

# **A stable isotope method to rapidly screen the foraging ecotype profiles of Australian sea lion subpopulations**



*Photo: A Lowther (SARDI)*

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## EXECUTIVE SUMMARY

The Australian sea lion was listed as a threatened species under the EPBC Act in February 2005, vulnerable in February 2008 under the National Parks and Wildlife Act (1972) South Australia and added to the IUCN Redlist in 2008. The reproductive biology of this species poses significant challenges in understanding the mechanisms that may be important in understanding population substructuring. Previous research has highlighted matrilineal population substructuring by adult females exhibiting one of two mutually exclusive foraging ecotypes (inshore or offshore). This project represents the first year of a three year PhD being undertaken by A Lowther to examine the role of social and genetic factors in shaping Australian sea lion population substructuring. The project was conducted at two pairs of breeding colonies (Blefuscus and Lilliput Islands in the Nuyts Archipelago, and The Pages Islands off Kangaroo Island) involving sampling at least 60% of all pups produced in a breeding season for genetic and stable isotope material. Pup stable isotope ratios would reflect maternal values but be enriched one trophic level due to their total dependence on maternal milk. Foraging ecotype validation was conducted by satellite tracking a random sample of adult female Australian sea lions and examining their stable isotope ratios ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ).

Three successive sampling trips were conducted at the Nuyts Archipelago during the 2007-08 breeding season resulting in 62% of all pups being sampled. Five adult female Australian sea lions were satellite tracked at each colony to provide validation of foraging ecotype isotope ratios. Due to the difficulty of ingress / egress at The Pages Islands and the high risk of equipment loss, a single sampling trip was conducted resulting in approximately 63% of all pups being sampled at each island and a pre-existing satellite telemetry data set was used to validate foraging ecotypes.

A lack of a priori knowledge of foraging ecotype prevented a balanced sample of inshore and offshore tracks, with only three offshore tracks recorded at Blefuscus I. and none at Lilliput I. Examination of pup stable isotope revealed a more complex subpopulation substructure than previously suggested, with highly significant inter-colony ecotype segregation in the Nuyts Archipelago, and an equally significant intra-colony division within each colony at the Pages Islands.

The mitochondrial DNA (mtDNA) control region (approximately 1200 base pairs in length) was targeted as a suitable gene to examine any genetic basis to population subdivision. Sequencing was hampered by the presence of PCR inhibitors and tandem repeats in the middle of the control region. Present analyses suggests limited alignment of mtDNA haplotype with foraging ecotype, however, the genetic variation determined from the present sequence data limits interpretation at this stage. However, the identification of tandem repeats within the control region may provide an opportunity to develop mtDNA markers similar to microsatellites that will greatly increase the resolution of maternal population substructuring. This will become a focus of genetic analyses in the final year of this project (2010) pending further funding from the AMMC.

This report represents the summary of the first year of a three year project. Support has been received by the AMMC to conduct extensive stable isotope sampling throughout the range of the species, and further support has been requested to examine the fine-scale molecular genetic substructuring of the entire species.

# 1. INTRODUCTION

## 1.1. Background

The Australian sea lion (ASL) is Australia's only endemic and least-abundant seal species. It is unique among pinnipeds in being the only species that has a non-annual breeding cycle that is also temporally asynchronous across its range (Gales et al. 1994, Gales and Costa 1997) and it has the longest gestation period of any pinniped and a protracted breeding and lactation period (Higgins and Gass 1993, Gales and Costa 1997). The evolutionary determinates of this atypical life-history remain enigmatic. Recent population genetic studies have indicated little or no interchange of females among breeding colonies, even those separated by short (20 km) distances (Campbell 2008). The important management implication of extreme philopatry is that each colony could represent a closed population. ASLs were listed as a threatened species (vulnerable category) under the EPBC Act in February 2005.

