	
62422	2006-07	62404M	1	#14	
62423	2006-07	*12		#15	
62424	2006-07	*13		#2	
62425	2006-07	62405M	1	78234F	1
62430	2006-07	*14		78252F	1
62431	2006-07	*15		93062F	1
62432	2006-07	*16		78258F	1
62433	2006-07	*17		#7	
62434	2006-07	*3		#15	
62435	2006-07	*8		#6	

Table A2-1 Continued

Pup ID*	Pup cohort	Father ID*	Paternity	Mother ID*	Maternity
62436	2006-07	*17		78257F	1
62437	2006-07	*18		78261F	1
62438	2006-07	*17		#10	
62439	2006-07	*19		#11	
62440	2006-07	62427M	1	78255F	1
62441	2006-07	*20		#3	
62442	2006-07	*17		78260F	1
74449	2007-08	*17		78258F	1
74450	2007-08	*17		78260F	1
74451	2007-08	*17		78257F	1
74452	2007-08	62427M	1	78255F	1
74453	2007-08	*19		#15	
74454	2007-08	62427M	1	78253F	1
74455	2007-08	*17		93062F	1
74456	2007-08	*15		78252F	1
74457	2007-08	*1		78261F	0.438
74458	2007-08	*20		78254F	0.975
74459	2007-08	62404M	1	#12	
74460	2007-08	*2		78271F	1
74461	2007-08	*10		78227F	1
74462	2007-08	*21		#16	
74463	2007-08	*21		#14	
74464	2007-08	62427M	1	#3	
74465	2007-08	*9		78233F	1
74466	2007-08	*13		#17	
74467	2007-08	*19		78230F	1
74468	2007-08	*16		#18	
74469	2007-08	*14		#11	
74470	2007-08	*22		#1	
78211	2008-09	*7		78234F	1
78212	2008-09	*23		78231F	1
78213	2008-09	62404M	1	78228F	1
78214	2008-09	*16		#10	
78215	2008-09	*23		78227F	1
78216	2008-09	62404M	1	78230F	1
78217	2008-09	62405M	1	78226F	1
78218	2008-09	62404M	1	#12	
78219	2008-09	*24		78229F	1

Table A2-1 Continued

Pup ID*	Pup cohort	Father ID*	Paternity	Mother ID*	Maternity
78220	2008-09	62404M	1	#17	
78221	2008-09	*8		78233F	1
78225	2008-09	*18		#17	
78235	2008-09	62427M	1	78255F	1
78237	2008-09	62427M	1	78260F	1
78238	2008-09	*20		78250F	1
78239	2008-09	*3		#5	
78240	2008-09	*25		78251F	1
78241	2008-09	*26		78262F	1
78242	2008-09	62427M	1	#15	
78243	2008-09	*12		78261F	1
78244	2008-09	62427M	1	78257F	1
78245	2008-09	62427M	1	78252F	1
78246	2008-09	62427M	1	93062F	0.974
78247	2008-09	62427M	1	78254F	1
78248	2008-09	62427M	1	78258F	1
78263	2008-09	*21		78271F	1
78264	2008-09	*4		78270F	1
78265	2008-09	*11		78269F	1
93043	2009-10	62406M	1	78230F	1
93044	2009-10	62406M	1	93056F	1
93045	2009-10	*5		93057F	1
93046	2009-10	78268M	1	78270F	1
93047	2009-10	*27		93059F	1
93048	2009-10	78267M	1	78269F	1
93049	2009-10	62427M	1	93061F	1
93050	2009-10	62427M	1	93062F	0.932
93052	2009-10	62427M	1	78261F	1
93053	2009-10	*28		93065F	1
93054	2009-10	*22		93066F	1

* Five-digit sample ID corresponds to accession numbers at the Marine Mammal and Marine Turtle Molecular Research Collection, at the Southwest Fisheries Science Center- NOAA Fisheries, La Jolla, CA.

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CHAPTER 3: Rematings are rare among Antarctic fur seals (*Arctocephalus gazella*) despite high levels of site fidelity and polygyny.

ABSTRACT

Genetic studies of polygynous mating systems have revealed unexpected complexities such as extra-pair paternities and alternative mating strategies. However, few studies have addressed mating patterns among individuals over time. Antarctic fur seals are polygynous and both genders present high levels of breeding site fidelity, so remating across breeding seasons could be common. To investigate this hypothesis, we monitored the reproductive output of 55 females, and opportunistically sampled a subset of their pups (n=280) born from 1997-2009 at Livingston Island, Antarctica (62°27'30"S, 60°47'17"W). These females had a mean of 5 ± 1.28 pups. All samples were genotyped using 17 microsatellite markers and a full likelihood pedigree inference method was used to estimate the number of full sibling pups born across years, which represent cases of remating between their parents. We found 12 full sibling pairs in our dataset, indicating 4.2% remating of parents. Although this number was significantly higher than what would be expected under random mating, it is relatively low if we consider the strong breeding site fidelity and high male reproductive skew observed in this species. We suggest that female movements within and among beaches over time reduce chances of remating. The relatively lower site fidelity of females, who are driven by suitable pupping and nursing habitat, is probably an important indirect factor contributing to the low remating rate observed in this species.

INTRODUCTION

Field observations of individual reproductive history allow for pedigree reconstruction and provide the basis of genealogical hypotheses, which can be subsequently tested through genetic analysis (Pemberton, 2008; Clutton-Brock & Sheldon, 2010). Indeed, an assortment of molecular techniques now allow investigators to couple genetics with field observations (see reviews by Blouin, 2003; Jones & Ardren, 2003; Jones et al., 2010; Jones & Wang, 2010b). These varied approaches have revealed extraordinary diversity within vertebrate mating systems. For example, the diversity among mating systems in bony fishes (reviewed by Avise et al., 2002) and discovery of high levels of extra-pair paternity in birds (reviewed by Griffth et al., 2002), were made possible by these advances. Such discoveries have had a direct impact on our assessment of fundamental ecological and evolutionary parameters such as demography, selection, inbreeding, and effective population size (Nunney, 1993; Sugg et al., 1996; Nomura, 2008; Lotterhos, 2011). As we try to predict how populations will respond to environmental change, an understanding of mating systems is essential for determining a species' susceptibility to changes in population size.

Polygyny is characterized as the breeding of one or more males (uni- or multi-male polygamy) with several females during a breeding interval (Clutton-Brock, 1989). Although this mating system has been well studied in a variety of mammals, rarely has the focus been on mate fidelity over time. An exception is the study of grey seals, *Halichoerus grypus*, which revealed that mate fidelity is more common than what would be expected by chance alone (Amos et al., 1995). Moreover, these authors hypothesized that some sort of female choice for highly heterozygous partners must occur in order to compensate for mate fidelity, which could lead to inbreeding (Amos et al., 2001). Detailed studies of polygyny in wild populations should reveal further complexities within this mating system, providing insights as to how and

why genetic diversity is maintained and thereby expand the scope of evolutionary theory (e.g. Pérez-González et al., 2009).

Several attributes of Antarctic fur seal, *Arctocephalus gazella*, biology make this a good model for exploring the complexities of polygynous mating systems. Male Antarctic fur seals arrive on traditional breeding beaches in early November and compete with other males to establish territory (McCann, 1980). Dominant, territorial bulls are responsible for siring the majority of offspring (terrestrial polygyny: Bonner, 1968; Hoffman et al., 2003). Females arrive at breeding sites a few weeks after the males, and typically give birth to a single pup within a few days of arrival; they become receptive once annually 6-7 days after giving birth (Doidge et al., 1986; Lunn & Boyd, 1991). Female Antarctic fur seals present high pupping site fidelity and, like males, are faithful to breeding territories across seasons (Lunn & Boyd, 1991; Hoffman et al., 2006). Female Antarctic fur seals can be captured, tagged, identified, and tracked. To date, the Antarctic fur seal mating system has been extensively documented at Bird Island (South Georgia; SG) where animals breed at very high densities, but geographic variability regarding the reproductive biology of this species is poorly known.

Antarctic fur seals have, at least in part, recovered from near-extinction that resulted from intense sealing in the 1800s. Breeding colonies are circumpolar, located on islands throughout the Southern Ocean. The SG population is the largest and most dense Antarctic fur seal colony in the world (possibly > 4 million; Hofmeyr et al., 2005). In the South Shetland Islands, the fur seal population apparently recovered from less than 40 animals (counted in the late 1950's; O'Gorman, 1961) to an estimated 21,000 animals by 2002 (Hucke-Gaete et al. 2004). Despite intense exploitation, high levels of genetic diversity remain within populations (Wynen et al., 2000). Genetic analyses have indicated a steep demographic decline, but clear evidence of a genetic bottleneck is lacking (Hoffman et al., 2011).

At the remote Livingston Island (LI), situated within the South Shetland Islands archipelago, the Antarctic fur seal population has been monitored by the US Antarctic Marine Living Resources Program (US AMLR) for nearly 15 years. The primary goal of this effort has been to assess life-history parameters over time (e.g. attendance behavior, pup production and foraging locations). Individual seals have been tracked for over a decade (1997-2010) and, tissue samples from known-age females and their offspring were collected. By genotyping these samples using 17 highly polymorphic microsatellites and making use of novel full pedigree inference methods, we have investigated mating patterns among individuals over time and examined this species' polygynous mating system in detail. Here we focused our study on the following questions: (i) Given that both females and males present high levels of breeding site fidelity, how often does remating occur among sampled individuals? and (ii) How does the observed proportion of remating compare to expectations of random mating over time?

MATERIALS AND METHODS

Field data collection and sampling

This study was conducted at Cape Shirreff (62°27'30"S, 60°47'17"W) (Figure 3-1) which is an ice-free peninsula located on the northern coast of LI, Antarctica. Fur seal observation and sampling encompassed 10 pupping beaches at Cape Shirreff (Figure 3-1) and were carried out during the austral summers (December to March) of 1997/1998 through 2009/2010.

The reproductive output of 55 adult female fur seals was monitored for purposes unrelated to this study, so although efforts were made to sample every pup born to these

females, tissue- sampling was opportunistic. Therefore, we observe: (i) that our dataset does not represent a sub-sample of a single beach at Cape Shirreff, (ii) that this study focuses on females whose movements and pupping records were monitored over time, and (iii) no Antarctic fur seal adult males were handled or tagged for this study so we cannot directly investigate male reproductive behavior. In spite of these caveats, extensive effort at this remote field location provided invaluable data regarding long-term mating patterns through a matrilineal pedigree, and allowed us to focus on individual seals throughout their lives in a fashion that has never before been possible.

In the field, individual seals were sampled according to the following scheme: (1) Adult female seals were captured using a net and gas anesthesia methods described in Gales and Mattlin (1998). (2) While captured, the seals were tagged with a unique identification number (Dalton Jumbo Rototags, Dalton ID systems, UK). The excess tissue resulting from tagging was collected using sterile material. (3) Also, most seals had a post-canine tooth extracted for aging by methods described in Arnbom et al. (1992) and McCann (1993). (4) Once captured and tagged, seals were monitored every year upon arrival at a breeding site. The study area was monitored daily from mid-November until early March. On first return, seals were visually checked for pregnancy. If a birth was not witnessed, nursing behavior and consistent non-aggressive interactions were used to verify maternity, avoiding the sampling of pups that could be mistakenly assigned as offspring of a given female. (5) Female pupping beaches were recorded during every breeding season. (6) Pups born to the tagged females had a tissue sample collected using sterile biopsy punches, taking 2mm of skin from a rear flipper. All tissue samples collected were stored in either 20% dimethylsulfoxide (DMSO) saturated with sodium chloride (NaCl) or 95% ethanol.

