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Population Genetics of Antarctic Seals

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Population Genetics of Antarctic Seals

by

Caitlin Curtis

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Biology
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University of South Florida

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Population Genetics of Antarctic Seals

Caitlin Curtis

ABSTRACT

I developed and tested a protocol for determining the sex of individual pinnipeds using the sex-chromosome specific genes *ZFX* and *ZFY*. I screened a total of 368 seals (168 crabeater, *Lobodon carcinophagus*; 159 Weddell, *Leptonychotes weddellii*; and 41 Ross, *Ommatophoca rossii*) of known or unknown sex and compared the molecular sex to the sex assigned at the time of collection in the Ross and Amundsen seas, Antarctica.

Discrepancies ranged from 0.0% – 6.7% among species. It is unclear, however, if mis-assignment of sex occurred *in situ* or in the laboratory. It also is possible, however, that the assigned morphological and molecular sex both are correct, owing perhaps to developmental effects of environmental pollution.

I sequenced a portion (ca 475 bp) of the mitochondrial control region of Weddell seals ($N = 181$); crabeater seals ($N = 143$); and Ross seals ($N = 41$). I resolved 251 haplotypes with a haplotype diversity of 0.98 to 0.99. Bayesian estimates of θ from the program LAMARC ranged from 0.075 for Weddell seals to 0.576 for crabeater seals. I used the values of

theta to estimate female effective population sizes (N_{EF}), which were 40,700 to 63,000 for Weddell seals, 44,400 to 97,800 for Ross seals, and 358,500 to 531,900 for crabeater seals. Weddell seals and crabeater seals had significant, unimodal mean pairwise difference mismatch distributions ($p = 0.56$ and 0.36 , respectively), suggesting that their populations expanded suddenly around 731,000 years ago (Weddell seals) and around 1.6 million years ago (crabeater seals). Both of these expansions occurred during times of intensified glaciations and may have been fostered by expanding pack ice habitat.

Autosomal microsatellite based N_{ES} were 147,850 for *L. Weddellii*, 344,950 for *O. rossii*, and 939,600 for *L. carcinophagus*. I screened one X-linked microsatellite (Lw18), which yielded a larger N_E estimate for *O. rossii* than the other two species. Microsatellite N_E estimates are compared with previously published mitochondrial N_E estimates and this comparison indicates that the Ross seal may have a serially monogamous system of mating. I find no sign of a recent, sustained genetic bottleneck in any of the three species.

Chapter 1: Introductory Remarks

Population size and endurance may be positively correlated, as larger populations often maintain larger amounts of genetic variability allowing for continual adaptation to changing environmental and biotic conditions (e.g., Hansson and Westerberg, 2002; Reed and Frankham 2003). Quantifying population sizes, however, may be difficult in species inhabiting logistically inaccessible aquatic Antarctic pack-ice and fast-ice habitats. In addition, simple counts of individuals may not accurately reflect the numbers of individuals contributing genetic information to subsequent generations, thus the long term genetic variability maintained within a population. Effective population size (N_E), as defined by Sewall Wright (1931) describes the number of individuals in an ideal population that would show the same dispersion of allele frequencies as the observed population. Estimates of N_E are often different (usually lower) than census size due to large variances in individual reproductive success, population size changes across generations, non-random systems of mating (e.g., polygeny and polyandry), and unequal sex ratios. When considering evolutionary processes, N_E is more important than a population count because N_E represents the actual numbers of individuals that commonly contribute to each generation over the long term. Here, I estimate genetic effective

population sizes of three of the four phocid carnivores that live in the seasonal fast ice and pack ice habitats of the western Amundsen and Ross seas in west Antarctica. These seals occupy important niches in the notoriously short Antarctic marine food web.

Due to their isolation around the Antarctic continent, Antarctic phocid seals have large population sizes and have persisted relatively free from anthropogenic disturbances. In addition, they are closely related, yet employ varying long-term mating strategies. My research focused on three species of Antarctic phocid seals. The Weddell seal, *Leptonychotes weddellii*, (Lesson 1826), named after the British sealing commander Sir James Weddell, tends to inhabit the land-fast ice surrounding the Antarctic continent. Adults are brown, lighter ventrally, and mottled with large darker and lighter patches, which tend to be silvery white on the ventral surface. Males can grow up to 2.9 m in length, whereas females may reach about 3.3 m, weighing around 400-600 kg (Jefferson *et al.* 1993). Weddell seals have a varied diet, consisting primarily of fish and cephalopods such as squid and octopi (Plotz *et al.* 1991), and are known to forage occasionally on Antarctic krill (*Euphausia superba*). Conversely, Weddells and most other seals become prey items of orcas (*Orcinus orca*). Leopard seals (*Hydrurga leptonyx*) are also known to feed on pups and subadults. Males use their large canine teeth to maintain breathing holes in the ice, and actively defend the three-dimensional surrounding territory (Kaufman *et al.* 1975, Bartsh *et al.* 1992), though prolonged scraping at the ice may wear teeth down to the pulp cavity over time, leading to mortality

(Stirling 1969). Weddell seals have a moderately polygynous breeding system, in which males may mate with as many females as may share his breathing hole (Gelatt *et al.* 2000). The Weddell seal is known for its very deep dives which may reach 700m, and may stay underwater for more than 60 minutes (Kanatous *et al.* 2002). Such deep dives typically involve foraging sessions (Mitani *et al.* 2004), as well as searching for cracks in the ice sheets that can lead to new breathing holes. The seal is thought to be able to remain submerged for such long periods of time in part due to low levels of aerobic lipid-based muscle metabolism (Kanatous *et al.* 2002).

The crabeater seal, *Lobodon carcinophagus* (Hombron and Jacquinot 1842), uses its highly specialized multilobed teeth to strain Antarctic krill (*Euphausia superba*), which form the majority of its diet (Laws 1977). Despite its name, the crabeater seal does not feed on crab. Dark yellowish brown to silvery grey, which is lighter ventrally, they have slender bodies and long muzzles (Jefferson *et al.* 1993). Crabeater seals are somewhat sexually dimorphic in size, with males up to 2.6 m and females 2.8 m (Laws *et al.* 2003). In late summer (i.e., post-breeding season) when surveys have been conducted, they are generally found near the outer edges of the pack ice. Crabeater seals are thought to be serially monogamous, whereby one males mates with a single female within a breeding season, though not necessarily the same female between seasons (Stirling 1983). Population size has been estimated at 7 to 15 million individuals (Laws 1977, Erickson and Hanson 1990) and the circumpolar population appears to be

panmictic with no indication of geographically localized breeding groups (Davis *et al.* 2000, 2008).

Relatively little is known about the Ross seal, *Ommatophoca rossii* (Gray 1844). Ross seals reach more than 2.4 m and 204 kg (Jefferson *et al.* 1993), making them the smallest of the Antarctic phocids, though females are usually slightly larger than males. Countershaded, they are dark grey dorsally and silvery grey underneath, often with brown or reddish brown stripes on the neck, sides and chest (Jefferson *et al.* 1993). They appear to be solitary, and utilize the stable ice floes on the exterior of the pack ice to molt, while they give birth on the more densely packed interior pack ice (Splettstoesser *et al.* 2000, Stewart 2007). Though they have a circumpolar distribution, higher numbers may be found in the Ross Sea, King Haakon VII Sea, and perhaps portions of the western Weddell Sea (Stewart 2007). Ross seals may spend much of the rest of their time to the north of the pack ice, alone in the open water (Stewart 2007). The mating system of this species is not known.

My research comprises four main elements, which have been organized into three chapters. Chapter one (Sexing Pinnipeds with *ZFX* and *ZFY* Loci) focuses on developing and testing a protocol for genetically determining the sex of pinnipeds in the laboratory through developing and employing sex-chromosome specific genetic markers located in the zinc-finger protein regions of the X and Y chromosomes (*ZFX* and *ZFY*, respectively). Presence of the Y chromosome specific *ZFY* marker, as determined by

presence of a PCR amplicon of the corresponding size, indicated a male tissue sample, whereas absence of the *ZFY* marker indicated a female sample. Laboratory confirmation of the sex of each seal sample was critical to the sex-specific analyses in later chapters. Genetic data were compared to the sex of the animals as it was described *in situ* upon collection. Chapter two (Pleistocene Population Expansions of Antarctic Pack-Ice Seals) uses mitochondrial DNA sequence data to estimate the current distribution of genetic variation among the three species of seals, which is in turn used to estimate female effective population size and long term population stability and demography. Chapter three (Autosomal and sex-linked patterns of genetic partitioning among three species of Antarctic seals) used nine autosomal DNA microsatellites and one X-linked microsatellite to estimate the current distribution of genetic variation among the three species of seals, which in turn was used to estimate total effective population sizes. Autosomal and X-linked effective population sizes were compared to the mitochondrial estimates from Chapter two, as well as previously published census estimates. The microsatellite data were also used to look for evidence of past genetic bottleneck events. Y chromosome data is presented in this chapter.

Chapter 2: Sexing Pinnipeds with *ZFX* and *ZFY* Loci

Introduction

The ability to accurately and reliably identify the sex of free-ranging animals is essential for estimating sex ratios, categorizing behavioral observations, and understanding almost every aspect of an animal's life history. For many species, it may be relatively easy to distinguish between adult males and females if they are sexually dimorphic in size or color. Distinguishing subadult or juvenile males from females, however, often can be challenging. Even adult sex can be difficult to determine in animals like the phocid pinnipeds that live in pack ice or fast ice habitats of the Antarctic and exhibit little to no sexual dimorphism. This difficulty is even more pronounced when individuals are viewed from a distance with no direct physical examination. In phocid pinnipeds, male genitalia are internal and, consequently, the only clue to sex in otherwise sexually monomorphic species is the presence (male) or absence (female) of a ventral penile opening. For animals with chromosomal sex determining mechanisms, molecular sex determination has the potential to unequivocally determine the genetic sex of individuals. This approach can circumvent many of the difficulties in identifying sex of

animals in the field and articularly useful in secretive species where only traces of the individual such as blood, hair, or scat are available.

Two sex-chromosome specific genes, *ZFX* and *ZFY*, are zinc-finger homologues located on the X and Y chromosomes, respectively (Pecon-Slattey and O'Brien 1998). Because it is typically located outside the pseudoautosomal region of the Y chromosome in eutherian mammals (Mardon and Page 1989, Page *et al.* 1987), *ZFY* only rarely recombines with *ZFX* (but see Pecon-Slattey *et al.* 2000), and together these genes have proven useful as a molecular method for determining the sex of many mammalian species (e.g., felids, Pilgrim *et al.* 2005; canids, Lucchini *et al.* 2002; sea otters, Hattori *et al.* 2003; pinnipeds, ungulates, and ursids, Shaw *et al.* 2003; cetaceans Morin *et al.* 2005; prosimians and humans, Fredsted and Villesen 2004; and rodents, Marchal *et al.* 2003). Shaw *et al.* (2003) developed a PCR-based *ZFY/ZFX* assay applicable in a variety of mammals, including one pinniped, the harbor seal (*Phoca vitulina*), by using a single generic primer pair to simultaneously amplify both homologues in a single PCR reaction, then verifying presence or absence of the amplicons on an agarose gel. This approach, however, relies on differences in the size of the X- and Y-specific regions that may be subjected to PCR competition and lead to allele dropout of the larger allele producing incorrect sex assignment. Although I have no empirical indication that this is happening with the Shaw *et al.* (2003) primers, it is nonetheless a well-documented phenomenon and one I wished to avoid (Piyamongkol *et al.* 2003, Sefc *et al.* 2003, Buchan *et al.*

2005). I expanded on the work by Shaw *et al.* (2003) and created primers specifically targeted to separately amplify a portion of the *ZFX* or *ZFY* gene in pinnipeds. This allowed me to genotype each seal to determine sex and to directly sequence one or both of these genes which could be used for forensic or species identification purposes.

Crabeater (*Lobodon carcinophagus*), Ross (*Ommatophoca rossii*), and Weddell (*Leptonychotes weddellii*) seals are phocid pinnipeds that live almost exclusively in fast ice or pack ice habitats around the Antarctic Continent (Reeves and Stewart 2003, Reeves *et al.* 1992). Males and females of each species are similar in size and color and are not easily distinguished most of the time without close inspection for the presence or absence of a ventral penile opening. Consequently a genetic method for determining or verifying sex may be useful to a variety of ecological studies in these species.

Methods

Tissue samples were collected from 168 free-living crabeater seals (*L. carcinophagus*), 159 Weddell seals (*L. weddellii*), and 41 Ross seals (*O. rossii*) via remote darting or direct handling during the 2000 Antarctic Pack Ice Seal (APIS) scientific cruise (Decker *et al.* 2002, Solls *et al.* 2005). Although all three species are circum-polar, most samples were collected from the pack-ice zone of the eastern

Amundsen and Ross Seas, approximately 67° – 78° S, 129° – 180° W (Figure 2.1) and some *L. weddellii* samples came from McMurdo Sound, Antarctica. Of the crabeater seals, there were 71 field identified males, 56 females, and 41 sex unknown. For Weddell seals there were 90 males, 64 females, and 5 unknown and for the Ross seals there were 25 males and 16 females. I also assayed four male and two female northern elephant seals and one male and one female California sea lion. Since samples from the latter two species were from captive individuals, I am highly confident of the true sex of the individuals. Most of the sampled Antarctic phocids, however, were free ranging and many were sampled remotely by biopsy dart and not directly examined.

I designed primers specifically targeting the last intron of the phocid *ZFX* and *ZFY* genes. To accomplish this, I used the two, previously published sets of nested generic *ZFX* and *ZFY* felid primers (Pecon-Slattey and O'Brien 1998; Table 2.1) to simultaneously amplify both gene regions from a male crabeater seal. PCR products were cloned (Original TA Cloning Kit, Invitrogen, Inc. Carlsbad, California, USA) and sequenced on an ABI 3730XL automatic sequencer (Macrogen Inc., Seoul, Korea). Sequences were aligned using Sequencher software (GeneCodes Corp., Ann Arbor, MI) and compared to published *ZFX* and *ZFY* sequences in GenBank. From the aligned crabeater seal and GenBank sequences, locus-specific primers were designed to target the *ZFX* or *ZFY* loci separately (Table 2.1). A subset of male crabeater, Weddell, and Ross seals were amplified and sequenced at both loci. I also assayed *ZFY* and *ZFX* in one

northern elephant seal (*Mirounga angustirostris*) and one California sea lion (*Zalophus californianus*). To determine the sex of individuals, I set up separate PCR reactions for the *ZFX* and *ZFY* genes for all individuals and visualized the amplifications side by side on an agarose gel. There are four possible amplification patterns. If both *ZFX* and *ZFY* or just *ZFY* alone amplified, the individual was assigned male. If the *ZFX* but not the *ZFY* amplified the individual was assigned female. If neither locus amplified, the individual was classified as unresolved. It should also be noted that individuals that amplify for the *ZFX* locus and not the *ZFY* could be either females or non-amplifying males (i.e., false negative for *ZFY*). The inclusion of a second male specific locus (e.g., *SRY*) can be helpful in confirming the results (Gilson *et al.* 1998).

DNA was extracted from frozen tissue samples using standard phenol-chloroform techniques and/or using a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA).

Amplification reactions generally were 25 μ l and contained 0.5 μ l total cell DNA, 1 X reaction buffer (Promega, Madison, WI, USA), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pmol of each primer, 6 mg BSA, and 1.25 U *Taq* polymerase (Promega, Madison, WI, USA). Thermocycling conditions were 95° C 2 min, 35 cycles of 95° C 1 min, annealing temperature (Table 2.1) 1 min, and 72° C 1 min, followed by a final extension at 72° C for 7 minutes. PCR products were visualized on a 2% agarose gel with ethidium bromide to assess quantity and fidelity of amplification and then purified using either Microcon[®] Centrifugal Filter Units (Millipore Corp., Billerica, MA, USA) or QIAquick spin

columns (Qiagen, Valencia, CA, USA). Approximately 100 ng of purified PCR product was directly sequenced in both directions on an ABI 3730XL automatic sequencer (Macrogen Inc., Geumcheon-gu, Seoul, Korea).

Results and Discussion

All five species successfully amplified for both loci. The length of the crabeater seal *ZFY* fragment, including the primer sequences, was 931 nucleotides (nt). The other species produced DNA fragments of similar length. The length of the crabeater seal *ZFX* fragment including the primer sequences was 1045 nt and the other species produced fragments of similar length. The *ZFX* fragment matches with 83% identity to the final *ZFX* intron of *Bos taurus* (Lawson and Hewitt 2002; GenBank accession AF241273) and my *ZFY* fragment was 79% identical to the final intron of the Amur leopard (AB211426). After removing segments for which reliable sequence data was not obtained from all individuals (usually at the beginning and end of the sequence), I was able to cleanly resolve 851 and 956 nucleotides of sequence for the *ZFY* and *ZFX* loci, respectively.

The genotypic sex of nearly all seals (95.8%) agreed with the sex assigned in the field (Table 2.2). Discrepancies between the field and laboratory assigned sex ranged from 0 to 6.7%. Conflicts between the laboratory and field assigned sex of an individual

can arise from either field sexing errors or an unidentified laboratory artifact. I am most confident in the laboratory assessment when both *ZFX* and *ZFY* amplifications produce strong, clear bands since non-homologous amplification of an appropriate sized fragment is unlikely given the specificity of the primers. Furthermore, the homology of the fragment can be verified by DNA sequence or RFLP analysis. Any male that amplifies for the *ZFX* gene but fails to amplify for the *ZFY* gene would appear to be a female by my genetic tests. That the *ZFX* gene amplified indicates that the failure of *ZFY* amplification was not due to poor template quality or other general amplifications problems. Given that I saw no intra-specific variation and that these primers generally worked well in all three species, I also believe that this type of locus specific artifact is unlikely. Nonetheless, I cannot definitively rule out that the eight individuals that were field identified as male but failed to amplify for the *ZFY* locus may indeed, be male. A field-identified female appearing to be male based on amplification of both the *ZFY* and *ZFX* loci, is a likely candidate for field misidentification. Sex of individuals in the field was determined in one of three ways. First, many crabeater and Weddell seals were closely approached so that skin samples could be taken from the trailing edge of their rear flippers while they slept. At this time, individuals were visually examined for the presence of a ventral penile opening or for distinctive scarring around the neck and fore flippers (suggesting male in crabeater seals). Six of the incorrectly identified individuals in Table 2.2 were sampled in this way, evenly split between the two possible

discrepancies (i.e., field male but genetic female and field female but genetic male). Second, a number of seals (particularly crabeater seals which are more difficult to approach) were remotely sampled by biopsy darting either using a crossbow or a hand held dart pole. The sex in these cases was assigned based on the quickly observed presence or absence of a penile opening or scarring on neck and near the fore flippers. Five of the total 15 incorrectly sexed individuals were sampled in this way and all but one of these five, a Weddell seal, were field males but genetic females. Finally, a smaller number of seals (13) were captured and anesthetized while samples were taken and telemetry instruments attached. These individuals were examined closely and assigned a sex by a wildlife veterinarian. Only one individual field-identified as male but failing to amplify the *ZFY* gene (a crabeater) was among the examined individuals. This may indicate that, although rarely, false negative amplification of *ZFY* has occurred.