Approximately 80% of the species breed in South Australia, where there are 39 known breeding sites that produce more than five pups, with a median pup production of only 25 per colony, 60% of which produce fewer than 30 pups per season (Goldsworthy and Page, 2008). However, information on the size and status of most subpopulations is poor and significantly hampers development of appropriate management strategies for species-recovery (McKenzie et al. 2005). These limitations are highly significant because management for the recovery of ASL will need to be underpinned by an ability to detect changes in the status of populations over time. To this end, recent research has focused on improving methods for obtaining quantitative estimates of pup production, and metapopulation analyses to determine the minimum subset of subpopulations (logistically feasible, cost effective, and safe to survey) that can form the basis of an ongoing monitoring program (through both NHT Goldsworthy et al. 2007, Goldsworthy et al. 2009 and ACAMMS - Project 13 and 27). The metapopulation analysis used a distance-matrix between subpopulations as a proxy for genetic distance (Campbell 2003, Campbell et al. 2008). However, Goldsworthy et al. (2007), cautioned that this approach may not accurately reflect the extent or level of subpopulation subdivision in the species, and recommended that genetic studies, additional to those of Campbell (2003) and Campbell et al. (2008), be undertaken to better resolve appropriate management units of the species. Finer scale knowledge of genetic subdivisions, especially within regions/archipelagos was also identified as a high priority/key knowledge gap, and one of eight targeted projects to address critical management needs for the species (McKenzie et al. 2005).



Two important issues have come to notice recently that heighten the need to enhance our understanding of genetic population structure within the species. First, satellite tracking studies have recently identified marked subpopulation differences in foraging strategies (ecotypes). In the Nuyts Archipelago, adult females demonstrate either inshore (shallow) or offshore (deep) foraging behaviours, with female body mass differing by 25% between colonies (large offshore foragers vs. small inshore foragers, Goldsworthy et al. unpublished data). Foraging differences appear to be relatively fixed among the six sites studied (three inshore, three offshore), with even adjacent colonies (eg. Lilliput and Blefuscu Islands, <5km apart), demonstrating marked differences in foraging behaviour (Goldsworthy et al. unpublished data). Tracking studies undertaken at The Pages have also identified inshore and offshore foraging behaviours, but in contrast to the Nuyts Archipelago, both strategies were equally represented among tracked females within the subpopulation (Goldsworthy et al. 2007). These data suggest that population subdivision may have more to do with foraging ecotypes within and between subpopulations, than geographic distances between them.

The second key issue is the recently recognized vulnerability faced by the majority of ASL subpopulations, to low-level fishery bycatch. Several recent studies (Goldsworthy and Page 2008, Goldsworthy et al. 2007a; 2007b) have identified that bycatch in the Commonwealth managed (Southern and Eastern Scalefish and Shark fishery) demersal gillnet fishery, is of greatest concern because fishing effort is substantial (especially in SA ~ 20,000 km net-set per year), occurs year-round and in close proximity to most ASL subpopulations, and recent fishery observer programs have identified significant fatal ASL bycatch (AFMA observer data).

A two-year FRDC supported project (PN 2007/041, Goldsworthy et al. 'Mitigating seal interactions in the SRL and gillnet SESSF in South Australia') aims to develop spatial management options (gillnet fisheries closures) to mitigate bycatch of critically threatened ASL subpopulations. Risk to bycatch varies considerably among subpopulations, depending on the spatial distribution of fishing effort, and the relative spread of ASL foraging effort in inshore or offshore regions. Improved quantitative assessments of the relative importance of inshore and offshore regions will be critical to enhancing our understanding of the risks posed to different subpopulations, and improve efforts to spatially manage fishing effort in proximity to ASL populations. Stable isotope methods provide a proven approach to enable rapid assessments of the foraging ecotypes of a subpopulation (Forero et al. 2005). A stepwise trophic enrichment of  $\delta^{15}\text{N}$  and latitudinal cline in  $\delta^{13}\text{C}$  allows us to predict expected offshore and onshore foraging ecotypes; the expression of high  $\delta^{15}\text{N}$ / low  $\delta^{13}\text{C}$  ratios reflect feeding offshore on prey items relatively high in the food web whilst a low  $\delta^{15}\text{N}$ / high  $\delta^{13}\text{C}$

indicates foraging closer to coastal areas on lower order prey items (Quillfeldt et al. 2005). When validated with tracking studies and analysed in conjunction with molecular genetic data, this novel approach will address a range of important management issues including the role of foraging ecotypes in defining population structure and the risk posed by fishery bycatch.