Genetic Data

Total genomic DNA was extracted from tissue samples using a NaCl precipitation method (adapted from Miller et al., 1988). After extraction, the genomic DNA was amplified for 17 microsatellite markers (Table 3-1): Ag10 (Hoffman et al., 2008), Agaz8, Agaz9 (Hoffman, 2009); Hl4, Hl16, Lc28 (Davis et al., 2002); Hg3.7 (Gemmell et al., 1997); M11A, M2B (Hoelzel, 1999); Pvc29, Pvc78 (Coltman et al., 1996); ZcCgDh1.8, ZcCgDh4.7, ZcCgDh48, ZcCgDh5.8, ZcCgDh7tg, ZcCgDhB.14 (Hernandez-Velasquez et al., 2005). Genotyping protocols, raw data editing, and basic information on these loci are detailed by Bonin et al. (2012).

An identity analysis of the dataset was conducted using CERVUS v.3.0.3 (Marshall et al., 1998) to verify the presence of duplicate individuals that may have been accidentally re-sampled in the field. Genotypes from a randomly collected sample of pups (N= 94; published in Bonin et al., 2012), and the fur seal mothers used this study (n=55), were combined to assess all basic statistics pertinent to population-level microsatellite data. Using this dataset (total n=149), deviations from expected Hardy-Weinberg proportions (heterozygote deficit) and linkage disequilibrium were calculated using 100,000 dememorizations and 10,000 iterations per batch within GENEPOP v.4.0 (Raymond and Rousset, 1995).

To estimate genotyping error rates, we searched for mismatched genotypes between putative mothers and offspring at each locus. Bonin et al. (2012), demonstrated that when maternity was excluded using a similar panel of markers, there were typically six or seven locus mismatches between Antarctic fur seal mother-offspring pairs. Thus, we considered that 1 or 2 mismatches out of the 17 total loci were likely to be genotyping errors. The genotyping error rate was then estimated as the number of mismatches between mother-offspring pairs

over the total number of calls for that locus. We compared the results obtained from our error rate assessment to the error estimated for 15 out of the 17 loci presented in Bonin et al. (2012).

Remating among individuals over time

The number of full sibling pups within a maternal family is indicative of whether a pair of pups shared the same set of parents. Thus, the identification of full siblings in our dataset would suggest remating over time. In order to detect full sibling pups in the dataset and confirm the maternities inferred in the field, we used the full pedigree inference method of COLONY2 (Wang & Santure, 2009; Jones & Wang, 2010a). This method makes most use of the available genetic information because it infers multiple relationships simultaneously, considering all samples jointly. This approach that has been shown to outperform methods that consider pairs of individuals in a step-wise fashion (Walling et al., 2010). COLONY2 partitions individuals based on their genotypes into clusters or "family groups". Clusters contain individuals linked by parentage, sibship or both. COLONY2 also infers the presence of “unsampled parents”, necessary to explain the relationships among the individuals within clusters. Considering this information, it is possible to estimate the number of unsampled parents (in our case, fathers) for a given progeny, especially when other relationships within the clusters are known (e.g. maternity). The likelihood of a certain data partition is estimated based on Mendelian rules, and it corresponds to the product of the likelihood of the clusters. Because there can be numerous configurations for a given dataset, a simulated annealing algorithm searches for the best configuration as relationship re-assignments are successively tested, until the configuration with the highest likelihood is reached. Within this method, uncertainty is estimated by calculating how often a true dyad relationship is not excluded at the 95% confidence interval; ideally this frequency should be ≥ 0.95.

To determine whether COLONY2 could infer families based solely on genetic data, an initial run of the dataset without any “a priori” relationship information (field data) was conducted (n=351). After an assessment of this preliminary run, genetic and field information were both included in the analysis (n=351). The analysis in COLONY2 was conducted using the full likelihood method (intermediate precision and length runs), including marker error rates. In order to secondarily assess COLONY2 relationship assignments, we also performed an independent relatedness coefficient (rxy) calculation for all pairs of individuals in the pedigree using COANCESTRY v.1.0 (Wang, 2011). We only report rxy values obtained using Milligan's algorithm (Milligan, 2003) as this estimator has had a comparatively better performance in a study utilizing a similar marker panel on the same species (Bonin et al., 2012).

Observed mate fidelity vs. expectations of random mating over time

In order to test whether observed mate fidelity rates differed from expectations of randomness, we performed a simulation according to the following steps. (1) We simulated Poisson distributions for the number of pups that 55 females would have if randomly mating with one male at a time, sampling from a pool of 153 males (the number of males, or unsampled parents, was estimated by the analysis in COLONY2, as described above). The mean number of pups per female was set to 5 (= the mean number of pups per female in our study). The total number of pups was not constrained and varied around a mean of five at each iteration. (2) We set the simulation for 100,000 iterations and summarized the results by counting the percentage of remating events and the total number of pups obtained at each iteration. Finally, we obtained the total expected percent of mate fidelity for a range of total

pups but focused on the results relevant to our dataset (n= 280 pups). This simulation was conducted in R v.2.14.0 (R Core Development Team, 2008).

Female pupping beach fidelity

After full sibling pairs were identified in our pedigree, we compiled and counted their mother's pupping beach records from all years. This was done to quantify the number of times females moved between beaches and assess overall levels of female beach pupping fidelity at LI.

RESULTS

Field data collection and sampling

We obtained genetic information on 59% of offspring born to our study females (μ= 5 ± 1.28; range= 2 to 8 pups genotyped per female; Table 3-2). In summary, the number of genotyped individuals available for the analysis included 55 females (54 of known age, born 1985-1998) and 280 pups (born 1999-2010; 115 females, 91 males and 74 unidentified gender).

We found 17 mismatched genotypes between putative mother-offspring pairs (out of 291 comparisons) and on average, mismatches occurred at 1.11 ± 0.33 of the 17 loci. We did not detect any errors for 10 loci (Ag10, Agaz9, Hl4, Hl16, M11A, Pvc29, Pvc78, ZcCgDh5.8, ZcCgDh7tg, ZcCgDhB.14). For the remaining loci the genotyping error rate varied: it was estimated at 0.003 for loci Hg3.7, M2B, ZcCgDh1.8 and ZcCgDg48 and 0.015, 0.013, and 0.01 for loci Agaz8, Lc28 and ZcCgDh4.7, respectively. Overall, these rates are comparable to

the findings of Bonin et al., (2012) for a very similar marker panel which were all estimated < 0.01, using blind sample replication. Because error assessments are typically underestimated when using mother-offspring pairs (Hoffman and Amos, 2005b), we opted to apply a minimum error rate of 0.01 to all loci (including the loci for which no error was detected among mother-offspring pairs).

Missing data were rare in our dataset, as we collected 97.4% of genotypes for all samples analyzed (all individuals were scored at a minimum of 10 loci). The mean number of alleles per locus was 13.94 (range: 6 - 29), and the mean expected heterozygosity (HE) was 0.82. Together, these yielded a combined exclusion probability (PE) of 0.9999. None of the loci presented significant deviations from Hardy-Weinberg equilibrium expectations (Table 3-1) and there was no evidence for linkage among the marker loci.

Estimating remating over time

COLONY2 recovered the same maternal families inferred by field observation, regardless of whether "a priori" relationship information was included in the analyses, indicating high marker power and robustness of the method. The analysis revealed 12 full siblings out of 280 pups sampled (Figure 3-2). This indicates that the same parents mated more than once 4.3% of the time. Full siblings were distributed among 10 maternal clusters (1 cluster had 3 full sibling pups- Table 3-3).

High confidence of the COLONY2 analysis was supported by high relationship assignment probabilities and results of the relatedness analysis using COANCESTRY. All maternities were reliably confirmed with a 100% probability of assignment. Similarly, full and half-sibling relationship assignments had high confidence: the mean probability of assignment was 99% ± 5% for half siblings and 98% ± 6% for full siblings. Uncertainty has higher for one

of the full sibling cases, where the probability of assignment was 78%. However, in this case, the alternative relationship (half sibling) had a much lower probability (21%), so this pair's relationship was counted as a full sibling case. The relatedness analysis in COANCESTRY revealed that the relationships assigned by COLONY2 matched theoretical expectations for each of the relationship categories as follows: all mother-offspring rxy values were narrowly distributed around the mean 0.51 ($\sigma 2$= 0.001), full sibling dyads had mean rxy of 0.49 ($\sigma 2$= 0.023) and half sibling dyads had mean rxy= 0.25 ($\sigma 2$= 0.012).

Testing against expectations of random mating

The simulation of random mating resulted in a proportion of full sibs = 0.7% (0.007 ± 0.006; 95% quantile = 0.017; Fig. 3). Therefore, the observed proportion of remating (4.3%) was significantly higher than what would be expected if mating occurred at random over time.

Male tenure

The time interval between full-sib births was variable. In four cases out of the 10, this interval was three or four years (Table 3-3). Therefore, full siblings were not born significantly more frequently in subsequent seasons than at longer intervals (Fisher exact test, p= 0.71), indicating that a considerable number of males who remated with our study females remained in their territories and successfully sired pups for at least three years. The total number of (unsampled) fathers inferred by COLONY2 for the entire dataset was 153. So on average males sired < 2 pups each in the data set.

Female beach pupping site fidelity

Pupping beach (location) records were available for 92% of the 280 offspring of our study. Considering the female movements, 65% of females presented absolute pupping beach fidelity, always returning to the same beach to give birth. Nineteen out of 55 females moved from one beach to another at least once and the mean number of pupping beaches used by females over time was 1.41 ± 0.62.

DISCUSSION

The exhaustive monitoring of female Antarctic fur seals over a decade at a remote Antarctic Island provided us with a unique opportunity to investigate the long-term mating patterns of a highly polygynous species and to assess how often individuals remate during the course of their life. Remating occurs at a significantly higher rate than it would be expected under a scenario of random mating. However, this rate can be considered unexpectedly low if we take into account the high breeding site fidelity and male reproductive skew observed in this species. We propose that some aspects of female and male behavior may preclude remating from occurring more often.

Given that Antarctic fur seals are highly polygynous and that both male and female Antarctic fur seals present high levels of breeding site fidelity led us to predict that remating should be common in this species. At the crowded colony of Bird Island, SG, a quarter of paternities were attributed to only 12 territorial males (660 pups and 415 males were sampled; Hoffman et al., 2003). Similarly, a study at LI demonstrated that nearly 30% of paternities at a small beach were attributed to only 2 territorial males (97 pups and 23 males were sampled; C.

