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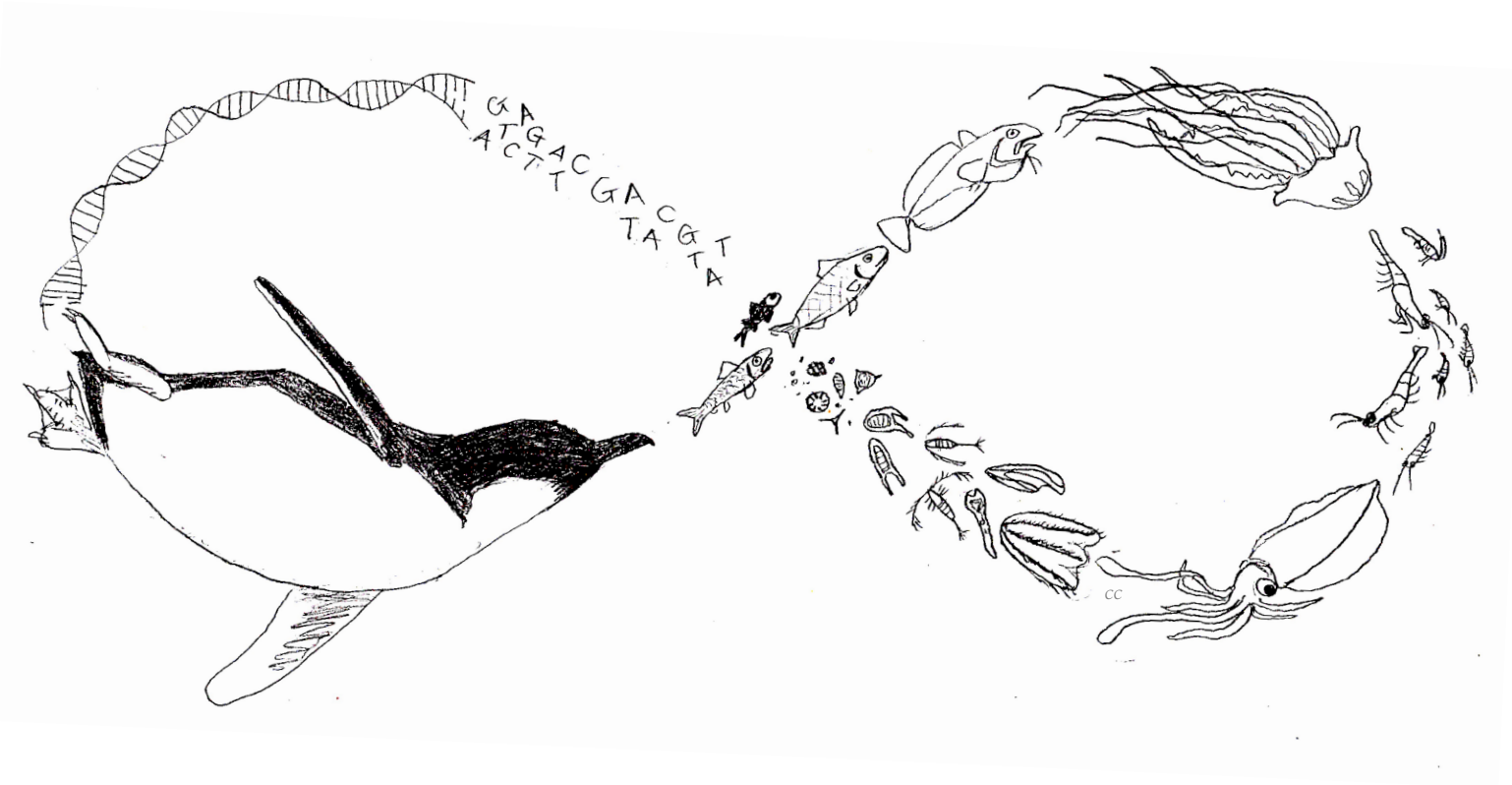

Plastic foraging behaviour of a marine top predator

A thesis submitted for the Degree of Doctor of Philosophy

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Plastic foraging behaviour of a marine top predator

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A thesis submitted for the degree of *Doctor of Philosophy* at
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Table of Contents

Table of Contents ii

Abstract iv

Publications during enrolment vi

Thesis including published works declaration vii

Acknowledgements ix

1 Chapter 1 General Introduction 1
- **1.1 Harnessing plastic seabird behaviours to monitor environmental change** 2
- **1.2 Measuring seabird diet** 4
- **1.3 Faecal DNA-metabarcoding** 5
- **1.4 Penguins as marine sampling units** 6
- **1.5 Aims and thesis structure** 8
- **1.6 References** 12

2 Chapter 2 Molecular analysis of predator scats reveals role of salps in temperate inshore food webs. 24
- **2.1 Abstract** 24
- **2.2 Introduction** 25
- **2.3 Methods** 27
  - 2.3.1 Experimental design 27
  - 2.3.2 Study sites, species and collection of breeding stage data 27
  - 2.3.3 Collection of scat samples 28
  - 2.3.4 Molecular methodology 29
  - 2.3.5 Statistical analysis 31
- **2.4 Results** 33
  - 2.4.1 Diet composition 35
  - 2.4.2 Dominant taxa in individual samples 35
  - 2.4.3 Diet in relation to month of the year 36
  - 2.4.4 Diet in relation to breeding stage 39
- **2.5 Discussion** 41
  - 2.5.1 The role of salps in a piscivorous diet 44
  - 2.5.2 Shedding light on temperate inshore food webs 45
- **2.6 Acknowledgements** 46
- **2.7 Author contributions** 46
- **2.8 Funding disclosure** 46
- **2.9 References** 47
- **2.10 Chapter 2 Supplementary Information** 55

3 Chapter 3 Quantifying prey availability using the plastic foraging ecology of marine top predators.. 58
- **3.1 Abstract** 59
- **3.2 Introduction** 60
- **3.3 Methods** 62
  - 3.3.1 Study site and system 62
  - 3.3.2 Foraging success and trip duration 62
  - 3.3.3 Field records of breeding stage 63
  - 3.3.4 Faecal DNA-metabarcoding 63
  - 3.3.5 Developing a predator-derived index 65
  - 3.3.6 Estimating biomass consumption 67
  - 3.3.7 Statistical analysis 68
- **3.4 Results** 69
  - 3.4.1 Penguin diet 69

3.4.2 pCPUE determination ... 72
3.4.3 Estimated breeding-season biomass consumption ... 76
3.5 Discussion ... 77
3.5.1 A useful and adaptable index for ecosystem management ... 79
3.5.2 Considerations ... 80
3.5.3 Conclusions ... 82
3.6 Acknowledgements ... 82
3.7 Ethics Statement ... 83
3.8 Author contributions ... 83
3.9 Funding ... 83
3.10 Data accessibility ... 83
3.11 References ... 84
3.12 Chapter 3 Supplementary Information ... 93

4 Chapter 4 Predator foraging efficiency related to local environmental conditions ... 100
4.1 Abstract ... 100
4.2 Introduction ... 100
4.3 Methods ... 103
4.3.1 Experimental design ... 103
4.3.2 Automated monitoring ... 104
4.3.3 Diet ... 105
4.3.4 Catch per unit effort (CPUE) ... 105
4.3.5 Environmental data ... 106
4.3.6 Comparing relevant CPUE and environment values ... 107
4.4 Results ... 109
4.4.1 The foraging environment ... 109
4.4.2 Penguin foraging outcomes ... 110
4.4.3 The effects of the environment on penguin foraging ... 111
4.4.4 The effects of environmental variables in the presence of thermal stratification ... 114
4.5 Discussion ... 116
4.5.1 Sea surface temperature ... 116
4.5.2 Wind and stratification ... 118
4.6 References ... 122

5 Chapter 5 General Discussion ... 129
5.1 General findings ... 129
5.2 Increased diet breadth ... 131
5.3 Patterns in little penguin diet ... 132
5.3.1 At the broad taxonomic scale ... 132
5.3.2 Patterns in the availability of key little penguin prey fish species ... 133
5.4 Responses to change in a temperate coastal system ... 134
5.5 Estimating biomass extraction by top and meso predators ... 135
5.6 Limitations ... 136
5.6.1 Limitations of experimental design ... 136
5.6.2 Limitations of the method ... 138
5.7 Management implications and suggestions ... 139
5.8 Concluding remarks ... 142
5.9 References ... 144

6 Appendices ... 153

Abstract

In this Anthropocene era of change, we would be in a better position to avoid extinctions and ecosystem collapse if we have a strong understanding of how species fit together in food webs and respond to environmental variation. Because marine environments are logistically difficult to survey, we often look to accessible marine top predators to shed light on changes in lower trophic levels. However, many predators exhibit flexible foraging strategies that may buffer the negative effects of these changes. This makes it difficult for us to identify functional links between predators, their food web and environment. As the trophodynamics of marine predators are often employed by marine biologists as proxies of resource availability, resolving these flexible "plastic" behaviours is an important focus of current marine research. High precision tools such as DNA-metabarcoding and automated monitoring systems are making it possible to track and define foraging traits at the high temporal resolution needed to infer relationships between foraging and environmental variation. To improve our understanding of foraging plasticity in marine top predators and increase their capacity as indicators of marine resources, I investigated the plastic foraging behaviours of little penguins, *Eudyptula minor*, which are generalist predators that are highly restricted in their foraging range when breeding. I used the information gained to develop methods to obtain predator-derived measures of prey availability and consumption.

To resolve potential gaps in our knowledge of little penguin diet, I used broad-spectrum faecal DNA-metabarcoding to avoid taxonomic biases associated with traditional methods. I recovered DNA sequences from gelatinous and crustaceous plankton groups that have not previously been detected in the little penguin diet. Gelatinous groups were present in 76% of faecal samples and comprised 25% of all sequences, presenting a new trophic link in the food web of little penguins. Broad diet varied both seasonally and over breeding stages, indicating both extrinsic and intrinsic drivers of penguin foraging.

I integrated fine-scale measures of foraging effort (trip duration) and success (mass change) from an automated weighbridge system with diet recorded by DNA-barcoding to develop a new index of predator foraging efficiency analogous to the catch per unit effort (CPUE) index used by fisheries to measure stock availability. I used this prey-specific index, the pCPUE, to infer the availability of key fish species to little penguins breeding at Phillip Island in south-eastern Australia during the

breeding season and to estimate biomass extracted within their foraging zone. Breeding little penguins extracted ~1300 tonnes of biomass from their coastal ecosystem over two seasons, including ~220 tonnes of commercially important sardine. Fish-specific pCPUE analysis indicated a highly opportunistic foraging strategy that progressed from a focus on a single prey species to a group of species over each breeding season, matching regional schooling and spawning patterns. Penguins maintained high foraging success, low foraging effort (trip duration) and high breeding success, demonstrating their capacity to buffer themselves against varying prey availability by switching prey species.

I also investigated whether little penguin foraging efficiency (as pCPUE) varied in response to key environmental variables known to drive penguin prey availability. Little penguins foraged more efficiently at sea surface temperatures <17.5°C, but caught more of their primary prey, fish, as sea surface temperature increased. This may point to a local adaptation that ensures chicks are raised during the peak in fish availability in the region.

In summary, I have extended the known foraging niche of little penguins, revealed gelatinous trophic links in coastal food webs and produced a method to resolve functional links between predators and prey resources, by employing the very plasticity that has formerly limited seabirds as bio-indicators. Together, these findings offer insight into the foraging strategies of predators that are tied to land during breeding attempts and therefore limited in foraging opportunities. Importantly, the predator-derived pCPUE and prey consumption measures I developed in this study are applicable across a range of inshore marine predators and reduce our reliance on fisheries data by providing a program for seasonal monitoring of prey resources.

Publications during enrolment

Cavallo, C, Chiaradia, A, Deagle, B, McInnes, JC, Sánchez, S, Hays, GC, Reina, RD (2018) Molecular analysis of predator scats reveals role of salps in temperate inshore food webs. *Frontiers in Marine Science* **5**, 381, 1–14, doi: 10.3389/fmars.2018.00381

Sánchez, S., Reina, R. D., Kato, A., Ropert-Coudert, Y., Cavallo, C., Hays, G. C., & Chiaradia, A. (2018) Within-colony spatial segregation leads to foraging behaviour variation in a seabird. *Marine Ecology Progress Series* **606**, 215-230.

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in a peer reviewed journal, one submitted publication and one intended for submission in a peer reviewed journal. The core theme of the thesis is *investigating the foraging ecology of marine central-place foragers as measures of prey availability*. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Biological Sciences under the supervision of Associate Professors Richard Reina and André Chiaradia.

(The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.)

In the case of chapters 2-4 my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N
2	Molecular analysis of predator scats reveals role of salps in temperate inshore food webs	*Published*	80%. Design, data collection, data analysis, and writing of manuscript.	*1.* André Chiaradia[ac] 4% *2.* Bruce E. Deagle[ac] 2.75% *3.* Julie C. McInnes[c] 2.75% *4.* Sonia Sánchez[bc] 2.75% *5.* Graeme C. Hays[ac] 2.75% *6.* Richard D. Reina[ac] 5%	Yes (Sonia Sánchez)
3	Quantifying prey availability using the plastic foraging ecology of marine top predators	Submitted	80%. Design, data collection, data analysis, and writing of manuscript.	*1.* André Chiaradia[ac] 3% *2.* Bruce E. Deagle[ac] 2.5% *3.* Graeme C. Hays[ac] 2.17% *4.* Simon Jarman[c] 2% *5.* Julie C. McInnes[c] 2% *6.* Yan Ropert-Coudert[c] 2% *7.* Sonia Sánchez[bc] 2% *8.* Richard D. Reina[ac] 4%	Yes (Sonia Sánchez)
4	Predator foraging efficiency related to local environmental conditions	Not yet submitted	90%. Design, data collection, data analysis, and writing of manuscript.	*1.* André Chiaradia[ac] 4% *2.* Richard D. Reina[ac] 4% *3.* Sonia Sánchez[b] 2%	Yes (Sonia Sánchez)

[a]*Aided with study design*
[b]*Helped collect field data*
[c]*Proof-read and contributed to manuscript*

I have renumbered sections of submitted and published papers in order to generate a consistent presentation within the thesis. I have also re-formatted in text citations and reference lists of submitted and published papers for consistency.

Student name: Catherine Rose Cavallo

Student signature: **Date:** 30/05/19

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor name: Richard Reina

Main Supervisor signature: **Date:** 31/5/19

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Chapter 1
General Introduction

The capacity of animals to respond to short-term variation and longer-term changes in their environment is an adaptive necessity. An important mechanism through which many species respond is phenotypic plasticity, which is the expression of an array of phenotypes from a single genotype in response to environmental conditions (DeWitt and Scheiner 2004; Sultan and Stearns 2005). This range of responses allows individuals to cope with wider variation than their genotype would suggest and, accordingly, levels of phenotypic plasticity among species and populations are expected to be an adaptation to differing degrees of environmental variability (Klopfer and MacArthur 1960). A special case of phenotypic plasticity is behavioural plasticity, that is, immediate and generally reversible behavioural changes over a continuous spectrum or discontinuous strategies (West-Eberhard 1989). Behavioural plasticity has been widely studied in regards to resource acquisition (e.g. Kause *et al.* 1999; Chiaradia and Nisbet 2006; Manenti *et al.* 2013), with a variety of organisms shown to employ foraging plasticity to maximise foraging efficiency over varying levels of resource availability (e.g. Greeff and Whiting 2000; Karkarey *et al.* 2017; Ratcliffe *et al.* 2018). Whether through flexible dietary modes or foraging strategies, foraging plasticity provides a way for animals to survive and reproduce under environmental variability (Ratcliffe *et al.* 2018), and is expected to enhance capacity to survive longer-term changes (Evans and Moustakas 2017).

Resource availability tends to be more temporally and spatially variable in marine environments than on land (Hyrenbach *et al.* 2000), suggesting that capacity to make the most of foraging opportunities might be particularly important for marine foragers. Marine foragers display diverse adaptations to deal with patchy and limited food supply, such as by having very large foraging ranges (Weimerskirch *et al.* 1993) or aggregating and breeding close to clumped resources (Oliver *et al.* 2019). As in terrestrial systems, diet modes vary from specialised to generalist strategies (Polito *et al.* 2015, van Denderen *et al.* 2018) and the addition of behavioural plasticity allows individuals, species and populations to move along a continuum between these extremes, allowing predators to exploit diverse foraging opportunities (Kuwae *et al.* 2010; Cardona *et al.* 2017). Evidence for the advantage posed by such dietary plasticity comes from sympatric populations of

highly plastic gentoo penguins (*Pygoscelis papua*) and more constrained, krill-specialist Adélie (*Pygoscelis adeliae*) and chinstrap penguins (*Pygoscelis antarcticus*), in which populations of the former have remained stable while the latter have declined under environmental change (Miller, Karnovsky and Trivelpiece 2009, Polito *et al.* 2015). Many marine predators are also constrained by the need to raise offspring on land, which makes them vulnerable to environmental and resource change within their limited foraging range (Coulson 2001). For these species, it is an advantage to be able to exercise plasticity in terms of foraging effort. For example, seabird central-placed foragers reliant on sandeel (*Ammodytes marinus*) availability can be ranked as more or less sensitive to prey collapse based on their degree of diet and foraging effort plasticity (Furness and Tasker 2000). As a further example, female northern fur seals (*Callorhinus ursinus*) are able to vary their diving effort to achieve constant reproductive output despite changing prey availability (Costa and Gentry 1986).

1.1 Harnessing plastic seabird behaviours to monitor environmental change

Assessing changes in marine ecosystems is logistically difficult and expensive, due to their inherent complexity, large spatial extent and dynamic boundaries – often necessitating the development of proxies, such as indicator species (Hilty and Merenlender 2000). Indicator species are organisms that are sensitive and responsive to ecosystem stresses in ways that can be recorded and interpreted (Carignan and Villard 2002). Seabirds are often employed as indicators in marine systems, because as top or meso predators they are responsive to changes in the functional levels below them (Iverson *et al.* 2007), as well as environmental influences that affect the whole system (Springer *et al.* 2007). They are central-place foragers during breeding attempts (Coulson 2001), which constrains their foraging range and thus their capacity to avoid poor foraging conditions. Many seabird species have been monitored for several decades (Wooller *et al.* 1992). The biological and ecological knowledge gained during those past studies now leaves us ideally placed to use seabirds to shed light on changes in the ecosystems they interact with.

Many seabirds are K-selected animals whose low fecundity and high adult survival are likely to be adaptations to low and unpredictable resource availability and high environmental variability, exacerbated by their inability to disperse from unprofitable foraging grounds while breeding (Weimerskirch 2001). Such life-history traits allow seabirds to maximise lifetime reproductive

output at the expense of individual breeding attempts, but many seabird species also display plasticity in their foraging traits, improving their capacity to raise young when food is scarce. When prey are less available, adult seabirds may maintain their condition and that of their chicks through flexible time budgets (Litzow and Piatt 2003), increasing foraging effort (Suryan *et al.* 2000), switching prey species (Abraham and Sydeman 2006) or increasing provisioning (Chiaradia and Nisbet 2006). As such, many commonly measured seabird demographic traits vary non-linearly in relation to food availability (Cairns 1987), requiring a critical threshold to be reached before effects manifest (Cairns 1987) or becoming apparent only after chronic exposure (Durant *et al.* 2009). For example, common guillemots (*Uria aalge*) have been described by Frederikson *et al.* (2006) as comparatively insensitive to changes in prey availability because plasticity buffers their foraging and breeding outcomes over all but the lowest prey densities (Furness and Tasker 2000). So far, this has largely limited seabirds to a "sentinel" or guiding role in ecosystem and fisheries management (reviewed in Montevecchi 1993; Einoder 2009) with qualitative, rather than quantitative outcomes (reviewed in Piatt and Sydeman 2007).

Foraging plasticity in seabirds could be seen to constrain (Grémillet and Charmantier 2010) or improve their suitability as indicators (Brisson-Curadeau *et al.* 2017) depending on our capacity to measure plastic behaviours. Cairns (1987) suggested a multi-tiered approach to contend with the non-linear responses of different seabird demographic parameters to resource-availability in order to estimate food supply across high to low availabilities. Yet, most seabird foraging plasticity occurs via either changes in diet or effort, both of which can be measured to provide information on prey availability. Though in the past investigators may have been limited in their capacity to capture this at-sea behaviour, we are now able to effectively and cheaply monitor seabirds during (e.g. Brisson-Curadeau *et al.* 2017) and after (e.g. Chiaradia and Kerry 1999; Deagle *et al.* 2007) foraging trips, allowing us to directly measure plasticity in foraging behaviour and diet choices. Because foraging seabirds are expected to maximise energy acquisition and minimise effort, measuring effort and diet provides insight into foraging opportunities and may be useful as an indirect measure of the availability of different prey species. Several studies have made use of this expectation, employing a catch per unit effort (CPUE) measure of foraging efficiency equivalent to that used by fisheries to monitor stock (e.g. Grémillet 1997; Wilson *et al.* 1994; Zimmer *et al.* 2008). Using CPUE, these and other studies shed light on predator foraging efficiency under differing environmental conditions (Wilson *et al.* 1994; Carroll *et al.* 2016; Zimmer *et al.* 2008), varying depths (Zimmer *et al.* 2010), at

different colonies (Hennicke and Culik 2005; Sala *et al.* 2012; Gómez-Laich *et al.* 2015), on schooling *vs* isolated prey items (McInnes *et al.* 2017a) and manipulated prey densities (Enstipp *et al.* 2007). However, no studies have yet included detailed diet information to investigate what seabirds can tell us about the patterns in availability of the individual species they target. By integrating measures of foraging success, effort and diet composition, it may be possible to improve the capacity of seabirds as indicators of resource availability as originally proposed by Cairns (1987; 1992).

1.2 Measuring seabird diet

Over the past decade, enormous advances have been made in the accuracy and precision of diet analysis tools and methods. Killing birds and dissecting their gastrointestinal tracts was once the primary method of obtaining diet data (Duffy and Jackson 1986), but this precluded long term studies of individuals and has become increasingly difficult to justify ethically (Barrett *et al.* 2007). Less invasive is lavage sampling, whereby water is pumped into the stomach of captured birds (Gales 1987) forcing them to regurgitate their stomach contents. Methods with comparable levels of taxonomic resolution of prey include the direct inspection of hard remains in voluntary regurgitates (digestible matter), regurgitated pellets (non-digestible matter) and faeces (Karnovsky *et al.* 2012). However, all these methods underestimate diet contribution of prey with soft bodies (Polito *et al.* 2011) and small prey with small hard parts (van Heezik and Seddon 1989) which may be quickly digested or rendered unidentifiable, while overestimating the contribution of animals such as cephalopods and large fish whose beaks and large otoliths are likely to remain in the stomach for extended periods (Furness *et al.* 1984; Jobling and Breiby 1986). They require expert knowledge of diagnostic hard parts, which is increasingly difficult and expensive due to a worldwide decline in the number of experts (Pearson *et al.* 2011).

In many cases, destructive and invasive methods have given way to non-invasive methods made possible by recent technological revolutions. The direct observation of prey items carried to the colony in seabird bills has become simpler and cheaper to implement since the advent of digi-scoping (cameras paired with high powered scopes), making it possible to record and measure the length of many individual prey to provide quantitative estimates (Larson and Craig 2006). It is also now possible to instrument birds with miniaturised cameras to directly observe prey capture at sea

(Watanabe and Takahashi 2013), although this is still limited by the cost of camera units, the need to directly instrument individuals and the relatively brief time frame that the cameras can capture (usually only a couple of hours). Biochemical methods such as stable isotope analysis and fatty acid analysis have greatly informed our understanding of seabird dietary niches and shifts (e.g. Iverson *et al.* 2007; Kowalczyk *et al.* 2014), and provide longer term dietary data (weeks and months: Hobson and Clark 1992; Quillfeldt *et al.* 2008) than hard parts analysis (hours to days). However, these biochemical methods are limited in their precision and ability to provide quantitative data (Bond and Jones 2009). In contrast, a technique with high taxonomic resolution and potential to inform quantitative estimates is faecal DNA-metabarcoding (Deagle *et al.* 2019).

1.3 Faecal DNA-metabarcoding

Faecal DNA-metabarcoding analysis is the identification of prey species from degraded DNA in the scats of a consumer. It works by targeting small standardized regions of DNA that are highly conserved within a target group, y*et al*low enough variability to differentiate between members of that group (Pompanon *et al.* 2012). From highly degraded scat DNA, an individual predator's whole recent diet can be reconstructed, without the biases created by hard and soft prey parts (Oehm *et al.* 2017). DNA metabarcoding is thus a tool that can recover unrecognized diet items that are lost or rendered unidentifiable by digestive processes and cannot be visually identified (McInnes *et al.* 2016). Many more samples can be amassed than with destructive or stomach contents sampling, since scats can be collected from around nest sites without ever having to handle an animal. Further, recent advances in next generation sequencing now allow hundreds to thousands of samples to be processed and analysed rapidly and without the need for taxonomic experts (Bourlat *et al.* 2013). A comprehensive dataset across many individuals, sites, species and at a fine temporal scale can thus be generated. Consequently, metabarcoding is now being used in marine and terrestrial environments to investigate in fine detail a wide range of ecological and conservation questions (Gebremedhin *et al.* 2016; Kartzinel *et al.* 2015; Thomsen and Willerslev 2015). Amongst marine predators alone this method has characterized the diet of cryptic pelagic species (Deagle *et al.* 2005; Sousa *et al.* 2016) uncovered new diet items (Gerwing *et al.* 2016; McInnes *et al.* 2017b), and revealed interactions between predators and fisheries (McInnes *et al.* 2017c), impacts on threatened prey populations (Thomas *et al.* 2017), diet partitioning between sympatric species (Hardy *et al.* 2017) and seasonal diet shifts (Komura *et al.* 2018). Though still in the early stages of

development and subject to its own inherent biases (Deagle *et al.* 2019), DNA-metabarcoding holds great promise for the tracking of marine ecosystem change via high trophic level central-place foragers, such as penguins.

1.4 Penguins as marine sampling units

Penguins have many traits that make them well-suited for use as biological data-collection units. They are relatively long-lived and many breed in dense, reliable colonies and nest either on the surface or in accessible, shallow burrows (De Roy *et al.* 2013), enabling the capture and instrumentation of large numbers of individuals over many years. Despite nesting on land, they spend the majority of their time in marine environments (De Roy *et al.* 2013), enabling the observation of physical and biological aspects of marine ecosystems, and the collection of diet samples back in the colony. During breeding attempts, they are limited in the distance they can travel to forage before needing to return to land to provision their chicks (Coulson 2001), allowing frequent transferral of information from marine environments to data collection systems or observers on land. For colonial-nesting species, their restricted terrestrial mobility facilitates the use of automated monitoring and weighing systems that can record presence in the colony, time spent at sea and mass change both over foraging trips and time in the colony (Kerry *et al.* 1993; Chiaradia and Kerry 1999; Ballard *et al.* 2010), providing information about the abundance and availability of prey in their local environment. Crucially, they are apex and meso predators and their foraging success reflects changes in the physical marine environment (e.g. Carroll *et al.* 2016; Lowther *et al.* 2018) and patterns in the availability of their prey (Chiaradia *et al.* 2003), making them effective indicators (Hilty and Merenlender 2000). Their biology is well understood and links between their environment and indicator traits are rooted in well-defined theory (Boersma 2008). There are equal numbers of specialist and generalist feeding strategies across the group, and in some locations specialists and generalists breed sympatrically, offering unrivalled information on prey patterns and the effects of environmental variability on these high trophic level predators (Polito *et al.* 2015).

Little penguins are opportunistic predators that sample many ecologically and economically important species (Chiaradia *et al.* 2003) and are highly responsive to cues from the marine environment (Afán *et al.* 2015; Kowalczyk *et al.* 2015; Saraux *et al.* 2016). Due to their accessibility

on the mainland of Australia and New Zealand (Marchant and Higgins 1990) they are the subject of long-term studies in several locations across their range (Birdlife International 2017; e.g. Sutherland and Dann 2014). Consequently, much of their biology, behaviour and ecology is well understood (e.g. Gales and Green 1990; Kato *et al.* 2008; Chiaradia *et al.* 2003), and so researchers are well equipped to address confounding factors pertaining to biology or ecology when employing little penguins as environmental indicators. They have one of the shortest foraging distances of any penguin (Collins *et al.* 1999), and return often to provision chicks (Saraux *et al.* 2011) and prey availability dictates the frequency of provisioning within the later breeding stage where large chicks can be left on their own (Chiaradia and Nisbet 2006). Once thought to be reliant on small clupeoid fish for reproduction, a major population decline in the key prey species of little penguins demonstrated that their nutritional needs were less specific, tied instead to the availability of small fish in general (Chiaradia *et al.* 2003). These traits suggest that little penguins might be useful indicators of resources in inshore environments.

There is a mega colony of little penguins on Phillip Island in south-eastern Australia for which land-based, anthropogenic threats have largely been eliminated, and prey availability remains the key limiting factor (Dann 1992; Sutherland and Dann 2014). The colony is within an ecologically important region currently experiencing rapid change. This area is the fastest warming oceanic region (Wernberg *et al.* 2011; IPCC 2014), already warming four times faster than the global ocean average (Ridgway 2007). The region is also a hotspot for marine biodiversity (Tittensor *et al.* 2010; Ramírez *et al.* 2017), but has high levels of anthropogenic impact (Davidson *et al.* 2012), indicating the potential for significant losses for global biodiversity. With primary productivity closely tied to climate, and prey and predator populations dependent on primary productivity, it is predicted that phenological and spatial mismatches will eventuate that will affect trophic interactions and biodiversity in this area (Fulton 2011). Rising sea surface temperature and changing wind patterns within Bass Strait may reduce the productivity and recruitment of prey species for little penguins, or uncouple penguin breeding from the peak in prey availability (Dann and Chambers 2013; Afán *et al.* 2015). The Phillip Island little penguin colony is appropriately placed to provide information both on how temperate, inshore, central-place foragers respond to short and long-term environmental change, and on the responses of lower trophic levels in these environments. However, before such investigations can be made, there are still factors to be determined in the ways in which these foraging range-restricted predators find and select their food. These include understanding the full

diet breadth of the species and identifying patterns of seasonality of different prey taxa, given that previous assessments may have overlooked cryptic or soft-bodied prey (Chiaradia *et al.* 2016; Chiaradia *et al.* 2010).

1.5 Aims and thesis structure

Considering the necessity of characterising species and community responses to variability and change, especially in this era of rapid environmental change, the principle aims of my study were two-fold. My first aim was to describe the full diet of this highly opportunistic species using a precise genomic technology to uncover previously unrecognised prey taxa and allow frequent sampling to identify seasonal patterns. The second was to use this technology to develop quantitative tools capable of capturing patterns in foraging efficiency and corresponding patterns of prey availability for little penguins and other opportunistic, inshore predators. This involved conducting weekly scat sampling and nest checks for reproductive status at two sites over two breeding seasons, coupled with continuous passive recording of foraging success (body mass change) and effort (foraging trip duration) by automated weighbridge systems. By integrating methods of measuring foraging behaviour (Chiaradia and Kerry 1999) with a new diet analysis technique (Deagle *et al.* 2019), I planned to advance the use of seabirds as quantitative indicators of resource availability and ecosystem change via the following specific questions and objectives (Figure 1).

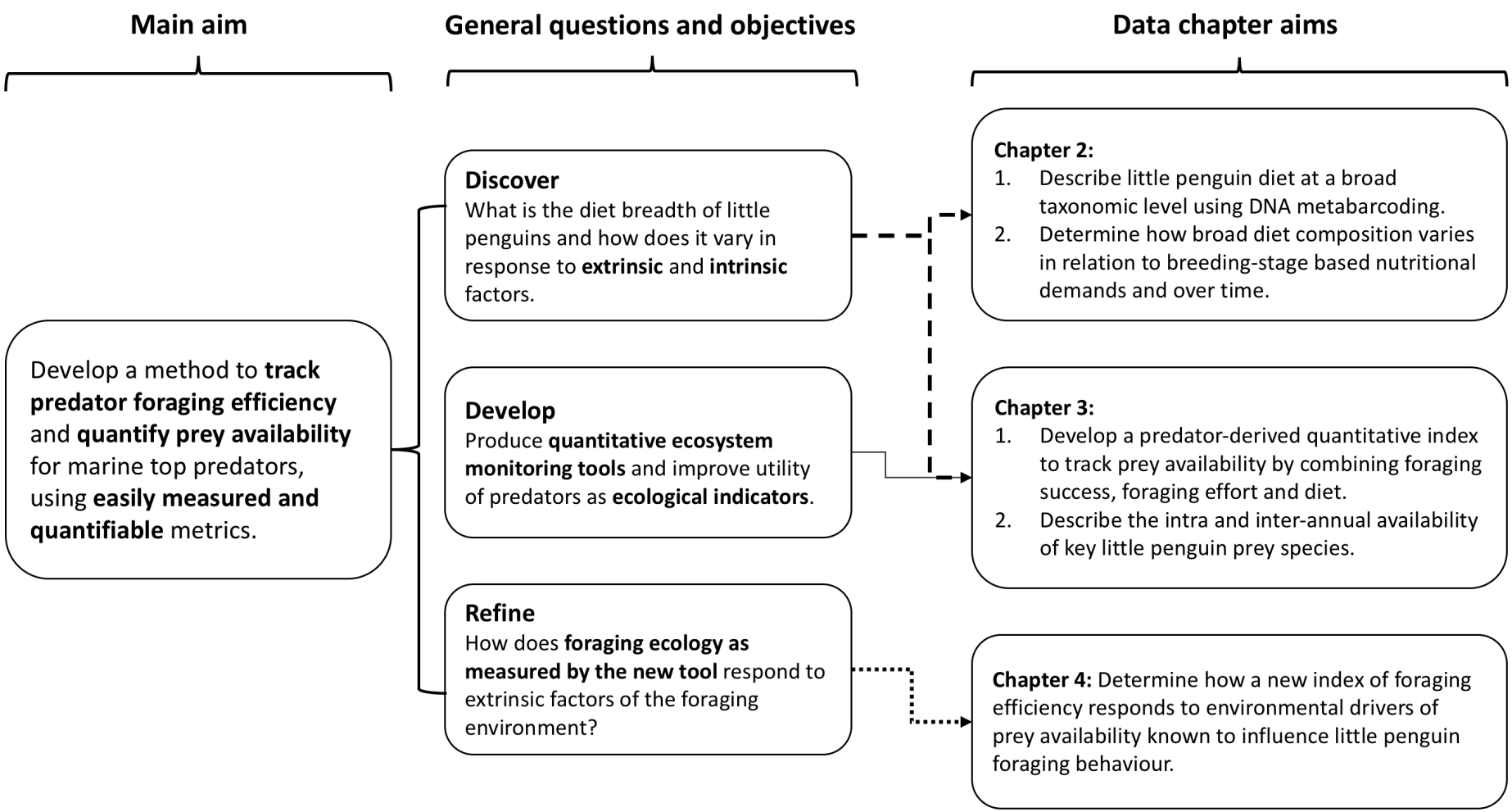

Figure 1| Research aims and general structure of the thesis.

This thesis consists of one published chapter (chapter 2, Cavallo *et al.* 2018), two unpublished manuscripts intended for peer-reviewed journals (chapters 3-4), an introduction (chapter 1) and general discussion (chapter 6).

Following this general introduction, chapter two details an investigation of little penguin diet using a broad-spectrum DNA primer to characterise diet at the class level. This chapter was published in the journal Frontiers in Marine Science in 2018 (Cavallo *et al.* 2018).

Chapter three describes the development of a quantitative index of foraging behaviour and diet, analogous to the Catch Per Unit Effort (CPUE) index employed by fisheries to monitor commercial stocks. The index is formed from the integration of foraging success, effort and diet. These measures are recorded by non-invasive techniques that permit high volumes of data to be collected at fine temporal scales, and processed and analysed at comparatively low cost, with the aim to generate comprehensive long term datasets for which to track resource availability in inshore environments. The new pCPUE index offers a method for producing informed estimates of species-level prey biomass extracted from the marine habitat by little penguins.

Together, the first two chapters aimed to present the first comprehensive DNA metabarcoding investigation of wild little penguin diet at both the broad-scale, highlighting previously unrecognised prey groups, and at the species level to provide information on their key prey of small fish.

In chapter four, foraging efficiency as measured by the enhanced CPUE index is related to local measures of environmental change, namely sea surface temperature, thermal stratification of the water column and wind speed. This study was undertaken to investigate how penguin diet and this new measure of foraging behaviour relate to environmental variables. In this I build upon prior knowledge about the way penguin foraging responds to local perturbations.

The findings from my three data chapters are synthesized in a general discussion chapter that highlights the broad applicability of the method I have defined, as well as focussing in on newly discovered aspects of little penguin foraging ecology and prey availability in north west Bass Strait. I

discuss suggestions for improvements to the method in light of potential limitations and provide recommendations for future research and the development of a time-series of prey availability for little penguins.

Attached to my thesis are several appendices. The first of these is the published PDF version of my second chapter. The second is a published study that ran concurrently with my research, for which I helped collect the data and provided comments on the manuscript, and on which I am named as a co-author (Sánchez *et al.* 2018). I also include scat collection protocol I developed for the ongoing study of penguin diet, which is currently being used by Phillip Island Nature Parks. Finally, I developed code in the R language and statistical environment to interpret DNA sequence data and calculate the prey-specific catch per unit effort by predators from records of foraging success, effort and diet and the code for these steps is included in my appendix.

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Chapter 2
Molecular Analysis of Predator Scats Reveals Role of Salps in Temperate Inshore Food Webs

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Chapter 2

Molecular analysis of predator scats reveals role of salps in temperate inshore food webs.

Running title (5 words): Metabarcoding reveals inshore predator diet

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2.1 Abstract

High precision, high coverage DNA-based diet analysis tools allow greater insight into the food web interactions of cryptic taxa. We used DNA faecal-metabarcoding to look for unrecorded taxa within the diet of a generalist centrally-placed predator, the little penguin *Eudyptula minor*. We examined 208 scats from 106 breeding pairs throughout August-February in a large colony at Phillip Island, Australia. While we confirmed a largely piscivorous diet, we also recovered DNA sequences from gelatinous and crustaceous plankton groups that have not previously been detected in the little penguin diet using other diet analysis methods. Gelatinous plankton, including salps, appendicularians, scyphozoans and hydrozoans were present in 76 % of samples and represented 25 % of all sequences. DNA recovered from minute copepods and appendicularians may indicate links between trophic levels through secondary predation. Percentage frequency of occurrence (%FOO) demonstrated that little penguin diet composition changed over months and stages (incubation, guard and post guard) of the breeding season (month: $\chi2 = 201.91$, df = NA, $p < 0.01$; stage: $\chi2 = 33.221$, df = NA, $p = 0.015$). Relative read abundance (RRA) uncovered variations in the relative abundance of taxa in the diet over months and stages (month: $F = 53.18$, df = 59, $p < 0.001$; stage: $F = 66.56$, df = 29, $p < 0.001$). The diet became progressively fish-focussed over months of the season and stages, while salps were only present in four out of six months, with a peak in

September. Based on their prevalence in this dataset, in this year of very high breeding success (2.15 chicks per pair), salps may constitute a food source for this largely piscivorous generalist. Our work highlights how DNA metabarcoding can improve our understanding of the trophic role of gelatinous plankton and other cryptic taxa.