## **1.2 Approach**

This project forms part of a PhD project being undertaken by A. Lowther examining the role of social and genetic factors in determining subpopulation structure in a pinniped with a unique reproductive strategy. Details presented in this report reflect a summary of research undertaken in the first year of this three year project. Second year support to build upon the promising foraging ecotype population subdivision reported here involves the expansion of sampling across the range of the species and has been confirmed by the AMMC (Project No.27). A final application to the AMMC to support the third year of the study was submitted in March 2009 and is intended to complete the study of factors affecting substructuring by performing a fine-scale molecular examination of subpopulations across the species range.

## **1.3 Objectives**

To address the above issues, this study aims to:

- a) Develop and validate stable isotope methods to distinguish different foraging ecotypes (inshore and offshore) among ASL adult females and their dependent pups, and use this approach (sampling of pups) to rapidly screen the foraging ecotype profiles of ASL subpopulations.
- b) Undertake molecular genetic analysis of the same pups (above) and assess the importance of geographic distance and foraging ecotype in determining the genetic structure among ASL subpopulations; and
- c) Use the above results to improve subpopulation based foraging models to assist spatial management of fisheries, improve our understanding of genetic population structure, and develop a more appropriate population survey design for the species.

## 2. MATERIALS AND METHODS

### 2.1 Study site

The current study was conducted at Lilliput and Blefuscu Islands in the Nuyts Archipelago (WGS84 32°27'973S, 133°38'715E), and The Pages Islands (WGS84 35°49'27S, 138°21'13E) near Kangaroo Island (Figure 1). Two 14 day-long sampling trips were conducted between January and March 2008 at Lilliput and Blefuscu I., and a single trip of 12 days to The Pages Islands in July 2008.



**Figure 1. Sampling site locations in South Australia. Blefuscu and Lilliput I. are separated by <5km of water, whilst North and South Pages are less than 1km apart.**

### 2.2 Sample collection

#### Foraging ecotype validation – satellite tracking

Ten adult females (Lilliput I. n=5, Blefuscu I. n=5) were sampled to provide validation data on foraging ecotype with stable isotope ratio. Females were captured, restrained and anaesthetised using isoflurane (5% induction, 0.5-3% maintenance) (Veterinary Companies of Australia, Artarmon, New South Wales) delivered through a portable gas anaesthesia machine. After anaesthetic induction a whisker was snipped at its base and stored in a plastic snap-loc bag, and a small biopsy (<1mm) was taken from a digit on the hind flipper and stored in a 2ml eppendorf tube containing 70% ethanol. All samples were stored at -30°C prior to analysis.

A Fastloc GPS transmitter (Sirtrack Ltd, Havelock North, NZ) was attached to each animal either to the pelage distal to the midpoint on the back using two-part Araldite<sup>®</sup>2014 epoxy resin. Each female was individually marked using plastic numbered tags (Dalton porcine Supertag<sup>®</sup>, Dalton Supplies Ltd, U.K.) on the trailing edge of the foreflipper. After one foraging cycle each adult female was recaptured and the transmitter removed.

**Sample collection – mitochondrial DNA (mtDNA) and stable isotopes**

Sampling was conducted at approximately six weeks of age (Figure 2). Pups were captured by hand and manually restrained. A whisker and DNA biopsy was taken and each pup was individually marked using numbered plastic tags as described for adult females; pups of adult females used in validation tracking at Lilliput and Blefuscu I. were also sampled (n=7 pairs) (an additional three mother-pup pairs sampled at Liguanea were included in the analysis of isotope fractionation from mother to offspring only)

Location	Number of samples
Lilliput I.	42 (62%)
Blefuscu I.	51 (62%)
North Pages I.	100 (62%)
South Pages I.	100 (64%)

**Table 1. Number of pup samples collected from each site. Percentage of estimated pup production sampled in parentheses.**

**2.3 Laboratory analysis**

**Stable isotope chemistry**

Whiskers were thawed to room temperature over six hours. All whiskers were cleaned in a method similar to that used for human hair (O'Connell & Hedges 1999) and southern elephant seal (*Mirounga leonina*) whiskers (Lewis *et al.* 2006). Each whisker was cleaned individually for 20mins with de-ionised water then with a solution of 2:1 methanol : chloroform for a further 20 mins to remove lipids, followed by a final clean with de-ionised water for an additional 20mins. Each whisker was checked under a stereo microscope for

any remaining tissue or dirt; contaminants were removed using a scalpel blade. All samples were then rinsed with distilled water and left to air dry overnight in a fume cupboard.