Bonin, unpublished data). This reproductive skew is similar to what is observed in Northern fur seals, *Callorhinus ursinus* (Kiyota et al., 2008), but not nearly as extreme as in elephant seals, *Mirounga leonina*, where harem holders can obtain nearly 90% of paternities (Fabiani et al., 2004). Regarding breeding site fidelity, Antarctic fur seal males are extremely faithful to their breeding site, as over half of them returns to within one body length of the territory they occupied during the previous season (Hoffman et al., 2006). Females are also site-faithful (Lunn & Boyd, 1991): 65% return to the previous pupping beach over multiple years (this study). Thus, conservatively, if at least 50% of males and females are returning to the same beach, there is roughly a minimum chance of re-encounter among parents of 25% from one breeding season to the next. Generally, if both parents tend to return to similar locations within the colony to breed full siblings are likely to be common (Amos et al., 1995), especially if only a few males are contributing to the total reproductive output. Our results did not provide strong support for our prediction, as the number of rematings detected in this study was only slightly higher than what would be expected from random mating over time.

We suggest that some details regarding the levels of female pupping site fidelity may partially explain the low number of remating occurrences in Antarctic fur seals. In this case, the distinction of fidelity to breeding site (specific location at a given beach) or breeding beach (broader spatial scale) is rather important. For example, female beach pupping fidelity can decrease over time: a dedicated study has shown that beach fidelity declined from 80% in the first two years of return to < 70% considering more years (Lunn and Boyd, 1991). Also, our own data show that a considerable number of females (35%) switched beaches at least once during the study. Interestingly, at a smaller spatial scale (= pupping site), when females do return to the same beach they do that with relatively high precision (6-7m; Lunn and Boyd, 1991), which is still reduced relative to the males' precision (< 2m; Hoffman et al., 2006). In combination, these studies suggest that Antarctic fur seal females are selective of suitable

partuition sites where they can give birth and nurse their young (Lunn and Boyd, 1991), and thus, their choices may be more susceptible to environmental conditions. It is possible that the variability of female site fidelity at broader (beach) and smaller scales (site) is fundamental in dispersing them from subsequent contact with previous mates.

In addition to shifts in female breeding site fidelity, the quick replacement of territorial males by their competitors likely reduces the chance of remating. A study at SG demonstrated that even though some highly successful territorial males are reproductive for up to six years, the vast majority of males conceive pups mostly during their first or second year of tenure and father in average 0.93 pups in their lifetime (Hoffman et al., 2003). The same was observed at LI independently of this study, where only 2 out of 23 sampled males successfully sired pups for more than three seasons (C. Bonin unpublished). Therefore, except for the few highly successful males, most returning females do not encounter the same male for more than two seasons on the pupping beach, reducing the probability of remating.

In addition to aforementioned factors, female mate choice could also affect the probability of remating. The idea that female choice for unrelated males promotes the increase in a population's overall fitness is not new in pinnipeds (Bartholomew, 1970). Some mechanism of choice for highly heterozygous mates has been proposed for grey seals (Amos et al., 2001), where remating occurs more frequently than can be accounted for by chance (Amos et al., 1995). Also, Hoffman et al. (2007) demonstrated that female Antarctic fur seals at SG might cross a crowded colony in search of highly heterozygous and unrelated mates. Nevertheless, this suggestion has faced some controversy, and debate continues about whether fundamental principles of sexual selection theory and mating system concepts (e.g., lek) can be applied to the Antarctic fur seal mating system (see discussion by Kotiaho et al., 2008 a, b). Furthermore, female mate choice has not been investigated at LI, where seals breed at much lower densities.

Long-term studies on male and female behaviors are key to our understanding of how genetic variability is maintained in highly polygynous populations, but few wild systems are suitable for detailed exploration of this issue. Our efforts tracking female Antarctic fur seals throughout most of their reproductive lives provided a unique opportunity to investigate mating patterns among individuals over time. Despite presenting high breeding site fidelity and reproductive skew, Antarctic fur seals remate at a rate only slightly higher than what would be expected with random mating. Among the proposed explanations for this finding, we suggest that female movements are particularly important (e.g. shifts in female pupping beach fidelity). Therefore, although most polygyny studies focus on males, female behavior seems to be determinant of many aspects of this mating system as revealed by other recent research (Hoffman et al., 2007; De Bruyn et al., 2010). As demonstrated here, the importance of long-term studies of natural populations should be prioritized, as they clearly have the potential to reveal geographic variability and complexity within polygynous mating systems, and by extension, expand our perspective of mammalian population dynamics and evolution.

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Table 3-1: Microsatellite loci used to genotype Antarctic fur seals, *Arctocephalus gazella*, sampled at Livingston Island Antarctica. Data includes 149 samples (55 adult females and 94 randomly sampled pups*). The table includes the following details: species where markers where designed, PCR annealing temperatures (Tm), number of alleles (k), expected and observed heterozygosities (HE and Ho), and Hardy-Weinberg equilibrium test p values (p HW; heterozygote deficit).

Locus	Source	Species	Tm	K	H_E	Ho	*p* (HW)
Ag10 t	Hoffman et al. 2007	*Arctocephalus gazella*	50	7	0.778	0.803	0.5458
Agaz8t	Hoffman et al. 2009	*Arctocephalus gazella*	52	17	0.882	0.885	0.4833
Agaz9t	Hoffman et al. 2009	*Arctocephalus gazella*	50	10	0.802	0.811	0.5356
Hg3.7 t	Gemmell et al. 1997	*Halichoerus grypus*	50	12	0.855	0.866	0.1025
Hl-16 t	Davis et al. 2002	*Hydrurga leptonyx*	56	14	0.824	0.824	0.2159
HI-4 t	Davis et al. 2002	*Hydrurga leptonyx*	52	4	0.568	0.584	0.7334
Lc-28 t	Davis et al. 2002	*Lobodon carcinophaga*	58	13	0.884	0.851	0.864
M2B t	Hoelzel 1999	*Mirounga angustirostris*	56	9	0.842	0.845	0.9966
M11C-t	Russ Hoelzel unpubl.	*Mirounga angustirostris*	55	18	0.904	0.885	0.4626
Pvc29	Coltman et al. 1996	*Phoca vitulina*	52	16	0.869	0.859	0.9865
Pvc78	Coltman et al. 1996	*Phoca vitulina*	55	11	0.818	0.812	0.9574
ZcCgDh1.8 t	Hernandez-Velazquez et al. 2005	*Zalophus californianus*	60	8	0.758	0.758	0.997
ZcCgDh4.7 t	Hernandez-Velazquez et al. 2005	*Zalophus californianus*	60	14	0.859	0.893	0.6388
ZcCgDh48 t	Hernandez-Velazquez et al. 2005	*Zalophus californianus*	55	9	0.688	0.635	0.0525
ZcCgDh5.8	Hernandez-Velazquez et al. 2005	*Zalophus californianus*	60	15	0.87	0.866	0.3247
ZcCgDh7tg t	Hernandez-Velazquez et al. 2005	*Zalophus californianus*	55	18	0.891	0.886	0.119

Table 3-2: Antarctic fur seal tagged females, *Arctocephalus gazella*, monitored for their reproductive output (Pups obs.) at Livingston Island, Antarctica, from 1997-2009. All mothers and the majority of their pups were genotyped for 17 microsatellite markers (Pups genotyped).

Female tag(s)	Cohort	Pups obs.	Pups genotyped
37	1991	11	5
41	1991	8	3
48	1985	8	3
61	1994	5	2
73	1987	10	4
78	1989	12	6
79	1988	10	4
80	1991	8	5
83	1991	11	7
90	1988	5	5
92	1992	11	4
93	1987	7	4
95	1987	8	6
97	1992	8	5
100	1988	8	4
102	1989	8	4
113	1994	8	5
115	1993	10	8
116	1995	8	6
118	1996	9	6
119	1995	10	5
120	1993	9	4
121	1992	9	6
130	1990	6	5
132	1991	8	8
147	1990	9	3
011/157	1992	8	5
169	1993	9	5
178	1995	11	4
184	1993	11	5
186	1989	9	4
191	1991	10	5
194	1991	10	6
199	1987	8	4
204	1992	9	8
216	1990	6	5
218	1986	6	5

Table 3-2 Continued

Female tag(s)	Cohort	Pups obs.	Pups genotyped
017/228	1987	12	5
684/229	1997	8	6
237	1992	9	7
238	1993	8	4
241	1988	8	5
249	1991	8	6
255	1997	9	5
256	1993	9	4
267	1997	9	7
275	1991	8	6
286	n/a	8	7
290	1997	8	6
291	1996	7	4
306	1992	7	5
309/1083	1998	6	5
321	1995	7	5
183/311/341	1994	11	4
382	1992	5	6

Table 3-3: Pupping beaches recorded (according to Figure 1) for 10 known-age Antarctic fur seal females, *Arctocephalus gazella*, that gave birth to full sibling pups. These females were monitored from 1997-2009 at Livingston Island, Antarctica. Note: Pups that were genotyped for 17 microsatellite markers are indicated by the grey shadow. Full sibling pups are indicated by "*".

	Year/Pupping location												
Female	**1997**	**1998**	**1999**	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**
83		F	F	F	F	F	F	G	G	G	F*	G*	G
95			G	G*	G	G*	G	H	H	H	H		H
157	F	F	F	F	F*	F*	F	F					
169			H	H	H	H*	H	H	H	H	H*	H	H
191				H	H	H	H	H	H	H	H	H*	H*
194				H	H	H*	H	H	H	H*	H*	H	H
218				E	E*	E*	E	E	E	E	E		E
237						E	E	E	E	E	E	E*	E*
306			H	H	H	H	H	H	H	H*	H*	H	
309					D	E*	E	E	D*	D	D	D	D