2.2 Introduction

Diet analyses form the foundation of many important community and species-specific time series used to inform models and plans for conservation and management. However, many diet analysis techniques are limited in their coverage and accuracy (Phillips *et al.* 2003; Braley *et al.* 2010), in some cases excluding whole taxonomic clades (Cardona *et al.* 2012). New techniques now enable us to fill in the gaps and update information being used to make important decisions about species and ecosystem management.

Gelatinous plankton form an abundant and vast group that has often evaded detection by past diet analysis methods. This polyphyletic group encompasses the cnidarian sea-jellies, the salps, appendicularians and other tunicates and the ctenophores, all of which we will hereafter refer to as "jellies". Our understanding of the contribution of jellies to food webs has been obstructed by the difficulty in recovering and identifying gelatinous material from predator diet samples, because the soft tissue is digested rapidly into an amorphous gel (Mianzan *et al.* 1996; Arai *et al.* 2003). Stable isotope and fatty acid analysis have uncovered specialist and opportunistic jelly predators (Wanless *et al.* 2005), challenging assumptions based on observed feeding (Syvaranta *et al.* 2012; Couturier *et al.* 2013) and demonstrating that scyphozoans can make up as much as ~90 % of the diet of some fish species that use them as shelter (D'Ambra *et al.* 2015). However, classification of gelatinous dietary items has been limited because fatty acid and isotopic signatures can vary within species, and may overlap among species in a region (Iverson *et al.* 2002).

Molecular technologies that identify prey DNA in the gut, faeces and pellets of consumers are revolutionising our understanding of organisms' diets and food web interactions (Gerwing *et al.* 2016; Hardy *et al.* 2017; Komura *et al.* 2018,), and providing new ways to capture biodiversity (Boyer *et al.* 2015). It is now possible to sample an animal's entire diet using DNA metabarcoding, which targets regions of DNA that are highly conserved within a target group and that offer enough

variability to allow distinction between taxonomic groups (Cristescu 2014). Furthermore, technological advances in Next Generation Sequencing allow hundreds of diet samples to be processed and analysed rapidly (Metzker 2009) maximising capacity for high temporal resolution, particularly when compared to visual analysis of prey remains in stomach, gut or faecal contents (Taberlet *et al.* 2018). Metabarcoding is now being used across marine and terrestrial environments to investigate in fine detail a wide range of ecological, community, and conservation questions (Kartzinel *et al.* 2015; Thomsen and Willerslev 2015; Thomas *et al.* 2016), and holds great promise for the description and understanding of predation on gelatinous taxa.

Despite their low energy density (Doyle *et al.* 2007; Cardona *et al.* 2012), true sea-jellies (Class: Scyphozoa), hydrozoans or salps are included in the diets of many marine and coastal birds and some mammals (Childerhouse *et al.* 2001; Arai 2005; Cardona *et al.* 2012; Phillips *et al.* 2017). These endotherms generally show wide diet composition concordant with a generalist foraging strategy, including jellies in small amounts, seasonally, in accordance with local abundance (Harrison 1984; Jarman *et al.* 2013). However, a recent DNA metabarcoding study demonstrated that the diets of two albatrosses, *Thalassarche melanophris* and *Thalassarche impavida*, included a considerable gelatinous component (McInnes *et al.* 2017a). Recent video footage shows penguins exploiting Scyphozoa as both a source of fish prey and an occasional prey item (Sutton *et al.* 2015; Thiebot *et al.* 2016) and metabarcoding of Adélie penguin *Pygoscelis adeliae* scats also reveals scyphozoan prey (McInnes *et al.* 2016). Whether or not they are important prey for these groups, jellies can constitute a large proportion of biomass in some areas (Lynam *et al.* 2006; Henschke *et al.* 2016) and many exhibit bloom and bust cycles that significantly affect trophic interactions (Pitt *et al.* 2014; Smith *et al.* 2016). Jellies are resilient across a range of physical conditions (Mills 2001; Purcell 2011), and some gelatinous groups readily invade ecosystems and favour environments characterized by anthropogenic disturbance (Richardson *et al.* 2009; Purcell 2012). They are capable of displacing fish and other taxa in the water column through competition and predation (Purcell and Arai 2001; Pakhomov *et al.* 2002; Henschke *et al.* 2016). Together, these observations indicate that gelatinous taxa warrant greater monitoring and further consideration in ecosystem management planning (Brodeur *et al.* 2016; Aubert *et al.* 2018).

Little penguins, *Eudyptula minor*, are generalist predators that prey mainly on small fish but also crustaceans and squid (Cullen *et al.* 1992; Chiaradia *et al.* 2010; Chiaradia *et al.* 2014; Kowalczyk *et*

al. 2015). Their generalist diet makes them useful for capturing information about the wider food web (Boyer *et al.* 2015), however during the breeding season they are central place foragers whose foraging range is constrained to differing degrees by the specific demands of each breeding stage, potentially affecting the types of prey they are able to exploit (Preston *et al.* 2008; Chiaradia *et al.* 2016; Poupart *et al.* 2017). Breeding is not synchronous across a colony, with some birds incubating eggs while others have chicks that are close to fledging and, depending on the availability and abundance of prey, pairs can raise one to three clutches in a single season (Chiaradia and Kerry 1999).

Little penguin reproductive output is thought to be determined by the availability and abundance of fish prey of a certain size (Chiaradia *et al.* 2003; Kowalczyk *et al.* 2014), but almost all that is known about the diet of this species comes from analysis of stomach contents that overlooks soft prey, and stable isotope analysis that does not provide species identification (Chiaradia *et al.* 2016). Hence important prey items may have been under-represented in previous studies. We use DNA metabarcoding of little penguin scats to refine the diet composition of little penguins so we can better understand their inshore food web.

2.3 Methods

2.3.1 Experimental design

We combined metabarcoding scat diet analysis with field records of little penguin breeding stages. To determine diet composition, we collected scats from little penguin nests on Phillip Island, Victoria, Australia, over the seven-month breeding season in the Austral summer 2015/16. We used a universal primer (18s_SSU rDNA, (McInnes *et al.* 2017b) capable of identifying multicellular animals to class and sometimes order or family level. We compared little penguin diet between months of the breeding season, and between breeding stages, to investigate extrinsic and intrinsic drivers of diet.

2.3.2 Study sites, species and collection of breeding stage data

Little penguins were monitored at two sites located ~ 2 km apart within the Phillip Island colony in south eastern Australia (38°31’S, 145°07’E). The two study sites were “Penguin Parade”, where monitored penguins breed in artificial wooden nest boxes, and “Radio-tracking Bay”, where

penguins breed in natural burrows. There is a high degree of foraging segregation between these sites (Sánchez-Gomez *et al. in press*) but this does not translate to dietary differences (Table S1). Around one hundred nests at each site were monitored to record the presence of adults, eggs or chicks; thrice weekly at Penguin Parade and once a week at Radio-tracking Bay due to the differing accessibility of the sites. Little penguins have three discrete breeding stages, which significantly influence their foraging range and effort. These include "incubation", in which adults take turns incubating their eggs for two to seven days while the partner forages at sea (Numata *et al.* 2000; Kato *et al.* 2008); "guard", where small chicks are guarded by one parent while the other forages at sea, swapping roughly daily (Chiaradia and Kerry 1999), and "post guard", where both adults forage to provision their large chicks, which are left unattended in the burrow (Saraux *et al.* 2011). Foraging trips during post guard can range from between one to two days when food is abundant and two to five days when food is scarce (Chiaradia and Nisbet 2006). Therefore, foraging range is most limited during the guard stage.

2.3.3 Collection of scat samples

Each week, we attempted to sample scats from fifty random nests with eggs or chicks at each site. Cardboard was placed on the floor of the entrance of nests, taking care not to block the exit, and pegged in place. The next morning, scats that had been deposited on cardboard were collected using a metal spatula or tweezers, placed in individual vials and mixed thoroughly with 75-80 % ethanol for preservation. We did not collect scat from substrate (soil, vegetation etc.) as humic substances in soil can inhibit DNA amplification (Schrader *et al.* 2012) and contaminant DNA from the environment can swamp dietary DNA in samples (McInnes *et al.* 2017b). Samples collected each week ranged from less than ten to 30, due to contamination by substrate, loss of sample freshness, and failure by penguins to defecate on the cardboard. Samples were stored in a freezer at -20°C within three hours of collection. For full details of the scat collection protocol see (McInnes *et al.* 2017b). Only around 100 nests were available at each site for monitoring, and of these less than fifty had eggs or chicks in any given week. This means that many nests were sampled more than once during the study. A total of 579 samples were successfully collected from 106 nests across the two sites.

2.3.4 Molecular methodology

2.3.4.1 DNA extraction

DNA extraction and marker amplification were performed at the Australian Antarctic Division in Kingston, Tasmania. DNA was extracted from approximately 30 mg of each scat sample, using a Promega Maxwell® 16 instrument and Maxwell® 16 Tissue DNA Purification Kits. PCR inhibitor concentrations in DNA extracts were reduced by mixing samples with 250µL Roche Stool Transport and Recovery (S.T.A.R.) Buffer (Roche Diagnostics, Basel, Switzerland) prior to extraction.

2.3.4.2 DNA metabarcoding

Dietary DNA metabarcoding involves amplification and sequencing of large numbers of short, unique regions of DNA (barcodes) present in the scats of consumers (Pompanon *et al.* 2012). We used a two-step PCR amplification process enabling amplification of a DNA barcode region, and subsequent attachment of unique 'index tag' sequences to each sample, allowing samples to be pooled for sequencing (Binladen *et al.* 2007). In this study, DNA extracts were amplified using broad range primers (18s_SSU: McInnes *et al.* 2017b), that are conserved in metazoans, and amplify the V7 region of the nuclear small subunit ribosomal DNA gene (Hadziavdic *et al.* 2014). In choosing a barcode marker, there is a trade-off between coverage and resolution. Highly conserved regions provide information covering the breadth of a generalist diet, but are less variable, limiting taxonomic resolution to the class or family level (Pompanon *et al.* 2012). The reaction mixture for the first amplification was 2 µL faecal DNA, 5 µL Phusion HF (Taq polymerase), 1 µL bovine serum albumin, 0.5 µL Evagreen (Biotium, Inc., USA), 0.1 µL of each of the 5µM 18s_SSU primers and 1.3 µL of water. We chose not to use a blocking primer to prevent amplification of penguin (host) DNA, because these can also mask DNA from some target species (Pinol *et al.* 2014). The amplification process was completed on a LightCycler 480 (Roche Diagnostics). Thermal cycling conditions were 98 °C for 2 min; followed by 35 amplification cycles of 98 °C for 5 s, 67 °C for 20 s, 72 °C for 20 s, with a final elongation of 72 °C for 1 min. Samples with a Cycle Threshold (CT) score greater than 30, indicating low DNA or inhibited amplification, were excluded from further analysis (Murray *et al.* 2015). Second stage amplification and sequencing steps were carried out at the Ramaciotti Centre for Genomics (Sydney, Australia). After purification with AMPure XP magnetic beads amplicons were labelled with Nextera Index Primers in unique combinations. The reaction mix for this stage was 2.5 µL DNA, 2.5 µL of each of the Nextera Index primers, 12.5 µL of KAPA HiFi HotStart ReadyMix (Taq polymerase) and 5 µL water. Thermal cycling conditions were 95°C for 3

min; then 8 amplification cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and then a final elongation of 72°C for 5 min. Finally, the pooled, indexed samples were loaded into an Illumina Miseq sequencer, and underwent 150 bp paired-end sequencing (Illumina Miseq v2 reagent kit).

2.3.4.3 Bioinformatics

Following sequencing, the separate forward and reverse reads were merged using the fastq_mergepairs function in USEARCH v8.0.1623 (Edgar 2010; 2013). Amplicons that did not exactly match one of the forward or reverse 18S_SSU primers and those shorter than 150 base pairs were excluded. The merged amplicons from all samples were clustered into molecular Operational Taxonomic Units (mOTUs) using the UPARSE algorithm (Edgar 2010; 2013) with a cut-off threshold of 97 % similarity. These mOTU clusters were then compared to a reference library (SILVA: Quast *et al.* 2013) containing DNA sequences linked to taxonomically verified specimens. The SILVA dataset is a comprehensive online resource containing > 3 million small subunit rRNA gene sequences (relevant for this study) within its more than 3.5 million sequences. Datasets within SILVA are curated, quality-controlled and kept up-to-date (Quast *et al.* 2013). Those with >95 % match to a reference sequence were assigned to that taxon at class level. Many but not all sequences could be assigned taxonomic information at the class level using the SILVA database. The ten most common classes (each accounting for more than 0.5 % of food reads in the dataset) were further resolved to the lowest possible level using the NCBI Basic Local Alignment Search Tool (BLAST: Johnson *et al.* 2008). This global mOTU library was then used to classify sequences in each of the samples. Following identification of sequences, data were filtered to retain only food sequences, defined as all metazoa that are neither obligate parasites nor known post-defecation contaminants. Then, samples with fewer than 100 food sequences were discarded (Jarman *et al.* 2013). In the remaining samples, sequence numbers were summed, and the percentage of each sequence within this total calculated. If a sequence (food item) constituted greater than 1 % of a sample total, it was considered 'present'. Table 1 gives the final samples numbers for each breeding stage and month.

Table 1| Number of samples across months and breeding stages during the 2015/2016 breeding season at Phillip Island. Note that January was not incorporated in analyses due to insufficient sample size.

	August	September	October	November	December	January	February	Total
Incubation	5	24	3	6	6	0	0	**44**
Guard	0	17	26	9	13	2	3	**70**
Post guard	4	9	13	24	24	0	20	**94**
Total	**9**	**50**	**42**	**39**	**43**	**2**	**23**	**208**

Diet composition was determined using two methods to account for biases in the diet summaries (Deagle *et al.* 2018). The two can give different results – although they are generated from the same dataset – because one functions as a record of presence or absence, and the other is used as a proxy for abundance. First, the number of reads of an individual taxon in a scat sample was divided by the total number of food reads in that sample, giving a relative read abundance (RRA) for each taxon in each sample. These read abundances were then scored as present (>1 % of food reads in a sample) or absent (<1 % of food reads in a sample) to measure the percentage frequency of occurrence (%FOO) of each taxon across the dataset. Relative read abundance describes the relative percentage of prey sequences deriving from a prey group across the dataset and although it does not represent exact proportions of prey items in the diet, this metric provides a more accurate summary of overall diet in many situations (Deagle *et al.* 2018). The percentage frequency of occurrence describes the percentage of samples in the dataset in which a given taxon is present, but, like all presence-absence records, can overestimate the importance of common prey items that are ingested in very small amounts, including in the digestive tract of targeted prey (secondary predation). Samples were also categorised according to the prey group represented by >70 % of the sequences (RRA) to further enable distinction between primary and secondary predation (McInnes *et al.* 2017a).

2.3.5 Statistical analysis

We initially explored a mixed effects modelling approach to analyse both percentage frequency of occurrence and relative read abundance data. However, due to unresolvable model convergence issues we chose to analyse the data via separate methods that were better suited to each data type. A Chi-squared analysis was applied to the percentage frequency of occurrence data, and a mixed modelling approach with multiple comparisons employed for the relative read abundance

data. Individuals breeding at different sites within the same colony sometimes (Ceia *et al.* 2015), but not always (Waggitt *et al.* 2014), exhibit foraging segregation and this may result in dietary segregation (Cleasby *et al.* 2015), but we found no evidence of dietary differences between sites in this dataset (Supplementary 1) and so chose to include site as a random, rather than fixed effect in the linear mixed effects models. Statistical analyses were executed in R statistical software (R 3.1.2, R Development Core Team).

We employed contingency tables with Chi-squared tests to detect associations between taxa detected in faecal samples and either breeding stages or months of the breeding season within the frequency of occurrence (%FOO) data. Two contingency tables were built with counts of samples for each combination of taxon and either month or stage (*xtabs* function, *vcd* package; Meyer *et al.* 2008). To control for some of the possible pseudoreplication attributable to repeated sampling of burrows we merged the data wherever there were multiple records for any combination of burrow, breeding stage and month. That is, if a taxon was recorded as present within any of the records for a single combination, it was retained as present in the merged record and if a taxon was absent from all records, it was retained as absent in the merged record. However, we were unable to fully account for repeated measures of burrows over multiple breeding stages and months and it is possible that this may inflate Type 1 error. Therefore, we have chosen to accept only chi-squared results with residuals greater than four (or > -4), equivalent to a p-value of 0.01 or less. Anything between a residual of 2 and 4 (or -4 and 2) represents p-values between 0.05 and 0.01 and should be interpreted with care. Each Chi-squared test was visualised as an association plot (*assoc* function*, vcd*).

To test for effects of breeding stage or month on the relative read abundance (RRA) of individual taxa, we constructed two linear mixed effects models with burrow nested within site as a random intercept to account for repeated measures (*lme* function, *nlme* package; Pinheiro *et al.* 2014). We applied a rank normal transformation to improve normality in the relative read abundance response. Each model contained only the interaction of taxon and either stage or month. As we were interested only in the interaction, we ignored the main effects (taxa, month and breeding stage), which represented only the proportion of the total sequences that were collected in each breeding stage or month, or for each taxon, overall. The variance attributable to each level of the

random effect was calculated by hand after retrieving estimated variances using the *VarrCorr* function in *lme4* (Bates *et al.* 2014)

Following the outcome of the above analysis, we then wrote individual linear mixed effects models to test the effect of month and breeding stage (separate models) for each taxon, again with rank-normalised responses and burrow nested within site as a random effect. We ran multiple comparisons using Tukey's Honestly Significant Difference tests with Bonferroni corrections to reduce the chance of Type I error (*glht* function, *multcomp* package; Hothorn *et al.* 2013). These were visualised in a compact letter display (*cld* function, *multcomp*).
Ethics approval statement: This research was conducted under an ethics permit No. 2.2014 approved by Phillip Island Nature Parks Animal Ethics Committee number and Research Permit No. 10006148 from the Department of Environment, Land, Water and Planning.

2.4 Results

Of 376 processed samples, 208 samples met the requisite 100 prey DNA sequences threshold for inclusion in subsequent analyses. Of these, 112 were from Penguin Parade birds, while 96 were collected from Radio-tracking Bay. A total of 465 molecular Operational Taxonomic Units were recognized, of which 353 contained non-food DNA including 31 obligate parasite OTUs, one penguin OTU and the remainder a range of non-food eukaryotes including fungi, unicellular organisms and plant DNA, a further 26 were unable to be identified (Table 2). A total of 86 mOTUs were identified as possible prey items, and pooled into their seventeen classes for downstream analyses. It is typical for scat samples to include a high proportion of DNA sequences from parasites and contaminants (McInnes *et al.* 2017b).

Table 2| Sequences uncovered in the faeces of little penguins *Eudyptula minor* on Phillip Island during the 2015-16 breeding season, using the universal eukaryote primer, 18S_SSU (18s rDNA, (McInnes *et al*. 2017b).

Phylum	Identity at lowest taxonomic ranking	Percentage frequency of occurrence(%FOO %)	Relative read abundance (RRA %)	Samples with greater than 70% RRA (%)‡
Annelida	Annelida	1.92	0.04	
Arthropoda	Malacostraca	10.10	2.12	0.96
	Branchiopoda	2.40	0.27	
	Maxillopoda			
	Calanoida	20.19	4.23	1.44
	Cyclopoida	6.25	0.22	
	Harpacticoida	23.08	2.84	0.96
	Other	7.21	1.09	
Chordata	Actinopterygii			
	Teleostei	100	63.56	49.52
	Appendicularia*			
	*Oikopleuridae**	40.87	5.39	1.44
	Ascidiaceae*			
	*Salpidae**	42.79	16.33	8.17
Cnidaria	Anthozoa*	0.48	0.01	
	Hydrozoa*			
	*Leptothecata**	9.13	0.96	
	*Other**	2.40	0.11	
	*Siphonophores**	13.46	0.66	
	Scyphozoa*	7.21	1.25	
Ctenophora	Ctenophora*	4.81	0.23	
Echinodermata	Echinodermata	0.48	0.01	
Mollusca	Bivalve	0.48	0.10	
	Cephalopod			
	Oegopsida	4.81	0.38	
	Other	1.92	0.09	
Chlorophyta	Green Algae	0.48	0.01	
Stramenopiles	Stramenopiles	0.48	0.02	
Tardigrada	Tardigrada	1.44	0.07	
Total prey		**100**	**18.00**	
Parasite		**88**	**5.7**	
Contaminant		**99.52**	**8.9**	
Penguin (host)		**100**	**66.3**	
Unidentified		**66.35**	**1.1**	

**gelatinous taxa*

‡In 37.5 % of samples, no single taxon dominated the sequence reads.

2.4.1 Diet composition

Fish (Actinopterygii) were present in all samples with prey DNA detected (frequency of occurrence; %FOO: Table 2) and accounted for 63.6 % of prey sequences across all samples (relative read abundance; RRA: Table 2). Of the sixteen other taxa detected in the food dataset, only nine accounted for more than 0.5 % of prey sequences each (Table 2). Gelatinous plankton accounted for five of the ten most common taxa in the samples, were present in 76 % of samples (%FOO) and represented almost 25 % of all prey sequences (RRA). Of these, salps were present in 43 % of samples, and comprised 16 % of prey sequences, while oikopleurids (appendicularians) were present in 41 % of samples but accounted for only 5 % of prey sequences. Siphonophore and leptothecate hydrozoans were present in 13 % and 9 % of samples respectively, and accounted for less than 1 % of prey sequences each. True jellies (Scyphozoa) were present in 7 % of samples and accounted for 1.25 % of prey sequences. Crustaceans and cephalopods accounted for four of the ten most common taxa. Crustaceans were present in 48 % of samples and accounted for 11 % of prey sequences. Of these, free-living calanoid and harpacticoid copepods were present in 20 % and 23 % of samples, but accounted for only 4 % and 3 % of prey sequences respectively. Malacostraca occurred in 10 % of samples but represented only 2 % of prey sequences. Squid (Oegopsida) were present in 5 % of samples and accounted for less than 1 % of prey sequences.

2.4.2 Dominant taxa in individual samples

62 % of samples were dominated by sequences from a single prey group that accounted for 70 % of the reads or more (Figure 1). Most of these samples were dominated by fish sequences. Almost 10 % of samples were dominated by gelatinous salps, while only 1-1.5 % of samples were dominated by copepods (calanoids and harpacticoids), oikopleurids or malacostracan sequences.

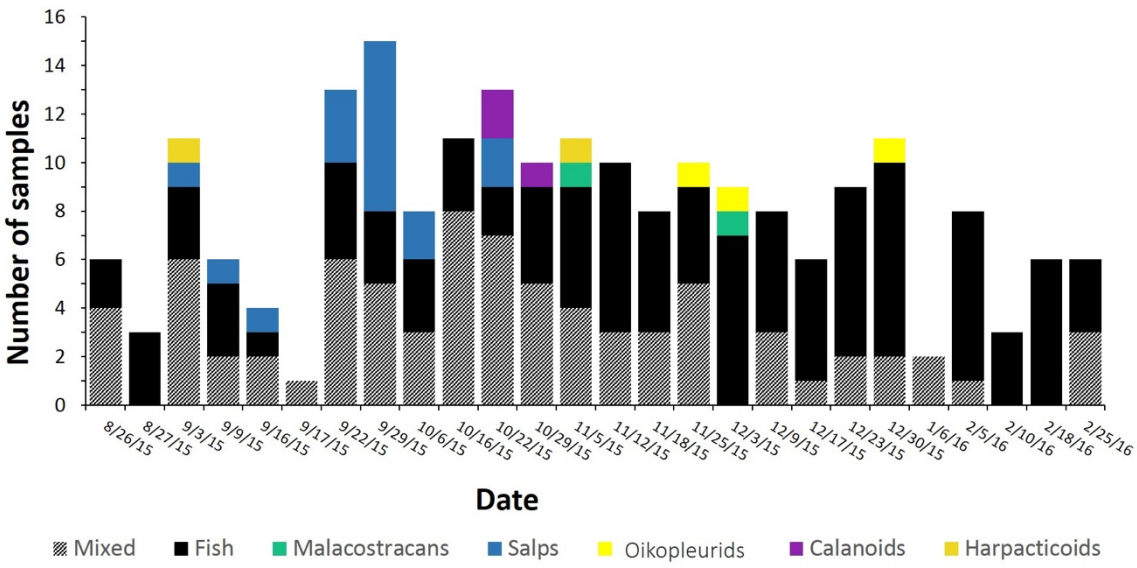


Figure 1 | Sample numbers for each sampling date, coloured according to dominant prey type. The dominant prey type was calculated as the taxon that constituted more than 70 % of DNA sequences in that sample. Samples where no single prey type dominated were classed as "mixed". On the y-axis, each tick mark represents one sample. Note that January was not incorporated in analyses due to insufficient sample size.

2.4.3 Diet in relation to month of the year

2.4.3.1 Frequency of occurrence (%)

The frequency of occurrence of different taxa varied over months of the breeding season (Figure 2a), however, based on an alpha level of 0.01, only two of the ten most common prey items varied significantly and these were salps and squid (Figure 2b; $\chi 2=201.91$, df= NA, $p<0.01$). Fish were the most common item present, occurring in all samples, and squid occurred the least often, appearing in only November and December. The free-living calanoid copepods and the oikopleurid tunicates were the only taxa other than fish to occur across all five months sampled. Salps were present during August to November only and were significantly more common than expected in September. Squid were only present in the diet in two months (November and December) and were significantly more common than expected in December.

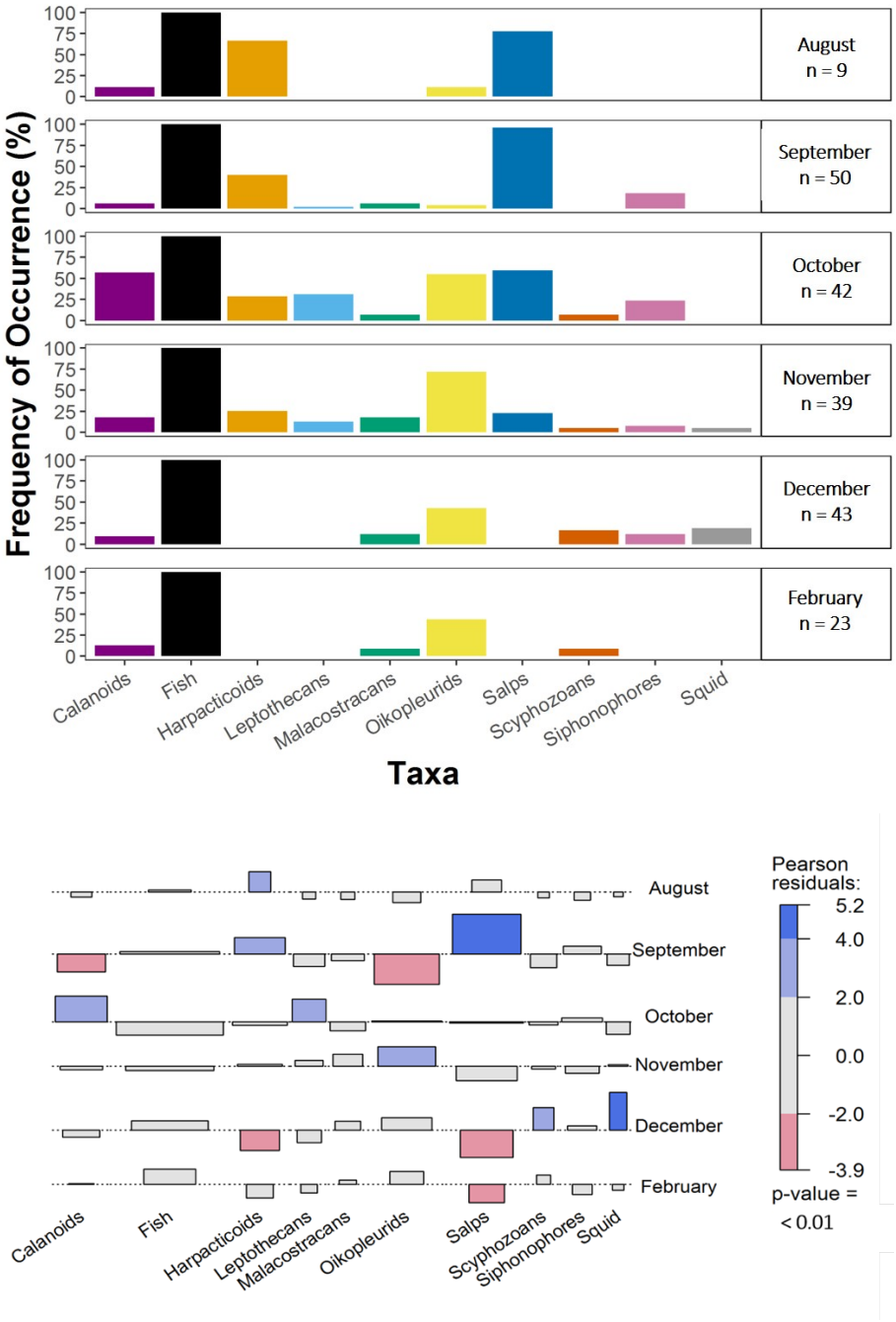
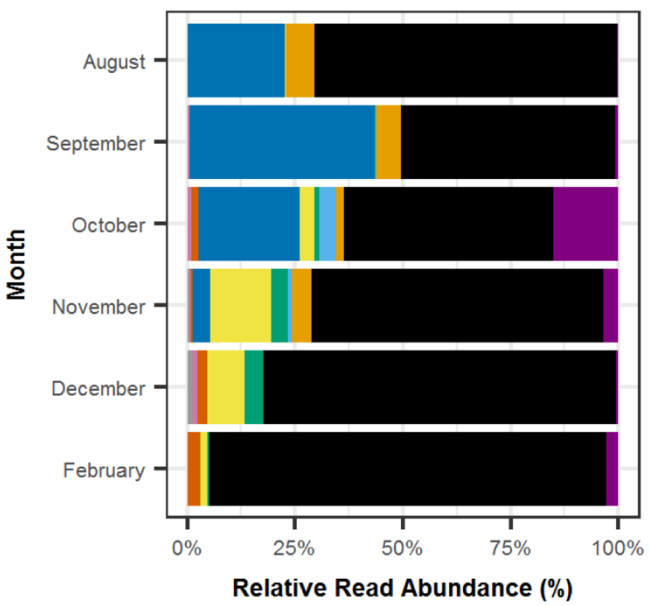


Figure 2| The frequency of occurrence (%) of the ten most common taxa in faecal samples over breeding stages. The percentages for each taxon over breeding stages are shown along with number of samples per stage (A) and the association plot (B) illustrates significant differences between observed and expected numbers of samples containing taxa at each breeding stage, based on Pearson residuals. Red or blue shading indicates significance level. Light shading indicates p-values between 0.05 and 0.01, while bold shading indicates p-values < 0.01. In our analysis, we have accepted only significant values less than 0.01. Note that January was not incorporated in analyses due to insufficient sample size.

2.4.3.2 Relative read abundance (%)

The relative abundance of individual taxa varied over months of the breeding season (Figure 3) with nine out of ten of the most common taxa showing a significant difference in abundance in one or more months (F=61.06, df=59, p<0.001). Fish sequences showed a highly significant trend over months, being least abundant in September and October, increasing in abundance from October to February and accounting for more than 90 % of the reads in February. Salp sequences were significantly more abundant in September, with raised abundance in August and September compared to November and December, when the taxon was in very low abundance, and February, when no salps were recorded. Oikopleurid sequences were most abundant in November and December, and calanoid copepods and Leptothecan hydrozoan sequences were most abundant in October. The random effect of burrow accounted for 0.5% of the variation in this data, while the random effect of site accounted for less than 0.001 %.



Prey type	A	S	O	N	D	F
Calanoids	*a*	*a*	*b*	*a*	*a*	*a*
Fish	*ab*	*a*	*a*	*a*	*bc*	*c*
Harpacticoids	*c*	*bc*	*bc*	*b*	*a*	*a*
Leptothecans	*a*	*a*	*b*	*a*	*a*	*a*
Malacostracans	a	a	a	a	a	a
Oikopleurids	*ab*	*b*	*c*	*d*	*c*	*ac*
Salps	*bc*	*c*	*b*	*a*	*a*	*a*
Scyphozoans	*ab*	*b*	*ab*	*ab*	*a*	*ab*
Siphonophores	*ab*	*b*	*b*	*ab*	*ab*	*a*
Squid	*ab*	*b*	*ab*	*ac*	*c*	*ab*

Figure 3| The relative read abundance (%) of the ten most common taxa in faecal samples over breeding stages. The percentages for each taxon over stages are accompanied by a compact letter display indicating significant differences between breeding stages. Breeding stages with the same letter are considered similar to each other, and months with different letters are considered significantly different from one another. Note that January was not incorporated in analyses due to insufficient sample size.

2.4.4 Diet in relation to breeding stage

2.4.4.1 Frequency of occurrence (%)

We conservatively conclude that no group varied significantly over stages in terms of frequency of occurrence, based on an alpha level of 0.01 (Figure 4; $\chi2=33.221$, df= NA, $p=0.0145$). Fish were recorded in all samples. Scyphozoa and squid occurred only during guard and post guard in a low number of samples, such that this was not significantly different to the zero counts in incubation.

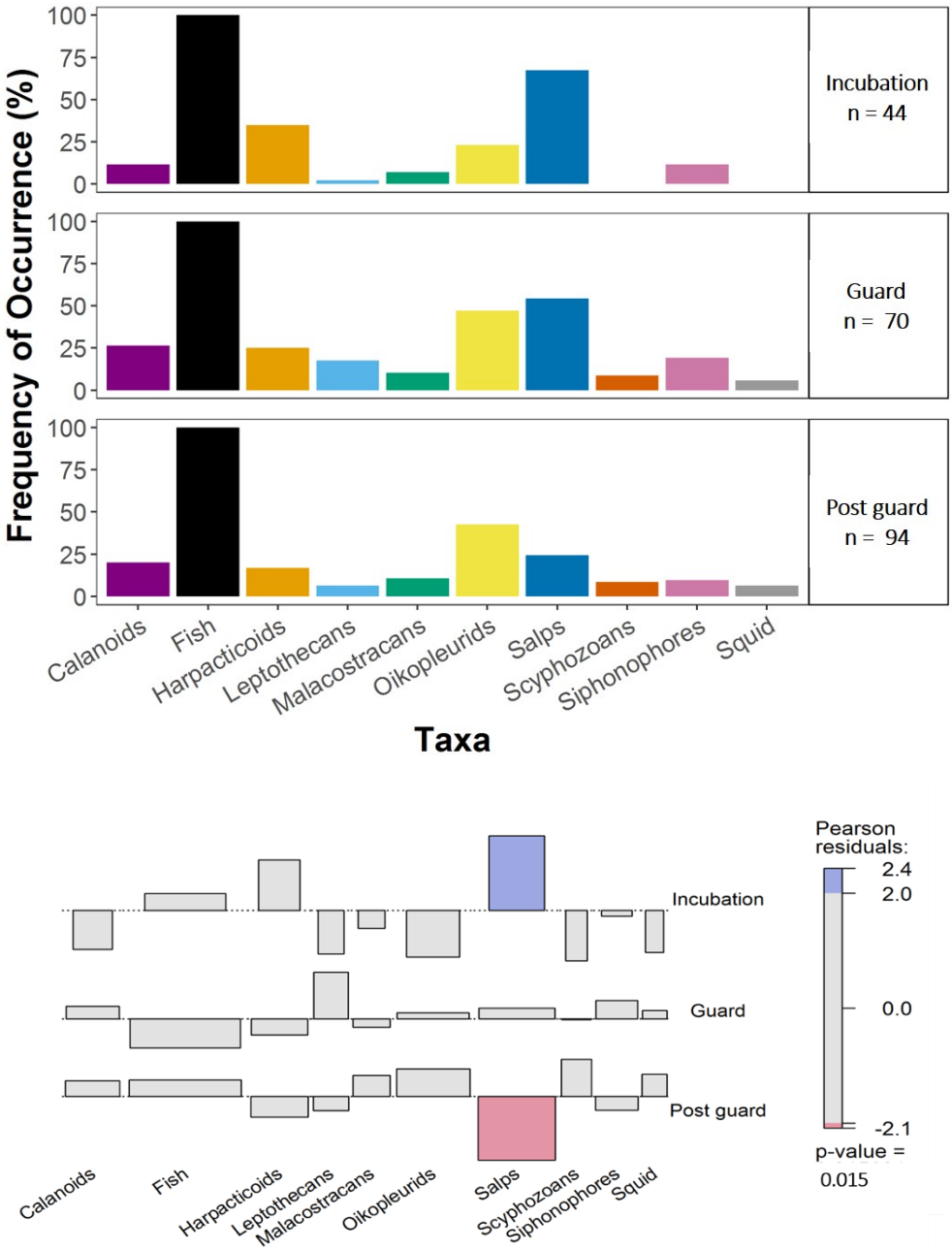
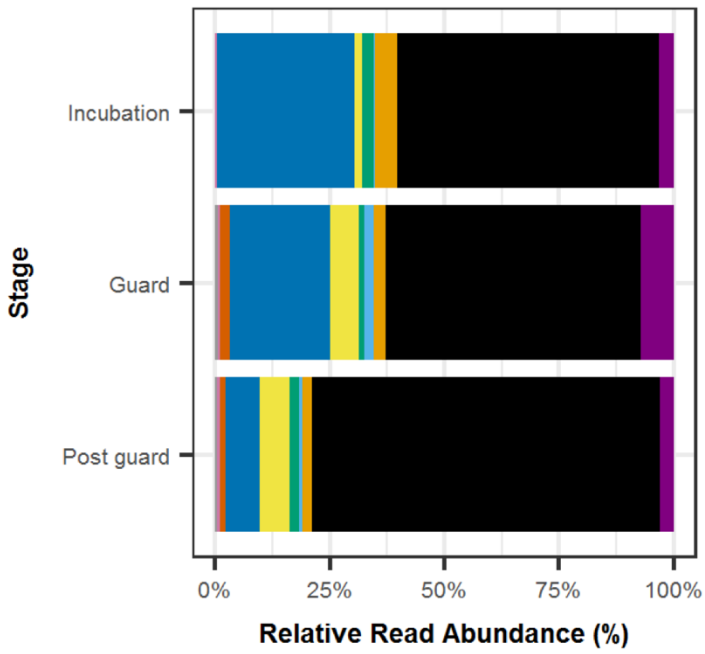


Figure 4| The frequency of occurrence (%) of the ten most common taxa in faecal samples over surveyed months of the breeding season. The percentages for each taxon over months are shown along with number of samples per month (A) and the association plot (B) illustrates significant differences between observed and expected numbers of samples containing taxa in each month, based on Pearson residuals. Red or blue shading indicates significance level. Light shading indicates p-values between 0.05 and 0.01, while bold shading indicates p-values < 0.01. In our analysis, we have accepted only significant values less than 0.01. Note that January was not incorporated in analyses due to insufficient sample size.