Interestingly, three of the examined individuals field-identified as female (two crabeater seals and one Ross seal) produced strong *ZFY* (and *ZFX*) amplifications. Given that I believe that false positive amplification is unlikely, it would seem logical that these individuals might have been misidentified in the field. To the contrary, however, given that these individuals received a close examination by a wildlife veterinarian, it also seems highly unlikely that the field identification is wrong. Although I cannot fully validate either the field or laboratory methods for sex identification, it is possible that the observed discrepancy of a field-identified female clearly being a genetic male does not

involve errors in either method. It has been observed that three northern elephant seals (*M. angustirostris*) in California clearly had secondary sexual characteristics of adult males yet lack a penile opening leading to ambiguous sexual assignment (Stewart BS personal observation). Unfortunately, I did not have access or samples from these individuals to determine genetic sex. Presumably, if I did, they would produce positive amplifications for *ZFY*.

Persistent organic pollutants (POPs) are nearly ubiquitous in the environment (Damstra, *et al.* 2002) and many of them (e.g., dithiothreitol, polychlorinated biphenyls, and tributyltin) are endocrine-disrupting chemicals. In vertebrates, some POPs act as estrogen mimics or androgen antagonists resulting in genetically male individuals possess female physical characteristics (as is seen here; Ayaki *et al.* 2005, Hayes *et al.* 2002, Penaz *et al.* 2005). Although the near absence of industrial development and its remoteness make the Antarctic appear to be an unlikely place for POPs, this is clearly not the case. While studying sediment cores in McMurdo Sound, Ross Sea, Antarctica, Nigri *et al.* (2004) documented detectable levels of butyltins (i.e., TBT, DBT, and MBT) at six of eight surveyed sites. Butyltins are commonly used in anti-fouling paints on large boats including the icebreakers that visit McMurdo Sound. Nigri *et al.* (2004) thought that butyltins might be introduced into the sediment from paint chips rubbed off of icebreakers. One of their sampling sites in McMurdo Sound, Cape Armitage, had extremely high levels of butyltin "...only exceeded in very busy harbours..." (Nigri *et al.*

2004). Goerke *et al.* (2004) also documented a 30 – 160 fold biomagnification of several POPs in Weddell seals and southern elephant seals (*Mirounga leonina*).

Although it is difficult to say at this time what, if any, effect Antarctic POPs are having on the health and sexual development of Antarctic pack-ice seals, there is a wealth of studies indicating that the presence of POPs in the environment can have long-lasting and dire consequences for wildlife. They also may have been contributing factors resulting in field sex identifications not agreeing with my genetic sex assignments. Regardless, being able to genetically assess the sex of free-ranging seals can provide a backup method for testing the veracity of visual designations, allow sex determination of DNA samples when individuals are not handled or even sighted, and provide a key to the understanding of the impacts that POPs might have in marine ecosystems. Furthermore, to fully assess the potential effects that POPs may be having on natural populations, it is necessary to have both genetic and morphological information along with some understanding of the individual exposure to POPs.

I found no intra-specific *ZFY* variation after sequencing 12 crabeater (GenBank accession number DQ493902), 10 Weddell (DQ493904), or 10 Ross seals (DQ493903). There also was no intra-specific variation in *ZFX* genes of those species after screening four (DQ811091), two (DQ811093), and two (DQ811092) individuals, respectively. In addition, I sequenced *ZFY* and *ZFX* from one each of northern elephant seals (*Mirounga angustirostris*; *ZFY*– DQ493906, *ZFX*– DQ811095) and California sea lion (*Zalophus*

californianus; *ZFY* – DQ493905, *ZFX* – DQ811094). Among all five species there was considerable inter-specific variation at both the *ZFY* and *ZFX* genes (Appendix A and B). At the *ZFX* locus, there were 50 variable nucleotide positions (~5%). Of those, 29 were transitions, 10 were transversions, and three were deletions (one 3 nt and two 4 nt). At *ZFY*, 61 of the nucleotide sites were variable (~7%). Of those, 33 were transitions, 13 were transversions, one had both a transition and a transversion, and one had both a deletion and a transition. There was a single 4 nt deletion in the Weddell sequence and there were two 2 nt and two 3 nt deletions in the *Z. californianus* sequence. It appears that all species can be uniquely identified from all others through DNA sequence by at least three (crabeater seal at *ZFY*) to at most 31 (California sea lion at *ZFY*) sites. My assessment of intra-specific variation, however, was limited and species designations should be made on multiple sites or a larger number of individuals from throughout the range of the species. Nonetheless, this level of variation is likely to be useful in identifying species from forensic samples and for phylogenetic analyses.

The results of this study have been threefold. First, I have indicated that the *ZFX* and *ZFY* loci are likely to be good nuclear markers for species identification. Second, I have demonstrated the utility of new *ZFX* and *ZFY* markers for assigning the sex of pinnipeds especially when access to the animal is highly limited. Third, I uncovered several cases where the sex designated based on morphology appears to be in conflict with molecular markers. In some of these cases, it likely is simply misidentification in the

field or technical artifact. Others, however, may be indicating that persistent organic pollutants are having significant impact on Antarctic fauna. Further targeted research, however, is needed before final conclusions about the likelihood and magnitude of the effect can be reached.

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Table 2.1. Primer sequence, annealing temperature, and fragment size for loci used in *ZFX/ZFY* screening. All amplifications were of the crabeater seal (*Lobodon carcinophagus*).

Locus	Primer Sequence (5' to 3')	°C	Size (bp) ^a	Reference
Zinc-finger Y (<i>ZFYF</i>)	F: CCAAACAGGTGAGGGTGCACA	60	931	This Study
Zinc-finger Y (<i>ZFYR</i>)	R: GTAATCACAGTCAGTACAGTGG			
Zinc-finger X (<i>ZFXF</i>)	F: TGAGGGCACATGAGTCCCACA	55	1045	This Study
Zinc-finger X (<i>ZF2RA</i>)	R: GGTGGTTGTGTAAACTTATCTT			
ZF1F	F: ATAGATGAGTCTGCTGGC	48	Multiple	Pecon-Slattery and O'Brian 1989
ZF1R	R: CGTTTCAAATCACTTGA			
ZF2F	F: GGTGATTCCAGGCAGTAC	52	~1200	Pecon-Slattery and O'Brian 1989
ZF2R	R: TGGTCAGCTTGTGGCTCTCCT			

^a Size of the fragment includes the primer sequence.

Table 2.2. Summary of sex determination of pinnipeds. The number correct refers to agreement between the field and laboratory sex determinations and the number incorrect refers to disagreement. Unknown refers to individuals where sex was not assigned in the field and unresolved is the number of samples that did not reliably amplify with either *ZFX* or *ZFY* primers.

Species	Field Identified As						Total ^a
	Male		Female		Unknown	Unresolved	
	Correct	Incorrect	Correct	Incorrect			
<i>Lobodon carcinophagus</i>	65 (94.2%)	4 (5.7%)	48 (96.0%)	2 (4.0%)	41	14 (8.3%)	168
<i>Leptonychotes weddellii</i>	86 (95.5%)	4 (4.5%)	57 (93.4%)	4 (6.6%)	5	5 (3.1%)	159
<i>Ommatophoca rossii</i>	25 (100%)	0 (0%)	14 (93.3%)	1 (6.7%)	0	1 (2.4%)	41
<i>Mirounga angustirostris</i>	4 (100%)	0 (0%)	2 (100%)	0 (0%)	0	0	6
<i>Zalophus californianus</i>	1 (100%)	0 (0%)	1 (100%)	0 (0%)	0	0	2
Total	181 (95.8%)	8 (4.2%)	122 (94.6%)	7 (5.4%)	46	19 (5.1%)	376

^a Row sum may not equal total because some individuals field-classified as unknown also were unresolved.

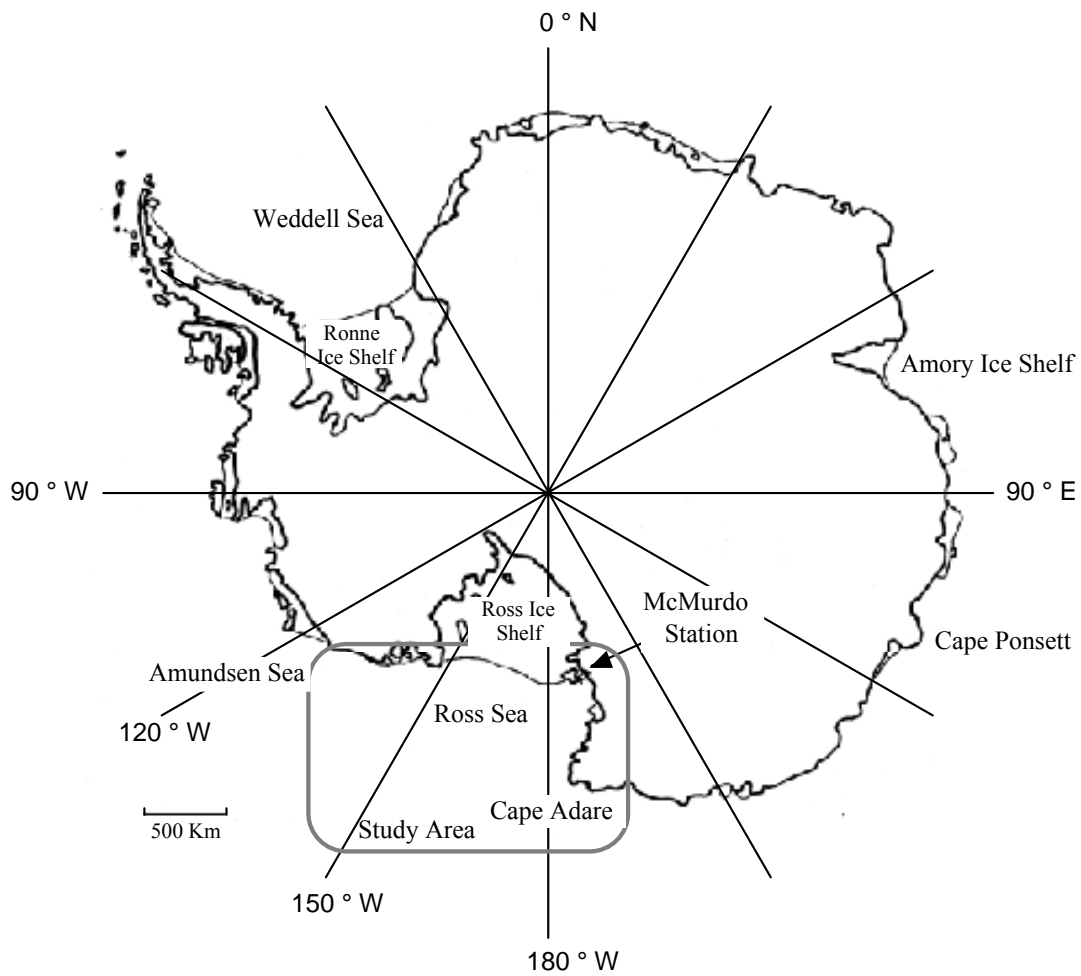


Figure 2.1. Map of the Antarctic continent showing the area where genetic samples were collected.

Chapter 3: Pleistocene Population Expansions of Antarctic Pack-Ice Seals

Introduction

Population abundance and persistence are often positively correlated, as larger populations are thought to be more resilient to ecological perturbations. Similarly, genetically variable populations are generally more fit than less variable ones presumably because greater genetic variability allows for adaptation to changing environmental and biotic conditions (e.g., Hansson and Westerberg, 2002; Reed and Frankham 2003). A simple count of individuals in a population may not, however, accurately reflect the numbers of individuals that are actually contributing to the next generation and thus the long-term evolutionary potential of the population. Wright (1931) defined the genetically effective population size (N_E) as the number of individuals in an ideal population that would have the same rate of genetic drift as the observed population. When considering evolutionary processes, N_E is more important than a population count because N_E represents the actual numbers of individuals that commonly contribute to each generation over the long term. Several different factors can conspire to result in N_E being different from census population size (almost always smaller). Large variance in individual reproductive success, population size changes across generations, non-random systems of

mating (e.g., polygeny and polyandry), and unequal sex ratios all work to reduce N_E compared to census size (Hartl and Clark, 1997). Consequently, to know the evolutionary potential of a population an understanding of the genetic effective population size is needed first. Here, I estimate genetic effective population sizes of three of the four phocid carnivores that live in the seasonal fast ice and pack ice habitats of the western Amundsen and Ross seas in west Antarctica. These seals occupy important niches in the notoriously short Antarctic marine food web.

Demography of Antarctic seals remains a critical but relatively little understood element of the Antarctic marine ecosystem, though it is important to management models and conservation plans for the Southern Ocean. This is particularly true for crabeater seals (*Lobodon carcinophaga*), which have a large circumpolar population and feed exclusively on Antarctic krill (*Euphausia superba*; e.g., Mori and Butterworth 2004). Owing principally to the remoteness and heavily ice-covered habitats of pack ice seals, there has been only *de minimus* anthropogenic exploitation and impact to their populations (e.g., Oritsland 1970, Stirling 1971, Laws *et al.* 2002). Abundance and population trend data for crabeater seals have been specifically identified by the International Whaling Commission as important, though deficient, when considering trophic interactions with Southern Ocean whale populations (International Whaling Commission 2005) and this is arguably true for all Antarctic seals.

My research focused on three closely related species of phocid carnivores that

have circumpolar Antarctic distributions, occupy distinct ecological niches, and have different mating tactics. The population of Weddell seals (*Leptonychotes weddellii*) has been estimated at around 730,000 to 800,000 (Laws 1977, Erickson and Hanson 1990) and typically inhabits the land-fast ice surrounding the Antarctic continent when breeding. They appear to be polygynous with males defending underwater territories and mating with up to five females during each breeding season (Gelatt *et al.* 2000). Regional populations apparently consist of separate, geographically disjunct breeding colonies (Stirling 1969). Adult female seals appear to be philopatric to breeding site and natal philopatry may also be strong (e.g., Stirling 1969). Relative to seals outside of the Antarctic, however, choice of breeding sites by Weddell seals is more variable (cf. Croxall and Hiby, 1983). Adjacent colonies may have substantial genetic exchange driven in part by intraspecific competition for prime space within or among breeding colonies (Hastings and Testa, 1998). Further, most estimates of site fidelity are based on tagging studies that generally reveal levels of exchange sufficient (i.e., on average, one migrant exchanged per generation) to genetically homogenize populations. There is some indication that at least distantly separated colonies (i.e., on opposite sides of the continent) might be genetically differentiated (Davis *et al.* 2000, 2008). Those assessments, however, have indicated only slight genetic subdivision among breeding colonies and no genetic subdivision when individuals are mixed in the pack ice in the Ross and Amundsen seas (Davis *et al.* 2008). But given the potential for philopatry and a

polygynous mating system, I would expect that N_E in this species would be smaller than the other species where these attributes were not found.

Crabeater seals are generally found near the outer edges of the pack ice in late summer (i.e., post-breeding season) when surveys have been conducted. Population size has been estimated at 7 to 15 million individuals (Laws 1977, Erickson and Hanson 1990) and the circumpolar population appears to be panmictic with no indication of geographically localized breeding groups (Davis *et al.* 2000, 2008). The abundance of crabeater seals was expected, by some, to have increased substantially following the decline and near collapse of populations of krill-eating baleen whales in the Antarctic in the early to mid 20th century (e.g., Laws 1977, Smetacek and Nicol 2005). Between the 1920's and the mid 1960's, the total abundance of baleen whales dropped from an estimate of 22 million metric tons to approximately 2 million metric tons (Macintosh 1970). Reduced competition for krill presumably would have promoted an increase in the abundance of crabeater seals. The population size of crabeater seals might have declined in recent years, however, owing to resource competition from rebounding whale populations and the beginning of commercial harvesting of krill (Bester *et al.* 1995). Crabeater seals are thought to be serially monogamous with males mating with only a single female each mating season but not necessarily the same female between seasons (Stirling 1983). Consequently, I expect that the combination of this breeding system with panmixia and an extremely large population size will result in a larger N_E and less of a

difference between N_E and census size.

Much less is known about the ecology or breeding biology of Ross seals (*Ommatophoca rossii*). They are usually found in deeper pack ice where medium and large ice floes are common. Their circumpolar abundance has been estimated at 131,000 to 220,000 (Laws 1977, Erickson and Hanson 1990) and there is no indication of geographic population subdivision (Davis *et al.* 2008). All three species, however, have evolved and existed in relative isolation and have experienced very little to no sustained hunting pressure. Historic population changes, therefore, should be a reflection of natural processes and not a result of anthropogenic disturbance. To better understand the ecology and breeding biology of these unique animals, I explored the degree of genetic variation in nuclear and mitochondrial loci of a relatively large number of seals sampled in the Ross and western Amundsen seas in west Antarctica from December 1999 through February 2000. Specifically, I have assayed DNA sequence variation in sex-linked nuclear loci (both X and Y; Curtis *et al.* 2007), autosomal and sex-linked microsatellite loci (data not shown), and maternally inherited cytoplasmic mitochondrial DNA. Here, I report the results from the maternally inherited mitochondrial DNA.

Methods

Samples

Biopsy samples of skin were collected from 181 free-living Weddell seals, 143 crabeater seals, and 41 Ross seals by remote darting or direct handling during the Austral summer from December 1999 through February 2000 (cf. Decker *et al.* 2002, Solls *et al.* 2005). Most samples were collected from the pack ice zone (i.e., not specific breeding colonies) of the eastern Ross and western Amundsen seas, between 67° and 78° S latitude and 129° to 180° W (Figure 3.1) though a few samples from Weddell seals were collected from a long-studied colony in Erebus Bay, McMurdo Sound. Sex of most individuals was determined and/or confirmed in the laboratory using *ZFX* and *ZFY* genetic screening (Curtis *et al.* 2007). Tissue samples were either stored in ethanol or frozen at -80°C until DNA extraction.

Genetic Methods

Total cell DNA (tDNA) was extracted using standard phenol-chloroform techniques or with a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA), and 5 μL of DNA

was visualized on a 0.8% agarose gel with ethidium bromide to assess DNA quality. I amplified a roughly 475 nucleotide (nt) fragment of the 5' portion of the mitochondrial control region using conserved, generic primers as follows: forward primer TDKD (Slade *et al.* 1994): 5'-CCTGAAGTAGGAACCAGATG-3', reverse primer L15926 (Kocher *et al.* 1989): 5'-TCAAAGCTTACACCAGTCTTGTAACC-3'. A 50 μ L reaction contained 0.5 μ L tDNA, 1 X reaction buffer (Promga, Madison, WI, USA), 2.0 mM MgCl₂, 0.2 mM of each dNTP, 10 pmol of each primer, 6 μ g BSA, and 1.25 U *Taq* polymerase (Promga, Madison, WI, USA). Thermocycling conditions were 94° C for 1 min, 35 cycles of 94° C for 1 min, 50° C for 1 minute, 72° C 1 min, and a final extension at 72° C for 7 min. PCR products were visualized on a 2% agarose gel with ethidium bromide to assess quantity and fidelity of amplification and then purified using either Microcon[®] Centrifugal Filter Units (Millipore Corp., Billerica, MA, USA) or QIAquick spin columns (Qiagen, Valencia, CA, USA). Approximately 100 ng of purified PCR product was used as template in the sequencing reaction, and sequences in both directions were run on an ABI 3730XL automatic sequencer (Macrogen Inc., Geumcheon-gu, Seoul, Korea).

