Diet of the Australian sea lion (*Neophoca cinerea*): an assessment of novel DNA-based and contemporary methods to determine prey consumption

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THESIS ABSTRACT

A fundamental prerequisite in the conservation and management of endangered species is knowledge of diet, because diet provides information on habitat use and resource requirements. However, understanding diet in marine mammals is difficult because direct feeding events are rarely observed. To overcome these limitations, many studies use the identification of skeletal remains (hard parts) recovered from faeces, or regurgitates. Yet, for the endangered Australian sea lion (*Neophoca cinerea*) (ASL), one of the rarest pinniped species in the world, diet remains a key knowledge gap that impedes our understanding of the species ecology and connectedness to other taxa in the marine ecosystem.

When this thesis commenced, knowledge of ASL diet was based on few hard part studies comprising small sample sizes, which were limited in temporal and spatial extent. Knowledge of prey utilised by ASL was poor because prey hard parts are completely digested, or, if recovered in faeces, heavily eroded. Therefore, traditional methods of dietary analysis are 'unreliable' and biased toward robust prey. However, limitations notwithstanding, the analysis of Australian sea lion diet via traditional methods still provides useful information on prey species consumed that cannot be readily obtained using other methods. For example, alternative biochemical methods, such as fatty acid and stable isotope analyses, have provided important insights into habitat use the broader trophic levels of prey consumed by ASL; however, they are yet to provide reliable taxonomic information on the diversity of prey species consumed, at least not without first having a thorough understanding of Australian sea lion prey.

Given the paucity of information on ASL diet, I initially aimed, as presented in Chapter 2, to investigate the diet of the ASL at different breeding colonies in South Australia. This initial study provided insights into some of the prey taxa consumed by ASL, which were

subsequently used to develop a range of DNA-based dietary analyses to determine consumption of different prey.

In order to apply DNA-based dietary analysis methods to wild populations, it was important to assess the application of different methods in a controlled environment to understand methodological constraints and refine the methods. In Chapter 3, I present feeding trials on captive ASL, with the aim to: i) assess end-point PCR and quantitative real-time PCR (qPCR) DNA-based techniques to determine their suitability to amplify and detect prey in ASL faeces and, ii) compare the DNA diet results with prey detected and identified using traditional hard-part methodology.

Having successfully applied faecal DNA-based methods in a controlled feeding experiment to identify different prey, I applied DNA-based methods to faecal samples collected from two ASL breeding colonies in South Australia and identified a range of prey. The aims of Chapter 4 were to: (i) determine the diversity of prey taxa by sequencing a large number of clones from a few individuals, (ii) compare the prey taxa recovered at two study sites, and (iii) determine whether pooling faecal DNA from multiple individuals provides a useful means to characterise diet at the colony/population level.

Finally, Chapter 5 utilised and extended the information gained from using the DNA-based faecal analyses presented in previous chapters, by integrating next-generation sequencing (NGS). Next-generation sequencing has the capacity to provide a greater depth of DNA sequencing than the cloning-sequencing approach, with the method potentially improving prey diversity information for the ASL. The aim of this study was to use DNA-based faecal analysis and NGS technology at one breeding colony to investigate seasonal and annual variation in prey consumed by ASL.

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"The very basic core of a man's living spirit is his passion for adventure. The joy of life comes from our encounters with new experiences, and there is no greater joy than having an endlessly changing horizon". McCandless

There are few opportunities as an ecologist that you get to spend time exploring the unknown. This has been particularly true for this project on the Australian sea lion (*Neophoca cinerea*); few have visited the island colonies and even fewer have had the opportunity to co-exist with the inhabitants. My PhD presented an opportune moment akin to historical exploration; though modern equipment has engaged a more comfortable existence in times of adversity, there is a certain allure that draws one back to these rugged, windswept, and captivating islands. Working on a project to assist the future conservation management of an endemic and endangered species has also been a humbling experience. The behavioural characteristics among individuals at different colonies presented many challenging moments. Some could be regarded as "lover's sunsets", others not so passionate endeavours to a point of "you take my DNA and I'll take some of yours". Battle scars aside, Australian sea lions exhibited an unusual tenacious resilient spirit, which deserves full respect and support through future conservation programs.

Many people need to be thanked for the folds of this PhD. First, I would like to thank my PhD supervisors Prof Simon Goldsworthy and Dr Kathy Ophelkeller. Simon, we have spent many years together, catching and tracking sea lions and had some of the most memorable adventures and encounters. Thank you for your ongoing support and guidance as program leader of Threatened, Protected, and Endangered Species (TEPS) at South Australian Research and Development Institute (SARDI). Ultimately, your passion of Australian sea lions and their conservation is the key reason I have been able to experience

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To my family. I am indebted for all your gracious support during my PhD. You have always told me anything is possible and that nothing is impossible. Mum, I have been away for some time now in pursuit of this dream- now I might need some dinner. Dad, thank you for taking time out of to assist with fieldwork. Your dream of oceanography, world mariners and marine ecology has finally come together.

Most importantly, to my wonderful partner, Jamie Hicks. Words cannot describe the level of encouragement and support you have provided over the years. I am blessed you see the world through the same aqua marine, and that Australian sea lions have captured your imagination. I am truly fortunate to have such a special person. This is a part of you as much as it is a part of me.

LIST OF PUBLICATIONS

Chapter 2

Peters, K. J., McIntosh, R. R., Shaughnessy, P. D., Baylis, A. M. M. and Goldsworthy S. D. Diet diversity and estimates of prey size of the endangered Australian sea lion (*Neophoca cinerea*) in South Australia. *In review.*

Chapter 3

Peters, K. J., Ophelkeller, K., Bott, N. J., Herdina, H., and S. D. Goldsworthy. (2014). PCR-based techniques to determine diet of the endangered Australian sea lion (*Neophoca cinerea*): a comparison with morphological analysis. *Marine Ecology,* **36** (4), 1428 – 1439. doi: 10.1111/maec.12242

Chapter 4

Peters, K. J., Ophelkeller, K., Bott, N. J., Deagle, B. E., Jarman, S. J., and S. D. Goldsworthy (2014). Fine-scale diet of the Australian sea lion (*Neophoca cinerea*) using DNA-based analysis of faeces. *Marine Ecology,* **36** (3), 1–21. doi: 10.1111/maec.12145.

Chapter 5

Peters, K. J., Ophelkeller, K., Bott, N. J., Deagle, B. E., Jarman, S. J., and S. D. Goldsworthy. Insights into seasonal prey use by the Australian sea lion (*Neophoca cinerea*) using faecal DNA and high-throughput sequencing. *In review.*

Additional publication containing results from thesis

Goldsworthy, S. D., Page, B., Rogers, P. J., Bulmand, C., Wiebkin, A., McLeay, L. J., Einoder, L., Baylis, A. M. M., Braley, M., Caines, R., Dalye, K., Huveneers, C., Peters, K., Lowther, A. D., Ward, T. M. (2013). Trophodynamics of the eastern Great Australian Bight ecosystem: Ecological change associated with the growth of Australia's largest fishery. *Ecological Modelling*, **255**, 38 –57.

CHAPTER 1

General Introduction

INTRODUCTION

For animals threatened with extinction, understanding the processes that influence the distribution and resource use of individuals is important, because it improves the efficacy of conservation efforts and projections of population change (Caughley and Gunn, 1996). Understanding diet is particularly important for threatened and endangered species, because changes in ecosystems can affect the availability of preferred prey and viability of small populations. The diets of many marine mammals, information that is more readily available for terrestrial animals, remains poorly understood, because their foraging typically occurs underwater and a long way from land (e.g. Fristrup and Harbison, 2002; Cherel *et al.* 2009, Tollit *et al.* 2010; Bowen and Iverson, 2013).

Sea lions (Pinnipedia: Otariidae) are of particular conservation concern because five of the six extant species have not yet recovered from population reductions caused by 18th and 19th century sealing (e.g. Gerber *et al.* 2001; Hoffman *et al.* 2015). The Californian sea lion (*Zalophus californianus*) is the only species that has recovered (Caretta *et al.* 2015). The Australian (*Neophoca cinerea*), Galapagos (*Zalophus wollebaeki*), Steller (*Eumetopias jubatus*), South American (*Otaria flavescens*), and New Zealand sea lion (*Phocarctos hookeri*) are classified as either *endangered* species or species that are *Vulnerable to depletion* (International Union for Conservation of Nature (IUCN, 2008-2012), and diet largely remains poorly understood.

Causes of recent sea lion population declines include fisheries-based mortality and disease (Goldsworthy *et al.* 2003; Page *et al.* 2004; Wilkinson *et al.* 2006; Castinel *et al.* 2007; Chilvers, 2008; Kovacs *et al.* 2012), predation (Springer *et al.* 2003), and the availability and quality of prey (Trites and Donelly, 2003; Estes *et al.* 2009; Robertson and Chilvers, 2011). For example, during El Niño years, unseasonal sea surface warming is associated with

nutritional stress of Galapagos and Californian sea lions and population declines (Trillmich, 1985; Trillmich and Dellinger, 1991; Alava and Salazar, 2006; Shirasago-German *et al.* 2015). The western stock of the Steller sea lion has experienced population declines over the past 30 years, with one of the causes suggested to be the substitution of energy dense prey with poor quality prey (Schaufler *et al.* 2006; Trites *et al.* 2007). Competition with fisheries for prey may have contributed to the decline of New Zealand sea lions (Robertson and Chilvers, 2011). Many of these studies indicate improved understanding of the diets of sea lions would inform the development of conservation policies, and potentially improve the probability that their populations will recover.

The Australian sea lion (*Neophoca cinerea*) (ASL) is classified an *endangered* species of high conservation priority by IUCN (Goldsworthy, 2015). The population, estimated at ~12,000 individuals comprising 78 fragmented breeding colonies, extends from the subtropical Houtman Abrolhos in Western Australia (WA) (28º 43’ S, 113º 47’ E) to temperate Pages Islands in South Australia (SA) (35º 45’ S, 138º 18’ E) (Ling, 1992; Shaughnessy *et al.* 2011; Goldsworthy *et al.* 2015) (Figure 1). The breeding biology of ASL is unusual among pinnipeds in that females have a non-annual reproductive cycle of 15−18 months, and breeding is temporally asynchronous among colonies (Higgins, 1993; Higgins and Gass, 1993; Gales *et al.* 1994). High natal site fidelity and limited dispersal of female ASL increases the risk that small subpopulations can be threatened with extinction as a result of either natural or anthropogenic pressures (Goldsworthy and Page, 2007; Campbell *et al.* 2008; Hamer *et al.* 2013; Goldsworthy *et al.* 2015; Ahonen *et al.* 2016).

Fishery by-catch of ASL off South Australia has slowed the recovery of ASL populations (Goldsworthy and Page, 2007; Goldsworthy *et al.* 2010; Hamer *et al.* 2013). Since 2010, management actions have attempted to reduce the impact of fishery-bycatch on ASL. This

has included expanding area closures supplemented with by-catch trigger limits based on ASL population sizes where operational interactions occur, and the implementation of different fishing gear (i.e. long lines/hooks) as a replacement for bottom set nets. These measures have effectively reduced by-catch of ASL, but ASL populations still are in decline. The recent population estimate of 42 South Australian breeding colonies indicated ASL populations have declined 24% between 2007 and 2015, with an average statewide decline of 2.9 % per year (Goldsworthy *et al.* 2015).

Most research on the foraging ecology of ASL has occurred in South Australia where 86% of the population resides (Shaughnessy *et al.* 2011). Tracking of ASL and dive profiles indicate individuals are benthic foragers and exhibit a high degree of fidelity to foraging locations. In South Australia, ASL forage across a range of habitats in the coastal and continental shelf waters (typically < 200 m water depth) of the Great Australian Bight and the adjacent Spencer Gulf and Gulf St Vincent (Figure 1) (Goldsworthy *et al.* 2010, 2014). Trophic-level diet studies using stable isotopes and fatty acids suggest ASL are individual foraging specialists, with long-term fidelity to either inshore areas (5 to 20m) or offshore areas (about 70 m) and repeatly target the same trophic-level of prey (Costa and Gales, 2003; Baylis *et al.* 2009; Fowler *et al.* 2006, 2007; Goldsworthy *et al.* 2009a, b; Lowther *et al.* 2011, 2012). These studies have advanced our understanding of the at-sea movements and dive behaviour of ASL, but comprehensive knowledge of the prey used by ASL is typically regarded as poor (Goldsworthy *et al.* 2009).

Figure 1. The distribution of ASL breeding colonies in Western Australia ▲ (A, B) and South Australia ○ (C-F).

To study the diet of pinnipeds, researchers have largely relied on methods by which they recover and identify the remains of prey in faeces and regurgitates. Hard parts that are relatively resistant to digestion such as cephalopod beaks, fish otoliths, and vertebrae are often utilised to identify prey, although instances of soft tissue identification have complemented such analyses (Gales and Pemberton, 1994; Tollit *et al.* 2006; Casper *et al.* 2007a; Mèheust *et al.* 2015). Hard part analyses have well-documented biases. For example, prey hard-parts are subject to differential and species-specific erosion during digestion and retention of remains may occur in the stomach (e.g. Pitcher, 1981; Murie and Lavigne, 1986; Bowen 2000; Staniland, 2002; Tollit *et al.* 1997, 2007, see Bowen and Iverson, 2013 for review). In addition, intraspecific differences in transit times of prey hard parts can confound recovery rates biasing diversity estimates of prey consumed (e.g. Tollit *et al.* 2007; Casper *et al.* 2007b). Despite these limitations, analyses of prey hard parts are thought to provide reasonable estimates of pinniped prey, as well as estimates of prey mass and size (e.g. Hyslop, 1980; Tollit *et al.* 1997; Bowen, 2000; Iverson *et al.* 2004; Page *et al.* 2005).

The diet of ASL has been studied using hard part analyses of faeces and regurgitates, the stomach remains from dead individuals, and direct observations of predation from animal-borne cameras (Marlow, 1975; Ling, 1992; Richardson and Gales, 1987; Gales and Cheal, 1992; McIntosh *et al.* 2006; Gibbs, 2008, Fragnito, 2013) (Table 1). Among early studies, Marlow (1975) identified beaks of squid in the stomachs of deceased ASL, and Ling (1992) reported benthic and demersal teleost fish including Australian salmon (*Arripis trutta*), whiting (Sillaginodes), bottom dwelling triakid shark and squid. Gales and Cheal (1992) assessed diet of ASL using a small number of faeces and stomach remains collected across ASLs breeding range. Although their collections were sporadic and small, prey comprised the eroded remains of benthic dwelling teleost fish, octopus, squid, and shark (Table 1). McIntosh *et al.* and Gibbs (2008) conducted independent diet analyses using regurgitate and

stomach remains from deceased ASL in South Australia. They noted prey items were highly digested or eroded, and that diet comprised largely beaks from octopus, giant cuttlefish (*Sepia apama*), and ommmastrephid squid, with few teleost fish and eggs of oviparous sharks (Table 1). One novel approach used animal-borne cameras and tracking equipment to study prey consumed by female ASL. Fragnito (2013) found different individuals adopted different foraging strategies in seagrass, sand and reef habitats to acquire a range of teleost fish, gastropods, crustaceans and rays (Table 1).

These studies indicate that ASL utilise a range of habitats and consume benthic and demersal fish, cephalopods, crustaceans, molluscs, and cartilaginous prey (Table 1). They have all noted that estimates of ASL diet are likely to underestimate the diversity of prey because collections have been limited to a small number of samples or individuals, or individuals that are deceased, which subsequently may not be representative of the diet of healthy individuals (e.g. Pierce *et al.* 2004). Studies based on the recovery and identification of prey remains have also concluded that few hard parts are present in ASL faeces and that the prey recovered from ASL stomachs are biased toward cephalopods (Richardson and Gales, 1987; Gales and Cheal, 1992; McIntosh *et al.* 2006; Gibbs, 2008; Chapter 2, Chapter 3). For example, Gales and Cheal (1992) found less than 2% of fish otoliths were recovered from faeces of two captive fed ASL and variable recovery of cephalopod beaks (9% and 98%) from a trial diet comprising cephalopod prey.

Table 1. ASL prey from colonies in South Australia (SA) and Western Australia (WA). Colony names and areas are: Seal Bay (SB) (Kangaroo Island), Yorke Peninsula (YP), Lewis Island (LE) (Eyre Peninsula), Dangerous Reef (DR) (Spencer Gulf), Lilliput Island (LI) (N Nuyts Archipelago) (see Fig. 1). Sample types are regurgitate (R), stomachs from dead ASL (S), faeces (F) and videos attached to ASL (V).

Prey type	Genus or species (if known)	Number of records	Sample location (colony)	Geographic region	Type of sample	Size and biomass estimates?	Author/s
Cephalopoda							
Gould's squid	*Nototodarus gouldi*	1	SB	SA	RS	Y	McIntosh *et al.* (2006)
Ommastrephid squid (other)		1	SB	SA	R	Y	McIntosh *et al.* (2006)
Giant cuttlefish	*Sepia apama*	2	SB	SA	RSF	Y	McIntosh *et al.* (2006), Gales and Cheal (1992)
Cuttlefish	*Sepia* spp.	3	SB, UK[1], YP	SA	RS	Y	McIntosh *et al.* (2006), Gales and Cheal (1992), Gibbs (2008)
Calamari squid	*Sepioteuthis australis*	3	SB, UK[1], YP	SA	RS	Y	McIntosh *et al.* (2006), Gales and Cheal (1992), Gibbs (2008)
unknown squid		1	WA[2]	WA	S	N	Richardson and Gales (1987), Gales and Cheal (1992)
Octopus	*Octopus* spp.	4	SB, UK[1], YP, DR	SA	RSFV	Y	McIntosh *et al.* (2006), Gales and Cheal (1992), Gibbs (2008), Fragnito (2013)
Birds							
Little penguin	*Eudyptula minor*	1	SB	SA	R	N	McIntosh *et al.* (2006)
Fish							
Leatherjacket	*Monocanthidae* spp.	2	SB, DR	SA	RS	N	McIntosh *et al.* (2006), Fragnito (2013)
Flathead	*Neoplatycephalus* spp.	1	SB	SA	R	N	McIntosh *et al.* (2006)
Swallowtail	*Centroberyx lineatus*	1	SB	SA	R	N	McIntosh *et al.* (2006)
Common bullseye	*Pempheris multiradiata*	1	SB	SA	R	N	McIntosh *et al.* (2006)
Eastern school whiting	*Sillago flindersi*	1	SB	SA	R	N	McIntosh *et al.* (2006)
Yellowtail mackeral	*Trachurus novaezelandiae*	1	SB	SA	R	N	McIntosh *et al.* (2006)
Rock Ling	*Genypterus tigerinus*	1	LE	SA	V	N	Fragnito (2013)
Estuary Cobbler	*Cnidoglanis macrocephalus*	1	LE	SA	V	N	Fragnito (2013)
Western fox fish	*Bodianus frenchii*	1	LE	SA	V	N	Fragnito (2013)
Red velvetfish	*Gnathanacanthus goetzeei*	1	LE	SA	V	N	Fragnito (2013)
Tommy ruff	*Arripis georgianus*	1	LE	SA	V	N	Fragnito (2013)
Puffer fish	Tetraodontidae	2	SB, DR	SA	SV	Y	Gales and Cheal (1992), Fragnito (2014)
Crustacea							
Southern rock lobster	*Jasus edwardsii*	1	SB	SA	RS	N	McIntosh *et al.* (2006)
Western rock lobster	*Panulirus cygnus*	2	WA[2]	SA	SF	N	Richardson and Gales (1987), Gales and Cheal (1992)
Sand crab		1	SB	SA	RS	N	McIntosh *et al.* (2006)
Stone crab		1	DR	SA			
Unknown crab[3] (small)		1	UK[1]	SA	F	N	Gales and Cheal (1992)
Elasmobranch					V	N	Fragnito (2013)
Oviparous shark egg		1	SB,	SA	R	N	McIntosh *et al.* (2006)
Catshark shark egg[3]	(Scyliorhinidae)	1	SB, UK[1]	SA	RF	N	Gales and Cheal (1992), McIntosh *et al.* (2006)
Port Jackson shark	*Heterodontus portusjacksoni*	2	UK[1]		F	Y	Gales and Cheal (1992)
Mollusca							
Greenlip abalone	*Haliotus laevigata*	1	LI	SA	V	N	Fragnito (2013)
Amphipoda							
unknown amphipod[3]		1	UK[1]		F	N	Gales and Cheal (1992)
unknown shrimp[3]		1	UK[1]		F	N	Gales and Cheal (1992)

[1]*Location not specified and unknown within regional (geographic) context,* [2]*Broad regional context only,* [3]*Possible secondary ingestion*

To overcome limitations associated with the analyses of prey hard parts several studies have used biochemical methods including fatty acid signature analyses and stable isotope analyses to assess the trophic-level of prey used by marine predators (Lea *et al.* 2002; Bradshaw *et al.* 2003; Hückstädt *et al.* 2012; Bowen and Iverson, 2013). Because prey fatty acids accumulate in the tissues of predators over time (e.g blubber), they are thought to provide information on prey consumed over several months, rather than days (e.g. Tollit *et al.* 2006). For marine predators, this advantage has enabled fatty acids to be applied to understand how different age/sex groups or species use different prey. For example, fatty acids have been used to define demographic and ontogenetic prey use by pinniped and seabird predators, whose cryptic and wide ranging foraging behaviour makes their diet difficult to study (Baylis *et al.* 2009; Meynier *et al.* 2008).

Diet studies based on fatty acids have several limitations (for review see Bowen and Iverson, 2013). For example, the identification of prey is dependent on prey species having different fatty acid signatures, which is not always the case, particularly in closely related taxa. Fatty acid profile libraries of prey species are also required, and these can vary between locations. Deposition, mobilisation and selective uptake of prey fatty acids are also known to differ among long and short chain fatty acid groups (Iverson *et al.* 2004, 2007; Nordstrom *et al.* 2008). Experiments on captive-fed predators indicate that the resolution provided by analyses of fatty acids varies depending on the composition of prey (e.g. Bowen and Iverson, 2013). These biases limit the conclusions that can be drawn from studies that are based on analyses of prey fatty acids.

Stable isotope analyses assume that isotopic signatures in a predator's metabolically active (e.g. blood, serum) and inert tissues (e.g. hair) are derived from equivalent signatures in their prey (DeNiro and Epstein, 1978, 1981, Roth and Hobson, 2000; Kelly *et al.* 2012). Stable

isotope ratios of $\delta^{15}N$ and $\delta^{13}C$ are typically used to assess the diets of predators (Bowen and Iverson, 2013). Enrichment of nitrogen typically occurs up the food chain, enabling the trophic position of prey consumed to be determined (Crawford *et al.* 2008; Ben-David and Flaherty, 2012). In contrast, $\delta^{13}C$ provides information on primary productivity, facilitating discrimination of the habitat used by primary and tertiary consumers (Post, 2002; Crawford *et al.* 2008; Ehrich *et al.* 2015). In marine systems, $\delta^{13}C$ typically decreases with distance from shore and it differs between benthic and pelagic habitats (Hobson *et al.* 1996; Miller *et al.* 2008). Consequently, $\delta^{13}C$ is often used to differentiate marine predators that use inshore versus offshore foraging areas (e.g. Hobson *et al.* 1996; Aurioles *et al.* 2006; Lowther *et al.* 2011). Stable isotope analyses have also been used to infer individual and population-level changes in the diets of marine predators (e.g. seasonal, annual, prey switching) and to track changes in entire ecosystems (Newsome *et al.* 2007; Hückstädt *et al.* 2012; Scherer *et al.* 2015). Like fatty acids, stable isotopes provide information on prey that have different isotopic signatures, which typically limits studies to trophic-level analyses and conclusions (e.g. Ehrich *et al.* 2015). Mixing models and trophic enrichment factors have been used to refine stable isotope analyses, but these models do not provide information on all prey consumed (Parnell *et al.* 2010; Kelly *et al.* 2012; Phillips, 2012).

DNA-based methods are now widely used to identify prey in the diets of many herbivore and predator species (King *et al.* 2008; Soininen *et al.* 2009; Riemann *et al.* 2010; Willerslev *et al.* 2014). The DNA-based approach assumes that DNA fragments of food survive digestion and can be identified when compared to the DNA from known animal or plant species (Sydmonson, 2002; Herbert *et al.* 2003, 2005; Ward *et al.* 2005, 2008). The polymerase chain reaction (PCR) (Mullis *et al.* 1986, 1987) underpins DNA-based diet studies because it can be used to amplify and produce multiple identical copies of prey DNA even when degraded and/or in very low concentrations (Deagle *et al.* 2006; Kohn and Wayne, 1997). This has

enabled DNA-based analyses to be widely applied to determine predator-prey interactions, because food items can be detected and identified irrespective of whether their morphological counterparts are present (e.g. Casper *et al.* 2007b; Soininen *et al.* 2009; Khanam *et al.* 2016).

One of the earliest studies to apply DNA-based methods determined diet of the European brown bear (*Ursus arctos*) from faeces collected in the Brenta region of Italy (Höss 1992). Using PCR, Höss (1992) amplified the chloroplast *rbc*L gene revealing that bears consumed the Christmas berry, *Photinia villosa,* which is a common summer plant in the region. DNA-based diet studies have since been conducted on many terrestrial and aquatic vertebrates and invertebrates (e.g. Asahida *et al.* 1997; Riemann *et al.* 2010; Willerslev *et al.* 2014). These studies confirm that DNA-based methods can be used to study diets based on analyses of either stomach contents or faeces (Deagle *et al.* 2005a,b; Sydmonson, 2002; Kvitrud *et al.* 2005; Deagle and Tollit, 2007; King *et al.* 2008; Riemann *et al.* 2010).

DNA-based methods have been increasingly used in marine systems to determine both simple and complex diets of marine predators (Tollit *et al.* 2009; Dunn *et al.* 2010; Ford *et al.* 2016). These studies have used species- and group- specific assays to amplify prey DNA, and in most instances, have improved information on the consumption of single taxa or the broad diversity of prey. Like other techniques used to analyse diet, DNA-based methods have biases. These largely stem from the fact that DNA of prey digest at different rates, which can vary the success of prey detection (Deagle and Tollit, 2007). Prey identification from degraded templates such as faeces is therefore highly dependent on the target size of the amplified gene: which the likelihood of success decreases with an increase in product size (Kohn and Wayne, 1997; Deagle *et al.* 2006; Marshall *et al.* 2010).

Despite such biases, DNA-based methods have greatly improved our understanding of the diets of marine predators, particularly where prey hard parts are not present or identifiable

(e.g. Casper *et al.* 2007b). In particular, DNA-based methods have been used to identify the remains of prey in the stomach contents of fish and cephalopods, neither of which typically contain identifiable prey remains (Rosel and Kocher, 2002; Deagle *et al.* 2005b; Braley *et al.* 2009; Dunn *et al.* 2010). Sousa *et al.* (2016) for example, used DNA derived from stomach material to determine the diet of the ocean sunfish, *Mola mola*, which were thought to be largely planktivores. DNA analyses revealed however, a diet comprised of crustacean and fish prey, with low proportions of plankton. That study further revealed diet partitioning between small and large individuals, whom used different coastal and pelagic prey.

Similarly, faecal DNA-based analyses have been applied to determine the diets of penguins, seabirds and pinnipeds. These have revealed a wide range of fish, cephalopod, and crustacean prey (Jarman *et al.* 2004, 2013; Casper *et al.* 2007a; Tollit *et al.* 2009; Deagle *et al.* 2007, 2010; McInnes *et al.* 2016). For example, Jarman *et al.* (2010) assessed the diet of Adelie penguins (*Pygoscelis adeliae*) using DNA recovered from faeces, which, like faeces of other seabirds, typically do not contain the hard parts of of prey (Hartley, 1948; Deagle *et al.* 2007). By amplifying a short section of mitochondrial DNA, the authors revealed a wide diversity of krill, fish, crustacean and jellyfish prey. They concluded Adelie penguins would be useful indicators of community structure of the coastal Southern Ocean. These studies demonstrate the utility of DNA-based methods, which have greatly improved our understanding of trophic interactions in marine ecosystems.

Pinnipeds (Phocidae, Otarridae and Odobenidae) are one of the most common marine predators for which DNA-based diet analyses have been applied. This is because pinnipeds are large consumers of marine prey resources and an accurate understanding of their use of prey assists in understanding how such predators function within marine ecosystems. Furthermore, comprehensive information of diet is an important component to determine the

food and habitat requirements for declining species, which includes the potential to assess competition with fisheries (e.g. Tollit *et al.* 2009). The tendency of pinnipeds to forage from a central place, and come ashore to rest, moult, and breed, enables a large number of samples to be collected. This however is not the case for ice-breeding seals, which subsist on mobile substrates to breed and haulout on ice. Several pinniped diet studies have compared the results of DNA-based to the analyses of hard parts, and shown that DNA-based methods improve prey detection, which can increase the information on frequency and use of different prey. For example, Purcell *et al.* (2000) used restriction fragment length polymorphism analyses (RFLP) to determine predation by Pacific harbour seals (*Phoca vitulina*) on salmonids in the Umpqua River, Oregon. In that study, mitochondrial DNA (mtDNA) from unidentified fish bones recovered from faeces were PCR amplified then identified. The authors found by using DNA, that harbour seals consumed coho, chinook and steelhead salmon, but also non-salmonid fish prey. They concluded that DNA was the only useful method to identify salmon to species, as bones and otoliths were similar in structure or typically digested. Similar studies using faecal DNA analyses have also been applied to other pinnipeds including Antarctic fur seals (*Arctocephalus gazella*) (Casper *et al.* 2007a), Australian fur seals (*Arctocephalus pusillus*) (Deagle *et al.* 2009) and Steller sea lions (*E. jubatus*) (Tollit *et al.* 2009). Casper *et al.* (2007a) in particular, improved dietary information on the frequency and use of squid, myctophid and mackerel ice (*Champsocephalus gunnari*) fish prey of the Antarctic fur seal (*A. gazella*) by combing faecal DNA with hard part analyses at Heard Island. They found, by amplifying nuclear and mtDNA that prey detection rates increased by ~30%, and by ~46% if used in combination with hard parts. They concluded that a combined approach provided better representation of prey consumed by *A. gazella*.

The study by Marshall *et al.* (2010) is a unique example where DNA and hard parts have been used to identify the stomach content of pinnipeds. Although destructive sampling is rarely used, Marshall *et al.* (2010) captured harp seals (*Pagophilus groenlandicus*) and directly sequenced their stomach DNA. They found DNA-based analyses improved information on how predation by harp seals effects recovering stocks of Arctic cod (*Boreogadus saida*) and Capelin (*Mallotus villosus*), and concluded that DNA-based methods present a viable means to improve the detection of different prey.

In this thesis, I investigated the diet of the ASL using traditional analyses of prey hard parts and three DNA-based methods: 1) PCR amplification followed by DNA cloning and sequencing, 2) PCR amplification using quantitative (real-time) PCR (qPCR) and 3) PCR amplification followed by next-generation sequencing (NGS).

The DNA cloning and sequencing method is essentially a barcoding approach that enables the DNA of different species or taxonomic groups of prey (e.g. fish, cephalopods, crustaceans) to be PCR amplified and identified by cloning and sequencing PCR amplicons within a library. The cloning approach has been widely used to assess diet in a range of marine predators including pinnipeds, as it can provide information of prey diversity without previous knowledge of diet (e.g. Deagle *et al.* 2005a; Jarman *et al.* 2004, Dunshea, 2009).

Quantitative PCR (qPCR) has been used in the assessment of pinniped diets both as a direct method of detection and as a quantitative tool to assess the amount of prey DNA recovered in a sample (Deagle *et al.* 2007; Bowles *et al.* 2011; Casper *et al.* 2007a, Matejusovà *et al.* 2008). Because of its sensitivity and efficacy to visualise low concentrations of DNA, the qPCR method can improve the frequency of prey detection particularly in pinniped faeces. For example, Matejusovà *et al.* (2008) detected the DNA of salmon to concentrations as low as 0.01% in spiked grey seal (*Halichoerus grypus*) faeces. This level of accuracy underpins

the qPCR approach, resulting in the technique being applied to faeces of captive fed seals in attempt to estimate the relative proportions of prey ingested (Deagle and Tollit, 2007; Bowles *et al.* 2011; Matejusovà *et al.* 2008). The results of such studies however have indicated differential prey digestion and copy number variability among tissue DNA affect DNA-based biomass estimates. Such biases require numerical correction factors to compensate for differences between tissue ratios of genomic and mitochondrial DNA (Bowles *et al.* 201; Hartmann *et al.* 2011).

Next-generation high throughput DNA sequencing (NGS) has improved the depth of sequencing information produced for dietary studies. Various sequencing platforms can now generate unprecedented amounts of taxonomic information from a range of environmental samples including pinniped faeces (Deagle *et al.* 2009, 2013; Pompanon *et al.* 2013; Quéméré *et al.* 2013). The ability to amplify then simultaneously characterise the broad diversity of prey consumed in mixed-species templates can improve the capacity to elucidate complex food-web interactions. Although early NGS studies were largely qualitative and based on presence/absence models (e.g. Deagle *et al.* 2009), more recent platforms (e.g Illumina, Ion torrent) are now using sequence abundances as a quantitative proxy to estimate prey biomass (Shokralla *et al.* 2012; Deagle *et al.* 2013; Pompanon *et al.* 2013). For ASL, DNA-based NGS technology presents a novel opportunity to address some of the knowledge gaps in diet such as the use of seasonal prey.

Using the range of methods outlined, I aimed to compare different diet methods, and improve information of diet for ASL.

THESIS ORGANISATION AND STRUCTURE

Chapter 2 reports on my analyses of prey hard parts, in an attempt to document baseline information on the diet of ASL. Chapter 3 examines the use of conventional and quantitative

PCR (qPCR) methods to identify the remains of prey using controlled feeding experiments on captive ASL. Chapter 4 uses DNA-based cloning and sequencing methods to examine individual and spatial differences in the diet of wild ASL. Chapter 5 is a pilot study that assesses the utility of next-generation sequencing (NGS) to estimate prey diversity using ASL faeces. Chapters 3 and 4 are published manuscripts. The other data chapters (2 and 5) have been submitted for publication. The citation and co-authorship details for these four chapters are provided at the end of Chapter 1.

All of the chapters are self-contained, except for the introduction (Chapter 1) and general discussion (Chapter 6). Because the focus of the thesis was to apply different DNA techniques to study the diet of ASL, there is repetition in chapters that overlap in content. The figures and tables refer only to the chapter in which they are contained and as a result, their numbers begin at one in each chapter.

Chapter 2 assessed the diet of ASL using prey hard parts recovered from faecal and regurgitate samples collected across the South Australian breeding range of ASL (Figure. 1). This study determined the diversity and biomass of ASL prey and addressed the following questions:

- Can analyses of prey hard parts found in ASL faeces and regurgitates provide adequate taxonomic information on the diet of ASL?
- Based on these analyses, what are the most important prey, and do they differ among seasons or colonies?
- Do ASL use prey that are commercially fished?

The prey taxa identified in this study and previous studies (e.g. Gales and Cheal, 1993; McIntosh *et al.* 2006; Gibbs, 2008) provided the baseline data that were used to develop and test group-specific DNA markers in Chapters 4 and 5.

To validate the use of DNA-based analysis methods for ASL, Chapter 3 explored their application using captive feeding trials. This follows successful amplification and identification of prey using DNA-based methods in the study of diet of captive Steller sea lions (*E. jubatus*), sub-Antarctic fur seals (*Arctocephalus tropicalis*) and New Zealand fur seals (*Arctocephalus forsteri*) (Deagle *et al.* 2006; Casper *et al.* 2007b). I evaluated conventional PCR and quantitative PCR methods and compared their prey detection limits. I also compared DNA-based detection to analyses of prey hard parts recovered from faeces. The feeding trial addressed the following questions:

- Are different prey species detectable in ASL faeces using DNA-based methods?
- Are there differences between PCR techniques (conventional and quantitative) in their prey detection limits? How do the results of these DNA-based methods compare to the results of analyses based on of identification of prey hard parts?
- Do quantitative PCR techniques provide reliable estimates of the amount of prey ingested?

Chapter 4 is the first field-based diet study to apply DNA-based analyses to the faeces of wild ASL following the successful amplification of prey DNA from faeces collected from captive fed ASL. In this study, I developed new and used existing mtDNA markers to PCR amplify prey DNA, and used the cloning and sequencing approach to develop representative prey libraries. The study established new information of diet for two ASL colonies, and identified a range undescribed prey of ASL. Specific questions I aimed to address were:

Can PCR clone libraries be used to improve our understanding of ASL diet?

- Does the diet of ASL differ among individuals from the same colony?
- Does the diet of ASL differ among colonies?
- Will clone libraries represent the full range of prey identified from individual diets if DNA of multiple faecal samples is pooled into a metasample?
- Does pooling DNA improve the efficacy of PCR sequencing methods?

Chapter 5 explores NGS as a novel sequencing method to assess seasonal variation in the diet of ASL. Using NGS technology can enable rapid screening of multiple samples and provide a greater depth of dietary information that would not be logistically affordable using the clone and sequence method (Chapter 4). This study collected faecal samples in different seasons from Seal Bay (Kangaroo Island) over a three-year period. Prey DNA was amplified using previously published PCR markers with PCR amplicons sequenced using the Roche 454 NGS platform. Although newer platforms are now available and provide even greater depth of sequencing (e.g. Illumina), at the time of this study, Roche 454 sequencers were amongst the forefront of NGS technology. Combined with the analyses of prey hard parts, this study aimed to identify some of the seasonal prey used by ASL. Questions this study aimed to address were:

- Does NGS provide comprehensive information of diet for ASL?
- What are important prey?
- How do results from conventional hard part analyses compare with NGS?
- Are there any seasonal patterns to the prey resources consumed by ASL at Seal Bay?

The thesis concludes with a discussion on the effectiveness of DNA-based methods to study the diet of ASL (Chapter 6). It includes a summary of the potential for DNA-based methods

to improve diet studies. This section highlights the implications and examines future directions of DNA-based analyses for research into the diet of ASL.

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CHAPTER 2

Diet of the endangered Australian sea lion (*Neophoca cinerea*) in South Australia.