2.4.4.2 Relative read abundance (%)

The relative read abundance of taxa varied significantly over breeding stages (Figure 5), with six out of the ten most common taxa showing a significant difference in abundance between two or more stages (F=66.56, df= 29, p<0.001). Most pronounced were the trends in fish and salps. Salp sequences were most abundant during incubation, and decreased to less than 20 % of the reads in post guard. Fish sequences were most abundant during post guard. Oikopleurids, harpacticoid copepods, leptothecans and scyphozoans also varied significantly over breeding stages, but there were no differences in calanoid copepods, malacostracans, siphonophores or squid. As random effects, both burrow and site accounted for less than 0.001 % of the variation in this data.



Prey type	I	G	P
Calanoids	a	a	a
Fish	***a***	***a***	***b***
Harpacticoids	***b***	***ab***	***a***
Leptothecans	***ab***	***a***	***b***
Malacostracans	a	a	a
Oikopleurids	***a***	***b***	***b***
Salps	***b***	***b***	***a***
Scyphozoans	***a***	***b***	***ab***
Siphonophores	a	a	a
Squid	a	a	a

Figure 5 | The relative read abundance (%) of the ten most common taxa in faecal samples over surveyed months of the breeding season. The percentages for each taxon over months are accompanied by a compact letter display indicating significant differences between months. Months with the same letter are considered similar to each other, and months with different letters are considered significantly different from one another. Note that January was not included in analyses due to insufficient sample size.

2.5 Discussion

We confirm a fish-dominated diet in little penguins that includes a wide variety of other items. We revealed the presence of gelatinous and crustaceous plankton taxa not recorded by previous little penguin studies (e.g. Klomp and Wooller 1988; Cullen *et al.* 1992; Flemming *et al.* 2013; Kowalczyk

et al. 2015; Chiaradia *et al.* 2016). Though DNA metabarcoding makes it possible to detect cryptic taxa, it cannot describe whether items were ingested intentionally or incidentally (Sheppard *et al.* 2005). Consequently, some taxa recorded in our study may represent secondary predation by penguins (Sheppard *et al.* 2005), but their presence indicates a trophic role for newly recorded taxa and how they may contribute to energy flow through food webs.

The most interesting result was the high frequency and abundance of salp sequences in the dataset. Not only did salps appear in >40 % of faecal samples and constitute >16 % of reads in the dataset, they were the dominant taxon in 8 % of samples, suggesting they were likely to have been targeted prey in many cases. Salps, and other gelatinous plankton, have traditionally been thought to be of minor importance as prey items to higher trophic levels owing to their poor nutritional content (e.g. Doyle *et al.* 2007). Due to this low nutritional content, animals known to routinely feed on gelatinous plankton, such as the leatherback turtle (*Dermochelys coriacea*) and the ocean sunfish (*Mola mola*), have a low metabolic rate (Davenport 1998; Houghton *et al.* 2006). In contrast, gelatinous plankton alone may not sustain endotherms such as marine mammals and birds that have high metabolic rates. However, despite being an order of magnitude lower in energy density than fish, cephalopods or crustaceans, salps can be equally or even more energy rich than scyphozoans (salps 0.43, scyphozoans 0.41, fish 8.4, squid 5.46 kJ g-1 wet mass: Cardona *et al.* 2012); krill 3.03 kJ g-1 wet mass: Gales and Green 1990), which many seabirds are known to eat (Thiebot *et al.* 2016; McInnes *et al.* 2017a; Thiebot *et al.* 2017) and on which predation by little penguins was recorded in recent video logging (Thiebot *et al.* 2017). The aforementioned study did not record predation on salps by little penguins, however the authors note that they “seldom, if ever” observed salps in videos, suggesting that there were few recorded opportunities for predation.

Salps are commonly targeted by larval and small pelagic fish (Young *et al.* 1997; Mianzan *et al.* 2001), and these are important prey items for many seabirds and marine mammals, including little penguins (Chiaradia *et al.* 2003). Since fish occurred in all samples, we cannot state unequivocally that little penguins in this study fed directly on salps, because salp presence could result from secondary predation. However, we would expect the relative read abundance to be much lower if their occurrence in the diet was due to secondary predation alone. Around half of the salp-dominated samples contained less than 10 % fish sequences, and many of these contained less than

5 % fish sequences. Further, there is no information to suggest that the DNA of salps or other ascidians are preferentially recovered by the DNA barcode marker we used. Indeed, the other ascidian in the dataset (*Oikopleuridae* sp.) is recorded only in low abundance and salps are rare in other seabird diet dataset generated with this DNA marker (McInnes *et al.* 2017a). Finally, because gelatinous tissue degrades so rapidly in comparison to other tissues, it is unlikely that enough salp DNA would persist through two rounds of digestion to create the signal seen in our dataset. Thus, little penguins do appear to directly feed on salps, which are known in the diet of other endotherms (Childerhouse *et al.* 2001; Cruz *et al.* 2001; Hedd and Gales 2001), and even fed to offspring (Cruz *et al.* 2001).

Small crustaceous copepods and gelatinous oikopleurids, as well as gelatinous Scyphozoa and Hydrozoa, were occasionally found (20 % to 40 %) but with very low abundance, each accounting for < 2-8 % of the reads in the dataset. These low records for scyphozoan jellies are surprising, given Sutton and colleagues 2015 video-logging dataset where 28 % of recorded little penguin predation events included scyphozoans from the Cyanea genus. It is possible that video-logging and DNA metabarcoding provide quite different summaries of diet, since video-logging provides real proof of predation in the immediate sense for individual observed events, and faecal material may comprise dietary DNA from up to the last four days (Deagle *et al.* 2010). Therefore, dietary barcoding may capture full diet more comprehensively, but prey that are more resilient to mechanical and biochemical digestion are likely to be overrepresented (Thomas *et al.* 2014). It is likely that the true proportion of gelatinous items eaten by penguins in our study is underestimated by our method. The presence of very small copepods and oikopleurids (<5 mm) may represent secondary predation events and so patterns observed in these tiny taxa may reflect their changing availability to fish, or predation by penguins on different fish species. Detection of these tiny and cryptic taxa can help us improve the trophic links within the broader little penguin food web.

We detected low presence of cephalopods and malacostraca in the diet of little penguins, although both have been detected previously (Klomp and Wooller 1988; Flemming *et al.* 2013; Kowalczyk *et al.* 2015; Sutton *et al.* 2015; Chiaradia *et al.* 2016). While the presence of malacostraca have never been high in little penguin diet analyses, cephalopods can be quite common in their diet (30-60 %: Chiaradia *et al.* 2016). High presence of cephalopods in penguins' diet has been associated with years of low food availability (Cullen *et al.* 1992; Chiaradia *et al.* 2003). Given our study season was

one of very high breeding success (2.15 chicks per pair), which suggests high food availability (Chiaradia and Nisbet 2006), this may explain the low presence of cephalopods. Another possibility is that predation on this group has been overestimated in the past, given the tendency for hard parts such as squid beaks to be retained in the stomachs and thereby overrepresented in stomach content analysis (van Heezik and Seddon 1989). Indeed, no little penguins were observed to prey upon cephalopods during Sutton and colleague's video-logging and only three predation events included krill (Sutton *et al.* 2015) although we do not know how often opportunities to prey on these sources presented themselves. Finally, it is unlikely but not impossible that low cephalopod records were the result of technical bias in the metabarcoding (see McInnes *et al.* 2017a). Further analysis across years should be undertaken to resolve this uncertainty, ideally alongside DNA markers designed for squid and concurrent stable isotope and stomach contents sampling to clarify method-specific biases.

2.5.1 The role of salps in a piscivorous diet

The high presence and relative abundance of salps shows they may have a role in the diet of this largely piscivorous species. However, the importance of that role is difficult to assess for salps and indeed all diet items in this study, since relative abundance does not necessarily reflect true mass proportions, though it is a useful proxy (Deagle *et al.* 2018). Fish increased over stages and were in highest proportion in the post guard stage; a period when a nutrient-rich diet is important for the body condition of chicks (Chiaradia *et al.* 2003). This pattern could indicate that adults were driven to attain increasingly high nutrient prey to provision growing chicks, but the concurrent increase in fish over months could similarly point to a general increase in fish availability (Cullen *et al.* 1992; Afan *et al.* 2015). Similarly, salp-dominated samples occurred in all three breeding stages but were restricted to September and October, which coincide with a peak in the incubation. An avoidance of lower nutrition prey during chick-raising stages by penguins would explain the negative trend we found between salps and breeding stage, but the fact that salp sequences constituted more than a third of the diet sequences during September, with raised sequence proportions in August and October, may signpost an extrinsic influence. Raised salp proportions may indicate the presence of a salp bloom during this time within the foraging range of penguins at our study sites. Salp blooms are notorious for blanketing large areas and excluding other planktonic species (Siegel and Harm 1996; Verity and Smetacek 1996; Lee *et al.* 2010) and other consumers have been shown to feed on salps as stomach fillers when other prey is scarce (Mianzan *et al.* 2001). It has been suggested that

predators may target the visible stomachs of salps, which contain densely packed diatoms and other phytoplankton (Gili *et al.* 2006), linking primary productivity to higher trophic levels through these direct grazers. They may even act as a useful source of calcium (Caron *et al.* 1989), by making concentrated diatomaceous calcium available to larger consumers. Furthermore, it is well known that in SE Australia salp blooms are most prevalent in August, September and October (Henschke *et al.* 2015), exactly the time of year when they appeared maximally abundant in the diet of little penguins (Figure S2). For example, *Thalia democratica* is the most abundant salp in the Tasman Sea with individual zooids, around 1cm in size, and has been reported to reach densities of up to 1312 individuals/m^3 (Henschke *et al.* 2014). In such dense blooms the low energy density of salps is presumably offset by the ease with which large numbers can be consumed. So the most parsimonious explanation of our findings is that penguins opportunistically feed on salps when they happen to encounter blooms.

2.5.2 Shedding light on temperate inshore food webs

Regardless of whether taxa uncovered by metabarcoding were obtained through primary or secondary ingestion, this method provides an avenue for capturing food web and population dynamics in gelatinous and otherwise cryptic taxa. Since the method is not taxon-specific, and is non-invasive, a wide variety of range-restricted, central-placed foragers could be used to capture inshore gelatinous patterns of abundance, and resolve trophic links. By pairing a short-range forager with sympatric long-range foragers, such as seals or shearwaters, it would be possible to capture whole-of-ecosystem patterns. Since jellies are expected to flourish under increasingly disturbed conditions while most other taxa are expected to suffer (Richardson *et al.* 2009; Purcell 2012) tracking their changing abundance and distribution is of utmost importance if we are to mitigate flow on effects to other taxa (Purcell and Arai 2001; Pakhomov *et al.* 2002; Henschke *et al.* 2016). This method also offers an efficient approach to track cycles of ecologically important taxa, such as small pelagic fish, in locations that are not subject to regular commercial fishing-based surveys, providing important data for ecosystem managers and those managing populations of threatened predators.

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2.7 Author contributions

CC, RR, AC, BD and GCH, conceived the project. CC and SSG performed the fieldwork. CC lead the laboratory analysis. BD and JM assisted with bioinformatics. CC conducted the statistical analysis and led the writing with contributions from all authors.

2.8 Funding disclosure

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2.10 Chapter 2 Supplementary Information

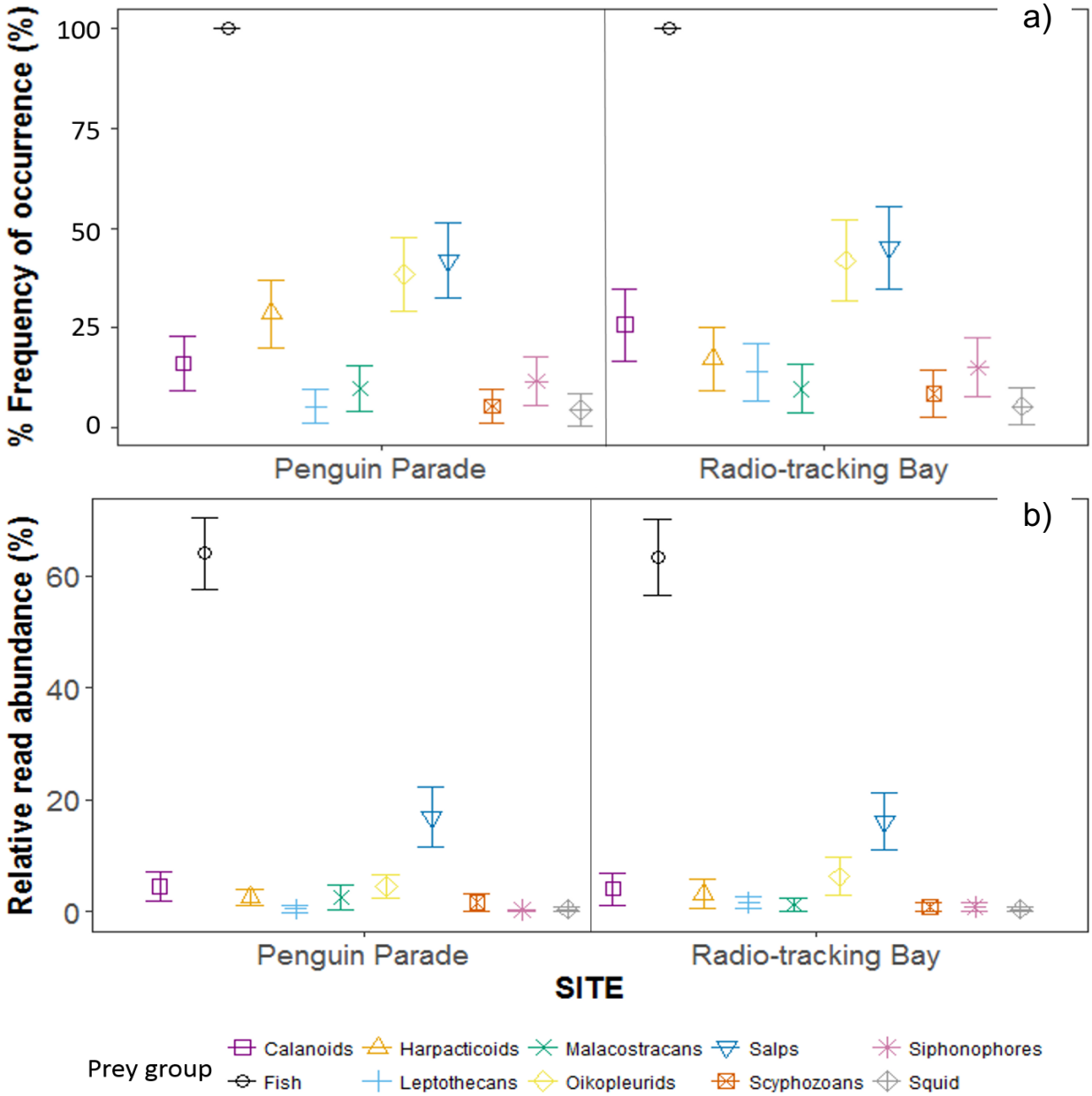


Figure S1| Both percentage frequency of occurrence (a) and relative read abundance (b) of the ten most common prey items in the dataset show no significant difference between sites during the 2015/2016 breeding season. Data points indicate the mean for each prey group in each site. Individual prey groups are indicated by shape and colour and error bars represent the 95% confidence intervals. Groups for which error bars overlap are not significantly distinct from one another.

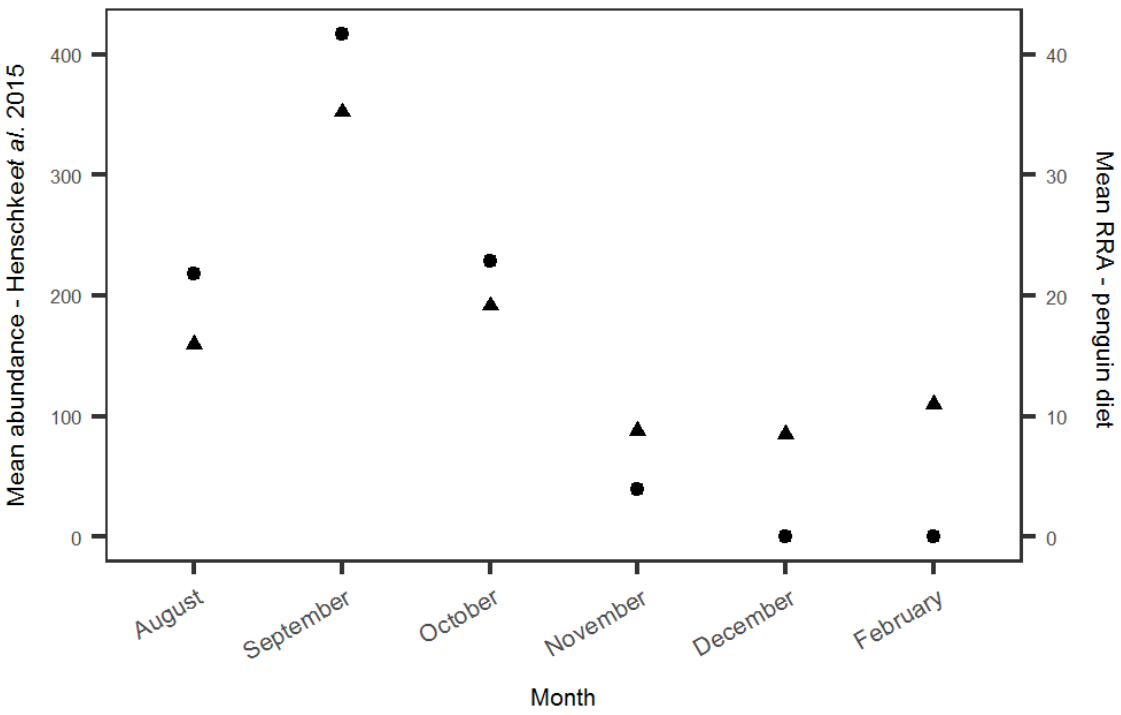
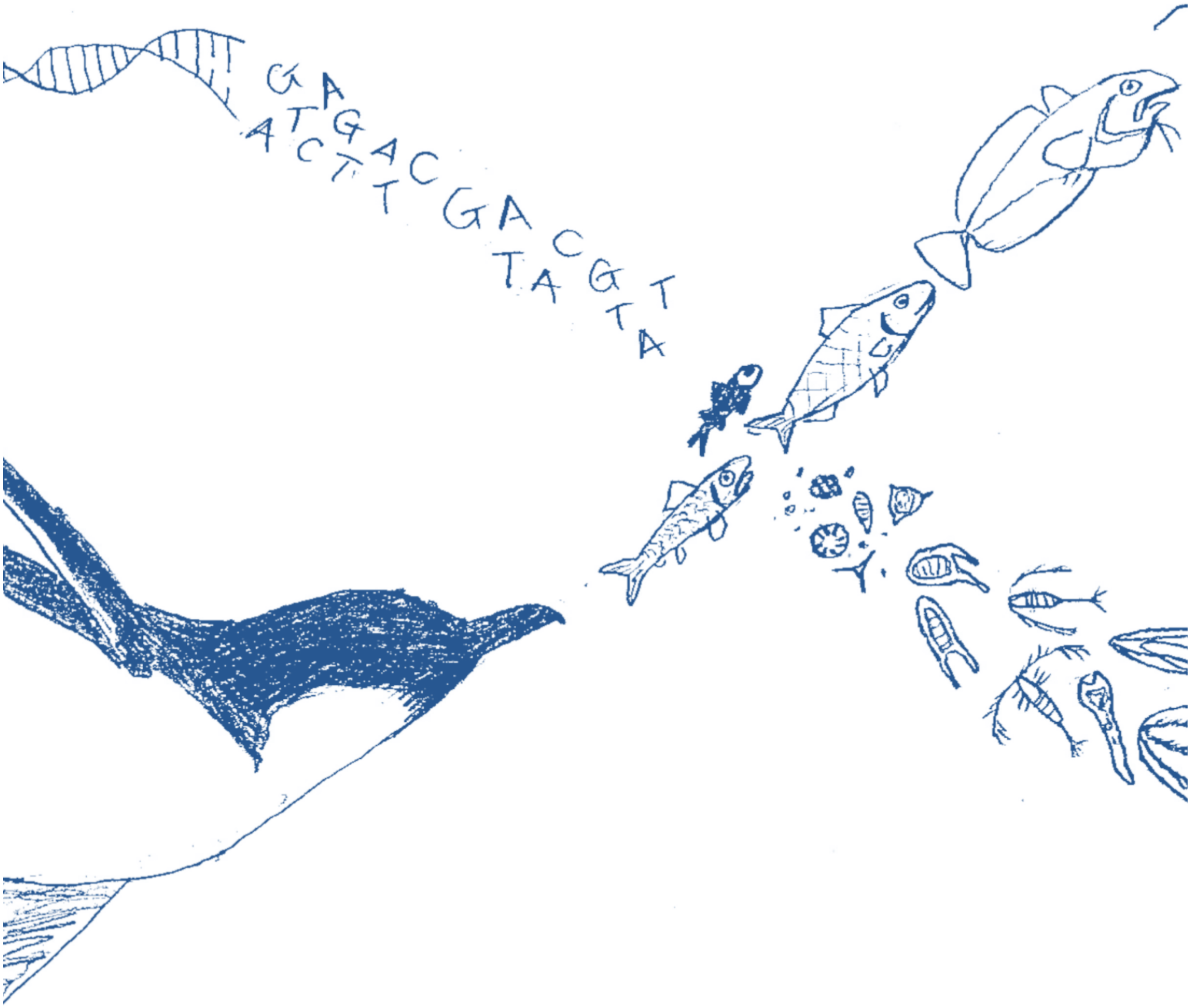


Figure S2| Comparison between the eight-year monthly mean abundance of the common salp Thalia democratica in Sydney from Henschke *et al*. 2015 (▲) and monthly mean RRA of salps in the present study (●). The former were collected via net sampling across the years 2003-2010. The two datasets were highly correlated, with an r2 value of 0.95. Henschke *et al*. 2015 data were digitized from Figure 6 in the published paper.

Chapter 3
Quantifying prey availability using the plastic foraging ecology of marine top predators

Submitted to Functional Ecology



Chapter 3

Quantifying prey availability using the plastic foraging ecology of marine top predators

Running title: Top predator-derived prey availability

Keywords: CPUE, ecosystem-based management, next generation sequencing, DNA metabarcoding, diet, eDNA, tracking ecosystem change, conditional inference trees, pCPUE, *Eudyptula minor*

Target journal: Functional Ecology

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3.1 Abstract

1. Tracking change in marine food webs is a complex task, but top predators can provide information on other trophic levels. However, many commonly-measured predator responses can be decoupled from prey availability by plasticity in predator foraging effort. This is overcome by directly measuring foraging effort and success and integrating these into a measure of foraging efficiency analogous to the catch per unit effort (CPUE) index employed by fisheries.
2. In this study, we extend existing CPUE methods so that they are applicable to the study of generalist foragers, which introduce another layer of complexity through dietary plasticity. Using this method, we infer species-specific patterns in prey availability and estimate taxon-specific biomass consumption.
3. We recorded foraging trip duration and body mass change of little penguins *Eudyptula minor*. We combined these traditional data with diet composition identified via non-invasive faecal DNA-metabarcoding to derive CPUE indices for individual prey taxa (pCPUE).
4. We captured weekly patterns of availability of key fish prey in the penguins' diet and identified a major prey shift from sardine *Sardinops sagax* to red cod *Pseudophycis bachus* between years. Predation on a dominant fish species (~150 g/day) early in each season was replaced by greater fish diversity in the diet as the season progressed. We estimate that the colony extracted 1300 tonnes of biomass from their coastal ecosystem over two breeding seasons, including 219 tonnes of the commercially important sardine and 215 tonnes of red cod.
5. The enhanced pCPUE is applicable to most central-placed foragers and offers a valuable alternative to existing metrics. Informed estimates of biomass of prey species extracted by apex and meso predators will be a useful input for mass-balance ecosystem models and for informing ecosystem-based management.

3.2 Introduction

Tracking change in marine food webs is a complex task, but vast and rapid anthropogenic changes make it crucial that we do so to manage and conserve biodiversity, ecosystems and ecosystem services (Worm 2006; Borja 2014). As environmental changes propagate up food webs, high-order predators are particularly vulnerable, making them sensitive indicators of changing food web patterns (e.g. Boyd and Murray 2001; Piatt and Sydeman 2007). However, many predator traits commonly used to measure predator-prey relationships can be obscured by behavioural plasticity of the predator, whereby individuals change their foraging approach to buffer themselves and their offspring against environmental variation (Grémillet *et al.* 2012). If predators mitigate low resource availability by increasing their foraging effort the imposed cost of this increased effort may not be measured by conventional methods unless food is so scarce that they reach the physiological limit of their plasticity resulting in observable demographic effects (Cairns 1987). In particular, seabird population parameters have limited value as indicators of prey resources because many of their traits vary nonlinearly in relation to food availability (Cairns 1987). Demographic responses, such as changes in population-wide survivorship, reproductive success, or recruitment, require a critical threshold to be reached (Cairns, 1987), or are latent (Durant *et al.* 2009). This masks or delays crucial signals needed by managers to detect declining resources and threatened populations (Piatt *et al.* 2007). When prey are less available, adult seabirds may maintain their body condition and that of their chicks through flexible time budgets (Litzow and Piatt, 2003), increasing foraging effort (Suryan *et al.* 2000), increasing provisioning (Chiaradia and Nisbet, 2006) or switching prey species (Abraham and Sydeman, 2006).

The confounding effect of foraging plasticity can be overcome by directly measuring foraging success and effort and integrating these into a measure of foraging efficiency (Grémillet, 1997). This measure is analogous to the catch per unit effort (CPUE) index used by fisheries to account for fishing effort when monitoring stock abundance (Maunder *et al.* 2006). CPUE indices have been applied in ecological studies by recording i) foraging success via direct measures of total prey mass ingested over a trip (Grémillet, 1997), or through proxies such as prey capture attempts (e.g. Ropert-Coudert *et al.* 2006, Zimmer *et al.* 2007), temperature drops in the stomach (Wilson *et al.* 1994) or oesophagus (e.g. Ropert-Coudert *et al.* 2001) as an indication of a cold food item being swallowed by a hot-blooded predator, or by directly observing food items caught by captive birds

(Enstipp *et al.* 2007), and dividing it by ii) time spent foraging to determine catch rates. These data identify functional relationships between predator behaviour and overall resource availability, and provide a more realistic view of predator responses for conservation managers (Enstipp *et al.* 2007). However, without taxon-specific diet data, a CPUE index could inform on the availability and abundance of prey species only if the diet of the predator studied is limited to a single prey species. Generalist predators add another level of complexity when they mitigate low resource availability by switching prey species (Abraham and Sydeman, 2006), making it impossible to infer patterns in lower trophic levels by using CPUE. However, when taxon-specific diet information is included in CPUE calculations the index becomes a powerful measure of both predator efficiency and the availability of individual prey species. Generalist predators, whose diet composition reflects prey availability (Cullen *et al.* 1992), could be used to index the availability of a wide range of prey species, providing a tool which would be especially valuable for prey species and areas that are not subject to commercial stock assessments, from which population information is traditionally obtained (Hutchings and Baum, 2005).

Little penguins *Eudyptula minor* are high trophic level generalist predators that consume many ecologically and economically important species. These include primary prey items sardine *Sardinops sagax* and anchovy *Engraulis australis*, alternative items red cod *Pseudophycis bachus*, barracouta *Thyrsites atun* and jack mackerel *Trachurus declivis* and a wide array of minor items (Cavallo *et al.* 2018; Cullen *et al.* 1992; Chiaradia *et al.* 2010). Diet composition varies temporally (Chiaradia *et al*, 2010, Chiaradia *et al.* 2016) and proportions caught match those of trawls (Klomp and Wooller, 1988; Cullen *et al.* 1992), suggesting that little penguins consume prey relative to its availability in the foraging zone. At the breeding colony, the penguins' predictable use of walking paths and fidelity to their breeding site facilitates the use of a transponder-based automated penguin monitoring system (APMS: Chiaradia and Kerry 1999). This allows mass, foraging trip duration and colony attendance to be monitored for individual birds in real time, avoiding the time lag and disturbance associated with many traditional seabird research methods (Kerry *et al.* 1993). Using an APMS, large datasets can be acquired over long time-series with little disturbance to the colony.

In this study, we enhance the CPUE index introduced by Wilson (1992) and Grémillet (1997) by integrating precise diet composition measured using faecal DNA-metabarcoding with trip duration

and body mass data from the APMS. We use this method to track the availability of key prey fish species of little penguins over time and to estimate the biomass consumed by breeding little penguins.

3.3 Methods

3.3.1 Study site and system

We monitored little penguins at two sites within the Phillip Island colony in Victoria, south eastern Australia (38°31'S, 145°07'E) during the 2015/16 and 2016/17 breeding seasons, hereafter referred to as the 2015 and 2016 breeding seasons. The sites were < 2 km apart and were "Penguin Parade®", where penguins breed in artificial wooden nest-boxes, and "Radio-tracking Bay", where penguins breed in natural burrows. We randomly selected one hundred nests at each site for monitoring (details in Sánchez *et al.* 2018).

3.3.2 Foraging success and trip duration

All penguins within the study site are equipped with passive identification transponders or PIT tags (Penguin Parade-Allflex, Australia and Radio Tracking Bay-Trovan, United Kingdom), which are injected subcutaneously between the scapulae (Chiaradia and Kerry, 1999). The Automated Penguin Monitoring System (APMS) reads these PIT tags, allowing us to identify and calculate both the length of foraging trips and the change in body mass of individuals during each foraging trip. We used the *R statistical language and environment* (R Core Team, 2013) to extract foraging trip information from the APMS dataset. We sorted the APMS data by PIT tag number and date, then compared each 'out' record with the previous 'in' record to identify distinct foraging trips made by individual birds. Two calculations could then be made from the mass and date data relating to these trips. *Foraging trip duration* was calculated as the return date minus the departure date (Saraux *et al.* 2011) and was recorded in days because little penguins typically leave on foraging trips just before dawn and return to the colony at dusk (Daniel *et al.* 2007), but can spend a considerable amount of time socialising and preening in the water and on the beach before crossing the weighbridge into the colony (CITE). Therefore, the hour of return recorded by the weighbridge may not accurately reflect the time at which foraging ended. The change in body mass of individual birds over each foraging trip (*body mass change*; grams) was then calculated as the

return mass minus the departure mass and was used as a proxy for *foraging success*, that is the total mass of food obtained during a foraging trip (Saraux *et al.* 2011; 2016).

3.3.3 Field records of breeding stage

We checked nests to record the presence of adults, eggs or chicks – thrice weekly at Penguin Parade and once a week at Radio Tracking Bay due to differing accessibility (Sánchez *et al.* 2018). This frequency was required to detect discrete breeding stages of the little penguin breeding, which significantly influence their foraging range and effort (Chiaradia and Kerry, 1999; Chiaradia and Nisbet, 2006; Kato *et al.* 2008). These include 'incubation', in which adults take turns incubating their eggs for one to seven days while the partner forages at sea (Numata *et al.* 2000; Kato *et al.* 2008); 'guard', where small chicks are guarded by one parent while the other forages at sea, swapping roughly daily (Chiaradia and Kerry, 1999), and 'post-guard', where both adults forage to provision their large chicks, which are left unattended in the burrow while parents are out at sea (Saraux *et al.* 2016). Foraging trips during the post-guard stage can range from one to two days when food is abundant and from two to five days when food is scarce (Chiaradia and Nisbet, 2006). Because of the need to frequently return to the colony to feed chicks and relieve partners, foraging range is most limited during the guard stage.

3.3.4 Faecal DNA-metabarcoding

Each week we sampled scats from approximately fifty nests of known birds with eggs or chicks at each site. Due to the limited number of active nests, most burrows were sampled repeatedly throughout the season and in both years. Scat samples were analysed for broad diet composition at the class level, and at the species level for fish, which make up the majority of the little penguin diet (Cullen *et al*. 1992; Flemming *et al.* 2013). For full details of the scat collection protocol see Cavallo *et al.* (2018). More than 800 scat samples were collected from 159 nests over the two sites. We used 384 samples from each year (randomised within month) for analysis and extracted DNA from approximately 30 mg of each scat sample, using a Promega Maxwell® 16 instrument and Maxwell® 16 Tissue DNA Purification Kits. PCR inhibitor concentrations in DNA extracts were reduced by mixing samples with 250µL Roche Stool Transport and Recovery (S.T.A.R.) Buffer (Roche Diagnostics, Basel, Switzerland) prior to extraction.

We used a two-step PCR amplification process enabling amplification of a DNA barcode region, and subsequent attachment of unique 'index tag' sequences to each sample, allowing samples to be pooled for sequencing (Binladen *et al.* 2007). We used a universal primer (18S_SSU; McInnes *et al.* 2016) to assess broad diet composition and a group-specific primer (16S_Fish; McInnes *et al.* 2017a) to provide fine taxonomic resolution on fish species composition. Detailed methodology can be found in Cavallo *et al.* (2018), and Supplementary Table 1.

Following sequencing, the separate forward and reverse reads were merged using the *fastq_mergepairs* function in USEARCH v8.0.1623 (Edgar 2010; 2013). Amplicons that did not exactly match both the forward or reverse primers (either 18S_SSU or 16S fish primer pairs) and those with number of expected errors > 1 (maxee = 1.0) were excluded. The primer sequences were removed. Sequences from each marker for all samples were then clustered into molecular operational taxonomic units (mOTUs) using the *UPARSE* algorithm (Edgar 2010; 2013) with a cut-off threshold of 97% similarity. The sequences from each sample were assigned to these mOTUs (-usearch_global -id .97) and an OTU table was generated using a custom *R* script. To assign taxonomy to these mOTUs we used different approaches for each marker. For 18S_SSU, we followed the procedure outline in Cavallo *et al.* (2018), which provided class level identification based on matches in the SILVA database (Quast *et al.* 2013). The SILVA dataset is a comprehensive online resource containing > 3 million small subunit rRNA gene sequences (relevant for this study) within its more than 3.5 million sequences. Datasets within SILVA are curated, quality-controlled and kept up-to-date (Quast *et al*. 2013). For 16S_Fish, OTUs were resolved to the lowest taxonomic level based on matches to sequences from locally distributed species in GenBank using the NCBI Basic Local Alignment Search Tool (BLAST: Johnson *et al.* 2008) and then we manually curated the output (Table S1).

Following taxonomic identification of sequences, samples were filtered to retain only food sequences (i.e. penguin, parasite and contaminant sequences were excluded). Samples with fewer than 100 food sequences were discarded since these either contained insufficient DNA to analyse or were dominated by non-target DNA so that inferences about diet would be unreliable. Similarly, samples that were slow to amplify because of low template concentration (based on real-time PCR critical threshold values) were also discarded (see Table S1 for cut-off values for each marker).

We used the relative read abundance (RRA) of amplicons within each sample (Deagle *et al.* 2019) for input as the diet variable in the CPUE. The RRA was calculated by dividing the number of sequence reads of an individual prey species in a scat sample by the total number of food sequence reads in that sample and multiplying by 100, giving a percentage for each taxon in each sample.

3.3.5 Developing a predator-derived index

We filtered APMS records so that only birds that returned on the day before each faecal sample collection were considered (Figure 1). We calculated foraging success (*body mass change* in grams) and effort (*foraging trip duration* in days) per trip. Occasionally, an untagged individual could cross simultaneously with a tagged individual (recording erroneously high mass) or individuals may avoid the weighbridge on entry or exit to the colony (giving erroneously long foraging trips). To account for these potential sources of error, we retained only positive *body mass change* results < 420 g (i.e. the maximum change in body mass for a foraging trip between 1 to 14 days long; Salton *et al.* 2015) and filtered out *foraging trip durations* > 7 days, which are rare for breeding little penguins as they can lead to nest desertion (Chiaradia and Kerry, 1999; Numata *et al.* 2000). The distribution of foraging trips before and after filtering are provided as supplementary information (Figure S1, S2). DNA in little penguin scats presents dietary information from at least the four previous days (Deagle *et al.* 2010) and trip durations of breeding little penguins are usually less than five days (Chiaradia and Nisbet, 2006), especially during chick rearing (e.g. Saraux *et al.* 2016). We built the pCPUE using the medians for both *foraging trip duration* and *body mass change*, because the data for each was skewed and the median is less affected than the mean by extreme values in the tail. Since the differential demands of breeding stages may affect diet, all median calculations were made per breeding stage and date and then paired with diet data for birds at that breeding stage and date. We then calculated the prey specific CPUE (g/day) of each fish (hereafter pCPUE) and overall CPUE independent of diet (hereafter CPUE) as described in Figure 1. Individual pCPUE values were scaled by the seasonal mean RRA of fish in the diet (18s_SSU) because monthly averages may have been affected by secondary predation (Sheppard *et al.* 2005). pCPUE was calculated only for fish species that accounted for more than 2% of the diet in one or more years. Code to replicate pCPUE and CPUE from weighbridge and diet data is included in the appendix of this thesis.