Sequence and Population Analyses

Forward and reverse sequences were aligned using Sequencher software (Gene Codes, Ann Arbor, MI, USA), and edited manually when necessary. All aligned

sequences were analyzed using Arlequin vers. 3.01 (Excoffier *et al.* 2005) to estimate standard genetic indices, including nucleotide diversity (π) and haplotype diversity (h ; Nei, 1987). I estimated Θ using the Bayesian, Markoff-chain, maximum-likelihood approach implemented in the program LAMARC (Kuhner 2006). All analyses used a general time reversible (GTR) model of evolution chosen as the most likely by the Akaike Information Criteria within MODELTEST v3.06 (Posada and Crandall 1998), two simultaneous searches with heating, and 100,000 steps with an initial burn-in of 10,000 steps. Analyses were repeated three times with different random number seeds to assess consistency. Assuming that the populations are in equilibrium, female effective population sizes (N_{EF}) for the three species were estimated by replacing Θ in the equation, $N_{EF} = \frac{\Theta}{2\mu}$, where μ is the mutation rate per site per generation.

I evaluated evidence for historic population expansion using two methods. First, I used Fu's F_S value, which is primarily based on the differences between expected numbers of alleles (estimated through 10,000 computer simulations based on the observed pair-wise differences in my samples) and observed numbers of alleles (Fu 1997). F_S is particularly sensitive to past population expansions, which typically generate large, negative numbers due to the predominance of new, rare haplotypes in the sample. I also analyzed the distribution of all pair-wise haplotype differences (mismatch distributions), and calculated the goodness of fit of the estimated distribution to that

predicted by a sudden expansion model using 16,000 computer simulations (Rogers and Harpending 1992, Rogers 1995, Schneider and Excoffier 1999, Excoffier 2004) and associated raggedness index (Harpending 1994) as performed in ARLEQUIN (Excoffier *et al.* 2005). Mismatch distributions tend to be unimodal, and smooth (i.e. wave-like) in populations that have undergone population size changes. Multimodal or random and rough distributions are characteristic of populations that have experienced long-term stability. The significance or goodness of fit of the observed data to the predicted distribution modeled for sudden expansion growth was assessed by using a sum of squares (SSD) method. When observed distributions fit the sudden expansion model ($p \geq 0.05$), I estimated, using ARLEQUIN, the number of generations since the expansion (t) from the peak of the distribution (τ) as $t = \frac{\tau}{2\mu}$ (re-arranged from Li 1977) where μ is the rate of mutation per gene per generation.

Results

MtDNA Variation

I obtained useable mitochondrial sequence from 365 seals, resulting in 251 unique

haplotypes. A 471 bp fragment of the control region was aligned for 181 Weddell seals (GenBank accession numbers EU653156 – EU653238), a 472 nt fragment was aligned for 143 crabeater seals (EU653021 – EU653155), and a 481 bp segment was aligned for 41 Ross seals (EU653239 – EU653272). DNA fragments from Ross seals were truncated to 420 bp due to poor sequence quality and missing data in several seals near the ends of the fragment. No polymorphic sites were observed in the truncated segments. There were large numbers of haplotypes in each species with 83 haplotypes identified in the Weddell seals, with 49 of them (59.0%) found in only a single seal (singletons; Table 3.1). There were 33 haplotypes in the Ross seals, with 26 singletons (78.8%). Notably, there were 135 haplotypes in the crabeater seals, with 127 singletons (94.1%). Consequently, estimates of haplotype diversity were large and approaching 1.0 in all cases (Table 3.1).

Effective Population Size

All LAMARC analyses gave very similar results within species and unimodal distributions for the likelihood distributions indicating that I have the true probability density functions. The estimated sampling sizes (ESS) were all greater than 100 and often much larger and when combined, the ESSs were all greater than 200. The estimates of θ across the three separate runs within a species were within ~1% or less of the

combined median value. The median Θ estimates ranged from 0.075 to 0.576 (Table 3.1). Using a mutation rate (μ) for the mitochondrial control region estimated from pinnipeds of 7.5×10^{-8} substitutions/site/year (Slade *et al.* 1998) and a conservative estimate of generation time of 9 years for each species (cf. Croxall and Hiby 1983, Bengtson and Laws 1985, Harding and Harkonen 1995, Hadley *et al.* 2006), the genetically effective female population sizes ranged from ~55,600 for Weddell seals to ~426,700 for crabeater seals, with Ross seals closer in number to Weddell seals (~65,200; Table 3.1).

Demographic History of Pack-Ice Seals

All three seal species had significant F_S values (i.e. $p < 0.01$) and all were less than -20.0 (Table 3.2), persuasive evidence of population expansion. I also tested for population expansion using mismatch distribution estimates. Weddell and crabeater seals were not statistically significantly different from a unimodal mean pair-wise difference distributions ($p = 0.56$ and 0.36 , respectively, Table 3.2, Figure 3.2), suggesting that they had previously increased rapidly in abundance. The Ross seal distribution was not unimodal ($p = 0.04$, Figure 3.2) and does not indicate any substantial historic change in population size (Rogers and Harpending 1992, Slatkin and Hudson 1991). This trend was reinforced with Harpending's raggedness values (Harpending 1994), which were

significantly different from ragged distribution in Weddell and crabeater seals but not so in Ross seals ($p = 0.84, 0.82, \text{ and } 0.05$, respectively).

The peak of the unimodal distribution (τ) for Weddell seals was 5.74 corresponding to a population expansion 81,246 generations ago (731,210 years). The τ for crabeater seals was twice as large (12.59) suggesting that their numbers increased about 177,825 generations (1.6 million years) ago. Both correspond to times of decreasing Antarctic temperatures and increasing seasonal ice extent (Petit *et al.* 1999).

Discussion

My assessment of genetic variation in the mtDNA control region indicates that Weddell, crabeater, and Ross seals all have levels of intraspecific haplotype and nucleotide diversity similar to, or greater than, those reported for other marine mammals that have not been substantially harvested and that do not have strongly matrilineal population structuring (e.g., Dalebout *et al.* 2005, Malik *et al.* 2000, Westlake and O’Corry-Crowe 2002). Haplotype diversity in these Antarctic seals is extremely high and indicates that populations of these species have been consistently large for long periods.

My conclusions are conditioned, however, on a couple of caveats. First, mutation rates have consistently been difficult to estimate and likely have large margins of error. Consequently, if my estimate of mutation rate is off by as much as an order of magnitude then my estimates of N_{EF} and time since population expansion are also off by an order of magnitude. I think, however, that the mutation rate that I used is reasonable and appropriate. I take this rate from Slade *et al.* (1998) who estimated the mutation rate using mtDNA d-loop divergence and fossil records of northern and southern elephant seals, the leopard seal, and the Weddell seal. As one of those is a species that I analyzed here and the others are closely related, I think that the estimate is not affected by phylogenetic peculiarities. Although the taxa used for the comparison were 4.5 million years divergent, at most, I also do not think that saturation had any effect. Slade *et al.* (1994) tested the relationship between genetic distance and divergence time in these and other pinnipeds that diverged from 4.5 to 40 million years ago and found a linear relationship in all comparisons except for the most distantly related pairs of taxa for both transitions and transversions. A non-linear relationship is characteristic of saturation in the sequence data. Consequently, they concluded that saturation wasn't present until 30 million years divergence. This means that the estimates of divergence with which the mutation rate was estimated are unlikely to be effected by homoplasy in the data.

A second caveat concerns the estimate of generation time. Only a few studies have examined the reproductive characteristics of these species. Croxall and Hiby (1983)

reported an extensive survey on breeding and survival in Weddell seals and estimated that the average age of first reproduction was 4 – 5 years and after about 6 years of age 80% or more of the females have pups. Lifespan in Antarctic seals is not generally more than 25 years and Croxall and Hiby found that over 80% of the females with pups were between the ages of 6 and 13 and the oldest reproducing female was 17. Harding and Harkonen (1995) estimated average of sexual maturity in crabeater seals at between 3.7 and 5 years old and Bengtson and Siniff (1981) estimated it at 3.8 years. Hadley *et al.* (2006) reported average age of first reproduction in a long-studied population of Weddell seals in the Ross Sea (McMurdo Sound) at 7.6 years. Consequently, I think that an estimate of generation time of 9 years is conservatively (i.e., underestimates N_E) appropriate. If this were an under- or overestimate by even two years then my estimates of N_{EF} would be around 20% lower (or higher) but, given the 95-percentile range, well within the estimate range of N_E using 9 years.

Given the above, I think that my estimates of N_{EF} and time since population expansion are reasonable. Moreover, the relative rank order of the estimates is not affected by errors in mutation rate or generation time estimates. Further, N_{EF} values reflect the magnitude of the breeding population over evolutionary time and may not correspond to contemporary values. It is important also to note that estimates of

genetically effective population size using $N_{EF} = \frac{\Theta}{2\mu}$ assume that the population under

consideration is in equilibrium. Given that I detected historic population expansions for the crabeater and Weddell seals, this is unlikely to be true in these species. How far from equilibrium and what effect this might have on my estimates of N_E , however, are unknown. It is reasonable to assume that the time since expansion has been relatively long (80,000 – 170,000 generations), the populations are converging on equilibrium levels. Nonetheless, that I still can detect the population expansion indicates that they have yet to completely reach equilibrium.

My estimate of N_{EF} for crabeater seals is around 426,700 females. The serially monogamous mating system of this species suggests that female and male long-term effective population sizes should be roughly equal and thus a total genetically effective population size of less than 853,400 seals. This compares with previous visual census estimates of circumpolar abundance of 7 to 15 million (Laws 1977, Erickson and Hanson 1990). The Ross and Amundsen seas are key areas of ice habitat and a larger effective population size would be expected if the prior circumpolar estimates are accurate. In a review of 192 empirically derived estimates, Frankham (1995) showed that the ratio of N_E to census size is often less than 0.5 and frequently less than 0.25. Considering just mammals (45 studies of 25 species), the N_E to census size was 0.45 ± 0.21 . My estimate, however, is 0.057 – 0.122 (Table 3.1); up to an order of magnitude smaller than commonly seen. Avise *et al.* (1988), however, showed that N_E could be two to three orders of magnitude lower than census size estimates.

Although few assessments of population genetic subdivision have been made, there is little indication of extant barriers to dispersal. In the two published genetic studies that I am aware of, there were no indications of population subdivision around the Antarctic for crabeater seals (Davis *et al.* 2000, Davis *et al.* 2008). As such, I would expect my estimates of N_E to be reasonably close to the visual census estimates. This is clearly not the case. Several factors can lead to a highly reduced N_E relative to census size. In spite of the genetic data, there may be some level of undetected geographic subdivision among crabeater seals, particularly between east and west Antarctica and perhaps other geographically disjunct areas like the Weddell Sea. Consequently, my estimates would then reflect the number of breeding seals in the immediately sampled and geographically defined area of the western Amundsen and Ross seas. In Erickson and Hanson's (1990) survey of seal densities in Antarctica, they defined the Ross Sea as bounded by 130° W to 160° E which roughly encloses the study area. The population estimate for the Ross Sea crabeater seal was 1.3 million individuals (Erickson and Hanson 1990). My estimate of as many as 853,400 individuals then would give a N_E to census size ratio of 0.657, which is more typical of wild animal populations. I have, however, no indication that the area I sampled constitutes a separate population. Although Davis *et al.* (2008) found no indication of population subdivision, my data set is only a partial subset of theirs and may be genetically structured. If this were the case and my samples represent more than one sub-population, I would expect that my

estimates of N_E would be overestimates. It might also be that the mitochondrial DNA variation is underestimating the effective population size (Bazin *et al.* 2006), though it is difficult to address the potential for this. Perhaps, it may be possible through examination of nuclear loci. The high levels of haplotype diversity do suggest, however, the mtDNA is evolving in a neutral fashion for these three species of Antarctic seals. There also is little indication that crabeater seals underwent a 4-10 fold population expansion in the last 30-50 years, as suggested by the 'krill surplus hypothesis' (Laws 1977; Mori and Butterworth 2004). If this were true, I would expect considerably lower levels of nucleotide and haplotype diversity. There may not, however, have been enough time for such a recent population size change to be reflected in the mtDNA data. Nonetheless, fluctuations in population size, high variance in female reproductive success, or recent expansion in population size all would result in an estimate of N_E that is substantially lower than actual population size.

Extrapolating total effective population size in Weddell seals may be less straightforward than a simple doubling of female numbers, which would imply a long term 1:1 sex ratio. Given the polygynous mating system of this species, this is unlikely to be the case. My genetically effective population size estimate is 55,600 females. Male effective population sizes may be lower and thus the total genetically effective population size may be less than 111,200. Even so, this number is considerably lower than the circumpolar estimate of 730,000 by Laws (1977) and ~800,000 by Erickson and Hanson

(1990). The geographic range of my effective population size estimate is not clear, though both genetic studies to date (Davis *et al.* 2000, 2008) do suggest that it is possible that I have sampled a subpopulation. Therefore, it may be that my estimate of effective population size reflects a regional geographic scale (i.e., eastern Ross and western Amundsen seas). Erickson and Hanson (1990) estimate that there were ~50,000 Weddell seals in the Ross Sea. If I was estimating the number of seals in just this area, the census size would then be smaller than the genetically effective size; an unlikely situation. Erickson and Hanson (1990), however, do caution that their population size estimates are very conservative and likely underestimate true abundances. My estimate of 111,200 also assumes a 1:1 sex ratio, which is most likely not the case and mine is an overestimate. My samples cover approximately 60° longitude, which is less than 20% of the circumference of Antarctica. If I assume that the distribution of Weddell seals around Antarctica is uniform and I have sampled a discrete subpopulation, that would put the total effective population size at ~556,000 individuals. Although still substantially smaller than the direct census estimate, the N_E to census size ratio is 0.67 – 0.76 and similar to the estimate of ~0.50 for other mammals (Frankham 1995)

Not enough information is known about Ross seals to speculate about their breeding system, however if a 1:1 sex ratio is assumed, total genetically effective population size may be ~130,400 reproducing individuals, which is very near the census estimate of ~130,000 to 220,000 made by Erickson and Hanson (1990) and Laws (1977),

respectively, and would result in the highest N_E to census size ratio of all three species. Census sizes, however, were estimates that the authors speculated may be underestimates due to the unknown numbers of seals in the water at any given time, even at times of peak haul out, and the fact that a significant part of the range of the seals was unsurveyed. Even so, the ratio of the genetically effective population size to census size for Ross seals is clearly larger than the other two species (Table 3.1). There are several factors or combinations of factors that can account for this. Unlike both Weddell and crabeater seals, Ross seals did not show indications of historical population size changes. With changing population size, the genetically effective size is better approximated by the harmonic mean of size across generations and as such is substantially reduced and influenced by the smallest population sizes. If Ross seal populations have been fairly stable relative to the other two species that show population expansions, then I would expect them to have the largest N_E to census size ratio. It is also possible that non-equilibrium states for the other two species are resulting in an underestimation of N_E . I have, however, no data with this to support or refute this argument.

A total of 11,414 seals were observed during the multi-disciplinary cruise in 1999-2000 when the samples reported here were collected (Ackley *et al.* 2003). Of the 7,781 seals that were identified to species, 4,817 were crabeater (53.8%), 2,852 were Weddell (36.7%), 79 were Ross (1%), and 33 (0.4%) were leopard seals (*Hydrurga leptonyx*). Though Ross seals were seen less often than Weddell seals, my estimate of

effective population size for Ross seals was higher than that for Weddell seals. This is likely due to differences in their primary habitats and distributions. Ross seals primarily live in pack ice habitats, where the surveys were conducted, whereas Weddell seals mostly occur close to the coast in fast ice habitats, at least in summer. Ross seals may be more difficult to sight than Weddell seals. A historic population bottleneck of Weddell seals followed by population expansion also would have resulted in a smaller genetically effective population size and may account for some of the difference. If Ross seals are monogamous, compared to the polygynous Weddell seal, then notwithstanding other influences, this would also result in larger estimates of Ross seals. This should not, however, be reflected in the maternally inherited mtDNA.

Crabeater and Weddell seals have apparently increased in abundance since prior glaciations. My mismatch analysis indicated that, historically, crabeater seal experienced a population expansion (Table 3.2) approximately 1.6 million years ago. The Pliocene – Pleistocene boundary is ~1.8 million years ago and roughly marks the start of the latest ice age. At this time sea levels were lower than now, and ice volume was significantly larger. Temperatures oscillated between warmer and colder periods on an approximately 41,000 year cycle until about 1.0 million years ago when the cycle switched to 100,000 years. Correlative changes in fast ice and pack ice extent and seasonal tenure might have provided larger and better habitats for breeding and molting and consequently promoted range and population expansions. My analyses indicate that Weddell seals increased in

abundance around 731,000 years ago. Expansions of fast ice and pack ice habitat may have provided more breeding habitat for Ross seals but had little influence on pelagic, non-ice habitats where Ross seals appear to spend most of each year foraging. This may explain the results of my analyses, which did not unambiguously indicate a sudden population expansion in the Ross seal.

Antarctic seals are important predators in the remarkably short food web of Southern Ocean ecosystems. Unlike most marine mammals, Antarctic seals have not been substantially affected directly by humans, and small local harvests during the past several decades could not explain the patterns of genetic variability that I observed or the derived estimates of population history. My results indicate that the N_E for pack ice seals is generally lower than census size but on par with what is seen for other animals. There are considerable amounts of genetic diversity (at least for mtDNA) in all three species indicating that, genetically, these species are healthy (Spielman *et al.* 2004). Two, if not all three, of the species I studied evidently increased substantially in abundance in prehistoric times correlative with expanding ice habitat during times of increased glaciation. The earlier speculation that crabeater seal populations might have increased substantially with the reductions in krill-eating competitors is not supported by my analyses. The rank order of my N_E estimates does not coincide with those of previous direct counts surveys suggests that population size of Ross seals is not adequately estimated by surveys as traditionally conducted.

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Table 3.1. Genetic diversity and female effective population sizes in pack ice seals. Number of individuals (N), number of haplotypes (n), number of singleton haplotypes (n singletons), percent singletons (%), haplotype diversity (h), mean pair wise differences between sequences (π) with standard deviation (SD), Bayesian estimate of diversity (Θ) with upper and lower 95 percentile, range of female effective population size estimated for $\Theta(N_{EF})$, and the ratio of genetically effective size to census size $\left(\frac{N_E}{N}\right)$.