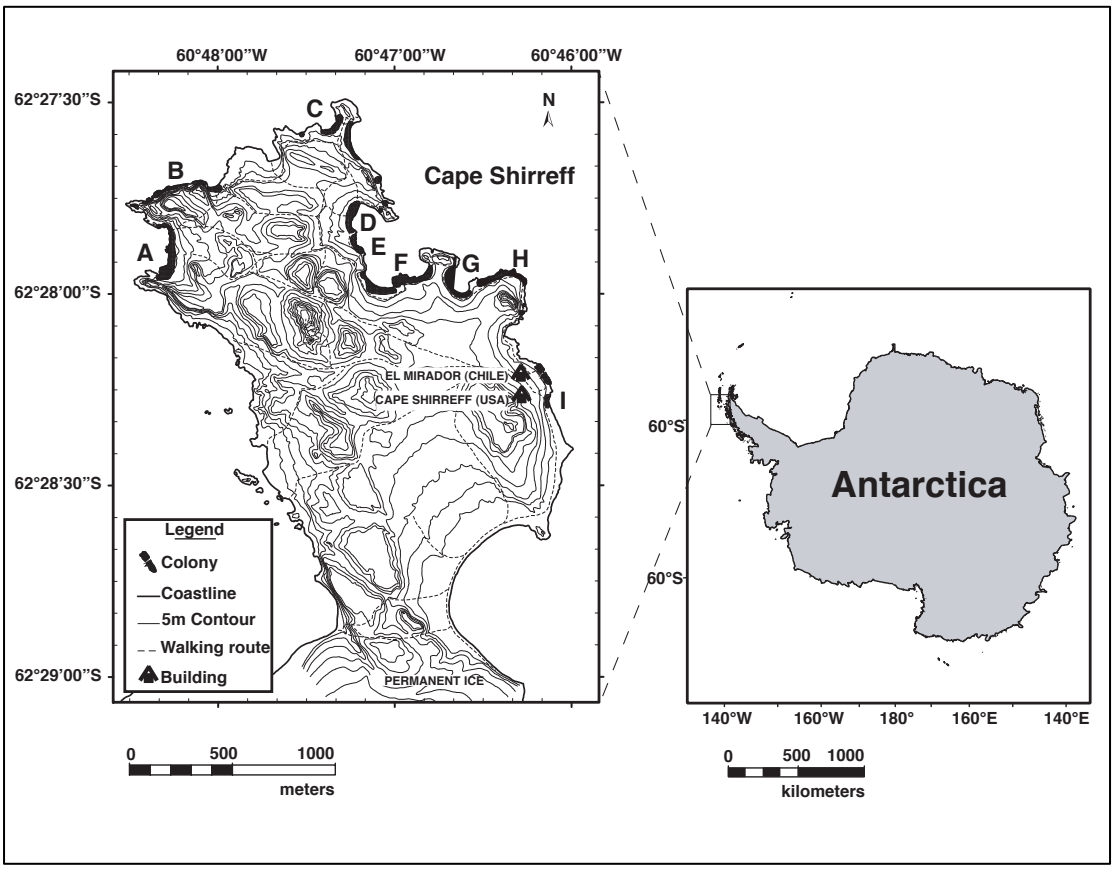

Fig. 3-1: Cape Shirreff, Livingston Island, Antarctica where the United States Antarctic Marine Living Resources Program (US-AMLR) monitors Antarctic fur seal, *Arctocephalus gazella*, populations. The insert shows Antarctic fur seal breeding beaches at Cape Shirreff, (re-designed from Torres, 1993 - INACH.

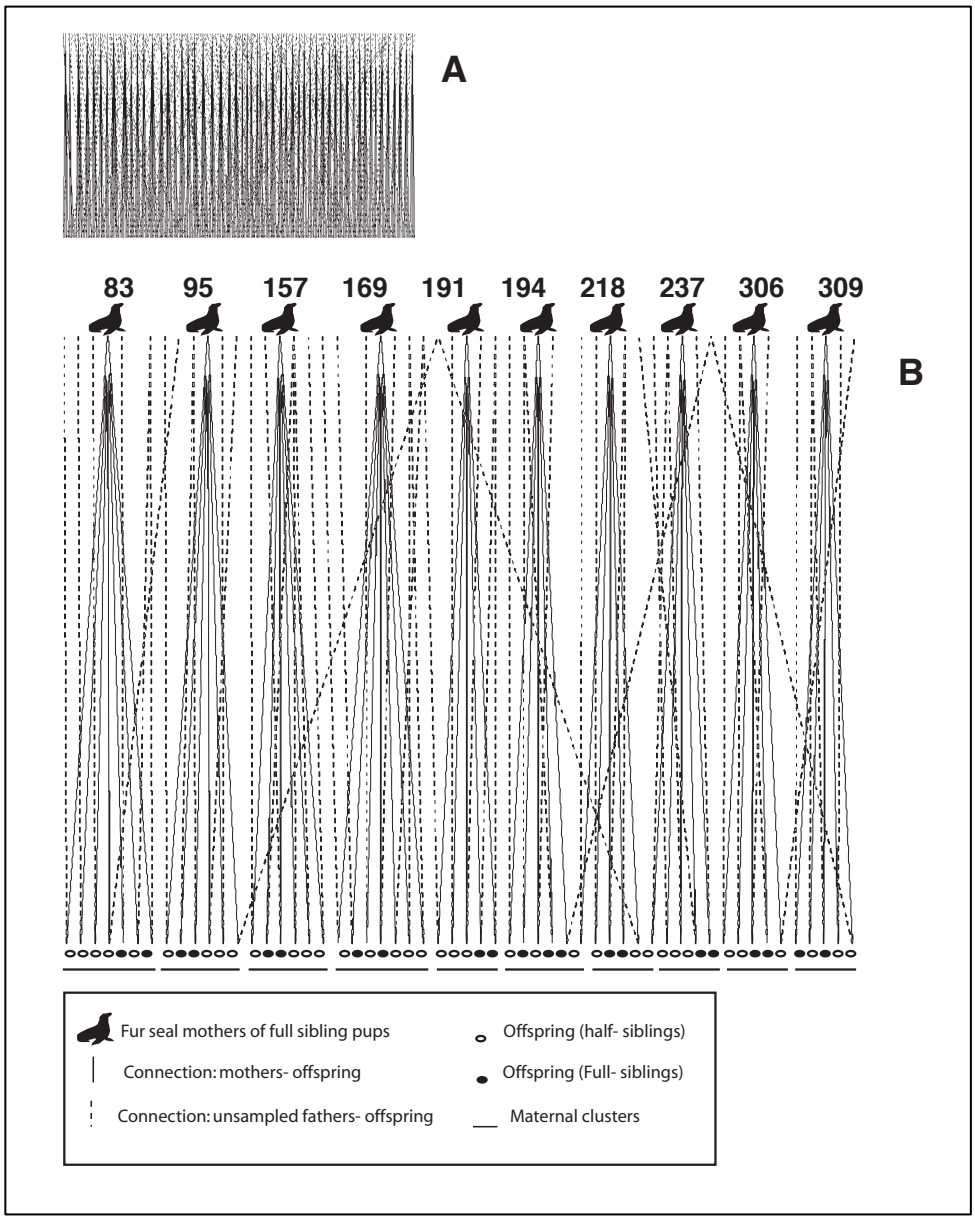

Fig. 3-2: A) Diagram illustrating the full pedigree configuration for 55 Antarctic fur seals, *Arctocephalus gazella*, and their offspring (n=280). B) Detail of configuration showing maternal clusters where full sibling pups were identified. Results were obtained using the full likelihood inference method of COLONY2 (Jones and Wang, 2010) using both genetic (individuals genotyped using 17 microsatellite markers) and field information input into the analysis.

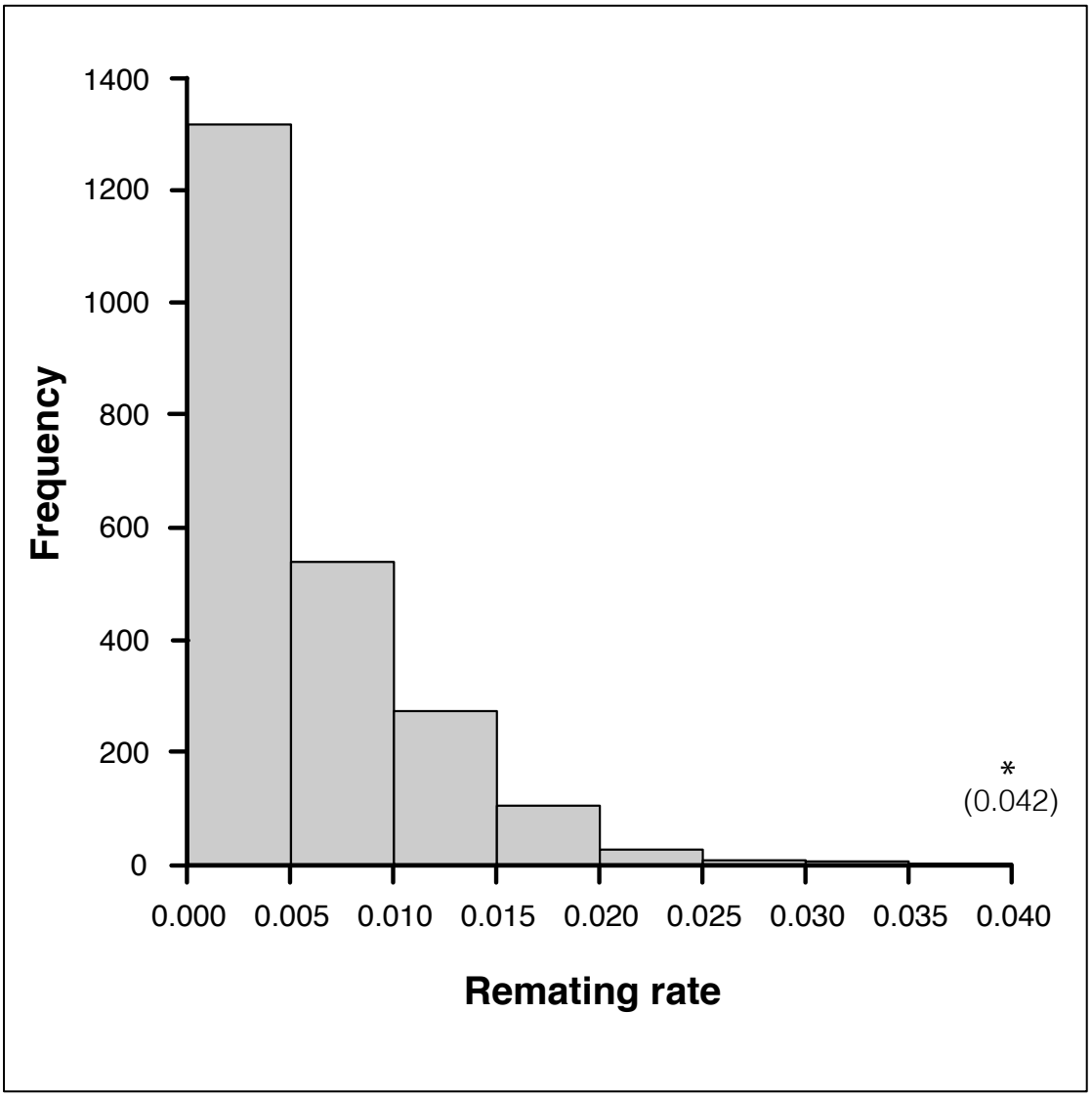

Fig. 3-3: Simulation results (n= 2,226 iterations) for the remating frequency obtained for 55 females randomly mating with 153 males with a mean number of pups per female of 5 (total pups=280). Maternal clusters configurations were drawn from a Poisson distribution.

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CHAPTER 4: Twins or not? Genetic analysis of putative twins in Antarctic fur seals, *Arctocephalus gazella*, on the South Shetland Islands.

ABSTRACT

Genetic analyses can reliably determine the relationships among putative cases of twins in pinniped species. These studies demonstrate that field observations of nursing twins may often be cases of adoption or foster nursing of unrelated pups. A recent study of Antarctic fur seals (*Arctocephalus gazella*) on South Georgia Island found that only 3 of 11 putative twin cases were truly twins. Here we report results of genetic testing of eight putative cases of twinning (twin siblings and mother) observed at Cape Shirreff (62°27'30"S, 60°47'17"W), Livingston Island, Antarctica. Parentage and relatedness analyses using 18 microsatellite markers confirmed six out of the eight cases as twins and two cases of adoption/foster nursing of unrelated pups. All twins analyzed were dizygotic and in five out of six cases, the twins were likely full siblings (relatedness coefficient, or rxyμ= 0.46, σ2=0.004). In one case, the twins were likely half-siblings (rxy=0.17), supporting a previous finding of heteropaternity in Antarctic fur seals. This result suggests that mate infidelity during estrus maybe common in Antarctic fur seals, which has implications for our understanding of this species mating system. The twinning rate estimated at Cape Shirreff (0.12% or 6 twins per 4,965 births) is consistent with the scarcity of twin births observed in pinnipeds, which is associated with the high cost of nursing multiple pups in these animals.