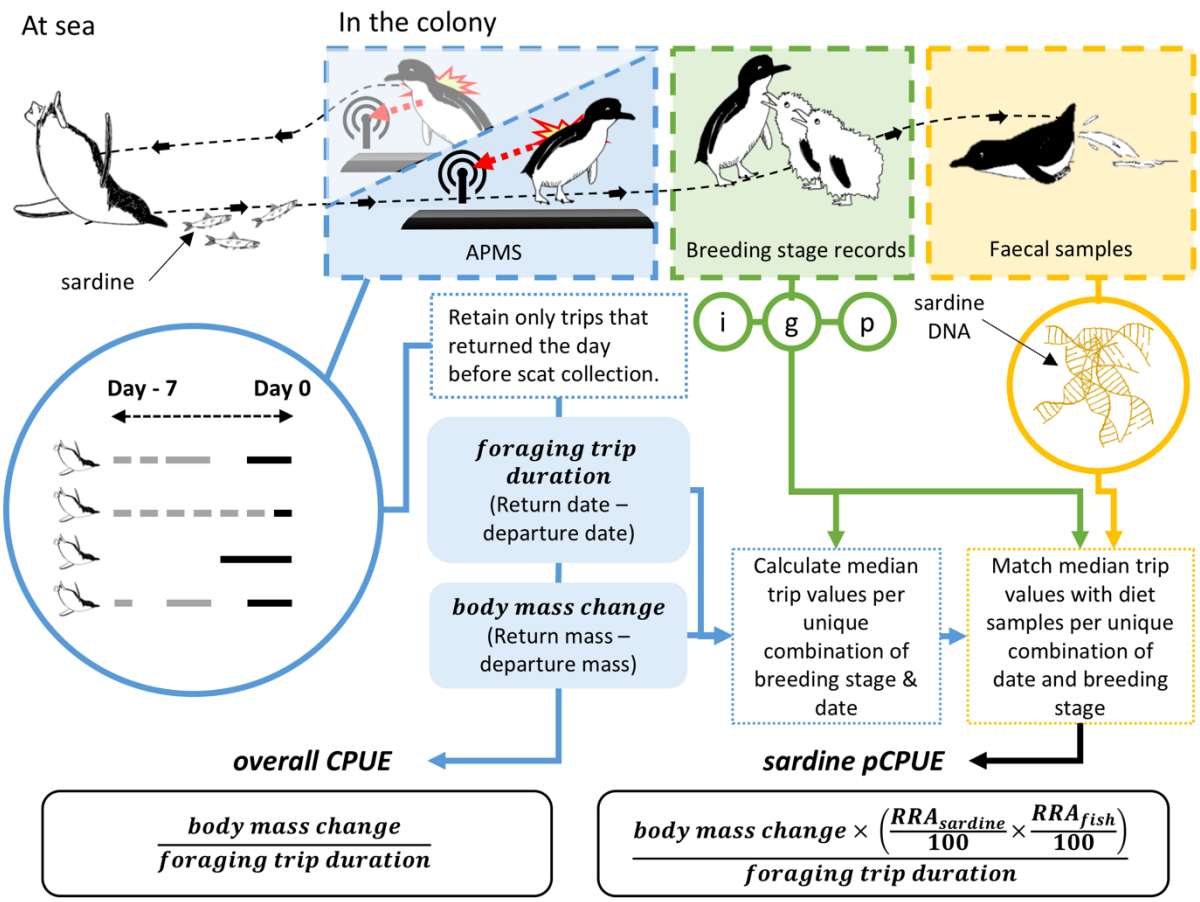

Figure 1| Details of the data generation and calculations of CPUE and pCPUE (for example, sardine). The method has three important data inputs: foraging success, which is recorded as mass change over a foraging trip, and foraging effort, which represents the duration of each foraging trip as obtained from the Automated Penguin Monitoring System (APMS). The third input is diet composition, summarised as relative read abundance (RRA %).

3.3.6 Estimating biomass consumption

We estimated biomass consumed by breeding little penguins in each season by multiplying the number of days spent foraging by an individual breeding bird by the number of birds breeding in a season and then by the mean pCPUE of each prey species in the diet.

We estimated the number of foraging days (D_j) undertaken by the average individual during a single nesting attempt per season j using

$$D_j = \left(\frac{I_j + G_j}{2}\right) + P_j \,,$$

where I_j, G_j and P_j are the average number of days spent in incubation, guard and post-guard, respectively, by studied birds in season j. Note that in incubation and guard stages only one parent forages at a time, while in the post-guard stage both parents forage to provision chicks.

We then estimated the total number of birds in the population (N_j) involved in one, two and three nesting attempts (i) in season j as

$$N_j = \sum_{i=1}^{3} (A_i \times 31{,}000),$$

where A_i is the percentage of studied nests that had at least one, two or three clutches, respectively, and 31,000 is the estimated size of the breeding population (Sutherland and Dann, 2014).

Finally, we calculated the consumption ($C_{p,j}$) of prey species p in season j using

$$C_{p,j} = CPUE_{p,j} \times D_j \times N_j,$$

where $CPUE_{p,j}$ is the mean pCPUE of species p for season j.

The same approach was used to derive an estimate of the total prey consumption for each season.

3.3.7 Statistical analysis

The availability and distribution of prey vary both between and within years (Hobday, 1991), and central-placed foragers often display foraging differences between breeding stages, due with the varying demands placed upon them by growing offspring (Sánchez *et al.* 2018; Shaffer *et al.* 2003). We used non-parametric conditional inference trees in *R 3.4.4* (R Core Team, 2013) to investigate patterns in the pCPUE for individual fish species over years, months, stages and sites and to determine the relative importance of these variables. Species were gathered into a single response (*gather* function, *tidyr* package; Wickham, 2017) and grouped by *Species*. August, February and March were dropped from analyses because they lacked observations for one or more combinations of month and stage or month and season. Zero-inflation and overdispersion are common in environmental DNA metabarcoding data wherein many rare species are present in only a few samples and a few species dominate most samples (Xu *et al.* 2015). Likelihood ratio tests (*lrtest* function, *lmtest* package; Zeileis and Hothorn, 2002) indicated that our pCPUE data were both zero-inflated and overdispersed. Hence, we elected to investigate the complex relationships between terms using Conditional Inference Trees (*ctree* function, *party* package; Hothorn *et al.* 2006a; 2006b; Johnstone *et al.* 2014).

We built a conditional inference tree that included *season, month, stage* and *site. Site* was included because birds at the two sites exhibit strong spatial foraging segregation, even though they are only two kilometres apart (Sánchez *et al.* 2018). *Species* was included as a structural term to allow the tree to split pCPUE by prey species, rather than performing the analysis on the total CPUE recorded. We also included *burrow ID*, to determine whether repeated measures at this level had any effect on explaining variation in the data. We set the conditional inference tree to split only for p-values < 0.05, a conservative measure, since conditional inference trees are generally fit with a splitting structure of $p < 0.1$. Then, to explore the relative importance of variables, we built non-parametric random forests using *cforest* (package *party*; Hothorn *et al.* 2006b) with controls (n = 100,000 trees, mtry = 2, replacement = FALSE) set using the *cforest_control* function. We retrieved the relative importance of variables from this random forest using the *varimp* function in *party* with arguments *conditional = TRUE*, *mincriterion = 0.95* and *threshold = 0.95* (Strobl *et al.* 2008).

3.4 Results

3.4.1 Penguin diet

The average relative read abundance (RRA) of fish in the diet, identified by the 18S_SSU, was 62 % in 2015 and 64 % in 2016 (Table 1), but individual samples varied widely. Crustacea, Mollusca, Cnidaria and Tunicata were also identified, the RRA of which varied over the two years. Fish had the highest RRA in diet, but in 2015 there was moderately high RRA of salps and in 2015 moderately high RRA of calanoid copepods.

Using the 16S_Fish primers, 82 individual fish mOTUs were isolated, with ~ 75 identified to genus or family levels and 50 identified to species level. Of these, eleven fish accounted for ≥ 2% of reads in the dataset in one or both years (Table 2). The full 16S_Fish diet composition can be found in Table S2.

Table 1| Number of samples analysed by 18S_SSU primer, with the relative read abundance (%) of classes that comprised ≥ 2% of the dataset in either year, split by season and month. *Low Actinopterygii RRA in January 2016 also coincided with high Siphonophore RRA, of which sequences were abundant in that month but did not account for ≥ 2% of the dataset in that year.

	2015/16							**2016/17**						
	Aug	**Sep**	**Oct**	**Nov**	**Dec**	**Jan**	**Feb**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan**	**Feb**	**Mar**
n Samples	*8*	*57*	*53*	*49*	*54*	*5*	*28*	*24*	*81*	*67*	*17*	*20*	*9*	*6*
Actinopterygii	79.5	54.3	44.1	62.2	82.8	44.8*	93.6	62	54.8	55.7	57	49.9	95.3	82
Calanoida	0.2	2.8	15.6	6.7	0.6	0	0.8	32.8	32.4	25.6	1.8	0	0.4	7.1
Copepoda (other)	0	0	0	0	0	0	0	0	0	0	34.3	18.3	0	0
Harpacticoida	2.1	7.1	1.5	1.4	0	0	0	0.1	0.3	0	0	0.2	0	0
Malacostraca	0	0	0.5	12.4	3.8	13.9	0.3	1.1	0.2	1.5	0.7	2.4	0.1	0.4
Oikopleuridae	0	0	3	7.6	7.7	11.5	2	0.8	6.2	2.9	2.5	28.8	2.8	6
Salpidae	11.9	33.1	24.4	3.3	0	0	0.9	0.2	0.5	2.5	0	0	0	0

Table 2| Number of samples analysed using the 16S_Fish primer, with the relative read abundance (%) of species that comprised ≥ 2% of the dataset in each season, split by season and month.

		2015/16							2016/17						
Common name	**Species name**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan**	**Feb**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan**	**Feb**	**Mar**
n Samples		*5*	*54*	*50*	*48*	*58*	*5*	*31*	*25*	*81*	*69*	*17*	*21*	*11*	*6*
Australian sardine	*Sardinops sagax*	81.3	86.8	70.3	75.8	24.6	3	7.6	25.5	10	5	12	1	23.1	26.6
Jack mackerel	*Trachurus declivis*	0	0	1.3	7.1	38.2	7.6	37.2	2.5	0	3.9	0	9.3	0.8	0.3
Blue warehou	*Seriolella brama*	1.5	0	15.2	5.4	10.1	8.7	0.6	10.1	5.9	24.8	4.2	0	0	0
Barracouta	*Thyrsites atun*	0	0	1.4	1.8	11	39.1	4.1	1.3	7.4	19	66.2	60.7	9.7	10.9
Blue-finned leatherjacket	*Thamnoconus degeni*	0	0	0.4	1.3	1.4	10.6	22.4	0	0	0	1.6	8.3	0	38.6
Spiny gurnard	*Lepidotrigla papilio*	3	10	2.7	0	0.9	0	0	2.5	0.5	3.2	0.1	0	0	0.3
Leatherjacket sp.	*Acanthaluteres* sp.	0	0	3.2	0.6	1.4	21.9	6.3							
Southern anchovy	*Engraulis australis*	0.4	0.9	0.3	4.2	1	2.6	6.9	0	0.2	0	0	1.7	14.4	2.2
Weedfish sp.	*Heteroclinus* sp.	0	0	1.8	0	5.2	0	1.7	0	0	0	0	9.2	0	0.4
Velvet leatherjacket	*Meuschenia scaber*	0	0	0	0	0	0.1	0	0	0	8.4	14.5	2.9	0	0
Red cod	*Pseudophycis bachus*	7.7	0	2.3	0.6	3	2.3	0	54.7	73.9	29.8	0.3	0	0	0
Bluespotted goatfish	*Upeneichythys* vlamingii								0	0	0	0	2	49.2	6.6
Leatherjacket sp.	*Acanthaluteres* sp.								0	0	0	0	1.5	0	0

3.4.2 pCPUE determination

Foraging success (*body mass change*) detected by the weighbridge was highly variable (Figure S3). In contrast, foraging effort was much less variable, with the median foraging effort (*trip duration*) consistently one day only (Figure S3). Consequently, the overall CPUE (Figure 2a, b) followed foraging success closely in the years studied. The relative read abundance of individual fish species in the penguin diet varied widely from month to month (Figure S3).

Little penguins maintained similar averaged overall CPUE in both years (2015: mean 271.0 ± S.E. 5.1 g/day per individual; 2016: 274.6 ± 5.8 g/day per individual) but the monthly pattern was more variable in 2016 than in 2015 (Figure 2a, b).

The pCPUE of the key fish prey revealed dynamic diet and foraging behaviour over two years (Figure 2c, d). In October 2015, a little penguin ate a mean of 148.4 ± 14.0 grams of sardine per day, but from December it ate < 40 grams of sardine per day and the fish component of the diet was spread across multiple species. For example, in February 2016, a little penguin ate a mean of 57.7 ± 12.9 g/day of jack mackerel, 41.3 ± 11.2 g/day of bluefin leatherjacket, 10.5 ± 4.1 g/day of barracouta and 21.5 ± 9.2 g/day of sardine (Figure 2). The pCPUE of red cod showed a brief peak in October 2016 (165.4 ± 7.8 g/ day) but the species was absent from December on. During February 2017 the average little penguin ate a mean of 59.8 ± 33.8 g/day of bluefin leatherjacket, 38.4 ± 38.1 g/day of barracouta, 31.6 ± 30.7 g/day of goatfish, and 30.5 ± 26.0 g/day of sardine.

The conditional inference tree (Figure 3c) illustrates the complex relationships between variables affecting the pCPUE of different penguin prey. The first split separates prominent prey items red cod and sardine from the secondary prey items. For these main prey items, the conditional inference tree indicates that pCPUE is strongly affected by month of the year. Summarising the combined effects of all variables, the variable importance analysis within years (Figures 3b, c) shows that month was the most important explanatory variable overall for determining pCPUE and that other variables were not important (Figure 3a, b). This is also evident when examining the subsequent splits in the conditional inference tree, remembering that the variable “species” is included only for structuring purposes.

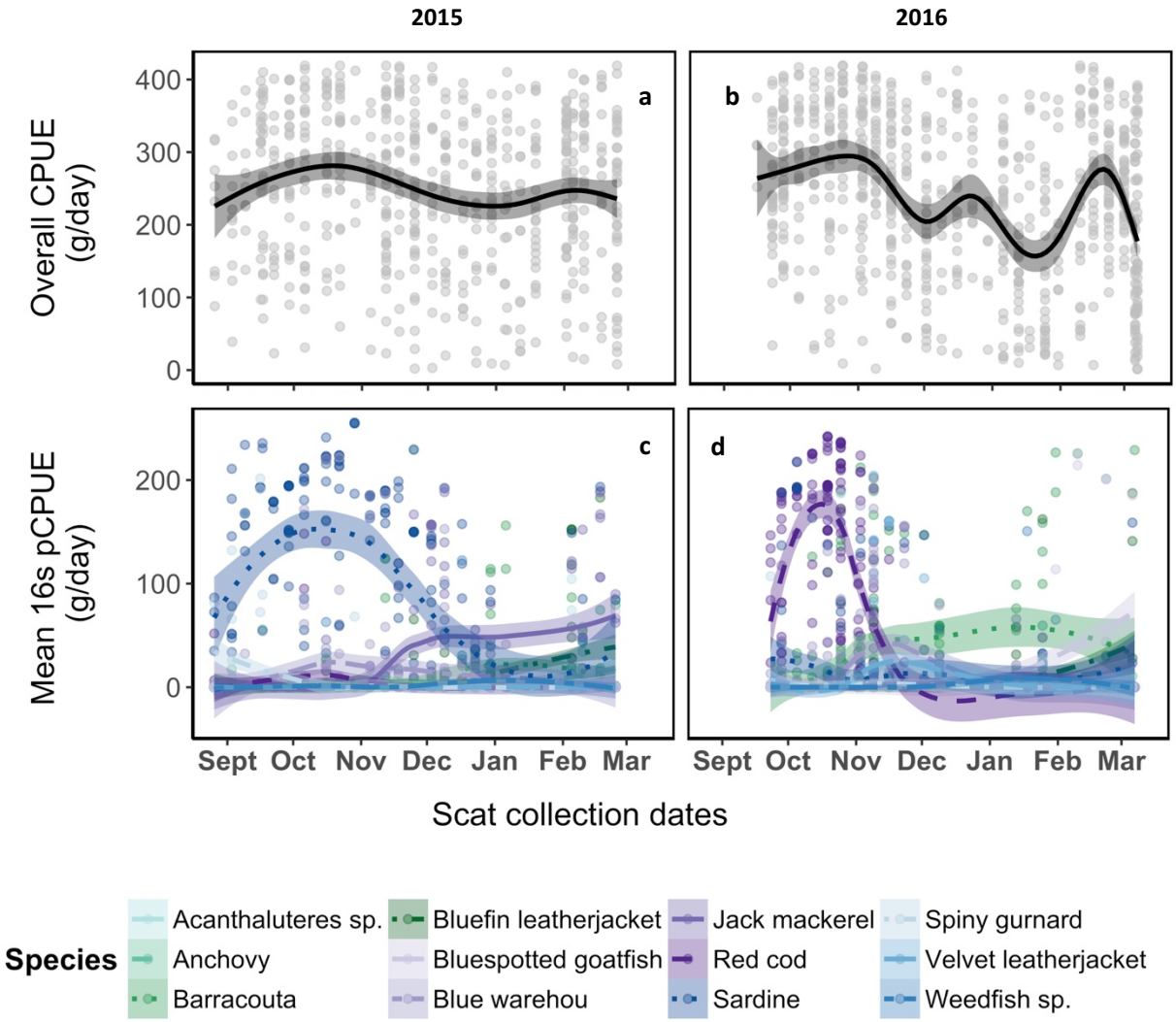

Figure 2| Overall Catch per Unit Effort (CPUE; a-b) and prey-specific Catch per Unit Effort (pCPUE; (c-d)) for the 2015/16 (a, c) and 2016/17 (b, d) breeding seasons fitted as Loess smoothers (lines) with span = 0.75 over observed data (points). The shaded area represents the 95% confidence intervals. Note that pCPUE has a lower bound at zero and cannot assume negative values, but the smoother may extend below zero if there is a rapid transition from high to low values.

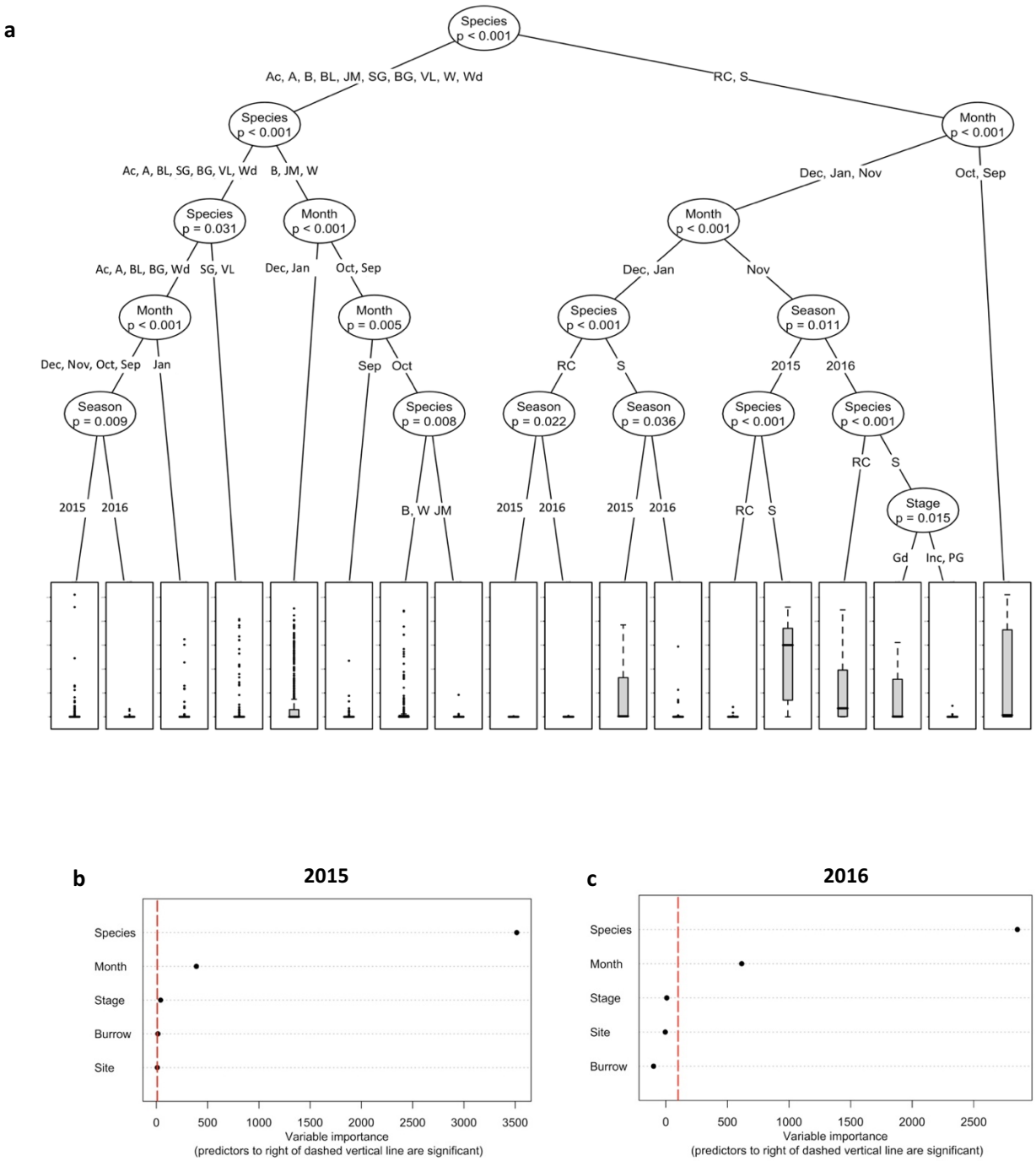

Figure 3| Variable importance analysis (a-b) and conditional inference tree analysis (c) of factors explaining patterns in pCPUE. In the upper panels, conditional relative variable importance is shown for the five explanatory variables used to build the conditional inference tree. Predictors to the right of the dashed vertical line are significant ($p < 0.05$). Species was included as a structural term in both the conditional inference tree and variable importance analysis to separate pCPUE by species, to prevent the analysis being performed on the total CPUE recorded. Consequently, the influence of species on variable importance is structural only. In the lower panel, species are Ac=Acanthaluteres sp., B = barracouta, BL = bluefin

leatherjacket, BG =bluespotted goatfish, JM = jack mackerel, RC = red cod, S = sardine, SG = spiny gurnard, VL = velvet leatherjacket, W = blue warehou and Wd = weedfish sp. Months are abbreviated to three letters and breeding stages are Inc = incubation, G = guard and PG = post-guard. The number of each node is shown in a small box inset in a larger oval bearing the relevant explanatory variable's name and associated p-value. Categories for each split are shown immediately below the variable name box e.g. Node 1 is the first split and splits results into groups based on species, grouping sardine and red cod into one population, and all other species into another with a p-value of <0.001. Boxplots show pCPUE (g/day) medians, ranges and upper and lower quartiles.

3.4.3 Estimated breeding-season biomass consumption

The colony consumed 618.0 ± 11.7 (mean ± S.E.) tonnes of fish during the 2015 breeding season, and 682.2 ± 14.4 tonnes in the 2016 season. The sardine biomass extracted by little penguins in this colony during the 2015 season was estimated at approximately 219.1 ± 13.4 tonnes. Red cod biomass consumed by the colony in that year was 7.7 ± 3.4. Over the 2016 season, we estimate that 215.3 ± 15.2 tonnes of red cod and 38 ± 7.6 tonnes of sardine were caught by Phillip Island penguins. Results for the twelve most common prey species and total catch across two breeding seasons are shown in Figure 4.

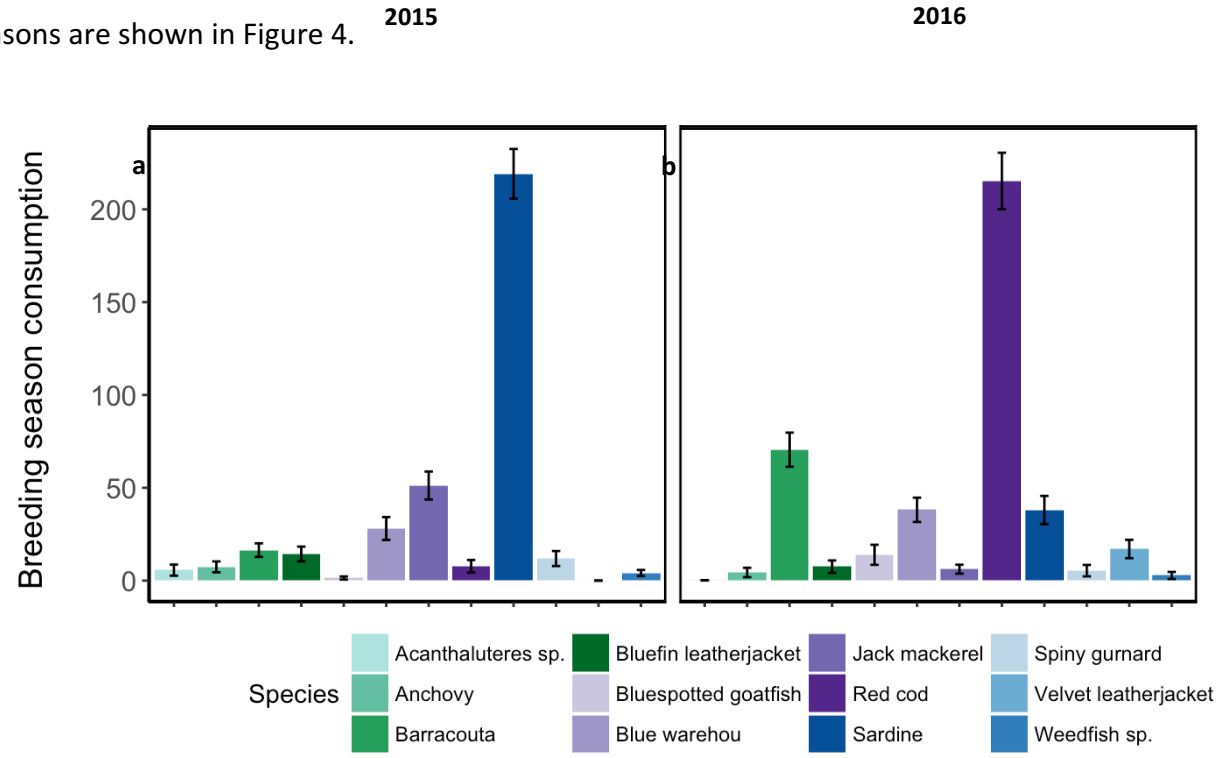

Figure 4| Estimated breeding-season consumption by penguins of fish species (tonnes per season ± S.E.) that each constituted ≥ 2% of the total fish consumption in 2015 (a) and 2016 (b).

3.5 Discussion

During years of high resource availability (high foraging success and/or low foraging effort) pCPUE analysis can reveal underlying patterns in species-specific prey availability that would be overlooked by conventional analysis. In our study, pCPUE indices varied significantly, including a major prey shift from sardine in 2015 to red cod in 2016. Despite this, mean overall CPUE varied little between seasons, indicating that the predator population was able to maintain stable foraging effort and success despite marked changes in diet (Holling, 1959; Baudrot *et al.* 2016). Corresponding chick productivity by the population was high in both seasons (2015: 2.18 chicks per pair, 2016: 1.76 chicks per pair). The very low variability in foraging trip durations in this study is consistent with the findings of Chiaradia and Nisbet (2006) who, in a five-year study of this population, observed that variation around mean daily foraging trip lengths was all but non-existent in years of high chick productivity.

We noted an interesting seasonal pattern where a diet dominated by a single fish species early in the breeding season was replaced by a diverse diet consisting of a group of species later in the season in two consecutive years. In each year, the transition from a narrow to broad diet in the fish component coincided with a drop in overall CPUE, after which overall CPUE values increased to a lower peak. Given that, in both years, effort and success remained favourable compared with past studies (Chiaradia and Nisbet, 2006, Saraux *et al.* 2011), we interpret this pCPUE variation to index patterns of prey availability within the penguins' foraging range. Higher CPUE values were attained for specific prey and overall foraging during periods when single species dominated the diet, which may indicate exploitation of a preferred food source that was abundantly available in the first half of the season (Davies, 1977; Lacher *et al.* 1982). This food source may have been depleted in the locality (Birt *et al.* 1987), causing penguins to transition to a wider diet base (Chiaradia *et al.* 2003). Given the biomass of sardine and red cod consumed by little penguins during our study, local depletion is plausible. It is also possible that the patterns reflect spawning and aggregation patterns in the fish consumed. Red cod spawn locally during spring (Kemp *et al.* 2012) and in our study the highest pCPUE values for this species were during October (mid-spring) in both years, when penguins likely fed on "age-0" larval and post-larval fish. Blue warehou *Seriolella brama* also spawn in this region from June to November (Bruce *et al.* 2001), Australian sardine and barracouta spawn during spring and summer (Blackburn, 1950) and jack mackerel in mid to late summer, with smaller

fish remaining inshore (Sexton *et al.* 2017). Nutrient-rich spawning sardines may be targeted by little penguins when available, and spent, nutrient-poor sardines ignored later in the season, since seabirds are highly dependent on prey quality (Diamond and Devlin, 2003; Wanless *et al.* 2005). Red cod, jack mackerel, blue warehou and barracouta grow rapidly (Grant *et al.* 1978; Marshall *et al.* 1993; Horn and Sutton, 1996, Beentjes and Renwick, 2001), so penguins probably consume age-0 young of that year. Given the high level of agreement between the timing of spawning recorded in historical spawning surveys and diet composition in our dataset, future pCPUE time series could be used to indicate changing breeding phenology by these species in this area that is not subject to direct stock assessment.

Given that little penguin breeding stage affects trip duration in little penguins (Chiaradia and Nisbet, 2006 , as well as diet at a broad taxonomic level (Cavallo *et al.* 2018), I expected that breeding stage might also influence CPUE and pCPUE. However, I found no evidence for such an effect in this study. Earlier studies have indicated a link between foraging success and breeding stage (Chiaradia and Nisbet, 2006; Saraux *et al.* 2016), but this appears to be less evident in years of high resource availability (Chiaradia and Nisbet, 2006). Our finding of no relationship between breeding stage and pCPUE may be explained by the fact that resource availability during our study was high relative to past breeding seasons (as inferred from measures of breeding success and foraging effort). In years of lower resource availability, when little penguins attempt to maintain foraging success by increasing trip lengths during incubation (Kato *et al.* 2008) and post-guard reproductive stages (Chiaradia and Nisbet, 2006), the effect of breeding stage may well be significant.

Measuring pCPUE enabled us to make an informed estimate of the biomass extracted from a coastal ecosystem by a colony of little penguins containing over 30,000 individuals. Our estimates illustrate the population's considerable influence on both pelagic and demersal environments in the Bass Strait system, where demersal fish prey were likely taken as pelagic larval and post-larval individuals (Cullen *et al.* 1992), but may also have been caught on the sea floor (Ropert-Coudert *et al.* 2006). There is a global lack of data on prey consumption rates by predators, which is important information for ecosystem-based management (Hansson *et al.* 2017), and locally-relevant consumption rates are even rarer. In terms of the north-west Bass Strait, penguins are not the only consumers, with large populations of fish-eating Australian fur seals *Arctocephalus pusillus*

doriferus, short-tailed shearwaters *Ardenna tenuirostris* and crested terns *Thalasseus bergii* (Bulman *et al.* 2012). All four groups display a high degree of niche overlap (Bulman *et al.* 2012) and understanding the individual and combined impact of these major consumers will help us to plan for the sustainable future of the ecosystem and its components. Estimates such as these are a key input in mass-balanced food web models used to predict changes in biomass and trophic interactions in time and space (Bulman *et al.* 2012; Christensen *et al.* 2009; Walters *et al.* 1997) and obtaining regionally informed estimates is important for the reliability of model output (Essington, 2007).

3.5.1 A useful and adaptable index for ecosystem management

We distilled complex foraging behaviour into a single index of prey availability that could be interpreted by managers, policy makers, fishers and other stakeholders to assist with resource management. Cury *et al.* (2011) showed that regardless of seabird life-history or population size, prey resources must be maintained at a third or more of their maximum long-term biomass to sustain seabird colonies and ecosystem resilience. Predator colonies located within highly localised fisheries (Pichegru *et al.* 2010) could provide information to inform the sustainable future of the fishery in true ecosystem-based management (e.g. Velarde *et al.* 2013). For example, red cod fisheries are strongly recruitment-driven and the key to successful management may lie in being able to predict abundance of red cod in the next year and set limits accordingly (Beentjes and Renwick, 2001). Red cod are a very fast-growing species, growing too large for little penguins to catch (14 cm; Hobday, 1991) within a few months of spawning, and recruit to the fishery at two years of age and a length of 50 cm (Beentjes and Renwick 2001). Therefore, a penguin-derived red cod pCPUE could be a strong predictor of cohort recruitment in the following years as fish grow beyond the capacity of the penguins to capture. Of course, this should be validated by concurrently sampling age-0 red cod and comparing penguin-derived predictions to independent measurements of red cod recruitment. Alternatively, several species with complementary ranges and water-column foraging depths could create a broad and highly informative picture of the prey species in a larger region. Small-scale, in-house and affordable DNA sequencing is now widely available, which will allow the analysis of diet and pCPUE at critical time-points within the season (e.g. during an acute decline in adult mass or reproductive success). This would provide key information on the current prey base, facilitating rapid response to shortages in terms of limiting or closing a fishery temporarily or managing other anthropogenic impacts such as pollutants or run-off. Conditional

inference trees informed by pCPUE time series and known local drivers of prey abundance and availability would resolve general patterns to generate useful and easily interpreted predictive decision-making trees, making it possible for managers to assess departures from expected pCPUE and act accordingly.

It is with generalist predators that the full potential of the pCPUE method can be realised. When prey is unreliable, generalists have an advantage over specialists, as prey-switching presents an extra dimension to their capacity to be plastic and hence buffered against resource scarcity (Terraube *et al.* 2010). Long-term pCPUE time-series of generalists could help us to monitor changing patterns in prey species distribution, including trends in invasive and threatened species, and to measure the effects of management implementation (Boyer *et al.* 2015). For example, we now know that several seabirds consume diverse gelatinous groups (Cavallo *et al.* 2018; McInnes *et al.* 2017b; Thiebot *et al.* 2017), some of which readily invade ecosystems and favour environments characterised by anthropogenic disturbance (Richardson *et al.* 2009; Purcell, 2012). We will also be able to measure the implications of changing prey distributions for predators. For example, cnidarians, tunicates and ctenophorae are prey with lower nutritional content than fish, crustaceans and squid (Gales and Green, 1990; Cardona *et al.* 2012). Rapid digestion of low energy-density gelatinous prey may still allow high levels of energy assimilation to be achieved by foraging adults (reviewed in Arai, 2005), but seabirds with limited provisioning rates may have reduced chick growth and fledging success if they deliver predominately nutrition-poor food to offspring (van Heezik and Davis, 1990). Linking pCPUE at the class level with levels of chick production and adult mass may detect these scenarios, while linking at the species level may be used to identify the impacts of consumption of anomalously low nutritional quality fish (‘junk food’, e.g. Wanless *et al.* 2005). Removing diet, success or effort from the equation would mask these effects.

3.5.2 Considerations

Though it does not apply to this study, there may be some loss of information on trips longer than four days. Faecal DNA-metabarcoding provides a longer snapshot of diet than regurgitates (Gales, 1988) by integrating diet over several days (Deagle *et al.* 2010), but the vast majority of the DNA present in a scat will be from the most recently digested meal (Deagle *et al.* 2010), strongly tying it to the measure of foraging effort we employ here. For species that regularly undertake trips longer than four or five days, this method may not be suitable. Additionally, over longer trips, we are

unable to account for food mass metabolized at sea, because birds do not digest food at a constant rate (Gales, 1988).

The pCPUE and consumption estimates produced by this study should be considered to be conservative. In some months, high RRA of crustaceous or gelatinous sequences (Table 1) may indicate either direct or secondary predation (Sheppard *et al.* 2005). Some likely represent targeted diet items (e.g. salps, Cavallo *et al.* 2018), while miniscule taxa are more likely to have been ingested within the gut of target prey (copepods and Oikopleurids, see Bowser *et al.* 2013). Therefore, the proportion of fish sequences in months dominated by these taxa is likely to underestimate predation on fish. We chose to lessen the uncertainty introduced in these months by scaling individual prey species pCPUE by the seasonal average of fish in the diet (RRA), rather than the monthly RRA. Consequently, we may underestimate the pCPUE of individual fish species in some months and the seasonal consumption of some species. This does not affect our estimates of total biomass extracted by the penguin population, only that of individual species. Other potential sources of bias between the DNA proportions recorded via scat analysis and the true diet proportions include potential differential digestion of prey groups and gene copy number within different taxa and tissue types (Deagle *et al*. 2018). In the absence of information on differential digestion rates and gene copy number between fish, crustaceans and gelatinous plankton, we have made the assumption of no differential digestion, which may incorporate error in those few samples that had a very high proportion of DNA from gelatinous taxa. However, because little penguins in this study predominantly preyed upon fish, we expect that the small biases potentially introduced from these sources will have been minimised by scaling individual prey species by the seasonal average of fish in the diet.

Prey availability to predators may be considered a proxy for fish abundance (Velarde *et al.* 2013) but there are behavioural and environmental factors that can confound interpretations. Generalists may disproportionately feed on the most abundant prey (Jaworski *et al.* 2013), and, when overall prey abundance is low, they may choose to target the most easily caught prey, termed ‘rank switching’, rather than sampling across the prey base (Baudrot *et al.* 2016). If pCPUE analysis indicates that species A has declined, it may be that species B has become more abundant or available. These behaviours are less of an issue if levels of diet ‘preferences’ are well understood within the study system. For example, sardine are considered to be a preferred primary prey for

little penguins, while barracouta, red cod and jack mackerel are considered alternative prey (Chiaradia *et al.* 2016). Therefore, we could interpret the lack of sardine in the second year of the study to indicate a decline in sardine abundance or availability, but we cannot discount a high abundance of red cod being the cause of the diet shift. Finally, environmental conditions can affect the availability of prey to predators (e.g. thermoclines: Ropert-Coudert *et al.* 2009) and schooling species are not distributed randomly or evenly in space, which can affect interpretation (Hilborn and Walters, 1992; Dunn *et al.* 2000). As long as these biases are consistent through time, these patterns can be compared to what we already know about prey species and used to identify how and why predators catch different prey types, especially those that may be increasing in ecosystems (Atkinson *et al.* 2004) or changing in distribution (Perry *et al.* 2005). Crucially, unlike population demographics and reproductive success, the CPUE index is not affected by drivers of change in the nesting environment such as significant storms, floods, heat waves, predation and human disturbance, making it possible to track change in the marine environment alone.