Parameter	Species		
	Weddell seal	crabeater seal	Ross seal
N (n)	181 (83)	143 (135)	41 (33)
n singletons (%)	49 (59.0%)	127 (94.1%)	26 (78.8%)
$h \pm SD$	0.98 ± 0.04	0.99 ± 0.01	0.99 ± 0.08
$\pi \pm SD$	0.012 ± 0.006	0.027 ± 0.014	0.020 ± 0.011
Θ (95 percentile)	0.075 (0.055 – 0.085)	0.576 (0.484 – 0.718)	0.088 (0.060 – 0.132)
N_{EF}	55,600	426,700	65,200
$\frac{N_E}{N}$	0.139 – 0.153	0.057 – 0.122	0.592 – 1.0

Table 3.2. Estimation of sudden population expansion in Antarctic seals. Shown are Fu's F_s test, sum of squared deviation (and significance) for the mismatch distribution, Harpending's raggedness index (and significance), and τ , number of generations (and years) before present when the expansion occurred.

Parameter	Species		
	Weddell seal	crabeater seal	Ross seal
Fu F_s (p)	-24.99 (0.00)	-24.09 (0.00)	-20.13 (0.00)
SSD (Mismatch p)	0.002 (0.56)	0.001 (0.36)	0.006 (0.04)
Raggedness (p)	0.007 (0.84)	0.002 (0.82)	0.017 (0.05)
τ	5.74	12.59	—
Generations (years)	81,246 (731,210)	177,825 (1,600,424)	—

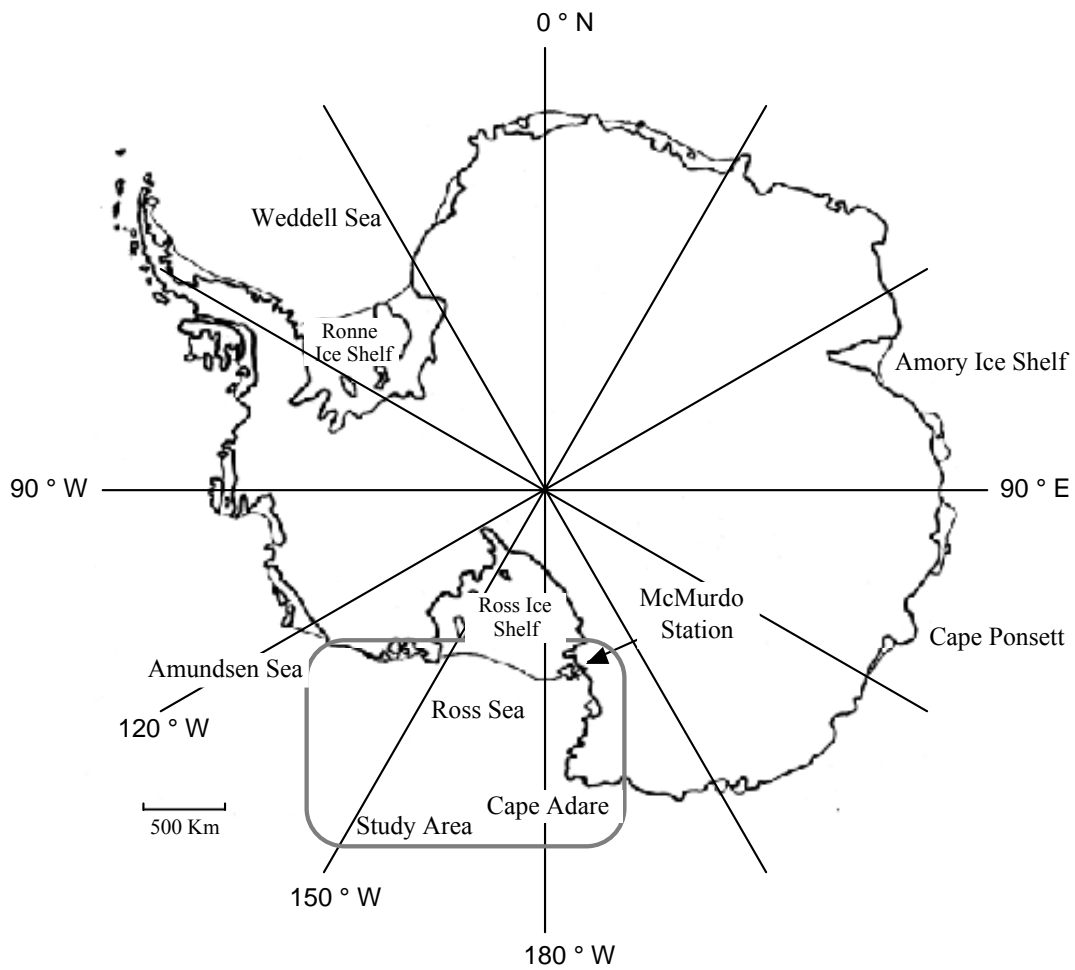


Figure 3.1. Map of the Antarctic continent showing the area where genetic samples were collected.

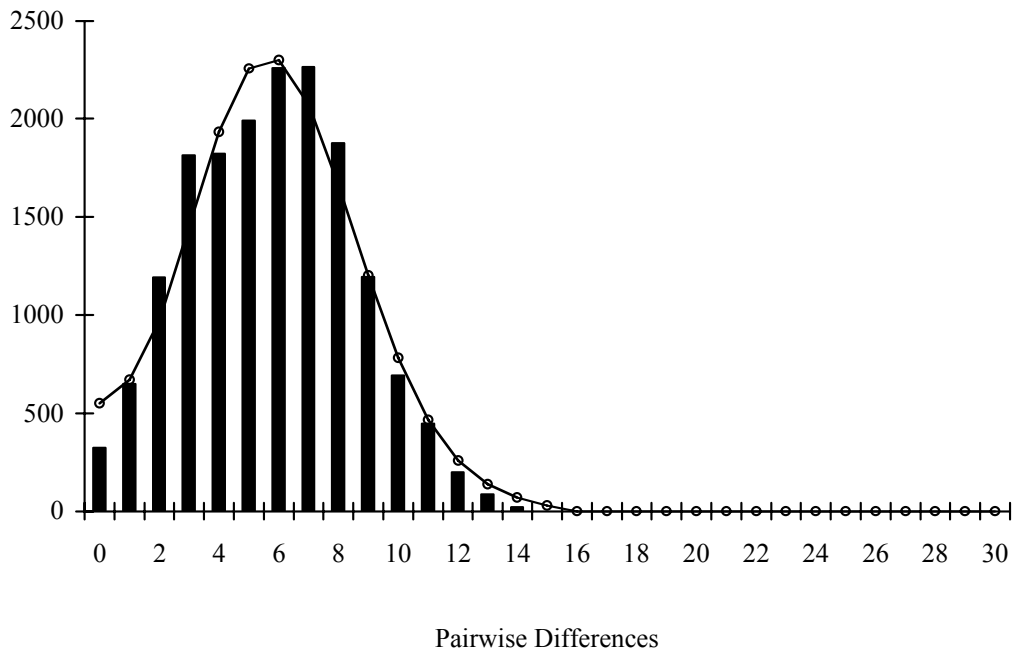


Figure 3.2a. Mismatch distribution for Weddell seals. Bars represent observed distribution of pair-wise differences among samples and the line shows the distribution modeled for sudden population growth. Note that the Y-axis scales differ among graphs. Conformance to the sudden growth model can only be rejected for Ross seals.

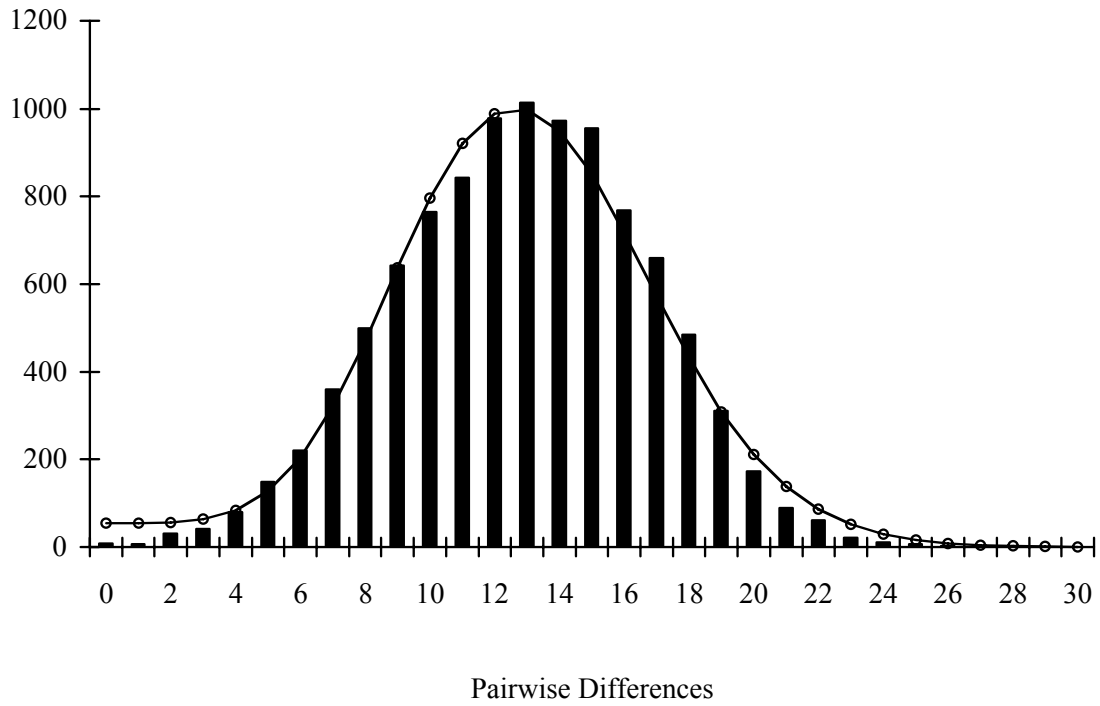


Figure 3.2b. Mismatch distribution for crabeater seals. Bars represent observed distribution of pair-wise differences among samples and the line shows the distribution modeled for sudden population growth. Note that the Y-axis scales differ among graphs. Conformance to the sudden growth model can only be rejected for Ross seals.

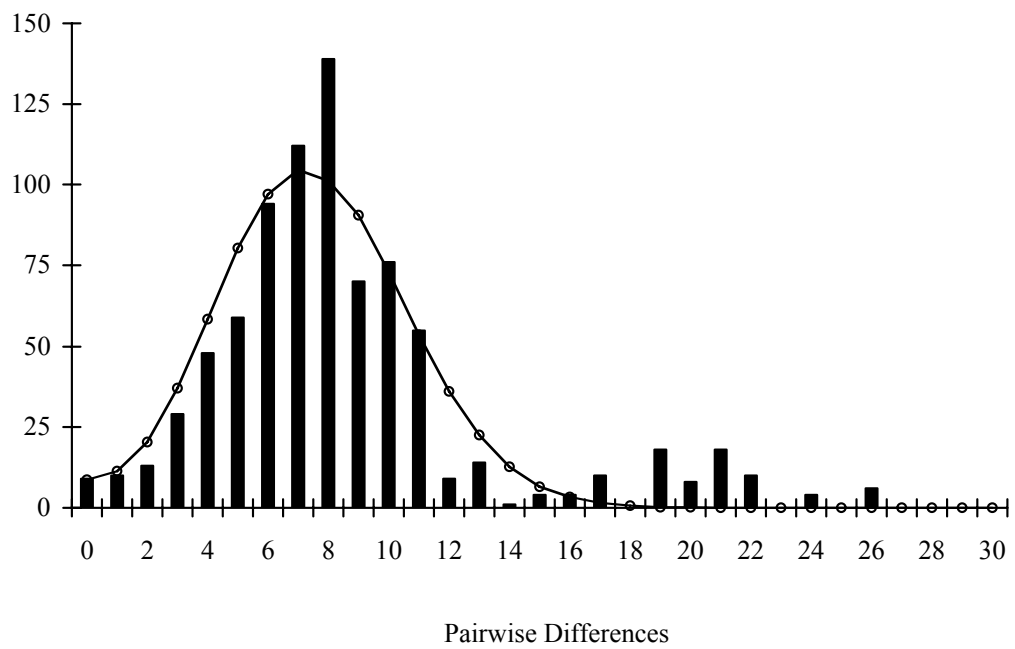


Figure 3.2c. Mismatch distribution for Ross seals. Bars represent observed distribution of pair-wise differences among samples and the line shows the distribution modeled for sudden population growth. Note that the Y-axis scales differ among graphs. Conformance to the sudden growth model can only be rejected for Ross seals.

Chapter 4: Autosomal and Sex-linked Patterns of Genetic Partitioning in Three Species of Antarctic Seals

Introduction

The level of genetic variation in a population (or species) is shaped primarily by the forces of mutation, drift, selection and migration, and can be an important clue to the evolutionary trends in population size, demography and long-term stability. While mutation serves to generate genetic variation, drift tends to counterbalance it by removing variation (Hartl and Clark 1997). In the absence of selection and migration, a mutation-drift equilibrium should be established. The parameter theta (θ) is the amount of neutral genetic diversity expected at equilibrium and equals $4N_E\mu$ (for nuclear genes) where N_E is the genetically effective population size and μ is the mutation rate per gene per generation. Theta is large when there is a large population size or high mutation rate (or both) and conversely, small θ with a small N_E or slow mutation rate (or both). The genetically effective population size is usually less than census size (N_C), but can provide a more useful description of the actual numbers of individuals contributing to each generation over evolutionary time. Several factors conspire to reduce N_E relative to N_C

and include deviations from 1:1 sex ratios, any form of non-random mating, variance in reproductive success, fluctuations in population size from one generation to the next, and overlapping generations (Hartl and Clark 1997). The ratio, $\frac{N_E}{N_C}$, on average, is often less than 0.5, and frequently less than 0.25 (Frankham 1995), with average mammalian ratios slightly lower than 0.45.

Antarctic seals provide an ideal system to capitalize on the relationship of θ and the neutral mutation rate to estimate effective population size for several reasons. Due to their relative inaccessibility, Antarctic seals have robust population sizes and have enjoyed a history relatively free from anthropogenic disturbances. Four species of Antarctic seals, the crabeater (*Lobodon carcinphagus*), the Ross (*Ommatophoca rossii*), the Weddell (*Leptonychotes weddellii*) and the leopard seals (*Hydrurga leptonyx*) are more closely related to each other than to any other species, though the relative placement of the crabeater and Ross seals is debated (see Higdon *et al.* 2007, Arnason *et al.* 2006). All four species appear to have diverged relatively recently, at three points in time between 4.3 and 7.1 million years ago (mya) (Higdon *et al.* 2007), or at the most up to ~9 mya (Arnason *et al.* 2006). This makes direct DNA analysis of homologous regions possible, including cross-species amplification of polymorphic microsatellites (Galbusera *et al.* 2000). Though they are closely related, the three species have significantly different life history characteristics and mating systems, including serial

monogamy and polygyny. This allows the opportunity to make predictions about female-only N_E estimates (mitochondrial estimates) in relation to biparentally based N_E estimates (those inferred with autosomal microsatellites).

Estimating N_E in Antarctic pack ice seals is not only useful from a theoretical perspective, but population abundance is a critical, but poorly understood, element of Antarctic ecology. Species abundance is particularly important due to the uniquely short Antarctic food web and its implications on management models and plans. For example, one of the species in my study, the crabeater seal, feeds exclusively on Antarctic krill (*Euphausia superba*), which is also the primary diet of the six species of baleen whales found in the Antarctic. An abundance or shortage of krill, through natural causes or commercial overharvesting (e.g. Bester 1995) or dramatic population size fluctuations of baleen whale stocks might result in changes in crabeater seal abundance, or vice versa. The decline and near collapse of krill-eating baleen whale populations in the early to mid 20th century (Macintosh 1970) was hypothesized to have spawned a significant increase in crabeater seal abundance (Laws 1977, Mori and Butterworth 2004). In a 2005 report, the International Whaling Commission highlighted the importance, and deficiency, of abundance and population trend data of the crabeater seal to the understanding of trophic interactions with Southern Ocean whale populations (International Whaling Commission 2005).

My research focused on three species of Antarctic seals that have circumpolar

distributions and occupy distinct niches. More is known about the Weddell seal than the other two species of seals, due to a breeding population near Ross Island, McMurdo Sound, which has been extensively studied since its discovery in 1907 (Wilson 1907), and to annual tagging and census studies which have been carried out since 1969 (Stirling 1969, Cameron and Siniff 2004). Though they are found in pack ice, Weddell seals typically inhabit the landfast ice regions around the continent. The extent of fast-ice fluctuates annually due to freeze patterns, changing influence from wind and tides, and glacial movement (Ushio 2006). Breeding-age adults return in the Austral spring to breeding-birthing colonies situated in the fast ice, to which they demonstrate a limited degree of philopatry. Davis *et al.* (2008) used a genetic approach to show some degree of genetic differentiation between populations around the continent and demonstrated a weak signal of isolation by distance. Copulation takes place under the ice (Cline *et al.* 1971). Males use their teeth to maintain breathing holes in the ice, and they actively guard the 3-dimensional territory surrounding these holes (Kaufman *et al.* 1975, Bartsh *et al.* 1992). This allows males to mate with as many females as may share the breathing hole and to thwarting access to these females by competing males. Genetic studies indicate that males successfully mate and produce pups with up to five females within a season (Gelatt *et al.* 2000). Successful males (i.e. those able to reproduce) have a mean seasonal reproductive success of 1.2 with a variance of 1.6 (Gelatt *et al.* 2000). Population size census estimates for this species range from 730,000 (Laws 1977) to

800,000 (Erickson and Hanson 1990) individuals. A mitochondrial DNA based female genetically effective population size (N_{EF}) estimate was $\sim 55,600$, suggesting as many as $\sim 111,200$ total individuals (Curtis, *et al.* 2009). While significantly lower than census estimates, this number may reflect a regional estimate encompassing roughly 20% of the continent (eastern Ross and western Amundsen seas), which was the approximate sample collection area (Curtis *et al.* 2009). If this were the case, the total N_E may be as high as $\sim 556,000$ individuals around the continent and would give a $\frac{2N_{EF}}{N_C}$ ratio of 0.70 – 0.76, similar to the ~ 0.5 estimate for other mammals (Frankham 1995). It is also possible that mtDNA estimate represents the circumpolar population size but the mitochondrial DNA, *per se*, is significantly underestimating N_{EF} (Bazin *et al.* 2006). In any case, given the potential for philopatry (and higher potential for inbreeding) and a polygynous mating system, I would expect that the ratio of neutral, biparentally inherited nuclear DNA estimate of N_E (N_{EA}) to $2N_{EF}$ would be smaller for Weddell seals than for the other species where these attributes are not present. In other words, the Weddell seal $\frac{N_{EA}}{2N_{EF}}$ ratio should be less than that for crabeater or Ross seals.

Crabeater seals (*L. carcinophagus*) are distributed throughout the perimeter of the Antarctic continent, and young seals occasionally travel in large groups and sometimes for great distances. No significant level of population genetic subdivision has been

detected (Davis *et al.* 2000, 2008), suggesting a single, panmictic population likely due to high levels of gene flow around the continent. Crabeater seals utilize the floating pack ice for breeding, and are thought to be serially monogamous (Siniff *et al.* 1979). Population size has been estimated at 7 to 15 million individuals (Laws 1977, Erickson and Hanson 1990). An estimate of N_{EF} was $\sim 426,700$, suggesting as many as $\sim 853,400$ total individuals (Curtis *et al.* 2009). Thus a $\frac{2N_{EF}}{N_C}$ ratio of 0.057 – 0.122; an order of magnitude smaller than commonly seen. Assuming crabeater seals are a single, panmictic population and are serially monogamous with a 1:1 sex ratio, I would expect $\frac{N_{EA}}{2N_{EF}} \approx 1.0$.