INTRODUCTION

Twins can be either monozygotic (MZ) or dizygotic (DZ), with MZ twins developing from one oocyte fertilized by a single sperm and DZ twins developing from two oocytes fertilized by two different sperms. In DZ twinning, the twins can share a single father or may have distinct fathers. In humans, single paternity is typical and as a result, DZ twins usually have the same genetic relationship as full siblings, sharing on average 50% of their genes (Hoekstra, 2008). However, the fertilization of two oocytes by sperm from different males, or DZ "heteropaternal superfecundation" (James, 1993) may also occur, which indicates partner infidelity during ovulation (Girela et al., 1997). In this case, DZ twins are fathered by distinct males and have the genetic relationship of half-siblings.

Twinning is considered rare among pinnipeds (Spotte, 1982). Nevertheless, twinning in phocids has been reported in elephant seals, *Mirounga leonina* (Arnbom et al., 1997; Galimberti & Boitani, 1999; McMahon & Hindell, 2003) and Weddell seals, *Leptonychotes weddellii* (Gelatt et al., 2001). In otariids, twinning has been reported for several species: northern fur seals (*Callorhinus ursinus*), Cape fur seals (*Arctocephalus pusillus*), Antarctic fur seals (*Arctocephalus gazella*), California sea lions (*Zalophus californianus*), Steller sea lions (*Eumetopias jubatus*), southern sea lions (*Otaria byronia*) and New Zealand sea lions (*Phocarctos hookeri*; Haase, 2007; Hoffman & Forcada, 2009; Maniscalco & Parker, 2009; Spotte, 1982). However, only a few pinniped twinning studies have confirmed relationships among mothers and pups using genetic analyses (Gellat et al. 2001; Hoffman & Forcada 2009).

Antarctic fur seal (*Arctocephalus gazella*) twinning has been recently examined by Hoffman & Forcada (2009). In their study at Bird Island, South Georgia, 11 putative cases of twins were analyzed and genetic twins were confirmed in only three cases. According to these

authors, field observations of more than one suckling pup per mother must be foster nursing in the majority of cases, which can be fairly common at South Georgia (Hoffman & Amos, 2005a; Lunn, 1992). To further investigate this phenomenon, genetic analyses should be routinely undertaken to verify putative cases of twinning in pinnipeds (Gellat et al., 2001; Hoffman & Forcada, 2009).

Antarctic fur seal breeding populations are circumpolar, occurring at several islands south of the Antarctic Polar Front. Around the South American continent they are found at South Georgia, South Sandwich, South Orkney and the South Shetland Islands (Hofmeyr et al., 2006). As a consequence of over-hunting, Antarctic fur seals were extirpated from the South Shetlands by the end of the 19th century; the population has rapidly recovered to nearly 21,000 animals since the first birth documented at Cape Shirreff during the austral summer of 1958/59 (Hucke-Gaete et al., 2004; O'Gorman, 1961).

Antarctic fur seals have been intensively studied at Cape Shirreff for over a decade by researchers of the United States Antarctic Marine Living Resources (US- AMLR) Program. In recent years, putative twin cases have been observed and recorded in the field, providing an opportunity to investigate twinning in a recently recovered population of this species.

The objectives of this study were to: (1) investigate our ability to infer relationships among individuals via simulations of genotypic data, (2) genetically analyze putative twinning cases at Cape Shirreff, and (3) estimate twinning rates for the Cape Shirreff population and evaluate results within the context of Antarctic fur seal reproductive strategies.

MATERIALS AND METHODS

Sampling

The samples for this study were collected at Cape Shirreff (62°27'30"S, 60°47'17"W) (Figure 4-1), located south of the Drake Passage and on the northern coast of Livingston Island, the second largest of the South Shetland group. More specifically, Cape Shirreff is a low, ice-free peninsula of approximately 3.1 km^2 located between Barclay Bay and Hero Bay (Anonymous, 1994).

All twinning cases were identified during the perinatal period (within one week of birth); they consisted of a pair of pups frequently observed nursing simultaneously on a single female. Candidate fathers were not sampled. Eight pairs of putative twins and their respective mothers (eight mothers and 16 pups, n=24) were sampled during the austral summers of 2006-07; 2008-09; 2009-10. For a brief description of the putative twinning cases sampled in this study refer to Table 4-1. For the purposes of estimating allele frequencies within the Cape Shirreff Antarctic fur seal population, 94 pups (n= 42 males and 52 females) were sampled randomly during the austral summer of 2009-10.

Fur seal pups were sampled using 2mm sterile biopsy punches, taking skin from the inter-digital membrane of the rear flippers. The biopsy punches were attached to a pole to collect tissue from 8 adult females (1 untagged, and 7 previously tagged; Dalton Jumbo Rototags, Dalton ID systems, UK). All tissue samples were stored in either 20% dymethylsulphoxide (DMSO) saturated with NaCl or 95% ETOH, and all procedures were conducted in compliance with Marine Mammal Protection Permit No. 774-1847-03 granted by the Office of Protected Resources, National Marine Fisheries Service, United States.

Laboratory procedures

Total genomic DNA was extracted from tissue samples using a NaCl precipitation method (adapted from Miller et al., 1988). After extraction, the genomic DNA was amplified for 18 microsatellite markers: Aa4, Hg3.7 (Gemmell et al., 1997); Ag10, Ag4, Ag7 (Hoffman et al., 2008), Agaz8, Agaz9 (Hoffman, 2009); Hl4, Lc28 (Davis et al., 2002); M2B (Hoelzel, 1999); Pvc29, Pvc78 (Coltman et al., 1996); ZcCgDh1.8, ZcCgDh4.7, ZcCgDh48, ZcCgDh5.8, ZcCgDh7tg, ZcCgDhB.14 (Hernandez-Velasquez et al., 2005). Amplification consisted of 15 µl reactions containing: ~ 30 ng of template DNA, 2.0 µM 1X ThermoPol reaction buffer (New England Biolabs, USA, catalog # B9013S), 1.5 µM of dNTPs, 0.45 µM of each primer (forward and reverse) and 0.5 u Taq DNA polymerase (New England Biolabs, USA, catalog # M0267L). The reactions were amplified in an ABI 2700 thermocycler (Applied Biosystems, Foster City, California, USA) through an initial denaturing step of 97°C for 3 minutes and 36 cycles of denaturing at 90°C for 20 seconds, an annealing step at specific primer annealing temperatures (Tm; see Table 4-2 for specific primer Tms) for 30 seconds, and an extension at 72°C for 20 seconds. Successful PCR reactions were processed following standard ABI protocols for fragment analysis. Samples were run on a 48-capillary, 3130xl ABI Genetic Analyzer, and resulting raw data files were analyzed and edited on ABI GeneMapper® v.4.0.

Data analysis

Microsatellite markers were assessed for the presence of null alleles using MICROCHECKER v. 2.2.3 (Van Oosterhout et al., 2004). The dataset was also tested for deviations from Hardy-Weinberg equilibrium and linkage disequilibrium (dememorization # = 100,000; 10,000 iterations per batch) using GENEPOP v. 4.0 (Raymond & Rousset, 1995). Marker scoring error rates were assessed by re-running 26% of the samples (samples were re-amplified and genotypes were re-scored): 12 samples from the 94 pups sampled for the population allelic frequency estimation and 24 samples corresponding to all putative twins and their mothers. The error rate was calculated as the number of mismatched calls divided by the total number of calls for the replicated samples per locus (as described by Bonin et al., 2004). Additionally, mismatched calls were triplicated to reduce error. An identity analysis was conducted on the 94 randomly collected samples used to estimate baseline allele frequencies within the population. This analysis was carried on to check for potential duplicate samples (animals mistakenly sampled twice in the field) within our dataset. A maternity analysis was performed to verify the maternity of all twins and to allow for an evaluation of marker power via computed exclusion probabilities. Identity analyses, maternity analyses, and the calculations of exclusion probabilities, allele frequencies, null allele frequencies and heterozygosities (observed and expected) were conducted using CERVUS v.3.0.3 (Kalinowski et al., 2007; Marshall et al., 1998; Slate et al., 2000).

Relatedness analysis simulation

Prior to the relatedness analysis of the empirical twin dataset, a simulation was performed to provide an assessment of different estimators of relatedness coefficients (rxy hereafter), as well as expected means and variances for relationship categories (as described in Ivy et al., 2009). The simulation determined the most appropriate rxy estimator for our dataset and the research questions addressed here.

Given the allelic frequencies within the population (based on n=94), 2,000 individual genotypes were simulated. From the simulated genotypes, 1,000 dyads (or comparisons between two simulated individuals) were drawn for four relationship categories (unrelated, half-siblings, full-siblings and parent-offspring) and rxy was calculated for each dyad within each relationship category. The calculation of rxy for each dyad, within the four relationship categories listed above, was performed using six separate estimators (Li et al., 1993; Lynch, 1998; Lynch & Ritland, 1999; Milligan, 2003; Queller & Goodnight, 1989; Ritland, 1996; Wang, 2002) as described in Wang, 2011). The estimator with the lowest variance across the relationship categories was chosen for subsequent analyses. Confidence intervals (95%) for the estimation of rxy for the twin groups were calculated using bootstrapping (1,000 samples). All simulations and calculations of rxy for the empirical dataset (including estimation of 95% CI) were conducted using COANCESTRY v.1.0.0.0 (Wang, 2011).

Initially, relationship assignment between twin siblings was based on the calculated rxy. However, in a few instances, discerning full and half-siblings was challenging since these relationship categories overlapped considerably in their rxy distributions. Therefore, a statistical approach for testing relationship hypotheses via the calculation of likelihood ratios of putative over alternative relationships was employed using the ML-Relate (Kalinowski et

al., 2006). For each twin sibling pair, we tested one of the following three hypotheses: full (putative) vs. half sibling (alternative), half (putative) vs. full sibling (alternative) or unrelated (putative) vs. half-siblings (alternative). The decision of which hypothesis to test relied on the rxy value obtained for the dyad. In these specific tests, 10,000 genotypes for the alternative relationships were simulated for the significance (p value) estimation. At $p < 0.05$ we accepted the putative relationship over the alternative.

The twinning rate at Cape Shirreff was estimated as the number of genetically confirmed twin births out of the total number of pups born, counted on the US-AMLR study area during the field seasons when the samples were collected.

RESULTS

Genetic marker assessment

The 18 microsatellite markers used in this study averaged 11.71 alleles per locus (range 4 to 23 alleles per locus) and the mean expected heterozygosity (HE) was 0.81 (n= 94). Most microsatellite markers were in agreement with Hardy-Weinberg equilibrium (HWE) expectations. One locus (ZcCgDh48) presented a possible heterozygote deficit (p= 0.0126). However, there was no indication of null alleles and the deviation from HWE lost its significance after a Bonferroni correction. Both error rate and missing data per locus were incorporated into all calculations for rxy. No indication of linkage disequilibrium was detected among the loci (153 pair-wise comparisons).