3.5.3 Conclusions

Predator-derived indices of prey availability reduce our reliance on limited fisheries data in inshore systems (Hutchings and Baum, 2005). By presenting the mass of each species caught for each unit of effort, the pCPUE offers a quantitative index that allows us to make inferences with respect to both predator and prey, including prey biomass consumption by predators. The pCPUE can be adapted for a wide range of central-place foragers and such time series will provide a baseline from which we can assess whether changes in food-webs are natural cycles or symptomatic of environmental change.

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3.7 Ethics Statement

This research was conducted under an Ethics Permit No. 2.2014 approved by Phillip Island Nature Parks Animal Ethics Committee number and Research Permit No. 10006148 from the Department of Environment, Land, Water and Planning of Victoria, Australia.

3.8 Author contributions

CC, RR, AC, SJ, BD, YRC and GH conceived the project. CC and SS performed the fieldwork. CC lead the laboratory analysis. BD and JM assisted with bioinformatics. CC conducted the statistical analysis and led the writing with contributions from all authors.

3.9 Funding

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3.10 Data accessibility

We intend to make the datasets generated and analysed for this study available in the FigShare repository. See for example https://figshare.com/authors/Catherine_Cavallo/5723279 where the datasets for our previous study paper are stored.

3.11 References

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3.12 Chapter 3 Supplementary Information

Table S1| Information specific to each of the two primers used in this study.

Primer	**18S_SSU**	***16S_Fish***
Development	McInnes *et al.* 2016	McInnes *et al.* 2017a
Coverage	Broad	Group-specific
	V7 region of the nuclear small subunit ribosomal 18s DNA gene	Mitochondrial large subunit ribosomal 16S DNA gene
Amplification		
1st round amplification reaction mixture (10µL)	2 µL faecal DNA 5 µL Phusion HF (Taq polymerase) 0.2 µL bovine serum albumin 0.5 µL Evagreen (Biotium, Inc., USA) 0.1 µL of each of the 18S_SSU (5µM) primers 2.1 µL of water.	
1st round amplification thermal cycling conditions	98 °C for 2 min; followed by 35 amplification cycles of 98 °C for 5 s, 67 °C for 20 s, 72 °C for 20 s, with a final elongation of 72 °C for 1 min.	98°C, for 2 min; followed by 45 cycles of 98°C for 5 s, 67°C for 20 s, 72°C for 20 s, with a final elongation of 72 °C for 1 min
Cycle Threshold (CT) for exclusion	30	40
2nd round amplification reaction mixture (20µL)	2.5 µL DNA 2.5 µL of each of the Nextera Index primers, 12.5 µL KAPA HiFi HotStart ReadyMix (Taq polymerase) 5 µL water	
2nd round amplification thermal cycling conditions	95°C for 3 min; then 8 amplification cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and then a final elongation of 72°C for 5 min.	
Sequencing methods were the same for each primer and can be found in Cavallo et al. 2018		
Bioinformatics		

Merging of forward and reverse reads	Separate forward and reverse reads were merged using the fastq_mergepairs function in USEARCH v8.0.1623 (Edgar, 2010; 2013)	
Filter point 1	Amplicons that did not exactly match one of the forward or reverse primers, and any shorter than 150 bp were discarded.	
Clustering	Merged amplicons from all samples were clustered into molecular operational taxonomic units (mOTUs) using the UPARSE algorithm (Edgar, 2010; 2013) with a cut-off of 97% similarity.	
Bioinformatics reference library	SILVA (Quast *et al.* 2013) Version: silva.de_2015-07-10	NCBI nucleotide database https://www.ncbi.nlm.nih.gov/nucleotide/ Accessed in December 2016.
Taxonomic assignment	18S mOTUs were assigned to broad taxonomic level (usually Class) due to the relatively low variability of this marker and the inability to reliably discriminate between more closely related species.	16S mOTUs matching fish were manually classified based on the best BLAST hits and their geographic distribution. We also considered closely related species not in the database and assigned broader taxonomy when uncertainties existed. 16S mOTUs that had a less than 97% match to a species in the database, or matched to more than one species were identified at the level to which they could be resolved. That is, if matching voucher specimens were all from the same genus, they were identified as that genus, if they were not in the same genus but were in the same family, they were assigned that family. Where a mOTU matched more than one family at equal genetic distance, the mOTU was assigned 'Unidentified Actinopterygii sp.)'.

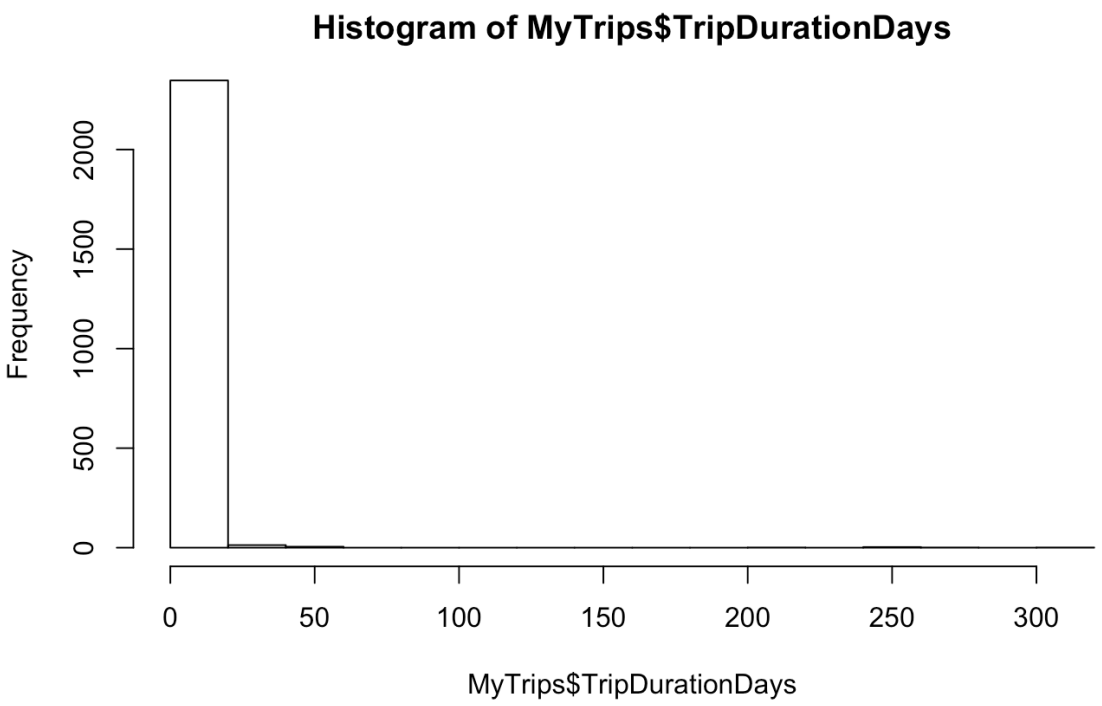


Figure S1| Histogram of all trips recorded by the weighbridge during my study that returned the day before scats were collected.

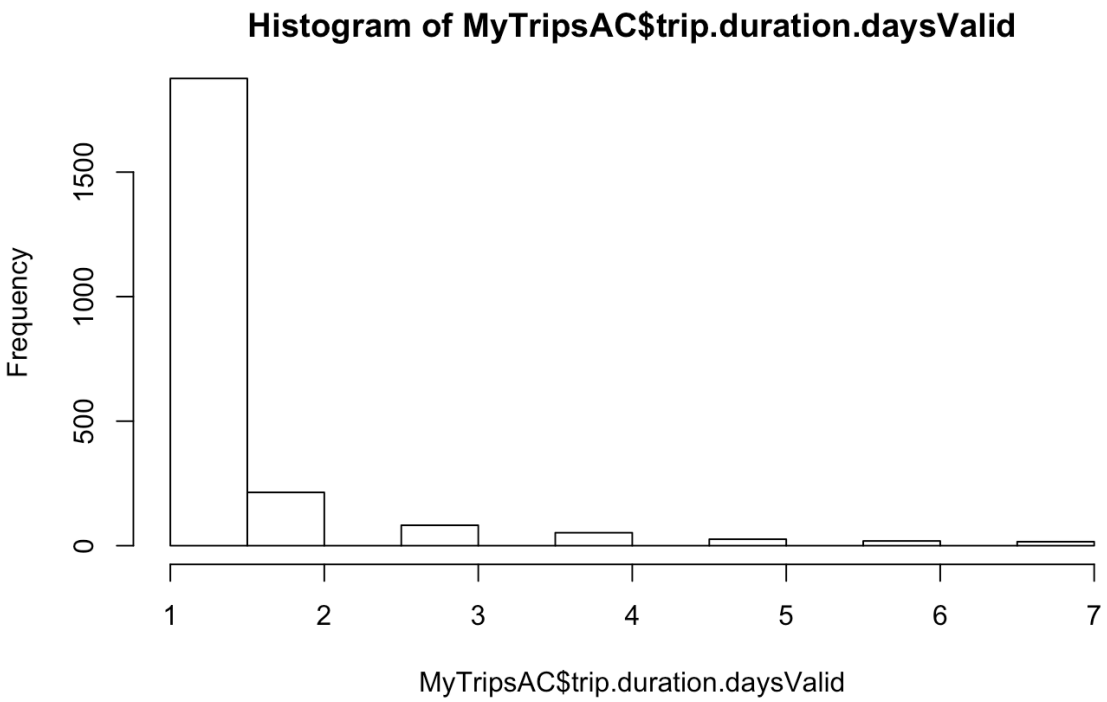


Figure S2| Histogram of trips filtered to equal or less seven days. These are the trips used in analysis.

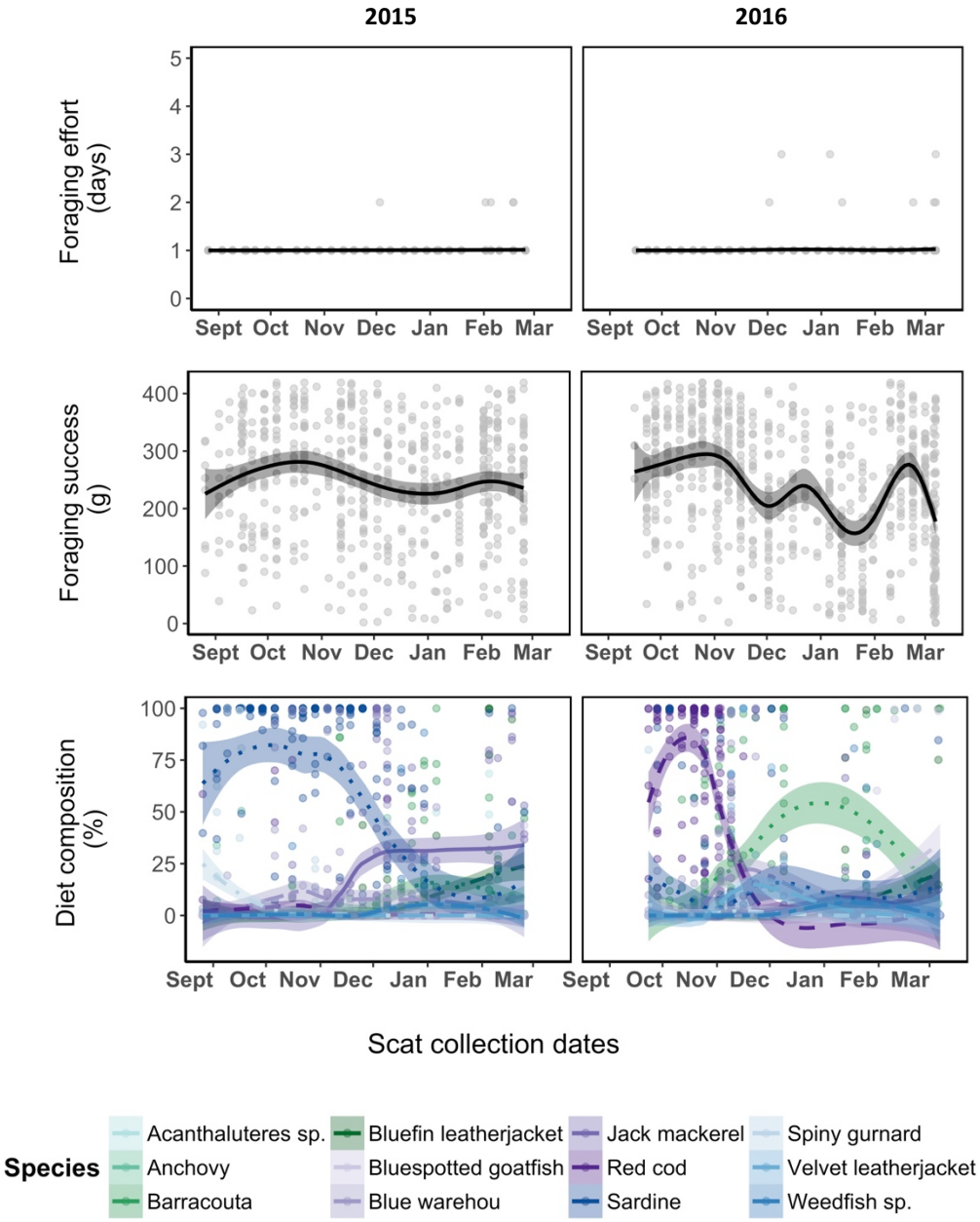


Figure S3| The above three panels are the inputs for the CPUE: foraging effort (top panel; foraging trip duration), foraging success (middle; mass gain) recorded by the weighbridge and the relative read abundance of individual fish species (bottom; RRA %) from scat DNA diet analysis.

Table S2| Fish diet composition of little penguins at Phillip Island over the 2015 and 2016 breeding seasons. Note that not all species could be resolved to species level, due to a lack of voucher species in the system. These species can be retrospectively identified whenever voucher species are entered into the NCBI GenBank (Johnson *et al*. 2008).

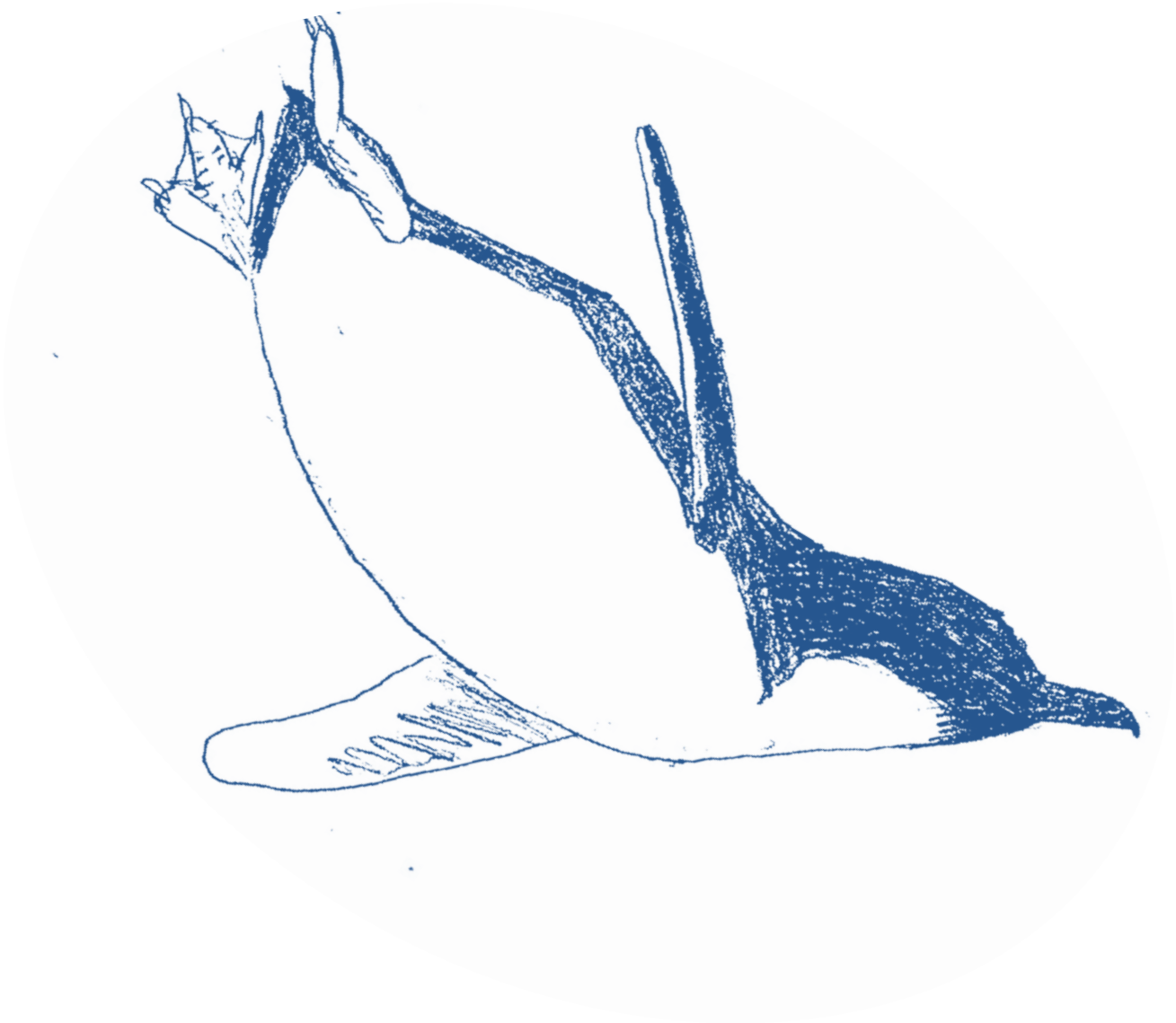
Common name	Species	Family	2015	2016
>1%				
Australian sardine	***Sardinops sagax***	**Clupeidae**	53.21	15.89
Red cod	***Pseudophycis bachus***	**Moridae**	0.76	36.96
Barracouta	***Thyrsites atun***	**Gemylidae**	5.39	18.1
Jack mackerel	***Trachurus declivis***	**Carangidae**	17.7	3.03
Blue warehou	*Seriolella brama*	Centrolophidae	6.27	10.02
Bluefin leatherjacket	***Thamnoconus degeni***	**Monacanthidae**	3.65	2
Spiny gurnard	***Lepidotrigla papilio***	**Triglidae**	2.98	1.31
Velvet leatherjacket	***Meuscenia scaber***	**Monacanthidae**	0.01	3.59
Weedfish sp.	*Heteroclinus* sp.	Clinidae	2.21	0.99
Bluespotted goatfish	*Upeneichythys vlamingii*	**Mullidae**	0.03	2.69
Australian anchovy	***Engraulis australis***	**Engraulidae**	1.67	1.32
Leatherjacket sp.	*Acanthaluteres* sp.	**Monacanthidae**	2.22	0.35
<1%				
Australian salmon	*Arripis trutta*	Arripidae	0.3	0.15
Blenny sp.	*Parablennius* sp.	Blennidae	0.01	0.01
Common stinkfish	*Foetorepus calauropomus*	Callionymidae	>0.01	>0.01
Skipjack trevalley	*Pseudocaranx georgianus*	Carangidae	0.07	>0.01
Warehou sp.	*Seriolella* sp.	Centrolophidae	>0.01	0.06
Morwong sp.		Cheilodactylidae	-	0.01
Adelaide weedfish	*Heteroclinus adelaidae*	Clinidae	>0.01	>0.01
Common weedfish	*Heteroclinus perspicillatus*	Clinidae	>0.01	>0.01
Heteroclinus kuiteri	*Heteroclinus kuiteri*	Clinidae	0.01	-
Weedfish sp.	*Heteroclinus* sp.	Clinidae	>0.01	-
Johnston's weedfish	*Heteroclinus johnstoni*	Clinidae	>0.01	>0.01
Kelp weedfish	*Heteroclinus eckloniae*	Clinidae	0.1	0.22
Longnose weedfish	*Heteroclinus tristis*	Clinidae	0.01	-
Ogilby's weedfish	*Heteroclinus heptaeolus*	Clinidae	0.04	-
Rosy weedfish	*Heteroclinus roseus*	Clinidae	0.4	0.01
Short-tassel weedfish	*Heteroclinus flavescens*	Clinidae	0.03	-
Southern crested weedfish	*Cristiceps australis*	Clinidae	0.01	>0.01
Weedfish sp.	*Heteroclinus* sp.	Clinidae	0.13	-
Weedfish sp.	Heteroclinus sp.	Clinidae	-	0.08
Wilson's weedfish	*Heteroclinus wilsoni*	Clinidae	-	0.09
Yellow crested weedfish	*Cristiceps aurantiacus*	Clinidae	>0.01	>0.01
Cottidae sp.		Cottidae	0.04	0.11
Silver Dory	*Cyttus australis*	Cyttidae	>0.01	0.48
Redbait	*Emmelichthys nitidis*	Emmelichthyidae	-	0.33
Old Wife	*Enoplosus armatus*	Enoplosidae	0.01	-
Common galaxias	*Galaxias maculatus*	Galaxiidae	>0.01	>0.01
Silverbelly	*Parequula melbournensis*	Gerreidae	>0.01	0.01
Red velvetfish	*Gnathanacanthus goetzeei*	Gnathanacanthidae	>0.01	0.01
Southern garfish	*Hyporhamphus melanochir*	Hemiramphidae	0.02	-
Blue weed-whiting	*Haletta semifasciata*	Labridae	0.01	-
Bluethroat wrasse	*Notolabrus tetricus*	Labridae	0.11	0.05
Herring cale	*Olisthops cyanomelas*	Labridae	0.03	0.01
Little weed whiting	*Neoodax balteatus*	Labridae	0.02	-
Notolabrus sp.		Labridae	>0.01	-
Purple wrasse	*Notolabrus fucicola*	Labridae	>0.01	>0.01
Senator wrasse	*Pictilabrus laticlavius*	Labridae	>0.01	>0.01
Tubemouth	*Siphonognathus argyrophanes*	Labridae	>0.01	-
Leatherjacket sp.	*Acanthaluteres* sp.	Monacanthidae	0.02	>0.01

Table S2 continued.

Common name	Species	Family	2015	2016
Leatherjacket sp.	*Meuschenia* sp.	Monacanthidae	0.03	0.21
Leatherjacket sp.	*Monacanthidae* sp.	Monacanthidae	0.39	0.44
Leatherjacket sp.	*Monacanthidae* sp.	Monacanthidae	0.02	0.04
Mosaic leatherjacket	*Eubalichthys mosaicus*	Monacanthidae	0.46	0.06
Bearded rock cod	*Pseudophycis barbata*	Moridae	0.02	0.01
Largetooth beardie	*Lotella rhacina*	Moridae	>0.01	-
Cod sp.	*Moridae* sp.	Moridae	-	0.01
Cod sp.	*Pseudophycis* sp.	Moridae	0.07	0.57
Yelloweye mullet	*Aldrichetta forsteri*	Mugilidae	0.02	-
Bartail goatfish	*Upeneus tragula*	Mullidae	0.01	-
Upeneichythys sp.	*Upeneichythys* sp.	Mullidae	-	0.02
Pegasidae sp.		Pegasidae	0.07	-
Elongate bullseye	*Parapriacanthus elongatus*	Pempheridae	0.13	-
Longsnout boarfish	*Pentaceropsis recurvirostris*	Pentacerotidae	0.05	0.03
Pomacentridae sp.		Pomacentridae	0.02	-
Longsnout flounder	*Ammotretis rostratus*	Rhombosoleidae	0.22	-
King gar	*Scomberesox saurus*	Scomberesocidae	0.02	-
Blue mackerel	*Scomber australasicus*	Scombridae	0.58	0.43
Butterfly perch	*Caesioperca lepidoptera*	Serranidae	0.01	0.01
Caesioperca sp.		Serranidae	0.13	0.04
Eastern school whiting	*Sillago flindersi*	Sillaginidae	0.11	>0.01
Bigbelly seahorse	*Hippocampus abdominalis*	Syngnathidae	0.02	0.01
Lepidotrigla sp.		Triglidae	0.02	>0.01
Common stargazer	*Kathetostoma laeve*	Uranoscopidae	-	0.03
Actinopterygii		Actinopterygii	>0.01	0.09
Actinopterygii		Actinopterygii	>0.01	0.04
Actinopterygii		Actinopterygii	>0.01	0.03
Actinopterygii		Actinopterygii	0.01	0.01
Actinopterygii		Actinopterygii	0.02	-
Actinopterygii		Actinopterygii	0.07	-
Actinopterygii		Actinopterygii	0.01	-

Chapter 4
Predator foraging efficiency related to local environmental conditions

Not yet submitted for publication



Chapter 4
Predator foraging efficiency related to local environmental conditions

4.1 Abstract

Top predators are sensitive to environmental changes mediated through the trophic web and so investigating how they respond to the physical characteristics in their foraging environment is important for understanding how local environmental and climate changes might affect their populations. Here, I investigated the effect of satellite-derived records of physical variables – wind speed, sea surface temperature and thermal stratification of the water column – on the foraging efficiency (foraging success as a function of effort) of a generalist, central-place marine forager with a restricted foraging range, the little penguin (*Eudyptula minor*). To account for the behavioural plasticity that can buffer seabirds against low resource availability, I used penguin-derived catch per unit effort (CPUE) as a proxy for foraging efficiency to determine the overall catch and the fish-only catch by little penguins in relation to effort expended. I showed that overall foraging efficiency regardless of diet composition was higher at lower sea surface temperatures, but that as temperatures rose, penguins became more efficient at capturing fish, their primary prey. I was unable to define a biologically meaningful relationship between wind speed and foraging efficiency, potentially due to averaging highly variable daily wind data over several days to match foraging data. The relationship I observed between stratification and foraging efficiency contrasted with earlier studies, possibly due to my use of satellite-derived measures of stratification, or to penguins foraging in shallower areas in these years. High foraging success and low foraging effort in the years studied may have further restricted my capacity to identify meaningful responses in these variables. My results add further evidence that this marine predator responds to the effect of sea surface temperature on local marine productivity, suggesting individuals time their breeding to best exploit spawning and juvenile fish.

4.2 Introduction

Marine predators are adapted to forage in a dynamic environment (Weimerskirch 2001) in which prey availability and composition are influenced by physical variables (e.g. Klein and Coste 1984;

Takahashi *et al.* 2008). Their environment is undergoing rapid changes in temperature, chemical composition, circulation, stratification and currents (IPCC 2014), which are likely to influence multiple levels of food webs (McFarlane, King and Beamish 2000; Richardson 2008; Sommer and Lengfellner 2008), potentially disrupting or improving foraging opportunities for predators. Given the important role of predators in regulating biodiversity (Madin, Madin and Booth 2011; Ritchie and Johnson 2009) and structuring food webs (Baum and Worm 2009), any positive or negative impacts on predator populations could also have a top-down effect on food webs, potentially compounding the effects of environmental change on marine ecosystems. Therefore, it is useful to examine how environmental variables influence predator foraging outcomes. Foraging efficiency, defined here as foraging success as a function of effort, is useful for understanding how predators cope with changing prey availability.

The physical characteristics of the foraging environment can alter the foraging efficiency of predators by directly influencing the location and abundance of prey (Lowther *et al.* 2018), by making prey harder or easier to locate and catch (Pink and Abrahams 2018; Ropert-Coudert *et al.* 2009; Strod *et al.* 2008), and by affecting where predators can travel and forage (Saraux *et al.* 2016; van Eeden *et al.* 2016; Weimerskirch *et al.* 2000). Studies have already detected broad-scale impacts of changing environmental characteristics on prey availability as prey distributions and reproductive phenology shift (e.g. Atkinson *et al.* 2004; Donaton *et al.* 2019; Green *et al.* 2015). In some cases, these impacts are due to changes in the position or presence of oceanographic features that have been predictable in the long term (Coetzee *et al.* 2008; Pichegru *et al.* 2010), disrupting foraging opportunities for populations that previously exploited them. However, the implications of environmental change are not just restricted to long-term and broad-scale patterns. Short-term local events can affect how easily animals can sense their prey, and may also affect the three-dimensional distribution of prey in the water column, making it harder or easier for predators to forage. For example, strong winds, large waves or low water clarity can make it difficult for predators to travel to or navigate foraging areas (Kowalcyzk *et al.* 2015a; Saraux *et al.* 2016), or find their prey (Baptist and Leopold 2010). Ephemeral, structural qualities of the water column, such as thermal stratification and dissolved oxygen levels, can concentrate prey, making it easier to catch (Pink and Abrahams 2018; Ropert-Coudert *et al.* 2009).

The capacity of predators to avoid or exploit environmental conditions depends on their foraging range and mobility (Schreiber 2001). For example, an intense tropical storm can be avoided by wide-ranging frigatebirds or boobies (Weimerskirch and Prudor 2019), and procellariformes can travel great distances following the scent of prey to locate more profitable foraging patches, (Nevitt 2008), while animals that are restricted to local inshore environments are much more limited (Frederikson *et al.* 2008). Therefore, it is expected that local changes in the foraging environment will disproportionately affect species with short foraging ranges, especially those with a terrestrial reproductive phase. In many cases, predators follow reliable signals from their physical environment to time breeding attempts within an optimum foraging window (Afan *et al.* 2015). Persistent breeding sites may be located within reach of predictable oceanographic features (Yen *et al.* 2004), which may disappear or be displaced outside the foraging area by changes in climate. In addition to foraging distance, the foraging specificity of predators will affect their sensitivity to environmental change. Predators with a generalist diet are typically more resilient than specialists to changes in prey, because generalists can target other prey when a preferred option is less abundant (Terraube *et al.* 2010).

Little penguins *Eudyptula minor* have one of the shortest foraging ranges of any seabird (< 20 km during reproductive bouts, Collins *et al.* 1999) and are high trophic level opportunistic predators that prey mainly on small fish, supplemented by a wide array of secondary prey items (Cavallo *et al.* 2018; Chiaradia *et al.* 2010; Cullen *et al.* 1992; Kowalczyk *et al.* 2015b). Their reproductive output is thought to be determined by the availability and abundance of fish prey of a certain size (Chiaradia *et al.* 2003), and they can lay up to three clutches of two eggs per season though typically only raise one or two chicks to fledging (Marchant and Higgins 1990). Populations across their range in southern Australia and New Zealand have highly variable breeding seasons (Gales 1985; Reilly and Cullen 1981) that are likely to be driven by their proximity to oceanographic features or abiotic and biotic conditions in the marine environment (Frederikson *et al.* 2004; Weimerskirch 2001), including a reliable annual signal from the marine environment to initiate breeding (Afan *et al.* 2015). While the species predominately preys on small pelagic fish, diet varies between breeding sites due to local prey availability, and some New Zealand colonies have been found to include a high proportion of relatively energy-poor cephalopod and crustacean prey (Flemming *et al.* 2013). These dietary differences are driven by local environment and oceanographic features (Chiaradia *et al.* 2012; Cullen *et al.* 1992; Gales and Pemberton 1990), suggesting that populations will be sensitive

to local environmental changes. Further, a large proportion of the total little penguin population is located within Bass Strait and the Tasman Sea (Birdlife International 2017), a region of rapid environmental change due to the strengthening East Australian Current (Wu *et al.* 2012).

Because the generalist foraging strategy of little penguins buffers them from environmental conditions that drive availability of their preferred prey items, monitoring their responses to environmental changes in their foraging environment is a complex task. In previous studies, various physical variables have been shown to influence little penguin foraging. For example, increased sea temperature triggers the breeding onset (Afan *et al.* 2015), high sea surface temperatures have been associated with higher foraging effort (Berlincourt and Arnold 2015) and reduced foraging success (Carroll *et al.* 2016). High wind speeds have been related to low body mass gain, longer foraging trips and smaller meals delivered to chicks, especially in response to brief, extreme events (Saraux *et al.* 2016). The presence of thermal stratification in the water column is associated with higher rates of prey encounter (Pelletier *et al.* 2012; Ropert-Coudert *et al.* 2009). Using catch per unit effort (CPUE), as a measure of foraging efficiency that integrates diet composition with foraging effort ($foraging\ trip\ duration$) and success ($body\ mass\ change$), I investigated the effect of wind speed, sea surface temperature and thermal stratification within the foraging area on the foraging efficiency of this central-placed forager. I expected that overall foraging efficiency (CPUE) would remain relatively stable over a range of conditions, but expected the foraging efficiency on the fish component of the diet to vary with changing environment, as penguins modified their foraging preferences.

4.3 Methods

4.3.1 Experimental design

I monitored little penguins at two sites (Penguin Parade and Radio-tracking Bay) on the Summerlands Peninsula of Phillip Island (38◦31’ S, 145◦07’ E), in southeastern Australia during the 2015/16 and 2016/17 breeding seasons. I used an Automated Monitoring System (Chiaradia and Kerry 1999) to measure the foraging trip duration and mass changes of individual birds and carried out DNA faecal metabarcoding using universal and group-specific primers to determine diet (Cavallo *et al.* 2018). I then integrated these records of foraging effort, success and diet into a weekly index of foraging efficiency, catch per unit effort (CPUE, detailed in Chapter 3) and tested

for relationships between CPUE and environmental variables known to affect little penguin foraging outcomes.

4.3.2 Automated monitoring

Penguins entering and exiting their colony tend to travel on consistent paths. I used automated weighbridges intercepting the main path used by the most birds at each site to capture data on a large proportion of the individuals in each study site. The Automated Penguin Monitoring System (APMS: Chiaradia and Kerry 1999) comprises a weighbridge, transponder reader and data logging computer and records time, mass and identity of birds crossing. All chicks and new birds within the study site are equipped with passive identification transponders or PIT tags (Penguin Parade-Allflex, Australia and Radio Tracking Bay-Trovan, United Kingdom) which are injected subcutaneously between the scapulae (Chiaradia and Kerry 1999). This system allows the passive collection of hundreds of thousands of records each year, and enables users to identify and calculate both the length of foraging trips and the change in mass of individuals over each foraging trip.

I also checked nests to record the presence of adults, eggs or chicks – thrice weekly at Penguin Parade and once a week at Radio Tracking Bay due to differing accessibility (Sánchez *et al.* 2018). This frequency of visits was required to detect discrete breeding stages of the little penguin breeding, which significantly influence their foraging range and effort (Chiaradia and Kerry 1999; Chiaradia and Nisbet 2006; Kato *et al.* 2008). These include 'incubation', in which adults take turns incubating their eggs for one to seven days while the partner forages at sea (Kato *et al.* 2008; Numata *et al.* 2000); 'guard', where small chicks are guarded by one parent while the other forages at sea, swapping roughly daily (Chiaradia and Kerry 1999), and 'post-guard', where both adults forage to provision their large chicks, which are left unattended in the burrow while parents are out at sea (Saraux *et al.* 2016). Foraging trips during the post-guard stage can range from one to two days when food is abundant and from two to five days when food is scarce (Chiaradia and Nisbet 2006). Because of the need to frequently return to the colony to feed chicks and relieve partners, foraging range is most limited during the guard stage.

To collect scats for diet analysis, each week I fixed cardboard plates into the entrance of nests to collect scats deposited overnight. These were collected early the next day, to minimise the risk of

sample contamination or deterioration. Due to the large numbers of viable scat samples collected, a stratified random sample (with stratification at the week level) was used for analysis.

4.3.3 Diet

Dietary DNA metabarcoding involves amplification and sequencing of large numbers of short, unique regions of DNA (barcodes) present in the scats of consumers (Pompanon *et al.* 2012). I used a two-step PCR amplification process enabling amplification of a DNA barcode region, and subsequent attachment of unique 'index tag' sequences to each scat sample, allowing samples to be pooled for sequencing (Binladen *et al.* 2007). I used a universal primer (18s_SSU; McInnes *et al.* 2016) to assess broad diet composition and isolate the fish component. Full molecular methodology and bioinformatics can be found in Cavallo *et al.* (2018) and Chapter 3.

4.3.4 Catch per unit effort (CPUE)

An overview of the calculation of the CPUE is given here, with full methods reported in Chapter 3. The CPUE combines data from the weighbridge and diet sampling. From the APMS I downloaded daily records of entry and exit of birds to the colony, as well as the body mass of individual birds at their time of crossing. I then calculated the foraging success ($body\ mass\ change$ in grams) and foraging effort ($foraging\ trip\ duration$ in days) from complete trips by birds that returned the day before scats were collected, and averaged these values by return date and breeding stage. I retained only positive *body mass change* results < 420 g (i.e. the maximum change in body mass for a foraging trip between 1 to 14 days long; Salton *et al.* 2015) and filtered out *foraging trip durations* > 7 days, which are rare for breeding little penguins as they can lead to nest desertion (Chiaradia and Kerry, 1999; Numata *et al.* 2000) to minimise potential sources of error discussed in Chapter 3. Diet samples were also averaged by breeding stage and the date of sample collection. I then calculated the CPUE of fish in the diet (hereafter "fish-only CPUE") using the 18s_SSU (McInnes *et al.* 2016) data as

$$CPUE_{Fish} = \frac{body\ mass\ change \times \left(\frac{RRA_{Fish}}{100}\right)}{foraging\ trip\ duration},$$

where (RRA_{Fish})is the relative read abundance of fish (class: Actinopterygii), ($body\ mass\ change$) is the median in grams and ($foraging\ trip\ duration$) is the median in days. I also calculated the overall CPUE irrespective of diet (hereafter "overall CPUE") as

$$CPUE_{Overall} = \frac{mass\ gain}{foraging\ trip\ duration}$$

Code to replicate CPUE from weighbridge and diet data is included in the appendices to this thesis.

4.3.5 Environmental data

Environmental variables were extracted from the foraging area of little penguins nesting on Phillip Island (Figure 1; latitudes -38.5 to -39.5, longitudes 144 to 146, Sánchez *et al.* 2018). Sea surface temperature, temperature at different depths and wind were downloaded from www.marine.copernicus.eu and extracted using the *ncd4* package (Pierce 2012). Spatial and temporal resolution of all environmental data are given in Table 1.