Comparatively little is known about the Ross seal (*O. rossii*). They appear to be solitary, and utilize the stable ice floes on the exterior of the pack ice to molt, while they give birth on the more densely packed interior pack ice (Spletstoeser *et al.* 2000, Stewart 2007). Though they have a circumpolar distribution, higher numbers may be found in the Ross Sea, King Haakon VII Sea, and perhaps portions of the western Weddell Sea (Stewart 2007). They may spend much of the rest of their time to the north of the pack ice, alone in the open water (Stewart 2007). There is no indication of population genetic subdivision (Davis *et al.* 2008). The mitochondrial DNA based N_{EF} , was $\sim 65,200$, suggesting as many as $\sim 130,400$ total individuals (Curtis *et al.* 2009), which is very similar to the circumpolar census estimates of 131,000 to 220,000 (Laws

1977, Erickson and Hanson 1990). Not enough information exists to speculate on their mating system, nor the ratio of N_{EA} to N_{EF} .

To gain a better understanding of the relationship of female genetically effective population sizes to the overall genetically effective population sizes within and among these three species, I compare and contrast N_{EA} based on biparentally inherited microsatellites with previously published mitochondrial $2N_{EF}$ and census size estimates. In addition, I examined one X-linked microsatellite and two Y-linked DNA sequences totaling approximately 740 nt. I also assess long-term population stability by looking for signs of recent, sustained population bottlenecks.

Methods

Samples

Tissue samples were collected from 214 free-living Weddell seals (*L. weddellii*), 175 crabeater seals (*L. carcinophagus*) and 41 Ross seals (*O. rossii*) via remote darting or direct handling during the Antarctic Pack Ice Seal (APIS) multidisciplinary cruise (Decker et al. 2002) in the Austral summer of 2000. Although all three species are circumpolar, most samples were collected from the pack-ice zone of the western Amundsen and eastern Ross seas, approximately $67^{\circ} - 78^{\circ}$ South and $129^{\circ} - 180^{\circ}$ West

(Figure 4.1). Some *L. weddellii* samples came from McMurdo Sound, in the western Ross sea. Collection date and location data was available for all individuals. Sex and other biometric data were available for some but not all individuals. Sex of most individuals was determined or confirmed using *ZFX* and *ZFY* genetic screening (Curtis *et. al.*, 2007). Samples were either stored in ethanol or frozen at -80° C until DNA extraction.

Laboratory methods

Autosomal and X-linked Microsatellites

DNA was purified using standard phenol-chloroform techniques or using a DNeasy Tissue Kit (Qiagen, Valencia, CA), and 5 μ l of total cell DNA was visualized on a 0.8% agarose gel stained with ethidium bromide to assess DNA quality. Dinucleotide microsatellites were amplified using the primers designed by Davis *et al.* (2002), and forward primers were fluorescently labeled (Integrated DNA Technologies, Inc., Coralville, IA). Polymerase chain reaction (PCR) amplification products were visualized on a 2% agarose gel stained with ethidium bromide to assess efficiency and fidelity of amplification. Microsatellite PCR amplifications took place in three multiplex reactions pooling loci Lw18, Hl15, Hl16, and Hl20 (annealing temperature of 56° C), Lw10, Lw11,

and Lw16 (57° C), and Hl4, Lc5, and Lw8 (55° C). Lw18, Hl15, Hl16 and Hl20 were screened simultaneously on an ABI 3100 Genetic Analyzer (Iowa State University Sequencing Facility), with the remaining six loci being pooled and screened separately. DNA amplifications were generally a 10 µl reaction containing 0.5 µl total cell DNA, 1 X reaction buffer (Promega, Madison, WI), 2.0 mM MgCl₂, 0.2 mM of each dNTP, 10 pmol of each primer, 6 µg bovine serum albumen, and 1.25 U *Taq* polymerase (Promega). Thermocycling conditions for microsatellites followed a standardized protocol of 94° C for 1 min, 30 cycles of 94° C for 45 sec, annealing temperature for 45 sec, and 72° C for 1 min followed by a final extension of 72° C for 7 min.

Y Chromosome Sequences

I amplified a portion of the 8th intron of the Y-linked DEAD-box Y gene (DBY8) using the mammalian primers DBY8F - 5'-CCCCAACAAGAGAATTGGCT-3' and DBY8R - 5'-CAGCACCACCATAKACTACA-3' (Hellborg and Ellegren 2004). I amplified approximately 560 nt of the 11th intron of the Y-linked Ubiquitously Transcribed Tetratricopeptide Repeat gene (UTY11) locus using the mammalian primers UTY11F: 5'-CATCAATTTTGTAYMAATCCAAAA-3' and UTY11R: 5'-TGGTAGAGAAAAGTCCAAGA-3' (Hellborg and Ellegren 2004). Amplifications were

as previously described except they were 50 μ l reactions. Thermocycling conditions for UTY11 followed a touchdown protocol of 94° C for 1 min and 35 cycles of 94° C for 1 min, an annealing temperature for 1 min starting at 55° C and decreasing 0.25° C every cycle, and 72° C for 1 min followed by a final extension at 72° C for 7 min.

Thermocycling conditions for DBY8 followed a similar touchdown protocol, except that all cycles except the final extension were 30 seconds and the touchdown annealing cycle started at 65° C. PCR products were visualized on a 2% agarose gel stained with ethidium bromide to assess efficiency and fidelity of amplification. For all Y chromosome specific primer sets, two female samples were also amplified in each batch of male amplifications to serve as a negative control. PCR amplification products were purified using either Microcon[®] Centrifugal Filter Units (Millipore Corp., Billerica, MA) or QIAquick spin columns (Qiagen, Inc., Valencia, CA) and quantified on an agarose gel stained with ethidium bromide. Approximately 100 ng of purified product was used as template in sequencing reactions and sequenced in one or both directions on an ABI 3730XL sequencer (Macrogen Inc., Seoul, South Korea or the University of South Florida Sequencing Facility, Tampa, FL). Due to the minimal amount of observed variation, sequences were sequenced in both directions only when there was any ambiguity or potential variable sites. Forward and reverse sequences were aligned and joined using Sequencher software (GeneCodes Corp., Ann Arbor, MI), and edited, when

necessary. Multiple sequences were aligned using Clustal W (v.1.83, Chenna *et al.* 2003).

Data Analyses

All loci were analyzed using Genescan software (Applied Biosystems, Inc., Foster City, CA). Number of alleles and heterozygosity were estimated using the Excel microsatellite toolkit v3.1 (Park, 2001). Hardy-Weinberg exact test and deviation from equilibrium frequency and linkage disequilibrium estimated for all autosomal loci were done using GENEPOP v3.4 (Raymond and Rousset 1995). The program BOTTLENECK (Piry *et al.* 1999) was used to look for signs of recent bottlenecks (heterozygosity excess) within each species. It is most sensitive in detecting bottlenecks $2N_E - 4N_E$ generations in the past depending on the severity of the bottleneck and the mutation rate of the loci being analyzed. This program calculates expected heterozygosity at mutation-drift equilibrium (H_{EQ}) based on the number of alleles at a locus and sample size using the infinite alleles model (IAM), the stepwise mutation model (SMM), and the two-phase model (TPM, an intermediary between the two). The H_{EQ} estimates are averaged across loci and compared with the observed levels of heterozygosity, with a null hypothesis of no heterozygosity excess. I did not use the IAM

model because the SMM and TPM are most appropriate for evaluating microsatellite data (DiRienzo *et al.* 1994, Luikart and Cornuet 1998, Piry 1999). I allowed 95% single stepwise mutations and 5% multi-step mutations in the TPM, with a 12% variance among multiple steps, as recommended by Piry *et al.* (1999). I focused on the Wilcoxon's signed rank test, which is suggested by the authors to be the most powerful with < 20 loci (minimum of four loci required). Locus specific θ_F values were estimated for all species at the ten loci (Xu and Fu 2004) using the ThetaF program (H. Xu, pers. comm.). To estimate the effective population size (N_{EA}), the mean of θ_F across all nine autosomal loci within each species was used as a surrogate for θ in the equation $N_{EA} = \frac{\theta}{4\mu}$ where μ is the mutation rate per gene per generation under the assumption of mutation-drift equilibrium. Because locus Lw18 is X-linked (Davis *et al.* 2002), the sex of all individuals was determined *a priori* through a combination of field and lab screening (Curtis *et al.* 2007) and females possessing a single Lw18 allele were designated as homozygotes. The equation $N_{EX} = \frac{\theta}{3\mu}$ (Yu *et al.* 2002) was used to estimate the genetically effective X-linked population size (N_{EX}) for Lw18 using female samples only. Several microsatellite studies of mammals, including seals, have estimated or assumed mutation rates of $10^{-3} - 10^{-6}$ mutations per gene per generation (Schlotterer 2000, Palo *et al.* 2001, Kretzmann *et al.* 2006, see Ellegren 2000 for review). Here, I use 10^{-5} to

estimate effective population size because it is the most frequently cited rate, is approximately in the middle of the cited range, and faster or slower rates change the genetically effective population size linearly.

Results

Microsatellites

All nine autosomal loci successfully amplified product in each species. Table 4.1 shows the number of individuals, number of alleles, and observed and expected heterozygosity at each locus for each species. *L. carcinophagus* showed the highest number of alleles and the highest heterozygosity at most loci and *L. weddellii* had the lowest heterozygosity (though not always the lowest number of alleles) at most loci (Table 4.1). Allele sizes and numbers of alleles at each locus were consistent with those observed in the same species by Davis *et al.* (2002, Table 4.1). The mean sizes of alleles did not differ significantly in pairwise comparisons among species (Student's one-tailed t-test, data not shown). Some loci did not amplify in every individual. All loci in all species were in Hardy-Weinberg genotypic frequency equilibrium except for two in *L. carcinophagus* (Table 4.1). Two sets of loci were statistically linked in *L. weddellii* and *O. rossii*, and four sets of loci were linked in *L. carcinophagus* (data not shown). The

linked loci differed, however, among species; therefore I include all available loci in the analyses.

I did not detect significant heterozygosity excess in any species using BOTTLENECK (Piry *et al.* 1999). Under the SMM or the TPM, the Wilcoxon test showed no patterns of excess heterozygosity in any species. The data do suggest heterozygosity deficiencies in all three species under the SMM, though under the TPM, *L. carcinophagus* was not significant ($P = 0.29$), while the other species were ($P = 0.00 - 0.01$).

Estimates of θ_F vary widely across loci within a species and across species at a locus (Table 4.2). Locus Lc5 had, by far, the lowest θ_F estimates for all species. The autosomal arithmetic means of θ_F were 5.91, 13.80, and 37.58 for *L. weddellii*, *O. rossii*, and *L. carcinophagus*, respectively (Table 4.2). Using a mutation rate of 10^{-5} resulted in genetically effective population sizes of 147,850 to 939,600 across species (Table 4.3). For the X-linked microsatellite locus, Lw18, no female homozygotes were observed in *O. rossii*, although they were seen in the other two species. For *L. carcinophagus* and *O. rossii*, the θ_F estimates at Lw18 were larger than the arithmetic mean across autosomal microsatellites, though this was not the case for *L. weddellii* (Table 4.2).

Y Chromosome Variation

Sequences from both Y specific primer pairs aligned with high sequence identities to Y-specific regions in the National Center for Biotechnology Information human genome database. UTY11 aligned with 83% identity to *Homo sapien* Ubiquitous TPR-motif Protein Y isoform (UTY) gene (Genbank accession number AF265575, Shen et al., 2000). DBY8 aligned with 84% identity to a Y chromosome region encompassing part of a DEAD-box protein gene, exons 9 and 10 and the intron between them (Genbank accession number AC004474, Birren *et al.* 1999 unpublished). DEAD-box proteins, named for the presence of the amino acid sequence ‘D-E-A-D’ (motif II or the Walker B motif), play an important role in RNA metabolism (Linder 2006). These high sequence identity alignments, coupled with sex-restricted amplification (in males only), support that my sequences were from the Y chromosome.

I sequenced a total of 224 male seals for the DBY8 locus, including 26 *O. rossii*, 88 *L. carcinophagus*, and 110 *L. weddellii*, discovering five new haplotypes, two in *L. weddellii*, and two in *L. carcinophagus* (Genbank accession numbers FJ813489-FJ813491). The two *L. weddellii* haplotypes differed by one G/C transversion, and the rare haplotype was shared by two, non-nuclear family individuals. These two individuals were genetically similar at the autosomal loci, but clearly had different mothers (i.e., mtDNA haplotypes) and fathers (i.e., different DBY8 sequences). The two *L.*

carcinophagus haplotypes differed by one A/G transition, and the rare haplotype was observed in a single individual. The DBY8 sequence, excluding primer sequences, was 141 nt in all three species. All three seal species differed by at least one base pair at DBY8 (Appendix 1).

I sequenced a total of 196 male seals for the UTY11 locus, including 25 *O. rossii*, 78 *L. carcinophagus*, and 93 *L. weddellii*, discovering four haplotypes (Genbank accession numbers FJ813492-FJ813494). There were two haplotypes in *L. weddellii* differing by one A/G transition. No variation was found in *O. rossii* or *L. carcinophagus* at either locus. When comparing the sequences among species, there are eleven variable sites, including six transitions, two transversions, and three indels (Appendix 1).

Discussion

My assessment of genetic variation in autosomal microsatellites suggests that Weddell, Ross, and crabeater seals have levels of intraspecific variation similar to those reported for other large mammals (Paetkau *et al.* 1995, Simonsen *et al.* 1998). The levels of diversity also fit into the range of published estimates for pinnipeds (Table 4.4), though direct comparisons should be made with caution due to differences in experimental design and population sampling among studies. At the low end of the range,

the critically endangered Mediterranean monk seal (*Monachus monachus*), whose population size has declined to an estimate maximum of 430 individuals divided between two remaining populations, has published heterozygosity estimates ranging from 0.23 to 0.35 (Pastor *et al.* 2007). A similarly low level of heterozygosity is seen in the Hawaiian monk seal, *Monachus schauinslandi*, (0.48) which has a current population size estimated of 1,247 seals (Schultz *et al.* 2008).

Of the species surveyed for this study, the Weddell seal has the lowest heterozygosity (Table 4.4). Similar levels were reported for northern European harbor seals, *Phoca vitulina*, (Goodman 1998, Table 4.4), whose population was nearly halved in 1988 due to a severe outbreak of phocine distemper virus (Goodman 1998), though it appears to have rebounded. Antarctic seals are thought to be minimally anthropogenetically impacted and the five lowest heterozygosity levels seen are from northern hemisphere seals (Table 4.4). Given the remoteness of the Antarctic, it seems unlikely that the Weddell seal has experienced significant recent population reductions unless they were naturally caused. An assessment of mtDNA in Weddell seals (Curtis *et al.* 2009) did, however, indicate that the Weddell seal went through a population expansion approximately 731,000 years ago possibly due to glaciation induced increases in fast and pack ice around Antarctica. Nonetheless, given that microsatellites mutate very quickly, I expect that there has been sufficient time in the ~81,000 generations since the population expanded for several new alleles to arise. High heterozygosities, similar

to those in the crabeater seals tended to be associated with seals of high population abundance (but see Twiss *et al.* 2006). Antarctic fur seals (*Arotocephalus gazella*) are estimated to number 4 – 7 million and (Scientific Committee on Antarctic Research, 2006) and have observed heterozygosities of 0.81, comparable to the crabeater frequency of 0.78. Interestingly, Curtis *et al.* (2009) showed that the crabeater seal went through a similar population size expansion. This expansion, however, occurred much earlier (i.e., ~1.6 mya) and it is possible that any loss of heterozygosity would have been regained. Heterozygosity for the Ross seal is high, and falls between that seen in the Weddell and crabeater. Interestingly, the rank order of heterozygosities matches the rank order of the genetically effective population sizes estimated from mtDNA (Curtis *et al.* 2009) and autosomal microsatellites (see below).

I found no evidence suggesting that any of the species in my study underwent a recent population bottleneck (data not shown), or at least not one in the past $2N_E$ to $4N_E$ generations, the reported detection range of the statistical analyses. A slow, gradual population decline would be unlikely to result in a statistically significant heterozygosity excess, and would not likely be detected using this type of analysis. Assuming N_{ES} of 111,200 for *L. weddellii*, 130,400 for *O. rossii*, and 853,400 for *L. carcinophagus* (Curtis *et al.* 2009), and an average age at reproduction (i.e., generation time) of 9 years (Curtis *et al.* 2009), the timeframe I examined would roughly correspond to 2 – 4 mya for *L. weddellii*, 2 – 5 mya for *O. rossii* and 15 – 30 mya for *L. carcinophagus*. If the average

age at reproduction or N_E estimates are off, this time frame would shift proportionally forward or backward in time, but the detection a bottleneck would not be effected. The BOTTLENECK analysis does indicate heterozygosity deficiencies (not shown) in all three species under the SMM and for two species in the TPM, which suggest the populations may have experienced a population expansion at some point in the recent past (Luikart and Cornuet 1998). This is consistent with the previous mtDNA estimates (Curtis *et al.* 2009) and the time of expansions are well within the above time frames. Mitochondrial DNA analysis, however, did not unambiguously reveal a population expansion for *O. rossii* (Curtis *et al.* 2009). It is possible that if this species underwent a significant expansion, it was smaller in scale than the others, or that it took place in a time range undetectable by mitochondrial signals. Nevertheless, as expected, these populations of Antarctic seals have not experienced dramatic declines in population sizes and are more likely to be increasing in size over evolutionary time.

Before I turn to estimates of genetically effective population size, I would like to address several caveats. It is possible that my genetic results include ascertainment bias in the microsatellite loci which can artificially deflate heterozygosity and θ_F . The microsatellite markers that I used were originally isolated from the three, closely related species, *L. carcinophagus* (Lc5), *L. weddellii* (Lw10, Lw11, Lw16, and Lw18) and the leopard seal, *Hydrurga leptonyx*, (Hl4, Hl15, Hl16, and Hl20). The leopard seal is believed to be the sister taxa of the Weddell seal. It has been shown that microsatellite

loci can show reduced heterozygosity and numbers of alleles when applied to species from which they were not originally isolated (i.e., cross species applications, Hutter *et al.* 1998). They also tend to have shorter pure repeat regions (i.e., are interrupted, Amos *et al.* 2003, Vowels and Amos, 2006) and shorter overall allele lengths in non-focal species (Amos *et al.* 2003). I do not, however, think that ascertainment bias is significantly affecting the analyses. One-tailed student t-test (data not shown) did not indicate statistically significant differences in the number of alleles in cross- versus non-cross species applications. Of the 30 locus by species comparisons, six are non-cross species applications. Of these six, the lowest relative per-locus allelism is seen in five of them and in all cases, the highest allelism is seen in cross-species applications. The same pattern generally holds for heterozygosity levels, as well (Table 4.1). Much of this pattern is due to the crabeater seal showing overall higher levels of variation than the other two species likely due to a larger population size. Even so, if the crabeater seal is excluded the highest levels of heterozygosity and allelism are still seen in the cross species applications.