A 0.3% error rate was estimated for the entire dataset based upon replication of PCR amplifications. In all cases, errors in calling alleles were due to weak amplification of a second allele (homozygote call versus a heterozygote call) rather than a complete miscall for that

individual at a given locus. The mean proportion of individuals genotyped was 0.9914. All missing data occurred in samples used to estimate population allelic frequencies, and not in the putative twin cases. For estimations of error and missing data rate per locus refer to Table 4-2.

The expected combined paternity exclusion probability (PE) calculated using CERVUS was 0.9999, indicating high power achieved by the microsatellite marker panel in parentage analysis. This conferred reliability for the genetic analysis of the putative twins as we were able to confidently verify whether they were born to a single mother.

Relatedness analysis simulation

The relatedness simulation results showed a strong correlation among the 6 relatedness estimators of rxy for the dataset and they all presented relatively low variances (σ2 range unrelated=0.0034-0.0133; σ^2 range half-siblings=0.018-0.0427; σ^2 range full-siblings=0.0126-0.0653; σ^2 range parent-offspring=0.0012-0.0573) within each relationship category. Among the estimators, Milligan's dyadic likelihood estimator (Milligan, 2003) had the least variance for all relationship categories (Table 4-3). Therefore, it was chosen for the following relatedness analysis of twin groups. The parent-offspring relationship category had the least variance and narrowest rxy distribution (rxy μ= 0.51, σ^2= 0.001) followed by the unrelated category (rxy μ= 0.04, σ^2= 0.003). Conversely, the half-siblings and full-siblings relationship categories presented broader rxy value distributions, with the observed rxy means for half- and full siblings respectively at 0.25 (σ^2= 0.011) and 0.50 (σ^2= 0.013), matching expected values for second and first order relatives. The simulation results are displayed as probability density distributions of rxy for each relationship category (Figure 4-2.).

Genetic analysis of twins

In six of the eight cases, putative twin pairs were confirmed; i.e., we were unable to exclude the females that were nursing them as mothers (each pup shared at least one allele per locus with its mother; parent-offspring μrxy = 0.52; σ^2=0.0014). In the two remaining cases (CS3 and CS7, see Table 4 for description) the parentage analysis indicated that one of the pups observed nursing along with its putative sibling on a female (case CS7 is shown on Figure 4-3.) was not a pup to that mother, and was adopted. In those cases the putative maternity was excluded based on mismatches at six and seven loci. The relatedness analysis revealed that these pups were not fathered by the same male and were unrelated to their adoptive mother regarding other relationship categories (i.e. second or third order relatives); in both cases, mother-adopted pup rxy= 0.

In the six confirmed cases, the twins were dizygotic (DZ). In five cases, the twins were likely full siblings (μ rxy = 0.46, σ^2=0.004) and were born to the same set of parents. In the remaining case, the twins were likely half siblings (rxy= 0.17) and this was interpreted as a case of heteropaternity, where the twins were born to a single mother but had different fathers. Refer to Table 4 for the significance of relationship hypothesis testing for all twin sibling dyads.

The twinning rate for the Cape Shirreff fur seal population was estimated at 0.12% (6 twins per 4,965 births). The twinning rate across years was 0.15% (3 twins per 2,067 births) for season 2006-07, 0.13% (2 twins per 1,513 births) in 2008-09, and 0.07% (1 twin per 1,385 births) in 2009-10. The inter-annual variation of twinning rate was not statistically significant (Fisher's exact test, p= 0.88). As well, the twinning rates estimated for Cape Shirreff presented

above and for South Georgia (0.06%, Hoffman & Amos, 2009) were not significantly different (Fisher's exact test, p= 0.07).

DISCUSSION

Genetic marker assessment and relatedness analysis simulation

The genotypes generated for the samples in this study allowed for confirmation of most putative twin cases (six out of eight) identified in the field. The microsatellite markers were highly effective in maternity assignments, as the expected non-exclusion probability was extremely low. Marker power was evidenced by the maternity exclusion at six and seven loci for the two adopted Antarctic fur seal pups.

The genotyping error rate of 0.3% for our dataset lies within an acceptable range of other reports in the literature (i.e. 0.8% Bonin et al., 2004). However, it has been demonstrated that even a low genotyping error can have a significant effect in parentage analysis. For example, a genotyping error rate of 1% can cause false paternity exclusion of 20% (Hoffman & Amos, 2005b). For that reason, the maternity analysis was also performed using a maximum likelihood approach offered by CERVUS v.3.0.3 that can incorporate genotyping errors, null alleles and mutations (Kalinowski et al., 2007). Both maternity assignment methods (exclusionary and maximum likelihood) yielded the same results, reinforcing the high power yielded by the 18 microsatellite markers used in this study.

The simulation of relatedness analysis was a useful tool for choosing the most appropriate relatedness estimator, as suggested by Csilléry et al. (2006) and Wang (2011). The same analysis also allowed for an assessment of the distribution of relatedness coefficient values (rxy) for each relationship category. The distribution of rxy values confirmed that the

parent-offspring category had the lowest variance followed by the unrelated category. Therefore, our analysis would be clearly reliable when used to assign "parent-offspring" or "unrelated" relationships for a pair of individuals. The most challenging assignments were in the full and half sibling categories. Although rxy means for full and half sibling categories matched expectations, they presented higher variance and overlapping distributions. This is not surprising, as it has been demonstrated that 20 microsatellite loci (HE= 0.75) in a vertebrate population are usually enough to discriminate unrelated from full siblings 97% of the time; however, up to 40 loci maybe required to distinguish between full and half siblings (Blouin et al., 1996). The high heterozygosity (HE= 0.81) found in Antarctic fur seal markers used in this study may confer some ability to distinguish full and half siblings, but the wide confidence intervals (95%) calculated for the twin siblings' rxy revealed that this assignment could benefit from additional loci. In this situation, additional statistical assessments, such as a priori hypothesis testing for relationship categories were an efficient way to assign the most likely relationship for a pair of individuals, as demonstrated by Zeyl's et al. (2009) relatedness study on polar bears (*Ursus maritimus*).

Twinning in Antarctic fur seals

Our ability to confirm most twinning cases (six out of eight) using genetic analysis indicates that although problematic (Gellat et al., 2001; Hoffman & Forcada, 2009), field observations of twins in our study area seem to closely reflect actual twinning rates. Therefore, field records of twinning can potentially be used to track changes in the reproductive strategies/ life history of Antarctic fur seals at Cape Shirreff.

Twinning in Antarctic fur seals can be considered rare, with no significant differences between the South Shetland Islands (0.12%) and South Georgia populations (0.06%, Hoffman

& Amos, 2009). This finding is consistent with reports for other pinniped species: 0.2-0.38% for elephant seals, *Mirounga leonina*, (Arnbom et al., 1997; Galimberti & Boitani, 1999; McMahon & Hindell, 2003) and 0.1% in Weddell seals (*Leptonychotes weddellii*; Gelatt et al., 2001). Twinning in pinnipeds is particularly scarce if compared to well-studied groups of mammals such as apes (i.e. chimpanzee, *Pan troglodytes*) with DZ twinning rates estimated at 2.36% (Ely et al. 2006) and ungulates, with twinning rates usually up to 20% (i.e. 2.5-20.7% for European mouflon, *Ovis sp*., Garel et al., 2005; 9-24% for moose, *Alces alces*, Testa, 2004). A remarkable annual twinning rate of >70% is observed in the Saiga antelope, *Saiga tatarica tatarica* (Kühl et al., 2007). The scarceness of twinning in pinnipeds can be explained by the overall high maternal investment for mothers who feed at sea but nurse on land (with the exception of walruses, *Odobenus rosmarus*; Oftedal et al., 1987). In the case of Antarctic fur seals, mothers take foraging trips to sea of two to seven days and spend one or two days nursing their pup ashore. They alternate these activities during the four-month lactation period (Doidge et al., 1986). During their foraging trips to sea fur seal mothers have to gather enough resources for themselves and their nursing pup. This constraint imposes high costs for nursing multiple pups. For example, after observing two female Antarctic fur seals rearing twins to weaning, Doidge (1987) estimated that the energy cost of pup rearing increased by 75% for those females compared to mothers rearing a single pup. Although possible, the rearing of two or more pups incurs a high cost that has influenced the evolution of reproductive strategies in pinnipeds, which rarely give birth to more than a pup.

Other factors related to demographic variables may also determine twinning, and they constitute a basis for interpreting rates of occurrence. These are generally called “maternal effects” and include advanced age, increased parity and matrilineal genetic inheritance (Bulmer, 1970; Bortolus et al., 1999; Hoekstra et al., 2008; Parisi et al., 1983). In humans, twinning rates increase four-fold between the ages of 15 and 37 years, because there is a rise

in the level of gonadotropins in females with age. Increased parity also affects the probability of twinning, and although age and parity are highly correlated, their effects are independent of each other (Bulmer, 1970). Pedigree studies in humans (Bulmer, 1970; Lichtenstein et al., 1996; Parisi et al., 1983) and chimpanzees (Ely et al., 2006) also reveal that DZ twinning is a familial trait, mainly inherited maternally. Twinning also has a high recurrence risk at the individual level: a female chimpanzee that has had twins once will have a recurrence risk five times greater than average (Ely et al., 2006). There is limited evidence for the influence of maternal effects in our study site. No twin birth recurrence has been observed at Cape Shirreff. As for age effects, the fur seals in this study were considered of advanced age (range from 11 to 16 years old), given that the female fur seals' peak in reproduction occurs at 7-9 years of age (Lunn et al., 1994). Thus, although sample size limits our ability to assess the significance of the age effect, our data could support the positive effect of increased parity and age on the twinning probability. As more samples become available, populations of Antarctic fur seals should represent an ideal case for studies on maternal effects of twinning rates in wild pinniped populations.

Zygosity, heteropaternity and Antarctic fur seal mating strategies

All twin cases confirmed in this study were DZ (fraternal twins). Given our sample size, we expected this result, since MZ twins usually occur at a very low rate in most human (Tong et al., 2007) and chimpanzee populations (Ely et al., 2006). Hoffman & Amos (2009) reported just one case of MZ twins in Antarctic fur seals, which is also the first confirmed case in pinnipeds.

One case of Antarctic fur seal heteropaternity at South Georgia has been previously reported by Hoffman & Amos (2009). Our results show that heteropaternity also occurs in the

South Shetlands population. One out of the six twinning cases examined here demonstrated that the twin siblings were likely half-siblings, meaning that one of our sampled females conceived from two different males on the same breeding cycle. Heteropaternal DZ twins have been well documented in humans (Bulmer, 1970; Girela et al., 1997; Verma, 1992; Wenk et al., 1992) and other primates (Bercovitch et al., 2002; Ely et al., 2006), but their occurrence is always considered rare. The fact that we were able to identify a case of shared paternity within a small sample set (as did Hoffman & Amos, 2009) indicates that mate infidelity during estrus maybe common in Antarctic fur seals, which has implications for our interpretation of this polygynous mating system.

In summary our study (1) demonstrates the utility of conducting simulations of relatedness analysis for an assessment of marker power and for choosing the most appropriate relatedness estimator, (2) shows that twinning appears to be rare across populations of Antarctic fur seals, indicating the strong constraint likely imposed by the high cost of lactation in this species and in pinnipeds in general and (3) confirms another case of heteropaternity in Antarctic fur seals suggesting that mating infidelity during estrus could be common, which has implications for our understanding of this species mating system.