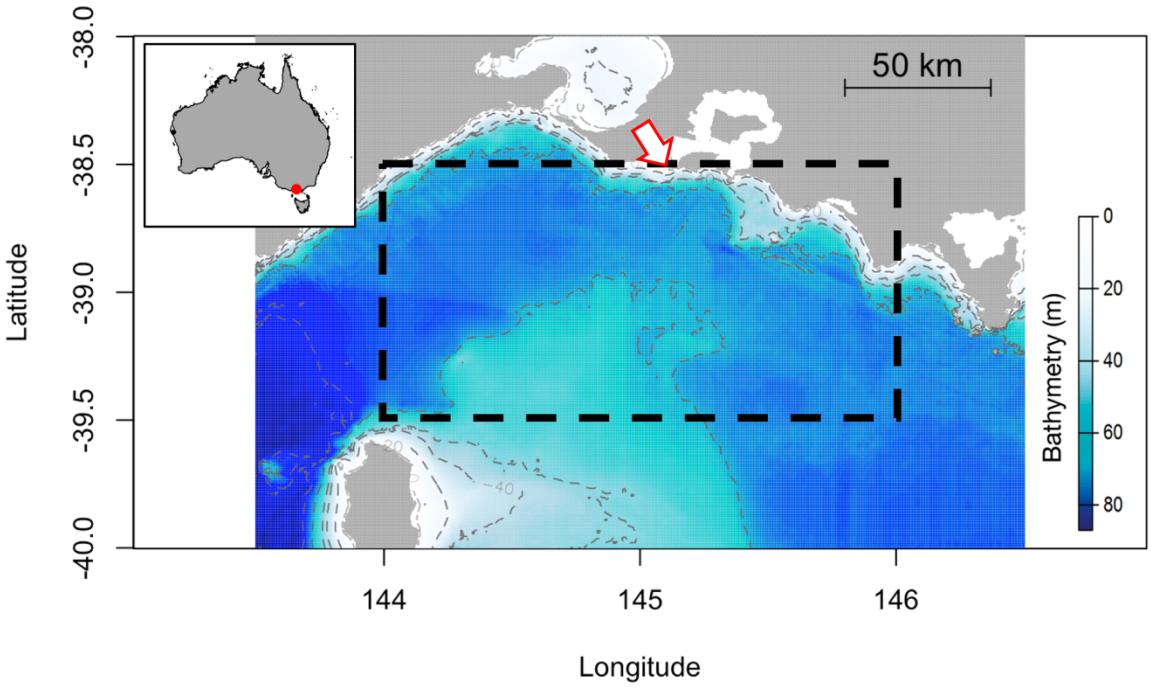


Figure 1| The foraging area (black rectangle) for little penguins breeding at Phillip Island (red and white arrow). Inset map indicates the location of the site within Australia. Environmental data were extracted for the area within the black rectangle.

Table 1| Spatial and temporal resolution of environmental data extracted from The Copernicus Marine Environment Monitoring System (www.marine.copernicus.eu).

Variable	Name	Units	Spatial res.	Temporal res.	Other
Sea Surface Temperature	thetao	°C		hourly	
Temperature at depth	votemper	K (converted to °C)	0.250°	daily	Recorded at 0, 5, 10, 20, 30, 40, 50 and 60 metres
Wind Speed	wind_speed	m/s	0.250°	six hourly	

Sea surface temperature and wind speed were both averaged per day over the foraging area. To determine the presence and depth of thermal stratification of the water column, temperature records were averaged over the foraging area for each depth on each day. Then, the value and depth of the greatest change in temperature over each ten metre section was calculated. This depth was designated as the mixed-layer depth. The temperature change values were filtered by a threshold of 0.1°C/1 m over a 10 m interval to determine whether a thermocline was present ($\Delta >$ 0.1°C/1 m) or absent on any given day. This rate of change is comparable in magnitude to the 0.12°C/1 m used in an earlier study with penguin-borne temperature-depth recorders (Ropert-Coudert *et al.* 2009). Chlorophyll A concentration was initially investigated as a variable of interest, but was found to co-vary with sea surface temperature, and henceforth was excluded from the analysis.

4.3.6 Comparing relevant CPUE and environment values

The maximum foraging duration recorded in this study was four days, and so environmental variables were averaged over the four days prior to each scat collection and paired with the corresponding CPUE data. This matched well with the diet data, which is expected to represent diet from at least the four previous days in little penguins (Deagle *et al.* 2010). Additive mixed models (GAMMs) were developed to accommodate nonlinear and non-normal data and relationships (Hastie and Tibshirani 1990), which are common when considering environmental variables (Richards *et al.* 2010). Mixed models were employed to handle the random effects of breeding site, month, season, breeding stage (incubation, guard or post-guard) and burrow. The data were investigated for autocorrelation by inspecting plots of residuals against time and were

found to satisfy the assumption of independence. No thermocline presence (Δ >1°C/10m) was detected until late December or January in the years studied, and so it was not possible to test the effect of thermocline presence across the whole season. Consequently, two sets of GAMMs were constructed (Table 2). The first model contained the fixed effects of average daily sea surface temperature and average daily wind speed, both fitted with a thin plate smoother, and all five random effects, fitted with a random effect smoother. This model was fitted with response data from across the breeding season. In the second model, a binary stratification variable (present/ absent) was added to the first model as a linear variable. This second model was fitted with data from only those months in which stratification was present in either year (December–March). All GAMMS were fitted to a Gaussian distribution with an identity link. To reduce the chance of Type II error I accept as significant only p-values < 0.01.

Table 2|Generalised additive models investigating the response of foraging efficiency (CPUE) to sea surface temperature (SST), wind speed (wind) and the presence of thermal stratification (StratificationPresence). Random effect variables are breeding season (Season), month (Month), breeding site (Site), breeding stage (BreedingStage) and burrow ID (Burrow).

Model set	**Responses**	**Model**
Model One	Overall CPUE, Fish-only CPUE	*CPUE ~ s(SST) + s(wind) + s(Season) + s(Month) + s(Site)+ s(BreedingStage) + s(Burrow)*
Model Two	Overall CPUE, Fish-only CPUE	*CPUE ~ StratificationPresence + s(SST) + s(wind) + s(Season)+ s(Month) + s(Site) + s(BreedingStage) + s(Burrow)*

4.4 Results

4.4.1 The foraging environment

The 2015/16 foraging environment (Figure 2) was characterised by a linear temperature increase from September to February, average wind speeds between 6 and 8 m/s and the presence of thermal stratification from late December onwards. Prior to this the water column was mixed across the recorded 0-50 metres. Once stratification was established, the bottom of the mixed layer stabilised at a depth between 30 and 40 m, before dropping to between 40 - 50 m in mid-February. The 2016/17 foraging environment (Figure 2) was characterised by two months of stronger average winds (8-10 m/s) during October and November, delayed incline in sea surface temperature and a well-mixed water column. Thermal stratification was observed on and off from late December, but never stabilized, progressing to deeper depths between January and March.

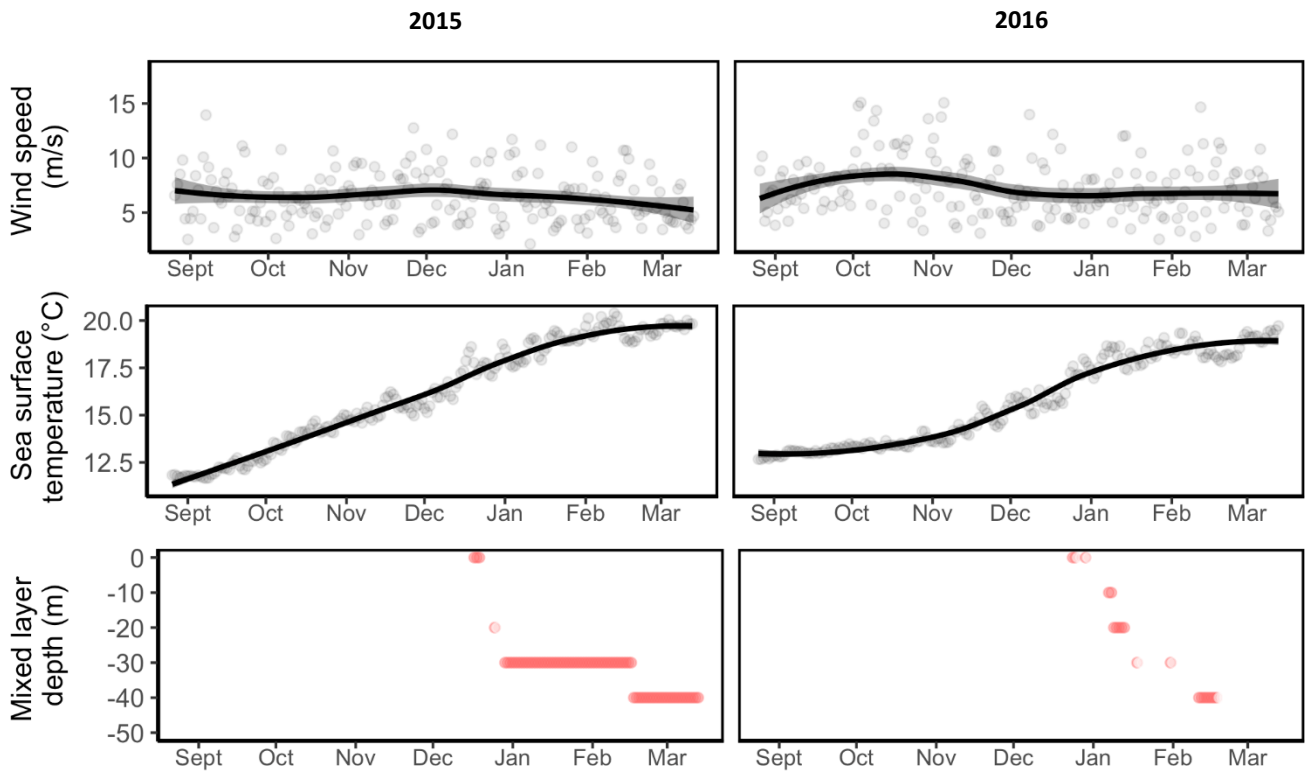


Figure 2| The foraging environment experienced by little penguins over the 2015/16 and 2016/17 breeding seasons on Phillip Island. Red dots in the lower panel signify presence of a thermocline (Δ > 1°C/10 m).

4.4.2 Penguin foraging outcomes

In both seasons, the overall CPUE peaked during October, decreased over November and December and increased again from early January (Figure 3). In 2015, the fish-only CPUE increased almost linearly throughout the season, while in 2016 an early increase in the fish-only CPUE peaked in October and then decreased, with a second increase from January. No foraging data was collected in March 2016 or in August or the first two weeks of September in 2016.

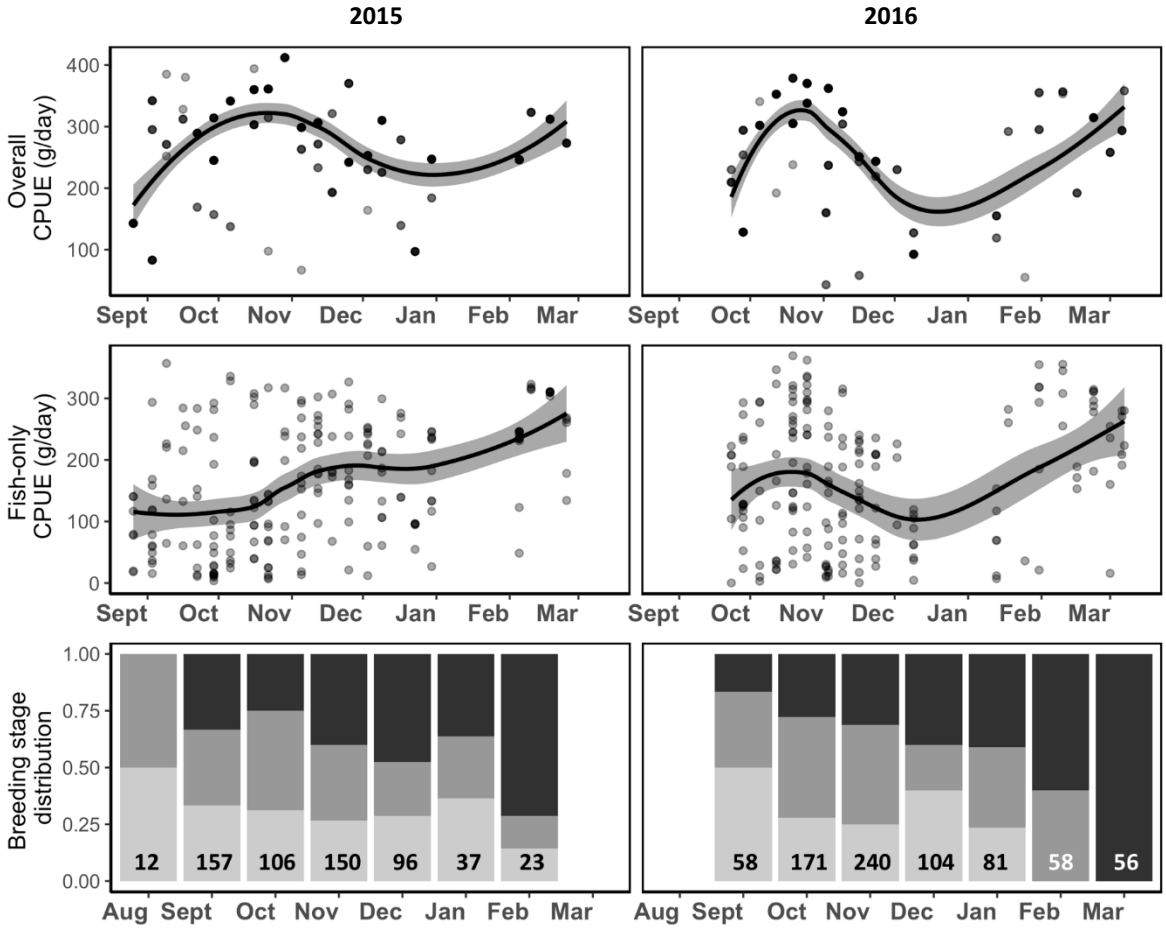


Figure 3| The CPUE (upper and middle panels) of foraging penguins in the 2015/16 (left panels) and 2016/17 (right panels) breeding season, along with the percentage and number of pairs in each breeding stage by month (lower panels). Breeding stages are incubation (lightest grey), guard (medium grey) and post guard (black). Note that no foraging data were collected in March 2016 or in August or the first two weeks of September in 2016.

4.4.3 The effects of the environment on penguin foraging

In generalised additive models that spanned the breeding season in each year studied, both wind speed and sea surface temperature affected overall CPUE and fish-only CPUE (Table 3). The overall CPUE brought in each day was high and stable when sea surface temperature was between 12 and 17.5°C, above which it fell steeply (Figure 4, top left). Conversely, there was a significant positive effect of sea surface temperature on the CPUE of fish, which increased linearly for sea surface temperatures between 12 and 20°C (Figure 4, bottom left). Wind had a complex relationship with both overall and fish-only CPUE. Overall CPUE had very low values between wind speeds of 6 and 7 m/s and speeds of 9 and 10 m/s and high values around 8 and 11 m/s (Figure 4, top right). Fish-only CPUE also had very low values between wind speeds of 6 and 7 m/s and at 12 m/s, with peaks

around 8 and 10.5 m/s (Figure 4, bottom right). As random variables, season, month and breeding stage had a significant effect on overall CPUE, but breeding site and burrow ID had no effect. For fish-only CPUE, only the random effect of breeding stage significantly affected the model.

Table 3| Generalized additive mixed model results for Model One. Parametric variable results are given as t values, and smoothed variable results are given as F values. Significant p-values are given in bold.

	CPUE				Fish			
	Estimate	**Std. Error**	**t value**	**p-value**	**Estimate**	**Std. Error**	**t value**	**p-value**
(Intercept)	269.24	43.88	6.14	**< 0.001**	155.1	17.37	8.93	**< 0.001**
	edf	**Ref. df.**	**F**	**p-value**	**edf**	**Ref. df.**	**F**	**p-value**
Fixed effects								
Sea surface temperature	8.70	8.95	5.05	**< 0.001**	1.00	1.00	15.67	**< 0.001**
Wind speed	8.86	8.98	9.45	**< 0.001**	8.48	8.86	4.92	**< 0.001**
Random effects								
Season	0.85	1.00	15.56	**< 0.001**	0.51	1.00	1.04	0.23
Month	7.11	8.00	8.62	**< 0.001**	3.04	7.00	1.38	0.03
Breeding stage	1.99	2.00	54.30	**< 0.001**	1.65	2.00	6.03	**< 0.01**
Breeding site	0.00	2.00	0.00	0.54	0.00	2.00	0	0.34
Burrow ID	30.66	151.00	0.27	0.03	32.88	151.00	0.29	0.03
Adjusted R^2	0.61				0.29			
Deviance explained	67.0%				37.6%			

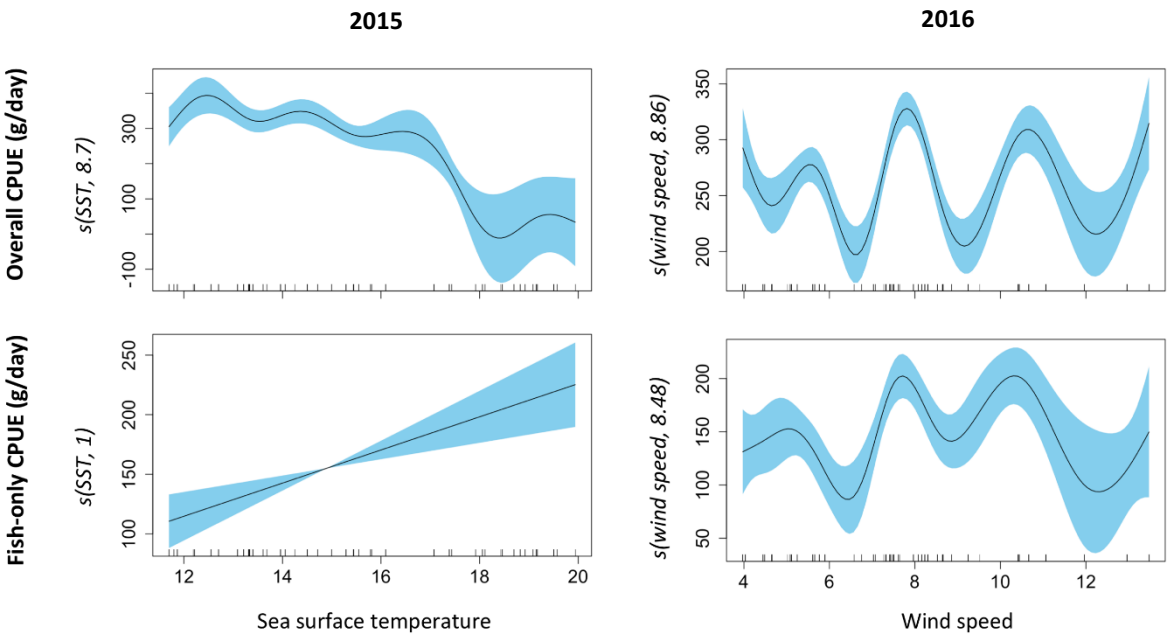


Figure 4| Partial effects plots illustrating the relationship between smoothed fixed parameters and CPUE in Model One. The shaded areas represent the 95% confidence intervals. The rug (tick marks) at the base of each plot indicates the spread of observations over the range of variable values.

4.4.4 The effects of environmental variables in the presence of thermal stratification

Across a smaller subset of the months of the breeding season (December – March), during which water column stratification was detected in one or both seasons, sea surface temperature had a significant effect on both overall and fish-only CPUE (Table 4). Here, both fish-only and overall CPUE were highest at sea surface temperatures between 18 and 19°C, and lowest at sea surface temperatures between 16 and 17°C (Figure 5, left panels). Wind speed also had a significant effect on CPUE values with low values for wind speeds between 6 and 7 m/s and at 9.5 m/s, and high values for wind speeds around 5 m/s and between 7 and 9 m/s. (Figure 5, right panels). The presence of stratification in the water column had a significant negative effect on both overall and fish-only CPUE (Table 4). As random effects, season and breeding stage had significant effects on both fish and overall CPUE and month had a significant effect on fish-only CPUE (Table 4).

Table 4 | Generalized additive mixed model results for Model Two. Parametric variable results are given as t values, and smoothed variable results are given as F values. Significant p-values are given in bold.

	CPUE				**Fish**			
	Estimate	**Std. Error**	**t value**	**p-value**	**Estimate**	**Std. Error**	**t value**	**p-value**
(Intercept)	139.14	132.83	1.05	0.30	113.16	68.97	1.641	0.1
Fixed effects								
Threshold present	-109.41	12.59	-8.69	**< 0.001**	-89.72	33.03	-2.72	**< 0.01**
	edf	**Ref. df.**	**F**	**p-value**	**edf**	**Ref. df.**	**F**	**p-value**
Sea surface temperature	6.87	6.93	67.76	**< 0.001**	5.98	6.67	4.256	**< 0.001**
Wind speed	8.60	8.60	61.50	**< 0.001**	9.00	9.00	3.83	**< 0.001**
Random effects								
Season	1.33	2.00	17.29	**< 0.001**	1.33	2.00	4.48	**< 0.001**
Month	2.46	4.00	6.02	**< 0.001**	0.52	3.00	0.43	0.05
Breeding stage	1.98	3.00	34.07	**< 0.001**	1.96	2.00	7.62	**< 0.001**
Breeding site	0.00	2.00	0.00	0.39	0.72	1.00	3.74	0.03
Burrow ID	2.20	67.00	0.04	0.39	0.00	68.00	0.00	0.99
Adjusted R2	0.95				0.60			
Deviance explained	96.0%				66.6%			

2015 **2016**

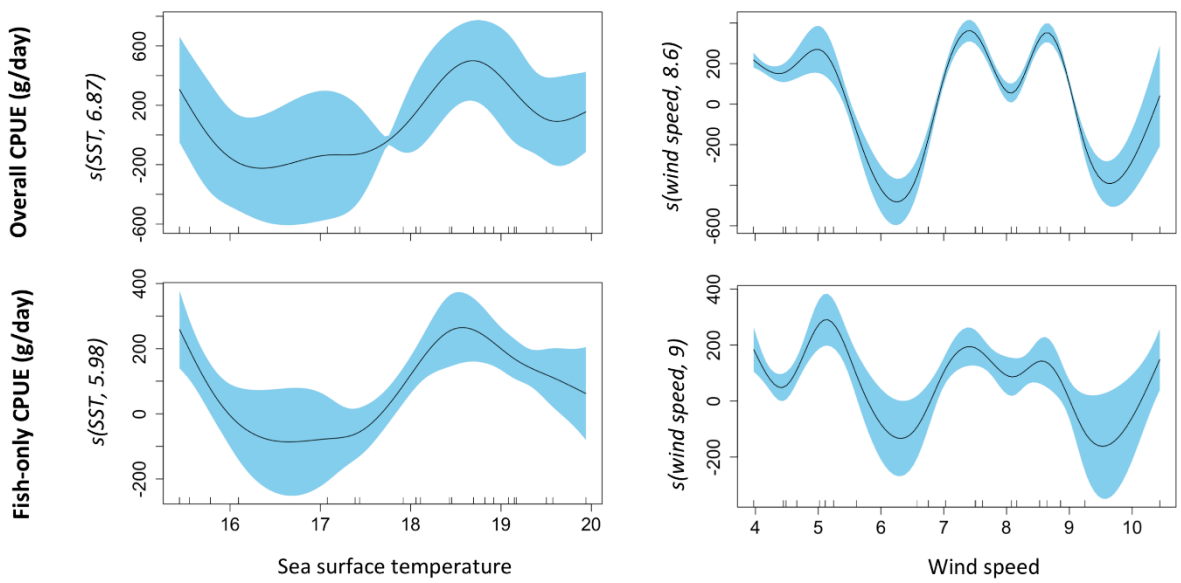


Figure 5| Partial effects plots indicating the relationship between smoothed fixed parameters and CPUE in Model Two. The shaded areas represent the 95% confidence intervals. The rug (tick marks) at the base of each plot indicates the spread of observations for over the range of variable values.

4.5 Discussion

Over two breeding seasons, foraging little penguins at Phillip Island experienced differing environments in terms of sea surface temperature, wind speed and thermal stratification of the water column. I found significant relationships between foraging efficiency (CPUE) and sea surface temperature, wind speed and stratification, although biological interpretation of the latter two was challenging, likely in part due to high prey availability in the years studied.

4.5.1 Sea surface temperature

Temperature is a major driver of three-dimensional distribution of low-trophic level organisms due to the importance of temperature for physiological function (Richardson 2008; Taylor *et al.* 1997). The CPUE of little penguins was significantly associated with sea surface temperature over the range 11.7 – 19.9°C observed during this study. When entire breeding seasons were considered, overall CPUE was consistently high when sea surface temperature was below 17°C, while temperatures above this were associated with lower overall CPUE. The temperatures over which overall CPUE was high in this study are lower

than the optimal thermal range for prey capture by little penguins reported by Carroll *et al.* (2016), but sea surface temperatures at Montague Island where Carroll's study took place are on average two degrees higher than at Phillip Island and in the present study temperatures and the seasonal thermal pattern in both years were close to the decadal average for the area (Figure 6). In contrast, fish-only CPUE rose almost linearly with temperature, although there were fewer records for sea surface temperatures above 16°C than below. This suggests that although penguins had lower overall foraging efficiency (CPUE) at higher sea surface temperatures, a larger proportion of the prey they ate at these temperatures was fish. Accordingly, I recorded a greater range of prey classes taken early in the season, when the temperatures were lower, and a greater focus on fish later in the season (further detail Chapter 2). A seasonal progression from mixed prey to fish over the breeding season has been recorded previously (Chiaradia *et al.* 2003) and may represent a common pattern at this penguin colony. Note that when fewer temperatures were considered during the latter half of the breeding season (Figure 4), a peak in both measures of CPUE was evident between 18 and 19 °C, suggesting that there may be an upper limit to the positive relationship between fish-only CPUE and sea surface temperature, similar to that recorded by Carroll *et al.* (2016).

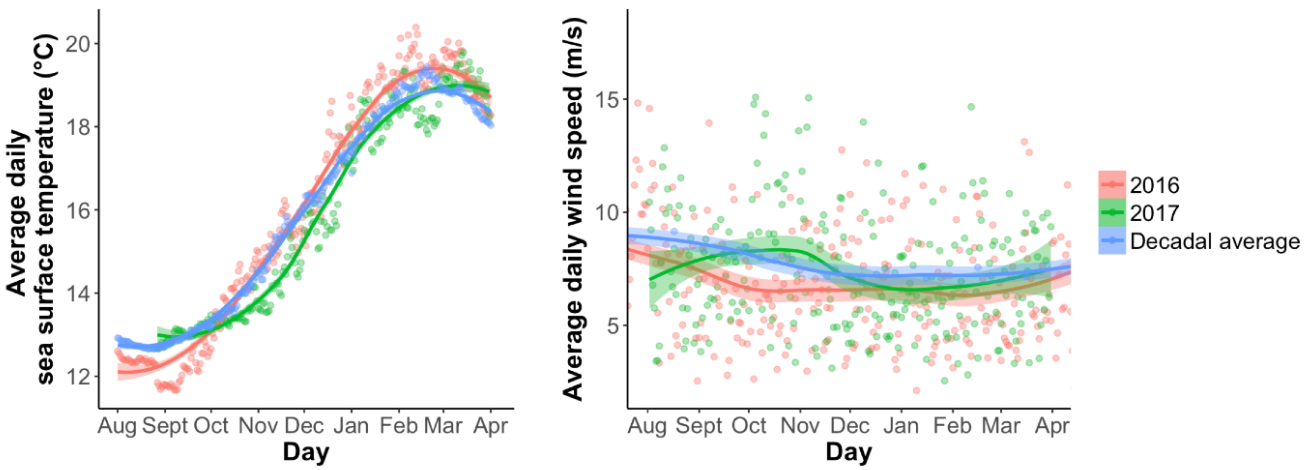


Figure 6| Sea surface temperature and wind speed for 2015/16 and 2016/17 season plotted against their decadal average. Shaded areas represent the 95 % confidence intervals.

The seasonal sea surface temperature pattern in this region is consistent across years and is highly correlated with marine productivity, such that the annual peak in chlorophyll A

concentration occurs no later than seven weeks after minimum sea surface temperatures are recorded (Afan *et al.* 2015). The rise in sea temperature is a signal for penguins to initiate courtship, so that mean laying date is always associated with the peak in productivity (Afan *et al.* 2015). The local spawning seasons for key little penguin prey species also show this pattern with red cod (*Pseudophycis bachus*) spawning in spring (Kemp *et al.* 2012), sardine (*Sardinops sagax*) and barracouta (*Thyrsites atun*) spawning in spring and summer (Blackburn 1950a; 1957), and anchovy (*Engraulis australis*) and jack mackerel (*Trachurus declivis*) spawning in summer (Blackburn 1950b; Bruce *et al.* 2001). The results of this study suggest that by using rising sea surface temperature as a cue to initiate breeding, little penguins might not only coincide their breeding effort with a period of high productivity, but may also time the subsequent raising of young with the period when highly nutritious fish prey are most efficiently exploited as a food source for growing chicks.

4.5.2 Wind and stratification

Although wind speed and thermocline presence had significant effects in my models, biological interpretation for these variables was challenging. Both fish-only and overall CPUE had several peaks and troughs across average wind speeds in the range of 4 m/s to 14 m/s. I anticipated a non-linear relationship between wind speed and CPUE, because seabird responses can differ across wind speeds such that foraging efficiency may improve from calm to moderate winds but high winds be associated with less efficient foraging (Dehnhard *et al.* 2013; Dunn 1973) – but this pattern of several peaks suggests either a complex mix of effects or an artefact of the sampling regime. Wind is an important driver of local productivity, influencing transport of nutrients throughout the water column (Klein and Coste 1984), and perhaps wind effects on penguin foraging behaviour (difficulty traveling to foraging grounds or seeing prey, disruption of stratification) were confounded by a positive effect of wind on local productivity and prey abundance. However, the pattern, or lack-thereof, may stem from limitations in my dataset. Despite the availability of daily wind speed data, I needed to average environmental variables over several days to match diet sampling frequency. Saraux and colleagues (2016) noted that the effects of strong wind on foraging variables were immediate, and so combining four day averages of highly variable daily wind data with averaged foraging measures in this study may have obscured any biological signal. For example, although there were several individual days above 14 m/s, no

four-day average wind speeds reached this level. Another point to consider is that the majority of wind speeds recorded were below 10 m/s, so it is possible that penguins in this study rarely experienced winds of sufficient speed to negatively affected their foraging efficiency. Saraux and colleagues (2016) observed a negative correlation between wind speed and mass gain (foraging success) per trip during the incubation and guard reproductive stages, but only at wind speeds greater than 10 m/s and they showed that wind speeds above 14 m/s during post-guard resulted in 28% longer foraging trip duration.

Thermal stratification in the water column can act as a physiological barrier to ectothermic prey (Kitagawa *et al.* 2001), and thus enhance foraging opportunities for endothermic predators (Ropert-Coudert *et al.* 2009). I expected to find a positive relationship between the presence of stratification and fish-only CPUE, if not overall CPUE, based on previous studies showing that little penguins are more efficient foragers in stratified water (Pelletier *et al.* 2012) and encounter more prey at the depth of the thermocline (Ropert-Coudert *et al.* 2009). Ropert-Coudert and colleagues also found a correlation between weaker stratification and reduced foraging and breeding success, and studies have recorded similar responses in other penguin species (Ballard *et al.* 2019; Scheffer *et al.* 2015) and fur seals (Kuhn 2010). So what does it mean that the model in this study found a negative effect of stratification on CPUE? A concurrent biologging study (Sánchez *et al.* 2018; Sánchez unpublished data) recorded that during the 2015/16 and 2016/17 breeding seasons little penguins foraged close to shore in waters shallower than the depth of the regional thermocline, which was between 30 and 50 m deep in this study. In shallow areas, the escape field of prey is already limited by bathymetry. Sánchez *et al.* also observed that mean prey encounter depths were generally less than 15 m. The results from Sánchez *et al.* suggest that in the years studied the presence or absence of stratification was not important in determining little penguin foraging strategies, because penguins largely foraged in areas without stratification. Further, an important difference between the current study and those by Pelletier *et al.* (2012) and Ropert-Coudert *et al.* (2009) is the use of satellite-derived temperature-at-depth data, averaged across the foraging range, rather than records of temperature directly recorded by temperature loggers attached to animals during foraging bouts. Any relationship between stratification and CPUE in this study may have been obscured by averaging temperature-at-depth values across a 200 by 100 km

foraging area that included a high proportion of shallow areas in which stratification was not present. The likelihood of detecting a relationship between stratification and CPUE will decline proportional to the number of grid cells that are shallower than the depth of the thermocline detected in deeper areas.

We observed high foraging success (foraging mass gained), efficiency (CPUE) and breeding success (chicks fledged per pair) in the two years studied (Chapter 3). These results point to high prey availability within the foraging range of little penguins, which likely affected our ability to capture relationships between environmental variables and foraging efficiency. It is widely acknowledged that unless prey availability is below the minimum threshold for offspring production, seabird responses to changing availability are difficult to interpret (Cairns 1987; Piatt *et al.* 2007). This is due to foraging plasticity that buffers seabirds and their offspring against low to moderate food limitation (Grémillet and Charmantier, 2010). A key advantage of CPUE analysis is the ability to account for this plasticity by integrating foraging success with diet and foraging effort, which are the behaviours through which generalist seabirds exercise their plasticity (Chapter 3). Little penguins may respond to low prey availability by extending trip duration (Chiaradia and Nisbet 2006) or switching prey (Chiaradia *et al.* 2003). In the seasons studied, I observed low foraging effort in terms of days at sea, suggesting that penguins were able to satisfy their needs and those of their chicks on the prey available to them. Therefore, prey availability may have been sufficiently high that it masked any negative effects of environmental variables, even for CPUE analysis. It is worth nothing that in the 2016/17, which was characterised by a higher number of strong wind days and a weaker thermocline, the colony produced almost 20% fewer chicks than the year before (2015: 2.15 chicks per pair, 2016: 1.76 chicks per pair, Chapter 3). I expect that the effect of environmental variables on foraging efficiency will be clearer in years of low to average breeding success.

A focus of this study was to increase the number of data points available for analysis by employing satellite-derived environmental data and using non-invasive measuring techniques that allowed us to monitor many individuals over an entire breeding season. Many biologging studies are limited by the proportion of the population they can collect data from (Aarts *et al.* 2008; Soanes *et al.* 2013), generally due to cost or logistics of

instrumentation (Ropert-Coudert and Wilson 2004). This study was designed to achieve the fine-scale temporal resolution usually associated with direct biologging, while retaining a very high sample size and seasonal coverage from which to draw relationships. In earlier studies, averaging environmental variables across the known foraging area has proved a strong predictor of little penguin behaviour (Afan *et al.* 2015; Saraux *et al.* 2016). Yet this study produced mixed results. Only the relationship between sea surface temperature and foraging efficiency was consistent with previous studies (Carroll *et al.* 2016). Recent studies that simultaneously analysed satellite-derived and locally-derived environmental data have showed that satellite-derived meteorological data may not resolve environmental variability at the local scale (Ballard *et al.* 2019; Lowther *et al.* 2018). Data collected in situ with the predators under observation is clearly preferable, yet can be limited in the number of subjects that can be equipped with biologging instruments (Soanes *et al.* 2013). To determine whether satellite-derived environmental data are sufficient to understand patterns in foraging efficiency in temperate, inshore environments, we will need to compare a greater number of seasons, especially those with lower foraging efficiency and breeding success. We are still in the early phases of recording prey-specific CPUE for this population, so I recommend revisiting this analysis once there is a decade-long CPUE time-series available. In the intervening years it would be useful to validate satellite-derived observations of temperature-at-depth against observations obtained directly from foraging penguins. Although there is clearly further investigation to be conducted, this study indicates that some environmental variables can influence CPUE and pCPUE, and that the environment should be considered when using pCPUE to estimate prey populations.

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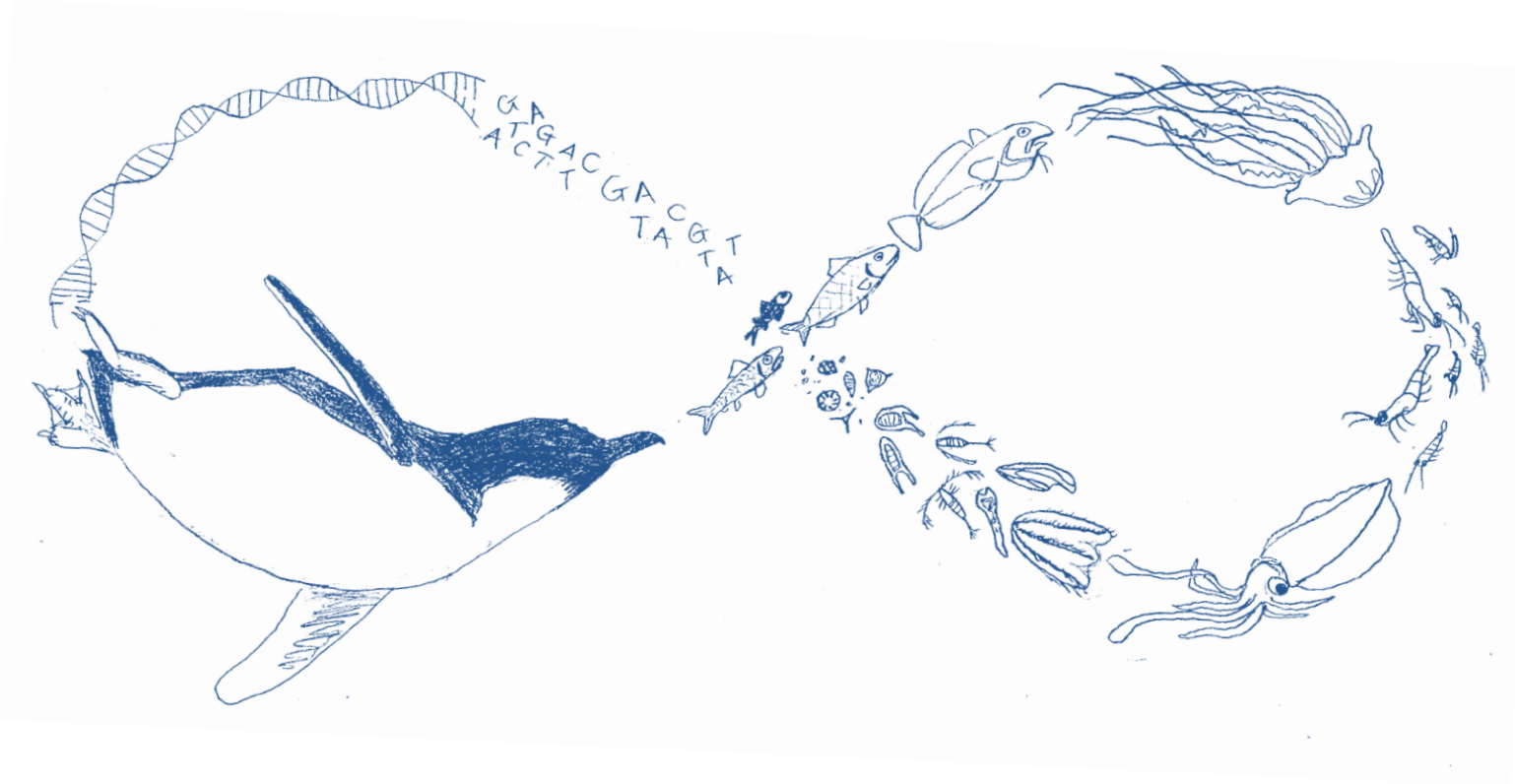
Chapter 5
General Discussion



Chapter 5
General Discussion

5.1 General findings

There are large knowledge gaps in our understanding of the trophodynamics of marine predators, particularly in reference to the effects of environmental variability (Young *et al.* 2015). These present a key barrier to the prevention of predator declines (Lewison *et al.* 2012) and management of biodiverse and productive marine environments (Heithaus *et al.* 2008; Young *et al.* 2015). The main aim of my thesis was to contribute to our understanding of the plastic foraging responses of marine predators and use these behaviours as tools for tracking variation in marine environments. I achieved this by using DNA metabarcoding as a precise new measure of diet composition and integrating this information with measures of foraging effort and success to produce estimates of prey availability and predator consumption. The key questions and findings of my thesis are presented in brief in Figure 1 and explored in the context of the literature in this discussion. My results will be useful 1) in a local context, to improve the use of little penguins as ecosystem indicators of the biologically-significant Northwest Bass Strait, 2) for the field of foraging ecology, in which knowledge of the responses of inshore foragers to environmental dynamics is still limited, and 3) for conservation, fisheries and ecosystem-based managers by providing a predator-derived index of prey availability that can be transferred for use with other accessible central-placed foragers to track food web patterns.