ABSTRACT

Management measures to assist the conservation of threatened and endangered species typically require knowledge of diet, because diet provides insights into resource and habitat requirements and threats to population persistence. Yet, for the endangered and endemic Australian sea lion (*Neophoca cinerea*) (ASL), diet is little studied and relatively unknown. To redress this fundamental knowledge gap, ASL diet was investigated using the most comprehensive dataset to date and provide information on the prey species, size, and biomass at breeding colonies throughout South Australia (where 86 % of the population breeds). Prey composition for the sites surveyed consisted of five species of cephalopods, ten teleost fish and a penaeid prawn; all benthic-demersal taxa that predominantly inhabit coastal and continental shelf seafloor. Cephalopods were the most frequently encountered and numerically abundant prey, accounting for ~80% of all dietary items identified. Octopodidae and Loliginidae squid were the most common prey and contributed the largest biomass, highlighting their importance as prey. Although fish were poorly represented by hard parts in the diet, most were medium to larger species (~ 200 mm to > 500 mm), and typically benthic dwelling. This study highlights the importance of cephalopods in ASL diet and the potential for competition between ASL and commercial fisheries. Given the continued decline of ASL in South Australia, resource competition with fisheries merits further investigation.

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INTRODUCTION

Harvesting and habitat loss have driven global declines in marine predators, many of which are now listed as threatened or endangered (Myers and Worm 2003; Myers *et al.* 2007; Lotze and Worm 2009). Contemporary conservation measures for threatened and endangered species typically require knowledge of diet, because diet provides insights into resource and habitat requirements that ultimately underpin individual fitness and reproductive success. Accordingly, knowledge of diet provides insights into threats to population persistence, and assists in disentangling the factors influencing population trends (Goodman-Lowe 1998; Matejusová *et al.* 2008; Meynier *et al.* 2009).

The Australian sea lion (*Neophoca cinerea*) (ASL) is an endangered species (IUCN, Goldsworthy, 2015), that is endemic to Australia, with a breeding distribution from the Houtman Abrolhos Islands, Western Australia (28.67° S, 113.82° E) to the Pages Islands (35.77° S, 138.29° E), South Australia (Goldsworthy and Gales, 2008; Campbell *et al.* 2008a; Goldsworthy *et al.* 2015). The population has been estimated at 14,780 individuals (Shaughnessy *et al.* 2011), but recently has been revised to approximately 12,000, making ASL one of the least numerous seal species in the world, and one that has shown limited recovery post 18th and 19th century sealing (Ling 1999; Gales *et al.* 2000; Kirkwood *et al.* 2010; Goldsworthy *et al.* 2015). Several unique life history characteristics have likely contributed to their protracted recovery. These include an extended gestation and lactation period (17.5-month) resulting in a low lifetime fecundity (Ling and Walker 1978; Higgins 1993; Higgins and Gass 1993; Gales *et al.* 1994), and breeding asynchrony with extreme female natal site philopatry, which limits dispersal among colonies (Gales *et al.* 1994; Campbell *et al.* 2008a; Lowther *et al.* 2011). In addition, incidental fisheries by-catch and episodic disease has impeded recovery at a number of breeding sites (Page *et al.* 2004;

Goldsworthy and Page 2007; Campbell *et al.* 2008b; Hamer *et al.* 2013; Goldsworthy *et al.* 2010; Marcus *et al.* 2014, 2015), with recent surveys indicating ASL populations are declining across their range (Goldsworthy *et al.* 2010, 2015). Their extreme life-history constraints, combined with stable or declining populations and fisheries induced mortality, indicates ASL remain at further risk of decline, highlighting the need to improve knowledge of their key conservation requirements.

ASL employs multiple foraging strategies to exploit a variety of habitats in search of predominantly demersal and benthic prey (Fowler *et al.* 2007; Costa and Gales 2003; Fragnito, 2013). In South Australia, ASL forage in continental shelf waters (typically <200 m) of the eastern Great Australian Bight (GAB) and adjacent Gulf St Vincent and Spencer Gulf, with recent studies revealing colony-level fidelity to local habitats and individual foraging specialisation (Goldsworthy *et al.* 2009; Fowler *et al.* 2007; Lowther *et al.* 2011, 2012; Lowther and Goldsworthy 2012). These studies have made considerable advances in understanding the at-sea movements and dive behaviour of ASL, but our understanding of ASL diet remains limited.

Information on the diet of otariid seals is typically derived from the identification of hard prey structures (such as fish otoliths, vertebrae and cephalopod beaks) obtained from faeces and stomach material. As central place foragers, otariids undertake foraging trips at sea, and return to haul-outs and breeding colonies that facilitate the collection of samples, which are typically cost effective to analyse and provide taxonomic prey information (Gales *et al.* 1993; Gales and Pemberton 1994; Tollit *et al.* 2006; Casper *et al.* 2006, 2007). Compared to biochemical (e.g. fatty acids, stable isotope) and DNA-based methods, analysis by hard parts is currently the only method able to accurately reconstruct prey biomass and also provides *a*

priori knowledge of diet that often underpins models used in the analysis of biochemical data (e.g. Iverson *et al.* 2004; Baylis and Nichols 2009; Deagle *et al.* 2007, 2009).

Based on information derived from limited diet studies, ASL target a range of benthic cephalopod, teleost fish, crustacean and cartilaginous prey species (Marlow 1975; Walker and Ling 1981; Richardson and Gales 1987; Gales and Cheal 1992; McIntosh *et al.* 2006; Gibbs 2008; Fragnito, 2013). However, little information exists on whether diet or prey size is consistent between ASL breeding colonies. Because hard prey parts are poorly preserved in faeces and regurgitates, such studies require large sample sizes, and previous dietary studies emphasise the limitations of using these samples to resolve ASL diet (Gales and Cheal 1992; McIntosh *et al.* 2006). However, given the lack of dietary information on ASL, such an approach is still important, because it provides a diagnostic method to identify prey taxa.

This study presents the most comprehensive and rigorous study of ASL diet to date across their South Australian breeding range. Here, hard-part analyses are utilised to examine whether diagnostic remains recovered from faeces and regurgitate can provide useful dietary information for ASL. Hard parts are known to digest at different rates, which can result in biased estimates of prey diversity (Hyslop 1980; Dellinger and Trillmich 1987; Gales and Pemberton 1994; Tollit *et al.* 1997, 2003; Bowen 2000; Staniland 2002; Yonezaki *et al.* 2003). Nevertheless, intact prey items and approximation of their proportional biomass can provide an alternative measure of relative importance that may differ from results derived from qualitative occurrence and abundance hard part indices (Tollit *et al.* 2009, 2015). As basic dietary information for ASL is limited with prey size estimates restricted to two small studies (McIntosh et al. 2006; Gibbs, 2008), any additional information on the use of prey resources will assist in ASL conservation management. The aims of the present study were to: i) describe the diet of ASL based on the analyses of hard parts from faeces and

regurgitates collected across the ASL South Australian breeding range and ii) using regression equations, estimate the length and biomass of the prey consumed.

MATERIALS AND METHODS

Sample collection

Faecal (n = 345) and regurgitate (*n* = 8) samples were collected from nine ASL breeding colonies in the eastern Great Australian Bight (GAB) in South Australia between 2003 and 2007 (Fig 1. and Table 1). Lounds Island (LoI) (32.27° S, 133.37° E), Breakwater Island (BwI) (32.32° S, 133.56° E), Lilliput Island (LI) (32.45° S, 133.67° E), Blefuscu Island (BI) (32.46° S, 133.64° E), and West Island (WI) (32.51° S, 133.25° E) are in the Nuyts Archipelago. Olive Island (OI) (32.72° S, 133.97° E) and Pearson Island (PI) (33.95° S, 134.26° E) are further south in eastern GAB. Dangerous Reef (DR) (34.82° S, 136.22° E) is in Spencer Gulf and Seal Bay is on Kangaroo Island (KI) (36.00° S, 137.33° E), which was the southern-most colony sampled. Seal Bay, DR, and LI colonies were sampled on multiple occasions, and all other colonies sampled once (Fig. 1, Table 1).

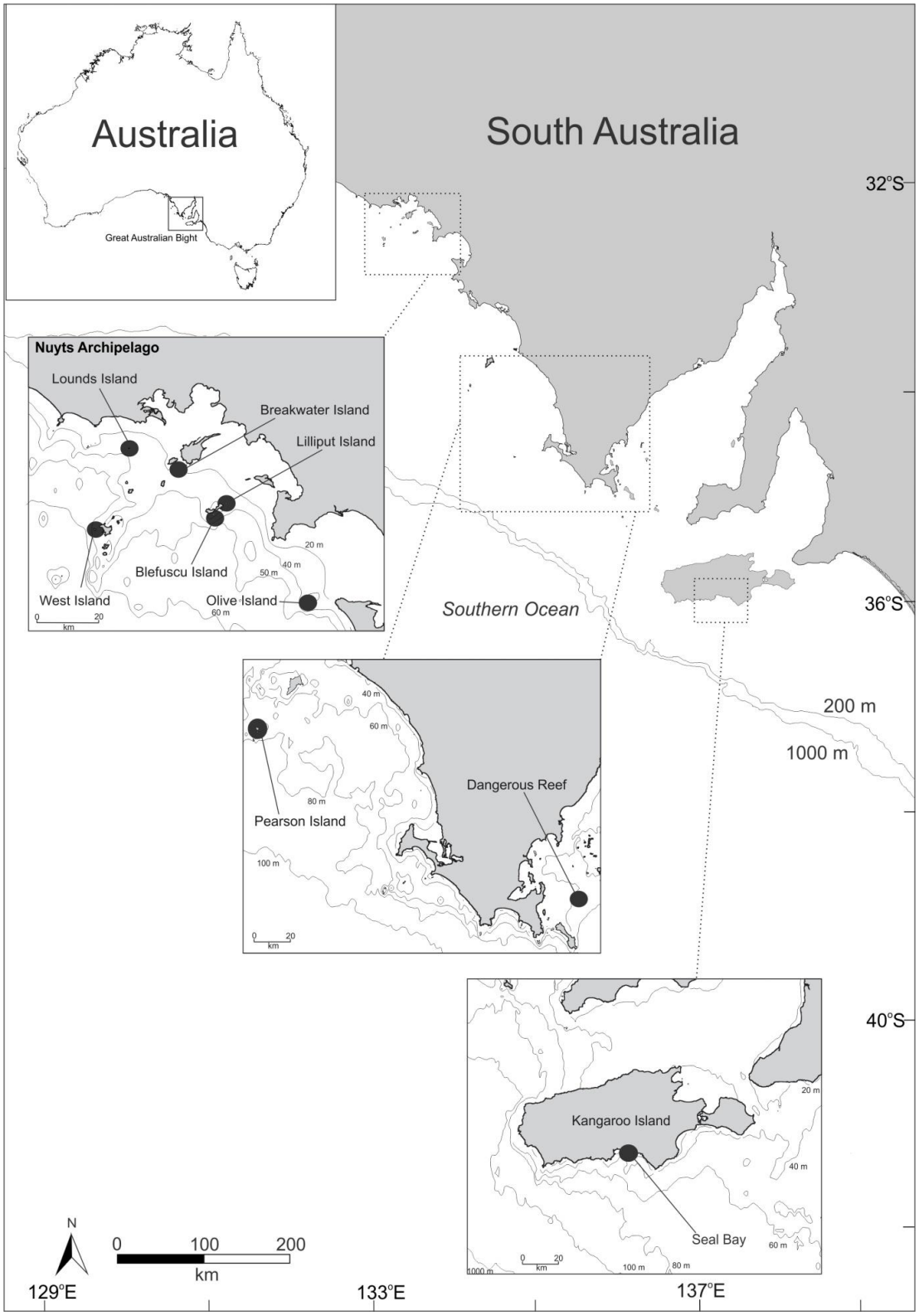

Figure 1. Location of ASL breeding colonies where faecal (n = 345) and regurgitate (n = 8) samples were collected in South Australia. Bathymetric contours are also shown.

Table 1. The distribution of sampling effort, frequency of occurrence (FO) and numerical abundance (NA) of diagnostic prey structures (cephalopod beaks, fish otoliths, vertebral processes and crustacean carapaces) recovered from faeces (n = 345) and regurgitates (n = 8) from nine breeding colonies of ASL in South Australia between 2003 and 2007. Unidentified fish otoliths (n = 20) are not included.

Sample type and site	*n*	Year	Season	FO	NA	Ratio of diagnostic items (cephalopod:fish:crustacea)
Faeces						
Dangerous Reef	26	2003	Spring	6	22	22 : 0 : 0
	7	2005	Winter	2	2	2 : 0 : 0
	6	2006	Spring	4	6	0 : 6 : 0
Pearson Island	49	2005	Winter	25	54	45 : 9 : 0
Kangaroo Island	38	2005	Spring	6	6	5 : 1 : 0
	36	2006	Summer	12	20	20 : 0 : 0
	14	2006	Autumn	1	1	0 : 1 : 0
	29	2006	Winter	4	3	1 : 2 : 0
	11	2007	Summer	1	1	1 : 0 : 0
	25	2007	Autumn-Winter	4	4	4 : 0 : 0
	23	2007	Spring	7	15	15 : 0 : 0
Olive Island	13	2007	Autumn-Winter	3	3	3 : 0 : 0
Lilliput Island	11	2005	Winter	6	50	49 : 1 : 0
	10	2007	Autumn	4	5	5 : 0 : 0
Blefuscu Island	4	2007	Autumn	1	1	1 : 0 : 0
West Island	33	2005	Winter	17	61	37 : 24 : 0
Lounds Island	10	2005	Spring	2	2	2 : 0 : 0
Total	**345**					
Regurgitates						
Breakwater Island	1	2007	Winter	1	38	35 : 1 : 2
Kangaroo Island	2	2006	Winter	2	6	6 : 0 : 0
	1	2007	Spring	1	3	3 : 0 : 0
Dangerous Reef	1	2005	Summer	1	12	9 : 0 : 3
	1	2005	Spring	1	1	1 : 0 : 0
Pearson Island	2	2005	Winter	2	8	7 : 1 : 0
Total	**8**					

Hard part analysis

Hard-parts of prey (fish otoliths, cephalopod beaks, bones, eye lenses, feathers, crustacean carapaces, and fragments) were isolated from each faecal sample by washing the sample through 1.0 and 0.5 mm nested sieves. Fish otoliths and cephalopod beaks were photographed and measured using digital microscopy supported by image analysis software Image Pro 5.1©. In all instances, otolith length (OL), upper and lower rostral length (URL, LRL), and

upper and lower hood length (UHL, LHL) of well-preserved cephalopod beaks were measured using digital callipers (0.01 mm) to supplement the microscopy results. Electronic scales (accuracy 0.0001 g) were used to determine otolith weight (OW). Diagnostic prey items were identified to the lowest taxon by comparison with reference collections and atlases (e.g. Lu and Ickeringill, 2002; Furlani *et al.* 2007). Where fish otoliths and cephalopod beaks were not differentiated to species level, the next closest taxonomic group was used (e.g., genus, family). Relative biomass, fish length (Furlani *et al.* 2007), and cephalopod mantle length (Lu and Ickeringill 2002) were determined from the fish otoliths and cephalopod beaks respectively using available regression equations (Lu and Ickeringill 2002; Furlani *et al.* 2007; McLeay *et al.* 2009; Wiebkin 2012) (Table 2). Where biomass or length regression equations were not available for a particular species, I used equations for a similar species. Otherwise, only counts of abundance were determined. For example, the size of unidentified octopus (*Octopus* spp.) was determined using regression equations for two sympatric species: *O. berrima* and *O. maorum*, which are common cephalopod taxa found in South Australia (Reid, 2016).

Table 2. Regression formulae used to estimate prey mass (g) and length (mm) from fish otoliths and cephalopod beaks recovered from faeces and regurgitates of ASL. Abbreviations: Mass (M), (Total Length (TL), Fork Length (FL), Standard Length (SL), Otolith Wei ight (OW), Otolith Length (OL) (fish only), and Mantle Length (ML), Upper Hood Length (UHL), Lower Hood Length (LHL), Upper Rostal Length (URL), Lower Rostal Length (LRL) (cephalopods only). Source of information for regression equations: (A) McLeay *et al.* 2009; (B) Furlani *et al.* 2007; (C) Wiebkin, A. (2012), and (D) Lu and Ickeringill, 2002.

Prey type	Species used for regression equation	Mass regression equation (g)	Length regression equation	Source
Fish otoliths				
Arripis georgianus	Same species	$M = 5e^{-06} \times FL^{3.25}$	$FL = 25.606 \times OL^{0.98}$	A
Foetorepus calauropomus	Same species	$M = 4.174 \times 10^{-5} TL^{2.65}$	$TL = 258.526\ OW^{0.247}$	B
Monacanthidae	*Meuschenia scaber*	$M = 5.114 \times 10^{-5} TL^{2.79}$	$TL = 250.385\ OW^{0.084}$	B
Parapriacanthus elongatus	Same species	$M = 3e^{-05} \times FL^{2.81}$	$FL = 27.3\ OL^{0.95}$	A, C
Platycephalidae	*Platycephalus richardsoni*	$M = 6.144 \times 10^{-6} TL^{3.029}$	$TL = 339.339\ OW^{0.084}$	A
Pseudophycis bachus	Same species	$M = 1.017 \times 10^{-7} TL^{3.838}$	$TL = 6.33 \times OL^{1.62}$	B
Sillago bassensis	Same species	$M = 1.11 \times 10^{-5} SL^{3.02}$	$SL = 16.78\ OL^{1.04}$	B
Triglidae	*Lepidotrigla modesta*	$M = 1.154 \times 10^{-4} FL^{2.576}$	$FL = 208.721\ OW^{0.002}$	B
Cephalopod beaks				
Upper				
Octopus berrima	Same species	$M = e^{0.44 + 3.53\ (\ln UHL)}$	ML = -11.58 +15.99 UHL	D
Octopus maorum	Same species	$M = e^{0.73 + 2.64\ (\ln UHL)}$	ML = -55.57 + 20.67 UHL	D
Octopus sp.	*O. berrima*	$M = e^{0.44 + 3.53\ (\ln UHL)}$	ML = -11.58 +15.99 UHL	D
Nototodarus gouldi	Same species	$M = e^{0.64 + 2.78\ (\ln URL)}$	ML = 57.75 + 29.90 URL	D
Sepioteuthis australis	Same species	$M = e^{2.07 + 2.26\ (\ln URL)}$	ML = -21.30 + 63.83 URL	D
Sepia apama	Same species	$M = e^{-5.78 + 3.6\ (\ln UHL)}$	ML = -8.4 + 7 UHL	D
Sepia sp. 2	*Sepia* sp.	$M = e^{-2.16 + 2.68\ (\ln UHL)}$	ML = 13.83 + 6.66 UHL	D
Ommastrephidae other	*N. gouldi*	$M = e^{0.64 + 2.78\ (\ln URL)}$	ML = 57.75 + 29.90 URL	D
Lower				
Octopus berrima	Same species	$M = e^{0.75 + 3.23\ (\ln LHL)}$	ML = -10.08 + 20.05 LHL	D
Octopus maorum	Same species	$M = e^{2.14 + 2.50\ (\ln LHL)}$	ML = -43.69 + 29.18 LHL	D
Octopus sp.	*O. berrima*	$M = e^{0.75 + 3.23\ (\ln LHL)}$	ML = -10.08 + 20.05 LHL	D
Nototodarus gouldi	Same species	$M = e^{0.80 + 2.86\ (\ln LRL)}$	ML = 41.88 + 33.99 LRL	D
Sepioteuthis australis	Same species	$M = e^{1.71 + 3.34\ (\ln LRL)}$	ML = -20.78 + 67.89 LRL	D
Sepia sp. 1	*Sepia* sp.	$M = e^{0.70 + 2.51\ (\ln LHL)}$	ML = 18.09 + 16.50 LHL	D
Ommastrephidae other	*N. gouldi*	$M = e^{0.80 + 2.86\ (\ln LRL)}$	ML = 41.88 + 33.99 LRL	D

Data analysis

Prey composition was assessed using frequency of occurrence (FO) (number of samples containing a given prey taxa) and numerical abundance (number of prey structures of each prey taxa), which were also represented as i) percentage of each functional taxonomic group and ii) percentage of all diagnostic prey items recovered. Mean, median and range estimates

of prey biomass (grams (g) ± standard deviation (sd)) and overall prey size (mm ± sd) consumed were determined by calculating the minimum number of individuals (MNI) in each faecal or regurgitate sample, which was estimated from the maximum count of right or left otoliths, and of lower or upper cephalopod beaks of each taxa. Where remains were identifiable but not quantifiable using regression equations (e.g. vertebrae of leatherjackets (Monacanthidae)), only qualitative counts of MNI were determined. For example, multiple Monacanthidae vertebrae in a sample were counted as one individual if otoliths of this taxonomic group were absent.

One-way analysis of variance (ANOVA), or the non-parametric equivalent were used to assess differences in the size of prey recovered between faeces and regurgitates, as well as any biomass differences between seasons and years. Differences in prey composition were assessed between locations using non-parametric analysis of similarity (ANOSIM) on a Bray-Curtis similarity matrix reinforced using the permutation multivariate analysis procedure (PERMANOVA) in Primer (Primer version 6.0, PRIMER-E Ltd., Plymouth, UK) ($P \leq 0.05$). Similarity or dissimilarity percentages (SIMPER) (Clarke and Warwick 1993) were used to identify prey responsible for group differences. To make data comparable between locations, species abundances were initially standardised by dividing the number of prey items of each species by the overall abundance data. The data for each location was then represented as a proportion of all species recovered.

RESULTS

Prey hard-parts

In total, 246 (71.3%) faeces contained skeletal structures of prey, as did all eight regurgitates. Except for cephalopod beaks (n = 273) and intact fish otoliths (n = 46), most prey structures were highly degraded or damaged. Altogether, 220 (63.8 %), 132 (38.3 %), and 17 (4.9 %)

faecal samples contained the remains of fish, cephalopods and crustaceans, respectively. Cephalopods were identified in all regurgitates, fish remains in four (50%) and crustaceans were recovered in two regurgitates (25%). Three-hundred and twenty-four prey items (cephalopod beaks, fish otoliths, vertebrae and crustacean carapace) recovered from faeces (n = 255) and regurgitates (n = 69) were identified to family, genus or species. Twenty fish otoliths were not identified because they were damaged (Table 3).

One-hundred and ten faecal samples (31.9 %) contained diagnostic prey items. Of these, 80 (23 %) contained cephalopod beaks (n = 212) and 23 (6.7 %) contained either fish otoliths (*n* = 54) and/or species-specific fish vertebrae (e.g., Monacanthidae) (n = 12). Prey represented ten families of fish and four families of cephalopod. Six fish and five cephalopod taxa were identified to species with three cephalopod taxa identified to genus (Table 3). Regurgitates comprised 61 cephalopod beaks (91 %), five prawn carapaces (7.5 %) and three fish otoliths. One fish, one crustacean, and four cephalopod taxa were identified to species, and two cephalopods to genus level.

Diet diversity

Fifteen taxonomic families of prey were identified across all samples collected (Table 3). Four fish (Platycephalidae, Monacanthidae, Triglidae and Serranidae) and two cephalopod taxa (Loliginidae and Ommastrephidae) were identified to family level, and two cephalopod taxa were identified to genus level (*Octopus* sp. and *Sepia* sp.) (Table 3). The most abundant prey identified from hard parts were cephalopods, which comprised 79 % (n = 273) of the total number of diagnostic items recovered and identified. Numerically, the most common cephalopod families were Octopodidae (65 %), Loliginidae (squid) (18%) and Sepiidae (cuttlefish) (8%), which was reflected in the frequencies of occurrence recovered (Table 3). A large quantity of Octopodidae beaks (n = 131, 48%) were damaged or eroded and could only

be assigned to the genus *Octopus* sp. The southern-keeled octopus (*Octopus berrima*) and Maori octopus (*Octopus maorum*) comprised 14 % and 3% (respectively) of the remaining octopus. *Sepioteuthis australis* (calamari squid) comprised 18% of all cephalopods, and *Sepia apama* and *Sepia* sp. (cuttlefish)), and Ommastrephids (*Nototodarus gouldi* (arrow squid)), comprised 8% each of the remaining cephalopods identified. The crustacean species recovered from two regurgitates was the western king prawn (*Melicertus latisulcatus*).

Fish and crustaceans

Numerically, the most common families of fish identified from otoliths or species-specific vertebrae were Platycephalidae (flatheads) (n = 15) and Monacanthidae (leatherjackets) (n = 13), which comprised only 8% of the diagnostic prey items recovered (Table 3). In most cases, the frequency of fish recovered from either faeces or regurgitates was low (≤ 3%), and comprised ≤ 1 % of all prey items recovered. Fish ranged in size (6.7 – 595.8 g and 67.1 – 433.3 mm) but were mostly small (mean 229.2 ± 251.5g and 247 ± 144.5 mm), except for Platycephalidae, which were the largest taxonomic group of fish recovered in terms of biomass and length (mean 542.4 ± 30.2 g and 419.9 ± 7.6 mm) (Table 4).

Table 3. Frequency of occurrence (FO) and numerical abundance (NA) of diagnostic prey items recovered from Australian sea lion faecal (n = 345) and regurgitate (n = 8) samples. Samples were collected across nine breeding sea lion colonies in South Australia (Da Dangerous Reef, Olive Is., Lounds Is., Breakwater Island, Lilliput Is., Blefuscu Is., Kangaroo Is., Pearson Is., West Is.). Symbols denote: †identified in faeces, †† identified in regurgitates.

Taxonomic group	Common name	FO	FO %	NA (*n*)	NA (% group)	NA (% of all diagnostic prey items)
Fish						
Platycephalidae	Flathead spp. †	3	1	15	23	4
Monacanthidae	Leatherjacket †	10	3	13	20	4
Sillaginidae						
Sillago bassensis	Silver whiting †	2	0	4	6	1
Pempheridae						
Parapriacanthus elongatus	Slender bullseye †	3	1	4	6	1
Triglidae						
Lepidotrigla spp.	Gurnard †	2	1	2	3	< 1
Moridae						
Pseudophycis bachus	Red cod †	2	0	2	3	< 1
Apogonidae						
Vincentia consperca	Southern cardinal fish ††	2	0	2	3	< 1
Arripidae						
Arripis georgianus	Tommy ruff †	2	1	2	3	< 1
Callionymidae						
Foetorepus calauropomus	Common stink fish †	1	0	1	2	< 1
Serranidae	Perch †	1	0	1	2	0
Unidentified fish otoliths		15	4	20	30	6
	Total			**66**	**100**	**19**
Cephalopods						
Octopodidae						
Octopus spp.	Octopus	43	12	131	48	38
Octopus berrima	Southern keeled octopus †	12	3	37	14	11
Octopus maorum	Maori octopus	5	1	9	3	3
Loliginidae						
Sepioteuthis australis	Southern calamari squid	17	5	49	18	14
Loliginidae sp.	Squid †	1	0	1	0	0
Sepiidae						
Sepia sp.	Cuttlefish	7	2	16	6	5
Sepia apama	Australian giant cuttlefish ††	2	1	6	2	2
Ommastrephidae	Squid	10	3	15	5	4
Nototodarus gouldi	Arrow squid	5	1	9	3	3
	Total			**273**	**100**	**79**
Crustacea						
Penaeidae						
Melicertus latisulcatus	Western king prawn ††	2	1	5	100	1
	Total			**5**	**100**	1

Table 4. Biomass (g) (mean ± SD), median, range, total mass) and length (mm) (mean ± SD), median, range) estimates of fish consumed by ASL based on prey items in faeces and regurgitates. Estimates are based on data using the minimum number of individuals determined from the maximum number otoliths recovered from each faecal sample or regurgitate. (See Table 2 for regression equations).

Genus / species	**Common name**	**Biomass estimate (g)**					**Length (mm)**		
		n	**Mean (± SD)**	**Median**	**Range**	**Total mass**	**Mean (± SD)**	**Median**	**Range**
Arripis georgianus	Tommy ruff	2	6.8 ± 0.3	-	6.7 - 7.0	13.7	77.1 ± 0.7	-	76.6 - 77.6
Foetorepus calauropomus	Common stink fish	1	-	-	90.1	90.1	-	-	233.0
Meuschenia scaber	Leatherjacket	1	-	-	35.3	35.3	-	-	145.9
Parapriacanthus elongatus	Slender bullseye	4	29.9 ± 20.5	30.24	5.6 - 53.5	119.6	155.3 ± 51.3	164.3	86.8 - 205.8
Platycephalus richardsoni	Flathead	6	542.4 ± 30.2	528.9	512.4 - 595.8	5965.9	419.9 ± 7.6	416.6	412.8 - 433.3
Pseudophycis bachus	Red cod	2	48.4 ± 58.3	-	7.2 - 89.6	96.8	162.6 ± 72.9	-	111.0 - 214.1
Sillago bassensis	Silver whiting	4	8.9 ± 5.3	8.1	3.6 - 15.7	35.5	88 ± 18.4	88.2	67.1 - 108.7
Lepidotrigla spp.	Gurnard	2	109.7 ± 0.8	-	109.1 - 110.3	219.4	209.3 ± 0.6	-	208.8 - 209.7
Vincentia consperca	Southern cardinal fish	2	NA				NA		

Cephalopods

There was high variability in the size of cephalopods consumed (Table 5). All cephalopod species recovered from regurgitates were significantly larger and estimated heavier than those in faeces (Mann-Whitney U: Z = - 4.7, P = < 0.001 (mass) and Mann-Whitney U: Z = - 6.24, P = < 0.001 (mantle length)). Overall, the median biomass of cephalopods was 23.3 g (mean 66.5 ± 152.7 g; range 0.1 – 1237.3 g) with mantle length of 37.9 mm (mean 62.6 ± 64.8 mm, range 4.1 – 384.7 mm). Ommastrephidae and Loliginidae squids were the largest cephalopods recovered (mean biomass 169.5 ± 260.3 g and mean mantle length 167.7 ± 75.3 mm, and 138.0 ± 200.1 g and 130 ± 79.1 mm, respectively). In comparison, Octopodidae and Sepiidae were typically small (mean 32.5 ± 64.3 g and 30.4 ± 14.6 mm, and 74.7 ± 261 g and 64.6 ± 43.9 mm, respectively) (Figs. 2, 3, 4, 5). Based on the MNI, Loliginidae and Octopodidae comprised the largest percentage biomass contributions of cephalopod consumed (36.9% and 31.9%, respectively), followed by Ommastrephidae (21.7%) and Sepiidae (9.5%) (Table 6).

For cephalopod taxa, diversity did not differ significantly between locations (ANOSIM: R = -0.042, *P* = 0.85; PERM: pseudo-F = 0.60164, df = 7, *P* = 0.96) and no seasonal differences were detected (ANOSIM: R = -0.057, P = 0.93) except for summer, which was weakly different in composition compared to winter and spring (ANOSIM $_{(winter-summer)}$: R = 0.168, *P* = 0.005) (ANOSIM $_{(summer-spring)}$: R = 0.111, *P* = 0.017). Cephalopod composition also differed weakly between years 2005 and 2006 (ANOSIM: R = 0.157, *P* = 0.07; PERM: pseudo-F = 2.995, df = 3, *P* = 0.002), and, 2006 and 2007 (ANOSIM: R = 0.223, *P* = 0.01; PERM: pseudo-F = 2.995, df = 3, *P* = 0.002) (41.7% and 40.3% dissimilarity, respectively).

Table 5. Biomass (g) (mean ± SD), median, range, total mass) and mantle length (mm) (mean ± SD), median, range) estimates of all cephalopods consumed by ASL based on prey items in faeces and regurgitates. Estimates are based on data using the minimum number of individuals determined from the maximum number of upper or lower beaks recovered from each faecal sample or regurgitate (See Table 2 for regression equations).

Genus / species		Biomass estimate (g)				Mantle length (mm)		
	n	**Mean (± SD)**	**Median**	**Range**	**Total mass**	**Mean (± SD)**	**Median**	**Range**
Upper beaks								
Nototodarus gouldi	3	642.8 ± 431.0	492.9	306.6 - 1128.7	1928.3	292.7 ± 57.0	278.7	244.0 - 355.4
Octopus berrima	21	34.3 ± 17.9	35.3	3.4 - 71.0	720.0	35.9 ± 8.9	38.1	14.0 - 49.0
Octopus maorum	3	339.5 ± 391.4	155.8	73.7 - 788.9	1018.4	71.5 ± 61./1	49.7	24.3 - 140.6
Octopus spp.	68	23.4 ± 20.6	18.7	1.1 - 143.1	1619.2	29.5 ± 10.8	30.3	6.8 - 62.3
Ommastrephidae (based on *N. gouldi*)	6	115.5 ± 171.8	30.8	0.1 - 430.7	692.9	151.6 ± 78.1	132.8	66.7 - 268.2
Sepia apama	6	223.6 ± 496.8	21.4	4.73 - 1237.3	1341.4	98.4 ± 72.6	73.3	45.29 - 243.6
Sepia sp.	6	34.8 ± 49.0	10.2	0.6 - 125.6	208.8	58.7 ± 28.7	49.4	26.4 - 104.4
Sepioteuthis australis	18	68.7 ± 119.6	25.6	2.07 - 518.6	1235.8	109.3 ± 82.1	91.0	14.0 - 384.7
Lower beaks								
Nototodarus gouldi	6	72 ± 64.2	57.1	4.9 - 174.9	432.0	145.5 ± 40.8	144.4	86.7 - 198.2
Octopus berrima	14	12.8 ± 14.0	8.0	1.4 - 56.3	179.1	22.0 ± 9.4	20.1	7.6 - 45.3
Octopus maorum	6	59.6 ± 11.7	61.2	44.6 - 76.63	357.6	19.7 ± 5.0	20.6	12.9 - 26.6
Octopus spp.	58	27.7 ± 30.3	18.3	0.7 - 163.3	1604.2	28.8 ± 15.2	29.0	4.1 - 68.5
Ommastrephidae (based on *N. gouldi*)	7	96.5 ± 129.1	46.7	3.7 - 372.3	679.0	147.1 ± 56.9	134.3	82.3 - 245.5
Sepia sp.	10	9.2 ± 3.3	9.4	3.7 - 14.42	92.2	47.9 ± 4.6	48.6	39.0 - 54.2
Sepioteuthis australis	28	182.6 ± 229.1	106.7	0.5 - 957.8	5111.8	143.3 ± 70.5	143.8	12.8 - 296.9

Table 6. Estimated mass (g) and percent biomass contribution (BM) (%) of cephalopods by taxonomic family consumed by ASL based on prey items in faeces and regurgitates. Biomass calculations for *Octopus* spp. are based on regression equations for *O. berrima* (A) and *O. maorum* (B). Cephlaopod biomass was significantly larger using estimate (B) than (A) ($P < 0.001$).

	A		B	
Family/species	**Total mass (g)**	**BM (%)**	**Total mass (g)**	**BM (%)**
Octopodidae	**5498.5**	**31.9**	**8113.7**	**40.9**
Octopus spp.	3223.4	18.7	5838.6	29.4
Octopus berrima	899.1	5.2	899.1	4.5
Octopus maorum	1376.0	8.0	1376.0	6.9
Loliginidae	**6347.6**	**36.9**	**6347.6**	**32**
Sepioteuthis australis	6347.6	36.9	6347.6	32
Ommastrephidae	**3732.2**	**21.7**	**3732.2**	**18.8**
Nototodarus gouldi	2360.3	13.7	2360.3	11.9
ommastrephid	1371.9	8.0	1371.9	6.9
Sepiidae	**1642.3**	**9.5**	**1642.3**	**8.3**
Sepia apama	1341.4	7.8	1341.4	6.8
Sepia sp.	300.9	1.7	300.9	1.5

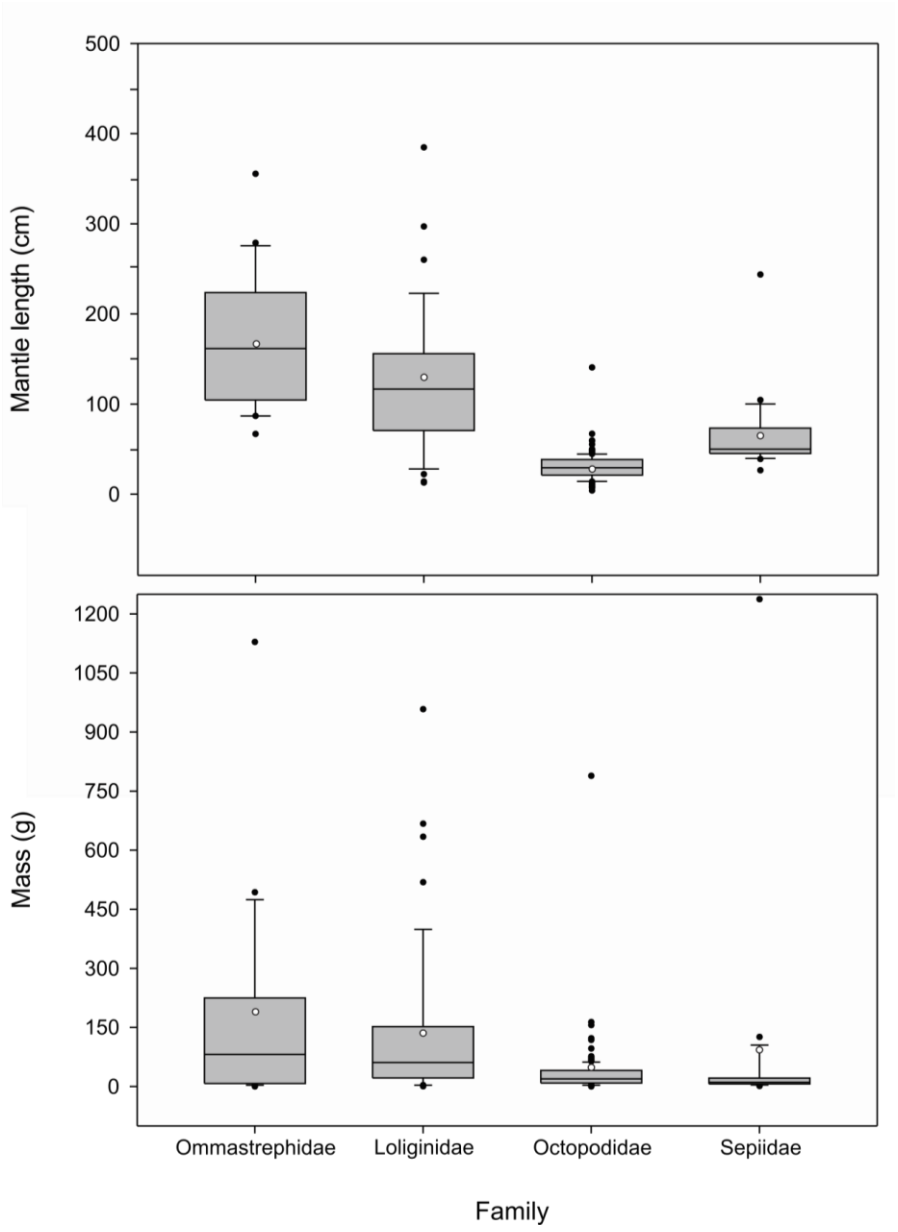

Figure 2. Box plots indicating size ranges of estimated mantle length (mm) and mass (g) of cephalopods based on regression equations from key families detected in this study (Ommastrephidae, Loliginidae, Octopodidae and Sepiidae). Estimates are based on the minimum number of individuals recovered from faeces (n = 345) and regurgitates (n = 8) collected from nine colonies in South Australia. Plots show the median, mean (open circle), 25th and 75th percentile, and outliers (filled circles).