A second caveat concerns the mutation rate. As with all population size estimates, those based on microsatellite diversity are reliant on mutation rates. Unlike most DNA sequences, however, microsatellite mutation rate estimates may vary by up to four orders of magnitude. Mammalian microsatellite mutation rates range from 10^{-2} to 10^{-6} (Schlotterer 2000). Other studies using microsatellite have estimated or assumed mutation

rates of $10^{-3} - 10^{-6}$ mutations per gene per generation (Schlotterer 2000, Palo *et al.* 2001, Kretzmann *et al.* 2006. See Ellegren 2000 for review). For my analyses, I used a mutation rate in the middle of this range (10^{-5} , Table 4.3). It is important to note that if my mutation rate estimate is off by an order of magnitude the N_E estimates would also be off by an order of magnitude. This would not, however, affect the rank order of population estimates among the species. I do, however, believe that the mutation rate I am using is a good approximation of the average mutation rate across the microsatellite loci in my study and is one that is commonly seen in large mammals.

Estimates of θ_F vary widely across loci within a species and across species at a locus (Table 4.2). Locus Lc05 had, by far, the lowest overall θ_F estimates. In *Le weddellii* and *O. rossii* this is likely due, in part, to a small number of alleles and very low heterozygosity levels. This cannot, however be the case for *L. carcinophagus* where Lc05 had the lowest θ_F but 12 alleles. In this case, the low θ_F was probably driven by highly unequal allele frequencies where one of the 12 alleles was seen at a frequency of 60%, and the next highest frequency was 1%. In general, however, single loci are poor estimators of θ and the accuracy of the overall estimation of N_E is proportional to the number of loci (Felsenstein 2005). As such, most of the remainder of the discussion will focus on estimates using all autosomal loci combined.

The rank order of microsatellite N_{EAS} are in agreement with the mitochondrial

estimates for the three species (Curtis *et al.* 2009), with *L. weddellii* showing the smallest effective population size, followed by *O. rossii* and *L. carcinophagus* the largest (Table 4.3). This is something of a surprise because the census estimates for *O. rossii* typically are lower than those of *L. weddellii* (Table 4.3). Even so, the agreement between the two types of markers (and, generally, at each microsatellite locus, individually) indicates that this is likely to be a genuine, biological attribute of the species. It is possible that the census sizes accurately reflect the true population sizes, but a sustained reduction in size occurred for *L. weddellii* lowering N_{EA} for this species. I did not, however, recover any indication that any of the species have undergone bottlenecks (data not shown). It is also interesting to note that *O. rossii* has a larger overall N_{EA} estimates than the Weddell seal as well as on a locus-by-locus basis. As with the mtDNA estimate, N_{EA} for the Ross seal is nearly as large if not larger than the census size (Curtis *et al.* 2009); an unlikely outcome. This supports the statement made by Curtis *et al.* (2009) that Ross seal population sizes may be significantly underestimated by traditional census survey techniques.

While the rank order of N_E estimates remains consistent between mitochondrial and microsatellite analyses, the estimates of N_{EA} were not the same as $2N_{EF}$. For the most part, however, N_{EA} and N_{EF} are fairly similar and both much lower than the census size.

For *L. carcinophagus*, $N_{EA} = 3939,600$ and $\frac{N_{EA}}{2N_{EF}} = 0.91$ (Table 4.3) indicating that both

types of markers agree that the N_E for this species is ~1,000,000 individuals. The per locus estimates of N_{EA} are also fairly consistent and indicate an effective size of about 1,000,000 individuals (Table 4.2). This is, however, significantly lower than the published circumpolar census estimates of 7 to 15 million individuals (Laws 1977), and results in an $\frac{N_{EA}}{N_C}$ ratio of 0.06 to 0.13, much lower than typically observed in mammals (Frankham 1995). There is a similar pattern for *Le weddellii* with an $\frac{N_{EA}}{2N_{EF}}$ of 0.75 and $\frac{N_{EA}}{N_C} = 0.185$. There are several factors that result in N_E being lower than N_C . In the Curtis *et al.* (2009) study, it was suggested that mtDNA might be underestimating the true N_E for these species. Given the general agreement of the nuclear and mitochondrial results, this is unlikely. Further, since mtDNA estimates only the female effective size, a 1:1 sex ratio was assumed and N_{EF} was doubled to approximate the full genetically effective population size. For the crabeater seal, this appears to have been a supportable assumption. If the sex ratio was not 1:1, then I would expect $N_{EF} > N_{EA}$. Although these two estimates are not identical they differ by only very little and are likely well within each other's range of deviation.

The Weddell seal is known to be polygynous and as such, it is expected that $\frac{N_{EA}}{2N_{EF}} < 1.0$ because, although all females can mate each season, not all mature males

can. Here, again the estimates are fairly similar, although the nuclear estimate is larger than the mitochondrial. There is no question that Weddell seals are polygynous so an explanation for this reversed results must lie elsewhere. It is possible, as mentioned previously, that the mtDNA can be underestimating N_{EF} . I doubt that this is generally the case given the similarity between the markers results and an expected pattern in the crabeater seal. Any underestimation would also have to be species specific since the mtDNA and microsatellite estimates for *L. carcinophagus* are remarkable similar as expected. It could be that the mutation rate I chose for the microsatellites is too slow and thus is overestimating N_{EA} . Changing the mutation rate from 1.0×10^{-5} to 2.0×10^{-5} lowers N_{EA} for Weddell seals to 73,900. This then would make $N_{EA} < N_{EF}$, as anticipated. This also, however, would result in $\frac{N_E}{N_C} = 0.092$, a value nearly an order of magnitude lower than is typically seen in mammals (Frankham 1995, but see Avise *et al.* 1988). This change, however, would also have to apply to the other species and would reduce N_{EA} for the crabeater seal to approximately half of $2N_{EF}$ estimated from mtDNA where N_{EA} is expected to, and does, equal $2N_{EF}$. It would also further reduce the census size to genetically effective size ratio in crabeaters making it even more unlike what is seen in mammals. It is possible that the microsatellites are overestimating N_{EA} in *L. weddellii*. There is considerable variation among the per-locus estimates of θ_F (0.01 – 28.34, Table 4.2), although most of the loci have values less than 10.0. It is possible that some form of

diversifying selection is acting on some loci but not others (Kashi and Soller 1999, Barker *et al.* 2009). If I remove the locus with the largest θ_F value (Lw10), $N_{EA} = 77,800$ (Table 4.2) for the Weddell seal and is now about 70% less than the value of N_{EF} , as would be expected from a polygynous species. There is, however, a corresponding reduction in the genetically effective and census size ratio. Even so, I think that the N_{EA} estimated excluding Lw10 is likely more accurate (reduced data, Table 4.3).

As with the mitochondrial estimate, the autosomal N_E for Weddell seals is lower than the published census size estimates of 730,000 (Laws 1977) to ~800,000 (Erickson and Hanson 1990), and results in an $\frac{N_E}{N_C}$ ratio of 0.10 – 0.11, also lower than typically observed. Given that population subdivision was noted in samples separated by more than 700 km (Davis *et al.* 2008), it is possible that I am sampling a regionally subdivided population, and therefore the N_{EA} and N_{EF} may be regional estimates encompassing only the western Amundsen and eastern Ross seas. In their 1990 survey, Erickson and Hanson estimated that there were ~50,000 Weddell seals in the Ross Sea. If I were estimating the number of seals in just this area, the census size would then be smaller than the genetically effective size; an unlikely situation. Erickson and Hanson (1990), however, do caution that their population size estimates are very conservative and likely underestimate true abundances. My samples cover approximately 60° longitude, which is less than 20% of the circumference of Antarctica. If I assume that the distribution of

Weddell seals around Antarctica is uniform and I have sampled a discrete subpopulation, that would put the total effective population size at $\sim 739,250$ individuals, which roughly corresponds to the direct census estimates.

The Ross seal autosomal estimate of nearly 350,000 is enigmatic for two reasons. First, N_{EA} for the Ross seals is 2.6 times larger than $2N_{EF}$. Second, N_{EA} is larger than the census size by a factor of ~ 1.6 (Table 4.2), an unlikely result. As with the Weddell seal, there are several factors that could account for this. Again, mitochondrial estimates may simply be underestimates of N_E (Bazin *et al.* 2006), though this would not explain why autosomal N_E exceeded the published census estimates (Erickson and Hanson 1990). Unlike both the Weddell and crabeater seals, *O. rossii* did not show a clear sign of population expansion with mitochondrial DNA (Curtis *et al.* 2009). It did, however, show a heterozygosity deficit in BOTTLENECK, suggesting that they may have experienced a more recent population expansion, detectable with faster mutating microsatellite markers, but not yet reflected in the mitochondrial DNA. A sudden, recent population expansion, however, would result in a smaller, not larger, N_{EA} . This also does not, however, explain why N_{EA} is so much larger than N_C . As with the Weddell seal, I believe that some of my data are obscuring the accurate results. The per locus estimates of θ_F seen in the Ross seal also have a considerable range of values (0.04 – 57.53, Table 4.2). Most loci, however, indicate small values of θ_F and are generally less than 11.0

(Table 4.2). If I remove the two autosomal loci with the largest θ_F values (Lw10 and Lw11, 57.53 and 34.18, respectively), $N_{EA} = 116,000$ and is only slightly smaller than N_{EF} . This would also mean that both N_{EA} and N_{EF} give genetically effective to census size ratios similar to other mammals of 0.53 and 0.59, respectively. If these new estimates are accurate I could then conclude that the Ross seal, like the crabeater, likely has a serially monogamous mating system with an even sex ratio.

Genetically effective population size (N_{EX}) estimated with the single X-chromosome marker (Lw18) were roughly in line with or slightly higher than both mitochondrial and autosomal estimates of N_E for *L. weddellii* and *L. carcinophagus* (Table 4.2). As mentioned earlier, caution should be used with inferences based on a single locus. The N_{EX} of *O. rossii*, however, was over 5 million, which is more than an order of magnitude higher than the N_{EA} or $2N_{EF}$. I am at a loss to adequately explain why this estimate is so different from all other estimates and believe that it is anomalous. Forces, such as selection, tend to have a greater impact removing variation in autosomes than in X chromosomes and hitchhiking may be a more powerful force on the X chromosome (Betancourt *et al.* 2004). Using a total of 133 microsatellites, Kauer *et al.* (2002) found statistically elevated average heterozygosity on the X chromosome ($H = 0.75$) than autosomes (0.62) in African *Drosophila* populations, and this trend has been seen in other studies as well (e.g. Andolfatto 2001, Payseur and Nachman 2002). Until more X-linked microsatellites or other X-linked markers are surveyed, I cannot know if

this is a quirk of a single locus or characteristic of the chromosome.

I found a limited intraspecific variation for Weddell seals and crabeater seals at the Y-linked DBY8 and UTY11 loci. There was, however, some intraspecific variation within Weddell seals at the UTY11 locus. Due to their polygynous mating system, however, male *L. weddellii* should have a reduced effective population size than females, and I expected to find reduced levels of male diversity among *L. weddellii* relative to mitochondrial diversity as well as to *L. carcinophagus* which is not the case. Overall, however, the level of variation is quite low and no statistical analysis of the data is possible. I did not find any variation in *O. rossii* Y chromosome loci, most likely due to small sample size and the apparent slow evolutionary rate for this marker. Given the higher diversity patterns (than *L. weddellii*) shown with both mitochondrial and microsatellite analyses for the Ross seal, I would expect further screening with Y chromosome markers to reveal genetic variation. I expect that further screening with these and other Y chromosome markers would reveal more genetic variation within all three species, which could render them useful in paternity analysis. These loci can, however, be used to identify samples to the species level and for future Y chromosome phylogenies of pinnipeds and higher order mammals.

In summary, my autosomal N_{EA} estimates were roughly in agreement with previous $2N_{EF}$ estimates with mitochondrial DNA for *L. weddellii* and *L. carcinophagus*, though both are less than census estimates and in the case of *L. carcinophagus* quite

significantly. This may suggest that some level of yet undetected circumpolar population structure exists for this species and my estimates are for a localized area. *O. rossii* N_{EA} was higher than that estimated with mitochondrial DNA, as well as being greater than census size estimates. The latter, however, is unlikely to be true and non-neutral forces on two loci may have resulted in anomalously high θ_F values. If I remove these loci from the analysis, the Ross seal N_{EA} estimate is less than the N_C and nearly equal to $2N_{EF}$. Similarly, when I removed one locus with an exceptionally large θ_F value from the analysis of the *L. weddellii*, autosomal estimates of N_E were, as expected for a polygynous species (i.e., lower than $2N_{EF}$). I find that an approach integrating genetic markers with different modes of inheritance (biparental, maternal, and paternal) as well as varying mutation rates is useful in focusing on evolutionary trends occurring at varying points in the evolutionary history of a species. Concordance among markers and expectations based on inheritance mode can more powerfully reveal biologically meaningful demographic patterns.

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Table 4.1. The number of individuals (N), expected (H_{exp}) and observed (H_{obs}) heterozygosities, and number of alleles (A) seen at microsatellite loci in three Antarctic pack-ice seals. Observed heterozygosity values in bold were statistically significant deficits ($p \leq 0.01$).

Species	Locus										Average \pm SD
	Hl4	Hl15	Hl16	Hl20	Lc5	Lw8	Lw10	Lw11	Lw16	Lw18 ¹	
<i>Le. weddellii</i>											
N	188	184	192	165	143	184	141	142	186	79(108)	163.3 \pm 28.3
H_{exp}	0.26	0.59	0.63	0.63	0.04	0.29	0.88	0.81	0.78	0.68	0.56 \pm 0.27
H_{obs}	0.24	0.61	0.65	0.63	0.04	0.29	0.92	0.82	0.80	0.62	0.56 \pm 0.28
A	7	22	8	7	3	4	11	13	10	10	9.5 \pm 5.4
<i>Lo. carcinophagus</i>											
N	141	147	154	135	140	145	153	86	148	65(126)	137.5 \pm 19.9
H_{exp}	0.81	0.90	0.90	0.84	0.65	0.91	0.69	0.91	0.94	0.91	0.85 \pm 0.10
H_{obs}	0.78	0.93	0.87	0.54	0.54	0.87	0.70	0.86	0.88	0.85	0.78 \pm 0.14
A	11	22	17	10	12	18	9	17	28	17	16.1 \pm 5.9
<i>O. rossii</i>											
N	41	40	41	22	18	40	40	40	41	15(23)	34.6 \pm 9.5
H_{exp}	0.52	0.65	0.68	0.82	0.11	0.69	0.92	0.89	0.82	0.95	0.71 \pm 0.25
H_{obs}	0.51	0.63	0.68	0.73	0.00	0.63	0.90	0.85	0.83	1.00	0.68 \pm 0.28
A	4	4	10	11	2	6	19	13	12	19	10.0 \pm 6.0

¹ This locus is X-linked so the number of females and total number (in parentheses) are listed separately and the heterozygosity represents only females. The total number of individuals regardless of sex was used in calculating the average and standard deviation of the number of individuals, as well as the number of alleles seen at each locus.

Table 4.2. Autosomal θ_F estimate (Xu and Fu 2003) and the genetically effective population size ($N_{EA} = \frac{\theta_F}{4\mu}$, and SD) for microsatellite loci surveyed in pack-ice seals. The mutation rate (μ) was assumed to be 10^{-5} per gene per generation.

Locus	<i>Le. weddellii</i>		<i>Lo. Carcinophagus</i>		<i>O. rossii</i>	
	θ_F	N_{EA}	θ_F	N_{EA}	θ_F	N_{EA}
H104	0.20	5,000	10.54	263,500	1.08	27,000
H115	1.74	43,500	42.30	1,057,500	2.54	63,500
H116	2.22	55,500	36.72	918,000	3.12	78,000
H120	2.27	56,800	15.68	392,000	11.10	277,500
Lc05	0.01	300	2.50	62,500	0.04	1,000
Lw08	0.26	6,500	47.09	1,177,300	3.47	86,800
Lw10	28.34	708,500	3.52	88,000	57.53	1,438,300
Lw11	10.30	257,500	52.32	1,308,000	34.18	854,500
Lw16	7.89	197,300	127.59	3,189,800	11.12	278,000
Lw18 ¹	3.36	112,000	44.34	1,478,000	161.2	5,373,300
Arithmetic mean \pm	5.91 \pm		37.58 \pm		13.80 \pm	
SD	9.15		38.78		19.50	

¹ This locus is X-linked so the effective population index is theta divided by three times the mutation rate and estimated using only female samples. The values for Lw18 are not included in the averages or standard deviations.

Table 4.3. Estimates of genetically effective population sizes and the ratio of N_E to census size (in parentheses) for maternally, paternally, and biparentally inherited loci in pack-ice seals based on θ_F (Xu and Fu 2003) or Θ as estimated using the program LAMARC (Kuhner 2006). Values for the autosomal microsatellites were calculated using the arithmetic mean of θ_F across all nine loci.

Species	Census Size ²	Mitochondrial $2N_{EF}$	Microsatellite			Y-linked yes/no
			X-linked ³ N_{EX}	Autosomal ³ N_{EA}	Autosomal ⁴ Reduced data	
<i>L. Weddellii</i>	800,000	111,200 (0.14)	112,000 (0.14)	147,850 (0.19)	77,800 (0.10)	Yes
<i>L. Carcinophagus</i>	15 million	853,400 (0.07)	1,478,000 (0.10)	939,600 (0.06)	N/A	Yes
<i>O. rossii</i>	220,000	130,400 (0.59)	5,373,300 (24.45)	344,950 (1.57)	116,000 (0.53)	No

¹ Based on Θ from Curtis *et al.* 2009. Estimated from mitochondrial DNA were doubled assuming that the sex ratio is 1:1.

² These are the largest of the published estimates and are from Erickson and Hanson (1990) and Laws (1977).

³ Mutation rate was assumed to be 10^{-5} .

⁴ Single loci with putatively aberrantly large θ_F values were removed (see text).

Table 4.4. Observed and expected microsatellite heterozygosities of seals. Number of individuals (N), observed heterozygosity (H_O), expected heterozygosity (H_E), and number of loci (A) used in the study. Studies without published observed heterozygosity were not included in this table.