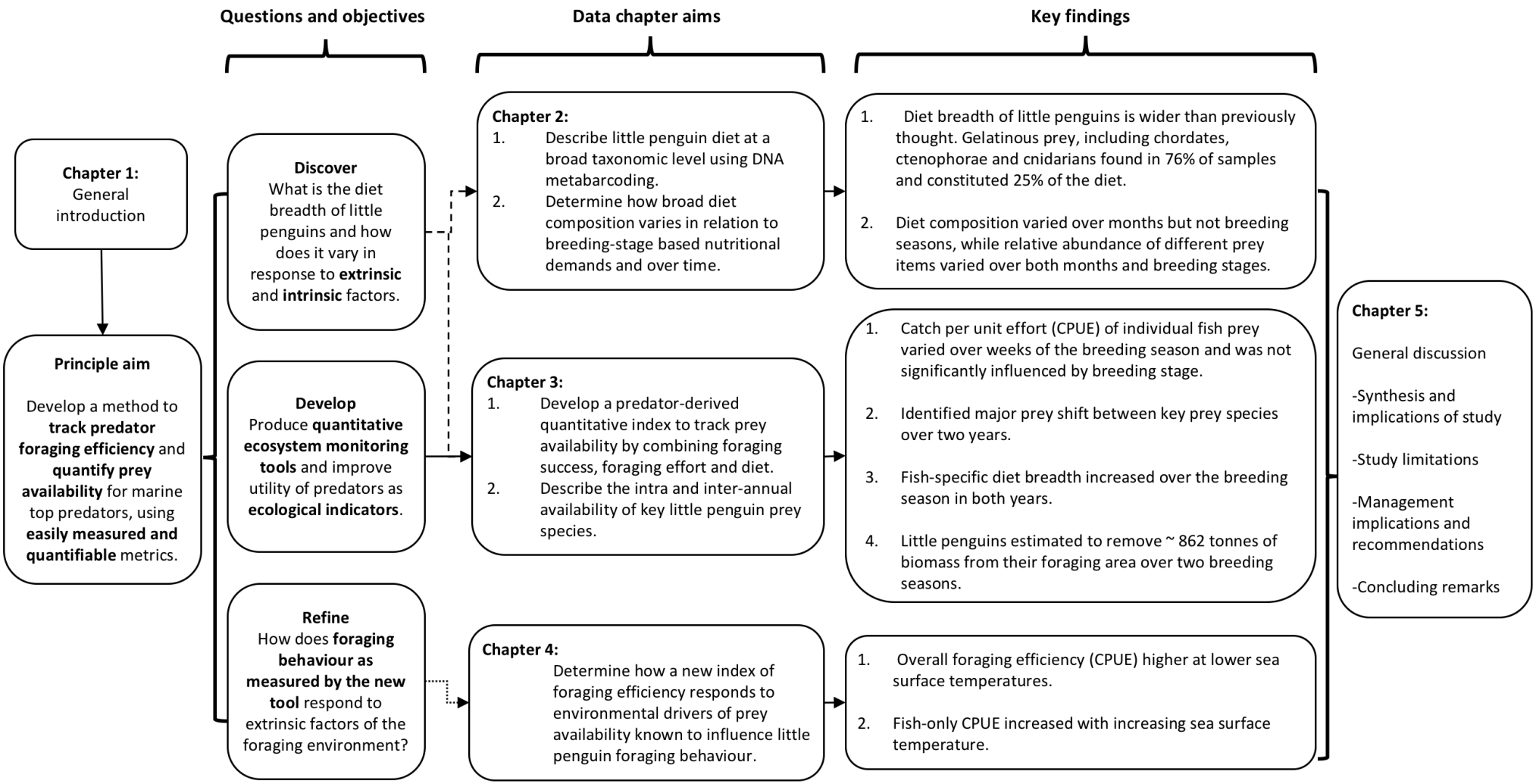


Figure 1| Schematic of the overarching aim, objectives and findings of the study.

5.2 Increased diet breadth

Using faecal DNA-metabarcoding to characterise the diet of wild little penguins, I uncovered prey groups that had not previously been described in this heavily studied seabird. DNA from gelatinous taxa from diverse phyletic lineages were found in 76% of samples and accounted for 25% of the diet in the 2015/16 breeding season, suggesting they may have an important role in the diet of little penguins (Chapter 2, Cavallo *et al.* 2018). Recent findings across diverse taxa suggest that gelatinous prey have been underestimated in the diet of marine vertebrates (Hays *et al.* 2018). Therefore, my results add to the growing body of evidence that gelatinous taxa are essential components of marine food webs that need to be considered in ecosystem management (Brodeur *et al.* 2016, Henschke *et al.* 2016). Our ability to uncover these items in the diet of marine predators is especially important now, with marine environments undergoing wide-scale change (Halpern *et al.* 2008; 2017). Gelatinous taxa are opportunistic colonists of damaged ecosystems (Richardson *et al.* 2009; Purcell 2012), so monitoring changes in their populations will be important in the near- to long-term future (Purcell 2012). Whether penguin predation on gelatinous prey is a result of environmental change or has long been a natural component of their diet is unclear due to a lack of prior records. Hence it is critical that this dietary time series is continued to determine whether the presence of gelatinous taxa in the diet is consistent, cyclical or increasing.

These findings follow recent observations of predation on gelatinous prey by penguins (Thiebot *et al.* 2017), including little penguins (Sutton *et al.* 2015), and albatross (McInnes *et al.* 2017a). However, mine is the first study to directly detect the presence of salps (phylum Chordata) in the penguin diet. Salps were the main gelatinous diet component in the penguins I studied, outweighing cnidarians and ctenophorae that were shown to be targeted by little penguins in the study by Thiebot *et al.* (2017). Considering that salps can present equal or higher nutritional and energetic rewards than Cnidarians or Ctenophorae (Percy and Fife 1981; Cardona *et al.* 2012), salps may constitute targeted prey items for little penguins. Salps may provide a direct link between primary production and predators like seabirds by concentrating phytoplankton into accessible mouthfuls (Gili *et al.* 2006), skipping intermediary trophic levels (Post 2002; Dickman *et al.* 2008). This could represent a significant increase in energy efficiency considering that approximately 90% of energy is lost from one trophic level to the next (Kerr and Dickie 2001). Discoveries of gelatinous taxa in the diets of diverse vertebrate predators have caused a paradigm shift in our understanding of these groups and their role in food webs (Hays *et al.* 2018). My results add weight to the need to further investigate the incidence of this “jellyvory” by marine vertebrates and include gelatinous trophic pathways in ecosystem modelling.

5.3 Patterns in little penguin diet

5.3.1 At the broad taxonomic scale

By sampling the diet of little penguins each week of the breeding season, I was able to identify seasonal and breeding stage-based patterns in diet composition and contribute to our understanding of how inshore predators forage. I observed increasing relative abundance of fish in the diet over months and breeding stages, underscoring the importance of fish prey for these generalists (Cavallo *et al.* 2018; Chapters 2-3). Breeding stage-based prey selection has been observed in little penguins in one study (Chiaradia *et al.* 2012) but not two others that have otherwise noted seasonal differences in diet composition (Chiaradia *et al.* 2010; 2016). Other seabirds are known to select prey specifically for offspring at different stages of development (Williams *et al.* 2008; Hipfner *et al.* 2013). Yet selecting prey to meet differential demands of different breeding stages is expected to be unlikely in little penguins due to their restricted foraging range and opportunistic diet (discussed in Chiaradia *et al.* 2016). My results raise questions on 1) the importance of reproductive timing for opportunistic generalists that forage on seasonal prey and 2) the consequences for successful reproduction if the phenology of locally adapted populations becomes mismatched from essential prey resources. It is important that future studies focus on investigating this aspect of little penguin foraging ecology, because if little penguin chicks share their parents' generalist diet, chick growth and fledging may be buffered against variable prey availability. However, if the trend identified between breeding stages in this study is the direct result of the phenological match between penguin reproduction and peak fish availability in the area, we may need to place safeguards on penguin prey availability, particularly as climate change threatens their Northwest Bass Strait coastal system (Wu *et al.* 2012) and in the event that commercial fisheries expand into the area.

The prevalence of non-fish prey in the penguins' diet in some months may tell us something about this species' opportunistic hunting strategy. The seasonal prevalence of salp in diet samples in 2015/16 matches regional salp phenology (Henschke *et al.* 2015, but in the 2016/17 season salps were all but absent from the diet (Cavallo *et al.* 2018; Chapter 2). Observations of penguin predation on jellies suggest that they are eaten on the upward phase of an otherwise unsuccessful dive (Sutton *et al.* 2015) so their absence in the second year could indicate a general absence of salps in the foraging area in 2016/17 or an abundance of other, more preferred prey. In general, jellies can be digested much faster than other prey (Arai *et al.* 2003), so if penguins are able to consume jellies when fish prey are less available, this may buffer them when preferred prey are scarce. However, salps are prone to boom and bust population dynamics (Henschke *et al.* 2014; 2015), can displace

other taxa from the water column and are known to outcompete fish of the size little penguins feed on (Purcell and Arai 2001; Pakhomov *et al.* 2002; Henschke *et al.* 2016). Understanding how, when and why penguins and other inshore foragers feed on salps, and the consequences for breeding and survival should be a key focus of upcoming monitoring.

5.3.2 Patterns in the availability of key little penguin prey fish species

At the species level, using penguin-derived catch per unit effort (pCPUE) index, I revealed a clear seasonal progression in each year from a diet predominated by a single fish species to a diet spread across a range of fish, regardless of the specific species in the diet (Chapter 3). Overall CPUE values were highest when penguins fed mostly on a single fish species, which was coincidental with a greater proportion of non-fish prey. In both years, the main fish prey early in the season was a schooling species; either sardine *Sardinops sagax,* (2015/16) or red cod *Pseudophycis bachus* (2016/17). High overall CPUE and fish-specific pCPUE values at this time may indicate high capture rates for predators foraging on schooling fish, or just indicate high abundance of these species at that time. African penguins achieve higher CPUE values when foraging on schooling prey, particularly when group foraging (McInnes *et al.* 2017b). Little penguins also achieve higher capture rates when feeding on schooling prey, but do not appear to coordinate to hunt prey, nor achieve higher capture success by foraging together (Sutton *et al.* 2015). Later in each season penguins foraged on both schooling fish (e.g. barracouta *Thyrsites atun*, jack mackerel *Trachurus declivus*) and demersal fish (e.g. bluespotted goatfish *Upeneichthys vlamingii,* bluefin leatherjacket *Thamnoconus degeni*), although they may have fed on pelagic larval stages of demersal prey (Kailola *et al.* 1993). Current little penguin breeding phenology in this region is strongly tied to regional patterns of marine productivity (Afan *et al.* 2015) and my results (Chapters 2-4) add weight to earlier suggestions (Chiaradia *et al.* 2016) that this may be in order to time late stage chick growth with peak fish prey availability, when many fish species come to the area to spawn. The monthly patterns in which different fish species peak in the diet of little penguins in this study closely match the regional schooling and spawning phenology of these species (Blackburn 1950; 1957; Bruce *et al.* 2001; Kemp *et al.* 2012). This reinforces that penguins exploit nutrient-rich breeding adults of some small pelagic species together with post-larval individuals of larger species (red cod, barracouta, jack mackerel etc.) (Cullen *et al.* 1992)

The spring phenology of key marine trophic groups is shifting earlier because of ocean warming, with the peaks in productivity of zooplankton, fish eggs and larvae and bony fish communities shifting faster than those of phytoplankton and seabirds (Poloczanska *et al.*

2016). If the peak in prey abundance and marine productivity becomes uncoupled with penguin phenology it could threaten breeding success and therefore population recruitment. However, the onset of breeding in little penguins is highly plastic and early onset appears to result in extended, rather than temporally-shifted, seasons and higher breeding success at this and other little penguin colonies (Reilly and Cullen 1981; Perriman and Steen 2000; Knight and Rogers 2004; Chambers 2004; Robinson *et al.* 2005; Cullen *et al.* 2009). Further, I observed a wide variety of demersal prey in the diet of little penguins, prey which are expected to be less variable in a seasonal context and in response to environmental variables (Cairns 1987; Litzow and Piatt 2003). So if penguins become mismatched from key, highly variable prey resources such as a spawning influx of pelagic species to the foraging zone, they may be able to sustain breeding and survival on the larvae and juveniles of demersal species. Little penguins are known to hunt in demersal environments (Ropert-Coudert *et al.* 2006), but previous stomach contents analysis suggested that predation on demersal prey at this site is largely on post-larval individuals (Cullen *et al.* 1992).

5.4 Responses to change in a temperate coastal system

Seabirds are well adapted to endure variability in their foraging environment (Weimerskirch 2001), but directional physical changes may test the extent of their plasticity (Grémillet and Boulinier 2009). Differing foraging strategies and diets can have diverse consequences in response to variables and may determine species resilience to climate change (Kitaysky and Golubova 2000). Consequently, understanding how the foraging efficiency of these predators relates to the physical variables of their foraging environment is key to predicting and managing potential effects on populations. In this study, when sea surface temperature was < 17.5°C little penguins gained more mass for effort expended (CPUE) regardless of diet composition (Chapter 4), but caught a higher proportion of fish as sea surface temperature rose between 12°C and 20°C. Although it is difficult to extricate the potentially confounding effects of sea surface temperature and time in this dataset, these results may shed light on the seasonal foraging opportunities available to inshore predators, and on penguin foraging opportunities. The majority of gelatinous and crustaceous prey were preyed upon at lower sea surface temperatures, generally associated with the earlier months of the breeding season, penguins.1 Individually, these non-fish prey items are likely to present lower nutritional value to penguins than fish, but can exist in the water column at very high abundances and are digested rapidly (Arai *et al.* 2003), providing a reasonable trade-off for quality (Mianzan *et al.* 2001; Henschke *et al.* 2014). Such strategies are common among generalists (e.g. Mianzan *et al.* 2001; Grémillet *et al.* 2008) but do not always result in favourable outcomes (Wanless *et al.* 2005). I have two alternate hypotheses

for the raised foraging success and wider diet breadth at low sea surface temperatures. First, predation across a wide range of gelatinous and crustaceous zooplankton early in the penguin breeding season and then fish later may indicate a high abundance of these groups as productivity is propagated up trophic levels (Vinogradov 1981), because cooler temperatures in this system co-occur with local peak of primary productivity (Afan *et al.* 2015). This bottom-up transition, wherein peaks in the productivity of subsequent trophic levels lag behind the peak in primary productivity has been observed in multiple systems (e.g. Vinogradov 1981; Croll *et al.* 2005). Alternatively, high foraging success and broad diet at lower temperatures may be a response to the low intrinsic demands on penguins during incubation (Gales and Green 1990; Ydenberg 1994). Penguins that need to sustain only themselves, albeit over 1-7 day fasts at the burrow (Numata *et al.* 2000; Kato *et al.* 2008), may forage with a focus on quantity rather than quality. This would agree with the increasing predation on fish later in the season (at higher temperatures) discovered in chapters two and four. Many more years of differing seasons will be needed to disentangle the effects of temperature, productivity and breeding stage on foraging efficiency. Again, this is a question of intrinsic vs extrinsic drivers of predator foraging success that should be investigated prior to assessing the sensitivity of different seabird species to directional environmental change.

5.5 Estimating biomass extraction by top and meso predators

The estimation of consumption and prey biomass is important information for mass-balanced food web models used by resource managers (Christensen *et al.* 2009; Walters *et al.* 1997), such as the Ecopath model in place for Northwest Bass Strait (Bulman *et al.* 2012). I estimated that this population of around 31,000 breeding little penguins (Sutherland and Dann 2014) consumed about 620 and 680 tonnes of prey from their foraging range during the 2015/16 and 2016/17 breeding seasons, respectively. Although coarse estimates, these values are within the same order of magnitude as those for breeding little penguins elsewhere, estimated using isotope turnover techniques to determine metabolic rate and energy requirements (Gales and Green 1990). It is important to understand the influence large populations of predators exert within their foraging range, especially when foraging is concentrated close to shore during breeding attempts (Ashmole 1963; Birt *et al.* 1987; Oppel *et al.* 2015). This population of little penguins is in a stage of growth, recovering from population decline caused by habitat destruction, predation by domestic and feral predators, roadkill and a massive mortality of its major prey, sardine (Dann 1992; Chiaradia *et al.* 2010; Sutherland and Dann 2014). It also co-exists with other ecologically important central-placed foragers at a local and regional scale (Chiaradia *et al.* 2002; Bulman *et al.* 2012; Berlincourt and Arnould 2015; McIntosh *et al.* 2018). The capacity to estimate biomass extracted from

the system by each of these top predators is important for ecosystem-based management of resources in this changing environment. Hence, estimates resulting from this study and from ongoing penguin-derived pCPUE time series will be used to inform Phillip Island Nature Parks' existing ecosystem model of the penguins' foraging area, the Northwest Bass Strait Ecopath with Ecosim (EwE) model (Bulman *et al.* 2012). This mass-balanced model is used to predict changes in biomass and trophic interactions in time and space (Fulton 2011; Christensen *et al.* 2009; Walters *et al.* 1997). The reliability of mass-balanced models is contingent on the quality of biomass and consumption data within its diet matrix (Essington 2007), and so these new estimates are significant for the future management of this system. Importantly, as well as deriving new estimates of consumption by little penguins, I was also able to add an additional trophic link between penguins and gelatinous taxa, which were not considered part of the penguin food web, and thus not part of current model simulations by the previous iteration of the Northwest Bass Strait Ecopath model (Bulman *et al.* 2012).

In the 2015/16 breeding season, penguins took about 220 tonnes of sardine from their foraging area. This equates to approximately a quarter of the sardine catch by the entire Victorian fishery in over that year (VFA 2018). Inquiries to both the Victorian Fisheries Authority (VFA) and the Australian Fisheries Management Authority (AFMA) revealed that there were no local fishing data to compare with my consumption estimates over the years studied, either from within or from the area surrounding the penguins' foraging area. This information would have provided a useful point of comparison for the trends observed. For example, in 2016, little penguins only extracted 40 tonnes of sardine from their foraging area, approximately 20% of the quantity the year before. Comparison with local CPUE data from fisheries active within the area would be useful for determining whether this decline was indicative of a decline in the abundance of sardines, or just their availability to little penguins. In the event that the state or Commonwealth fisheries increase effort in the area surrounding this important colony, prey species-specific consumption and biomass estimates will be useful for indicating the colony's needs, and provide decision support for ecosystem-based fisheries management. More will be understood about the needs of this thriving, recovering colony once the penguin-derived pCPUE dataset contains several years of poor, average and high breeding success.

5.6 Limitations

5.6.1 Limitations of experimental design

With two years of data from breeding seasons with above average breeding success, and where diet clearly differed, I was in a good position to investigate what the pCPUE of

different prey species meant for reproductive measures such as chick growth and fledging weights. I visited ~100 nests up to three times weekly throughout each breeding season to produce growth curves for individual chicks. However, I was unable to analyse enough repeated scat samples from individual burrows to determine the consequences of specific diets. This is due to sample attrition at multiple steps of the collection and metabarcoding process. The first point represents a trade-off between reducing the invasiveness of sampling and the likelihood of successful sample collection. Although I placed scat collection plates in all active nests within the study site across both seasons, whether a scat was deposited before collection the following day relied on penguins returning to their burrow that night, defecating on the cardboard and then not interfering with the sample. Due to the loose sand and soil substrate of both sites and the digging activity of the birds, many samples were contaminated and not usable. Laboratory analysis introduced a second filtering point where sample size was reduced. Funding constraints meant that not all of the samples collected in the field could be analysed, and a randomisation method was used to select the samples retained for analysis and consequently reduced my capacity to investigate individual-level effects. Finally, as is common in next-generation sequencing, many samples did not amplify sufficient DNA for sequencing. Thus, I was not able to perform any individual or burrow (family unit) level analysis within this study. Fortunately, preliminary analysis suggested that individuals were highly opportunistic and did not show consistent dietary choices, so I was able to deal with burrow ID as a random factor in subsequent analyses in the chapters of my thesis. However, I was unable to investigate the reproductive consequences of foraging on different prey groups due to the lack of strong temporal match between my diet, weighbridge and growth datasets. An investigation of the reproductive consequences of feeding on specific taxa such as jellies versus fish may require a different experimental design, perhaps focusing on fewer individuals and using a more active scat collection technique, such as swabbing the cloaca to induce defecation.

In Chapter 4, I intended to investigate the CPUE responses of key fish prey, as well as fish, jellies and crustacea in general, to environmental variables. From this, I may have been able to infer the potential for rising sea temperatures to affect penguins foraging on specific prey species and on high quality vs lower quality prey. However, due to extreme zero-inflation and overdispersion in the responses of each fish species, jellies and crustaceans I was unable to appropriately analyse this question. Zero-inflation and overdispersion are common in DNA library data (Xu *et al.* 2015), and presented many points of difficulty within my research. Unfortunately, in the case of Chapter 4 I was unable to resolve the issues within the data and so was only able to provide inferences on penguins' total foraging efficiency and their foraging efficiency when feeding on fish in general. I think that this is an important

line of investigation because coastal food webs are underrepresented in terms of research on functional ecological responses to environmental variables and these systems are disproportionately affected by human impacts compared to pelagic environments (Halpern *et al.* 2008).

5.6.2 Limitations of the method

As with all methods of diet analysis, DNA faecal-metabarcoding is not without inherent limitations (Zinger *et al.* 2019) and it is important to consider these in the light of our interest in quantitative estimates. Firstly, amplification via the polymerase chain reaction is a competitive process, and sequences from some taxonomic groups may amplify more efficiently than others (Deagle *et al.* 2014). Other potential sources of error include different rates of assimilation by penguins of different prey items and variable gene copy numbers between cells, tissue types and species that can introduce biases into relative read abundance (Pompanon *et al.* 2012). Importantly, both of these sources of error remain constant over time and correction factors can be developed to minimise their effects (Thomas *et al.* 2014).

Using the current metabarcoding technique, it is not yet possible to distinguish direct predation from secondary predation, that is, taxa inadvertently ingested within the gut of targeted prey (Pompanon *et al.* 2012). This obviously presents a limiting issue where the discovery of new diet items is concerned. A reasonable strategy is to make inferences based on the prevalence of a taxon in individual samples and across the dataset and look for co-occurrence with other prey species (e.g. McInnes *et al.* 2017a). Fish DNA was present in all samples collected during this study, making it impossible to rule out secondary predation as a source of sequences from other taxa. Instead, I investigated the prevalence of sequences from newly described prey species in individual samples. Salps dominated samples in 10% of the dataset, second only to fish in terms of relative abundance. In contrast, calanoid and harpacticoid copepods, oikopleurid chordates and malacostracans (decapod crustaceans and their relatives) were the dominant taxon in less than 1.5 % of samples and cephalopods, scyphozoans and other taxa little penguins prey upon never dominated samples. A strong secondary predation signal may result in underestimation of the importance of fish in the diet. For example, between September and November in 2016, calanoid copepods represented a high proportion of sequences in the diet, and in January 2017 oikopleurid chordates represented a high proportion of sequences. In these months, fish sequences were constrained to between 50 and 62% of sequences in the diet, compared with the 57 to 93% in months with low copepod and oikopleurid read abundance. Given their small size, it

is unlikely that penguins targeted these invertebrate taxa directly. Therefore, predation upon fish may have been underestimated during these times and consequently, species-specific estimates of fish biomass extracted by little penguins may also be underestimated, because the seasonal mean of fish in the diet was used in each year in the calculations. Confirming primary and secondary predation in this food web may require simultaneous video-logging and scat metabarcoding, or alternatively, simultaneously analysing the stomach contents of key little penguin prey, as has been done with Atlantic puffin *Fratercula arctica* and their Atlantic herring *Clupea heranga* prey (Bowser *et al.* 2013). However, regardless of whether taxa in the diet were targeted directly or ingested secondarily, they constitute a source of nutrition to little penguins. If secondary predation is revealed then diet monitoring of little penguins may present an avenue to monitor the food web across three trophic levels, rather than two.

DNA metabarcoding relies on the existence of reference sequences in an accessible database. In this project, most of the key prey species of little penguins were represented within an accessible library (NCBI GenBank: Johnson *et al.* 2008), likely because many of them are important commercially. However, there were many incidences of un-named weedfish *Heteroclinus* spp., leatherjacket (Monacanthidae spp.) and other reef-fish sequences that could not be resolved to a local species. Inability to resolve these to the species level should not present a problem in terms of understanding of penguin nutrition, since sequences could mostly be resolved to the genus level and nutritional content of different fish species should be comparable within their genera, and may actually be more variable over seasons and within cohorts than between species (Wanless *et al.* 2005). However, if we are to use little penguins as monitors of the species in the area, we will need to increase reference library representation of sequences of local fish fauna and of species whose distribution may move southward with the extension of the East Australian Current. Because the dataset from this study is stored digitally as sequences it would be simple to compare the sequences afresh once the library has been updated. The low representation of southeast Australian demersal fish in these databases may also indicates gaps in the knowledge of fish communities in this threatened marine biodiversity hotspot (Ramírez *et al.* 2017).

5.7 Management implications and suggestions

Coastal and shelf ecosystems are subject to high and increasing cumulative anthropogenic impacts (Halpern *et al.* 2015) and are a key priority for marine conservation and management (Selig *et al.* 2014). Therefore, a key aim of this thesis was to enhance our

capacity to monitor change in these ecosystems. This was achieved through the development of an enhanced penguin-derived CPUE method to track prey availability of predators and shed light on their trophodynamics. I sought to distil complex foraging behaviour into an index that could be interpreted by managers, policy makers, fishers and other stakeholders, to assist with resource management. The resulting prey-specific pCPUE method can be adapted for a variety of inshore central-place foraging seabirds to provide information on the general and region-specific trophodynamics of shelf and coastal ecosystems. Importantly, instrumentation and measures of foraging success, effort and diet can be adapted to suit the behaviour and biology of the predator in question. For flying species, or those that cannot be funnelled through monitoring stations like weighbridges, natural or artificial nesting shelves or platforms can be instrumented with individual weighbridges and passive integrated transponder readers or associated cameras for determining individual identity from sexually dimorphic features or coloured tags. Nest-based weighing platforms have been developed for seabirds ranging from tiny prions to albatross, nesting on a wide range of substrates and even in burrows (Reid *et al.* 1999; Ochi *et al.* 2016; Sugishita *et al.* 2017; Prince and Walton 1984). The metabarcoding approach can be developed to capture the diet of other consumers, whether they specialise on a single group or are generalists (Pompanon *et al.* 2012).

In some cases, such as for species that carry prey to the nest in their bill, cameras can be used to capture data on diet with a similar resolution (Gaglio *et al.* 2018), though this approach limits the information to provisioning data only, not adult self-foraging. The large quantity of detailed information that can be collected by automated weighbridge technology and non-invasive scat metabarcoding, without disturbance to the species or system (Chiaradia and Kerry 1999; McInnes *et al.* 2016; Gaglio *et al.* 2018), provides a clear advantage over more invasive or intensive methods that restrict sampling effort (Deagle *et al.* 2010; Soanes *et al.* 2013). Monitoring pCPUE at a weekly or monthly scale will allow conservation and resource managers to identify seasonal trophic patterns to better plan management actions for the coming year. The decreasing costs of small-scale next-generation sequencing equipment mean that managers may even be able to adapt their plan within a season, making informed decisions at trigger points in response to predator-derived CPUE information.

Populations of marine fish species are variously monitored based upon their economic importance and most species receive no monitoring at all (Hutchings and Baum 2005). The capacity to produce predator-derived estimates of prey-specific CPUE and consumption reduces reliance on fisheries data and fills a key data gap in areas where commercial

fisheries are either not active or do not report catches (Montevecchi 1993). This is particularly pertinent for the monitoring of marine reserves and inshore areas, from which fishing and research activities may be absent and data on lower trophic levels limited (Barrett *et al.* 1990; Montevecchi and Berruti 1991) compared to pelagic environments, which have so far received more dedicated research effort (Hunsicker *et al.* 2016). The prey-specific CPUE also provides a reasonable method to track the abundance of “age-0” pre-recruitment fish, an age-class generally missing from fisheries stock assessments (Diamond and Devlin 2003). Multiple researchers have pointed to the potential for seabirds to fill data gaps in fisheries and thus enhance stock and ecosystem management (Cairns 1992; Velarde *et al.* 2013). Inshore foraging penguins, terns, auks and other seabirds could predict future catch rates by fisheries and provide an early warning system when cohorts can be deemed to have failed or suffered very low recruitment. Further, given the high level of agreement between the timing of spawning recorded in historical spawning surveys and diet composition in our dataset, future CPUE time series could be used to indicate changes in spawning phenology, an expected and observed consequence of climate change (Poloczanska *et al.* 2016).

Another scenario in which such consumption estimates could be useful is within colony restoration plans for threatened central-placed foragers. Conservationists have attempted to restore locally extinct colonies of species such as Atlantic Puffin *Fratercula arctica* and Short-tailed Albatross *Phoebastria albatrus* using call playback and translocation, but such programs often fail, with varying success (Jones and Kress 2012). One aspect that may be important for recolonisation success is prey availability in the foraging area available to the colony and to competitors (Ewen and Armstrong 2007; Destro *et al.* 2018). Per capita consumption by the species in question could be determined by calculating CPUE and estimating consumption by established colonies and then compared with estimates of prey availability at the new site to determine whether the needs of the new colony can be met. Assessments of consumption by sympatric central-placed foragers could indicate potential competition between these established and new colonies and determine the appropriateness of the site choice, before any management actions are undertaken.

It is important to note that this method has been developed for use with central-placed foragers that are relatively predictable within time and space and that visit the colony frequently enough for weighbridge-based measures of success and effort to provide reasonable information on foraging trips. The pCPUE will not provide useful data for species that frequently spend more than a week at sea while provisioning offspring, such as procellariiformes, frigatebirds and offshore-foraging penguins (Schreiber and Burger 2001).

The method is best-suited to seabirds that visit the nest frequently, such as inshore-foraging penguins, terns, gulls, boobies, cormorants and alcids (Schreiber and Burger 2001). When appropriating this method for another species, ensure that enough is understood about the foraging behaviour, including dietary specialization, of that species to minimize the introduction of unknown bias into the index. If the potential biases are well understood, these can be taken into consideration when interpreting pCPUE results. For example, species that return to the colony to provision chicks multiple times throughout the day, and that do not spend time 'loafing' at sea will provide a more accurate measure of foraging effort than little penguins, which are restricted to returning at night, regardless of when they finish foraging. But, such species may introduce a greater bias when matching scat samples analysis to single foraging trips if dietary DNA persists in the gut for longer than a day, as it does in little penguins (Deagle *et al*. 2010).

5.8 Concluding remarks

In this Anthropocene era of change, we would be better prepared to avoid predator extinctions and ecosystem collapse if we have a strong understanding of how species fit together in food-webs and respond to environmental variation. By combining predator-derived measures of foraging success, effort and diet, I used the behavioural plasticity that has so far constrained the use of seabirds as indicators of food resources. I uncovered new items in the diet of little penguins, expanding their known foraging niche to include a variety of gelatinous taxa. I determined that the relative abundance of different items in the diet change over months and breeding stages, underscoring the importance of fish-availability to these generalist predators. I developed a method to obtain predator-derived measures of catch per unit effort and prey consumption, providing an avenue to track prey availability and monitor change in food webs. Importantly, this method extends to species not subject to commercial stock assessments and the outputs can inform mass-balanced trophic models for ecosystem-based management. By contributing to the field's understanding of inshore central place foragers and food-webs, and developing new tools to track ecosystem change and biomass extraction I think I have enhanced the capacity of scientists and managers to track, understand and respond to change in these environments. Locally, these results will set the base-line in a long-term study of the biodiversity in the little penguin diet, allowing us to identify changing patterns in important groups such as commercially targeted species, endangered species and invasive species, and groups that may be exerting increasing ecological influence in temperate coastal food-webs. Limitations in sample collection and inherent in DNA-metabarcoding meant that I was unable to investigate the consequences of diet and CPUE for chick growth and adult mass, or the specific responses of key prey items

to environmental variability. I recommend these areas as priorities for future research, particularly where new diet components have been recognised. Further, I encourage the prioritisation of defining trophic links between gelatinous taxa and other seabirds and predators to fill key knowledge gaps in marine energy pathways and better inform trophic models that are used within ecosystem-based management. Developments in commercial DNA sequencing over the next decade should address the needs of ecologists and managers so that other existing and sustainable monitoring programs can incorporate these tools to collect information for ecosystem-based management in terms of trigger points and ecosystem steady-state models. Finally, I recommend that the diet-informed pCPUE method I have described be validated against concurrent prey stock assessment. There is limited fishing activity within the foraging area of little penguins at Phillip Island, and data requests made to both state and commonwealth fisheries authorities showed that there was no relevant data for the years studied. I recommend collecting pCPUE data from an area with a high level of fishing activity, where data can be obtained for comparison. Alternatively, and at greater cost, the study could be repeated at Phillip Island, or a similar population, concurrent with at-sea measures of local fish abundance.

Although my thesis has highlighted areas for further investigation, the method I devised has demonstrated the potential to investigate them and enhance our capacity to employ seabirds and other central-place foragers as indicators of marine resources.

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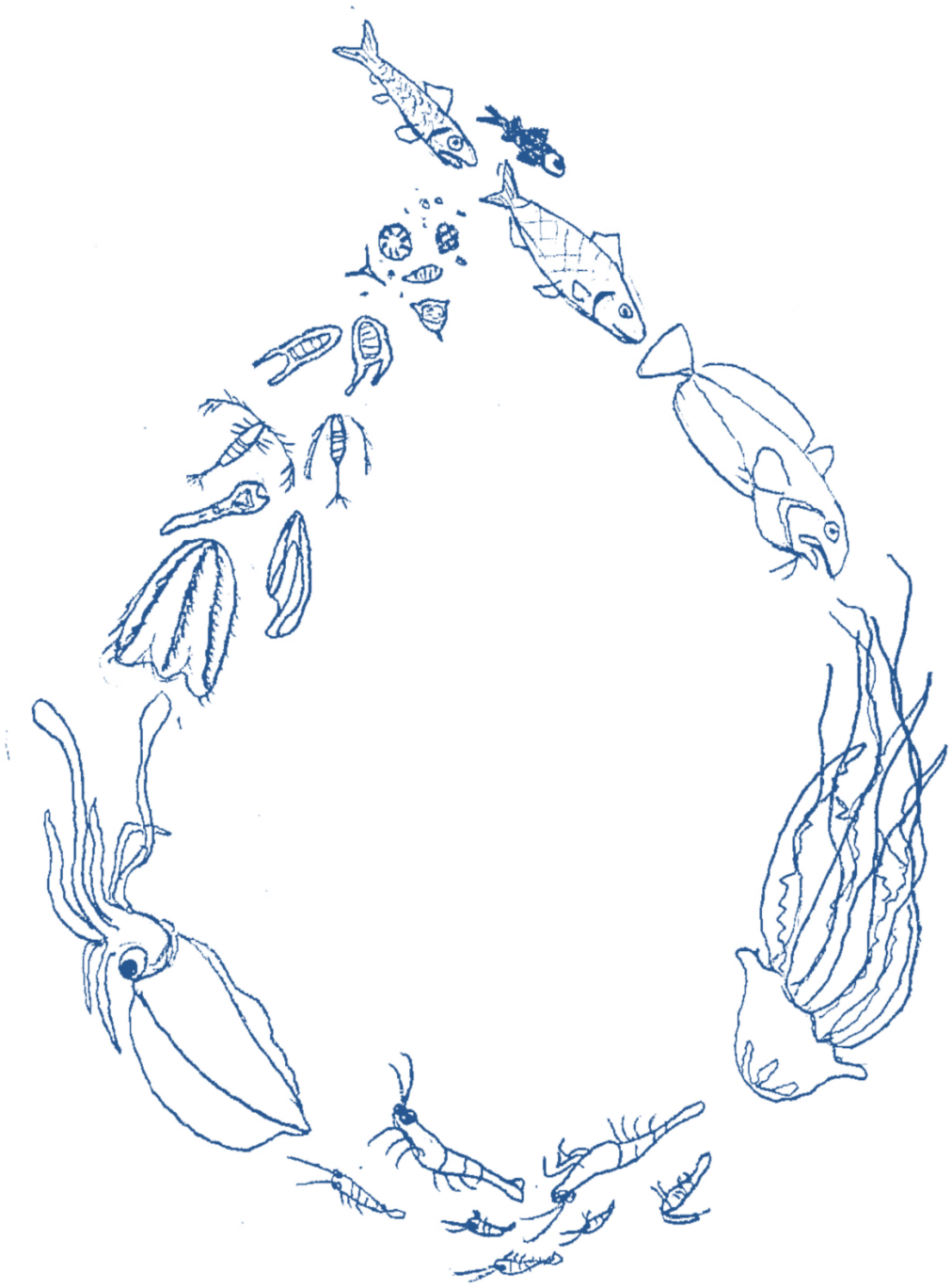
Appendices



Appendix A: Cavallo et al. (2018) Frontiers in Marine Science publication PDF

frontiers in Marine Science

ORIGINAL RESEARCH
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Molecular Analysis of Predator Scats Reveals Role of Salps in Temperate Inshore Food Webs

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High precision, high coverage DNA-based diet analysis tools allow great insight into the food web interactions of cryptic taxa. We used DNA fecal-metabarcoding to look for unrecorded taxa within the diet of a generalist central-placed predator, the little penguin *Eudyptula minor*. We examined 208 scats from 106 breeding pairs throughout August–February in a large colony at Phillip Island, Australia. While we confirmed a largely piscivorous diet, we also recovered DNA sequences from gelatinous and crustaceous plankton groups that have not previously been detected in the little penguin diet using other diet analysis methods. Gelatinous plankton, including salps, appendicularians, scyphozoans, and hydrozoans were present in 76% of samples and represented 25% of all sequences. DNA recovered from minute copepods and appendicularians may indicate links between trophic levels through secondary predation. Percentage frequency of occurrence (%FOO) demonstrated that little penguin diet composition changed over months and stages (incubation, guard, and post-guard) of the breeding season (month: $\chi^2 = 201.91$, df = NA, $p < 0.01$; stage: $\chi^2 = 33.221$, df = NA, $p = 0.015$). Relative read abundance (RRA) uncovered variations in the relative abundance of taxa in the diet over months and stages (month: $F = 53.18$, df = 59, $p < 0.001$; stage: $F = 66.56$, df = 29, $p < 0.001$). The diet became progressively fish-focused over months of the season and stages, while salps were only present in 4 out of 6 months, with a peak in September. Based on their prevalence in this dataset, in this year of very high breeding success (2.15 chicks per pair), salps may constitute a food source for this largely piscivorous generalist. Our work highlights how DNA metabarcoding can improve our understanding of the trophic role of gelatinous plankton and other cryptic taxa.