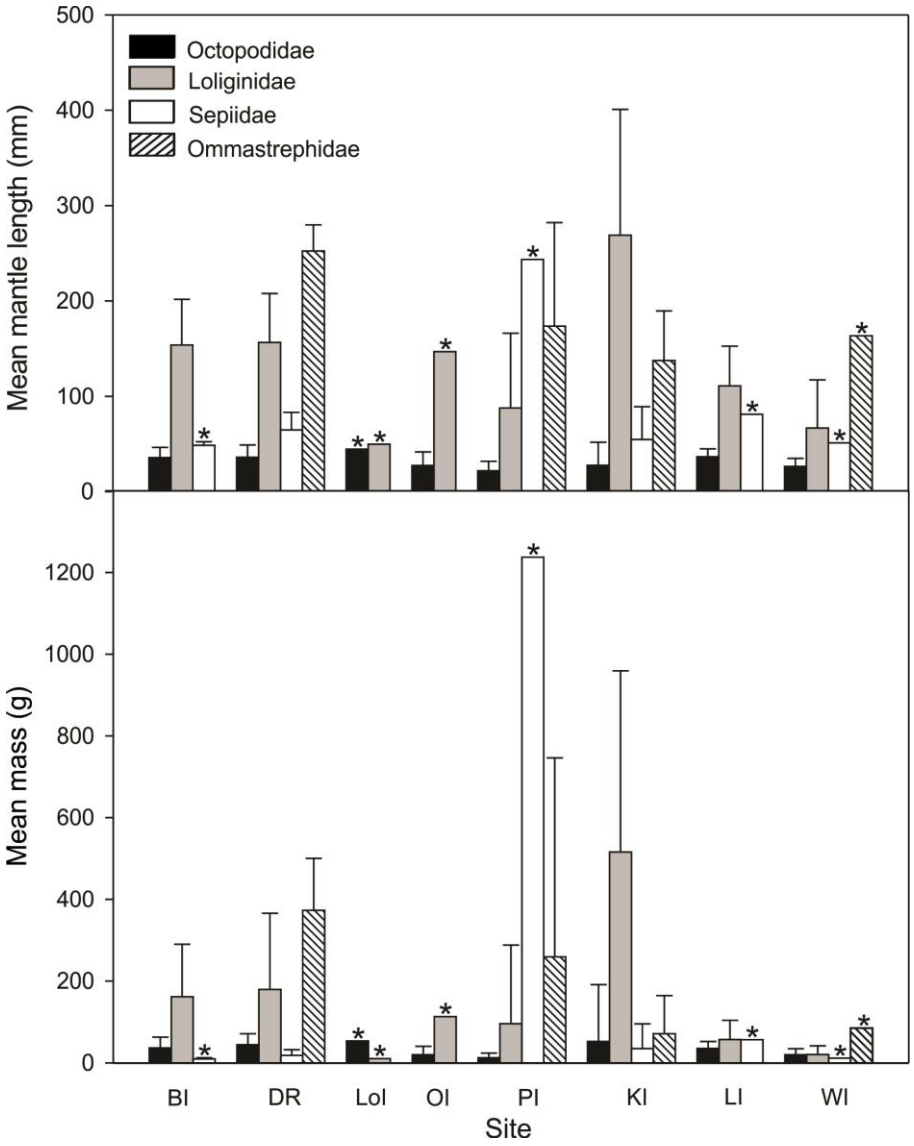

Figure 3. Mantle length (mm) and mass (g) (mean ± SD) of cephalopods (Octopodidae, Loliginidae, Sepiidae and Ommastrephidae) based on data from the minimum number of individuals recovered from faeces (n = 345) and regurgitates (n = 8) collected at nine breeding colonies of ASL in South Australia between 2003 and 2007. Sites are; Breakwater Is. (BI), Dangerous Reef (DR), Lounds Is. (LoI), Olive Is. (OI), Pearson Is. (PI), Seal Bay on Kangaroo Island (KI), Lilliput Is. (LI) and West Is (WI). *Denotes single individual only.

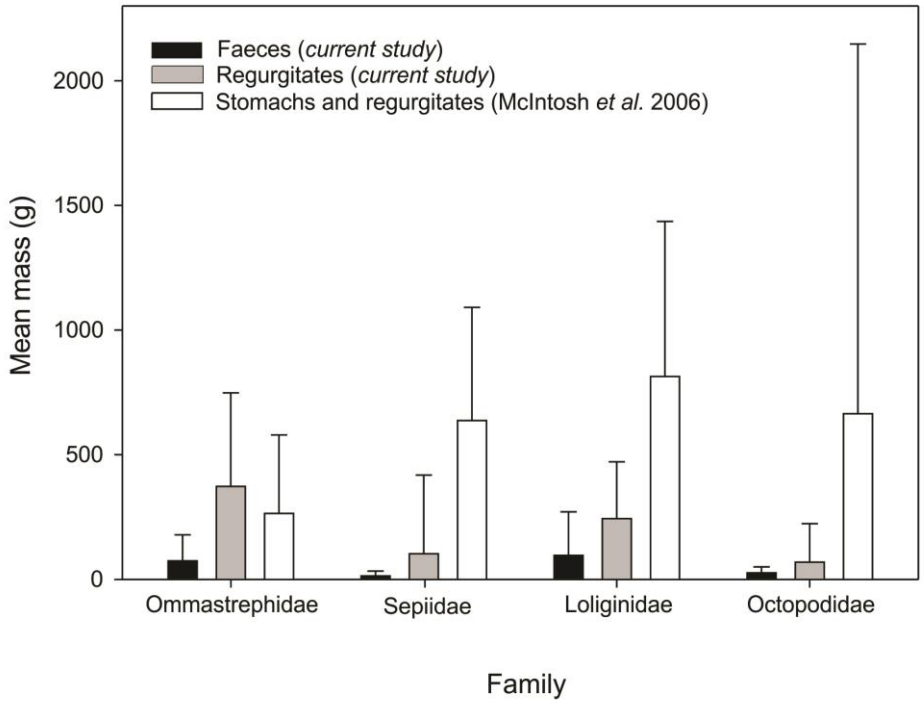

Figure 4. Mass (mean ± SD) of cephalopods consumed by ASL by taxonomic family estimated in the current study and from regurgitates and stomachs derived by McIntosh *et al.* (2006) from the Seal Bay colony. Cephalopods were significantly smaller from faeces than regurgitates and stomachs in both studies. For Sepiidae, Loliginidae and Octopodidae, beaks derived from regurgitates in the current study where significantly smaller than reported by McIntosh *et al.* (2006).

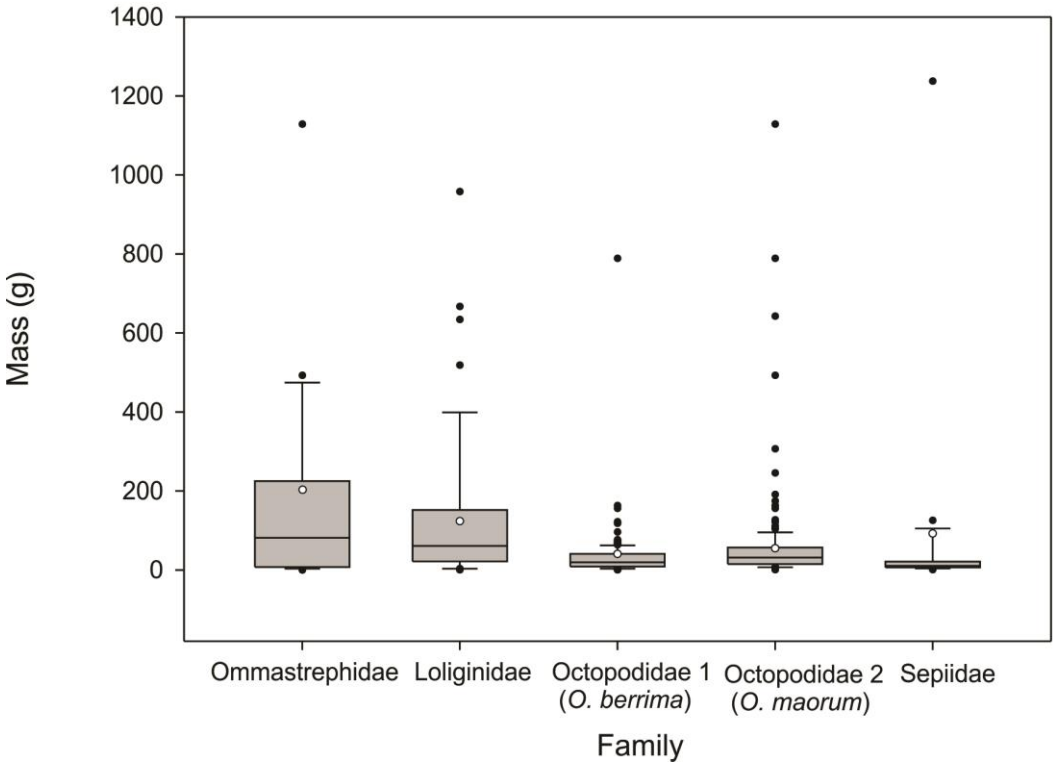

Figure 5 (supplementary). Box plots indicating size ranges estimated from mass (g) regression equations of key cephalopod families detected in this study (Ommastrephidae, Loliginidae, Octopodidae and Sepiidae). Estimates are based on data from the minimum number of individuals recovered from faeces (n = 345) and regurgitates (n = 8) collected from nine breeding colonies of ASL in South Australia between 2003 and 2007. Plots show the comparison between overall size range of Octopodidae estimated from mass (g) regression equations based on *O. berrima* (Octopodidae 1) and regressions equations based on *O. maorum* (Octopodidae 2). Data show the median, mean (open circle), 25th and 75th percentile, and outliers (filled circles).

DISCUSSION

Diet Diversity

Hard part diet analysis was a reliable method to estimate the size and biomass of a small range of prey species consumed by ASL that would not have been possible using other diet

methods (e.g., fatty acids and DNA). Consistent with previous diet studies (Gales and Cheal 1992; McIntosh *et al.* 2006; Gibbs 2008) these findings confirm the ASL are benthic foragers that consume a range of predominantly demersal teleost fish, crustacean and cephalopod prey. This is the most geographically comprehensive ASL diet study to date. Given the paucity of dietary information for ASL, these findings are an important step in gaining a broader geographic understanding of ASL prey diversity. In particular, the use of cephalopods, which were the most frequent prey consumed across sites by ASL.

Studies that use hard-parts recovered from faeces and regurgitates have inherent biases because the resulting data emphasise the remains of taxa with robust hard parts and are less reliable for identifying highly digestible prey (e.g. Tollit *et al.* 2006). Similar to previous studies that used hard parts to determine ASL diet, hard parts from faeces and regurgitates in this study provided limited information on the range of prey consumed (Gales and Cheal 1992; McIntosh *et al.* 2006; Gibbs 2008). Species level identifications of fish from otoliths for example, were constrained by structural damage caused by digestive erosion and many items remained unidentified in this prey group. In addition, small otoliths are more susceptible to erosion and complete digestion (Fea and Harcourt 1997), and few small otoliths were found in the current study. Even though fragments of carapace from what were probably decapods crustaceans were recovered, the identification of crustaceans was limited to the penaeid prawn (*M. latisulcatus*). These two prey groups (fish and crustaceans) represented less than 4% of the overall abundance of prey items recovered and hard remains of cartilaginous taxa were absent (Gales and Cheal 1992; McIntosh *et al.* 2006). In contrast, cephalopod beaks were structurally well conserved, numerically abundant and frequently encountered.

Diet diversity comprised 15 families with 11 genera identified. Among these, cephalopods comprising octopus, loliginid and ommastrephid squids and cuttlefish were the dominant prey type recovered. Most of the taxa identified typically occur over the continental shelf at depths from 10 m to 200 m (Edgar *et al.* 2001; Gomon *et al.* 2008). This is consistent with the foraging behaviour reported for ASL, which utilise coastal and on-shelf waters and employ continuous U-shaped dives characteristic of foraging at, or near to the seabed (Costa and Gales 2003; Fowler *et al.* 2007; Goldsworthy *et al.* 2009; Lowther *et al.* 2011, Fragnito 2013).

Many of the taxa identified in this study have been reported in previous ASL diet studies (Gales and Cheal 1992; McIntosh *et al.* 2006), although the geographic extent of their distribution has remained poorly studied. Octopodidae, Sepiidae, Ommastrephidae and to a lesser extent Loliginidae, were common cephalopod prey recorded in stomachs and regurgitates of ASL at Kangaroo Island (McIntosh *et al.* 2006). Otoliths from fish families: Monacanthidae (leatherjackets), Platycephalidae (flathead), Sillaginidae (whiting), and Pempheridae (bullseyes) were also identified in that study, although the size of specimens could not be determined. Cephalopods (Octopodidae, Sepiidae and Loliginidae) and unidentified fish were also typical of remains recovered from a small number of faeces and stomachs collected across a broad geographic range assessed by Gales and Cheal (1992). The remains of Triglidae (gurnard), Apogonidae (cardinal fish), Serranidae (perch), Callionymidae (stink fish), and Penaeidae (king prawn) are novel prey to this study, and have not been previously recorded in diet of ASL. The collective range of prey identified across different diet studies suggests individual ASL probably utilise a broad range of habitats to acquire prey. Given temporal and spatial information on diet is limited in ASL further studies are needed to ascertain the importance of different prey.

In southern Australia, ASL coexists with two other pinnipeds; the Australian (*Arctocephalus pusillus doriferus*) and long-nosed fur seal (*A. forsteri*). There is a high level of spatial overlap in the foraging areas used by ASL (Fowler *et al.* 2007, Lowther *et al.* 2012) and long-nosed (adult males and females) and Australian fur seals (Page *et al.* 2007). The level of inter-specific dietary competition between ASL and fur seals has not been studied in detail. Diet studies on long-nosed fur seals indicate that adult females and males have similar diets, but adult males consume larger prey, including penguins (Page *et al.* 2005; Reinhold, 2015). The diets of both adult female and male long-nosed fur seals comprise fish and cephalopods, including Gould's squid (*N. gouldi*), redbait (*Emmelichthys nitidus*) and southern garfish (*Hyporhamphus melanochir*) (Page *et al.* 2005; Reinhold, 2015) which are all typically found in the water column. Juvenile long-nosed fur seals preyed on small myctophids (*Symbolophorus* sp.), which occur south of the shelf break in pelagic waters (Page *et al.* 2005), which are not preyed on by ASL. The diet of Australian fur seals includes several benthic species of rock cod (*Pseudophycis* spp.), flathead (*Platycephalus* spp.) and leatherjacket (Monacanthidae) (Page *et al.* 2005), which are also used by ASL, but Australian fur seals also utilise pelagic prey, including redbait. This study supports the findings of Page *et al.* (2005), which indicated that despite high degrees of spatial overlap in foraging grounds, the three pinniped species only share a few common prey.

Insights into habitat use

Recent studies using animal-borne cameras and biotelemetry suggest ASL exploit a range of habitats in search of prey (Lowther *et al.* 2012; Fragnito, 2013). Preferential habitat selection by ASL cannot be confirmed from results of this study, but a number of taxa identified suggest ASL use a variety of habitats to obtain prey. For example, Pempheridae, Triglidae, Moridae, Monacanthidae, Apogonidae and Serranidae are closely associated with algal-reef

and rocky reef systems that are widely distributed throughout the GAB, and Platycephalidae, Sillagindae and Callionymidae tend to inhabit sandy substrates that are common amongst seagrass and low relief broken bottom, but they also can occupy reefs (Gomon *et al.* 2008). Similarly, Sepiidae and Octopodidae are benthic cephalopods that brood and actively forage on algal-rocky reef systems (Mather *et al.* 1985). Cryptic octopods such as *O. berrima* and *O. maorum* use soft-sediments between rocky habitats, burying to avoid predation (Anderson 1997, 1999; Stranks and Norman 1992). Biotelemetry studies indicate ASL prospect such habitats (Lowther *et al.* 2011, 2012), suggesting they may be important foraging locations for ASL in South Australia.

As found in the study by McIntosh *et al.* (2006), the loliginid squid, *S. australis*, was scarce at Seal Bay, but these species were well represented in samples from colonies within the Nuyts Archipelago (94% of all loliginids recovered). Benthic habitats adjacent to the latter colonies comprise seagrass and low relief shallow algal reef systems (Edyvane 1999), which provide important breeding and foraging habitat for *S. australis* (Smith 1983; Triantafillos 2001; Miller *et al.* 2006; Pecl *et al.* 2006; Steer *et al.* 2006). This contrasts with the heterogeneous rocky and sandy reef complexes utilised by ASL adjacent to the Seal Bay colony (Fowler *et al.* 2006, 2007). The variety of prey taxa observed in the diet of ASL among colonies suggests they use a range of different habitats. This likely reflects the individual specialisations in foraging areas (e.g., coastal shallow seagrass regions, vs. offshore rocky reefs) previously described (Baylis *et al.* 2009; Goldsworthy *et al.* 2009; Lowther *et al.* 2011).

This study indicates that benthic dwelling and demersal cephalopods are important in the diet of ASL (Ling 1992; Costa and Gales 2003). Results indicated that Octopus and loliginid squids were the most important cephalopod prey taxa identified, as previously reported in

other studies (Gales and Cheal 1992; McIntosh *et al.* 2006). Octopus contributed the highest abundance (~ 65% of all cephalopods) and, despite being one of the smallest cephalopod taxa recovered, their proportional biomass was greater than loliginid and ommastrephid squid. Overall, the size of cephalopod prey is smaller than reported in previous studies (McIntosh *et al.* 2006; Gibbs, 2008). Faeces are more likely to contain smaller cephalopod beaks than regurgitates, because large beaks tend to remain in the stomach or be regurgitated rather than being defecated (Richardson and Gales, 1987; Bowen, 2000; Pitcher, 1980; Childerhouse *et al.* 2001).

Potential overlap with fisheries

The relative importance of octopus in the diet of ASL raises interesting questions regarding resource competition with local GAB fisheries. For example, octopus by-catch in the South Australian Northern Zone Rock Lobster Fishery (NZRLF) (Linnane *et al.* 2014) suggests there may be competition in areas where ASL and the NZRLF overlap. By-catch data indicate the take of octopus has sharply declined in the NZRLF since 1998 (Brock *et al.* 2004, Linnane *et al.* 2012), however current catch remains at ~0.005 octopuses per pot lift (~2730 octopuses annually) (Linnane *et al.* 2014, Brock *et al.* 2004). A reduction in cephalopod biomass can lead to decreased levels of recruitment (André and Hartmann, 2014). If octopus is indeed important prey of ASL, it would be useful to understand the level of interaction with the NZRLF particularly at ASL colonies that are in decline. Such an approach should use a finer-scale colony-level analysis than that used here, which could account for regional differences in seasonal abundances of octopus production (Katsanevakis and Verriopoulos 2006; Steer *et al.* 2006; Stark *et al.* 2008).

Conclusions and future directions

This study provides detailed information on the diet of ASL at several breeding colonies in South Australia. Future diet studies should complement analyses of prey hard parts with alternative methods, such as faecal DNA-based analyses (Deagle *et al.* 2005; Casper *et al.* 2007; Bowles *et al.* 2013; Peters *et al.* 2015). Feeding trials using captive otariids and several studies on the diets of wild otariids have shown DNA-based faecal analyses can improve prey detection rates compared with other methods (Deagle *et al.* 2005a; Casper *et al.* 2007b; Bowles *et al.* 2011). Combined with biotelemetry studies, faecal DNA-based analyses may improve understanding of the habitats used by ASL, as well as interactions with commercial and recreational fisheries.

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CHAPTER 3

PCR-based techniques to determine diet of the endangered Australian sea lion (*Neophoca cinerea*): a comparison with morphological analysis

ABSTRACT

Comprehensive dietary information for the endangered Australian sea lion (*Neophoca cinerea*) is currently limited by the deficiency and poor quality of identifiable prey remains recovered from regurgitate and faeces and the difficulty of observing feeding in the wild. In this study, DNA-based prey detection methods were assessed using conventional (end-point) and quantitative real-time PCR (qPCR) on faeces collected from two captive Australian sea lions fed experimental diets of whole teleost fish, squid and shark tissue. PCR prey detection methods using the mitochondrial cytochrome oxidase subunit I (COI) and 16S genes combined with clone sequencing were compared with prey identified using traditional hard part analysis. The molecular results indicated that prey DNA was degraded. However, prey amplification was successful by targeting short (71 bp) DNA fragments. Both conventional PCR and qPCR techniques significantly increased prey detection compared with analysis of hard parts. For both sea lions, the hard part analysis was constrained by sporadic and extremely low recovery of fish otoliths (<2%), and cephalopod beaks were not recovered from the 116 squid fed. Comparisons between PCR techniques indicated comparable prey detection frequencies for all species tested; however, the sensitivity and greater resolution of qPCR improved prey detection by ~25% in one sea lion fed the experimental squid and perch. The detection of squid DNA ≤ 6 day post-ingestion by qPCR further exhibits the ability and potential of this method to detect low concentrations of infrequent or pulse prey. This study highlights the use of DNA-based analysis to detect prey taxa in the absence of identifiable hard prey remains.

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INTRODUCTION

Obtaining comprehensive knowledge of diet in high trophic level marine predators is often difficult because direct feeding events are rarely observed. For marine mammals such as pinnipeds, identification of prey skeletal remains recovered from faeces, stomach or regurgitate offer a reasonable and relatively non-invasive substitute, providing valuable information on foraging habits, the distribution and abundance of prey, and their relative biomass consumed (Fea *et al.* 1999; Childerhouse *et al.* 2001; McIntosh *et al.* 2006; Casper *et al.* 2007a; Longenecker 2010).

Dietary analyses based on the acquisition of prey hard parts are subject to well-documented biases (Boyle *et al.* 1991; Gales and Pemberton 1994; Bowen 2000; Staniland 2002). These largely stem from differences in digestive tolerance between diagnostic structures that may remain within the gastrointestinal tract, and remains that may be egested, fragmented and passed in faeces (Harvey 1989; Needham 1997; Tollit *et al.* 2003; Page *et al.* 2005; McIntosh *et al.* 2006). Marine predators may also consume only fleshy components or soft-bodied prey devoid of hard bony structures (i.e. cartilaginous fish). As a result, dietary analysis using these methods is challenged by the potential to under- or over-estimate the importance of the prey taxa consumed, leaving estimates of diet often incomplete (Dellinger and Trillmich 1987; Tollit *et al.* 1997; Kvitrud *et al.* 2005; Casper *et al.* 2006; Baylis and Nichols 2009a).

The Australian sea lion (*Neophoca cinerea*) is a top-level marine predator endemic to Australia and globally considered one of the rarest otariids. The endangered population (Goldsworthy and Gales, 2008; Goldsworthy et al. 2015), currently estimated at ~14,780 individuals (Shaughnessy *et al.* 2011), is distributed amongst 76 breeding colonies extending from subtropical Houtman-Abrolhos (113°47’E, 28°43’S), Western Australia, to the cool

temperate Pages Islands (35°45'S, 138°18'E), South Australia (McKenzie *et al.* 2005; Shaughnessy *et al.* 2011). The unusual life history of the Australian sea lion (characterised by protracted 17–18 month reproductive cycles, breeding asynchrony among colonies and extreme female natal site fidelity), and a population in recovery from historical sealing has effectively shaped a genetically autonomous substructure across most of their range (Campbell *et al.* 2008; Lowther *et al.* 2011). As a result, intrinsically slow population growth, low abundances and recorded subpopulation declines (Goldsworthy *et al.* 2009) have been met with considerable concern. Understanding the factors that sustain population viability will therefore probably be key to the conservation of the species.

One of the fundamental gaps in Australian sea lion biology and a critical step for their future management rests in understanding their trophic habits and defining key prey. Diet is not well understood, predominantly as a result of the deficiency and poor quality of prey remains recovered from regurgitate and faeces. Dietary analyses based on hard parts have indicated that Australian sea lions exploit a range of benthic species including cephalopods, fish, crustaceans and occasional elasmobranchs, but few remains survive digestion and, of those which do, are rarely defined to species (Walker and Ling 1981; Richardson and Gales 1987; Gales and Cheal 1992; Ling 1992; McIntosh *et al.* 2006; Gibbs 2008). Other non-destructive dietary methods such as fatty acid and stable isotope analysis have offered broader trophic-level insights into prey utilisation and habitat use, but owing to broad similarities in chemical signals amongst prey, these have provided little fine-scale taxonomic information on the species consumed (Baylis and Nichols 2009a; Baylis *et al.* 2009b; Lowther and Goldsworthy 2010; Lowther *et al.* 2012).

DNA-based dietary analysis through sequence analysis of prey DNA resolved from faecal and stomach remains has provided a reliable and non-invasive approach to evaluate prey consumed by a range of marine predators including seals (Purcell *et al.* 2004; Casper *et al.* 2007b; Deagle and Tollit 2007; Braley *et al.* 2009; Dunn *et al.* 2010; Marshall *et al.* 2010). Identification of prey using this method relies on the assumption that remnant fragments of DNA from food consumed survive the digestive process, and are identified through amplification of conserved sequences using PCR. Although reliability of DNA identification can depend upon the comparability and quantity of sequence information in the gene region of interest (e.g. Ward *et al.* 2005, 2008), the extent of DNA prey detection as a complementary method or alternative to traditional dietary analysis methods has rapidly grown (Sydmonson 2002; King *et al.* 2008).

In this study, feeding trials on captive Australian sea lions were used to explore the potential for DNA-based faecal analysis to provide preliminary assessments of prey consumption. Feeding trials on captive species can facilitate a progressive means to investigate new dietary techniques in a controlled environment. By removing some of the methodological constraints imposed by traditional diet analysis, captive-based assessments using molecular diet analyses can potentially pilot a step to quantifying trophic interactions in species and wild populations that lack dietary information (Tollit *et al.* 2009; Bowles and Trites 2013). The aims of this study were to (i) assess end-point PCR and quantitative real-time PCR (qPCR) DNA-based techniques to determine their suitability to amplify and detect fish, shark and cephalopod prey DNA recovered from Australian sea lion faeces, and (ii) compare the DNA diet results with prey detected and identified using traditional hard-part (HPA) methodology.

MATERIALS AND METHODS

Trial animals

At the time of the study, few Australian sea lions were available in captivity. I conducted the feeding experiment on one adult male and an adult female Australian sea lion held at the Adelaide Zoological Gardens, South Australia, during January and February 2006. Each sea lion was housed in a separate main enclosure during daylight hours (~8-10 h) and overnight (~14 h) housed separately in an evening pen. Evening pens contained a shallow pool and sprinkler system to limit heat stress to each animal. Each pool was fitted with a 100µm outlet filter.

Daily Feeding and experimental diet

The feeding trial was conducted over 48 days. Meals weighing 6 kg were fed by hand in two allocations per day to each sea lion (0.3 kg morning; 5.7 kg evening). For each sea lion, diets contained at least one prey taxon fed at 60% (3.6 kg), 50% (3.0 kg), 40% (2.4 kg), 30% (1.8 kg) or 10% (0.6 kg) (Table 1). The range of prey taxa fed was fresh whole calamary squid (*Sepioteuthis australis*), fresh gummy shark (*Mustelus antarcticus*) boneless fillets and two frozen fish: tommy rough (*Arripis georgianus*) and striped perch (*Pelates octolineatus*).

Table 1. Contribution of diet, prey species, number of days fed, and number of scats collected for hard-part and DNA-based diet analyses for the adult male and female Australian sea lion experimental trial. Prey taxa are: striped perch (*Pelates octolineatus*) (SP), tommy rough (*Arripis georgianus*) (TR), gummy shark (*Mustelus antarcticus*) (GS) and squid (*Sepioteuthis australis*) (SQ).

Sex	Diet	Prey species fed	Proportion (%) of prey / 6 kg daily diet	No. days fed	No. scats collected
Male	**C1**	SP, TR	50-50	5	2
	A	SP, TR, SQ	10-30-60	7	3
	B	SP, TR, SQ	30-60-10	7	4
	C	TR, GS, SQ	60-30-10	5	0
	D	TR, GS, SQ	40-30-30	9	9
	C2	SP, TR	50-50	14	10
Female	**C1**	SP, TR	50-50	47	30

Male and female diets

Two different diets were fed to the male and female. The female was fed a single diet containing an equal mass of the two fish taxa (3:3 kg, 50%) for the duration of the trial. Four different experimental diets (A, B, C, D) were fed to the male (Table 1). Diet A (60% squid, 30% tommy rough, 10% striped perch) and diet B (10% squid, 60% tommy rough, 30% striped perch) were fed for 7 days. Diet C (10% squid, 60% tommy rough, 30% shark) and diet D (30% squid, 40% tommy rough, 30% shark) were fed for 5 and 9 days, respectively. A diet containing an equal mass of the two fish taxa (3:3 kg, 50%) was fed for 5 days (diet C1) and 14 days (diet C2) prior to and after experimental diets, respectively. This facilitated initial and final detection times of experimental prey. The boneless shark fillets fed to the male sea lion (diets C and D) were used to assess the accuracy of DNA-based prey detection in the absence of skeletal remains.

Faecal collection and preparation

Faecal samples were collected for a total of 48 days. Each morning, the evening enclosures were checked for faeces, and the pools were drained and hosed clean. Day enclosures were checked hourly for faeces between 07:00 and 12:00 h and then every 2 h until animals were returned to the evening holding enclosures. Collected faeces were stored in sterile bags containing 95% ethanol, homogenised and then stored at -20 °C.

DNA preparation and extraction

Prior to DNA extraction, faecal-ethanol mixtures were re-homogenised and a subsample taken. The ethanol supernatant was removed from each subsample and the faecal slurry was freeze-dried and finely macerated. DNA was then isolated from 200 mg of each faecal subsample using a QIAamp DNA Stool Mini Kit (QIAGEN, Venlo, Netherlands). DNA yield was initially quantified using PicoGreen™ dsDNA Quantitation reagent (Molecular Probes™, Invitrogen, Mulgrave, Victoria, Australia) on a Wallac1420 multilabel fluorometer. Prior to PCR, DNA extractions were diluted to 3000 $pg \cdot \mu^{-1}$. Genomic DNA for the PCR controls was extracted from 10 mg of prey muscle tissue using QIAGEN DNeasy Tissue kits (QIAGEN). A DNA extraction blank (containing no faeces) was included in each batch of extractions (n = 12) to monitor sample contamination.

Morphological analysis preparation

Prey hard parts were isolated from the remaining ethanol/ faecal mixtures by washing through 1.0- and 0.5-mm nested sieves. Fish otoliths were photographed using digital microscopy supported by the image analysis software IMAGE PRO 5.1® (Media Cybernetics Inc, Rockville, MD, USA). Where possible, fish otoliths were identified to species by comparison with a reference collection of otoliths obtained from freshly dissected specimens.

For each fresh specimen, otolith size (mm), mass (mg) and robustness (otolith mass/otolith length) was calculated (Tollit *et al.* 1997). Remains identified to species were presented as numerical abundance (NA; percentage of total prey items made up by each prey taxon) and frequency of occurrence (FOO; percentage of samples containing a given prey taxa).

Primer design

I conducted preliminary PCR analyses testing a range of 100-250 base-pair (bp) target fragments but these did not amplify prey DNA. Three primer sets (gummy shark, striped perch and squid) were then designed to target a short fragment for each species tested. The gummy shark and squid primer sets target a 71-bp region of the mitochondrial cytochrome oxidase subunit I (COI) gene (Table 2). The striped perch primer set amplifies a 71-bp region of the 16S mitochondrial DNA gene (Table 2). Each primer set was constructed using ABI (Applied Biosystems, Waltham, MA, USA) PRIMER EXPRESS v 2.0® software (Warrington, UK) from published sequences and genomic DNA of prey species sequenced at the Australian Genomic Research Facility (AGRF) Brisbane, Australia (GenBank accession nos: AF075401, AF075386, DQ108315, DQ108311, GU205407). Optimal primers were re-assessed by eye and tested against an alignment of sequence profiles from the experimental species, species of taxonomic similarity, Australian sea lion and other marine taxonomic groups using DNAMAN v. 6.0© (Lynnon Corporation, Point Claire, Quebec, 2005). Each primer set was screened using BLASTn in GenBank to determine primer specificity (Altschul *et al.* 1990).

Table 2. Primer sequences used to amplify prey DNA from ASL faeces in this study.

Primer name	Target taxon	Target species	Gene region	Primer sequence (5' - 3')	Product size
SPerch16sF	Fish	striped perch	mtDNA 16s	GGCACTCCCCTATCACCAAG	71bp
SPerch16sR	Fish	striped perch	"	GGCTATGCCGGATCTGTTTG	
SepioCO171F	Cephalopod	calamary squid	mtDNA (COI)	CCCCTTTATCAAGTAACCTCTCACA	71bp
SepioCO171R	Cephalopod	calamary squid	"	AGCTAAGTGGAGGGAAAAAATGG	
GummyCO171F	Shark	gummy shark	"	TTGGTGCATGAGCAGGCATA	71bp
GummyCO171R	Shark	gummy shark	"	TCCTGGTTGTCCCAGTTCG	

Conventional PCR

For shark and striped perch, PCR amplifications (25 µl) contained 2 µl template DNA (3 ng), 2.0 mM $MgCl_2$, 1 x QIAGEN PCR buffer, 1 x bovine serum albumin (0.01%), 10 µM 9 deoxyribonucleotide triphosphates (DNTP), 10 µM primers and 1 x 0.625 unit·μl^{-1} HotStar Taq DNA polymerase (QIAGEN). Reagents for squid PCR amplification were similar to this, except that 1.5 mM MgCl2 was used. Thermal cycling conditions for shark amplifications were: 95 °C for 15 min, 94 °C for 2 min, followed by 32 cycles of: 94 °C for 10 s, 64.1 °C for 30 s and 72 °C for 30 s. Thermal cycling conditions for squid and striped perch amplifications were similar to the shark amplification conditions except for the annealing temperature being 60 °C. A final extension step of 72 °C for 10 min was included in all assay runs. PCR amplifications were detected by gel electrophoresis on 1.5% agarose gel stained with Gelred™ (Biotium, Hayward, ward, California, USA) and visualised using Gel-DOC UV illuminator (Bio-Rad©, Hercules, California, USA) supported by QUANTITY ONE software (Bio-Rad®). Replicate PCRs were performed to confirm results from the first PCR attempt. Positive PCR reactions were quantified using PicoGreen™ dsDNA Quantitation reagent (Molecular Probes™ Invitrogen) on a Wallac 1420 multi-label fluorometer. PCRs included negative controls to check for contamination. All PCR preparations used UV-sterilised aerosol-resistant filter tips, consumables and equipment. PCR's were prepared in DNA-free laminar flow UV sterilisation hoods.

Sequencing

Positive PCRs were purified using Nucleospin™ (Machery-Nagel, Bethlehem, PA, USA) Extract II PCR cleanup gel extraction kits (Macherey-Nagel, Easton, PA, USA), cloned using pGEM™-T easy cloning vector (Promega, Madison, WI, USA) and grown overnight on 5-Bromo- 4-chloro-3-indolyl β-D-galactopyranoside (X-gal) media. Transformed colonies (white colonies) were selected and PCR amplified using vector primers Sp6/T7. Clones that yielded PCR product were purified using NucleoSpin™ Plasmid DNA purification kits (Machery-Nagel) and sequenced at AGRF. Sequences were identified using BLASTn in GenBank and compared by alignment with reference sequences of the experimental species (GenBank accession nos. GU216244-GU216246, GU216247-GU2 16251, GU216252-GU216254).

Real-time quantitative PCR (qPCR)

To estimate mtDNA content of each prey species in seal faecal samples, a DNA standard curve was created for each qPCR assay. Firstly, DNA was extracted from each prey species. DNA concentration was then estimated using a known amount of Lambda DNA (Promega™, Lambda DNA/HindIII markers) and Quant-iT™ PicoGreen™ (Invitrogen). DNA concentration of each target DNA was adjusted to 200 ng·µl^{-1} and eightfold dilution series (200–0 ng·µl^{-1}) were prepared and used as a reference standard on each PCR run. The mtDNA content of each faecal sample was then interpolated from the standard curve.

qPCR amplifications (total of 20 µl) contained 0.4 µM primers, 1 x Power SYBRTM Green master mix (Applied BioSystems) and 4 µl template DNA (12 ng). Reactions were performed in triplicate on an ABI prism™ Real-time 7900HT sequence detection system (Applied BioSystems) with the fractional cycle (C_t) set to 10 deviations above mean fluorescence

threshold (0.1). Thermal cycling conditions for each PCR reaction were: 95 °C for 10 min, followed by 32 cycles of 95 °C for 15 s, 60 °C for 60 s. A dissociation stage of 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s was included in all assay runs. Melt curves for each sample reaction were assessed and compared against the inflection point (Tm)/melt curves for each species standard curve. Samples were not included in the analysis if melt curves deviated from the amplicon peak.