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Table 4-1: Summary information on putative Antarctic fur seal (*Arctocephalus gazella*) twin groups collected in Cape Shirreff, Antarctica. Results of maternity and relatedness are also summarized.

Twin Group	LABID*	Seal description, age	Field season	Sex
CS1	62444	Adult Female (Tag# 392, 11 yo)	2006-07	F
	62445	Pup- putative twin (Mother 392)	2006-07	M
	62446	Pup- putative twin (Mother 392)	2006-07	M
CS2	62447	Adult Female (Tag # 395, 15 yo)	2006-07	F
	62448	Pup- putative twin (Mother 395)	2006-07	M
	62449	Pup- putative twin (Mother 395)	2006-07	F
CS3	62450	Adult Female (Tag# 391)	2006-07	F
	62451	Pup- putative twin (Mother 391)	2006-07	F
	62452	Pup- putative twin (Mother 391)	2006-07	F
CS4	62453	Adult Female (Tag# 412)	2006-07	F
	62454	Pup- putative twin (Mother 412)	2006-07	F
	62455	Pup- putative twin (Mother 412)	2006-07	M
CS5	78410	Adult Female (Untagged)	2008-09	F
	78408	Pup- putative twin	2008-09	M
	78409	Pup- putative twin	2008-09	M
CS6	78423	Adult Female (Tag# 440)	2008-09	F
	78405	Pup- putative twin (Mother 440)	2008-09	F
	78406	Pup- putative twin (Mother 440)	2008-09	M
CS7	92464	Adult Female (Tag# 448, 16 yo)	2009-10	F
	92546	Pup- putative twin (Mother 448)	2009-10	F
	92547	Pup- putative twin (Mother 448)	2009-10	M
CS8	92467	Adult Female (Tag# 450)	2009-10	F
	92550	Pup- putative twin (Mother 450)	2009-10	M
	92551	Pup- putative twin (Mother 450)	2009-10	M

*LABID corresponds to sample accession numbers for the Marine Mammal and Sea Turtle Molecular Research Collection, at the Southwest Fisheries Science Center, La Jolla, CA, USA.

Table 4-2: Microsatellite markers used to genotype Antarctic fur seals (n= 94 randomly sampled pups) from Cape Shirreff, Livingston Island, Antarctica. Table contains: marker names (a "t" after marker name indicates use of a 7 bp tail: GTTTCTT on 5' of reverse primer), literature source (source), repeat motif (when available), annealing temperatures (Tm), number of alleles (K), number of observed heterozygotes (Ho), number of expected heterozygotes (He), Hardy-Weinberg p values (p HW), frequency of null alleles (Freq Null), marker missing data rate (Miss.), marker error rate over 13% replication (Error).

Locus	Source	Repeat Motif	Tm	K	Ho	He	p (HW)	Freq (Null)	Miss.	Error
Aa4 t	Gemmell et al., 1997	not available	55	7	0.75	0.75	0.5635	-0.0048	0.02	0.02
Ag10 t	Hoffman et al., 2008	$(AC)_{13}$	50	7	0.796	0.796	0.8543	-0.0036	0.01	0
Ag4 t	Hoffman et al., 2008	$(GT)_6GA(GT)_{12}$	60	23	0.904	0.908	0.5679	0.0005	0	0
Ag7 t	Hoffman et al., 2008	$(GT)_8AT(GT)_{13}$	52	8	0.734	0.778	0.7898	0.0273	0	0
Agaz8t	Hoffman, 2009	$(AC)_{22}$	52	17	0.915	0.88	0.5199	-0.0241	0	0
Agaz9t	Hoffman, 2009	$(GT)_{17}$	50	10	0.764	0.801	0.2586	0.0189	0.05	0
Hg3.7 t	Gemmell et al., 1997	$(CT)_{10}(CA)_5CT(CA)_{15}$	50	12	0.862	0.845	0.7289	-0.0127	0	0
HI-4 t	Davis et al., 2002	$(GT)_{12}$	52	4	0.596	0.559	0.9111	-0.0411	0	0
Lc-28 t	Davis et al., 2002	$(GT)_{11}$	58	12	0.828	0.855	0.6799	0.0134	0.01	0.01
M2B t	Hoelzel, 1999	not available	56	9	0.849	0.844	0.831	-0.0032	0.01	0
Pvc29	Coltman et al., 1996	not available	52	15	0.883	0.869	0.8315	-0.0119	0	0.02
Pvc78	Coltman et al.,1996	$(AC)_{15}$	55	9	0.819	0.818	0.9803	-0.004	0	0
ZcCgDh1.8 t	Hernandez-Velazquez et al. 2005	$(GT)_{14}(GC)_2(GT)_8$	60	8	0.787	0.771	0.9081	-0.0148	0	0
ZcCgDh4.7 t	Hernandez-Velazquez et al. 2005	$(GT)_{16}(GA)_{15}$	60	12	0.862	0.85	0.9556	-0.0095	0	0
ZcCgDh48 t	Hernandez-Velazquez et al. 2005	$(TC)_9(AC)_{14}$	55	9	0.581	0.652	0.058	0.0369	0.01	0
ZcCgDh5.8	Hernandez-Velazquez et al. 2005	$(GT)_{21}$	60	14	0.851	0.866	0.8408	0.0071	0	0
ZcCgDh7tg t	Hernandez-Velazquez et al. 2005	$(TG)_{10}(AG)_{19}$	55	16	0.883	0.888	0.0581	0.0002	0	0
ZcCgDhB.14 t	Hernandez-Velazquez et al. 2005	$(TGGA)_4GC(GATC)_6$	60	6	0.777	0.763	0.5029	-0.011	0	0

Table 4-3: Summary of simulation results (n=1,000 dyads per relationship category) for each relatedness coefficient (rxy) estimator. Population allelic frequencies were obtained from 94 Antarctic fur seal pups randomly sampled at Cape Shirreff, Livingston Island, Antarctica.

	Relationship category			
	Unrelated μ	Half-siblings μ	Full-siblings μ	Parent-Offspring μ
Wang rxy	-0.0070	0.2433 (0.0122)	0.4969 (0.0135)	0.4946 (0.0032)
Lynch & Li	-0.0066	0.2453 (0.0129)	0.4973 (0.0129)	0.4936 (0.0045)
Lynch & Rit.	-0.0032	0.2403 (0.0183)	0.4954 (0.0237)	0.4935 (0.0143)
Ritland rxy	-0.0007	0.2490 (0.0427)	0.4936 (0.0653)	0.4989 (0.0573)
QG rxy	-0.0062	0.2449 (0.0130)	0.4951 (0.0136)	0.4933 (0.0048)
Milligan rxy	0.0402	0.2552 (0.0108)	0.5015 (0.0126)	0.5172 (0.0012)

Table 4-4: Relatedness coefficients (rxy*) estimated for Antarctic fur seal twin groups sampled at Cape Shirreff, Livingston Island, Antarctica.

Case	Individual 1	Individual 2	Milligan's	rxy CI (95%)		p	Results
CS1	Mother	Twin 1-	0.5	0.5	0.5534		
	Mother	Twin 2-	0.5856	0.5	0.7356		
	Twin 1-	Twin 2-	0.5157	0.3466	0.6853	0.0059	Full
CS2	Mother	Twin 1-	0.5	0.5	0.6508		
	Mother	Twin 2-	0.5	0.5	0.6436		
	Twin 1-	Twin 2-	0.1753	0	0.3419	0.0001	Half
CS3	Mother	Twin 1-	0.5377	0.5	0.6699		
	Mother	Twin 2-	0.5422	0.5	0.6323		
	Twin 1-	Twin 2-	0.376	0.1181	0.6091	0.0411	Full
CS4	Mother	Twin 1-	0	0	0		
	Mother	Twin 2-	0.5	0.5	0.5711		
	Twin 1-	Twin 2-	0	0	0	0.0001	Adoption
CS5	Mother	Twin 1-	0.6177	0.5	0.7556		
	Mother	Twin 2-	0.5	0.5	0.5936		
	Twin 1-	Twin 2-	0.4151	0.1336	0.6134	0.0125	Full
CS6	Mother	Twin 1-	0.5	0.5	0.5451		
	Mother	Twin 2-	0.5001	0.5	0.627		
	Twin 1-	Twin 2-	0.5124	0.357	0.7775	0	Full
CS7	Mother	Twin 1-	0.5	0.5	0.5708		
	Mother	Twin 2-	0	0	0.2775		
	Twin 1-	Twin 2-	0	0	0.3087	0.0016	Adoption
CS8	Mother	Twin 1-	0.5	0.5	0.6252		
	Mother	Twin 2-	0.5	0.5	0.6586		
	Twin 1-	Twin 2-	0.5249	0.2433	0.7146	0.0008	Full

* rxy was estimated according to Milligan, 2003 (in COANCESTRY v. 1.0.0.0 by Wang, 2011). Confidence intervals were generated using 1,000 bootstrap samples. p values represent the significance of the likelihood ratio test calculated for two *a priori* relationships (putative relationship: full sibship, alternative relationship: half sibship); small p values indicate that the putative relationship fits the data significantly better.