The distal 10-15mm of pup whiskers and proximal 10-15mm of maternal whiskers were used for stable isotope analysis (SIA). The distal portion of a whisker represents the oldest section and should characterise either the in-utero or early lactation growth phase of the pup whisker. Similarly, the proximal portion of an adult female whisker characterises the most recent growth and thus the most recent isotopic record of feeding. Each whisker portion was clipped into  $\approx 1$ mm segments and stored within a uniquely-numbered glass scintillation vial.

Whisker samples were analysed at the Australian National University Environmental Biology Stable Isotope Facility. Analysis was performed using a Microass isoChrom CFIR mass spectrometer coupled to a Carlo Erba EA-1100 CHN-O analyser.

### **Mitochondrial DNA analysis**

Genomic DNA was extracted from all hair samples using the technique outlined in the Puregene® DNA purification kit. 5-10mg of thawed tissue was placed in a 1.5ml microfuge tube with 300 $\mu$ l of cell lysis solution and 3 $\mu$ l of Proteinase-K solution (20mg/ml) and allowed to homogenize at 55°C overnight.

Protein Precipitate Solution (100 $\mu$ l) was added and each sample centrifuged for 5 minutes. The supernatant was pipetted into a clean 1.5ml microfuge tube containing 300 $\mu$ l 100% isopropanol. All samples were centrifuged for 15 minutes and the resultant supernatant was poured off. The resultant DNA pellet was washed off in 300 $\mu$ l 70% ethanol before being centrifuged for an additional 5 minutes. The ethanol was removed and the microfuge tube allowed to air dry for 30 minutes. DNA hydration solution was added (50 $\mu$ l) and all samples stored in -20°C.

Polymerase Chain Reaction (PCR) reagents were added and 1:10 dilutions of extracted DNA samples were placed in an Applied Biosystems GeneAmp PCR Instrument machine set to 34 cycles at 58°C. PCR inhibitors present in some samples could not be removed by column filtration or the use of Bovine Serum Albumen. When samples could not be successfully amplified, a new sample representative of the same ecotype was extracted and amplified. Upon completion subsamples were visualised using gel electrophoresis and successful amplifications underwent a sequencing reaction prior to being sent to the Australian Genomic Research Facility (AGRF) for sequencing. Sequences were aligned and haplotypes identified using Sequencher v.4.8 (Gene Codes Corporation).

## Statistical analysis

Archival Fastloc GPS data recorded from adult female foraging trips were downloaded and processed using Sirtrack Ltd proprietary software. The resultant location data were plotted using MapInfo Professional 8.5.

Stable isotope ratio values ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) were graphed using OriginPro 8 and all statistical analysis conducted using R statistical software (R Development Core Team 2008). Unless otherwise stated, data are presented as mean  $\pm$  Standard Error and results were considered significant at  $p < 0.05$ .

## Stable isotope analysis

Maternal geospatial data were separated into 'offshore' and 'inshore' ecotypes by depth (if archival time and depth recorders were available) or the location of foraging grounds and significant differences in maternal isotope ratios between ecotypes were tested for using students t-tests. A linear regression to determine the strength and significance of maternal transfer of carbon and nitrogen isotopes to offspring was conducted.