Data analysis

Prey detection for each dietary method was compared using FOO. For qPCR, estimates for each amplified sample were expressed as $fg \cdot \mu l^{-1}$ mtDNA represented as mean ± SD. Owing to the small experimental sample size (n = 1 in most cases) and limited comparative data, differences amongst diet treatments (male only) were compared using paired t-tests providing that data assumptions of normality and variance were met (SPSS v. 19, 2010) (IBM Australia Ltd, St Leonards, NSW, Australia). Data were otherwise transformed or the non-parametric equivalent performed. Comparisons of qPCR estimates between the male and female sea lions were only performed for striped perch (50% diet) using the Mann-Whitney U-test.

RESULTS

Sample collection

Thirty faecal samples were collected from the female sea lion. Twenty-eight faecal samples were collected from the male (Table 1). Ninety-seven per cent of faeces were collected from the evening enclosures. For the male, 2/10 (43%), 3 (11%), 4 (14%) and 9 (32%) faeces were collected from diets C1/C2, A, B and D, respectively (Table 1). Faeces were not collected during diet C. Multiple faecal samples were collected on three consecutive days during diet D (day 29 (n = 3), day 30 (n = 2), day 33 (n = 2)).

Prey hard-parts

Prey hard parts were recovered from 23 (82%) faeces collected from the male and 30 (100%) faeces from the female sea lion. Fish otoliths were the only remains recovered that were identifiable to species or genus. Only one striped perch otolith was recovered from 6674 otoliths (3337 fish) fed to the female. Seventeen faecal samples produced by the male contained fish otoliths but only 15 faeces contained otoliths that were identified to species. Otolith recovery was sporadic (1–22 per faecal sample). In total, 110 (~2%) otoliths were recovered (Table 3). The percentage of otoliths recovered for each fish species fed ranged between 0.7 and 6.5% per diet (Table 3). Of these, tommy rough (n = 70) were detected in 13 faeces and striped perch (n = 13) in five faeces. Highly eroded otoliths (n = 27) were recovered from 10 faecal samples but not assigned a species (Table 3 and Table 4). The larger size, mass, robustness (r) and number of tommy rough fed compared with striped perch (6.59 ± 0.44 and 5.93 ± 0.14 mm; Kruskal-Wallis test: χ^2= 22.4, df = 86, P = <0.01; 25.5 ± 0.48 and 20.2 ± 1.9 mg; Kruskal-Wallis test: χ^2 = 16.01, df = 86, P = <0.01; 3.85 ± 0.05mm and 3.47 ± 0.25mg; Kruskal-Wallis test: χ^2 = 12.52, df = 86, P = <0.01; feed ratio ~3:1; Wilcoxon test, Z = -5.039, P = <0.05; respectively) probably contributed to the greater number of tommy rough otoliths recovered. Cephalopod beaks were not recovered from the 116 squid consumed by the male.

PCR optimisation

PCR primers showed high specificity in amplifying target DNA of the three taxa tested. Assays did not amplify sea lion DNA. For qPCR, all assay standard curves showed linearity, with r^2 values ≥0.984 and slopes (Δs) between -3.26 and -3.38. The linear range for each prey standard was 0.2–20 fg·µl^{-1} (squid), 0.2–2.0 fg·µl^{-1} (shark) and 0.2–20 fg·µl^{-1} (striped perch). Melt curve profiles complemented standard curves for each species tested (77.5 ± 0.2 and

77.3 ± 0.2 °C (striped perch), 75.6 ± 0.3 and 75.3 ± 0.4 °C (squid), 77.2 ± 0.4 and 77.7 ± 0.3 °C (shark), respectively).

Table 3. Total number of fish otoliths ingested and the number recovered from faecal samples collected during experimental diets fed to the male Australian sea lion. Overall percentage (%) (bold) is represented as a function of (i) diet for each fish species ingested and (ii) the total percentage of otoliths recovered for both fish species ingested.

Prey type	C1 (2)	A (3)	B (4)	C (0)	D (9)	C2 (10)	Total
Tommy rough							
Total otoliths ingested	420	288	752	558	702	1176	3896
Total otoliths recovered	5	16	4	0	11	34	70
Recovered / diet (%)	**1.2**	**5.6**	**0.5**	**0.0**	**1.6**	**2.9**	**1.8**
Striped perch							
Total otoliths fed	290	84	230	0	0	812	1416
Total otoliths recovered	0	3	0	0	0	10	13
Recovered / diet (%)	**0.0**	**3.6**	**0.0**	**0.0**	**0.0**	**1.2**	**0.9**
Unidentified otoliths	0	5	1	8	6	7	27
Recovered / diet (%)	**0.0**	**1.3**	**0.1**	**1.4**	**0.9**	**0.4**	
Total recovered combined (%)	**0.7**	**6.5**	**0.5**	**1.4**	**2.4**	**2.6**	

Table 4. Frequency of occurrence (FOO) and numerical abundance (NA) of prey items recovered from faeces produced by the male Australian sea lion.

Common name	Prey species	FOO		NA	
		n	%	*n*	%
Tommy rough otoliths	*Arripis georgianus*	13	46.4	70	63.6
Striped perch otoliths	*Pelates octolineatus*	5	17.9	13	11.8
Unidentified otoliths		10	35.7	27	24.6
Unidentified fish remains (vertebrae, scales, eye)		23	82.1	-	-
Calamary squid	*Sepioteuthis australis*	-	-	-	-
Gummy shark	*Mustelus antarcticus*	-	-	-	-

Prey detection by PCR and comparison to hard parts

Conventional PCR and qPCR assays equally amplified target DNA for each of the prey species tested at the percentages fed (10%, 30%, 50% and 60%). In total, striped perch DNA was detected and identified in 86% (n = 24) of faecal samples, squid in 71% (n = 20) and shark in 32% (n = 9) of faecal samples produced by the male sea lion (Table 5). Striped perch DNA was equally detected in all faeces (n = 30) produced by the female using either PCR technique. For all test species, the frequency of detection using PCR was significantly greater than detection and identification by prey hard parts (all species and PCR tests: P = <0.001 male and female).

Comparison of prey detection between PCR techniques

Detection of striped perch and squid by qPCR provided better detection frequencies than conventional PCR in faeces collected from the male. Compared with conventional PCR, detection of DNA by qPCR increased by 25% (n = 24 faeces versus n = 17 faeces) for striped perch and 21% (n = 20 faeces versus n = 14 faeces) for squid (Table 5). For both taxa, higher qPCR FOO resulted from low qPCR estimates for each target species (56.5 ± 44.7 and 50.7 ± 30.0 $fg \cdot \mu l^{-1}$, respectively). For striped perch, additional qPCR detections occurred at the lowest percentage fed (10%) and from four consecutive faecal samples recovered 6 days after its final ingestion at the conclusion of diet B. For squid, additional qPCR detection occurred at the lowest percentage fed (10%, diet B, n = 3), and from three faecal samples collected 9 days after the final ingestion of squid. Six faeces collected prior to these did not test positive for squid DNA.

qPCR prey comparisons

The qPCR estimates were highly variable amongst faeces of each test species for the percentages fed in both the male and female sea lion (Figures 1 and 2). For the male, comparison between striped perch qPCR estimates decreased significantly with a decrease in percentage fed (mean 2071.3 ± 1468.3 $fg \cdot \mu l^{-1}$ (50%), 226.6 ± 146.5 $g \cdot \mu l^{-1}$ (30%), 34.51 ± 51.8 $fg \cdot \mu l^{-1}$ (10%); all comparisons $P = \leq 0.006$). For squid, qPCR estimates were only significant for higher dietary percentages (60% and 30%) compared with low percentages fed (10%) ($Z = -1.99$, $P = 0.04$, and $Z = -2.201$, $P = 0.027$, respectively) but qPCR estimates were similar at the highest percentages fed (60% and 30%; $Z = -0.314$, $P = 0.75$). Faeces collected from the female sea lion exhibited significantly higher qPCR estimates than the male when dietary percentages containing 50% striped perch were fed (female: median 10596 $fg \cdot \mu l^{-1}$, mean 9518.2 ± 6633.2 $fg \cdot \mu l^{-1}$; male: median 1384 $fg \cdot \mu l^{-1}$, mean 2071.3 ± 1468.3 $fg \cdot \mu l^{-1}$; Mann-Whitney U:$Z = -5.380$, $P = <0.001$; Fig. 2).

Table 5. Diet assessment methods used to detect prey (presence / absence) in faeces collected from the adult male Australian sea lion. Samples (1 - 28) correspond to faeces collected during the experimental diet periods (see text). Prey contributions for each diet were: tommy rough (TR), striped perch (SP), squid (SQ), and gummy shark (GS). Detection methods used for the analysis were: Conventional PCR (PCR), quantitative PCR (qPCR), and hard part analysis (HPA). * Species not tested.

Experimental diet fed	Diet composition	Scat sample	Tommy rough (TR)			Striped perch (SP)			Squid (SQ)			Gummy shark (GS)		
			PCR	qPCR	HPA	PCR	qPCR	HPA	PCR	qPCR	HPA	PCR	qPCR	HPA
C1	TR : SP	1	*	*	+	+	+	-	-	-	-	-	-	-
C1	TR : SP	2	*	*	-	+	+	-	-	-	-	-	-	-
A	SQ : TR : SP	3	*	*	+	+	+	-	+	+	-	-	-	-
A	SQ : TR : SP	4	*	*	+	-	+	+	+	+	-	-	-	-
A	SQ : TR : SP	5	*	*	+	-	+	-	+	+	-	-	-	-
B	SQ : TR : SP	6	*	*	+	-	+	-	+	+	-	-	-	-
B	SQ : TR : SP	7	*	*	-	+	+	-	-	+	-	-	-	-
B	SQ : TR : SP	8	*	*	-	+	+	-	+	+	-	-	-	-
B	SQ : TR : SP	9	*	*	-	+	+	-	-	+	-	-	-	-
D	SQ : TR : GS	10	*	*	+	+	+	-	+	+	-	-	-	-
D	SQ : TR : GS	11	*	*	-	-	+	-	+	+	-	+	+	-
D	SQ : TR : GS	12	*	*	+	+	+	-	+	+	-	+	+	-
D	SQ : TR : GS	13	*	*	+	+	+	-	+	+	-	+	+	-
D	SQ : TR : GS	14	*	*	-	-	+	-	+	+	-	+	+	-
D	SQ : TR : GS	15	*	*	-	-	-	-	+	+	-	+	+	-
D	SQ : TR : GS	16	*	*	+	-	+	-	+	+	-	+	+	-
D	SQ : TR : GS	17	*	*	-	-	-	-	+	+	-	+	+	-
D	SQ : TR : GS	18	*	*	-	-	-	-	+	+	-	+	+	-
C2	TR : SP	19	*	*	-	-	-	-	-	+	-	+	+	-
C2	TR : SP	20	*	*	+	-	+	+	-	-	-	-	-	-
C2	TR : SP	21	*	*	-	+	+	-	-	-	-	-	-	-
C2	TR : SP	22	*	*	+	+	+	-	-	-	-	-	-	-
C2	TR : SP	23	*	*	+	+	+	+	-	-	-	-	-	-
C2	TR : SP	24	*	*	+	+	+	+	-	-	-	-	-	-
C2	TR : SP	25	*	*	-	+	+	-	-	-	-	-	-	-
C2	TR : SP	26	*	*	-	+	+	+	-	+	-	-	-	-
C2	TR : SP	27	*	*	-	+	+	-	-	+	-	-	-	-
C2	TR : SP	28	*	*	-	+	+	-	-	+	-	-	-	-
		Total	**-**	**-**	**13**	**17**	**24**	**5**	**14**	**20**	**0**	**9**	**9**	**0**

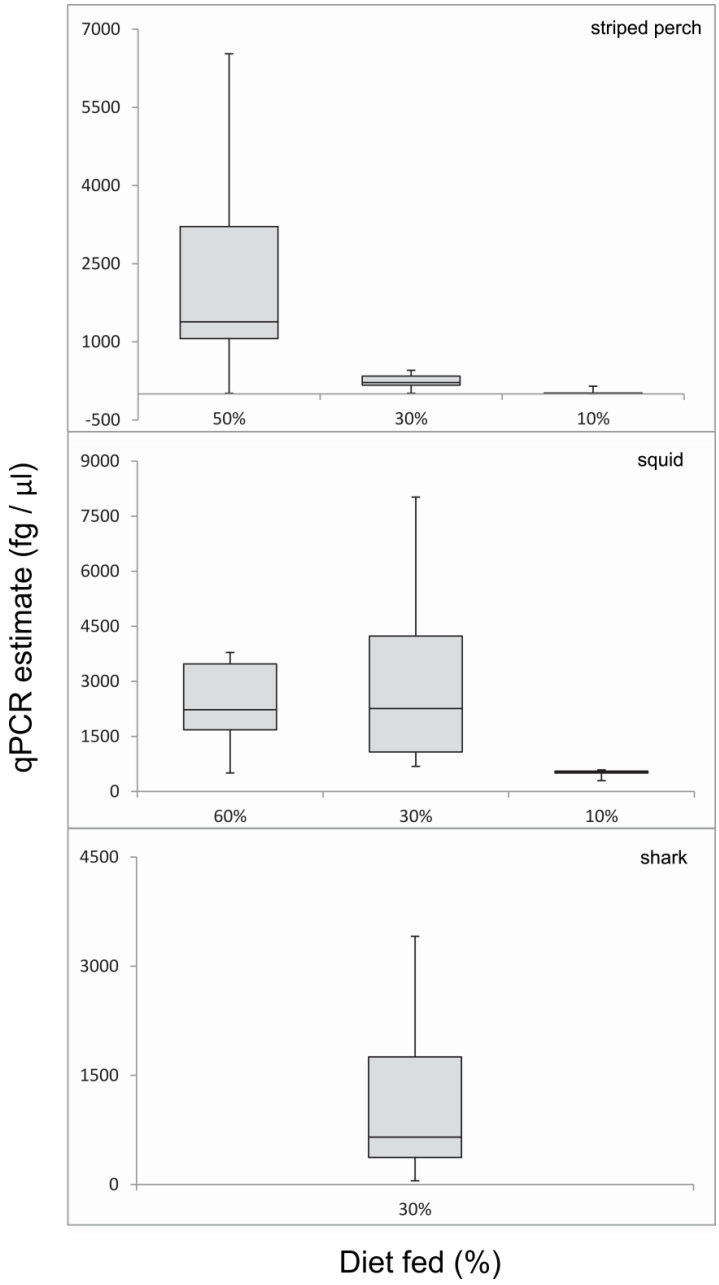

Figure 1. Striped perch, squid and shark qPCR estimates from faeces collected from the male. Quantitative estimates were compared for faeces collected during the dietary proportions fed (60%, 50%, 30% and 10%).

Figure 2. Comparison of striped perch qPCR estimates from faeces collected from the male and female Australian sea lion when daily dietary proportions contained 50% (3 kg) striped perch.

DISCUSSION

Similar to DNA-based diet studies on other captive marine predators such as penguins, and otariid and phocid seals (Deagle *et al.* 2005; Deagle and Tollit 2007; Matejusová *et al.* 2008; Bowles *et al.* 2011), PCR-based diet analyses were successfully applied in this study to faeces collected from two captive Australian sea lions fed mixed diets comprising fish, shark and squid. The use of taxon-specific PCR greatly improved detection rates of all prey taxa tested, providing a more reliable technique to determine prey ingestion than prey detection by traditional hard-part analysis. Here, shark, squid and striped perch DNA were respectively amplified and identified in 32%, 71% and 86% of faeces collected from the male and striped perch was detected in all faeces collected from the female. In comparison, prey identification by hard remains such as otoliths or squid beaks was poor, limiting faecal detection to <2% for both fish taxa in the male, and <1% in the female. Shark and squid were not identified by

hard parts, further highlighting the advantage of DNA-based techniques to detect and identify prey that may be retained in the gut or that have soft bodies or fragile digestible remains.

Hard part analysis

The low recovery of prey hard parts from my analyses is consistent with Gales and Cheal (1992), who documented limited recovery of fish otoliths (<2%) and cephalopod beaks from captive Australian sea lions fed a range of fish prey and squid. Expansion of their diet study to faecal analysis of free-living Australian sea lions further emphasised severe constraints in otolith recovery and condition, favouring a dietary bias toward the most highly robust remains. In this study, shark was the only taxon not anticipated to produce hard part results. However, the disparity illustrated between the recovery of fish otoliths and complete loss of cephalopod remains highlights several biases associated with (i) prey-specific and (ii) animal-specific digestion.

First, the absence of squid beaks recovered from the male sea lion confirms that amongst skeletal remains, cephalopods containing large beaks such as *Sepioteuthis australis* may be retained within the stomach for prolonged periods. In the present study, cephalopods were consumed for 28 consecutive days with no evidence of expulsion through regurgitation or faeces until the experimental trial ended (42 days). Although retention of cephalopod mouthparts is reasonably common in wild and captive otariid seals (Richardson and Gales 1987; Gales and Cheal 1992; Casper *et al.* 2006; McIntosh *et al.* 2006), their recovery was unreliable using the faeces collected here, imposing severe constraints for diet estimation and overall underestimating their importance in the diet. If traditional methods are solely used to infer prey ingestion in free-living seals, such biases could be partly improved if large sample sizes and complementary sampling of faeces and regurgitate are used (e.g. Page *et al.* 2005).

Second, although consumption of both fish species was significantly underestimated, the discrepancy between the ratio of tommy rough to striped perch ingested by the male (~3:1, respectively) compared with the overall recovery ratio of each species' otoliths (~ 5:1, respectively) suggests that there are differences in otolith digestion between the two fish species (Table 1). Variability in otolith digestion is common amongst fish species fed to captive seals (Tollit *et al.* 1997; Marshall *et al.* 2010); in particular, Arripidae (tommy rough) are known to survive digestion well (Casper *et al.* 2006). The dissection of fresh otoliths confirm this finding, indicating that tommy rough contained significantly larger, more robust otoliths than striped perch.

Lastly, despite being fed 20% more fish than the male sea lion, the disparity in otolith recovery between the female compared with the male sea lion possibly highlights intraspecific differences associated with digestion or sex differences between the individuals studied. In this case, the near-complete digestion of both striped perch and tommy rough otoliths by the female could have resulted from increased gut retention and assimilation of prey induced by the female's lengthy periods of inactivity compared with elevated activity (and greater otolith recovery) in the male. In other captive pinnipeds, such as Californian sea lions (*Zalophus californianus*), Steller's sea lion (*Eumetopias jubatus*) and South American fur seals (*Arctocephalus australis*), activity level has been demonstrated to be implicated in the recovery of fish remains, biasing diet estimates (Dellinger and Trillmich 1987; Bowen 2000; Orr and Harvey 2001; Tollit *et al.* 2003). Intra-specific differences amongst individual captive seals also appear to have some influence on digestive behaviour (Helm 1984; Casper *et al.* 2006). The low recovery of remains seem reasonably concordant with results reported by Gales and Cheal (1992) for other captive Australian sea lions; however, because otoliths were virtually absent in the female's faeces, this may indicate the level of digestion is unique

to the animal studied or an artefact of inactivity in the species *per se*. Further captive feeding studies on Australian sea lions would benefit our understanding of digestive physiology in this species.

Limitations of DNA-based study

Although the primary goal was to test the capability of prey detection using DNA-based analysis, clear technical and methodological biases inherent in DNA-based feeding trial studies must be acknowledged here. At the time of the study, few Australian sea lions were available for captive feeding experiments, which constrained the experimental sample size and restricted the ability to perform replicate trials. As a result, the small sample size (n = 1 in most cases) limited the qPCR dietary comparisons, and the irregularity and timing of faeces produced by the male possibly resulted in the large variation of prey DNA recovered amongst faeces. As a consequence and unlike other captive DNA-based seal diet studies (e.g. Deagle and Tollit 2007; Bowles *et al.* 2011), qualitative data was used omitting development of the primers for tommy rough although this would have probably provided better comparable data for the prey species ingested. Furthermore, digestive degradation of DNA can vary intrinsically amongst species (Deagle and Tollit 2007). It is unclear the level of digestive variability amongst the prey taxa tested in this study; however, frozen- then-thawed fish fed to each animal may have differences in cellular breakdown and some effect on DNA quality of these species prior to PCR amplification. Nonetheless, despite these reservations, short 71-bp amplicons of each prey tested were still achievable and provided reasonable information of prey ingestion.

PCR techniques

One objective for this study was to assess conventional and qPCR techniques in order to evaluate their success in detecting target DNA from faeces primarily devoid of identifiable

prey remains. Qualitatively, both PCR techniques demonstrated similar capabilities to detect prey DNA from faeces collected across the range of prey percentages fed (10%, 30%, 50% and 60%; Table 5). However, the sensitivity of qPCR ($\sim\leq 0.2 \times 10^{-5}$ ng·species^{-1}) supported by the sample melt curve analyses provided a higher detection frequency than conventional PCR for striped perch and squid in faeces collected from the male. In each case, the observed differences (n = 7 and n = 6 faeces, respectively) resulted from low qPCR estimates of target DNA, with most detections occurring after the prolonged absence of each prey in the diet (~6-9 days). The propensity of qPCR to detect such low concentrations of target DNA from faeces of other pinnipeds (e.g. Steller sea lions (5.2×10^{-4} ng·species^{-1}), grey seals (0.01%, salmonids) (Matejusová *et al.* 2008; Bowles *et al.* 2011), suggests these prey detections probably resulted from amplification of remnant DNA from tissue remaining in the gut. Furthermore, as faecal DNA for both conventional and qPCR tests were derived from the same samples, the discrepancy observed between the two PCR techniques therefore probably reflects a limitation in end-product resolution (gel-visualisation) of conventional PCR (e.g. Marshall *et al.* 2010; Bowles and Trites 2013).

Despite the accuracy of the qPCR standard curves, the limitation of small sample sizes affected the qPCR results, providing little quantitative information for the diets tested. Although targeted amplicons of the same size were used to partly control for differential digestion, the faecal qPCR estimates for each species tested varied widely (Figs 1 and 2) bearing little congruence with the different percentages of prey fed. The only meaningful comparison amongst diets (male only) indicated larger prey percentages (i.e. 60%, 50% and 30%) produced higher qPCR estimates compared with small dietary percentages (e.g. 10%); however, differences between higher prey percentages (60% versus 30% striped perch, 50% versus 30%, squid) were negligible, with each exhibiting extensive overlap.

Interestingly, there was a marked increase in qPCR estimate between the male and female when fed 50% striped perch (Fig. 2). This result was surprising, as it was expected the female's qPCR estimates to be lower than that of the male given the animal's inactivity and the limited recovery of prey hard parts. One possible explanation could be physiological digestive differences between each animal studied; however, one would expect DNA degradation to be somewhat consistent with the high level of digestion of prey remains. Alternatively, this result may indicate some methodological bias, possibly related to greater binding efficiency of striped perch that occurred during PCR amplification. It is difficult to draw inference from this limited data; thus, future attempts at quantifying the diet of captive sea lions using this method should where possible, focus efforts to include additional individuals and either longer feeding periods or a greater number of replicates of each diet.

Amplifying a small 71-bp fragment of mtDNA using prey-specific primers clearly provided a reliable method to elucidate prey ingestion in this study. However, the relative ease of designing short-fragment species-specific PCR tests compared with amplifying broader taxonomic groups using a group-specific PCR approach (e.g. Jarman *et al.* 2004, 2006) may impose some challenges for faecal- based analysis of Australian sea lions where DNA is likely to be degraded (Kohn *et al.* 1995; Kohn and Wayne 1997; Deagle *et al.* 2006; Casper *et al.* 2007a). The likelihood of detecting prey DNA recovered from faeces has been shown to increase with a decrease in target amplicon size (Deagle *et al.* 2006); thus, targeting larger homologous binding regions that amplify across multiple species (i.e. fish) may miss prey DNA in template that is highly fragmented. Conversely, targeting smaller prey fragments may be taxonomically less informative, limiting the ability to distinguish amongst closely related prey. The unsuccessful attempts to amplify 100-250-bp prey amplicons tend to

suggest a degraded starting template, although whether this also applies to other individuals or samples collected from the wild is yet to be determined. Upcoming dietary analysis of the Australian sea lion will invariably consider genetic-based faecal techniques following the fine taxonomic scale of dietary information obtained for other pinniped species (Deagle *et al.* 2005; Casper *et al.* 2007a). However, the resolution of prey diversity may depend on the PCR product size obtainable.

Conclusion and future directions

The consideration of dietary techniques to determine prey consumption in free-living marine predators often depends on the accessibility and availability of individuals to acquire samples. In active mobile foragers such as seals, traditional faecal analysis has provided a reliable, non-invasive source of dietary information to ascertain predator–prey relationships and emphasise interactions with fisheries (Gales and Pemberton 1994; Kirkwood *et al.*2008; Marshall *et al.* 2010). DNA-based diet assessments are now routinely adapted to seal faecal analysis, and although not free of bias, are providing highly resolved information of specific prey groups and targeted taxa (Parsons *et al.* 2005; Casper *et al.* 2007a,b; Tollit *et al.*2009). More informative, next-generation high-through put sequencing is further contributing substantial trophic information enabling greater inference at broader ecological scales (Deagle *et al.* 2009, 2010; Soininen *et al.* 2010).

Prey differentiation utilising genetic information obtained from either faecal or stomach contents presents a number of unique challenges because intrinsic biases resulting from differences in tissue digestibility and the susceptibility of DNA to degradation can be influenced by the behavioural and physiological characteristics of the consumer. The three techniques explored here exhibited notable differences; however, the consistent DNA signals and resolution obtained by PCR compared with limited detection by hard-part analysis clearly

highlight its suitability as a method to successfully detect and identify prey. In light of these results, future studies utilising PCR-based analysis that incorporate broader taxonomic groups and/or next-generation sequencing will provide valuable information of prey consumed by Australian sea lions.

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CHAPTER 4

Fine-scale diet of the Australian sea lion (*Neophoca cinerea*) using DNA-based analysis of faeces

ABSTRACT

DNA-based faecal analyses were applied to determine the diet of female Australian sea lions (n = 12) from two breeding colonies in South Australia. DNA dietary components of fish and cephalopods were amplified using the polymerase chain reaction (PCR) and mitochondrial DNA primers targeting the short (~100 base pair) section of the 16S gene region. Prey diversity was determined by sequencing ~ 50 amplicons generated from clone libraries developed for each individual. Faecal DNA was also combined and cloned from multiple individuals at each colony and fish diversity determined. Diets varied between individuals and sites. Overall, DNA analysis identified a broad diversity of prey comprising 23 fish and five cephalopod taxa, including many species not previously described as prey of the Australian sea lion. Labridae (wrasse), Monacanthidae (leatherjackets) and Mullidae (goat fish) were important fish prey taxa. Commonly identified cephalopods were Octopodidae (octopus), Loliginidae (calamary squid) and Sepiidae (cuttlefish). Comparisons of fish prey diversity determined by pooling faecal DNA from several samples provided reasonable but incomplete resemblance (55% -71%) to the total fish diversity identified across individual diets at each site. Interpretation of diet based on the recovery of prey hard-parts identified one cephalopod beak (*Octopus* sp.) and one fish otolith (*Parapriacanthus elongatus*). The present study highlights the value of DNA-based analyses and their capabilities to enhance information of trophic interactions.

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INTRODUCTION

Understanding feeding preferences is often the focus of ecological studies (Myers *et al.* 2007; Chiaradia *et al.* 2010). For high-order marine predators, information of diet is pivotal in the development of management strategies, as diet provides an insight into mitigating interactions between fisheries and elucidates important foraging habitat (Tollit *et al.* 1998; McConnell *et al.* 1999; Childerhouse *et al.* 2001).

Pinniped dietary information has been derived from analyses of prey hard-parts recovered from regurgitates, faeces or stomach contents and from predator tissues containing dietary fatty acids (FAs) or stable isotopes (SIA). Analysis of digesta for hard-parts is mostly non-invasive and can provide species-level taxonomic identification from undigested prey remains, and composition ratios of FAs and SIAs incorporated into a predator's tissues can be used to distinguish prey consumed over time, but these methods have well understood problems (Childerhouse *et al.* 2001; Iverson *et al.* 2004; Meynier *et al.* 2008, 2009; Baylis and Nichols 2009a; Baylis *et al.* 2009b). For example, the remains of prey hard-parts are subject to species-specific erosion and digestive retention, which can bias results (Tollit *et al.* 1997, 2003). Further, for consumers with broad dietary habits, FAs and SIAs are of limited use because they have low taxonomic and temporal resolution (Tollit *et al.* 1997; Dalsgaard *et al.* 2003; Baylis *et al.* 2009b; Meynier *et al.* 2009).

Polymerase chain reaction (PCR)-based DNA approaches, which aim to characterise the composition of prey DNA recovered from stomach and faecal remains, have become widely used for studying trophic interactions in marine environments (see Deagle *et al.* 2005b; King *et al.* 2008; Nejstgaard *et al.* 2008; Barnett *et al.* 2010; Marshall *et al.* 2010). DNA-based methods also have problems; for instance, prey identification is dependent on the survival of DNA during the digestive process, which may vary between species and quantity or tissue

type consumed (Deagle and Tollit 2007; Hartmann *et al.* 2011) and limit resolution to the most recent meals ingested (Deagle *et al.* 2005a; Casper *et al.* 2007b). Nevertheless, these methods are well suited to studying the diet of species where few prey remains can be recovered. For otariids such as fur seals and sea lions, DNA-based diet studies provide both complementary and alternative approaches to conventional hard-part analysis (Casper *et al.* 2007b; Deagle *et al.* 2009; Tollit *et al.* 2009).

Australian sea lions (*Neophoca cinerea*) are one of the rarest otariid seals in the world. The current population estimate of ~14,780 individuals (Shaughnessy *et al.* 2005, 2011) is distributed among 76 breeding colonies extending from Houtman-Abrolhos, Western Australia (113°47′ E, 28°43′ S) to the Pages Islands (35°45′ S, 138°18′ E), South Australia (McKenzie *et al.* 2005; Campbell *et al.* 2008; Shaughnessy *et al.* 2011; Lowther *et al.* 2012). Females exhibit an atypical, non-annual (~17.5 months) reproductive cycle coupled with high maternal site fidelity and low dispersal between breeding colonies (Higgins 1993; Higgins and Gass 1993; Gales *et al.* 1994; Campbell *et al.* 2008). This reproductive strategy may reflect reduced dependency on seasonal productivity typical of many annual breeding otariids (Lea *et al.* 2002; Baylis *et al.* 2008; Lowther *et al.* 2012), a theory supported by recent studies highlighting temporal resource stability and habitat specialisation (Lowther *et al.* 2011).

The diet of the Australian sea lion is poorly understood. Previous studies indicate Australian sea lions consume a range of benthic prey comprising teleost fish, crustaceans and cephalopods, but few identifiable remains have been recovered (Richardson and Gales 1987; Gales and Cheal 1992; McIntosh *et al.* 2006; Gibbs 2008; Baylis *et al.* 2009b; Lowther and Goldsworthy 2010). Feeding trials of captive sea lions indicate that less than 2% of otoliths are recovered and that larger prey items such as cephalopod beaks are often retained within

the digestive tract (Gales and Cheal 1992; McIntosh *et al.* 2006; Gibbs 2008; K.J. Peters, unpublished data).

In this study, faecal samples were collected from two Australian sea lion colonies in the Great Australian Bight (GAB) South Australia, and identify prey taxa from both hard-parts and prey DNA. The DNA of prey was PCR amplified using 'group-specific' genetic markers for fish and cephalopods, which are common prey (Gales and Cheal 1992; McIntosh *et al.* 2006). My aims were to: (i) determine the diversity of prey taxa by sequencing a large number of clones from a few individuals, (ii) compare the prey taxa recovered at two study sites, and (iii) determine whether pooling faecal DNA from multiple individuals provides a useful means to characterise diet at the colony/population level.

MATERIALS AND METHODS

Sample collection

Faecal samples were collected from female Australian sea lions during July-August 2006 at two breeding colonies in South Australia, Lilliput Island (LI), Nuyt's Archipelago (32° 26' 4.44" S, 133° 41' 34.79" E) and Seal Bay, Kangaroo Island (KI) (35º 59' 49" S, 137º 18' 21" E) (Fig. 1). For KI, fresh samples (soft and collected within ~12 hours of deposition) were collected whole. Samples from LI were collected via passive enema from individuals that had been recaptured to remove satellite tracking equipment. These individuals were caught by hoop net and anaesthetised using Isoflurane® (Veterinary Companies of Australia, Artarmon, New South Wales) administered via Cyprane Tec III gas-anaesthetic vaporiser (Advanced Anaesthetic Specialists, Melbourne). Enemas used distilled water and the faecal matter was stored in sterile jars. When samples settled, the water was removed. For both KI and LI, faecal samples were homogenised in sterile bags (Nasco-Whirl-Pak®;Nasco, Fort Atkinson, WI, USA) containing 95% ethanol and stored at -20ºC. Prior to DNA extraction, prey hard-

parts were isolated from ethanol/scat mixtures by washing through 1.0 and 0.5 mm nested sieves. Fish otoliths and cephalopod beaks were photographed using digital microscopy supported by image analysis software Image Pro 5.1©. All prey items were identified to the lowest taxonomic level by comparison with reference collections and atlases (Lu and Ickeringill 2002; Furlani *et al.* 2007).

To assess the number of prey taxa identified from individual samples, I utilised faecal DNA from 6 individuals per site (total 12 individuals for analysis) (Fig. 2). I also compared individual data to a pooled dietary approach for the fish component of the diet. In the pooled approach, extracted DNA samples were pooled at each site before analysis.

Molecular analysis

For DNA extraction, ethanol / faecal mixtures were re-suspended within each bag and a sub-sample taken. The ethanol supernatant was removed from the soft- matrix and the remaining faecal material was freeze-dried. Samples were macerated with sterile mortar and pestle, homogenised, and a 200-mg sample of dry faecal material was removed for DNA extraction. DNA was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN, Venlo, the Netherlands) and eluted in 100 µl buffer. DNA yield was initially quantified on a Wallac1420 multi-label fluorometer using PicoGreen® dsDNA Quantitation reagent (Molecular Probes-Invitrogen®; Invitrogen, Mulgrave, Vic, Australia). DNA was diluted to 3000 $pg \cdot \mu l^{-1}$ (~1:5) in distilled water prior to PCR. Extraction blanks were used to monitor DNA extraction contamination for each batch of 12 extractions.

Extracted DNA from each sample was re-homogenised and a 25-µl sub-sample was removed. Each DNA sub-sample was then divided into two equal 12.5-µl volumes with one volume used for the individual diet PCR- sequence analysis and the other one was combined (i.e. pooled) with the five remaining DNA sub-samples from the same site to form a single DNA

meta-sample. This ensured that clone sequence library results from each individual and pooled diet analysis were drawn from the same volume of DNA.

16S Fish primer set

The fish primer set (PCR A) amplifies ~100 bp fragment of the 16S mtDNA of chordates (Table 1). This primer set was designed for the DNA-based study by Deagle *et al.* (2009) to describe prey in the diet of the Australian fur seal (*Arctocephalus pusillus doriferus*). These primers were chosen for their potential to identify a large diversity of fish prey species, their short target amplicon size, and the large range of 16S prey sequence information available in GenBank. The specificity of the primer set was tested using amplification of genomic DNA extracted from a range of fishes, elasmobranchs, crustaceans and cephalopods obtained from benthic trawl experiments in the GAB conducted by the South Australian Research and Development Institute (SARDI). These were also used as positive controls during PCR (Table 1). This primer set also amplifies DNA of other chordates including the Australian sea lion. As faeces contain host DNA from epithelial cells (Albaugh et. al. 1992; Kohn and Wayne, 1997), the addition of a mitochondrial blocking primer was used to reduce binding and preferential amplification of sea lion DNA over prey DNA (Vestheim and Jarman, 2008). The blocking primer was added at 10 times the concentration of the fish primer set for PCR amplification (see Vestheim *et al.* 2011) (Table 2).

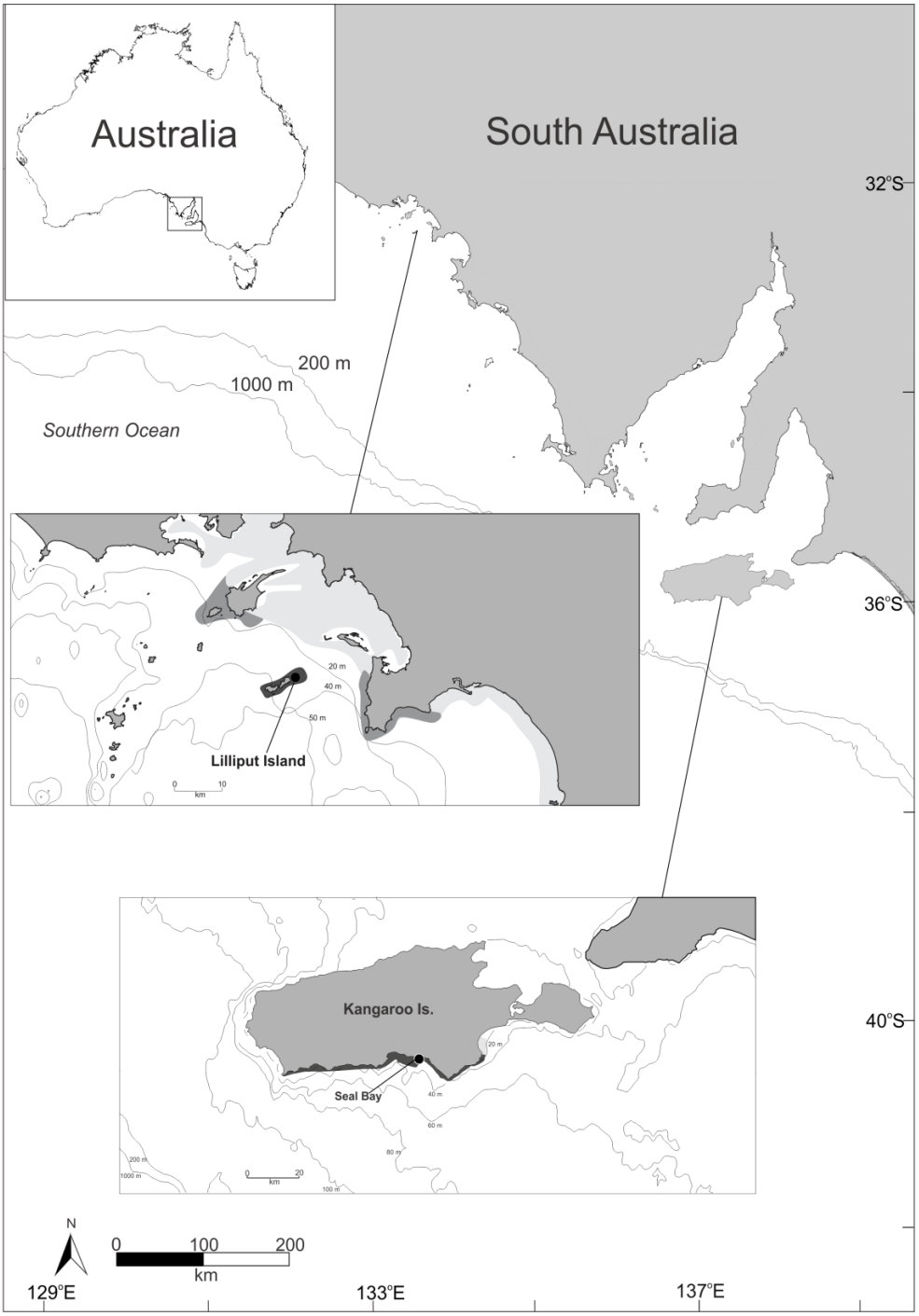

Figure 1. Study sites of two Australian sea lion colonies Lilliput Island and Kangaroo Island. Local benthic habitat is seagrass and unvegetated soft bottom (light grey), mixed reef, seagrass and unvegetated soft bottom (mid grey), and low profile reef (dark grey) (Edyvane *et al*. 1999; Bryars, 2003). Bathymetric contours are shown.