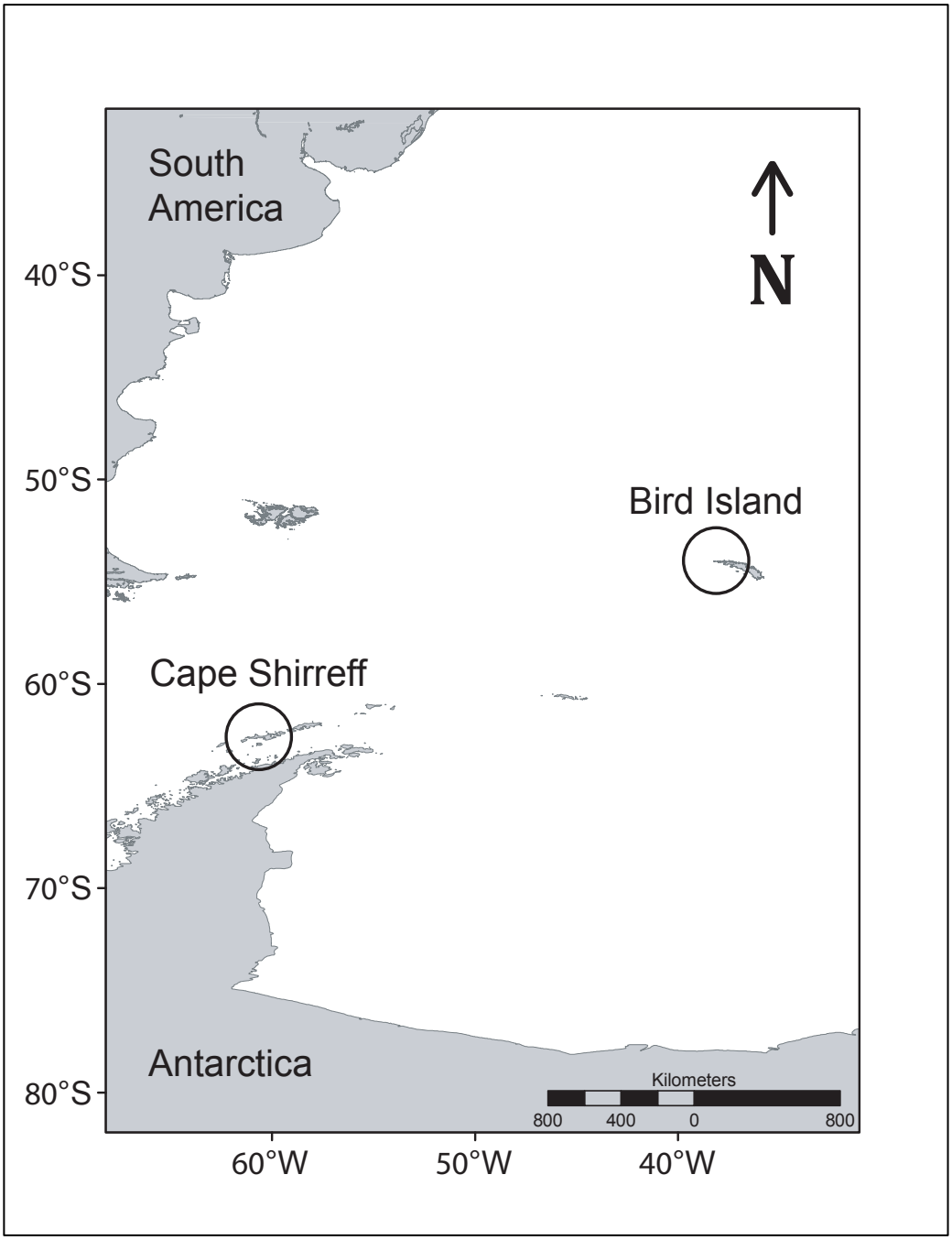

Fig. 4-1: The United States Antarctic Living Marine Research Program (US-AMLR) study site: Cape Shirreff, in Livingston Island, Antarctica indicated by the star. Note: British Antarctic Survey Program study site: Bird Island, South Georgia indicated by the circle.

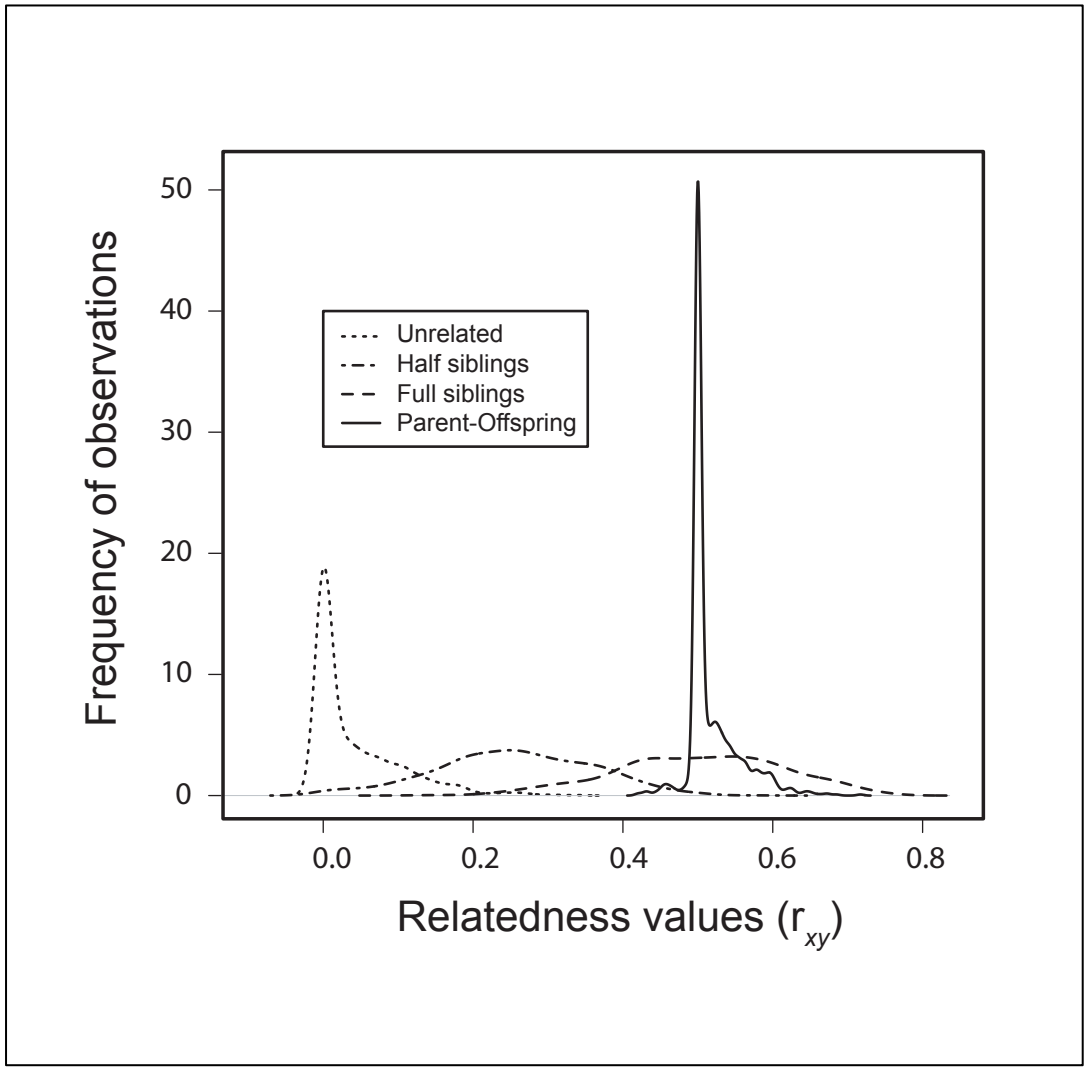

Fig. 4-2: Probability density of relatedness coefficients (rxy) calculated for 4 relationship categories: unrelated, half siblings, full siblings and parent-offspring (n= 1,000 simulated dyads per relationship category). Allelic frequencies were calculated from 94 Antarctic fur seal pups genotyped for 18 microsatellite markers.

Fig. 4-3: Putative Antarctic fur seal twin case "CS7" indentified at Cape Shirreff, Livingston Island, Antarctica. Photo taken in January 2010. Photo credit: Carolina Bonin.

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CONCLUSION

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The Antarctic fur seal, *Arctocephalus gazella*, was hunted to near-extinction in the early 1800's and was one of the most exploited marine mammal species in history. Nevertheless, the species remarkably recovered to re-colonize most of its original breeding range and despite its past demographic decline, currently holds high levels of genetic diversity. With the main objective of exploring this conundrum, this thesis: (i) investigated population-level patterns of genetic structure, interpreting it in light of the species re-colonization history and (ii) examined this species' polygynous mating system in detail, as it relates to the maintenance of genetic diversity. Efforts to accomplish the research objectives of each chapter included two seasons of intensive field work at a remote field camp in Antarctica, the establishment of an international collaboration including researchers from three countries, the processing of over 1,000 individual samples for the collection of data at 17 highly polymorphic microsatellite markers and mtDNA, and exhaustive genetic analyses.

Regarding the population genetics component of this research, results strongly support the hypothesis that LI was re-colonized by one or more unsampled source population(s), in addition to South Georgia. This emphasizes the potential of satellite populations, which may be demographically less significant, but harbor high levels of genetic diversity - a particularly relevant observation for the prediction of future scenarios in Polar regions, which face unquestionable environmental change. The Antarctic Peninsula is one of three areas undergoing the most rapid warming on Earth: atmospheric warming exceeded 0.1°C per decade over the last 50 years (Steig et al., 2009) and sea ice has decreased 10% per decade (Clarke et al., 2007). These changes have caused long-term declines in krill stocks (Atkinson et al., 2004), which have cascaded throughout the Antarctic food web to influence krill-dependent predators such as penguins and seals. For example, population declines exceeding

50% have occurred for the past 30 years for Adélie (*Pygoscelis adeliae*) and chinstrap (*Pygoscelis antarcticus*) penguins at the South Shetland Islands (Trivelpiece et al., 2011). Similarly, a recent decline in fur seal numbers has been detected at South Georgia and Livingston Island with a consistent reduction in annual pup production and juvenile recruitment rates for the past three years (J. Forcada personal comm.; Goebel et al., 2011). Although some short-term effects were recently observed, little is known about how Antarctic krill reductions will influence its dependent fur seals populations long-term (Forcada et al., 2008). The study of how genetic diversity is geographically partitioned within the species is paramount in directing management and monitoring objectives as it can provide unprecedented insights into the ability of these organisms to adapt to environmental change. In that regard, expanding the scope of this study to include additional breeding colonies throughout the species distribution range will be most valuable, as this will allow inferences of historical migration rates, global effective population size and more precise estimates of gene flow. Further, inclusion of pre-exploitation samples in genetic analysis would provide information on historical levels of genetic diversity, and help define baseline population sizes for Antarctic fur seals in this ecosystem (i.e. what were their population sizes before anthropogenic disturbance). Interestingly, ice-preserved samples have been discovered for other species of Antarctic seals and are currently being analyzed by researchers elsewhere, which highlights the urgency and timeliness of this study.

Considering the Antarctic fur seal mating system, this research has unraveled some key complexities within male and female breeding behavior. For example, remarkably high male reproductive skew was observed at a low-density breeding site suggesting that males seem to hold their territories for more seasons and/or exert stronger control over female's movements than is observed at a high-density site. Demonstrated plasticity in the species breeding behavior means that caution should be taken when extrapolating findings from the

high-density colony of Bird Island, South Georgia (i.e. female choice; Hoffman et al, 2007) to other Antarctic fur seal populations. Another surprising finding was a low percentage of rematings among individuals over time within a dataset that spanned a decade, despite the high levels of breeding site fidelity and male reproductive skew observed in Antarctic fur seals. This finding highlights the importance of nuances within male and female behavior as potential indirect mechanisms of inbreeding avoidance in highly polygynous species with typical breeding site fidelity. Lastly, the genetic analysis of twin cases confirmed the occurrence of multiple paternity within the same breeding cycle for the species, demonstrating that females can escape control from territorial males. Together, these results demonstrate the high complexity of pinniped mating systems, pointing out interesting future avenues of research that would refine our understanding of the how population density generally affects mating systems. For example, a study dedicated to sampling and observing all males, females and pups born at a medium-size colony at Livingston Island for multiple seasons, including the collection of fine-scale breeding site fidelity (within beaches), would allow for valuable and unprecedented comparisons of mating strategies across pinniped populations.

In summary, the monitoring of female Antarctic fur seals by the US AMLR Program for over a decade, the intensive dedicated sampling at a low-density breeding site during four breeding seasons and an inter-population comparisons between Livingston Island and South Georgia, allowed for unprecedented investigations of the re-colonization process and the mating system of the species. These investigations have revealed that Livingston Island was likely re-colonized by immigrants from more than one source and that this species mating system is highly complex: mating behavior seems to vary with population density and individuals do not commonly remate despite returning to the same breeding sites year after year. This work points to some interesting future avenues of research including circumpolar

estimations of gene-flow, historical DNA studies and further investigations of how density may influence mating systems through dedicated inter-population comparison studies.

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