Cluster analysis of pup  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  were performed for each site.  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  clusters were examined by Mann-Whitney U test to determine whether they reflected significant differences in stable isotope ratios (and thus foraging ecotype) (Aurioles et al. 2006). A likelihood ratio test for goodness-of-fit was performed to determine inter-colony differences in proportional representation of each ecotype

## 3. RESULTS

### 3.1 Foraging ecotype geospatial data

GPS and dive data on foraging trips were collected for 10 adult female Australian sea lions (Lilliput I.  $n=5$ , Blefuscu I.  $n=5$ ), with a pre-existing geospatial data set being used for The Pages Islands (South Pages I.  $n=4$ ) coupled with additionally collected adult female whiskers ( $n=6$ ). No *a priori* information on foraging ecotype existed with adult females being selected at random for ecotype tracking. This resulted in only three offshore foraging tracks for Blefuscu Island (Figure 2). Mean dive depth of animals foraging inshore was 20.4m ( $\pm 3.8$ m) (Lilliput and Blefuscu I.  $n=5$ ) with female foraging locations seldom more than 10km from the coast. No TDR data was available for offshore-tracked animals. Adult females of similar ecotypes shared similar  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  ratios however Blefuscu inshore  $\delta^{13}\text{C}$  was significantly higher than Lilliput I. (Figure 3 and Table 2) (students t-test,  $t=-0.4$ ,  $p=0.04$ ).  $\delta^{15}\text{N}$  was 2.8‰ higher in offshore ecotypes at South Pages (students t-test,  $t=3.93$ ,  $p < 0.02$ )

though  $\delta^{13}\text{C}$  displayed no significant variation with ecotype (Table 2) (students t-test,  $t=-0.44$ ,  $p=0.33$ ).



**Figure 2. Foraging ecotype tracking data for Lilliput (left) and Blefuscu (right) Islands. Only three 'offshore' foraging tracks at Blefuscu I. (turquoise, blue and red tracks).**

**Figure 3. Isotope biplot of whiskers sampled from adult female Australian sea lions tracked with GPS loggers. Note the distinct inverse relationship between  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  that characterises alternate foraging ecotypes most apparent at Blefuscu I. The Pages I. showed no relationship between  $\delta^{13}\text{C}$  and ecotype which may be due to the admixing effects of nearby cold-water upwellings and the narrowness of the continental shelf. Further validation experiments of inshore and offshore ecotype signals are being conducted as part of AMMC 0809/27.**

Location	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
<b>Blefuscu (inshore n=2)</b>	-12.8 ( $\pm 0.51$ )	13.8 ( $\pm 0.35$ )
<b>Blefuscu (offshore n=3)</b>	-15.6 ( $\pm 0.17$ )	17.3 ( $\pm 0.21$ )
<b>Lilliput (inshore n=4)</b>	-15.3 ( $\pm 0.99$ )	14.7 ( $\pm 0.42$ )
<b>South Pages (inshore n=3)</b>	-14.7 ( $\pm 0.16$ )	13.3 ( $\pm 0.56$ )
<b>South Pages (offshore n=7)</b>	-14.7 ( $\pm 1.29$ )	16.6 ( $\pm 0.98$ )

**Table 2. Mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  ratios for animals foraging inshore and offshore calculated after cluster analysis of adult female stable isotope data. Standard errors in parentheses. Blefuscu showed significant differences in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  stable isotope ratios attributable to alternate foraging ecotypes. Alternate ecotype values of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values were the same at each site.**

### 3.2 Maternal transfer of stable isotopes to offspring

Pup values of  $\delta^{13}\text{C}$  were not significantly different to maternal levels (students t-test,  $t=0.04$ ,  $d.f=16$ ,  $p=0.48$ ), however  $\delta^{15}\text{N}$  was significantly higher in pups (1.5‰, students t-test,  $t=-2.35$ ,  $d.f=16$ ,  $p<0.02$ ). Maternal  $\delta^{15}\text{N}$  was significant in explaining 47% of the variance in pup  $\delta^{15}\text{N}$  ( $r^2=0.47$ ,  $F_{[1,9]}=7.14$ ,  $p<0.05$ ).