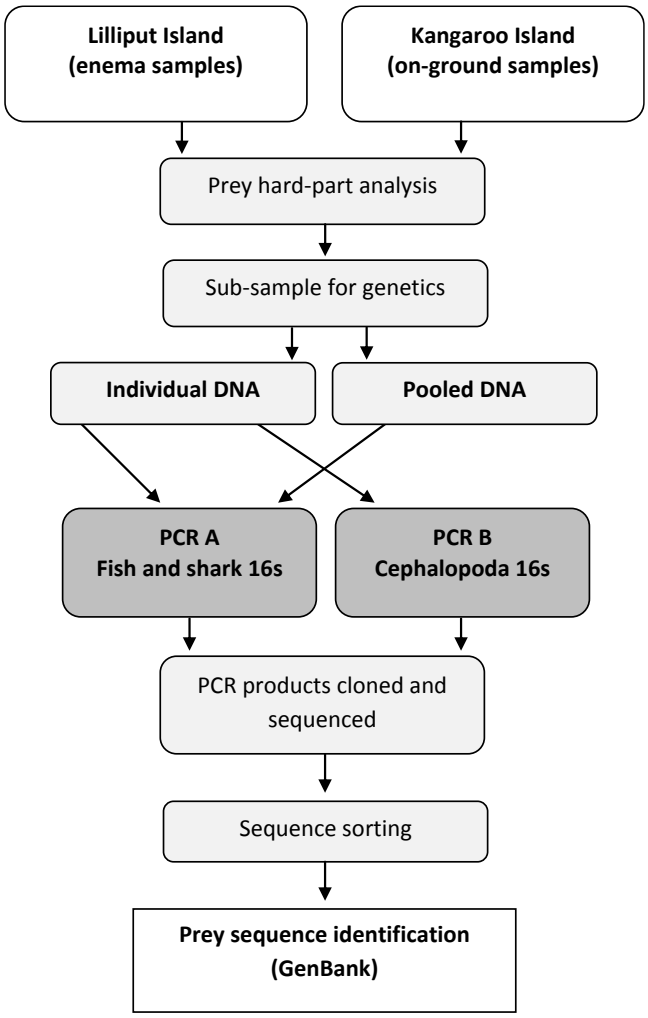

Figure 2. Outline of experimental procedure used to generate prey sequence data from Australian sea lion faeces collected from Kangaroo and Lilliput Island, South Australia.

Table 1. DNA extracted from fish, crustacean and cephalopod species used as positive controls to test the suitability of the mitochondrial 16S fish and cephalopod and primer sets.

Common name / Scientific name
Osteichthyes (Bony fish)
Jack mackerel (*Trachurus declivis*)
Silver trevally (*Pseudocaranx dentex*)
Sand flathead (*Platycephalus bassensis*)
Toothy flathead (*Neoplatycephalus aurimaculatus*)
Long head flathead (*Leviprora inops*)
Southern bluespotted flathead (*Platycephalus speculator*)
Common gurnard perch (*Neosebastes scorpaenoides*)
Silverbelly (*Parequula melbournensis*)
Southern school whiting (*Sillago bassensis*)
King George whiting (*Sillaginodes punctatus*)
Swallowtail (*Centroberyx lineatus*)
Horse-shoe leatherjacket (*Meuschenia hippocrepis*)
Spotted stink fish (*Repomucenus calcaratus*)
Blue-throated wrasse (*Notolabrus tetricus*)
Bulldog stargazer (*Xenocephalus armatus*)
Common bullseye (*Pempheris multiradiata*)
Rock Ling (*Genypterus tigerinus*)
Striped Perch (*Pelates octolineatus*)
Chondrichthyes (Cartilaginous fish)
Elephant shark (*Callorhinchus milii*)
Ornate wobbegong (*Orectolobus ornatus*)
Rusty catshark (*Parascyllium ferrugineum*)
Banded stingaree (*Urolophus cruciatus*)
Australian Angel shark (*Squatina australis*)
Crustacea
Mantis shrimp (*Erugosquilla grahami*)
Southern rock lobster (*Jasus edwardsii*)
Balmain bug (*Ibacus alticrenatus*)
Western King Prawn (*Penaeus laticulcatus*)
Cephalopoda
Southern calamary squid (*Sepioteuthis australis*)
Southern keeled octopus (*Octopus berrima*)
Hammer octopus (*Octopus australis*)
Southern dumpling squid (*Euprymna tasmanica*)
Striped pyjama squid (*Sepioloidea lineolata*)
Giant cuttlefish (*Sepia apama*)
Arrow squid (*Nototodarus gouldi*)

Table 2. Primer sequences (5' - 3') used to amplify fish and cephalopod prey DNA from Australian sea lion faecal samples. A blocking primer was used to reduce the amplification of sea lion DNA relative to prey DNA (Deagle et al. 2009). Shaded area indicates overlap region of 3'-end of forward fish primer and 5'-end of blocking primer.

Primer name	Target taxon	Target species	Gene region	Primer sequence (5' - 3')	Approximate product size (bp)
Fish and shark (F)	Fish + Chordates	Bony fish + shark + ray	mtDNA 16s	CGAGAAGACCCTRTGGAGCT	~100
Fish and shark (R)	"	"	"	CCTNGGTCGCCCCAAC	
Chordata blocking primer	Otariidae	Australian sea lion		ATGGAGCTTCAATTAACTTACCCAATCAGAACC	
S_Cephalopoda (F)	Cephalopoda	Squid + Octopus + Cuttlefish	mtDNA 16s	GCTRGAATGAATGGTTTGAC	~112
S_Cephalopoda (R)	"	"	"	GGACGAGAAGACCCTAWTGA	

16S Cephalopod primer set

A minimally degenerate cephalopod group-specific primer set was designed for this study (Table 2). The primer set amplifies a ~112 bp region of the 16S mitochondrial DNA gene of cephalopods that are found in southern Australia. The primer set was designed on alignment using a range of 16S mtDNA of cephalopods sequenced for this study at the Australian Genomic Research Facility (AGRF). Primer specificity was tested using sequences of fish, elasmobranch, crustacea and otariid taxon groups obtained from genomic DNA from specimens collected and sequenced at AGRF, other studies (Deagle *et al.* 2009; Braley *et al.* 2010) and sequences available in GenBank. Sequences were aligned using DNAMAN version 6.0© (Lynnon Corporation 2005, Pointe-Claire, QC, CAnada) and Clustal X© (Thompson *et al.* 1997). The primer set was screened using BLAST*n* in GenBank to determine target group specificity (Altschul *et al.* 1990). Initial PCR assays were conducted to assess the specificity of the primer set using genomic DNA of southern calamary squid (*Sepioteuthis australis*), southern keeled octopus (*Octopus berrima*), hammer octopus (*Octopus australis*), southern dumpling squid (*Euprymna tasmanica*), striped pyjama squid (*Sepioloidea lineolata*), Australian giant cuttlefish (*Sepia apama*) and arrow squid (*Nototodarus gouldi*).

PCR reactions

PCR amplifications (10µl) contained 2µl template DNA, 2.0 mM $MgCl_2$, 10 x QIAGEN PCR buffer, 1 x BSA, 10 µM DNTP, 10 µM primers, 100 µM blocking primer (if present) and 1 x 0.625 units HotStar *Taq* DNA polymerase (QIAGEN). Thermal cycling conditions for fish amplifications were 95 ºC for 15 min, followed by 32 cycles of: 94 ºC for 15s, 57 ºC for 90s, and 72 ºC for 30 s. Thermal cycling conditions for cephalopod amplifications were: 95 ºC for 15 min, 94 ºC for 2 min, followed by 32 cycles of: 94 ºC for 10 s, 62.0 ºC for 35 s and 72 ºC

for 30 s. A final extension step of 72 ºC, 10 min was included in all assay runs. PCR amplifications were detected by gel electrophoresis on 1.5% agarose gel stained with Gelred™ and visualised using Gel-DOC UV illuminator (Bio-Rad®, Hercules, CA, USA) supported by QUANTITY ONE QUANTITATION analysis software (Bio-Rad®). Replicate PCRs were performed to confirm positive results from the first PCR attempt. Positive PCR reactions were quantified using PicoGreen® dsDNA Quantitation reagent (Molecular Probes ®) on a Wallac1420 multilabel fluorometer. PCRs included negative controls to check for contamination. All PCR preparations used UV sterilised aerosol resistant filter tips, consumables and equipment, and were prepared in DNA free laminar flow UV sterilisation hoods.

Clone sequencing

Amplicons from positive PCRs were purified using a Nucleospin® Extract II PCR cleanup gel extraction kits (Macherey-Nagel, Easton, PA, USA), cloned into the TOPO TA cloning vector then transformed into TOP10 chemically competent *Escherichia coli* cells and grown overnight on X-gal media. Positive DNA inserts (white colonies) were selected and PCR amplified using vector primers M13 F/R primers. Positive clone PCR inserts were purified and sequenced using ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits on a ABI PRISM® 3730XL Analyzer (96 capillary type) by Macrogen Inc. (South Korea). For individual diets, a total of 53 positive clones were selected for sequencing from both fish and cephalopod primer sets (n = 106 per individual). For the pooled diet analysis (fish only), 100 clones were chosen for sequencing.

Sequence screening

Sequences obtained from each clone library were sorted initially by sequence read length. Sequences that showed read lengths significantly longer or shorter than the expected

sequence read length encompassed by each primer set were excluded. Sequences were then clustered by similarity using the neighbour-joining Kimura 2-parameter correction method (Kimura, 1980) incorporated in the program DNAman version 6.0©. Gap opening and extension parameters were set to 10 and 5 respectively with sequence alignments bootstrapped for 10,000 iterations. Sequences within each homologous cluster were then aligned to determine nucleotide polymorphisms. Representative sequences of each cluster were then assigned to GenBank and identified using the BLAST program (Altschul *et al.* 1990). Sequences that showed nucleotide divergences from within each aligned cluster were identified in GenBank. As a precautionary step, a conservative approach was used and restricted the final sequence identification to mostly high similarity (≥ 97%) matches in GenBank. Discarding potential prey sequences with lower alignment scores may underestimate prey diversity, but higher stringency means that identification of prey is less ambiguous. If a common recurrent sequence was identified in a clone library but not found in the GenBank database, identification was restricted to either the lowest taxonomic group or the sequence was listed as unknown. Sequence clusters were then compared by similarity. Where BLAST returned multiple sequence matches of closely related species, the geographic distribution and depth preference of each species was considered prior to the final sequence identification (see Deagle *et al.* 2009).

Data analysis

Sequences were represented as percent numerical abundance (NA %) (% prey sequences of each prey taxa per individual). Variation in dietary composition between sites was tested for significance with non-parametric analysis of similarity (ANOSIM) on a Bray-Curtis similarity matrix in Primer version 6.0 (Clarke and Warwick, 2001). A similarity percentage analysis (SIMPER) was used to identify prey that were responsible for the significant

differences. The R_{ANOSIM} statistic provides a relative measure of separation between groups with R values of zero (0) supporting the null hypothesis, and a value of one (1) indicating samples within defined groups are more similar to one other than samples from other groups. For comparisons of diets between sites, individual prey items (sequence data) were standardised as a proportion of the prey identified (sequences recovered) by different individuals. The data were then standardised as a proportion of prey identified across all individuals from different sites. Comparisons between the data from pooled and individual samples were also standardised using these methods. Data were transformed where necessary prior to analyses. For the multivariate analysis, a hierarchical similarity cluster analysis based on multidimensional scaling plot (MDS) was performed to exhibit the relationship between sites and individuals. Stress values were calculated in two- and three- dimensions to determine how well the data were representative for the ordination. Stress values of < 0.1 provided interpretable information of the separation between groupings (Schiffman *et al.* 1981; Page *et al.* 2005).

To evaluate if each clone library provided representative coverage of the potential number of prey species identified, power analyses were performed in R (version 2.12, R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria). Sequence clusters within each library were selected at random and the total number of unique clones represented across the sequence library was calculated. A second cluster was chosen at random from the sequence library, calculating the total number of clones that it represented within the sequence library. The procedure was replicated until all unique prey taxa, represented by their respective sequences had been selected and the cumulative number of prey species was calculated. Mean number of clones and their standard deviation was estimated for each sequence cluster by Monte Carlo bootstrapping the data 10,000 iterations

and for each prey species, a calculation of the cumulative number of prey species represented by *j* clones ± standard deviation ($\hat{\sigma}_{boot}$):

$$\hat{\sigma}_{boot} = \sqrt{\frac{n-1}{n}}\left(\sqrt{\frac{1}{n(n-1)}\sum_{i=1}^{n}(x_i - x_j)^2}\right)$$

where n is the number of iterations, x is the mean number of prey species identified by j^{th} clones at iteration i (Chernick, 2007).

The resulting data (mean number of prey species identified by each clone library) was plotted using CURVE EXPERT (Curve Expert v 1.4) (Hyams, 1995-2009, www.curveexpert.net). The Gompertz function (y=a*exp(-exp(b-cx)) was applied to calculate the asymptotic number of prey species identified in each library interpreted as the maximum number of prey species identified by *j* clones. I then calculated the number of clones required to be sequenced to achieve 95% coverage of the prey species used by sea lions from each site.

RESULTS

Prey hard-parts

All samples from KI and one sample from LI contained skeletal remains of fish but these could not be identified. A single otolith from the slender bullseye (*Parapriacanthus elongatus*) was recovered from individual 1 at KI. Two cephalopod beaks were recovered from individual 3 at KI (*Octopus berrima*) and individual 6 at LI (*Octopus* spp.). Cephalopod beak fragments recovered from individuals 2 and 3 from LI and individual 3 from KI could not be identified.

Clone library overview

PCR amplicons of fish were generated from all individuals from KI, but only from five individuals at LI (Table 3). The cephalopod primer set produced PCR amplicons from all individuals at both sites (n = 12). In total, 1329 sequences (638 from LI and 691 from KI) were generated from the individual and pooled DNA clone libraries (Table 3). After removal of truncated sequences, 1171 potential prey sequences (88%) were used for the data analyses. The final datasets for individual diets comprised 280 and 202 fish sequences from KI and LI, respectively. The cephalopod primer set recovered 238 (83%) and 262 (91%) sequences from KI and LI, respectively. In total, 97 (LI) and 92 (KI) sequences were obtained from the pooled DNA amplified with the fish primers. Sequence data file: doi:10.5061/dryad.d417q (201300401) (http://datadryad.org/).

Inter-colony and individual diet comparisons

16S Fish primer set

Overall, 23 species of fish across three genera were identified (Table 3). Of the 19 families identified, Labridae (wrasse) and Monacanthidae (leatherjackets) were the most commonly encountered fish prey taxa recovered from 10 individuals. Mullidae (goatfish) were recovered from seven individuals and Platycephalidae (flatheads), Serranidae (ocean perch) and an unknown teleost (Unknown Teleostei A) in five samples.

Table 3. Taxonomic assignment and numerical abundance of prey sequences obtained from Australian sea lion faeces collected from KI and LI, South Australia. Numbers 1-6 represent individuals sampled per site. Sequences obtained by combining DNA from individuals 1- 6 are represented as pooled DNA (fish primer only).

			Kangaroo Island															Lilliput Island													
			1		2		3		4		5		6		Total	Pooled		1		2		3		4		5		6		Total	Pooled	
	Prey taxa	Common name	count	%	count	%	count	%	count	%	count	%	count	%	count	DNA	%	count	%	count	%	count	%	count	%	count	%	count	%	count	DNA	%
Osteichthyes																																
Arripidae	*Arripis geogianus*	tommy rough	0	0	0	0	**2**	**4.0**	0	0	0	0	0	0	**2**	**2**	**2.1**	0	0	0	0	0	0	0	0	0	0	-	-	0	0	0
Berycidae	*Centroberyx australis*	yellow-eyed nannygai	0	0	0	0	0	0	**19**	**51.4**	0	0	**12**	**23.5**	**31**	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0	0	0
Carangidae	*Pseudocaranx wrighti*	skipjack trevally	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**15.0**	**44**	0	0	-	-	**15**	0	0
Carangidae	*Trachurus declivis*	jack mackerel	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1.0**	**2.9**	0	0	-	-	**1**	**2**	**2**
Cheilodactylidae	*Nemadactylus macropterus*	jackass morwong	0	0	0	0	0	0	0	0	0	0	**1**	**2.0**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0	0	0
Clupeidae	*Sardinops sagax*	Australian sardine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**2.0**	**5.9**	0	0	-	-	**2**	0	0
Gempylidae	*Thyrsites atun*	barracouta	0	0	**1**	**2.1**	0	0	0	0	0	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0	0	0
Gerreidae	*Parequula melbournesis*	silver belly	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**31**	**66**	0	0	0	0	0	0	-	-	**31**	**15**	**16**
Labridae	*Notolabrus tetricus*	blue throated wrasse	0	0	0	0	**46**	**92.0**	0	0	0	0	0	0	**46**	**70**	**72.2**	**1**	**2.8**	**5**	**11**	**12**	**28**	**8.0**	**24**	**3**	**7**	-	-	**29**	**9**	**10**
	Pictilabrus laticlavius	senator wrasse	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	**1.0**	0	0	**3**	**6.4**	**8**	**19**	0	0	0	0	-	-	**11**	**6**	**7**
	Pseudolabrus sp.	wrasse	0	0	**3**	**6.4**	**2**	**4.0**	0	0	0	0	0	0	**5**	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0	0	0
Monacanthidae	*Acanthaluteres brownii*	spiny-tailed leatherjacket	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**5**	**12**	0	0	0	0	-	-	**5**	0	0
	Meuschenia scaber	velvet leatherjacket	**13**	**31.0**	**1**	**2.1**	0	0	**2**	**5.4**	**42**	**79.2**	**1**	**2.0**	**59**	**5**	**5.2**	**10**	**28**	0	0	0	0	**1.0**	**2.9**	**3**	**7**	-	-	**14**	0	0
	Scobinichthys granulatus	rough leatherjacket	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**2**	**5.6**	0	0	0	0	0	0	0	0	-	-	**2**	1	1
Moridae	*Lotella rhacina*	bearded rock cod	**11**	**26.2**	0	0	0	0	0	0	0	0	0	0	**11**	**2**	**2.1**	0	0	0	0	0	0	0	0	0	0	-	-	0	0	0
	Pseudophycis barbata	southern bastard codling	0	0	0	0	0	0	**2**	**5.4**	0	0	0	0	**2**	**1**	**1.0**	0	0	0	0	0	0	0	0	0	0	-	-	0	0	0
Mullidae	*Upeneichthys vlamingii*	red mullet	**1**	**2.4**	**4**	**8.5**	0	0	0	0	**9**	**17.0**	**1**	**2.0**	**15**	**2**	**2.1**	**4**	**11**	**8**	**17**	0	0	**2.0**	**5.9**	0	0	-	-	**14**	0	0
	Upeneichthys sp.		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0	**4**	**4**
Platycephalidae	*Neoplatycephalus richardsoni*	tiger flathead	0	0	**10**	**21.3**	0	0	0	0	0	0	0	0	**10**	**2**	**2.1**	**2**	**5.6**	0	0	**2**	**5**	0	0	**36**	**86**	-	-	**40**	**36**	**39**
	Platycephalus sp.	flathead	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**4**	**11**	0	0	0	0	0	0	0	0	-	-	**4**	0	0
Rhinobatidae	*Trygonorrhina guanerius*	southern fiddler ray	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	**2**	0	0	0	0	-	-	**1**	0	0
Sebastidae	*Helicolenus* sp.	ocean perch	0	0	0	0	0	0	0	0	0	0	**34**	**66.7**	**34**	**5**	**5.2**	0	0	0	0	**1**	**2**	**3.0**	**8.8**	0	0	-	-	**4**	**3**	**3**
Serranidae	*Caesioperca lepidoptera*	butterfly perch	0	0	**9**	**19.1**	0	0	**10**	**27.0**	**2**	**3.8**	**2**	**3.9**	**23**	**1**	**1.0**	0	0	0	0	**5**	**12**	0	0	0	0	-	-	**5**	0	0
Sillaginidae	*Sillaginodes punctatus*	king george whiting	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**2**	**5.9**	0	0	-	-	**2**	**1**	**1**
	Sillago bassensis	silver whiting	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**7**	**19**	0	0	0	0	0	0	0	0	-	-	**7**	**6**	**7**
Soleidae	Fam. *Soleoidei*	flounder	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**5**	**14**	0	0	0	0	0	0	0	0	-	-	**5**	**0**	**0**
Tetrarogidae	*Gymnapistes marmoratus*	South Australian cobbler	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**2**	**4.7**	0	0	0	0	-	-	**2**	**0**	**0**
	Unknown Teleostei A		**17**	**40.5**	**19**	**40.4**	0	0	**4**	**10.8**	0	0	0	0	**40**	**6**	**6.2**	**1**	**2.8**	0	0	**7**	**16**	0	0	0	0	-	-	**8**	**9**	**10**
	Total		**42**	**100**	**47**	**100**	**50**	**100**	**37**	**100**	**53**	**100**	**51**	**100**	**280**	**97**	**100**	**36**	**100**	**47**	**100**	**43**	**100**	**34**	**100**	**42**	**100**	-	-	**202**	**92**	**100**
Cephalopoda																																
Octopodidae	*Octopus berrima*	southern keeled octopus	**6**	**14.0**	0	0	**3**	**7.1**	0	0	**2**	**11.1**	0	0	**11**	-	-	**43**	**94**	0	0	**28**	**72**	**1**	**2.4**	0	0	**36**	**84**	**108**	-	-
	Octopus maorum	maori octopus	**4**	**9.3**	0	0	**35**	**83.3**	**1**	**2.3**	**8**	**44.4**	0	0	**48**	-	-	0	0	0	0	**4**	**10**	**12**	**29**	0	0	0	0	**16**	-	-
	Octopus kaurna	southern sand octopus	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0	0	0	0	**1**	**2.6**	0	0	0	0	**1**	**2.3**	**2**	-	-
Loliginidae	*Sepioteuthis australis*	southern calamary squid	**2**	**4.7**	**48**	**100**	**2**	**4.8**	**13**	**30.2**	0	0	**8**	**18.2**	**73**	-	-	**3**	**6.5**	**47**	**100**	**4**	**10**	**28**	**67**	**1**	**2**	0	0	**83**	-	-
Sepiidae	*Sepia apama*	giant cuttlefish	**28**	**65.1**	0	0	**2**	**4.8**	**29**	**67.4**	**8**	**44.4**	**36**	**81.8**	**103**	-	-	0	0	0	0	0	0	0	0	**44**	**98**	0	0	**44**	-	-
Sepiolidae	*Euprymna tasmanica*	southern dumpling squid	**3**	**7.0**	0	0	0	0	0	0	0	0	0	0	**3**	-	-	0	0	0	0	**2**	**5.1**	**1**	**2.4**	0	0	**6**	**14**	**9**	-	-
	Total		**43**	**100**	**48**	**100**	**42**	**100**	**43**	**100**	**18**	**100**	**44**	**100**	**238**	-	-	**46**	**100**	**47**	**100**	**39**	**100**	**42**	**100**	**45**	**100**	**43**	**100**	**262**	-	-

Composition of the sequence clusters obtained at both sites showed a high level of variation in fish prey taxa between individuals (Table 3). A total of 14 sequence clusters were identified from KI. Eleven (79%) of these were identified to species, two (14%) to genus and one cluster remained unidentified. The median number of fish taxa detected per individual at KI was 4.5 (mean 4.7 ± 1.6; range 4 - 7). The most common species identified was the velvet leatherjacket (*Meuschenia scaber*), detected from five individuals. Red mullet (*Upeniechthys vlamingii*) and butterfly perch (*Caesioperca lepidoptera*) were both detected in samples, and the unknown teleost (Unknown Teleostei A) was detected in three samples. The results from SIMPER analyses agreed with these findings, with *M. scaber*, *U. vlamingii*, and *C. lepidoptera* contributing ~ 40% of overall similarity between KI individuals (17.7%, 11.1% and 10.6%, respectively). Ten of the prey species were either specific to single individuals or were detected in only two individuals: yellow-eyed nannygai (*Centroberyx australis*) and wrasse (*Pseudolabrus* sp.). Clone libraries contained sequences of two or three fish taxa with the exception of individual 3, for which a single species (flathead) comprised 92 % of the clone library. Two prey species, barracouta (*Thyrsites atun*) and jackass morwong (*Nemadactylus macropertus*), were represented by a single sequence.

Twenty fish sequence clusters were identified from the six individuals at LI. The median number of fish taxa detected at LI was 8.0 (mean 6.6 ± 2.9; range 0 - 9). Sixteen fish taxa, represented by 12 families, were identified to species (Table 2). One sequence cluster was identified only to genus (*Helicolenus* sp.), one to family (Soleoidei) and one cluster remained an unidentified teleost (Unknown Teleostei A). A single sequence of the southern fiddler ray (*Trygonorrhina guanerius*) was detected in one individual. Blue throated wrasse (*N. tetricus*) was the most common species with results from the SIMPER analysis confirming ~ 25 % of

the similarity between individuals resulted from consumption of this species. Velvet leatherjacket, tiger flathead (*Platycephalus richardsoni*) and red mullet were detected in three individuals but each contributed only small percentage (~ 7 %). Thirteen (65%) of the prey taxa identified were specific to single individuals. Of these, six sequence clusters were represented with less than two sequences: Australian pilchard (*Sardinops sagax*), rough leatherjacket (*Scobinichthys granulatus*), King George whiting (*Sillaginodes punctatus*), jack mackerel (*Trachurus declivis*), southern fiddler ray (*T. Guanerius*) and South Australian cobbler (*Gymnapistes marmoratus*).

16S Cephalopod primer set

Sequences recovered from cephalopods showed reasonable variation (≤14 bp) to differentiate between species. Cephalopod diversity was low and consisted six sequence clusters identified across four families (Table 3). Of these, Octopodidae was the most commonly identified cephalopod family, followed by Loliginidae (calamary squid), Sepiidae (cuttlefish) and Sepiolidae (dumpling squid).

The cephalopod primer set showed differences in prey taxa between females and sites (Table 3). The number of cephalopod taxa was similar at both sites (KI: median 3.0, mean 3.0 ± 0.6, range 1 to 5, LI: median 2.5, mean 2.8 ± 0.6, range 1 to 5). Cephalopod prey were represented by three species of octopus: southern keeled octopus (*Octopus berrima*), Maori octopus (*Octopus maorum*) and Southern sand octopus (*Octopus kaurna*), two squid species: Southern calamary squid (*Sepioteuthis australis*) and Southern dumpling squid (*Euprymna tasmanica*), and the giant cuttlefish (*Sepia apama*). *Octopus berrima*, *O. maorum*, *S. apama* and *S. australis* were detected at both sites but *O. kaurna* was only detected in two samples from LI. *Sepioteuthis australis* was the most common cephalopod, detected in five individuals from each site. For KI, *S. apama* was equally important in diets of individuals (n

= 5), but not at LI where it was detected in the diet of only 1 individual. Octopods *O. berrima* and *O. maorum* were important in diets of KI individuals (n = 4) but *O. maorum* was not as common in diets from LI (n = 2). A difference in the composition of cephalopods between sites was also reflected in SIMPER. The data indicated *S. australis*, *S. apama* and *O. maorum* contributed 46.4 % to overall similarity between individuals at KI (18.1%, 17.4% and 10.9%, respectively) but differed from LI where *S. australis* and *O. berrima* contributed 38.1% (24.7% and 13.4%, respectively) to overall site similarity. *E. tasmanica* and *O. kaurna* were less common at both sites.

Dietary comparison between sites

The prey taxa differed significantly between sites (global R_{ANOSIM} = 0.30, P = < 0.05). Hierarchical similarity cluster analysis separated individuals into 2 distinct groups (KI and LI) with the exception of 1 individual from LI (individual 5), which was grouped with KI (Fig. 3) (3D stress = 0.05). Two dimensional stress was also determined for the same dataset and produced similar groupings but with slightly higher values (stress = 0.09). SIMPER analyses indicated a dissimilarity of 74% between locations with the main differences resulting from giant cuttlefish (*S. apama*), dumpling squid (*E. tasmanica*), and blue throat wrasse (*N. tetricus*).

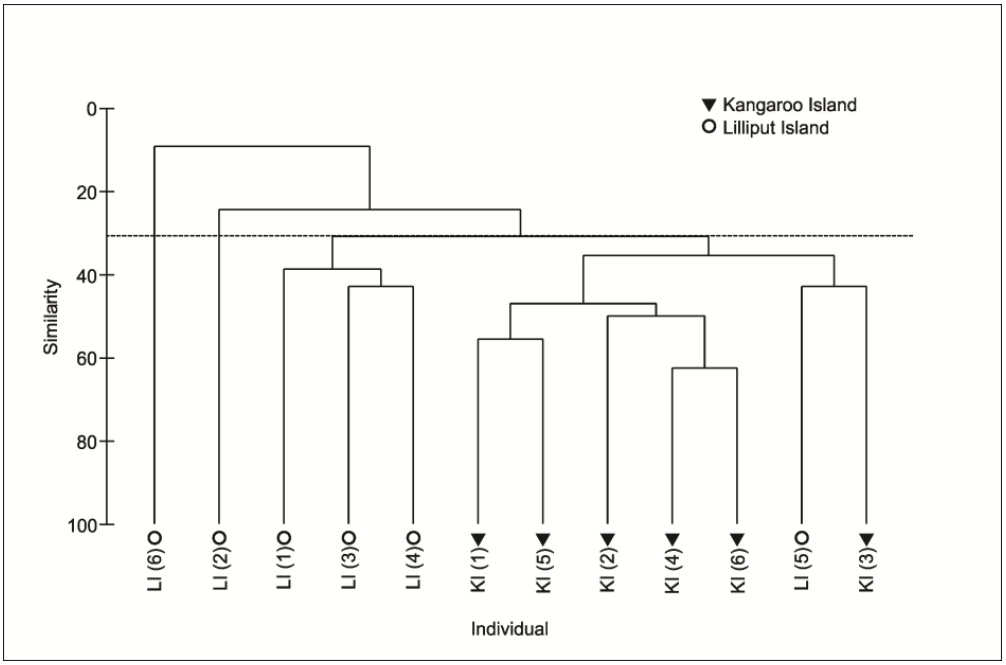

Figure 3. Hierarchical similarity cluster analysis of fish and cephalopod prey sequences obtained from Australian sea lions at Kangaroo Island (KI ▼) and Lilliput Island (LI ○).

Comparisons between individual and pooled DNA data sets

Ten prey taxa from KI (71%) and 11 (55%) from LI were represented in the pooled datasets. These were not significantly different from the combined total number of prey species identified from individuals at each site (χ^2 = 0.36, d.f. = 24, $P = \geq 0.05$ (KI), χ^2 = 2.61, d.f. = 30, $P = \geq 0.05$ (LI)) with the ANOSIM ranked data indicating negligible differences between pooled and individual prey datasets (KI global R_{ANOSIM} = 0.33, P = 0.3, LI global R_{ANOSIM} = -0.6, $P = \geq 0.1$). However, the pooled datasets showed differences in prey composition compared to the total prey taxa identified from individuals. Four prey taxa (21%) identified across individual diets from KI, and nine species from LI (45%) were absent from pooled datasets. There was no apparent pattern to these omissions; for example, rarer taxon sequences (≤ 5 sequences) such as King George whiting detected in one individual from LI

was represented in the pooled dataset, whereas velvet leatherjacket, which was abundant across individual samples from LI, was not. Conversely, velvet leatherjacket, which was relatively abundant across five individuals at KI, and also detected in the pooled datasets. Yellow-eyed nannygai (*Centroberyx australis*), was abundant in two individuals but was not in pooled samples. A single sequence of senator wrasse (*Pictilabrus laticlavius*) was detected in the pooled DNA dataset from KI but was not detected across the individual diets. Seven prey sequence clusters containing relatively low abundance sequences (≤ 5) from individuals at LI were absent from the pooled data.

Assessment of prey diversity from individual and pooled datasets

In most cases, identifying 95% of an individual's prey diversity required fewer clones than the total number sequenced (n = 53). The data demonstrated the typical shape of a sigmoidal curve and the Gompertz function fitted the data well in most cases (r^2= 0.961 - 0.999) (Table 4, Figs. 4 and 5) with exception of two datasets that exhibited linear curves (KI (individual 6, fish) and LI (individual 5, cephalopod) (Table 4). The sequence libraries in these individuals indicated they used a single prey species and contained a small number of single sequence taxa. Based on the asymptotes, the number of clones to be sequenced did not differ significantly between individuals at KI and LI (fish; 35.8 ± 20.8 and 24.1 ± 5.2, respectively; one-way analysis of variance (ANOVA): F = 1.48, d.f. = 9, P = 0.25), cephalopods (28.6 ± 12.88 and 37.0 ± 13.02, respectively, ANOVA: F = 0.93, d.f. = 7, P = 0.37), nor overall (ANOVA: F = 1.33, d.f. = 17, P = 0.27). The pooled fish data also exhibited sigmoidal curves (r^2 = 0.997 and 0.990) with 95% prey diversity reached with fewer clones for KI (n = 88.1) and LI (n = 51.4) than the total number sequenced (n = 103) (Table 4, Fig. 6).

Table 4. Estimated number of clone sequences required to achieve 95 % coverage of the asymptotic prey diversity for each clone library. The number of clones sequenced was higher than the asymptotic number of clones required except animal 6 (KI) and animal 5 (LI). Individual (~ n = 53 clones) and combined (pooled DNA) for 6 individuals per site (~ n = 110) are shown. Pooled estimates are for fish taxa.

	Cephalopods				Fish			
Region / Animal	**Prey Species (n)**	**Asymptote (a)**	**r^2**	**No. clones required**	**Prey Species (n)**	**Asymptote (a)**	**r^2**	**No. clones required**
Kangaroo Island (KI)								
1	5	4.74	0.997	24.5	4	3.54	0.961	15.3
2	1	-	-	-	7	6.40	0.988	30.6
3	4	3.94	0.999	35.4	3	3.03	0.999	49.2
4	3	2.79	0.962	33.5	5	4.71	0.993	21.4
5	3	2.80	0.993	8.3	3	2.82	0.992	27.1
6	2	1.90	0.997	41.5	6	6.34	0.994	71.2
DNA combined	-	-	-	-	11	10.57	0.997	88.1
Lilliput Island (LI)								
1	2	1.93	0.999	27.6	9	8.34	0.994	25.2
2	1	-	-	-	4	3.78	0.996	19.0
3	5	4.74	0.997	30.2	9	8.28	0.992	18.9
4	4	4.09	0.984	56.1	8	7.72	0.996	31.1
5	2	3.65	0.999	206.5	3	2.89	0.998	26.3
6	3	2.81	0.991	34.2	0	-	-	-
DNA combined	-	-	-	-	11	10.06	0.990	51.4

Effectiveness of sample size (number of individuals) and prey diversity

Estimated asymptotes of the total number of prey used at each site indicated more samples were needed to obtain 95% of the overall prey diversity (Table 5, Fig. 7). At KI, 92% of the asymptotic prey diversity required 7.4 individuals and 1.7 prey taxa to be sampled, at LI, 88% required 8.3 individuals and 3.5 prey taxa; for combined fish prey datasets 93.8% required 14.2 individuals.