Species	Location	N	H_O	H_E	A	Citation
Mediterranean monk seal (<i>Monachus monachus</i>)	Eastern Mediterranean	~60	0.23	0.32	24	Pastor <i>et al.</i> 2007
Mediterranean monk seal (<i>Monachus monachus</i>)	Western Mediterranean	~60	0.35	0.38	24	Pastor <i>et al.</i> 2007
Harbor seal (<i>Phoca vitulina</i>)	Strandings along Dutch coast	204	0.34	0.34	27	Rijks <i>et al.</i> 2008
Hawaiian monk seal (<i>Monachus schauinslandi</i>)	Hawaiian archipelago	2409	0.48	0.49	8**	Schultz <i>et al.</i> 2008
Harbor seal (<i>Phoca vitulina</i>)	Western Atlantic, Northern Europe	~972	0.50	?	7	Goodman 1998
Weddell seal (<i>Le. weddellii</i>)	Western Ross Sea, Antarctica	~163	0.56	0.56	10	This study
Leopard seal (<i>Hydrurga leptonyx</i>)	Bird Island, Antarctica	25	0.64	0.66	24	*Davis <i>et al.</i> 2002
Weddell seal (<i>Le. weddellii</i>)	Big Razorback Island, Antarctica	96	0.66	0.67	24	*Davis <i>et al.</i> 2002
S. elephant seal (<i>Mirounga leonina</i>)	Faukland Islands	263	0.66	0.66	9	Fabiani <i>et al.</i> 2004
Ross seal (<i>O. rossii</i>)	Western Ross Sea, Antarctica	~35	0.68	0.71	10	This study
Leopard seal (<i>Hydrurga leptonyx</i>)	Bird Island, Antarctica	21	0.70	0.72	18	Davis <i>et al.</i> 2000
Weddell seal (<i>Le. weddellii</i>)	East coast and west coast Antarctica	158	0.72	0.73	18	Davis <i>et al.</i> 2000
Gray seal (<i>Halichoerus grypus</i>)	North Rona, Scotland	309	0.76	?	up to 11	Poland and Pomeroy 2008
Gray seal (<i>Halichoerus grypus</i>)	North Rona, Scotland	~400	0.76	?	up to 11	Twiss <i>et al.</i> 2006
Crabeater seal (<i>Lo. carcinophagus</i>)	Western Ross Sea, Antarctica	~138	0.78	0.85	10	This study
Crabeater seal (<i>Lo. carcinophagus</i>)	RV Nathaniel B Palmer, 1994	25	0.79	0.83	24	*Davis <i>et al.</i> 2002
Antarctic fur seal (<i>Arctocephalus gazella</i>)	Isle de Crozet (in AA circle)	~102	0.80	0.82	6	Kingston and Gwilliam 2007
Subantarctic fur seal (<i>Arctocephalus tropicalis</i>)	Isle de Crozet (in AA circle)	~102	0.81	0.82	6	Kingston and Gwilliam 2007
Crabeater seal (<i>Lo. carcinophagus</i>)	Around Antarctic continent	49	0.81	0.87	18	Davis <i>et al.</i> 2000
Harp seal (juveniles) (<i>Phoca groenlandica</i>)	Northern United States	65	0.83	?	12	Kretzmann <i>et al.</i> 2006

*We estimated means of H_O and H_E from published data in Davis *et al.* 2002.

**A total of 154 were screened in the study (Schultz *et al.* 2008), we refer here to the data of the eight polymorphic loci.

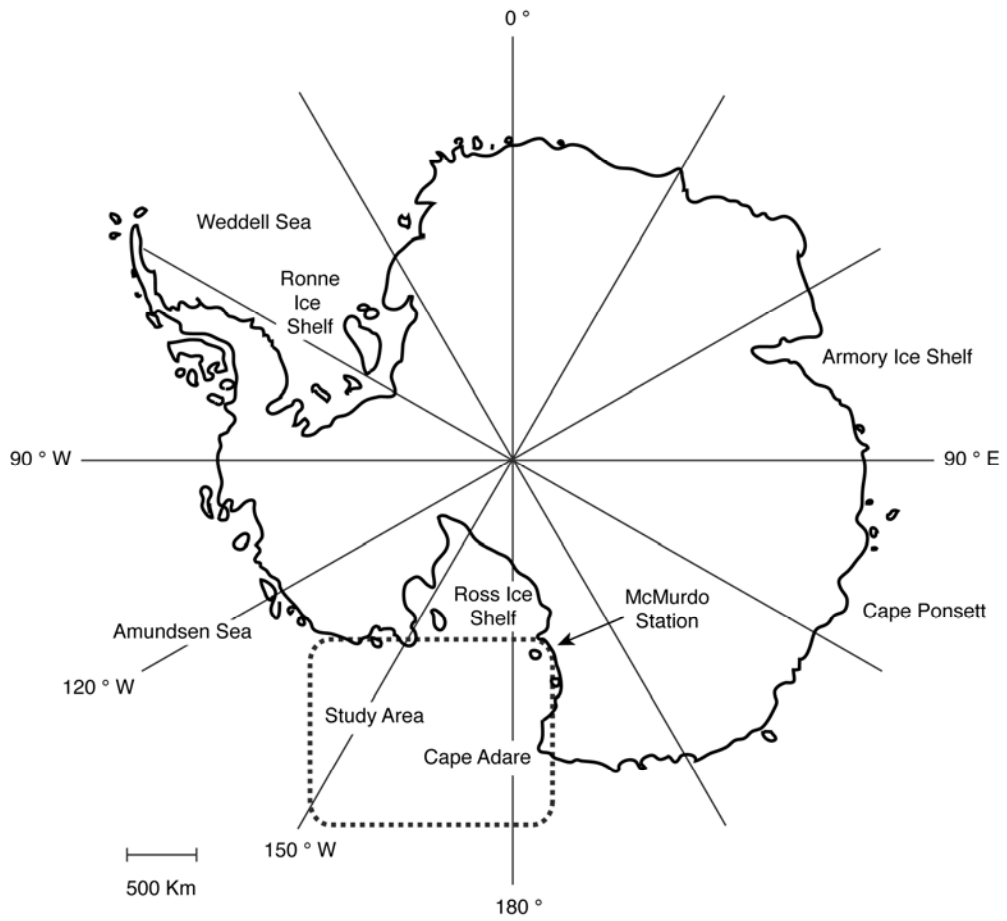


Figure 4.1. Map of the Antarctic continent showing the area from which the samples were collected.

Chapter 5: Conclusion

The major importance of this dissertation stemmed from estimating effective population sizes through several types of genetic markers, those with maternal inheritance (mitochondrial DNA), biparentally inherited markers (autosomal microsatellites) and sex-linked markers. In addition, these markers encompassed both sequence fragments as well as microsatellites, which have differing modes and rates of mutation. This allowed for several independent lines of investigation with the available samples, to the extent that is possible with genetic analyses on the same sample set. Concordance between expectations and markers can be a powerful tool in revealing biologically meaningful demographic patterns, and discord between markers of varying inheritance patterns may provide insight into sex-specific processes including mating systems and long term effective sex ratios. Despite an enormous screening effort, however, I was unable to find Y chromosome specific markers with enough variability to allow any sort of meaningful statistical analyses, so sex-linked estimations of effective population size were limited to a single X-linked microsatellite. It is likely that developing a Y chromosome specific microsatellite library would yield more variable markers for this purpose.

When my data are considered together, it seems likely that Weddell seal effective population size is between 111,200 and 147,850 individuals, and reflects a regional

population estimate in the western Amundsen and eastern Ross seas. It seems reasonable to postulate this to be the case, as genetic subdivision has been noted in samples separated by more than 700 km (Davis *et al.* 2008). If I assume that the distribution of Weddells is uniform around the continent, and roughly extrapolate this regional estimate, it gives an estimate of 556,000 to 739,250 animals, which approaches the census size estimates of about 730,000 (Laws 1977) to 800,000 (Erickson and Hanson 1990). The mitochondrial data suggest that these seals underwent a rapid population expansion roughly 731,000 years ago, a time of expanding ice habitat and lowering sea levels. Populations may have become more robust and expanded their ranges as new and better fast ice habitat became available for breeding and molting.

The effective population size estimate for crabeater seals 853,400 and 939,600 individuals. This number is significantly lower than published census estimates of 7 to 15 million individuals (Laws 1977, Ericson and Hanson 1990). Given their migratory behavior and the present lack of evidence of genetic sub-structuring around the continent (Davis *et al.* 2008), it is possible that this reflects a circumpolar estimate, in which case there exists a large discrepancy between census and genetic estimates. Further investigation may reveal some level of undetected geographic subdivision among crabeater seals, particularly between east and west Antarctica and perhaps other geographically disjunct areas like the Weddell Sea in which case our estimate may be limited to a portion of the perimeter of continent. Both Weddell seals and crabeater seals

appear to have undergone rapid population expansions during times of expanding ice habitat. One may postulate that dramatic, long term reductions in ice habitat off the coast of Antarctica may have an inverse effect on their population sizes.

Ross seal effective population sizes varied significantly between mitochondrial estimates (130,400 individuals) and autosomal estimates (344,950 individuals), though when single loci with putatively aberrantly large θ_F values were removed the autosomal estimate was closer to that of the mitochondria (116,000 individuals), close to the circumpolar census estimates of 131,000 to 220,000 individuals (Laws 1977, Erickson and Hanson 1990). Due to the large discrepancies between the mitochondrial, autosomal, and X-linked microsatellite data, this species effective population size warrants further investigation. The Ross seal did not show any signs of rapid population expansion, as did the crabeater and Weddell seals. It is interesting to note that the rank order for the three species remained consistent with both mitochondrial and autosomal markers, and that Ross seal effective population sizes appear to be larger than Weddell seals, which is not consistent with published census data. This may be due to greater genetic subdivision in Weddell seals, whereby Weddell seal effective population size estimates reflect a more regional estimate (that of the western Amundsen and eastern Ross seas) whereas Ross seals reflect a more circumpolar estimate. Conversely, it is possible that Ross seals are undercounted using traditional census techniques.

These data and effective population size estimates are good starting points, but

caution should be used with respect to these estimates and their application in management strategies. Ross seal estimates, based on 41 samples, may not have captured a true picture of the genetic variation in the population. The analyses of more samples in this species is critical, and this is arguably true for all the species in the study.

Additionally, an equal sampling effort from equidistant points around the continent would help to discern circumpolar estimates for the species in this study.

In addition to more samples, future studies should employ a greater number of both autosomal microsatellites (ideally more than 20 variable, unlinked microsatellites) and X-linked microsatellites, to reduce the variation around the mean estimates of θ . As research into pinniped biology continues, generation times of phocids should be revisited and confirmed. A better understanding of generation times may alter effective population size estimates. All other factors remaining constant, decreasing generation times will increase effective population size estimates and vice versa. Future research into Ross seal biology is important, and may help explain why Ross seal populations appear to have remained stable through the Pleistocene epoch, while the other two phocid seals in this study appear to have rapidly expanded during this time. It is possible that behavioral traits specific to the Ross seal may explain this difference, perhaps the Ross seals propensity to use the open ocean to a greater extent, causing them to be less influenced by expanding ice habitat. Food source may be another consideration, as invertebrates, the primary food source of Ross seals, may have been a factor limiting their expansion.

References

- Ackley SF, Bengtson JL, Boveng P, Castellini M, Daly KL, Jacobs S, Kooyman GL, Laake J, Quetin L, Ross R, Siniff DB, Stewart BS, Stirling I, Torres J, Yochem PK (2003) A top-down, multidisciplinary study of the structure and function of the pack-ice ecosystem in the eastern Ross Sea, Antarctica. *Polar Record*, **39**, 219-230.
- Amos W, Hutter CM, Schug MD, Aquadro CF (2003) Directional evolution of size coupled with ascertainment bias for variation in *Drosophila* microsatellites. *Molecular Biology and Evolution*, **20**(4), 660-662.
- Andolfatto P (2001) Contrasting patterns of X-linked and autosomal nucleotide variation in *Drosophila melanogaster* and *Drosophila simulans*. *Molecular Biology and Evolution*, **18**(3), 279-290.
- Arnason U, Gullberg A, Janke A, Kullberg M, Lehman N, Petrov EA, Vainola R (2006) Pinniped phylogeny and a new hypothesis for their origin and dispersal. *Molecular Phylogenetics and Evolution*, **41**(2), 345-354.

- Avise JC, Ball RM, Arnold J (1988) Current versus historical population sizes in vertebrate species with high gene flow - a comparison based on mitochondrial-DNA lineages and inbreeding theory for neutral mutations. *Molecular Biology and Evolution*, **5**, 331-344.
- Barker JS, Frydenberg J, Gonzalez J, Davies HI, Ruiz A, Sorensen JG, Loeschcke V (2009) Bottlenecks, population differentiation and apparent selection at microsatellite loci in Australian *Drosophila buzzatii*. *Heredity*, (2009 electronic-publication ahead of print).
- Bartsh SS, Johnston SD, Siniff DB (1992) Territorial behavior and breeding frequency of male Weddell seals (*Leptonychotes weddellii*) in relation to age, size, and concentrations of serum testosterone and cortisol. *Canadian Journal of Zoology*, **70**, 680-692.
- Bazin E, Glemin S, Galtier N (2006) Population size does not influence mitochondrial genetic diversity in animals. *Science*, **312**(5773), 570-572.
- Bengtson JL, Laws RM (1985) Trends in crabeater seal age at maturity – an insight into

Antarctic marine interactions. Pp. 669-675. In: *Antarctic nutrient cycles and food webs* (Siegfried WR, Condy PR, Laws RM eds.). Springer-Verlag, Berlin.

Bengtson JL, Siniff DB (1981) Reproductive aspects of female crabeater seals (*Lobodon carcinophagus*) along the Antarctic Peninsula. *Canadian Journal of Zoology*, **59**, 91-102.

Bester MN, Erickson AW, Ferguson JWH (1995) Seasonal change in the distribution and density of seals in the pack ice off Princess Martha Coast, Antarctica. *Antarctic Science*, **7**, 357-364.

Betancourt AJ, Kim Y, Orr HA (2004) A pseudohitchhiking model of X vs autosomal diversity. *Genetics*, **168**, 2261-2269.

Buchan JC, Archie EA, Van Horn RC, Moss CJ, Alberts SC (2005) Locus effects and sources of error in noninvasive genotyping. *Molecular Ecology Notes*, **5**, 680-683.

Cameron MF, Siniff DB (2004) Age-specific survival, abundance, and immigration rates of a Weddell seal (*Leptonychotes weddellii*) population in McMurdo Sound,

Antarctica. *Canadian Journal of Zoology*. **82**, 601-615.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD
(2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic
Acids Research*, **31**(13), 3497-3500.

Cline DR, Siniff DB, Erickson AW (1971) Underwater copulation of the Weddell seal.
Journal of Wildlife Management, **52**, 216-218.

Croxall JP, Hiby L (1983) Fecundity, survival and site fidelity in Weddell seals,
Leptonychotes weddelli. *The Journal of Applied Ecology*, **20**, 19-32.

Curtis C, Stewart BS, Karl SA (2007) Sexing pinnipeds with *ZFX* and *ZFY* loci. *Journal
of Heredity*, **98**, 280-285.

Curtis C, Stewart BS, Karl SA (2009). Pleistocene population expansions of Antarctic
pack-ice seals. *Molecular Ecology*, in press.

Dalebout ML, Robertson KM, Frantzis A, Engelhaupt D, Mignucci-Giannoni AA,
Rosario-Delestre RJ, Baker CS (2005) Worldwide structure of mtDNA diversity
among Cuvier's beaked whales (*Ziphius cavirostris*): implications for threatened

populations. *Molecular Ecology*, **14**, 3353-3371.

Damstra T, Barlow S, Bergman S, Kavlock R, Van Der Kraak G (2002) Global assessment of the state-of-the-science of endocrine disruptors. World Health Organization publication WHO/PCS/EDC/02.2 (visited last June 22, 2006) http://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/index.html

Davis CS, Stirling I, Strobeck C (2000) Genetic diversity of Antarctic pack ice seals in relation to life history characteristics. In: *Antarctic ecosystems: models for a wider ecological understanding*. (eds. Davison W, Howard-Williams P, Broady P), pp 56-62. Christchurch, New Zealand: Caxton Press.

Davis CS, Gelatt TS, Siniff D, Strobeck C (2002) Dinucleotide microsatellite markers from the Antarctic seals and their use in other Pinnipeds. *Molecular Ecology Notes*, **2**(3), 203–208.

Davis CS, Stirling I, Strobeck C, Coltman DW (2008) Population structure of ice-breeding seals. *Molecular Ecology*, **17**, 3078-3094.

Decker D, Stewart BS, Lehman N (2002) Major histocompatibility complex class II DOA sequences from three Antarctic seal species verify stabilizing selection on the dog locus. *Tissue Antigens*, **60**, 533-537.

Di Rienzo A, Peterson AC, Garza JC, Valdes AM, Slatkin M, Freimer NB (1994) Mutational processes of simple sequence repeat loci in human populations. *Proceedings of the National Academy of Science USA*, **91**, 3166-3170.

Ellegren H (2000) Microsatellite mutations in the germline: implications for evolutionary inference. *Trends in Genetics*, **16**(12), 551-558.

Erickson AW, Hanson MB (1990) Continental estimates and population trends of Antarctic ice seals. *In: Antarctic ecosystems, ecological change and conservation.* (eds. Kerry KR and Hempel G), pp. 253-264. Berlin and Heidelberg: Springer-Verlag.

Excoffier L (2004) Patterns of DNA sequence diversity and genetic structure after a range expansion: lessons from the infinite-island model. *Molecular Ecology*, **13**, 853-864.

- Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47-50.
- Fabiani A, Galimberti F, Sanvito S, Hoelzel AR (2004) Extreme polygyny among southern elephant seals on Sea Lion Island, Falkland Islands. *Behavioral Ecology*, **15**(6), 961-969.
- Felsenstein, J (2005) Accuracy of coalescent likelihood estimates: Do we need more sites, more sequence, or more loci? *Molecular Biology and Evolution*, **23**, 691-700.
- Frankham R (1995) Effective population size/adult population size in wildlife: a review. *Genetics Research*, **66**, 95-107.
- Fredsted T, Villesen P (2004) Fast and reliable sexing of primate and human DNA. *American Journal of Primatology*, **64**, 345-350.
- Fu Y (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, **147**, 915-925.

- Galbusera P, van Dongen S, Matthysen E (2000) Cross-species amplification of microsatellites in passerine birds. *Conservation Genetics*, **1**(2), 163-168.
- Gelatt T, Davis C, Cameron M, Siniff D, Strobeck C (2000) The old and the new: integrating population ecology and population genetics of Weddell seals. In: *Antarctic ecosystems: models for a wider ecological understanding*. (eds. Davison W, Howard-Williams P, Broady P), pp. 63-70. Christchurch, New Zealand: Caxton Press.
- Gilson A, Syvanen M, Levine K, Banks J (1998) Deer gender determination by polymerase chain reaction: validation study and application to tissues, bloodstains, and hair forensic samples from California. *California Fish and Game*, **84**, 159-169.
- Goerke H, Weber K, Bornemann H, Ramdor S, Plötz J (2004) Increasing levels and biomagnification of persistent organic pollutants (POPs) in Antarctic biota. *Marine Pollution Bulletin* **48**, 295-302.
- Goodman SJ (1998) Patterns of extensive genetic differentiation and variation among European Harp Seals (*Phoca vitulina vitulina*) revealed using DNA microsatellite

polymorphisms. *Molecular Biology and Evolution*, **15**(2), 104-118.

Hadley GL, Rotella JJ, Garrott RA, Nichols JD (2006) Variation in probability of first reproduction of Weddell seals. *Journal of Animal Ecology*, **75**, 1058-1070.

Hansson B, Westerberg L (2002) On the correlation between heterozygosity and fitness in natural populations. *Molecular Ecology*, **11**, 2467-2474.

Harding KC, Harkonen T (1995) Estimating mean age of sexual maturity in the crabeater seal (*Lobodon carcinophagus*). *Canadian Journal of Fisheries and Aquatic Sciences*, **52**, 2347-2352.