### 3.3 Foraging ecotypes of colonies

Cluster analysis of adult female whisker isotope ratios revealed significant groupings of high  $\delta^{13}\text{C}$  / low  $\delta^{15}\text{N}$  and low  $\delta^{13}\text{C}$  / high  $\delta^{15}\text{N}$  samples characterised as inshore and offshore foraging ecotypes respectively by geospatial data (students t-test,  $t>8.5$ ,  $p<0.01$  for both isotope means) and a significant grouping at South Pages for  $\delta^{15}\text{N}$  (students t-test,  $t=5.6$ ,  $p<0.01$ ). Stable isotope analyses of whisker samples collected from pups exhibited a bimodal distribution of  $\delta^{15}\text{N}$  within colonies at the Pages Islands and between colonies at the Nuyts Archipelago. North and South Pages Islands  $\delta^{13}\text{C}$  were normally distributed, with North Pages showing a small though significant enrichment (-15.9‰ and -16‰ respectively) (students t test,  $t=1.8$ ,  $p<0.05$ ). When data were clustered by  $\delta^{15}\text{N}$  there was highly significant within-colony segregation (Mann-Whitney  $U=4950$ ,  $p<0.001$ ) with median inshore / offshore  $\delta^{15}\text{N}$  values of 13.6‰ / 15.3‰ (South Pages) and 14.1‰ / 16.3‰ (North Pages) respectively. Each ecotype was equally represented at each colony (North Pages= 39% onshore, 61% offshore; South Pages = 46% onshore, 54% offshore) ( $G = -0.08$ ,  $p>0.05$ ).

When stable isotope data from Lilliput and Blefuscu I. were clustered there were significant groupings of 'offshore' (high  $\delta^{15}\text{N}$  / low  $\delta^{13}\text{C}$ ) and 'inshore' (low  $\delta^{15}\text{N}$  / high  $\delta^{13}\text{C}$ ) ecotypes identified using adult female data (Table 2) (Mann-Whitney  $U>1128$ ,  $p<0.001$  in all cases). Cluster analysis highlighted a third intermediate ecotype at Lilliput (20%) and Blefuscu (6%). The offshore ecotype predominated at Blefuscu I. (69%) whilst inshore ecotypes were more prevalent at Lilliput I. (53%)( $G= 65.1$ ,  $d.f=2$ ,  $p<0.001$ ). Inshore and offshore foraging ecotypes differed significantly by 1.8‰ and 0.88‰ in mean  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  respectively ( $\delta^{15}\text{N}$  students t-test,  $t=4.39$ ,  $p<0.001$ ;  $\delta^{13}\text{C}$  students t-test,  $t=2.51$ ,  $p<0.05$ ).

### 3.4 Foraging ecotypes and genetic substructuring

The presence of tandem repeats within the control region prevented a single-reaction sequencing of the entire control region, therefore a 540bp fragment (5'-3' reaction) was isolated and sequenced from 40 samples representing offshore and inshore foraging ecotypes at The Pages Islands (identified by  $\delta^{15}\text{N}$ ) and at Lilliput and Blefuscu Islands



(identified by  $\delta^{13}\text{C}$ ). Lilliput and Blefuscu Islands displayed no matrilineal association with foraging ecotype as both islands were characterised by a single haplotype. Two distinct mitochondrial haplotypes were identified at North Pages however intra-colony segregation did not appear linked to foraging ecotype.

**Figure 4. Isotope biplots of pup whiskers analysed for  $^{13}\text{C}$  and  $^{15}\text{N}$  stable isotopes. Alternate foraging ecotypes are clearly visible at Lilliput and Blefuscu I. (top), with low  $\delta^{15}\text{N}$  and high  $\delta^{13}\text{C}$  denoting inshore feeding. Ecotype segregation is visible using  $\delta^{15}\text{N}$  at The Pages I. only (bottom) – the lack of distinct  $\delta^{13}\text{C}$  signature may be due to the proximity of cold-water upwellings and the continental shelf promoting admixture of inshore and offshore  $^{13}\text{C}$ .**