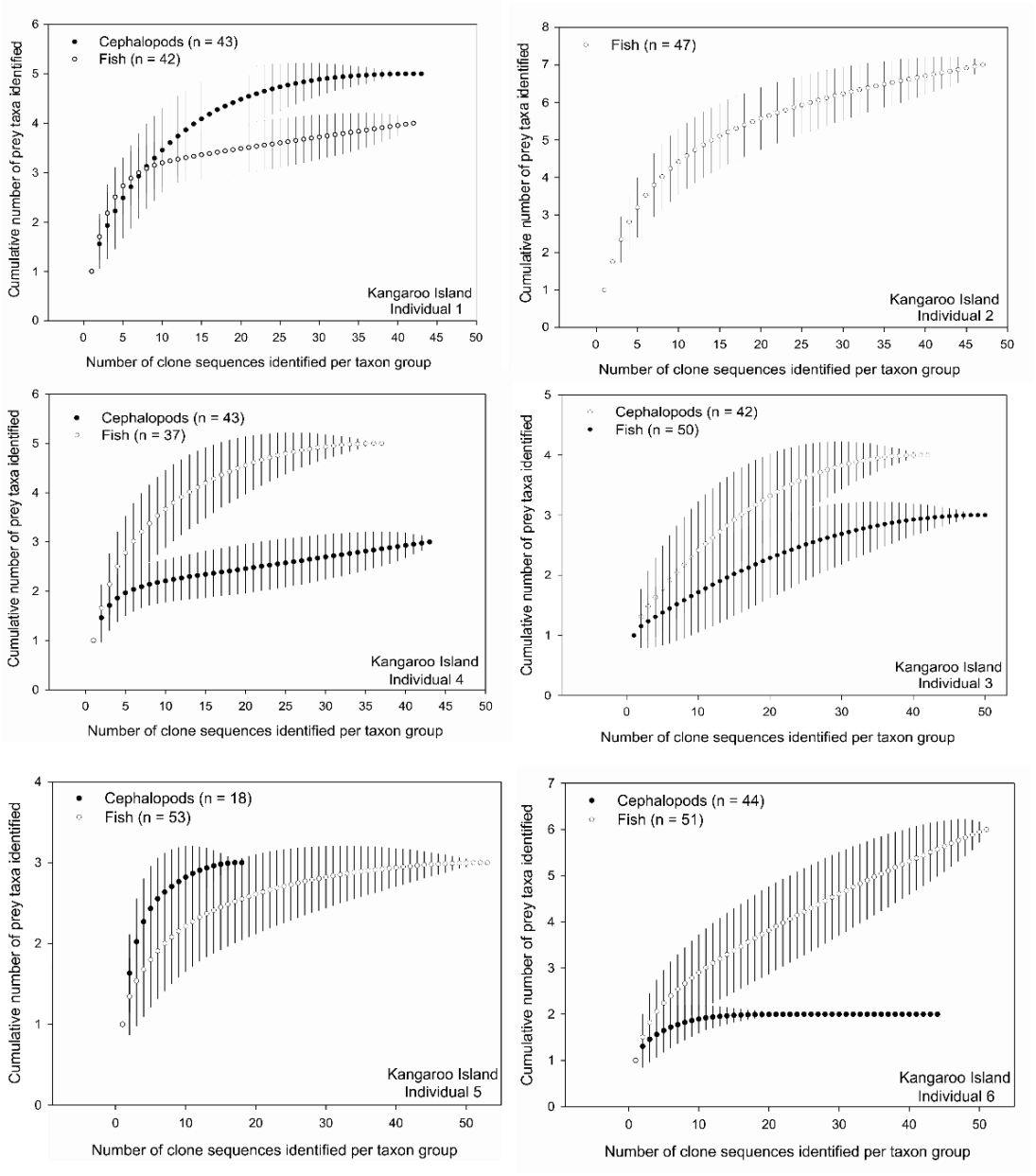

Figure 4. Asymptotic curves of prey sequence diversity obtained for fish (○) and cephalopods (●) from individual clone libraries. Individuals 1- 5 were representative of the asymptotic (a) number of clones required to sample 95 % of the potential prey identified per individual. Fish prey of animal 6 were 87.8% of the asymptotic number of clones required to sample 95% of fish prey identified (refer to Table 3). Curves were not estimated where < 2 prey species were identified (Individual 2). Values are mean ± SD. Data are for Kangaroo Island (KI).

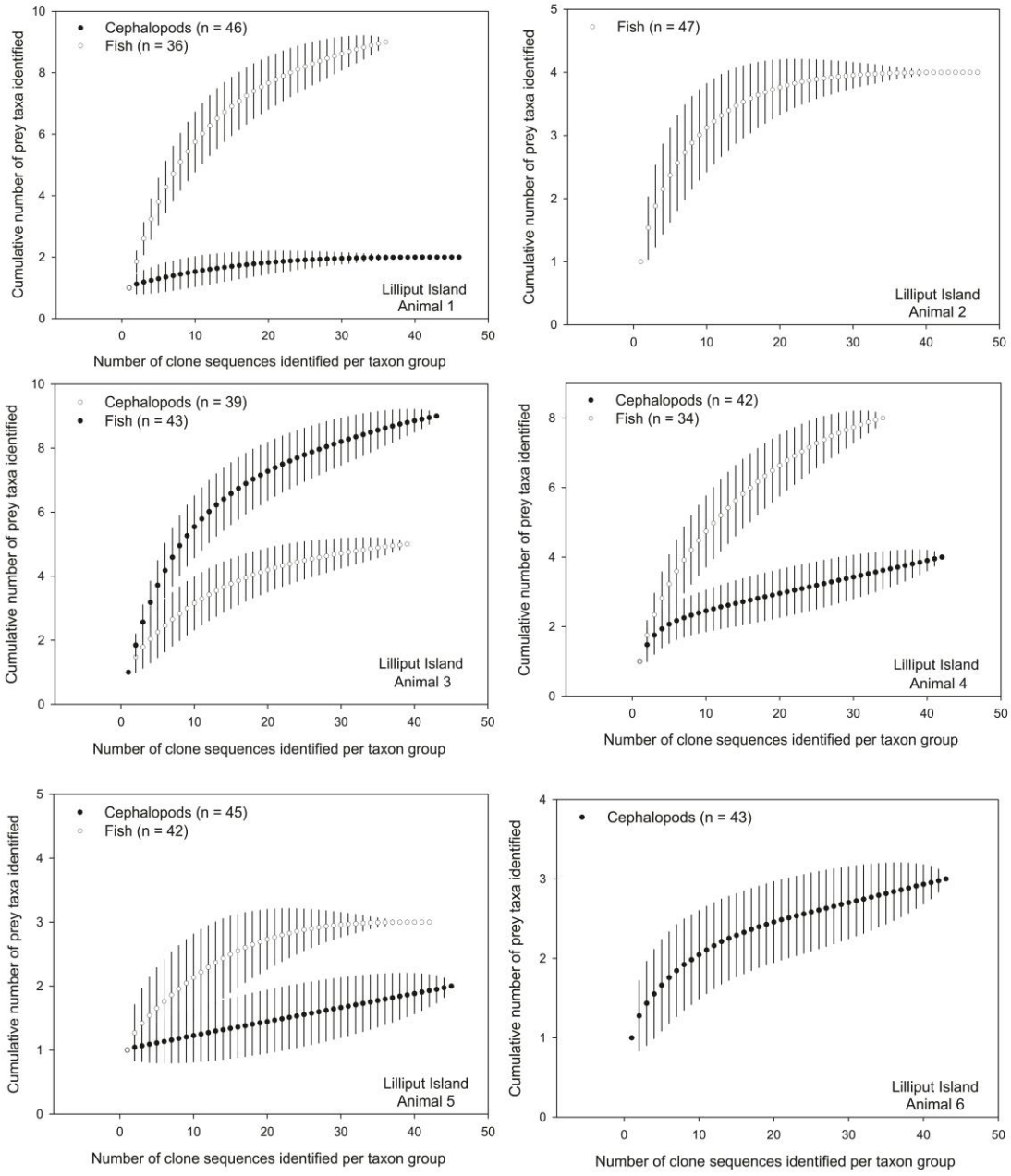

Figure 5. Asymptotic curves of prey sequence diversity obtained for fish (○) and cephalopods (●) from individual clone libraries. The asymptotic number of clones (a) required to sample 95 % of the potential prey diversity was representative for each individual. Curves were not estimated where only one prey species was identified (Individual 2) (refer to Table 4.4). Values are mean ± SD. Data are for Lilliput Island (LI).

Table 5. Total number of prey identified per site and estimate of the number individuals required to be sampled per site and combined sites to achieve 95 % coverage of the asymptotic number of prey taxa. Data indicate more individuals were required to be sample sampled to achieve 95 % of the asymptotic number of prey.

Site	Prey species (*n*)	Asymptote (*a*)	r^2	Prey coverage (%)	No. Individuals required (α = 95 %)
Kangaroo Island	19	20.7	0.998	91.8	7.4
Lilliput Island	26	29.5	0.999	87.9	8.3
Combined sites	33	35.2	0.996	93.8	14.2

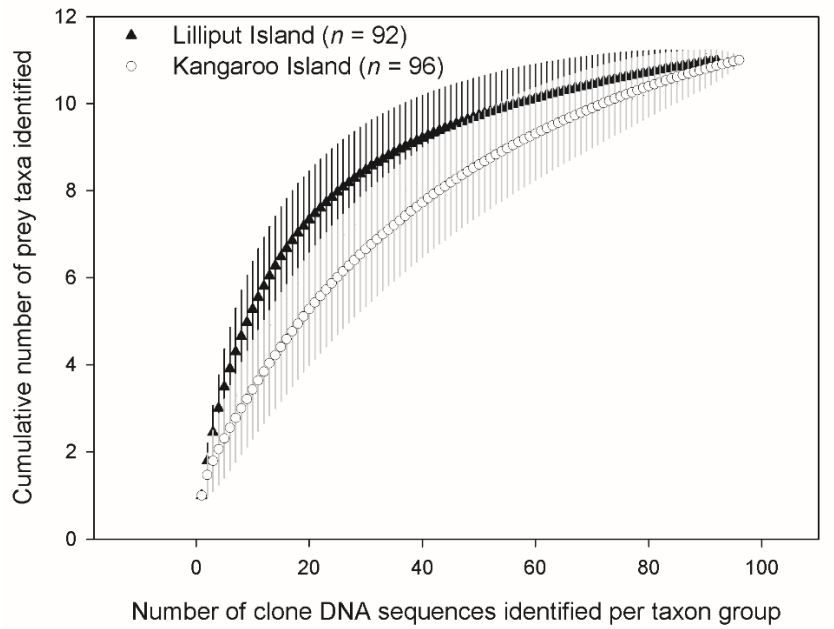

Figure 6. Asymptotic curves of fish prey sequence diversity obtained for combined (pooled) DNA from 6 individuals at KI (o) and LI (▲). The number of clones sampled was representative of the asymptotic (a) number of clones required to sample 95 % of potential prey identified per site (refer to Table 4). Values are mean ± SD.

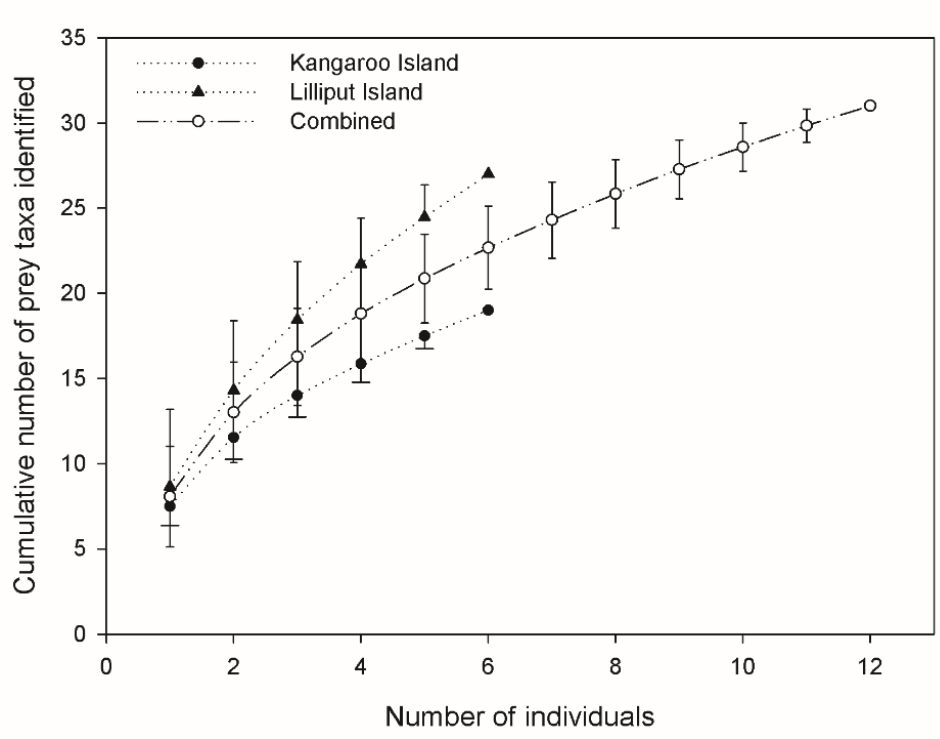

Figure 7. The relationship between the number of prey taxa identified from cloning PCR products and the number of individuals sampled. For KI, LI and combined datasets the asymptotic number of individuals required to be sampled to represent 95% of the asymptotic number of individuals required to be sampled to represent 95% of the asymptotic prey diversity was greater than the number sampled in the study (92 %, 88 %, and 94%, respectively; Table 5).

DISCUSSION

Amplification of prey DNA from scats using short sequence fish and cephalopod group-specific primers generated comprehensive detail of these two important dietary components of a threatened species, the Australian sea lion. Previous studies utilising hard-part analyses of faeces and regurgitates have indicated Australian sea lions consume a range of cephalopod, fish and crustacean prey, but to date only nine species of fish and five cephalopods have been described owing to the poor quality and limited number of prey remains recovered (Walker and Ling, 1981; Gales and Cheal, 1992; Ling 1992; McIntosh *et al.* 2006; Gibbs *et al.* 2008).

By applying the DNA-based cloning-sequencing approach, a diverse range of prey species were identified, many of which have not been previously described in diet of the Australian sea lion. As a result of this study, the sea lion prey spectrum has been extended by ~30 species. The majority of species identified here are benthopelagic or inhabit the sea floor, confirming the predominant benthic mode of foraging of the Australian sea lion (Costa and Gales, 2003; Fowler *et al.* 2006, 2007; Goldsworthy *et al.* 2009; Lowther *et al.* 2011).

Limitations

While the DNA analysis provided good taxonomic resolution, identifying prey by extracting DNA from faeces is subject to some of the inherent biases that accompany diet studies that utilise traditional hard-part methodology (Tollit *et al.* 1997; Staniland, 2002). For example, similar to the identification of prey-parts, the digestion of prey DNA is variable between species and, as a result, this study may only reflect prey whose DNA remained sufficiently intact after digestion to be amplified. In addition, even though the faeces from KI were fresh (collected within ~12h) DNA continues to degrade after deposition affecting DNA quality and quantity (e.g. Deagle et al. 2005a). This may have limited the detection of certain prey species from KI compared to those identified from direct enema samples collected from LI. The species identified here are also representative of only one single winter foraging event and can only imply recent prey ingestion that may not reflect feeding behaviour at other times of the year. Clear discrepancies in tissue mtDNA content can further equate to downstream differences in prey amplification rates (Soininen *et al.* 2010; Hartman *et al.* 2011). The strength of this statement is tempered by the fact that seals, like other large marine predators may not ingest whole specimens but parts of prey that contain tissues rich or poor in DNA content (i.e. liver versus pectoral fins) (Hartman *et al.* 2011; Dalziel *et al.* 2004). This factor alone could significantly influence interpretation of dietary data using these methods.

Prey diversity

Based on the DNA analysis, 23 fish and five cephalopod prey taxa were identified, some of which appeared specific to individual diets, but many species were consumed among individuals, within and between sites. This result suggests a large proportion of the species consumed were relatively abundant prey with either wide spatial distributions or were common to areas where females forage. Fish were dominated by six main groups; leather jacket (Monacanthidae), flathead (Platycephalidae), scorpion fish (Sebastidae), butterfly perch (Serranidae), red mullet (Mullidae) and wrasse (Labridae). The last four groups have not previously been reported as prey of the Australian sea lion.

Cephalopods have previously been described as important prey of the Australian sea lion (Walker and Ling 1981, Richardson and Gales 1987, Gales and Cheal 1992, Ling 1992, McIntosh *et al.* 2006; Gibbs, 2008). The results from this study support this, indicating that octopus, calamary squid (Loliginidae) and giant cuttlefish (Sepiidae) are common cephalopod prey. This study reports *O. kaurna* and *E. tasmanicus* as two new cephalopod prey of the Australian sea lion. The low number of sequences and low frequency with which each species was detected could reflect their relative abundance within the regions studied, low electivity, or that foraging areas where cephalopods were consumed were discrete and spatially limited.

The absence of arrow squid DNA in this study surprisingly contrasts results by McIntosh *et al.* (2006), where it was a relatively common prey item in the diets of sea lions at Seal Bay. Given this result, the molecular assay was tested by spiking a faecal sample with arrow squid DNA, which confirmed detection of arrow squid in the sample. I therefore suspect this result highlights the limitation of sampling a small number of individuals, or alternatively indicates

some seasonal prey variation that possibly coincides with the winter dispersal of arrow squid to deeper oceanic habitats where they mature (Triantifillos *et al.* 2004; Stark *et al.* 2008; Steer *et al.* 2006). Further work is required employing larger sample sizes over greater spatial and temporal scales to evaluate any seasonal variations in diet.

One of the advantages of DNA-based analysis is its ability to provide taxonomic resolution to prey that may be consumed but not defined using hard-part morphological analysis. The hard-part analysis indicated cephalopods such as octopus could not be taxonomically defined to species from beaks or beak fragments recovered in this study. McIntosh *et al.* (2006) and Gibbs (2008) also found reduced morphological integrity a constraint in assessing fish and cephalopod dietary components from the stomachs of deceased Australian sea lions. Furthermore, it was apparent from the lack of cephalopod and fish remains recovered in this study, that many prey items are either retained within the gut and digested or evacuated over time. Small species such as the Southern keeled octopus (*O. berrima*) are rarely recovered from digesta of Australian sea lions probably because they are small (≤ 40 mm mantle length) and solitary, with a rostrum that is unlikely to survive digestion intact (Carter *et al.* 2009; Peters *unpub. data*). Morphological similarity and damage incurred to beaks during digestion also makes differentiation between octopus species problematic (Roper, 1983; Stranks and Norman, 1992; Gibbs, 2008).

On the contrary, the DNA sequence data provided taxonomic separation between cephalopod species suggesting closely related species such as *O. berrima* and *O. maorum* were consumed by sea lions at each site. The higher prevalence of *O. maorum* at KI and of *O. berrima* at LI possibly reflects each species' preference for particular habitat types. Substrates of mud and sand for example, are preferred habitats of *O. berrima* and are consistent with the

predominant substrate near the LI colony. Conversely, *O. maorum* tend to inhabit soft sediment rocky reefs, which are widespread across foraging grounds utilised by sea lions at Seal Bay (Stranks and Norman, 1992; Anderson, 1999; Edyvane, 1999; Bryars, 2003; Fowler *et al.* 2007).

The sequence diversity curves indicated the clone-library approach provided adequate coverage of diet item diversity in the clone libraries. In most cases, 95% of the estimated diversity of fish and cephalopod prey consumed by individual animals and across the pooled DNA datasets was achieved. This relationship was slightly weaker (< 95%) when broad-scale diversity was considered (site and combined sites) (Table 4), indicating my study may have underestimated the potential range of fish and cephalopod prey taxa available; a bias potentially induced by the low sample size and small number of clones sequenced per library. The diversity curves indicated more comprehensive dietary information could have been achieved by sequencing ~150 clones per library and assessing ~30 samples per site. However, the cost of sequencing larger clone libraries limits such studies, and is the primary reason why only two key prey components were targeted here. Australian sea lions also consume crustaceans (McIntosh *et al.* 2006) that are notably absent from this study. While the benefits of employing the clone-sequencing approach to generate fine-scale dietary data is apparent in the diversity of prey identified, denaturing grade gel electrophoresis (DGGE), single strand chain polymorphism (SSCP) or high throughput sequencing technologies may be more appropriate where predators may consume a broad range of prey or where qualitative data is required (see Hiss *et al.* 2007; Tollit *et al.* 2009; Deagle *et al.* 2009).

Pooled and individual clone library datasets

The results from pooling DNA from multiple individuals at each site did not provide an accurate representation of the total number of fish prey identified. At each site, ten fish species were concurrently detected in the individual and pooled datasets, however a third of the species from KI (29%, n = 4) and almost half of the species from the LI pooled datasets (45%, n = 11) were missing. Neither the abundance of sequences represented within individual clone libraries, nor the frequency each species was detected could clearly explain these omissions, as species with high sequence counts (e.g. yellow-eyed nannygai) and those with few sequences (e.g. barracouta, jackass morwong) were absent (i.e. KI).Furthermore, while the most common fish sequences from the pooled datasets coincided with those accumulated across individual diets, each clone library was skewed by a small number of species with high sequence counts, an artefact also observed among individual clone libraries. While factors such as DNA degradation and copy number bias can influence outcomes of clone sequence analysis (Deagle *et al.* 2005a; Passmore *et al.* 2006), these results possibly highlight strong PCR amplification biases toward dominant intact prey DNA (e.g. blue-throated wrasse (KI)), or the methodological approach of standardising the quantity of faecal DNA prior to PCR possibly biased the clone libraries. Given prey DNA is a small and inconsistent component of faeces that comprise predominantly host and bacterial cells (Kohn and Wayne, 1997), standardising by pooling DNA post PCR would have eliminated the non-target DNA components and provided more equal representation in the final sequence libraries.

The single otolith of *P. elongatus* recovered in the hard-part analysis yet not detected by the PCR tests may reflect a limitation of the DNA analysis. For example, feeding trials on captive otariids indicate prey detection by PCR is limited to within ~48 hrs of prey ingestion

(Deagle *et al.* 2006; Casper *et al.* 2007*b*; Peters *et al.* 2013, *unpublished data*). This highlights the advantage of utilising a combined methodological approach for dietary analysis particularly morphological analysis of prey consumed beyond 2 d. Furthermore, for marine predators such as seals and sharks that consume large portions of their prey, combining dietary techniques can increase the frequency of prey detection (Casper *et al.* 2007*a*; Barnett *et al.* 2010; Marshall *et al.* 2010). The reason for the discrepancy in this study is uncertain, however otoliths of 'bullseyes' such as *P. elongatus* are robust and having previously been recovered intact from the stomachs of Australian sea lions (McIntosh *et al.* 2006; Peters *unpub. data*), may suggest the species was consumed prior to sample collection. Alternatively, this result may expose the shortcomings of sequencing small clone libraries or indicates a constraint related to the quantity or quality of *P. elongatus* DNA. Future application emphasises the importance of cross-validation using multiple but targeted primer sets or, as is now practical, generating a greater depth of dietary sequencing by using 'next generation' parallel sequencing technology (Deagle *et al.* 2009; Soininen *et al.* 2009; Valentini *et al.* , 2009).

Ecological implications

The array of fish and cephalopod prey identified from the individuals in this study indicates Australian sea lions are generalist predators that consume a wide range of demersal prey types. This strategy possibly accommodates their extreme foraging patch and natal site fidelity (Goldsworthy *et al.* 2009; Lowther *et al.* 2011). Unlike the seasonal cues that are implicit as breeding regulates in other otariid species, targeting a wide range of prey in this manner could reduce the dependency on single prey types facilitating their protracted 17.5 *mo* breeding and extended gestational period.

The range of species identified in this study are similar to the demersal and some pelagic prey consumed by the Australian fur seal (*Arctocephalus pusillus doriferous*) and New Zealand fur seal (*Arctocephalus forsteri*) that sympatrically breed and forage over parts of the Australian sea lion range (Page *et al.* 2005; Shaughnessy, 2010). In particular, the recent discovery of a breeding colony of *A. pusillus doriferous* at North Casuarina Island adjacent to Kangaroo Island could pose significant competition for the Australian sea lion at Seal Bay (*this study*), as both species are predominantly benthic foragers and target similar prey resources (Page et al 2005; Deagle *et al.* 2009). Studies investigating dietary overlap between these species will be important in advising future conservation management of otariids within this region.

Finally, Australian sea lions inhabit a broad range of habitats from cool temperate to the warm near sub-tropics of Houtman-Abrolhos (Goldsworthy *et al.* 2009b; Shaughnessy *et al.* 2011). Whilst a range of prey taxa were identified here, these results reflect diet from individual foraging behaviour at two colonies. Habitat heterogeneity and localised influences from regional boundary current systems in structuring biodiversity will likely imply spatial differences in the diet of the Australian sea lion (Bryars, 2003; Muhling and Beckley, 2007; Beckley *et al.* 2009).

Conclusions and future directions

This study confirms the value of DNA-based analysis to reveal fine-scale dietary information from faecal remains of the Australian sea lion that were undetectable using traditional hard-part methodology. While only two key prey components are represented here, the sequence data has provided new insights into the broader diet of the Australian sea lion that would not have been possible using other current dietary techniques (Costa and Gales, 2003; Fowler *et al.* 2006; Goldsworthy *et al.* 2009). Further application of the DNA-based methodology will

be central in developing a better understanding of their key prey components. Future studies should utilise greater sample sizes over broader temporal and spatial scales to minimise some of the limitations of the methods identified in this study.

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CHAPTER 5

Insights into seasonal prey use of the Australian sea lion (*Neophoca cinerea*) using faecal DNA and high-throughput sequencing

ABSTRACT

The endangered Australian sea lion (*Neophoca cinerea*) (ASL) has a small, fragmented population (~12,000 individuals) that is widely distributed, extending from Houtman Albrolhos, Western Australia, to the Pages Islands, South Australia. Recent declines at breeding colonies across their range, has prompted a range of conservation initiatives to address knowledge gaps in the species' biology. Biologging studies indicate that ASL utilise various benthic habitats to acquire prey. However, information on diet and the spatial and temporal use of prey resources remains poor. In this study, prey hard parts and DNA-based next-generation sequencing (NGS) analyses were used to determine seasonal diversity of fish and cephalopod prey of ASL. Faecal samples were collected from the Seal Bay colony (n = 176), on Kangaroo Island South Australia across seasons. PCR amplification using short (~100 bp) 16S mtDNA PCR primers and NGS sequencing of 110 samples revealed 65 unique sequence clusters that comprised a wide range of benthic and demersal bony fish, cartilaginous fish, and cephalopod prey. Combined with prey identified by hard parts (2 fish and 5 cephalopod prey species), these findings indicate that ASL use similar resources throughout the year. These results highlight the value of DNA-based faecal analysis and NGS in identifying ASL prey.

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INTRODUCTION

Marine mammals often perform fundamental roles within marine ecosystems, from influencing the abundance and distribution of other species, to recycling of nutrients that are invaluable for ecological processes (Trites *et al.* 1997). With the increasing human demand for marine food resources, identification of key trophic interactions in marine ecosystems, including interactions with high trophic-level species, is important to ensure their management is sustainable (Block *et al.* 2011; Goldsworthy *et al.* 2011, 2013). Where species are of high conservation concern, identifying population processes that underpin distribution and abundance such as identifying important habitat or prey resources is particularly important because even small changes can result in population declines or instability (Boyd *et al.* 1995; Goodman-Lowe, 1998; Farrell *et al.* 2000; Lee *et al.* 2013; Quéméré *et al.* 2013). Hence, dietary information can assist in understanding threatening processes, and informing the development of appropriate conservation, mitigation and management strategies.

Among pinnipeds, sea lions are of particular conservation concern because five of the six extant species have not recovered from the population reductions caused by sealing in the 18th and 19th centuries (e.g. Gerber *et al.* 2001; Hoffman *et al.* 2015). The Californian sea lion (*Zalophus californianus*) is the only species that has recovered (Caretta *et al.* 2015). The Australian (*Neophoca cinerea*), Galapagos (*Zalophus wollebaeki*), Steller (*Eumetopias jubatus*), South American (*Otaria flavescens*), and New Zealand sea lion (*Phocarctos hookeri*) are classified as either *Endangered* or *Vulnerable* (International Union for Conservation of Nature, 2008-2012).

Causes of recent sea lion population declines may include fisheries-based mortality and disease (Goldsworthy *et al.* 2003; Page *et al.* 2004; Wilkinson *et al.* 2006; Castinel *et al.*

2007; Chilvers, 2008; Kovacs *et al.* 2012), predation (Springer *et al.* 2003), and the availability and quality of prey (Trites and Donelly, 2003; Estes *et al.* 2009; Robertson and Chilvers, 2011). For example, during El Niño years, unseasonal sea surface warming is associated with nutritional stress of Galapagos and Californian sea lions and population declines (Trillmich, 1985; Trillmich and Dellinger, 1991; Alava and Salazar, 2006; Shirasago-German *et al.* 2015). Similarly, the western stock of the Steller sea lion has experienced population declines over the past 30 years, with one of the causes suggested to be the replacement of energy rich prey with poor quality prey (Schaufler *et al.* 2006; Trites *et al.* 2007). Competition with fisheries for prey may have also contributed to the decline of New Zealand sea lions (Robertson and Chilvers, 2011). Given that abundant and available trophic resources are essential to sustain marine predator populations (Boyd *et al.* 2000; Bluhm and Gradinger, 2008), understanding of diet, especially for threatened or endangered species, is central to improving conservation and management strategies to facilitate species recovery.

The Australian sea lion (*Neophoca cinerea*) (ASL) is an endemic species and one of the rarest otariids in the world. The population comprises ~12,000 individuals (Shaughnessy *et al.* 2011; Goldsworthy, 2015) and extends from Western Australia (Houtman Albrohos, 113º 47’ E, 28º 43’ S) to South Australia (Pages Islands, 35º 45’ S, 138º 18’ E) (Ling, 1992; Shaughnessy *et al.* 2011). The breeding biology of ASL is unusual among pinnipeds in that females have a non-annual reproductive cycle of 15-18 months, and breeding is temporally asynchronous among colonies (Higgins, 1993; Higgins and Gass, 1993; Gales *et al.* 1994). Females typically return to breed at the colony where they were born, increasing the risk that small subpopulations will become extinct as a result of either natural and anthropogenic pressures (Goldsworthy and Page, 2007; Campbell *et al.* 2008; Goldsworthy *et al.* 2009a;

Shaughnessy *et al.* 2011; Lowther *et al.* 2012; Hamer *et al.* 2013; Goldsworthy *et al.* 2015; Ahonen *et al.* 2016).

While tracking and biochemical diet analyses suggest ASL are individual specialists with high fidelity to foraging areas and prey (Costa and Gales, 2003; Fowler *et al.* 2006, 2007; Baylis *et al.* 2009; Goldsworthy *et al.* 2009a, b; Lowther *et al.* 2011, 2012), little taxonomic information exists on the geographic or temporal variation in diet across their range. Hard-part diet analyses indicate that ASL consume cephalopods, teleost fish, cartilaginous and crustacean prey (Richardson and Gales, 1987; Gales and Cheal, 1992; McIntosh *et al.* 2006; Gibbs, 2008; Baylis *et al.* 2009; Peters *et al.* 2014a). However, the results of such studies overestimate the importance of cephalopods because biases associated with hard-part analysis of ASL faeces mean that few other prey remains are able to be identified (Gales and Cheal, 1992; Childerhouse *et al.* 2001; Tollit *et al.* 2003; McIntosh *et al.* 2006; Gibbs, 2008). Recent studies have also used faecal DNA-based methods to assess ASL diet and highlight the promise of DNA-based methods to more accurately represent ASL diet, and improve information on the temporal-spatial use of resources. This information is currently limited in ASL to trophic-level inference of prey (Baylis *et al.* 2009; Lowther *et al.* 2012, Peters *et al.* 2014a, b).

In this study, I expand on previous work to explore the use of DNA-based faecal analyses as an alternative approach to determine diet in ASL. Amplification of prey DNA using the polymerase chain reaction (PCR) has become a useful method to elucidate prey from faeces that often contain ambiguous or unidentifiable remains (Casper *et al.* 2007a; Deagle and Tollit, 2007; Bowles *et al.* 2011). Although prey resolution using DNA-based methods is dependent on DNA surviving digestion (Sydmonson, 2002; 2006; King *et al.* 2008; Hartmann *et al.* 2011), the ability to identify ingested taxa with or without hard parts has broadened

information of diet in many species (Blankenship and Yayanos, 2005; Passmore *et al.* 2006; King *et al.* 2008; Deagle *et al.* 2007). More recently, the increased depth of sequencing offered by 'next generation' DNA sequencing (NGS) technology has improved the capacity for researchers to identify a broader range of species in the diets of predators and herbivorous grazers (Rongahi 2001; Buee *et al.* 2009; Pompanon *et al.* 2012; Shokralla *et al.* 2012; Rayé *et al.* 2011; Brown *et al.* 2012; Willerslev *et al.* 2014). Because NGS can simultaneously identify thousands of sequences per PCR-DNA sample, this approach is now commonly adapted to characterise faecal and stomach remains of marine mammal predators such as seals and seabirds, which are traditionally used to study diet (Deagle *et al.*2009, 2010; Jarman *et al.* 2013; McInnes *et al.* 2016). For ASL, applying NGS to faeces collected over a wide temporal scale may provide greater understanding of their long-term use of resources, enhancing knowledge of prey distributions and composition; information that is currently lacking for ASL.

Seal Bay on Kangaroo Island, South Australia (Fig. 1) is the third largest breeding site for the ASL with accessibility that is well suited to monitoring temporal variation in diet. This study aimed to apply novel NGS and hard part prey analyses to i) determine seasonal variation and prey diversity of ASL at Seal Bay and, ii) determine their broad use of prey.

MATERIALS AND METHODS

Sample collection

Fresh faecal samples (collected within ~12 hours of deposition) were collected over seven seasons (summer, autumn, winter, spring) from the Seal Bay sea lion colony, Kangaroo Island, South Australia (35º 59' 49" S, 137º 18' 21" E) (Fig. 1) between September 2005 and September 2007. Whole samples were collected; each was stored in a sterile bag (Nasco-Whirl-Pak®) containing 95% ethanol and homogenised prior to storage at -20ºC.

Hard part analysis

Hard-parts of prey (fish otoliths, bones, eye lenses, feathers, crustacean remains, cephalopod beaks and fragments) were isolated from each faecal sample by washing through 1.0 and 0.5 mm nested sieves. Fish otoliths and cephalopod beaks were photographed using digital microscopy supported by image analysis software Image Pro 5.1©. Prey items were identified to the lowest taxonomic group with the aid of reference collections and atlases (e.g., Lu and Ickeringill 2002; Furlani *et al.* 2007).

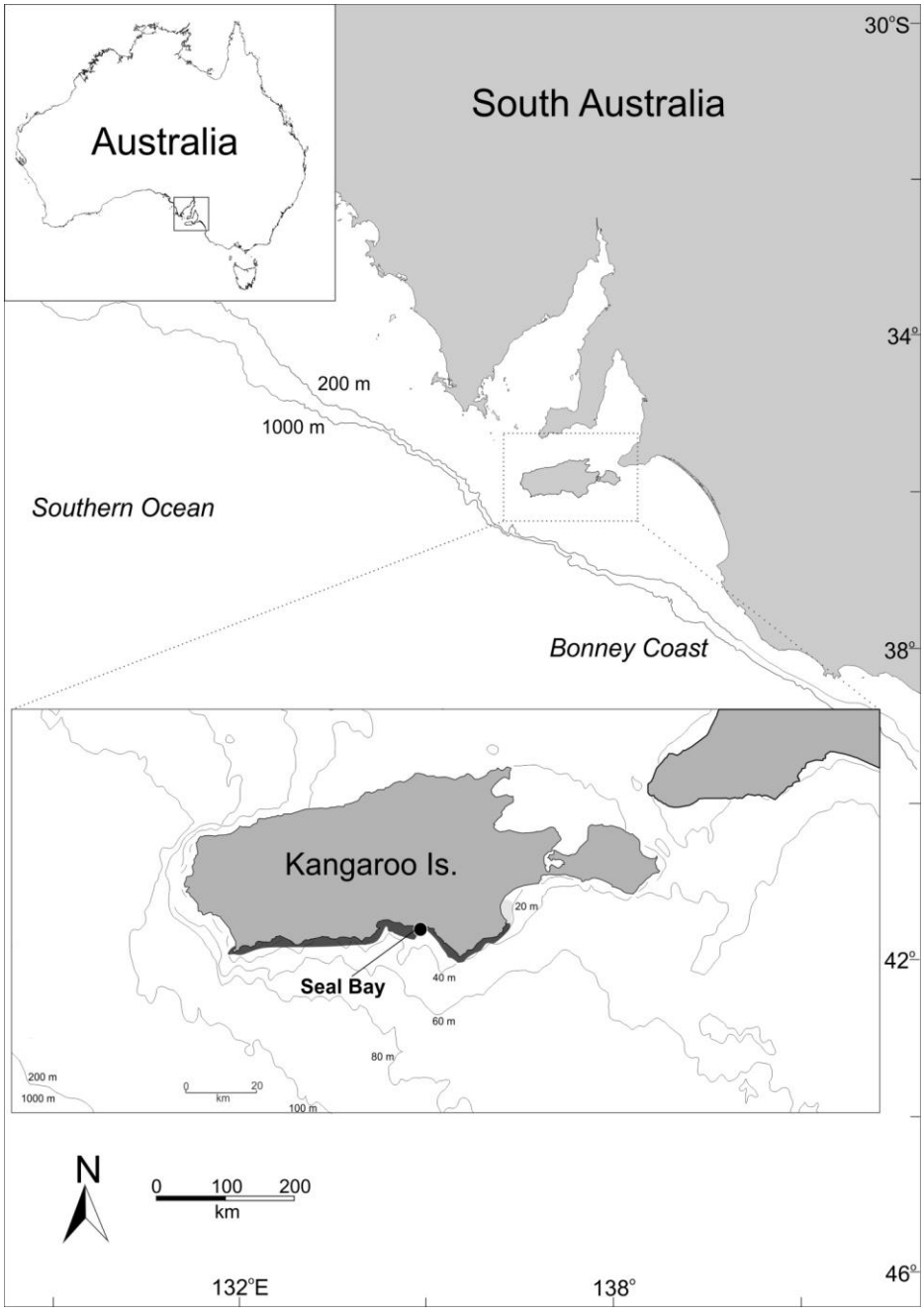

Figure 1. Location of study site, Seal Bay on Kangaroo Island. Local benthic habitat is indicated low profile reef (dark grey) and sea grass meadow with unvegetated soft bottom (light grey) (Edyvane *et al.* 1999; Bryars, 2003). Bathymetric contours are shown.

DNA extraction

DNA was extracted from a subset of the faecal samples (n = 110) prior to removal of prey hard parts. For the extraction process, ethanol-faecal mixtures were re-suspended in each bag and a 200 mg sub-sample taken. The remaining faecal material was used for the hard-part analysis. For each sub-sample the ethanol supernatant was removed from the soft-matrix and DNA extracted using the QIAamp DNA Stool Mini Kit (QIAGEN). DNA yield was quantified on a Wallac1420 multi-label fluorometer using PicoGreen® dsDNA Quantitation reagent (Molecular Probes-Invitrogen®). DNA was diluted to 3000 pg / µl (~1:5) in distilled water prior to PCR. Extraction blanks were used to monitor DNA extraction contamination for each batch of 12 extractions.