Harpending HC (1994) Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. *Human Biology*, **66**. 591-600.

Hartl DL, Clark AG (1997) *Principles of population genetics*, 3rd ed. pp. 289-294. Sinauer Associates, Inc, Sunderland Massachusetts.

- Hastings KK, Testa JW (1998) Maternal and birth colony effects on survival of Weddell seal offspring from McMurdo Sound, Antarctica. *Journal of Animal Ecology*, **67**, 722-740.
- Hattori K, Burdin AM, Onuma M, Suzuki M, Ohtaishi N (2003) Sex determination in the sea otter (*Enhydra lutris*) from tissue and dental pulp using PCR amplification. *Canadian Journal of Zoology*, **81**, 52-56.
- Hellborg L, Ellegren H (2004) Low levels of nucleotide diversity in mammalian Y chromosomes. *Molecular Biology and Evolution*, **21**(1), 158-163.
- Higdon JW, Bininda-Emonds ORP, Beck RMD, Ferguson SH (2007) Phylogeny and divergence of the pinnipeds (Carnivora: Mammalia) assessed using a multigene dataset. *BMC Evolutionary Biology*, **7**, 216.
- Hutter CM, Schug MD, Aquadro CF (1998) Microsatellite variation in *Drosophila melanogaster* and *Drosophila simulans*: a reciprocal test of the ascertainment bias hypothesis. *Molecular Biology and Evolution*, **15**(12), 1620-1636.
- International Whaling Commission Report, Annex G (2005) Document SC/57/021.

Report of the Sub-committee on In-depth Assessment (IA). p.9.

Jefferson TA, Leatherwood S, Webber MA (1993) *FAO Species Identification Guide, Marine Mammals of the World*. United Nations Food and Agricultural Organization. Rome. 320 p.

Kanatous SB, Davis RW, Watson R, Polasek L, Williams TM, Mathieu-Costello O (2002) Aerobic capacities in the skeletal muscles of Weddells seals: key to longer dive durations? *The Journal of Experimental Biology*, **205**, 3601-3608.

Kashi, Y, Soller M (1999) Functional Roles of Microsatellites and Minisatellites. In: *Microsatellites: Evolution and Applications*. Edited by Goldstein and Schlotterer. Oxford University Press.

Kauer M, Zangerl B, Dieringer D, Schlotterer C (2002) Chromosomal patterns of microsatellite variability contrast sharply in African and non-African populations of *Drosophila melanogaster*. *Genetics*, **160**, 247-256.

Kaufman GW, Siniff DB, Reichle R (1975) Colony behavior of Weddell seals, *Leptonychotes weddellii*, at Hutton Cliffs, Antarctica. Rapport et Proces-Verbaux

des Reunions du Conseil International Pour L'Exploration de la Mer, **169**, 228-246.

Kingston JJ, Gwillium J (2007) Hybridization between two sympatrically breeding species of fur seals at Iles Crozet revealed by genetic analysis. *Conservation Genetics*, **8**(5), 1133-1145.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo A, Villablanca FX, Wilson AC (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences, USA*, **86**, 6196-6200.

Kretzmann M, Mentzer L, DiGiovanni R, Leslie MS, Amato G (2006) Microsatellite diversity and fitness in stranded juvenile harp seals (*Phoca groenlandica*). *Journal of Heredity*, **97**(6), 555-560.

Kuhner, MK (2006) LAMARC 2.0: maximum likelihood and Bayesian estimation of population parameters. *Bioinformatics*, **22**, 768-770.

Laws RM (1977) Seals and whales of the southern ocean. *Philosophical Transactions of*

the Royal Society, London, B, **279**, 81-96.

Laws RM, Baird A, Bryden MM (2003) Size and growth of the crabeater seal, *Lobodon carcinophagus* (Mammalia: Carnivora), *Journal of Zoology*, **259**, 103-108.

Laws RM, Baird A, Bryden MM (2002) Age estimation in crabeater seals (*Lobodon carcinophagus*). *Journal of Zoology* 258, 197-203.

Lawson LJ, Hewitt GM (2002) Comparison of substitution rates in *ZFX* and *ZFY* introns of sheep and goat related species supports the hypothesis of male-biased mutation rates. *Journal of Molecular Evolution*, **54**, 54-61.

Li W-H (1977) Distribution of nucleotide differences between two randomly chosen cistrons in a finite population. *Genetics*, **85**, 331-337.

Linder P (2006) Dead-box proteins: a family affair- active and passive players in RNP remodeling. *Nucleic Acids Research*, **34**(15), 4168-4180.

Lucchini V, Fabbri E, Marucco F, Ricci S, Boitani L, Randi E (2002) Noninvasive

molecular tracking of colonizing wolf (*Canis lupus*) packs in the western Italian Alps. *Molecular Ecology*, **11**, 857-868.

Luikart G, Cornuet J-M (1998) Empirical evaluation of a test for identifying recently bottlenecked populations from allele frequency data. *Conservation Biology*, **12**(1), 228-237.

Macintosh NA (1970) Whales and drill in the twentieth century. *In. Antarctic ecology*, vol. 1 (eds. Holdgate, MW) pp 194-212. Academic Press, New York, New York.

Malik S, Brown MW, Kraus SD, White BN (2000) Analysis of mitochondrial DNA diversity within and between North and South Atlantic right whales. *Marine Mammal Science*, **16**, 45-558.

Marchal JA, Acosta MJ, Bullejos M, de la Guardia RD, Sanchez A (2003) Sex chromosomes, sex determination, and sex-linked sequences in Microtidae. *Cytogenetic and Genome Research* **101**, 266-273.

Mardon G, Page DC (1989) The sex-determining region of the mouse Y chromosome encodes a protein with a highly acidic domain and 13 zinc fingers. *Cell*, **56**, 765-

770.

Mitani Y, Watanabe Y, Sato K, Cameron MF, Naito Y (2004) 3D diving behavior of Weddell seals with respect to prey accessibility and abundance. *Marine Ecology*, **281**, 275-281.

Mori M, Butterworth DS (2004) Consideration of multispecies interactions in the Antarctic: a preliminary model of the mink whale – blue whale – krill interaction. *African Journal of Marine Science*, **26**, 245-259.

Morin PA, Nestler A, Rubio-Cisneros NT, Robertson KM, Mesnick SL (2005) Interfamilial characterization of a region of the *ZFX* and *ZFY* genes facilitates sex determination in cetaceans and other mammals. *Molecular Ecology*, **14**, 3275-3286.

Negri AP, Hales LT, Battershill C, Wolff C, Webster NS (2004) TBT contamination identified in Antarctic marine sediments. *Marine Pollution Bulletin*, **48**, 1142-1144.

Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press.

- Oritsland T (1970) Sealing and seal research in the south-west Atlantic pack-ice, September-October 1964. Pp. 367-376, In: Antarctic Ecology, Vol. 1 (Holdgate MW, ed.). Academic Press.
- Paetkau D, Calvert W, Stirling I, Strobeck C (1995) Microsatellite analysis of population structure in Canadian Polar Bears. *Molecular Ecology*, **4**(3), 347-354.
- Page DC, Mosher R, Simpson EM, Fisher EMC, Mardon G, Pollack J, McGillivray B, de la Chapelle A, Brown LG (1987) The sex-determining region of the human Y chromosome encodes a finger protein. *Cell*, **51**, 1091-1104.
- Palo JU, Makinen HS, Helle E, Stenman O, Vainola R (2001) Microsatellite variation in ringed seals (*Phoca hispida*): genetic structure of history of the Baltic Sea population. *Heredity*, **86**, 609-617.
- Park SDE (2001) Trypanotolerance in West African Cattle and the Population Genetic Effects of Selection. Ph.D. thesis. University of Dublin.
- Pastor T, Garza JC, Aguilar A, Tounta E, Androukaki E (2007) Genetic diversity and

differentiation between the two remaining populations of the critically endangered Mediterranean monk seal. *Animal Conservation*, **10**(4), 461-469.

Payseur BA, Nachman MW (2002) Natural selection at linked sites in humans. *Gene*, **300**, 31-42.

Pecon-Slattery J, O'Brien SJ (1998) Patterns of Y and X Chromosome DNA Sequence Divergence During the Felidae Radiation. *Genetics*, **148**, 1245-1255.

Pecon-Slattery J, Sanner-Wachter L, O'Brien SJ (2000) Novel gene conversion between X-Y homologues located in the nonrecombining region of the Y chromosome in Felidae (Mammalia). *Proceedings of the National Academy of Science, USA*, **97**, 5307-5312.

Petit JR, Jouzel J, Raynaud D, Barkov NI, Barnola J-M, Basile I, Bender M, Chappellaz J, Davis M, Delaygue G, Delmotte M, Kotlyakov VM, Legrand M, Lipenkov VY, Lorius C, Pépin L, Ritz R, Saltzman E, Stievenard M (1999) Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. *Nature*, **399**, 429-436.

Pilgrim KL, McKelvey S, Riddle AE, Schwartz MK (2005) Felid sex identification based

on noninvasive genetic samples. *Molecular Ecology Notes*, **5**, 60-61.

Piry S, Luikart G, Cornuet J-M (1999) BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity*, **90**, 502-503.

Piyamongkol W, Bermudez MG, Harper JC, Dagan W (2003) Detailed investigation of factors influencing amplification efficiency and allele drop-out in single cell PCR: implications for preimplantation genetic diagnosis. *Molecular Human Reproduction*, **9**, 411-420.

Plotz J, Ekau W, Reijnders PJH (1991) Diet of Weddell seals *Leptonychotes weddellii* at Vestkapp, Eastern Weddell Sea (Antarctica), in relation to local food supply. *Marine Mammal Science*, **7**(2), 136-144.

Poland VF, Pomeroy PP, Twiss SD, Graves JA (2008) A fine scale study finds limited evidence of kin clustering in a grey seal colony. *Marine Mammal Science*, **24**, 371-387.

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution.

Bioinformatics, **14**, 817-818.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248-249.

Reed DH, Frankham R (2003) Correlation between fitness and genetic diversity. *Conservation Biology*, **17**, 230-237.

Reeves RR, Stewart BS (2003) Marine Mammals of the World: An Introduction. In: Walker's Marine Mammals of the World (Nowak RM ed). Baltimore MD: The Johns Hopkins University Press: 1-64.

Reeves RR, Stewart BS, Leatherwood S (1992) *The Sierra Club Handbook of Seals and Sirenians*. San Francisco, CA: Sierra Club Books.

Rijks JM, Hoffman JI, Kuiken T, Osterhaus ADME, Amos W (2008) Heterozygosity and lungworm burden in harbor seals (*Phoca vitulina*). *Heredity*, **100**, 587-593.

Rogers A (1995) Genetic evidence for a Pleistocene population explosion. *Evolution*, **49**, 608-615.

Rogers AR, Harpending H (1992) Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution*, **9**, 552-569.

Schlotterer C (2000) Evolutionary dynamics of microsatellite DNA. *Chromosoma*, **109**(6), 365-371.

Schultz JK, Baker JD, Toonen RJ, Bowen BW (2008) Extremely low genetic diversity in the endangered Hawaiian monk seal (*Monachus schauinslandi*). *Journal of Heredity*, **100**(1), 25-33.

Scientific Committee for Antarctic Research (2006) Proposal to de-list Antarctic fur seals as specially protected species. Antarctic Treaty Consultative Meeting, 2006.
http://www.scar.org/treaty/atcmxxix/atcm29_wp039.pdf

Schneider S, Excoffier L (1999) Estimation of demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: Application to human mitochondrial DNA. *Genetics*, **152**, 1079-1089.

Sefc KM, Payne RB, Sorenson MD (2003) Microsatellite amplification from museum feather samples: effects of fragmentation size and template concentration on genotyping errors. *Auk*, **120**, 982-989.

- Shaw CN, Wilson PJ, White BN (2003) A reliable molecular method of gender determination for mammals. *Journal of Mammology*, **84**, 123-128.
- Shen P, Wang F, Underhill PA, Franco C, Yang WH, Roxas A, Sung R, Lin AA, Hyman RW, Vollrath D, Davis RW, Cavalli-Sforza LL, Oefner PJ (2000) Population genetic implications from sequence variation in four Y chromosome genes. *Proceedings of the National Academy of Science USA*, **97**(13), 7354-7359.
- Simonsen BT, Siegismund HR, Arctander P (1998) Population structure of African buffalo inferred from mtDNA sequences and microsatellite loci: high variation but low differentiation. *Molecular Ecology*, **7**, 225-237.
- Siniff DB, Stirling I, Bengston JL, Reichle RA (1979) Social and reproductive behavior of crabeater seals (*Lobodon carcinophagus*) during the austral spring. *Canadian Journal of Zoology*, **57**, 2243-2255.
- Slade RW, Moritz C, Heideman A (1994) Multiple nuclear-gene phylogenies: applications to pinnipeds and comparison with a mitochondrial DNA gene phylogeny. *Molecular Biology and Evolution*, **11**, 341-356.

Slade RW, Moritz C, Hoelzel, AR, Burton HR (1998) Molecular Population Genetics of the Southern Elephant Seal *Mirounga leonina*. *Genetics*, **149**, 1945-1957.

Slatkin M, Hudson RR (1991) Pairwise comparisons of mitochondrial-DNA sequences in stable and exponentially growing populations. *Genetics*, **129**, 555-562.

Smetacek V, Nicol S (2005) Polar ecosystems in a changing world. *Nature*, **437**, 362-368.

Solls S, Stewart BS, Lehman N (2005) Conservation of MHC class I sequences among carnivores. *Tissue Antigens*, **65**, 283-266.

Splettstoesser J, Gavrilov M, Field C, Harrison P (2000) Notes on Antarctic wildlife: Ross Seals, *Ommatophoca rossii*, and emperor penguins, *Aptenodytes forsteri*. *New Zealand Journal of Zoology*, **27**, 137-142.

Spielman D, Brook BW, Frankham R (2004) Most species are not driven to extinction before genetic factors impact them. *Proceedings of the National Academy of Science*, **101**, 15261-15264.

Stewart BSS (2007) Current status of the Ross seal (*Ommatophoca rossii*): a specially protected species under Annex II (Appendix I). XXX Antarctic Treaty Consultative Meeting, Scientific Committee on Antarctic Research, May 2007.

Stirling I (1969) Ecology of the Weddell seal in McMurdo Sound, Antarctica. *Ecology*, **50**, 573-586.

Stirling I (1969) Tooth wear as a mortality factor in the Weddell seal, *Leptonychotes weddellii*, *Journal of Mammalogy*, **50**(3), 559-565.

Stirling I. (1971) Population aspects of Weddell seal harvesting of McMurdo Sound, Antarctica. *Polar Record*, **15**, 653-667.

Stirling I (1983) The evolution of mating systems in pinnipeds. In: *Advances in the study of mammalian behavior* (eds. Eisenberg JF, Kleiman DG), pp. 489-527. The American Society of Mammalogists, Special Publication No. 7, Shippensburg Pennsylvania.

- Twiss SD, Poland VF, Graves JA, Pomeroy PP (2006) Finding fathers: spatio-temporal analysis of paternity assignment in grey seals (*Halichoerus grypus*). *Molecular Ecology*, **15**, 1939-1953.
- Ushio S (2006) Factors affecting fast-ice break-up frequency in Lutzow-Holm Bay, Antarctica. *Annals of Glaciology*, **44**(1), 177-182.
- Vowles EJ, Amos W (2006) Quantifying ascertainment bias and species-specific length differences in human and chimpanzee microsatellites using genome sequences. *Molecular Biology and Evolution*, **23**(3), 598-607.
- Westlake RL, O’Corry-Crowe GM (2002) Macrogeographic structure and patterns of genetic diversity in harbor seals (*Phoca vitulina*) from Alaska to Japan. *Journal of Mammalogy*, **83**, 1111-1126.
- Wilson EA (1907) Mammalia. Pgs 1-66 in National Antarctic Expedition 1901 – 1904, natural History II. Zoology. British Museum, London. Vol 2, pt 1.

Wright S (1931) Evolution in Mendelian populations. *Genetics*, **16**, 97-159.

Xu H, Fu Y-X (2004) Estimating effective population size or mutation rate with microsatellites. *Genetics*, **166**, 555-563.

Yu N, Fu Y-X, Li W-H (2002) DNA polymorphisms in a worldwide sample of human X chromosomes. *Molecular Biology and Evolution*, **19**, 2131-2141.

APPENDICES

Appendix 1. Among species variability in the *ZFX* gene. Variable site position is relative to GenBank sequence (see text for accession numbers).

	Variable Site
	112222222333344444444444444445555555566666666677777889 66155788902590000001133779466678890166668823688663 78419139766273456786705066201234537445670161706784
Crabeater	CAATGGTTTTGCTAATACCTGCCCGTATAACAGCGGTTTAACTGTTTAT
RossG.....C.CGG.
WeddellCA.....T....G---.G.....C.....
ElephantA....C.T.....CG.....CC.....
Sealion	AGGC...CC.A.G---TGCA.GTC.G...GA.AAC---TGTCC....C

Appendix 2. Among species variability in *ZFY* gene. Variable site position is relative to GenBank sequences (see text for accession numbers).

	Variable Site
	00000000111111222222223333333334444445555566666666666667777778
	1246778915789901133367445556777467789266680001112244681144682
	4269015346230940312751291231347711390778940890181412310169727
Crabeater	TGGGTCCAACCTGCTGGGATTTATAATTAAGCCAGAATGCACAATGAAAATCGTTCTCCTGC
Ross	GCAA..T.....A.....CG.....T.....T.....
Weddell	.T.A.....C.A.....C.....T.T..----.T..A.....
Elephant	...A...T.....A.....C...G...TT.....GT.....G.....T....
<u>Sealion</u>	<u>..A---.C.TCAG.AAA--CCCC.---.GTT..A.--A..TGG....G.AT.ACGC.TAAT</u>

Appendix 3. DBY8 Sequence Variable Sites.

Haplotype	SITE NUMBER			
	1	33	63	69
<i>L. weddellii</i> common	G	A	T	C
<i>L. weddellii</i> alternate	G	A	T	G
<i>O. rossii</i>	A	T	T	C
<i>L. carcinophagus</i> common	G	T	T	C
<i>L. carcinophagus</i> alternate	G	T	C	C

Appendix 4. UTY11 Sequence variable sites.

Haplotype	SITE NUMBER														
	26	85	101	102	103	104	125	140	199	254	280	281	387	391	465
<i>L. weddellii</i> common	C	T	T	A	C	T	T	G	G	T	—	—	A	G	T
<i>L. weddellii</i> alternate	C	T	T	A	C	T	T	G	G	T	—	—	A	A	T
<i>O. rossii</i>	T	—	T	A	C	T	T	A	A	C	A	T	G	G	C
<i>L. carcinophagus</i>	T	T	—	—	—	—	A	A	G	T	A	T	A	G	C

About the Author

Caitlin Curtis completed this research as a portion of her requirement for the degree of Ph.D in Biology at the University of South Florida. She is interested in population genetics and evolution, especially in marine organisms. She is currently working on genetic tracking of Florida manatees, and population genetics of smalltooth sawfish and spiny lobsters with the Florida Fish and Wildlife Research Institute.