## 4. DISCUSSION

### 4.2 Foraging ecotype validation

A lack of *a priori* data on individual foraging ecotypes prevented the targeting of animals known to forage either offshore or inshore resulting in only three offshore-foraging animals being tracked. Data from a previous study conducted at South Pages (Goldsworthy et al. 2007a) identified offshore foraging animals, and SIA of those whiskers demonstrated a significant and expected difference in  $\delta^{15}\text{N}$  signatures between inshore- and offshore-foraging animals equivalent to one trophic level (Crawford et al. 2008). Inshore-foraging animals at the Nuyts Archipelago forage at the same trophic level as an ecotype identified at the Pages, suggesting that the median values of  $\delta^{15}\text{N}$  reflect animals from an inshore foraging ecotype at both study sites. The use of  $\delta^{13}\text{C}$  to differentiate ecotypes at the Pages is impaired possibly due to the relative narrowness of the continental shelf and presence of annual austral summer cold-water upwelling events in close proximity to the colonies promoting mixing of inshore and mid-shelf/offshore waters and reducing  $^{13}\text{C}$  differentiation (Clementz & Koch 2001, Hill et al. 2006, Crawford et al. 2008). The significantly higher inshore ecotype  $\delta^{13}\text{C}$  observed in two inshore foragers at Blefuscu I. may be due to the small sample size, the nature of the foraging behaviour exhibited (consistent foraging in seagrass beds just below the low-water mark may be the most extreme form of inshore foraging) or a combination of both. In light of a more complex subpopulation structure than suggested by prior study (Campbell et al. 2008), and now that accurate information on foraging ecotype is available, it will be possible to target offshore-foraging adult females to track in order to further validate the offshore foraging isotope signature. Further validation of foraging ecotype stable isotope ratios is also being performed throughout the range of Australian sea lions in South Australia as part of AMMC Project 27.

### 4.3 Mother-pup isotope transfer

To accurately assess the alternate utilisation of foraging space by adult female Australian sea lions within and between colonies it is necessary to quantify the magnitude of isotopic fractionation between mother and offspring. The results of this study indicate a 1.5‰ increase in  $^{15}\text{N}$  from mother to pup which is similar to those reported in Stellar sea lions (Hobson & Sease 1998), polar bears (Polischuk et al. 2001) and is in line with the interspecific average of other arctoid carnivores such as hibernating bears (Jenkins *et al.* 2001), yet below the expected average recorded for a trophic level (2-5‰)(Crawford *et al.* 2008). One possible explanation is an ontogenetic decrease in  $\delta^{15}\text{N}$  attributed to offspring independently supplementing their own diet as they move towards weaning (Jenkins *et al.* 2001, Aurioles *et al.* 2006). The weaning process in Australian sea lion pups is poorly

understood and is assumed to occur just before the mother gives birth after 17.6 months (Higgins & Gass 1993). Pups have been recorded swimming over 20 km at 88 days of age (Lowther 2006) and the exact date of pup birth was unknown in the current study. With no data in respect to foraging ontogeny it is possible some temporal variation in offspring age (and subsequent differences in reliance on maternal milk) may have diluted the mean  $^{15}\text{N}$  signature below one trophic level. Sampling was only performed on the smallest pups, and the distal portion of each pup whisker was used to reduce this effect. It is also possible that the distal portions of whiskers from pups used were formed *in utero*, and therefore would exhibit the maternal  $^{15}\text{N}$  signature. Without knowing the growth rates of adult and pup whiskers and having a small sample size, it is also possible that temporal decoupling of mother-offspring isotope signatures may also have contributed to reducing the strength of the relationship (Hobson et al. 2000). As part of AMMC Project 0809/27 we intend to quantify pup whisker growth rates so that *ex-utero* whisker segments will be used to characterise fractionation. Other studies suggest that prolonged fasting between feeding bouts in northern fur seal (6-10 days) (Kurle & Worthy 2002) and Wilson's storm petrel (4 days) (Quillfeldt *et al.* 2005) offspring may elevate  $^{15}\text{N}$  ratios by up to 2‰ potentially rendering the increase of 1.5‰  $^{15}\text{N}$  in the current study non-significant. We argue that nutritional stress (observed in other species) would not be significant to the results obtained here due to the comparatively short fasting periods endured by Australian sea lion pups (2-3 days) (Higgins & Gass 1993).