Primer sets

Two primer sets were used to amplify prey DNA in the study. The universal fish primer set (Univ Fish) is well conserved among marine fish and amplifies a ~100 bp fragment of the 16S mtDNA of fish and elasmobranchs. This primer set has been used previously to amplify fish prey from faecal DNA (Table 1) (Deagle *et al.* 2009; Peters *et al.* 2014*a*). The addition of a pinniped blocking primer to each PCR reaction reduced amplification of sea lion DNA and increased amplification of prey (Vestheim and Jarman, 2008, Vestheim *et al.* 2011). The blocking primer was added at 10 times the concentration of the primer set for PCR amplification (see Deagle *et al.* 2009) (Table 1).

The cephalopod primer set (Ceph) amplifies a ~112 bp region of the 16S mtDNA gene of common cephalopod taxa found in southern Australia (Table 1) (Norman and Reid, 2000). The primer set was designed for my previous study (Peters *et al.* 2014*a*). Primer specificity was examined using fish, elasmobranch, crustacean sequences derived from GenBank and other DNA-based diet studies (Deagle *et al.* 2009; Braley *et al.* 2010). Sequences were aligned using DNAman version 6.0© (Lynnon Corporation 2005) and Clustal X© (Thompson *et al.* 1997). Primer specificity was checked using BLASTn (Altschul *et al.* 1990) and searches of GenBank restricted to target and non-target taxonomic groups. PCR assays containing genomic DNA of southern calamary squid (*Sepioteuthis australis*), southern-keeled octopus (*Octopus berrima*), hammer octopus (*Octopus australis*), southern dumpling squid (*Euprymna tasmanica*), striped pyjama squid (*Sepioloidea lineolata*), Australian giant cuttlefish (*Sepia apama*) and arrow squid (*Nototodarus gouldi*) were initially conducted to confirm the specificity of the primer set.

Table 1. Primer sequences (5' - 3') used to amplify fish and cephalopod prey DNA from ASL faecal samples collected from Seal Bay, Kangaroo Island. Roche GS-FLX adaptor sequences* and the blocking primer†. Underlined area indicates overlap region of 3′-end of the forward fish primer and 5′-end of blocking primer.

Primer Name	Primer sequence (5'-3')	Annealing temp (^{o}C)	Product size (bp)
Univ Fish F†	CGAGAAGACCCTRTGGAGCT*1	57	~100
Univ Fish R	CCTNGGTCGCCCCAAC*2		
Ceph F	GCTRGAATGAATGGTTTGAC*1	62	~112
Ceph R	GGACGAGAAGACCCTAWTGA*2		

GS-FLX adaptor sequence: GCCTCCCTCGCGCCATCAG*1, GCCTTGCCAGCCCGCTCAG*2
†Blocking primer: ATGGAGCTTCAATTAACTTACCCAATCAGAACC

Primer adjustments for GS-FLX sequencing

To differentiate prey sequences generated for each season by the GS-FLX 454 sequencer, barcode sequence tags were added to the 5'-end of the forward and reverse primer of each primer pair. Adaptor sequences were added to the 5'-end of each primer pair to facilitate the GS-FLX sequencing process (Table 1).

PCR reactions

PCR amplifications (10µL) contained 2µL DNA, 2.0 mM $MgCl_2$, 10 x QIAGEN PCR buffer, 1 x BSA, 10 µM DNTP, 10 µM HPLC purified amplification primers, 100 µM blocking primer and 1 x 0.625 units HotStar Taq DNA polymerase (QIAGEN). Thermal cycling conditions for fish amplifications were 95 ºC for 15 min, followed by 32 cycles of: 94 ºC for 15s, 57 ºC for 90s, and 72ºC for 30 s. Thermal cycling conditions for cephalopod amplifications were: 95 ºC for 15 min, 94 ºC for 2 min, followed by 32 cycles of: 94 ºC for 10 s, 62.0 ºC for 35s and 72 ºC for 30 s. A final extension step of 72 ºC for 10 min was included in all assays. PCR amplifications were detected by gel electrophoresis on 1.5% agarose gel stained with Gelred™ and visualised using Gel-DOC UV illuminator (Bio-Rad®) supported by Quantity One Quantitation analysis software (Bio-Rad®). Positive PCR reactions were quantified using PicoGreen® dsDNA Quantitation reagent (Molecular Probes-Invitrogen®) on a Wallac1420 multilabel fluorometer. PCRs included negative controls to check for contamination. All PCR preparations used UV sterilised aerosol resistant filter tips, consumables and equipment, and were prepared in DNA free laminar flow UV sterilisation hoods.

Roche GS-FLX sequencing and analysis

Prior to GS-FLX sequencing, equimolar quantities of all amplicons for each seasonal set of samples generated were pooled to form a single metasample. Seven seasonal metasamples for each primer set were then sequenced at the Australian Genome Research Facility (AGRF) using the Roche GS-FLX (454) platform sequencer. Sequencing data for each season were obtained using a PicoTitre Plate separated by gaskets. All reads started from GS-FLX adapter primer A (Table 1).

Sequences were initially sorted by read length following Deagle *et al.* (2009). Sequences longer or shorter than the expected read length encompassed by each primer set were excluded from the analysis. Sequences were then sorted by the 5' end tag of the forward sequence followed by the reverse 5' tag sequence for each primer pair to differentiate sequences between seasons and years.

Clusters of similar sequences were identified following the method used by Deagle *et al.* (2009). Briefly, pairwise similarity was determined among all sequences using Kimura 2-parameter distance measures and clusters of similar sequences identified. Sequences within each cluster were then taxonomically identified by searches of GenBank using BLASTn. A high stringency was applied to the GenBank searches, using ≥ 97% matches and discarded sequences with lower alignment scores. Where recurrent sequences appeared common in the dataset but could not be clearly identified in GenBank, these were assigned to either the nearest lower taxonomic group or the sequences remained unidentified. In each pooled meta-sample the number of sequences per cluster was used as a proxy for species abundance (Deagle *et al.* 2009; Jarman *et al.* 2013; Willerslev *et al.* 2014).

Data analysis

Prey composition for each season and year were represented as percent numerical abundance (NA %) (proportion of total prey sequences of each prey taxa per season). Differences in dietary composition among seasons and years were assessed using non-parametric analysis of similarity (ANOSIM) on a Bray-Curtis similarity matrix with results reinforced using the permutation multivariate analysis procedure (PERMANOVA) in Primer (Primer version 6.0, PRIMER-E Ltd., Plymouth, UK). Similarity percentages (SIMPER) (Clarke, 1993) were used to identify prey responsible for group differences. The R_{ANOSIM} statistic provides a relative measure of separation between groups with R values of zero (0) supporting the null hypothesis, and a value of one (1) indicating samples within defined groups that are more similar to one other than samples from other groups. To make data comparable between seasons, sequence clusters for each prey taxa were standardised across all taxa by dividing the number of sequences of each prey item recovered by the total number of sequences recovered for that season. The data for each season was then represented as a proportion of all sequence data recovered. Yearly comparisons were also standardised using this method. Data were bootstrapped (Monte Carlo) 10,000 iterations and where necessary, transformed prior to analyses. For the multivariate analysis, a hierarchical similarity cluster analysis based on multidimensional scaling plot (MDS) was performed to exhibit the relationship between seasons or years. Stress values were calculated in three dimensions to determine how well the data were representative for the ordination. Stress values of < 0.1 provided interpretable information of the separation between groupings (Schiffman *et al.* 1981; Page *et al.* 2005). Species diversity was further calculated for each season and taxonomic group using Shannon's diversity index (H').

Assessment of sampling effort

To assess if the sequence data provided an effective representation of potential prey diversity, prey accumulation analyses were performed in R (Foundation for Statistical Computing, version 2.12, R Development Core Team, 2010) to determine the asymptotic prey diversity for overall seasonal and total prey taxa identified for each taxonomic group (fish and cephalopods). For each dataset, unique sequence clusters within each library were randomised and the total number of unique sequences represented across the sequence library was calculated. A second cluster was chosen at random, calculating the total number of sequences that it represented within the sequence library. The procedure was replicated until all unique prey taxa, represented by their respective sequences had been selected and the cumulative number of prey species calculated. The mean number ± standard deviation (SD) of prey sequences per season was calculated for each sequence cluster. The data was bootstrapped (Monte Carlo) 10,000 iterations and for each prey species, I then calculated the cumulative number of prey species represented by *j* sequences / season ± standard deviation ($\hat{\sigma}_{boot}$):

$$\hat{\sigma}_{boot} = \sqrt{\frac{n-1}{n}}\left(\sqrt{\frac{1}{n(n-1)}\sum_{i=1}^{n}(x_i - x_j)^2}\right)$$

where n is the number of iterations, x is the mean number of prey species identified by the j^{th} sequence or season at iteration i (Chernick,1999).

The mean number of prey species identified in each library was then plotted using Curve Expert (v 1.4) (Hyams, 2009). The accumulation curves were fitted using the Gompertz function to calculate the asymptotic number of prey species identified in each library interpreted as the maximum number of prey species identified by *j* sequences per season.

From the model, I then calculated the number of sequences and seasons required to represent 95% of the asymptotic prey diversity.

RESULTS

Prey hard parts

One-hundred and seventy-six faecal samples were collected for the diet analysis. Of these, a random subset comprising 110 faecal samples were selected for the DNA and hard-part analysis. Of the 110 samples, only 19 samples (17%) contained the remains of prey that were identifiable to a lower taxonomic level (genus and species) (Table 2). Ninety-one samples (83%) contained no remains, or prey remains were severely digested and damaged. To increase information on diet across seasons, 66 additional faecal samples were assessed for hard-parts. Of the 176 samples, prey hard-parts were absent in 72 (40.9 %) samples. In total, 174 prey items were recovered from 103 (58.5%) samples. Most prey items were highly eroded and only identified to phyla. In all, 72 (40.9 %) samples contained fish remains, 27 (15.3 %) cephalopods, and 4 (2.3 %) samples contained remains of small decapod crustaceans (~10 mm in length). Thirty-three samples (18.7 %) contained 56 diagnostic structures that were identified to genus or species. Two fish and four cephalopod taxa were identified to species, two cephalopods to genus, and prey items of one fish and one cephalopod to family (Table 2). Six eroded fish otoliths were recovered but these could not be identified to a lower taxonomic level.

DNA analysis overview

In all, 20,961 sequence reads were generated from the 110 faecal samples comprising 9,577 and 11,384 sequence reads from the universal fish and cephalopod datasets, respectively. For fish, the mean number of sequence reads generated per season was 1,368.1 ± 1,265.8 (median

1,294 sequences, range 10 to 3,806) and for the cephalopod dataset it was 1,626.1 ± 2,550.1 (median 239, range 10 – 5,651). For each dataset, the cumulative model for the number of sequences produced per taxonomic group exhibited a sigmoidal curve that reached an asymptote (Fig. 2). The corresponding r^2 values for each model (0.971 – 0.999) indicated sampling effort was sufficient to represent a minimum of 95% of the asymptotic diversity of potential population prey likely to have been encountered over all seasons (Table 3).

Fish primer set and prey composition

In total, 99 (90%) samples produced PCR amplicons of fish species. Four summer and seven spring samples did not produce PCR amplicons. Of the 9,577 sequences generated from GS-FLX sequencing, 6,356 (66.3%) sequence reads failed initial screening (truncated or long sequence reads) and were eliminated from the analysis. Post screening, 3,072 unique fish sequences remained in the dataset. With the exception of the winter season, the total number of fish sequences recovered ranged from 36 to 1, 415 per season (Table 4). Winter produced only one fish sequence even though all samples from this season produced PCR amplicons. This anomaly was attributed to sequencing error and the single sequence removed from the analysis. Overall, 61 unique sequence clusters were obtained and 59 of these were considered potential prey items (Table 4). Sequences in this dataset comprised predominantly bony fish (86.4%) with the remainder of sequences (13.6%) obtained from eight cartilaginous taxa.

Forty-four clusters were identified to species, 11 to genus, and 1 cluster to family (Trachichthyidae). In the last two cases, sequence clusters could not be assigned to a lower taxonomic level because these groups matched multiple species within the same genera. For example, within the sampling region, sequences assigned to *Platycephalus* sp. (flatheads) correspond to barcodes of four potential flathead species. Three clusters remained

unidentified (unknown teleosteii), as clear sequence matches could not be obtained from GenBank.

Table 2. Numerical abundance and frequency of occurrence (in parentheses) of diagnostic prey items identified from hard-parts recovered from ASL faecal samples (n = 176). Samples were collected across seasons from Seal Bay, Kangaroo Island between 2005 and 2007. Highlight (grey) with asterisk * represent prey taxa identified from faecal samples used for the DNA analysis (n = 110). No diagnostic prey items were identified from samples collected in the 2007 summer.

Genus / species	Summer	Autumn		Winter	Spring		Total
	2006	2006	2007	2006	2005	2007	
Osteichthyes							
Paraprioacanthus elongatus (slender bullseye)				2 (2)*			2 (2)
Pseudophycis bachus (red cod)		1 (1)*					1 (1)
Fam. Serranidae							1 (1)
unidentified otolith				2 (2)*	1 (1)*	3 (2)	6 (5)
Cephalopoda							
Octopus maorum (maori octopus)	1 (1)					4 (1)	5 (2)
Nototodarus gouldii (arrow squid)	3 (2) *1 (1)					1 (1)	4 (3)
Octopus berrima (southern keeled octopus)	1 (1)				1 (1)*		2 (2)
Octopus spp.	7 (5) *5 (3)	4 (4)	1 (1)*		1 (1)*	9 (4)	22 (15)
Sepioteuthis australis (southern calamary squid)						1 (1)*	1 (1)
Sepia spp. (cuttlefish)	1 (1)			1 (1)*	1 (1)*		3 (3)
Fam. Ommastrephidae	9 (7) *3 (3)						9 (7)

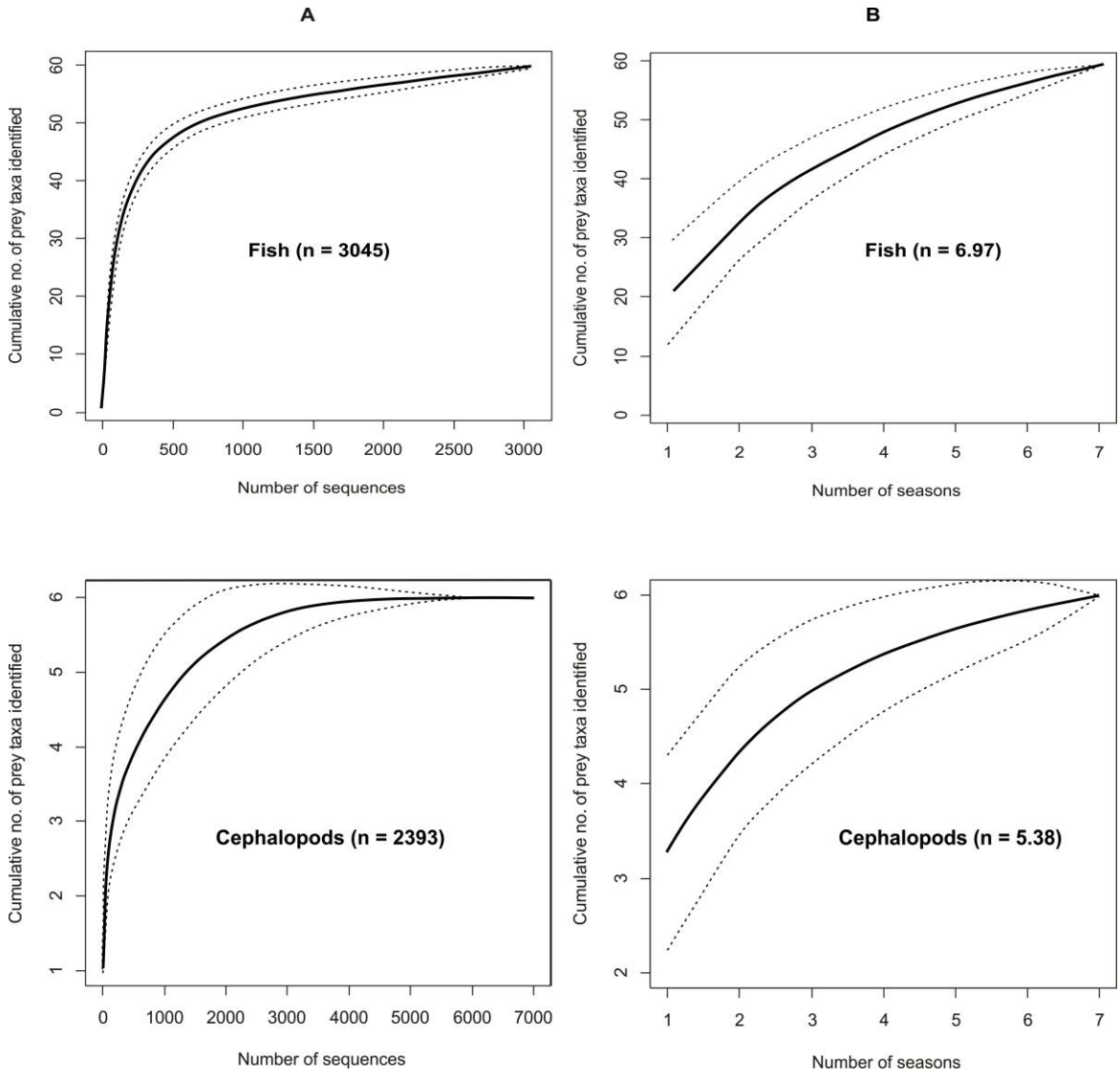

Figure 2. Asymptotic curves of prey sequence diversity obtained using next-generation sequencing for fishes (top left) and cephalopods (bottom right) from ASL faecal DNA. Asymptotes were calculated as a function of (A) total number of sequences and (B) the number of seasons sampled. Values are mean (solid line) ± SD (dotted line). In brackets are the number of sequences or seasons estimated to achieve 95% asymptotic prey diversity (see Table 3). The seasonal fish curve (B) is shown for 7 seasons (data for six seasons is presented in Table 3). Prey DNA sequences were generated from ASL faecal samples obtained at Seal Bay, Kangaroo Island between 2005 and 2007.

Table 3. The number of sequences and seasons analysed, and estimate of the asymptotic number of sequences or seasons required to achieve 95% prey diversity. All data excluding the winter analysis† indicated the mean asymptotic number of sequences or seasons sampled was similar to, or fewer than the number sampled for both fish and cephalopod datasets. Prey DNA sequences were generated from ASL faecal samples obtained at Seal Bay, Kangaroo Island between 2005 and 2007.

Prey type	Sequences or seasons sampled (*n*)	r^2	Asymptote (*a*)	Asymptotic number (95%) sequences or seasons required
Fish				
Total prey sequences	3072	0.971	57.01	3045
Season	7	0.998	61.3	6.97
Season excl. winter†	6	0.999	61.7	6.12
Cephalopods				
Total prey sequences	7019	0.991	5.98	2393
Season	7	0.998	6.07	5.38

Table 4. Taxonomic assignment and numerical abundance of prey DNA sequences obtained from ASL faecal samples collected seasonally from Seal Bay Kangaroo Island, South Australia. Combined seasonal data are the percentage of sequences for broad taxonomic group (Osteichthyes and Chondrichthyes). Numbers in parentheses are samples used for NGS. †Denotes prey taxa not previously identified in ASL diet. Winter data (*n* =1) not shown.

Genus / species	Common name	Summer		Autumn		Spring		Seasons combined	
		2006 (17)	2007 (9)	2006 (13)	2007 (16)	2005 (16)	2007 (16)		
		Count		Count		Count		Total Count	% Taxa Group
Osteichthyes									
Pseudophycis barbata	bearded rock cod	1	0	259	3	52	58	373	12.1
Platycephalus richardsoni	tiger flathead	18	14	107	2	93	27	261	8.5
Centroberyx australis †	yellow-eyed nannygai	0	0	201	1	0	18	220	7.2
Notolabrus tetricus	blue-throated wrasse	0	2	166	3	29	0	200	6.5
Genypterus blacodes †	pink ling	12	0	171	1	6	3	193	6.3
Lepidotrigla papilio †	Australian spiney gurnard	88	8	49	4	10	25	184	6.0
Thyrsites atun	barracouta	9	25	95	1	5	2	137	4.5
Kathetostoma spp. †	stargazer	7	101	0	0	0	4	112	3.6
Eubalichthys mosaicus †	mosiac leatherjacket	16	0	63	0	0	2	81	2.6
Dinolestes lewini †	long-finned pike	0	0	10	1	0	60	71	2.3
Platycephalus spp.	flathead	0	1	25	0	35	0	61	2.0
Arripis georgianus	tommy ruff	0	0	53	0	0	0	53	1.7
Nemadactylus macropterus	jackass morwong	0	0	20	0	32	0	52	1.7
Emmelichthys nitidus nitidus †	red bait	1	34	0	0	14	0	49	1.6
Parapriacanthus elongatus †	slender bullseye	0	0	44	0	0	0	44	1.4
Pictilabrus laticlavius †	senator wrasse	0	2	0	0	40	1	43	1.4
Polyprion oxygeneios †	hapuku	0	0	0	0	38	4	42	1.4
Lepidotrigla grandis †	supreme gurnard	14	26	0	0	0	0	40	1.3
Lotella rhacina	bearded rock cod	1	1	19	0	0	16	37	1.2
Allomycterus pilatus †	Australian burrfish	0	0	9	0	0	24	33	1.1
Gnathanacanthus goetzeei †	red velvet fish	0	3	12	0	16	0	31	1.0
Caesioperca lepidoptera	butterfly perch	0	0	13	0	1	11	25	0.8
Scobinichthys granulatus †	rough leatherjacket	0	22	0	1	0	2	25	0.8
Aulopus purpurissatus †	sargeant baker	0	0	18	6	0	0	24	0.8
Helicolenus spp.	ocean perch	5	0	16	0	1	1	23	0.7
Olisthops cyanomelas †	herring cale	0	0	11	0	9	1	21	0.7
Upeneichthys vlamingii	red mullet	1	0	17	2	0	0	20	0.7
Arripis trutta †	australian salmon	2	16	0	0	0	0	18	0.6
Mueschenia scaber	velvet leatherjacket	0	0	0	0	16	0	16	0.5
Pseudophycis bachus †	red cod	0	1	14	0	1	0	16	0.5
Threpterius maculosus †	silver spot	0	0	0	0	15	1	16	0.5
Conger spp. †	eastern conger eel	0	0	1	1	12	0	14	0.5
Upeneichthys spp.	goat fish	0	2	0	0	11	0	13	0.4
Trachichthyidae		0	0	7	0	0	3	10	0.3
Neosebastes pandus †	big head gurnard perch	0	8	0	0	0	2	10	0.3
Heteroscarus acroptilus †	rainbow cale	2	0	1	0	6	0	9	0.3
Meuschenia hippocrepis †	leatherjacket	0	0	0	0	0	9	9	0.3
Parequula melbournensis †	silver belly	6	0	1	1	0	0	8	0.3
Trachurus declivis †	jack mackeral	2	6	0	0	0	0	8	0.3
Scorpis lineolata †	silver sweep	2	3	0	0	0	0	5	0.2
Chironemus georgianus †	tassled kelp fish	0	0	0	4	0	0	4	0.1
Acanthaluteres brownii †	spiney-tailed leatherjacket	0	0	0	3	0	0	3	0.1
Girella spp. †	zebra fish	0	1	0	0	0	0	1	< 0.1
Cyttus australis †	sun dory	0	0	1	0	0	0	1	< 0.1
Gnathophis spp. †	conger eel	0	0	1	0	0	0	1	< 0.1
Maxillicosta spp. †	gurnard perch 2	0	1	0	0	0	0	1	< 0.1
Parapercis allporti †	barred grubfish	0	0	0	1	0	0	1	< 0.1
Pseudolabrus spp.	wrasse	0	0	0	1	0	0	1	< 0.1
Sardinops sagax †	Australian sardine	0	1	0	0	0	0	1	< 0.1
Unknown teleostii 1		0	0	8	0	0	0	8	0.3
Unknown teleostii 2		0	0	3	0	0	0	3	0.1
Unknown teleostii 3		0	0	0	0	22	0	22	0.7
Chondrichthyes									
Myliobatis sp.†	eagle ray	125	0	0	1	17	68	211	6.9
Urolophus cruciatus †	banded stingaree	30	0	86	0	0	11	127	4.1
Pristiophorus nudipinnis †	southern saw shark	0	0	0	2	0	34	36	1.2
Asymbolus spp. †	cat shark	2	0	7	0	12	3	24	0.8
Squatina australis †	Australian angel shark	10	0	0	0	0	0	10	0.3
Mustelus antarcticus †	gummy shark	9	0	0	0	0	0	9	0.3
Trygonorrhina guanerius †	southern fiddler ray	0	0	0	1	0	0	1	<0.1
	Total	**363**	**278**	**1508**	**40**	**493**	**390**	**3072**	**100**

Table 4. Continued.

Genus / species	Common name	Summer		Autumn		Winter	Spring		Seasons combined	
		2006	2007	2006	2007	2006	2005	2007		
		(17)	(9)	(13)	(16)	(23)	(16)	(16)		
Octopus maorum	maori octopus	18	5	2878	106	65	7	3631	6710	95.6
Nototodarus gouldi †	arrow squid	0	0	25	8	0	0	204	237	3.4
Sepia apama	Australian giant cuttlefish	4	0	15	7	23	1	3	53	0.8
Sepioteuthis australis	southern calamary squid	1	1	0	0	2	0	3	7	0.1
Euprymna tasmanica	southern dumpling squid	0	0	0	0	0	0	5	5	0.1
Octopus berrima	southern keeled octopus	1	0	1	1	0	0	0	3	< 0.1
Octopus spp.	octopus	0	0	0	3	1	0	0	4	< 0.1
	Total	**24**	**6**	**2919**	**125**	**91**	**8**	**3846**	**7019**	**100**

The most common sequences of fish species recovered in terms of sequence abundance were bearded rock cod (*Pseudophycis barbata*) (Moridae) (12.1%), tiger flat head (*Platycephalus richardsoni*) (Playcephalidae) (8.5%), yellow-eyed nannygai (Berycidae) (*Centroberyx australis*) (7.2%), blue-throated wrasse (*Notolabrus tetricus*) (Labridae) (6.5%), pink ling (*Genypterus blacodes*) (Ophidiidae) (6.3%), Australian spiney gurnard (*Lepidotrigla papilio*) (Triglidae) (6.0%), barracouta (*Thyrsites atun*) (Gempylidae) (4.5%), and stargazer (*Kathetostoma* sp.) (Uranscopidae) (3.6%). Sequences of the eagle ray *Myliobatus* sp. (Myliobatidae) and banded stingaree (*Urolophus cruciatus*) (Urolophidae) were the most common cartilaginous taxa detected (6.9% and 4.1%, respectively). Combined data from these 10 taxa comprised 65.7% of the total fish sequences obtained. When sequence abundances were combined and represented at taxonomic family, ten of the 39 families obtained appeared important: Moridae (cod), Platycephalidae (flathead), Labridae (wrasse), Triglidae (gurnard), Berycidae (red snapper), Myliobatidae (ray), Ophidiidae (ling), Gempylidae (barracouta), Monacanthidae (leatherjacket), and Urolophidae (stingaree) representing 74.6 % of the overall sequences obtained (Table 5). One contaminating sequence of human DNA (*Homo sapiens*) and 148 sequences of ASL were recovered indicating the blocking primer was not completely efficient at prohibiting amplification of non-target DNA.

Cephalopod primer set and prey composition

PCR amplicons of cephalopods were detected across all seasons and recovered in 92 (84%) of samples. Of the 11,384 sequences generated 4,365 (38.3%) were truncated or contained errors. Post screening 7,019 (61.7%) cephalopod sequences remained in the dataset (Table 4). The cephalopod dataset comprised six sequence clusters with five taxa identified to species and one to genus. The most common sequences comprised Maori octopus (*Octopus maorum*), which contributed overall 95.6% (n = 6710 sequences) of all but seven of the cephalopod sequences recovered (Table 4 and Table 6).The largest contributions to this sequence cluster were predominantly obtained in spring 2005 and autumn 2006 (42% and 53% of sequences, respectively). The number of sequences recovered for other cephalopod taxa identified was small: arrow squid (*Nototodarus gouldi*) (n = 237, 3.4%) and Australian giant cuttlefish (*Sepia apama*) (*n* = 53, 0.8%), with 4 other sequences from *Octopus* spp. Three were considered rare taxa, contributing < 0.1% to the cephalopod dataset: calamary squid (*S. australis*) (n = 7), southern dumpling squid (*E. tasmanica*) (n =5) and southern keeled octopus (*O. berrima*) (n = 3).

Seasonal and yearly comparisons

For each taxonomic data set, the seasonal cumulative models indicated that the number of sequences and the number of seasons sampled was adequate to represent at least 95% of the potential asymptotic prey diversity (Fig. 2, Table 3). The seasonal asymptote for fish was estimated with winter also excluded due to paucity of sequence data obtained. The resulting model indicated greater seasonal sampling effort would have provided more information of dietary diversity (6.12 asymptotic seasons vs. 6 seasons (actual)) (Table 3).

Table 5. Number of taxa, total sequences and overall percent of DNA sequences obtained for each family of fish prey taxa. †Denotes cartilaginous taxa. Prey DNA sequences were generated from ASL faecal samples obtained at Seal Bay on Kangaroo Island between 2005 and 2007.

Class / Family	Number of taxa (genus or species)	Number of sequences	Percent of sequence library
Osteichthyes / Chondrichthyes			
Moridae (morid cod)	3	426	13.9
Platycephalidae (flathead)	2	322	10.5
Labridae (wrasse)	5	274	8.9
Triglidae (gurnards, sea robbin)	2	224	7.3
Berycidae (red snapper)	1	220	7.2
Myliobatidae† (eagle ray)	1	211	6.9
Ophidiidae (ling)	1	193	6.3
Gempylidae (barracouta)	1	137	4.5
Monacanthidae (leatherjacket)	5	134	4.4
Urolophidae† (stingaree)	1	127	4.1
Uranoscopidae (stargazer)	1	112	3.6
Arripidae (Australian salmon, tommy rough)	2	71	2.3
Dinolestidae (pike)	1	71	2.3
Cheilodactylidae (morwong)	1	52	1.7
Emmelichthtyidae (rover)	1	49	1.6
Pempheridae (bullseye, sweeper)	1	44	1.4
Polyprionidae (hapuku, giant sea bass)	1	42	1.4
Pristiophoridae† (sawshark)	1	36	1.2
Diodontidae (porcupinefish)	1	33	1.1
Mullidae (red mullet, goatfish)	2	33	1.1
Gnathanacanthidae (velvetfish)	1	31	1.0
Serranidae (sea perch)	1	25	0.8
Aulopidae (treadsail)	1	24	0.8
Scyliorhinidae† (catshark)	1	24	0.8
Sebastidae (ocean perch)	3	23	0.7
Chironemidae (kelpfish)	2	20	0.7
Congridae (conger eel)	2	15	0.5
Neosebastidae (gurnard perch)	3	11	0.4
Squatinidae† (angel shark)	1	10	0.3
Trachichthyidae (roughies)	1	10	0.3
Triakidae† (hound shark)	1	9	0.3
Carangidae (trevally, jack, scad)	1	8	0.3
Gerreidae (silver biddy, mojarra)	1	8	0.3
Kyphosidae (drummer)	1	5	0.2
Clupeidae (herring, pilchard, sardine)	1	1	< 0.1
Cyttidae (dory)	1	1	< 0.1
Girellidae (zebrafish)	1	1	< 0.1
Pinguipedidae (grubfish)	1	1	< 0.1
Rhinobatidae† (shovelnose ray)	1	1	< 0.1
Unknown teleosts	3	33	1.1
Total		**3072**	**100**

Table 6. Number of taxa, total sequences, and overall percent of DNA sequences obtained for each family of cephalopod prey taxa. Prey DNA sequences were generated from ASL faecal samples obtained at Seal Bay on Kangaroo Island between 2005 and 2007.

Class / Family	Number of taxa (genus or species)	Number of sequences	Percent of sequence library
Cephalopoda			
Octopodidae (octopus)	2	6717	83.9
Ommastrephidae (arrow squid)	1	237	10.0
Sepiidae (cuttlefish)	1	53	5.2
Loliginidae (pencil squid)	1	7	0.8
Sepiolidae (dumpling squid)	1	5	< 0.1
Total		**7019**	**100**

Excluding winter, the mean number of fish taxa identified across all seasons was 21.7 ± 9.6 (median = 23, range 1- 32). The largest number of prey sequences were generated in autumn 2006, which included a high proportion of species with low sequence counts. The diversity of fish and the number of taxa consumed showed little variation among seasons and between years (Fig. 3) (ANOSIM $_{\text{FISHES (season)}}$: R = 0.281, *P* = 0.6; PERMANOVA $_{\text{FISHES (season)}}$: pseudo-F = 1.4, df = 3, *P* = 0.32; ANOSIM $_{\text{FISHES (year)}}$: R = -0.267, *P* = 0.9; PERMANOVA $_{\text{FISHES (year)}}$: pseudo-F = 0.94, df = 2, *P* = 0.54). Diet composition was dominated by seven key fish prey groups: Perciformes, Scorpaeniformes, Myliobatiformes, Gadiformes, Tetradontiformes, Beryciformes, and Ophidiiformes (Fig. 4).

Cephalopod diversity was much lower than fish (mean 3.7 ± 1.25 taxa, median = 4, range 2-5) (Fig. 3). Cephalopod sequence data for each season was dominated by the octopod, *O. maorum*. The seasonal diversity of cephalopods varied minimally (Shannon diversity index ranging between 0.1 and 0.8) (Fig. 3 and Fig. 5), although diversity H'values in autumn 2006 and spring 2007 were lower than other seasons (driven by large sequence counts of *O. maorum*). The similarity matrices indicated there were no significant differences in cephalopod prey diversity between seasons or years (ANOSIM $_{\text{CEPHALOPOD (season)}}$: R = 0.148,

P = 0.33; PERMANOVA $_{\text{CEPHALOPOD (season)}}$: pseudo-F = 0.746, df = 3, P = 0.68; ANOSIM $_{\text{CEPHALOPOD (year)}}$: R = 0.01, P = 0.47; PERMANOVA $_{\text{CEPHALOPOD (year)}}$: pseudo-F = 0.388, df = 2, P = 0.80).

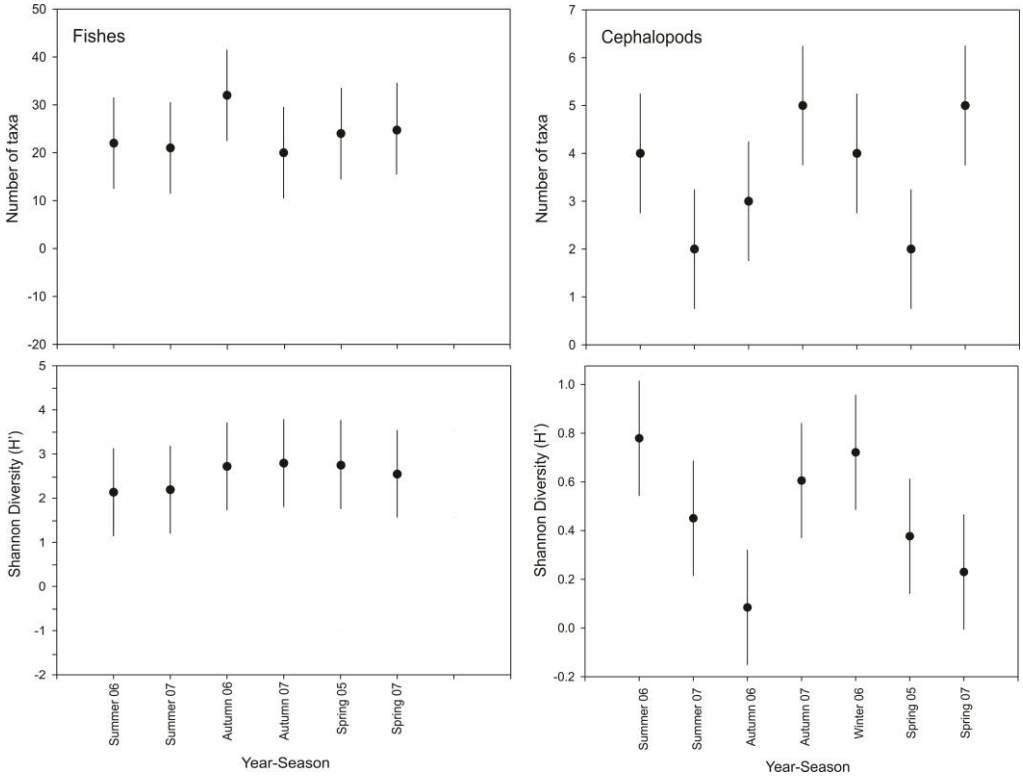

Figure 3. Number of taxa (upper) and Shannon diversity index (lower) of fish and cephalopod prey identified from DNA sequences and hard parts recovered from ASL faeces collected across seasons at Seal Bay, Kangaroo Island between 2005 and 2007. Error bars are 95% confidence intervals.

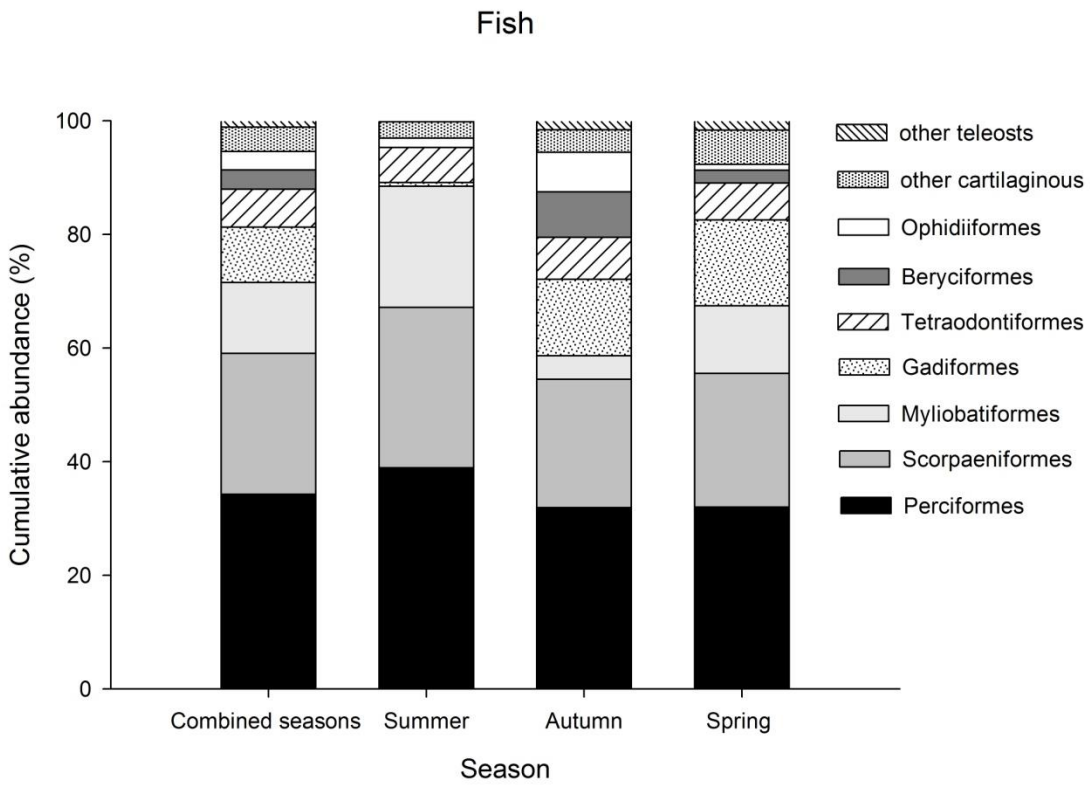

Figure 4. Cumulative percent (%) of bony fish and cartilaginous fish prey sequences by taxonomic order for each season, and for the total number of sequences recovered (combined seasons). Data were standardised within seasons and across seasons and years. * Winter data excluded from the analysis. Prey DNA sequences were generated using ASL faecal samples obtained at Seal Bay, Kangaroo Island between 2005 and 2007.

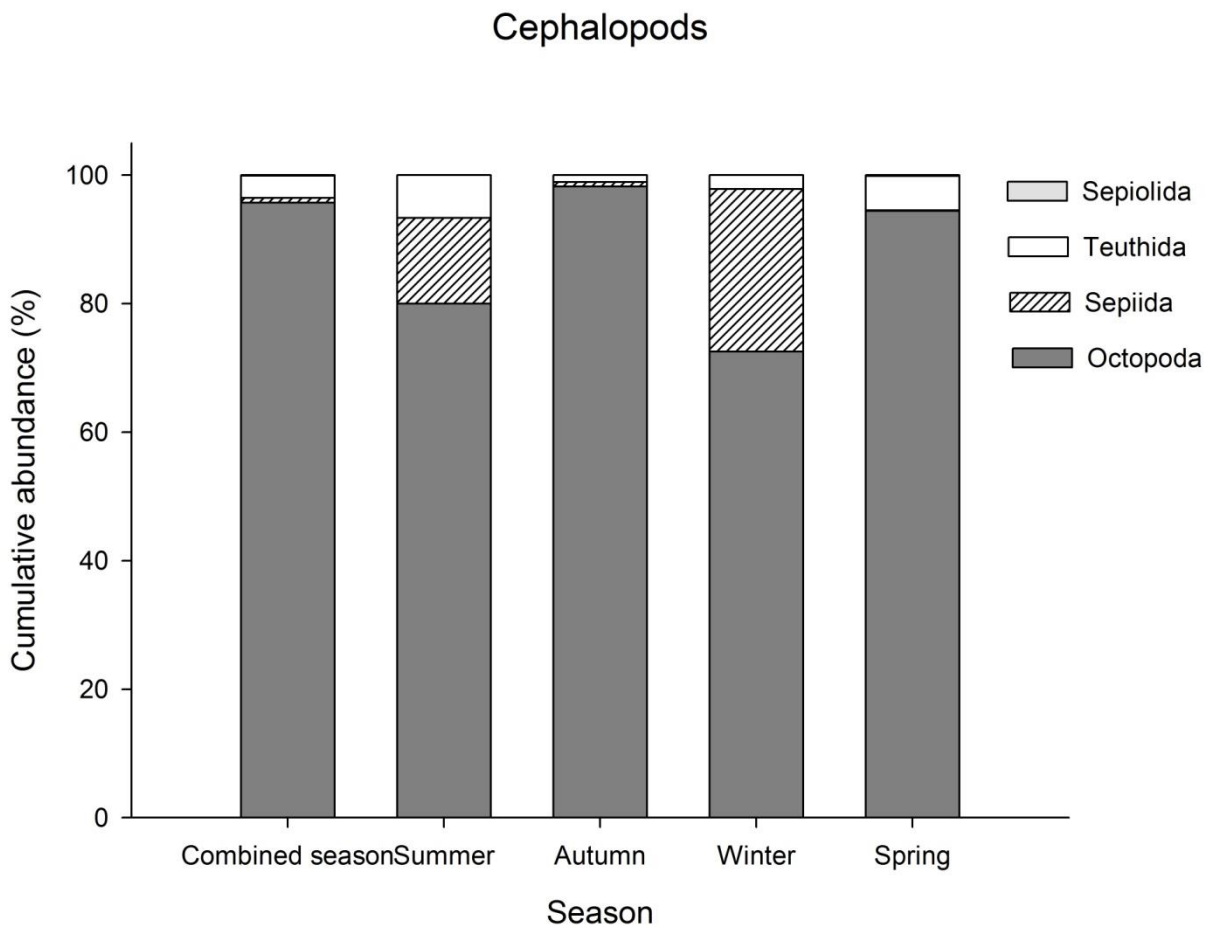

Figure 5. Cumulative percent (%) of cephalopod prey sequences by taxonomic order for each season, and for the total number of sequences recovered (combined seasons). Data were standardised within seasons and across seasons and years. Prey DNA sequences were generated from ASL faecal samples obtained at Seal Bay, Kangaroo Island between 2005 and 2007.

DISCUSSION

The analysis of faecal DNA is now widely used to determine diet across a range of terrestrial and marine organisms. More recently, next-generation high-throughput DNA sequencers have improved the depth of sequence production, providing greater resolution to individual samples and better information on system dynamics (reviewed in Shokralla *et al.* 2012). In particular, NGS technology is well suited to study the diets of seals because, as high trophic-level predators, their diets often comprise a wide range of species that can be readily

identified by amplifying and sequencing the breadth of available DNA (Deagle *et al.* 2009; Jarman *et al.* 2013).

In the present study, PCR amplification followed by NGS enabled greater depth of information on the prey consumed by ASL at one of the key breeding sites, Seal Bay than in previous studies. Sequence and hard part data indicated sea lions at Seal Bay fed on a broad range of benthic and demersal fish and cephalopod taxa, which over seasons appeared to show limited variation in prey diversity. Over the study period, 55 different fish and six cephalopod taxa were identified. When differentiated by taxonomic family, they revealed a wide diversity of prey that comprised 32 teleosts, six cartilaginous taxa, and five families of cephalopod. Similar to previous diet studies, hard parts of unidentified crustaceans were detected in this study (McIntosh *et al.* 2006; Fragnito, 2013; Chapter 2) but were not assessed using the DNA-PCR based method. Because ASL is a benthic forager (Costa and Gales 2003; Fowler *et al.* 2006, 2007; McIntosh *et al.* 2006; Goldsworthy *et al.* 2009a, b; Fragnito, 2013), benthic crustaceans (e.g. crabs, crayfish and prawns) are likely to be an important component of their diet and should be included in future DNA-based diet studies. Nevertheless, this study contributes 37 new taxa to the overall range of prey identified for ASL, of which 33 are novel to Seal Bay (Walker and Ling 1981; Gales and Cheal 1992; Ling, 1992; McIntosh *et al.* 2006; Gibbs, 2008; Peters *et al.* 2014*a*).

Study limitations

Reconstructing diet using amplified faecal DNA presents inherent biases that can limit prey identification and estimates of diversity. Similar to hard parts, DNA of different species degrades differentially which affects the likelihood of prey detection for different taxa. Detection is also dependent on the type or quality of prey tissue consumed (Dalziel *et al.* 2005; Prokopowich *et al.* 2011). Furthermore, because faeces consist largely of degraded

template DNA (Kohn and Wayne, 1997), sources of bias can stem from type II errors (failing to detect prey DNA), particularly if the predicted size of the target amplicon exceeds that of the available template (Deagle *et al.* 2006; Marshall *et al.* 2010). This is an important consideration for faecal-based DNA diet studies because DNA of taxa that is damaged and fragmented may go undetected and bias importance indices toward those species with intact DNA (Tollit *et al.* 1997; Casper *et al.* 2007b; Dunn *et al.* 2010). Degradation of faecal DNA also increases post evacuation (e.g. Deagle et al. 2005a), which, even with samples considered fresh (collected within ~12 hours of deposition), DNA quality and quantity may be compromised limiting detection of some prey. In this study, conserved PCR primers with short variable target regions (~100 bp) were used to amplify prey and improve prey detection if the sample DNA was degraded. Previous DNA-based diet studies on ASL, Australian fur seals (*Arctocephalus pusillus doriferus*) and the New Zealand fur seal (*Arctocephalus forsteri*) indicate short markers are effective in amplifying a range of taxa from degraded faecal template DNA and span a wide enough region to differentiate species-level prey (Deagle *et al.* 2009; Casper *et al.* 2007a, b; Peters *et al.* 2014a, b).

Pooling PCR amplicons from multiple samples prior to NGS did not allow sequencing of individual PCR products and improved the capacity to sample over a greater temporal scale. However, this approach has the potential to omit a range of rarer taxa that could be components of individual ASL diets. To reduce this source of sequencing error, all PCR products were standardised by molarity prior to amplicon pooling to reduce the potential of amplicon swamping. This method can eliminate contributions of non-target DNA (e.g., Deagle *et al.* 2010), but cannot account for variability in copy number among species and therefore does not ensure amplicons of all prey will be equally presented in the final sequence library (e.g. Pochon *et al.* 2013). Given these limitations, the results likely portray a representation of a range of prey taxa consumed by ASL at Seal Bay; however, as some taxa

may have gone undetected, future studies should consider sequencing individual samples to improve the potential to increase information on diet diversity.

DNA analysis

Using PCR followed by NGS improved the potential to increase the information on the range of prey taxa consumed that may not have been achievable using the clone-sequencing approach trialled in the only other DNA diet study on ASL (Peters *et al.* 2014a). Although comparisons between these two studies indicated a greater diversity of prey was achieved using NGS, a number of species that were detected by previous hard part studies at Seal Bay were absent from both, implying there are a number of biases that may limit the detection of prey by either DNA method. Early NGS platforms, such as the Roche 454, have higher sequence error rates than more recent Illumina platforms (e.g., Luo *et al.* 2012). The present study used the Roche platform, which may have resulted in the disparity in sequences produced between seasons and limited the calculation of asymptotic diversity to analyses with overall seasonal prey. Sequence counts are commonly used as a proxy for species abundance (e.g. Willerslev *et al.* 2014). It is unclear when sampling wild populations, whether these sequence counts represent single or multiple individuals, or taxa with more intact DNA. Furthermore, a number of studies have found multiple sources of sequencing bias using NGS that can influence prey sequence counts (Deagle *et al.* 2013; Thomas *et al.* 2014). Using recent NGS platforms will likely improve information on diet of ASL; however, the costs to sample multiple sites at greater temporal scales may limit its usefulness.

Prey diversity at Seal Bay

Strong foraging site fidelity revealed by satellite tracking and isotopic data suggest ASL exploit familiar habitats in search of local prey (Costa and Gales 2003; McIntosh *et al.* 2006; Fowler *et al.* 2006, 2007; Goldsworthy *et al.* 2009a,b; Lowther *et al.* 2011, 2013). At Seal

Bay, foraging by adults, juveniles and older pups (~14 mo) is concentrated in coastal and on-shelf waters adjacent to the colony, where individuals dive to depths of ~ 40 – 80 m (Costa and Gales, 2003; Fowler *et al.* 2006, 2007; Lowther *et al.* 2013). Benthic habitats in the Seal Bay region comprise a heterogeneous mix of rocky reef and sandy substrate (Edyvane, 1999; Bryars, 2003), thus, colony-level diet should reflect those species found within the adjoining foraging region. Our results are consistent with benthic foraging, as the majority of taxa identified were demersal species that are commonly associated with macro-algae reef and sand complexes that intermittently occur from the intertidal zone to the shelf slope (Shepherd and Edgar, 2013). Many of the taxa identified are located at a range of depths on the shelf out to the shelf slope, which likely reflects different sex and age groups (and depth profiles) of the individual samples collected, although these were not identified here. Interestingly, hapuku (*Polyprion oxygeneios*), yellow-eyed nannygai (*C. australis*) and ling (*G. blacodes*) are deepwater species (~90-556 m) that are commonly found closer to the shelf break (Gomon *et al.* 2008). For ASL to obtain prey in deeper water requires increased physiological capabilities such as increased body mass and oxygen storage, which nominally improve in pinnipeds with age (Costa, 1993). These taxa (e.g. *P. oxygeneios* and *C. australis*) are therefore, more likely preyed on by adult males or females, which can attain greater foraging depths than juveniles and pups (Fowler *et al.* 2006, 2007; Lowther *et al.* 2013). In the benthic foraging New Zealand sea lion (*P. hookeri*), foraging is partitioned by dive ability (e.g. depth, duration, bottom time), whereby bottom depth and habitat accessibility increases with size and age (Leung *et al.* 2014). Young, small male juveniles (2-3 years) for example, are restricted to foraging in the shallow benthic interface ≤100 m deep until the age of five, after which adult dive depths ≥ 250 m and access to deeper foraging grounds is achieved. Ontogenetic differences in diving ability and diet have also been reported in other pinniped species (e.g. elephant seal, Antarctic fur seal, and Galápagos fur seal) (Horning and

Trillmich, 1997; Le Bouf *et al.* 1996; McCafferty *et al.* 1998). Although individual diet and foraging behaviours were not examined here, the prey items identified tend to support the extent of foraging and dive depths observed in ASL for Seal Bay and broader southern Kangaroo Island region (Costa and Gales, 2003; Fowler *et al.* 2006, 2007; Lowther *et al.* 2013).

The fact that reef habitats are important aggregation sites for demersal and early life stages of some pelagic species could explain why the diet of ASL at Seal Bay comprised a consistent and higher proportion of reef species throughout the year. Overall, reef fish belonging to Perciformes (sea perch), Scorpaeniformes (scorpion fish), Gadiformes (cods), Myliobatiformes (rays), Tetradontiformes (leatherjackets), Ophiidiformes (ling), Beryciformes (snappers) accounted for ~75% of fish sequences recovered, and similarly, *O. maorum*, a common benthic reef octopus, appeared the key cephalopod prey. Aspects of reef topography, namely the density of refuges (e.g. crevices, overhangs and holes) and relief are common features of reef patches found on the shelf (Shepherd and Edgar, 2013), which are known to support a high density and abundance of species (Fréon and Dagorn, 2000). Some of the more common fish taxa identified, such as wrasse, gurnards, scorpion fish and leatherjackets, also exhibit strong site-attached fidelity to reef patches and have small home ranges (Barnett, 1995; Shepherd and Edgar, 2013; Baker *et al*. 2007, 2011). Some species including leatherjacket and wrasse, which were reasonably common prey groups, also reproduce at localised scales with some laying demersal eggs (Barnett, 1995; Baker *et al.* 2008). Such life history characteristics may increase the vulnerability of such taxa to continual predation by ASL.

This study further revealed a small, and perhaps an important range of cartilaginous taxa that have been rarely characterised as dietary components of ASL (McIntosh *et al.* 2006; Gales

and Cheal, 1992; Baylis *et al.* 2009; Peters *et al.* 2014*a*). Among these, the eagle ray (*Myliobatis* sp.) and banded stingaree (*U. cruciatus*), and to a lesser extent southern sawshark (*Pristiophorus nudipinnis*) emerged as potentially important prey. These taxa often feed in sandy substrate among low profile rocky reefs, implying ASL probably utilise such habitats to acquire these prey. Given very little is known of the cartilaginous diet of ASL, it is unclear if individuals specifically target these cartilaginous fish, or whether these species are common to the habitats where sea lions forage at Seal Bay. Rays such as *M. australis* are also benthic prey of the Australian fur seal (*A. doriferus*) (Deagle *et al.* 2009) and are reasonably common in temperate waters of South Australia (Gomon *et al.* 2008; Last and Stevens, 2009). Such taxa are largely suctorial bottom feeders that grow to 120cm with a short dorsal tail barb (Parry *et al.* 1995; Last and Stevens, 2009), which may increase its susceptibility to predation by benthic feeders such as ASL.

Finally, the benthic prey resources used by ASL at Seal Bay typically comprised low-energy taxa (Eder and Lewis, 2005) that appeared consistently available year-round. Occurrences of higher-energy bentho-pelagic species, such as redbait (*Emmelichthys nitidus*) and barracouta (*T. atun*) occurred in the diet possibly implying they are opportunistic prey obtained during the ascent or descent phases of diving. Redbait and barracouta are important seasonal prey of the Australian (*A. doriferus*) and the long-nosed fur seal (*A. forsteri*) that also forage in the shelf waters near Seal Bay (Page *et al.* 2005). Although it is not possible to determine fish size or number consumed by ASL, the low abundance of red bait and barracouta sequences relative to other taxa in the current study could suggest some partitioning of resources among these predators. ASL may have also developed a foraging strategy that favours long-term resource reliability over high energetic return. Long-term benthic foraging on familiar, lower-quality prey may confer the ecological benefit of lowering nutritional risk (Bradshaw *et al.* 2004; Lowther *et al.* 2011). For lactating ASL, such as strategy could be associated with the

prolonged gestation and investment into emergent and developing offspring. This contrasts with the reproductive strategy employed by the long-nosed and Australian fur seal, which reproduce during seasonally productive periods (e.g. coastal upwelling) that coincide with predictable and abundant, energy-rich prey (Baylis *et al.* 2008a, b; Gales *et al.*1994; Page *et al.* 2005; Deagle *et al.* 2009).

Conclusion and future directions

Determining the range of prey utilised by large marine predators is challenging because many forage in cryptic environments and target elusive prey. For pinnipeds, analyses using prey hard-parts and DNA have provided a valuable non-invasive means to obtain dietary information from faeces and regurgitates. NGS technology has greatly improved the capacity to differentiate a wider range of prey than previous methods, which has broadened information on how prey resources are utilised by predators in marine ecosystems.

In the current study, the amplification of prey DNA followed by NGS provided a new method to elucidate prey consumed by ASL at Seal Bay throughout the year. The colony-level results indicated a diet comprising mostly benthic species which, given the wide, yet repeated range of prey detected over seasons, could imply a localised dependence on these prey taxa. The high natal and foraging site fidelity of ASL, and that of some of the prey identified here, suggests conservation management should focus on strategies that protect local foraging habitats, particularly those adjacent to ASL colonies where many individuals forage. Future studies of ASL diet should aim to improve the spatial and temporal information of diet at different colonies, as individuals are likely to consume a range of prey that differs from Seal Bay. This could entail a similar DNA-based approach, and should include an assessment of crustaceans, to determine the full complement of prey used by ASL.

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CHAPTER 6

General Discussion and Future Directions

GENERAL DISCUSSION

Pinniped diets have been studied by analysing prey items recovered from stomach and faecal samples, fatty acids, stable isotopes, and more recently, using DNA analyses. These methods continue to evolve and improve, but each has well documented shortcomings (reviewed in Chapter 1). Despite these shortcomings, diet studies have provided new insights into the ecology of pinnipeds, and conservation managers have benefited from the information (e.g., Goldsworthy *et al.* 2013).

Determining the diet of Australian sea lions (*Neophoca cinerea*) (ASL) has been particularly problematic because the hard parts of their prey are completely digested, heavily eroded, or are retained within the stomach as found by other authors (Richardson and Gales, 1987; Gales and Cheal 1992; McIntosh *et al.* 2006). The small numbers of samples that have been analysed (Gales and Cheal 1992, McIntosh *et al.* 2006; Gibbs *et al.* 2008; Fragnito *et al.* 2013) have also limited the conclusions from these studies. Unlike other pinnipeds, limited detailed information of ASL diet has constrained an important aspect of understanding their biology and ecology, which is critical for conservation management to determine their role as predators within marine ecosystems. The continued decline of ASL across their South Australian range (Goldsworthy *et al.* 2015), further imparts the need to determine the extent of resource competition with sympatric otariids, the Australian fur seal (*Arctocephalus pusillus doriferus*) and long-nosed fur seal (*Arctocephalus forsteri*), and with commercial and recreational fisheries (Goldsworthy and Page, 2007).

The unique life history characteristics of ASL (protracted 17-18 mo gestation, aseasonal breeding and asynchronous reproductive cycles among colonies) (Higgins, 1993; Higgins and Gass, 1993; Gales *et al.* 1994) combined with limited natal site dispersal increases their

vulnerability to depletion from diffuse (e.g., extractive) and direct sources (e.g., by-catch). In addition, the fact that ASL display long-term site fidelity to foraging locations and a temporal persistence to particular trophic levels of prey, make them more susceptible to local prey depletions if foraging behaviour is rigid and limited flexibility to search for alternate sources of food (e.g., Lowther *et al.* 2011).

In the current study, the diet of ASL was determined from faeces and a limited number of regurgitate samples collected from several ASL breeding colonies in South Australia. This is one of the largest, fine-scale studies of ASL diet to date, which, by using traditional hard part and novel DNA-based analyses, aimed to improve the information available on the diet of ASL. A comparison of the methods is provided in Table 1.

The initial hard part analyses (Chapter 2) conducted across the range of ASL colonies in South Australia, confirmed the results of Gales and Cheal *et al.* (1992) who classified the approach as 'unreliable'. This study indicated hard parts recovered from faeces and regurgitates of ASL are mostly digested, biasing results towards prey items such as cephalopod beaks that are highly resistant to digestion. Even though a large sample size was examined in this study (n = 345), the hard part approach yielded poor information of ASL prey diversity other than cephalopod prey. Here, only 344 prey items (273 cephalopod, 66 fish and 5 crustaceans) were identified. In comparison, the study by Reinhold *et al.* (2015), identified 789 prey items from 110 faecal samples of the long-nosed fur seal (*A. forsteri*), exhibiting the magnitude of difference in level of digestion that must occur in ASL compared with the fur seal. It is unlikely that this relates to the types of prey ingested because digestion transit rates of prey hard parts are similar between these pinniped species (Richardson and Gales, 1987; Casper *et al.* 2007*a*). In addition, small, fragile otoliths such as those of redbait and sardine that are prone to digestion are common in long-nosed fur seal faeces. Information

on the cephalopod component of ASL diet from this study is however, invaluable. Four key taxa: octopus (*O. maorum*), cuttlefish (*Sepia* spp.), and loliginid (*S. australis*) and ommastrephid squids (*N. gouldi*) were identified as important prey of ASL. The distribution patterns and abundance of demersal Loliginidae and Ommastrephidae cephalopods is well understood in South Australia (e.g. Steer *et al.* 2006; Stark, 2008), however, little is known of the spatial extent of distribution of benthic species such as octopus in the GAB and Gulf ecosystems (M. Steer *pers. com*). In this study, the latter were the most frequent and abundant ASL cephalopod prey. Sampling multiple ASL colonies across South Australia provided useful information on the spatial extent of these cephalopods and their consumption by ASL. This information could be important to determine the level of competition with sympatric foraging Australian and long-nosed fur seals, and fisheries (Page *et al.* 2005; Reinhold *et al.* 2015). By calculating cephalopod biomass, this study also found smaller octopuses were as important in the diet as larger Loliginidae and Ommastrephidae squids. Biomass was also determined for a small number of teleost fish species. Although this information was limited, it provided information of the size of fish prey handled by ASL. Analysis of hard parts improved the baseline data of potential prey groups examined in subsequent DNA- based analyses.

Table 1. Advantages and disadvantages of different DNA-based analyses and hard part analyses to determine diet in ASL.

Diet method	Advantages	Disadvantages
Visual identification of hard remains		
Faeces, regurgitates	Some species-level identification of prey items	Damage to hard parts limits prey identification
	Good resolution of cephalopod taxa	Poor information of prey diversity
	Biomass estimates of prey (particularly cephalopods)	Poor taxonomic resolution for some taxa within same family or genus
	Can complement DNA-based diet methods	Prey identification time-consuming
	Regurgitates can provide information of prey ingested over many days	Requires diagnostic hard components to be ingested and survive digestion
		Prey items can be retained in the stomach and not present in faeces
		Limited to recent meal ingestion (~ 48 hours) (except for regurgitates)
Conventional PCR with clone sequencing		
Faeces	Identification of most prey species ingested	Differential digestion and damage to DNA may limit prey identification
	Can identify single species and groups of prey	Qualitative
	Does not require hard parts for prey identification	Limited resolution. Prey may not be detected at low DNA concentrations
	Identification of prey from soft tissues (e.g., muscle tissue) ingested	Expensive (cost and time) if a large number of samples need to be cloned
	No observer bias in prey identification	Requires DNA reference database for samples from the wild to be identified
	Samples can be pooled to obtain colony-level information on diversity	Limited to recent meal ingestion (~ 48 hours)
Real-time Quantitative PCR (qPCR)		
Faeces	Highly sensitive. Can detect prey ingested at very low concentrations	Differential digestion and damage to DNA may limit prey identification
	Useful for single-species detection	Limited to single species tests rather than groups of species
	Quantitative for single species (but may not reflect amounts of species ingested)	Expensive (cost and time) to develop DNA calibration tests for each species
	Can be quantitative if calibration curves are developed	Limited to recent meal ingestion (~ 48 - 72 hours)
	Does not require hard parts for prey identification	
	Identification of soft prey tissues (e.g., muscle tissue) ingested	
	No observer bias in prey identification	
	Pulse prey can be detected	
DNA barcoding with next generation sequencing		
Faeces	Can detect and identify DNA from most species present in sample	Differential digestion and damage to DNA may limit prey identification
	Samples can be pooled to obtain colony-level information on diversity	Presently qualitative (requires calibration trials for quantitave information)
	Lower costs (and time) to develop equivalent diversity profiles than cloning	Expensive (cost and time) to develop and run plates
	Does not require hard parts for prey identification	Contamination can influence diversity outcomes
	Identification of prey from soft tissue (e.g., muscle tissue) ingested	Requires DNA reference database for samples from the wild to be identified
	No observer bias in prey identification	

Captive feeding trials are often useful to validate new methods of diet analysis and have been important in the development of DNA-based analysis of pinniped faeces. DNA validation trials have been conducted on several pinniped species including harbour seals (*Phoca vitulina*) (Thomas *et al.* 2015), sub-Antarctic fur seals (*A. tropicalis*), long-nosed fur seals (Casper *et al.* 2007*a*), and Steller sea lions (*Eumetopias jubatus*) (Deagle *et al.* 2005; Bowles *et al.* 2011), with such studies indicating DNA-based analysis improves prey detection. A few studies have also attempted DNA quantification from meals ingested, with varying degrees of success (e.g. Bowles *et al.* 2011). The ASL feeding trial (Chapter 3) is the only pinniped DNA-based diet study to assess simultaneously, the efficacy of conventional PCR and real-time quantitative PCR (qPCR) prey detection, enabling a broader comparison of results to hard part analysis (Chapter 3). The results indicated qPCR was more sensitive and detected prey DNA more frequently than conventional PCR methods (by about 25%). However, when compared to hard parts, both DNA methods significantly improved the detection of fish, shark, and squid that had been fed to ASL by ≥ 50 -100%. The importance of all prey ingested was significantly underestimated by hard part analysis and some species remained undetected (shark and cephalopods). Furthermore, there were large discrepancies in the recovery of DNA between individual ASL, suggesting intraspecific biases may limit detection of some prey taxa if these techniques were applied to ASL faeces collected in the wild. The results of this study indicated DNA-based methods are reliable and can readily detect prey from ASL faecal DNA in the absence of hard parts. These findings will improve understanding of the limitations and biases of different studies and different methods to determine the diet of ASL and other pinnipeds.

A general conclusion from previous DNA-based diet studies of pinnipeds is that compared to hard part analyses, DNA-based methods can improve information on the diversity of some

prey species (Casper *et al.* 2006; Matejusová *et al.* 2008; Deagle *et al.* 2009). This study reinforced these interpretations. I employed species-specific and universal primers to amplify prey DNA, and trialled the clone sequence and next-generation (NGS) sequencing methods to identify individual species and different taxonomic groups of prey (Chapter 3, 4, 5). Following the success of the feeding trial (Chapter 3), the clone sequencing approach was used to study the diet of a small number of individuals (n = 12) from two breeding ASL colonies (Chapter 4). Clone sequencing revealed ASL consumed a wide diversity of benthopelagic and demersal prey, and 23 species of fish and five cephalopod prey were identified. Approximately 28 species identified were novel, having not been previously described in the diet of ASL. In comparison, the study of diet by hard parts identified one fish and one cephalopod prey. Diets of individuals varied between sites, and colonies varied in overall diversity, but there were several prey families (e.g. Labridae, Monacanthidae, Platycephalidae, Octopodidae) common to both colonies. This suggests individual diet of ASL probably reflects their individual foraging patterns, supporting the conclusion of foraging fidelity (e.g. Lowther *et al.* 2011), while at the same time could indicate ASL target common habitats or region-specific prey. To my knowledge, this is the first study to assess fine-scale individual diet variation in ASL using DNA-based methods, which provided intriguing insights into ASL prey. Clone sequencing has been a useful approach to assess diet diversity of other aquatic and terrestrial consumers (e.g. Bradley *et al.* 2007; Carreon-Martinez, 2011). However, the time and costs to clone and sequence large numbers of samples may limit such an approach to smaller diet studies of single individuals and groups of prey, particularly if diet comprises a wide diversity of prey.

The fact that ASL repeatedly target the same foraging locations and forego others that may be potentially viable has been suggested a response to long-term individual specialisation and familiarisation to local habitat and food resources (Lowther *et al.* 2011). In ASL, benthic

foraging in patchy heterogeneous seafloor environments could be expensive if the trade-off of viable resources is not met. Because benthic species are unlikely to escape vertically to the mid-water layer to avoid predation (Shepherd and Edgar, 2013), the predictability of certain patches and their associated prey is likely to be an important factor in decision making for ASL when feeding, as also observed in northern fur seals (Benoit-Bird *et al.* 2013).

I trialled novel next-generation sequencing (NGS) to examine seasonal diet variation in ASL by repeat sampling of faeces at the Seal Bay colony over time (Chapter 5). The NGS approach enables thousands to millions of DNA fragments to be sequenced simultaneously (Deagle *et al.* 2009; Rayé *et al.* 2011; Yoccoz, 2012; Sousa *et al.* 2016). By combining individual samples this method provided an unparalleled depth of information of the prey diversity used by ASL throughout the year. Diet of ASL at Seal Bay comprised 39 families of fish and five families of cephalopod prey, of which many were demersal species found in reef and sandy habitats that are common in shelf waters near Seal Bay. The consistency of common prey groups consumed over each season, suggested ASL consumed some prey that are available throughout the year. These results provide a cautionary tale of seasonal diet because colony-level synthesis generalises prey use and may not reflect the diets of all individuals or sex and age classes at the colony. Anomalies such as the disparity in sequence generation between seasons could have also influenced these results. Sequencing error, which is indicative of older Roche NGS platforms, could be resolved by conducting parallel analysis on newer sequencers such as Illumina. This would still require an assessment of the biases in a controlled environment such as a captive feeding trial, because of intraspecific differences in digestion between individuals and the digestive variability of DNA among prey. Such influences will ultimately exclude some prey if DNA-based approaches are employed to determine diet at ASL breeding colonies. Nevertheless, these methods are effective in providing fine-scale information.

Although independent foraging behaviour was not tracked in any of these studies, the results appeared consistent with biologging and animal borne videography diet studies, which indicate ASL target specific foraging patches and trophic levels of prey (Lowther and Goldsworthy, 2011, Lowther *et al.* 2011; Fragnito, 2013). Studies of the diets of other sea lion species have typically identified differences between individuals, colonies, and seasons (Villegas-Amtmann *et al.* 2008; Drago *et al.* 2016). The spatial differences observed in the diets of ASL in this study could therefore be explained by the differences in the benthic habitats near each colony that are known to support different species of prey (Bryars *et al.* 2003; Lowther and Goldsworthy 2011; Lowther *et al.* 2011; Shepherd *et al.* 2013). Many pinniped diet studies have concluded that diets reflect prey availability but few have measured the distribution or abundance of prey. Guinet *et al.* (2001) measured the abundance of prey in locations used by Antarctic fur seals (*A. gazella*) and found that diet did not reflect prey availability. They concluded that factors other than prey availability could influence their diet. Call *et al.* (2008), for example, proposed individual foraging site fidelity in northern fur seals (*Callorhinus ursinus*) was a function of route choice to reduce competition with conspecifics. In contrast, Benoit-Bird *et al.* (2013) found that northern fur seal foraging behaviour matched aggregations of preferred prey. A compelling avenue of research for ASL diet studies would be to compare the diet and feeding locations of ASL and the availability of their prey. The combination of NGS and biologging technology for example, could greatly improve understanding of how local-scale prey densities and diversity influence the foraging fidelity of ASL. Adult male ASL tracked by Lowther *et al.* (2013), for example, showed temporal fidelity to foraging grounds and isotopic data indicated seasonal trophic-level shifts in their use of prey. They concluded that although individuals may exhibit strong fidelity to sites, oceanographic events such as coastal upwellings could influence the diversity of available prey. Finer-scale studies of ASL diet using NGS could therefore assist in

understanding the foraging patterns of individuals and their trophic requirements. Such studies would improve understanding of ASL foraging ecology, as well as their interactions with commercial and recreational fisheries.

FUTURE STUDIES OF ASL DIET

The aim of this study was to develop and assess different DNA-based techniques to determine diet in wild populations of ASL. Although small-scale individual diet variability of female ASL was assessed (Chapter 3), this study did not investigate demographic differences such as sex or age in the diets of ASL, or spatial differences across their entire breeding range. These factors are likely to drive differences in the diet of ASL. In fur seals and sea lions body mass and diving capability are intrinsically correlated to the capacity to store oxygen, which determine the depth of water and habitats at which prey can be accessed (Kooyman 1989; Fowler *et al.* 2006; Weise *et al.* 2010; Jeglinski *et al.* 2012). Larger seals are also able to handle and consume relatively larger prey (Page *et al.* 2005). Future ASL diet studies should determine whether different age and sex groups use different prey, which could potentially reduce intra-specific competition as noted for northern fur seals (Call *et al.* 2008). For example, during lactation female fur seals and sea lions behave as central place foragers, alternating foraging with regular provisioning of their dependent pups on shore (Orians and Pearson, 1979; Robson *et al.* 2004; Lowther *et al.* 2011; Villegas-Amtmann *et al.* 2016). The need to commute between foraging grounds and the colony is one factor that influences their foraging behaviours. ASL are particularly interesting in this regard because they provision their young for approximately 18 months (Higgins, 1993), and during lactation ASL pups may travel between colonies and haul-out sites in the processes of foraging with their mother (Lowther *et al.* 2012). Because DNA-based studies can identify the diet of individuals, future diet studies could investigate fine-scale differences in the foraging ecology

of individual lactating females and their pups. Trophic level studies have partly quantified such relationships between mother and pup pairs (Lowther *et al.* 2012); if the availability of different prey was also quantified such studies could determine the extent to which pups learn foraging sites and skills from their mothers and improve our understanding of the habitat requirements of ASL.

Spatial differences in the diet of ASL are highly likely because they breed over a wide range, from South Australia to Western Australia. This entire area is influenced by the low nutrient environment of the Leeuwin Current particularly in the west of their range (Creswell and Golding, 1980), but other oceanographic features are also important, and may drive differences in the diets of ASL (Lowther *et al.* 2011). For example, seasonal upwellings increase productivity offshore from southwestern Australia (Gersbach *et al.* 1997; Rennie *et al.* 2009) and in the Kangaroo Island area (Butler *et al.* 2002; Middleton, 2007; Middleton and Bye, 2007; Van Ruth *et al.* 2010; Lowther *et al.* 2013). These increases in productivity may influence the prey available to ASL and increases in productivity have been associated with increased survival of ASL pups at Seal Bay (McIntosh *et al.*, 2013). To inform the management of ASL, including the species' potential trophic interactions with commercial and recreational fisheries, future studies should seek to quantify the diet of ASL across their range. This should also include an analysis of crustaceans that are likely important prey of ASL (McIntosh *et al.* 2006), but were not addressed here. Recent advances in DNA-based methods have improved the capacity to undertake such studies, because samples can be combined for analyses to examine spatial differences.

CONCLUSION

Recent improvements in DNA-based methods are likely to increase the efficacy of pinniped diet studies. In particular, continual growth of DNA sequence libraries and greater use of

NGS technology to characterise environmental sources of DNA (e.g., faeces, soil), will likely improve species-level information available to determine diet diversity of terrestrial and aquatic consumers. This information, combined with methods that quantify next-generation sequence counts to determine the relative abundance of prey will improve estimates of biomass consumed by pinnipeds (Deagle *et al.* 2013). Such studies will be able to quantify interactions between ASL and other marine predators and prey, and with commercial fisheries. These studies are required to address one of the most compelling questions facing conservation and fisheries managers – what is preventing the recovery of ASL populations across their range? This question is particularly interesting because sympatric populations of the Long-nosed fur seal and Australian fur seal are now rapidly recovering, and studies indicate that their populations could continue to increase in number over the next 15 to 20 years (Shaughnessy *et al.*, 2014, 2016). These data will also improve ecological models (e.g. Goldsworthy *et al.* 2013), which are used to inform the management of ASL populations and the ecological sustainable development of fisheries.

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