The lack of change in  $^{13}\text{C}$  from mother to pup has been recorded in several other otariid species including the northern fur seal and Californian sea lion (Newsome & Koch 2006). Otariid milk is rich in  $^{13}\text{C}$ -depleted lipids and pups will preferentially incorporate these lipids into their own carbon pathways (De Niro & Epstein 1978, Aurioles et al. 2006, Newsome & Koch 2006), and as such a non-significant difference in  $\delta^{13}\text{C}$  between mother and pup in this study was expected.

To more accurately characterise mother-pup isotope transfer values we will be collecting blood plasma (which has a more rapid isotope turnover rate) (Tierney *et al.* 2008) from mothers and pups simultaneously at a range of sites as part of AMMC Project 27.

#### **4.4 Foraging ecotypes of colonies**

Prior to this study it was necessary to deploy Platform Terminal Transmitters (PTT's) on a small subset of adult female Australian sea lions in a colony (often  $n < 10$ ) for up to one month. At colonies where there may be several hundred adult females this method will not be statistically powerful enough to accurately reflect the mode of foraging of the entire colony. The results described here suggest a more complex level of subpopulation structure

than previously described by tracking studies at the Nuyts Archipelago (Goldsworthy et al. unpublished data). SIA of pup whiskers and maternally-sampled tissues has been used to successfully infer use of foraging habitat in northern elephant seals (Aurioles *et al.* 2006), harp and grey seals (Lesage et al. 2001), harbor seals (Smith *et al.* 1996), short-tailed shearwaters (Baduini *et al.* 2006) and free-ranging manatees (Reich & Worthy 2006). The current study has successfully demonstrated the use of stable isotope analysis of whiskers from milk-dependent offspring provides a cheaper and more comprehensive means of weighting the representation of foraging ecotypes within and between Australian sea lion breeding colonies. The detection of intermediate isotope signals similar to that seen in the Nuyts Archipelago has been reported in many species of fish and marine mammals and appears to represent opportunistic and complex feeding behaviours involving prey at both high and low trophic levels (Davenport & Bax 2002). Australian sea lions commence diving as soon as they depart the colony (Costa & Gales 2002) and it is possible that adult females opportunistically feed on prey items at both ends of the food web resulting in the average isotope ratio described here. Although  $\delta^{13}\text{C}$  was not sufficiently varied to delineate foraging area at The Pages, alternate foraging ecotypes are still clearly discernable using  $\delta^{15}\text{N}$  as a surrogate however further validation is being conducted as part of AMMC Project 27.

#### **4.5 Foraging ecotypes and genetic population substructuring**

Tandem repeated regions in the *control region* has prevented us from achieving the fine-scale examination of population sub-structuring and potential relationship with foraging ecotypes through the use of the entire 1.2kb control region within the time allotted. However, we have successfully amplified and sequenced the 5' half of the control region from animals whose foraging ecotype was inferred from our SIA.

The presence of PCR inhibitors in some hair samples also significantly hampered our analysis. The failure of standard techniques (addition of BSA or column filtration) to remove inhibitors in hair such as melanin and eumelanin has required dilution titration trials and the use of different thermostable DNA polymerases which may be more robust in the presence of inhibitors. This has limited the number of sequences that we were able to obtain.

Present analyses suggests limited alignment of mtDNA haplotype with foraging ecotype, however, the genetic variation determined from the present sequence data limits interpretation at this stage. However, the identification of tandem repeats within the control region may provide an opportunity to develop mtDNA markers similar to microsatellites that will greatly increase the resolution of maternal population substructuring.

Given the low degree of genetic variation within Australian sea lion populations and the complex nature of subpopulation subdivision along foraging ecotypes shown here, an application to the AMMC has been made to conduct further fine-scale molecular genetic study across the range of the species upon completion of AMMC Project 27. The presence of tandem repeats within the *control region* presents an opportunity to develop higher resolution mitochondrial markers, as tandem repeats tend to show higher levels of variability in comparison with nucleotide substitutions (Curtis *et al.* 2001; Godbout *et al.* 2005). Development of data from the *control region* tandem repeats may provide a significantly finer-scale molecular marker to identify population sub-structuring than used previously on Australian sea lions (e.g. Campbell *et al.* 2008).

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