Keywords: next-generation sequencing, foraging ecology, DNA barcoding, eDNA, jellyfish

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INTRODUCTION

Diet analyses form the foundation of many important community and species-specific time series used to inform models and plans for conservation and management. However, many diet analysis techniques are limited in their coverage and accuracy (Phillips et al., 2003; Braley et al., 2010), in some cases excluding whole taxonomic clades (Cardona et al., 2012). New techniques now enable

us to fill in the gaps and update information being used to make important decisions about species and ecosystem management.

Gelatinous plankton form an abundant and vast group that has often evaded detection by past diet analysis methods. This polyphyletic group encompasses the cnidarian sea-jellies, the salps, appendicularians and other tunicates and the ctenophores, all of which we will hereafter refer to as "jellies." Our understanding of the contribution of jellies to food webs has been obstructed by the difficulty in recovering and identifying gelatinous material from predator diet samples, because the soft tissue is digested rapidly into an amorphous gel (Mianzan et al., 1996; Arai et al., 2003). Stable isotope and fatty acid analysis have uncovered specialist and opportunistic jelly predators (Wanless et al., 2005), challenging assumptions based on observed feeding (Syvaranta et al., 2012; Couturier et al., 2013) and demonstrating that scyphozoans can make up as much as ∼90% of the diet of some fish species that use them as shelter (D'ambra et al., 2015). However, classification of gelatinous dietary items has been limited because fatty acid and isotopic signatures can vary within species, and may overlap among species in a region (Iverson et al., 2002).

Molecular technologies that identify prey DNA in the gut, feces and pellets of consumers are revolutionizing our understanding of organisms' diets and food web interactions (Gerwing et al., 2016; Hardy et al., 2017; Komura et al., 2018), and providing new ways to capture biodiversity (Boyer et al., 2015). It is now possible to sample an animal's entire diet using DNA metabarcoding, which targets regions of DNA that are highly conserved within a target group and that offer enough variability to allow distinction between taxonomic groups (Cristescu, 2014). Furthermore, technological advances in next-generation sequencing allow 100s of diet samples to be processed and analyzed rapidly (Metzker, 2010) maximizing capacity for high temporal resolution, particularly when compared to visual analysis of prey remains in stomach, gut or fecal contents (Taberlet et al., 2018). Metabarcoding is now being used across marine and terrestrial environments to investigate in fine detail a wide range of ecological, community, and conservation questions (Kartzinel et al., 2015; Thomsen and Willerslev, 2015; Thomas et al., 2016), and holds great promise for the description and understanding of predation on gelatinous taxa.

Despite their low energy density (Doyle et al., 2007; Cardona et al., 2012), true sea-jellies (Class: Scyphozoa), hydrozoans or salps are included in the diets of many marine and coastal birds and some mammals (Childerhouse et al., 2001; Arai, 2005; Cardona et al., 2012; Phillips et al., 2017). These endotherms generally show wide diet composition concordant with a generalist foraging strategy, including jellies in small amounts, seasonally, in accordance with local abundance (Harrison, 1984; Jarman et al., 2013). However, a recent DNA metabarcoding study demonstrated that the diets of two albatrosses, *Thalassarche melanophris* and *Thalassarche impavida*, included a considerable gelatinous component (McInnes et al., 2017b). Recent video footage shows penguins exploiting Scyphozoa as both a source of fish prey and an occasional prey item (Sutton et al., 2015; Thiebot et al., 2016) and metabarcoding of Adélie penguin *Pygoscelis adeliae* scats also reveals scyphozoan prey (McInnes et al., 2016). Whether or not they are important prey for these groups, jellies can constitute a large proportion of biomass in some areas (Lynam et al., 2006; Henschke et al., 2016) and many exhibit bloom and bust cycles that significantly affect trophic interactions (Pitt et al., 2014; Smith et al., 2016). Jellies are resilient across a range of physical conditions (Mills, 2001; Purcell, 2012), and some gelatinous groups readily invade ecosystems and favor environments characterized by anthropogenic disturbance (Richardson et al., 2009; Purcell, 2012). They are capable of displacing fish and other taxa in the water column through competition and predation (Purcell and Arai, 2001; Pakhomov et al., 2002; Henschke et al., 2016). Together, these observations indicate that gelatinous taxa warrant greater monitoring and further consideration in ecosystem management planning (Brodeur et al., 2016; Aubert et al., 2018).

Little penguins, *Eudyptula minor*, are generalist predators that prey mainly on small fish but also crustaceans and squid (Cullen et al., 1992; Chiaradia et al., 2010, 2014; Kowalczyk et al., 2015). Their generalist diet makes them useful for capturing information about the wider food web (Boyer et al., 2015). However, during the breeding season they are central place foragers whose foraging range is constrained to differing degrees by the specific demands of each breeding stage, potentially affecting the types of prey they are able to exploit (Preston et al., 2008; Chiaradia et al., 2016; Poupart et al., 2017). Breeding chronology is not synchronous across a colony, with some birds incubating eggs while others have chicks that are close to fledging. Depending on the availability and abundance of prey, pairs can raise one to three clutches in a single season (Chiaradia and Kerry, 1999).

Little penguin reproductive output is thought to be determined by the availability and abundance of fish prey of a certain size (Chiaradia et al., 2003; Kowalczyk et al., 2014), but almost all that is known about the diet of this species comes from analysis of stomach contents that overlooks soft prey, and stable isotope analysis that does not provide accurate species identification (Chiaradia et al., 2016). Hence important prey items may have been under-represented in previous studies. We use DNA metabarcoding of little penguin scats to refine the diet composition of little penguins so we can better understand their inshore food web.

MATERIALS AND METHODS

Experimental Design

We combined metabarcoding scat diet analysis with field records of little penguin breeding stages. To determine diet composition, we collected scats from little penguin nests on Phillip Island, Victoria, Australia, over the 7-month breeding season in the Austral summer 2015/16. We used a universal primer (18S_SSU rDNA, McInnes et al., 2017a) capable of identifying multicellular animals to class and sometimes order or family level. We compared little penguin diet between months of the breeding season, and between breeding stages, to investigate extrinsic and intrinsic drivers of diet.

Study Sites, Species, and Collection of Breeding Stage Data

Little penguins were monitored at two sites located ∼2 km apart within the Phillip Island colony in south eastern Australia (38°31′ S, 145°07′ E). The two study sites were "Penguin Parade," where monitored penguins breed in artificial wooden nest boxes, and "Radio-tracking Bay," where penguins breed in natural burrows. There is a high degree of spatial foraging segregation between these sites (Sánchez et al., in press). Around 100 nests at each site were monitored to record the presence of adults, eggs or chicks; three times a week at Penguin Parade and once a week at Radio-tracking Bay due to the differing accessibility of the sites. Little penguins have three discrete breeding stages, which significantly influence their foraging range and effort. These include "incubation," in which adults take turns incubating their eggs for 2–7 days while the partner forages at sea (Numata et al., 2000; Kato et al., 2008); "guard," where small chicks are guarded by one parent while the other forages at sea, swapping roughly daily (Chiaradia and Kerry, 1999), and "post-guard," where both adults forage to provision their large chicks, which are left unattended in the burrow (Saraux et al., 2011). Foraging trips during post-guard can range from between one to two days when food is abundant and 2–5 days when food is scarce (Chiaradia and Nisbet, 2006). Therefore foraging range is most limited during the guard stage.

Collection of Scat Samples

Each week, we aimed to sample scats from all nests with eggs or chicks at each site. Cardboard was placed on the floor of the entrance of nests, taking care not to block the exit, and pegged in place. The next morning, scats that had been deposited on cardboard were collected using a metal spatula or tweezers, placed in individual vials and mixed thoroughly with 75–80% ethanol for preservation. We did not collect scat from substrate (soil and vegetation, etc.) as humic substances in soil can inhibit DNA amplification (Schrader et al., 2012) and contaminant DNA from the environment can swamp dietary DNA in samples (McInnes et al., 2017a). Samples were stored in a freezer at −20°C within 3 h of collection. For full details of the scat collection protocol (see McInnes et al., 2017a).

Only around 100 nests were available at each site for monitoring, and of these fewer than fifty had eggs or chicks in any given week. This means that many nests were sampled more than once during the study. Samples collected each week ranged from less than 10 to 30, due to contamination by substrate, loss of sample freshness, and failure by penguins to defecate on the cardboard. A total of 579 samples were successfully collected from 106 nests across the two sites.

Molecular Methodology

DNA Extraction

DNA extraction and marker amplification were performed at the Australian Antarctic Division in Kingston, TAS, Australia. DNA was extracted from approximately 30 mg of each scat sample, using a Promega Maxwell® 16 instrument and Maxwell® 16 Tissue DNA Purification Kits. PCR inhibitor concentrations in DNA extracts were reduced by mixing samples with 250 μL Roche Stool Transport and Recovery (S.T.A.R.) Buffer (Roche Diagnostics, Basel, Switzerland) prior to extraction.

DNA Metabarcoding

Dietary DNA metabarcoding involves amplification and sequencing of large numbers of short, unique regions of DNA (barcodes) present in the scats of consumers (Pompanon et al., 2012). We used a two-step PCR amplification process enabling amplification of a DNA barcode region, and subsequent attachment of unique 'index tag' sequences to each sample, allowing samples to be pooled for sequencing (Binladen et al., 2007). In this study, DNA extracts were amplified using broad range primers (18S_SSU: McInnes et al., 2017a), that are conserved in metazoans, and amplify the V7 region of the nuclear small subunit ribosomal DNA gene (Hadziavdic et al., 2014). In choosing a barcode marker, there is a trade-off between coverage and resolution. Highly conserved regions provide information covering the breadth of a generalist diet, but are less variable, limiting taxonomic resolution to the class or family level (Pompanon et al., 2012). The reaction mixture for the first amplification was 2 μL fecal DNA, 5 μL Phusion HF (Taq polymerase), 1 μL bovine serum albumin, 0.5 μL Evagreen (Biotium, Inc., United States), 0.1 μL of each of the 5 μM 18S_SSU primers and 1.3 μL of water. We chose not to use a blocking primer to prevent amplification of penguin (host) DNA, because these can also mask DNA from some target species (Pinol et al., 2014). The amplification process was completed on a LightCycler 480 (Roche Diagnostics). Thermal cycling conditions were 98°C for 2 min; followed by 35 amplification cycles of 98°C for 5 s, 67°C for 20 s, 72°C for 20 s, with a final elongation of 72°C for 1 min. Samples with a Cycle Threshold (CT) score greater than 30, indicating low DNA or inhibited amplification, were excluded from further analysis (Murray et al., 2015). Second stage amplification and sequencing steps were carried out at the Ramaciotti Centre for Genomics (Sydney, NSW, Australia). After purification with AMPure XP magnetic beads amplicons were labeled with Nextera Index Primers in unique combinations. The reaction mix for this stage was 2.5 μL DNA, 2.5 μL of each of the Nextera Index primers, 12.5 μL of KAPA HiFi HotStart ReadyMix (Taq polymerase) and 5 μL water. Thermal cycling conditions were 95°C for 3 min; then eight amplification cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and then a final elongation of 72°C for 5 min. Finally, the pooled, indexed samples were loaded into an Illumina Miseq sequencer, and underwent 150 bp paired-end sequencing (Illumina Miseq v2 reagent kit).

Bioinformatics

Following sequencing, the separate forward and reverse reads were merged using the fastq_mergepairs function in *USEARCH* v8.0.1623 (Edgar, 2010, 2013). Amplicons that did not exactly match one of the forward or reverse 18S_SSU primers and those shorter than 150 bp were excluded. The merged amplicons from all samples were clustered into molecular operational taxonomic units (mOTUs) using the *UPARSE* algorithm (Edgar, 2010,

2013) with a cut-off threshold of 97% similarity. These mOTU clusters were then compared to a reference library (SILVA: Quast et al., 2013) containing DNA sequences linked to taxonomically verified specimens. Those with >95% match to a reference sequence were assigned to that taxon at class level. Many but not all sequences could be assigned taxonomic information at the class level using the *SILVA* database. The 10 most common classes (each accounting for more than 0.5% of food reads in the dataset) were further resolved to the lowest possible level using the NCBI Basic Local Alignment Search Tool (BLAST: Johnson et al., 2008). This global mOTU library was then used to classify sequences in each of the samples. Following identification of sequences, data were filtered to retain only food sequences, defined as all metazoa that are neither obligate parasites nor known post-defecation contaminants. Then, samples with fewer than 100 food sequences were discarded (comparable to McInnes et al., 2016), since these either contained insufficient DNA to analyze or were dominated by non-target DNA such that inferences about diet would be unreliable. **Table 1** gives the final number of samples analyzed per each breeding stage and month.

Diet composition was determined using two methods to account for biases in the diet summaries (Deagle et al., 2018). The two can give different results – although they are generated from the same dataset – because one functions as a record of presence or absence, and the other is used as a proxy for abundance. First, the number of reads of an individual taxon in a scat sample was divided by the total number of food reads in that sample and multiplied by 100, giving the percentage, or relative read abundance (RRA), of each taxon in each sample. These read abundances were then scored as present (>1% of food reads in a sample) or absent (<1% of food reads in a sample) to measure the percentage frequency of occurrence (%FOO) of each taxon across the dataset. RRA describes the relative percentage of prey sequences deriving from a prey group across the dataset and although it does not represent exact proportions of prey items in the diet, this metric can provide a more accurate summary of overall diet than frequency of occurrence in many situations (Deagle et al., 2018). The percentage frequency of occurrence describes the percentage of samples in the dataset in which a given taxon is present, but, like all presence–absence records, can overestimate the importance of common prey items that are ingested in very small amounts, including in the digestive tract of targeted prey (secondary predation). Samples were also categorized according to dominant prey group (i.e., the group comprising > 70% of the sequences in the sample) to further enable distinction between primary and secondary predation (McInnes et al., 2017b).

Statistical Analysis

To accommodate differing structure and assumptions between FOO (presence/absence) and RRA (percentages), we chose to analyze the data via separate methods that were better suited to each data type. A Chi-squared analysis was applied to the percentage frequency of occurrence data, and a mixed modeling approach with multiple comparisons employed for the RRA data. Statistical analyses were executed in *R* statistical software (*R 3.1.2*, R Development Core Team, 2013).

We employed contingency tables with Chi-squared tests to detect associations between taxa detected in fecal samples and either breeding stages or months of the breeding season within the frequency of occurrence (%FOO) data. Chi-squared tests were applied to the raw frequencies of occurrence, rather than percentages, as applying chi-squared tests to percentages is not valid. However, results are reported as percentages, as this is in line with conventions around this data type. Two contingency tables were built with counts of samples for each combination of taxon and either month or stage (*xtabs* function, *vcd* package; Meyer et al., 2008). To control for some of the possible pseudoreplication attributable to repeated sampling of burrows, we merged the data wherever there were multiple records for any combination of burrow, breeding stage and month. That is, if a taxon was recorded as present within any of the records for a single combination, it was retained as present in the merged record and if a taxon was absent from all records, it was retained as absent in the merged record. However, we were unable to fully account for repeated measures of burrows over multiple breeding stages and months and it is possible that this may inflate Type 1 error. Therefore, we have chosen to accept only chi-squared results with residuals $> +4$ or < -4 (equivalent to a p-value of 0.01 or less). We have not interpreted residuals between $+2$ and $+4$ (equivalent to significance at the 0.05 level). Each Chi-squared test was visualized as an association plot (*assoc* function, *vcd*).

To test for effects of breeding stage or month on the RRA of individual taxa, we constructed two linear mixed effects models with burrow nested within site as a random intercept to account for repeated measures (*lme* function, *nlme* package; Pinheiro et al., 2014). We applied a rank normal transformation to improve normality in the RRA response. Each model contained only the interaction of taxon and either stage or month. As we were interested only in the interaction, we

TABLE 1 | Number of analyzed samples across months and breeding stages of the 2015/2016 breeding season at Phillip Island.

	August	September	October	November	December	January	February	Total
Incubation	5	24	3	6	6	0	0	44
Guard	0	17	26	9	13	2	3	70
Post-guard	4	9	13	24	24	0	20	94
Total	9	50	42	39	43	2	23	208

Note that January was not incorporated in analyses due to insufficient sample size.

ignored the main effects (taxa, month, and breeding stage), which represented only the proportion of the total sequences that were collected in each breeding stage or month, or for each taxon, overall. The variance attributable to each level of the random effect was calculated by hand after retrieving estimated variances using the *VarrCorr* function in *lme4* (Bates et al., 2014).

Following the outcome of the above analysis, we then generated individual linear mixed effects models to test the effect of month and breeding stage (separate models) for each taxon, again with rank-normalized responses and burrow nested within site as a random effect. We applied multiple comparisons using Tukey's *post hoc* comparisons with Bonferroni corrections to reduce the chance of Type I error (*glht* function, *multcomp* package; Hothorn et al., 2013). These were visualized in a compact letter display (*cld* function, *multcomp*).

Ethics Statement

This research was conducted under an Ethics Permit No. 2.2014 approved by Phillip Island Nature Parks Animal Ethics Committee number and Research Permit No. 10006148 from the Department of Environment, Land, Water and Planning of Victoria, Australia.

RESULTS

Of 376 processed samples, 208 samples met the requisite 100 prey DNA sequences threshold for inclusion in subsequent

TABLE 2 | Sequences uncovered in the feces of little penguins *Eudyptula minor* on Phillip Island during the 2015–2016 breeding season, using the universal eukaryote primer, 18S_SSU (18S rDNA, McInnes et al., 2017a).

Phylum	Identity at lowest taxonomic ranking	Percentage frequency of occurrence (%FOO; %)	Relative read abundance (RRA; %)	Percentage of samples with > 70% RRA per prey group (%)‡
Annelida	Annelida	1.92	0.04	
Arthopoda	Malacostraca	10.10	2.12	0.96
	Branchiopoda	2.40	0.27	
	Maxillopoda			
	Calanoida	20.19	4.23	1.44
	Cyclopoida	6.25	0.22	
	Harpacticoida	23.08	2.84	0.96
	Other	7.21	1.09	
Chordata	Actinopterygii			
	Teleostei	100	63.56	49.52
	Appendicularia*			
	*Oikopleuridae**	40.87	5.39	1.44
	Ascidiaceae*			
	*Salpidae**	42.79	16.33	8.17
Cnidaria	Anthozoa*	0.48	0.01	
	Hydrozoa*			
	*Leptothecata**	9.13	0.96	
	*Other**	2.40	0.11	
	*Siphonophores**	13.46	0.66	
	Scyphozoa*	7.21	1.25	
Ctenophora	Ctenophora*	4.81	0.23	
Echinodermata	Echinodermata	0.48	0.01	
Mollusca	Bivalve	0.48	0.10	
	Cephalopod			
	Oegopsida	4.81	0.38	
	Other	1.92	0.09	
Chlorophyta	Green Algae	0.48	0.01	
Stramenopiles	Stramenopiles	0.48	0.02	
Tardigrada	Tardigrada	1.44	0.07	
Total prey		100	18.00	
Parasite		88	5.7	
Contaminant		99.52	8.9	
Penguin (host)		100	66.3	
Unidentified		66.35	1.1	

*Gelatinous taxa.
‡In 37.5% of samples, no single taxon dominated the sequence reads.

analyses. Of these, 112 were from Penguin Parade birds, while 96 were collected from Radio-tracking Bay. A total of 465 mOTUs were recognized, of which 353 contained non-food DNA including 31 obligate parasite OTUs, one penguin OTU and the remainder a range of non-food eukaryotes including fungi, unicellular organisms and plant DNA, a further 26 were unable to be identified (**Table 2**). A total of 86 mOTUs were identified as possible prey items, and pooled into their 17 classes for downstream analyses. It is typical for scat samples to include a high proportion of DNA sequences from parasites and contaminants (McInnes et al., 2017a).

Diet Composition

Fish (Actinopterygii) were present in all samples with prey DNA detected (frequency of occurrence; %FOO: **Table 2**) and accounted for 63.6% of prey sequences across all samples (RRA: **Table 2**). Of the 16 other taxa detected in the food dataset, only nine accounted for more than 0.5% of prey sequences each (**Table 2**). Gelatinous plankton accounted for 5 of the 10 most common taxa in the samples, were present in 76% of samples (%FOO) and represented almost 25% of all prey sequences (RRA). Of these, salps were present in 43% of samples, and comprised 16% of prey sequences, while oikopleurids (appendicularians) were present in 41% of samples but accounted for only 5% of prey sequences. Siphonophore and leptothecate hydrozoans were present in 13 and 9% of samples respectively, and accounted for less than 1% of prey sequences each. True jellies (Scyphozoa) were present in 7% of samples and accounted for 1.25% of prey sequences. Crustaceans and cephalopods accounted for four of the 10 most common taxa. Crustaceans were present in 48% of samples and accounted for 11% of prey sequences. Of these, free-living calanoid and harpacticoid copepods were present in 20 and 23% of samples, but accounted for only 4 and 3% of prey sequences respectively. Malacostraca occurred in 10% of samples but represented only 2% of prey sequences. Squid (Oegopsida) were present in 5% of samples and accounted for less than 1% of prey sequences. No differences were found between the Penguin Parade and Radio-tracking Bay in terms of broad diet composition (**Supplementary Figure S1**) and so site was not considered in further analyses, other than as a random effect.

Dominant Taxa in Individual Samples

Sixty two per cent of samples were dominated by sequences from a single prey group that accounted for 70% of the reads or more (**Figure 1**). Most of these samples were dominated by fish sequences. Almost 10% of samples were dominated by gelatinous salps, while only 1–1.5% of samples were dominated by copepods (calanoids and harpacticoids), oikopleurids, or malacostracan sequences.

Diet in Relation to Month of the Year

Frequency of Occurrence (%)

The frequency of occurrence of different taxa varied over months of the breeding season (**Figure 2A**), however, based on an alpha level of 0.01, only 2 of the 10 most common prey items varied significantly and these were salps and squid (**Figure 2B**; $\chi^2 = 201.91$, df = NA, $p < 0.01$). Fish were the most common item present, occurring in all samples, and squid occurred the least often, appearing in only November and December. The free-living calanoid copepods and the oikopleurid tunicates were the only taxa other than fish to occur across all 5 months sampled. Salps were present during August to November only and were significantly more common than expected in September. Squid were only present in the diet in 2 months (November and

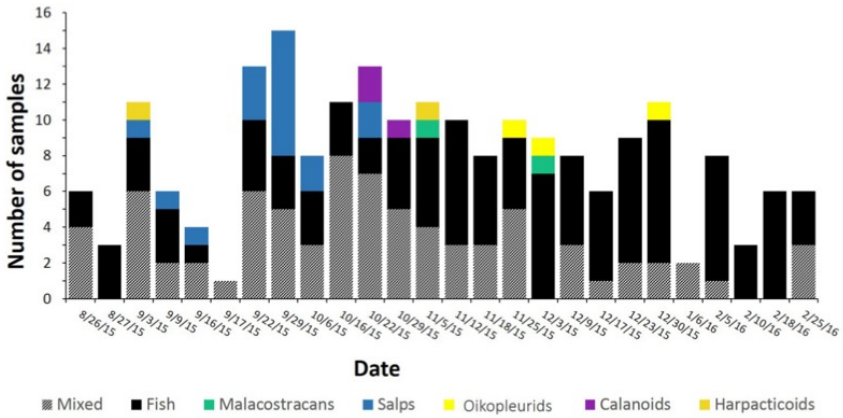


FIGURE 1 | Sample numbers for each sampling date, colored according to dominant prey type. The dominant prey type was calculated as the taxon that constituted more than 70% of DNA sequences in that sample. Samples where no single prey type dominated were classed as "mixed." On the y-axis, each tick mark represents one sample. Note that although samples from January are included here, these were not incorporated in further analyses due to insufficient sample size.

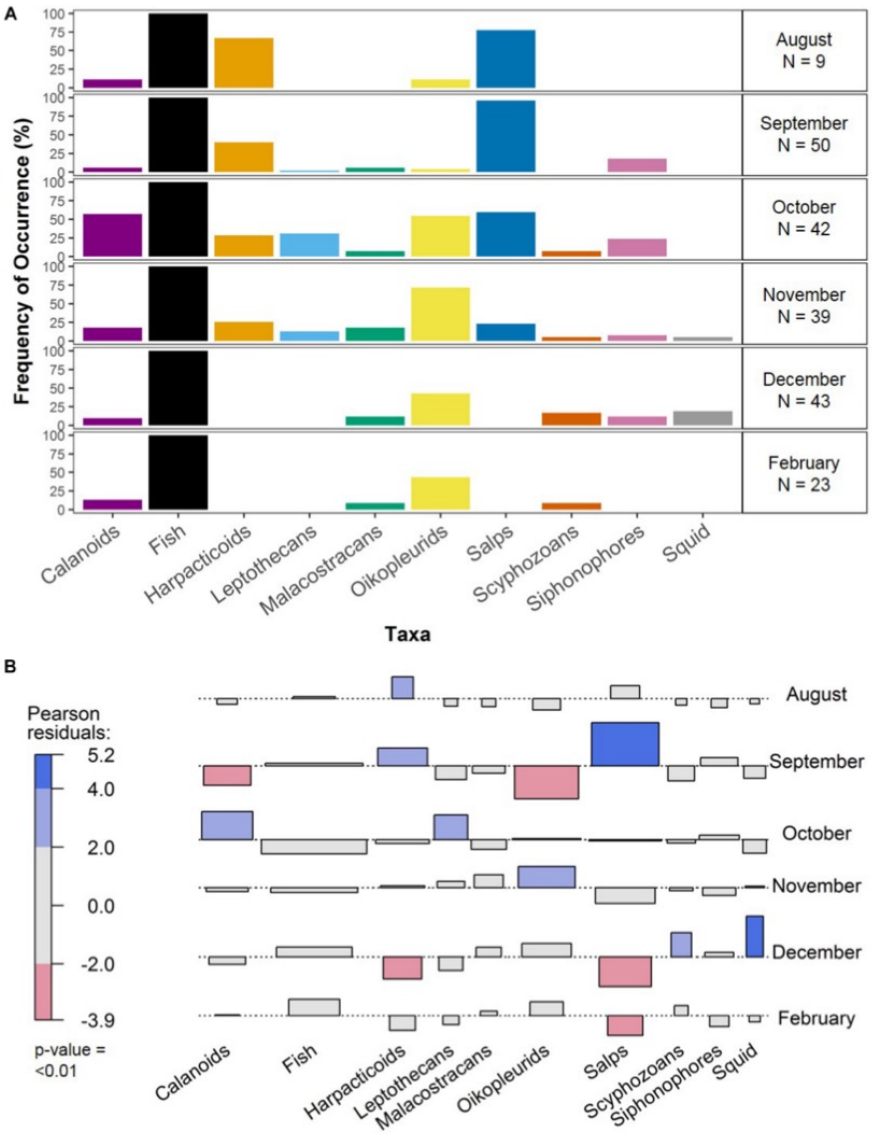
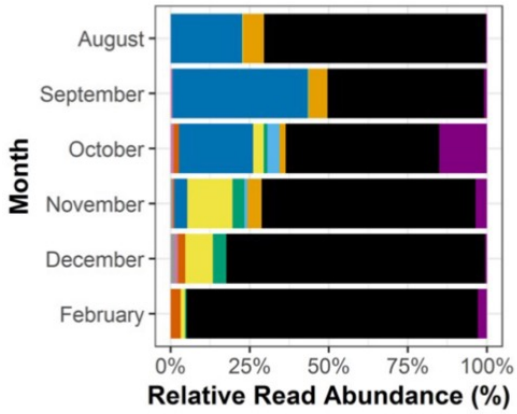


FIGURE 2 | The frequency of occurrence (%) of the 10 most common taxa in fecal samples over surveyed months of the breeding season. The percentages for each taxon over months are shown along with number of samples per month **(A)** and the association plot **(B)** illustrates significant differences between observed and expected numbers of samples containing taxa in each month, based on Pearson residuals. Red or blue shading indicates significance level. Light shading indicates *p*-values between 0.05 and 0.01, while bold shading indicates *p*-values < 0.01. In our analysis, we have accepted only significant values less than 0.01. Note that January was not incorporated in analyses due to insufficient sample size.

December) and were significantly more common than expected in December.

Relative Read Abundance (%)

The relative abundance of individual taxa varied over months of the breeding season (**Figure 3**) with nine out of 10 of the most common taxa showing a significant difference in abundance in one or more months ($F = 61.06$, df $= 59$, $p < 0.001$). Fish sequences showed a highly significant trend over months, being least abundant in September and October, increasing in abundance from October to February and accounting for more than 90% of the reads in February. Salp sequences



Prey type	Aug	Sept	Oct	Nov	Dec	Feb
Cala	***a***	***a***	***b***	***a***	***a***	***a***
Fish	***acd***	***ab***	***a***	***bc***	***cd***	***d***
Harp	a	a	a	a	a	a
Lept	***ab***	***b***	***a***	***b***	***b***	***b***
Mala	a	a	a	a	a	a
Oiko	***ab***	***a***	***ab***	***c***	***bc***	***ab***
Salps	***acd***	***d***	***c***	***ab***	***b***	***ab***
Scyph	a	a	a	a	a	a
Siph	a	a	a	a	a	a
Squid	***ab***	***b***	***b***	***ab***	***a***	***ab***

FIGURE 3 | The RRA (%) of the 10 most common taxa in fecal samples over surveyed months of the breeding season. The percentages for each taxon over months are accompanied by a compact letter display indicating significant differences between months. Months with the same letter are considered similar to each other, and months with different letters are considered significantly different from one another. Significant differences are also denoted by bold, italic text. Note that January was not included in analyses due to insufficient sample size. Taxa are Cala = Calanoids, Fish, Harp = Harpacticoids, Lept = Leptothecans, Mala = Malacostracans, Oiko = Oikopleurids, Salps, Scyph = Scyphozoans, Siph = Siphonophores, and Squid.

were significantly more abundant in September, with raised abundance in August and September compared to November and December, when the taxon was in very low abundance, and February, when no salps were recorded. Oikopleuran sequences were most abundant in November and December, and calanoid copepods and Leptothecan hydrozoan sequences were most abundant in October. The random effect of burrow nested in site accounted for 0.5% of the variation in this data, while the random effect of site accounted for less than 0.001%.

Diet in Relation to Breeding Stage

Frequency of Occurrence (%)

We conservatively conclude that no group varied significantly over stages in terms of frequency of occurrence, based on an alpha level of 0.01 (**Figure 4**; $\chi^2 = 33.221$, df = NA, $p = 0.015$). Fish were recorded in all samples. Scyphozoa and squid occurred only during guard and post-guard in a low number of samples, such that these were not significantly different to the zero counts in incubation.

Relative Read Abundance (%)

The RRA of taxa varied significantly over breeding stages (**Figure 5**), with 6 out of the 10 most common taxa showing a significant difference in abundance between two or more stages ($F = 66.56$, df = 29, $p < 0.001$). Most pronounced were the trends in fish and salps. Salp sequences were most abundant during incubation, and decreased to less than 20% of the reads in post-guard. Fish sequences were most abundant during post-guard. Oikopleurids, harpacticoid copepods, leptothecans, and scyphozoans also varied significantly over breeding stages, but there were no differences in calanoid copepods, malacostracans, siphonophores, or squid. As random effects, both burrow nested in site and site accounted for less than 0.001% of the variation in this data.

DISCUSSION

We confirm a fish-dominated diet in little penguins that includes a wide variety of other items. We revealed the presence of gelatinous and crustaceous plankton taxa not recorded by previous little penguin studies (e.g., Klomp and Wooller, 1988; Cullen et al., 1992; Flemming et al., 2013; Kowalczyk et al., 2015; Chiaradia et al., 2016). Though DNA metabarcoding made it possible to detect these cryptic taxa, it cannot describe whether items were ingested intentionally or incidentally (Sheppard et al., 2005). Consequently, some taxa recorded in our study may represent secondary predation by penguins (Sheppard et al., 2005), but their presence indicates a trophic role for newly recorded taxa and how they may contribute to energy flow through food webs. Further, getting an accurate measure of a predator's diet is always challenging, and in the case of DNA-based results, some biases could occur due to variation in trip duration, time since consumption and differential digestion rates among prey taxa (Thomas et al., 2014; Deagle et al., 2018). However, dietary DNA persists in the gut and feces of predators for four days (Deagle et al., 2010), and should reflect full diet.

We recorded a surprisingly high frequency and abundance of salp sequences in the diet of this predominately piscivorous predator. Not only did salps appear in >40% of fecal samples and constitute >16% of reads in the dataset, they were the dominant taxon in 8% of samples, suggesting they were likely to have been targeted prey in many cases. Salps, and

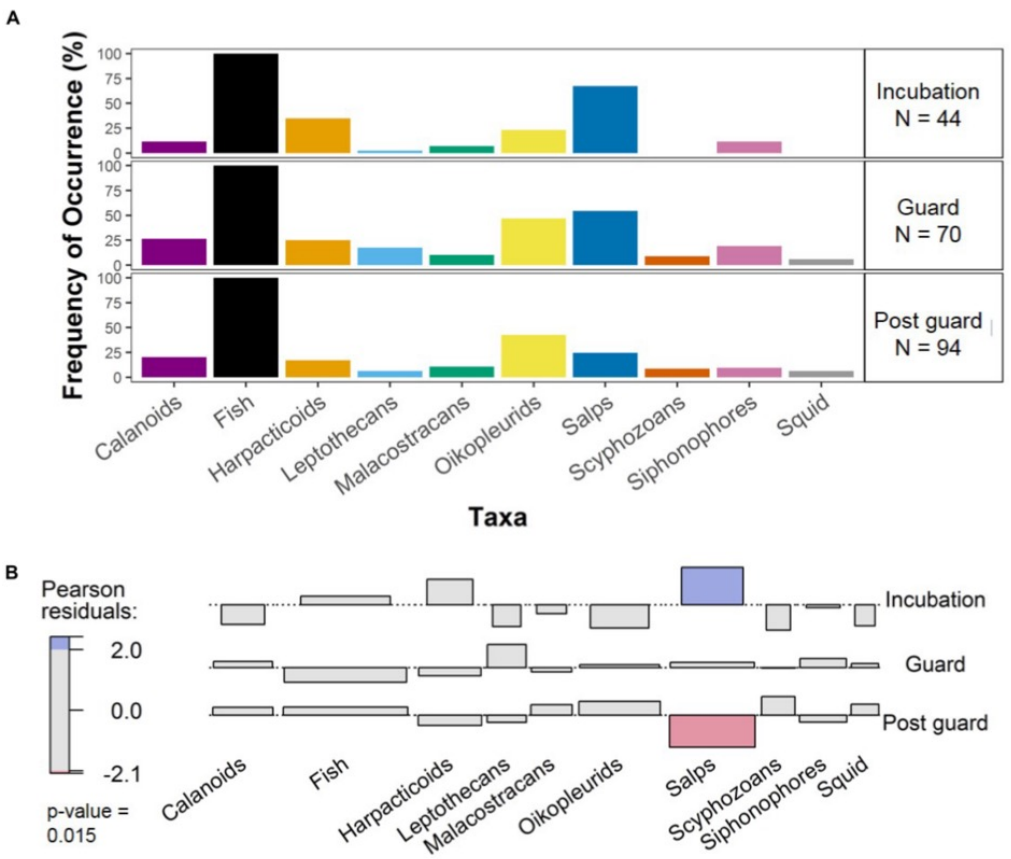
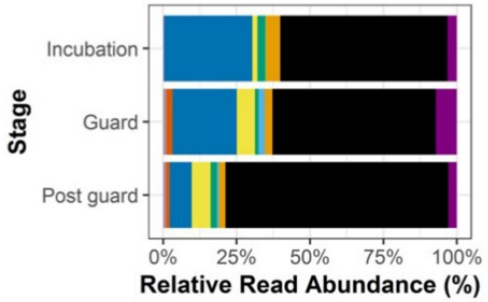


FIGURE 4 | The frequency of occurrence (%) of the 10 most common taxa in fecal samples over breeding stages. The percentages for each taxon over breeding stages are shown along with number of samples per stage **(A)** and the association plot **(B)** illustrates significant differences between observed and expected numbers of samples containing taxa at each breeding stage, based on Pearson residuals. Red or blue shading indicates significance level. Light shading indicates *p*-values between 0.05 and 0.01, while bold shading indicates *p*-values < 0.01. In our analysis, we have accepted only significant values less than 0.01. Note that January was not incorporated in analyses due to insufficient sample size.

other gelatinous plankton, have traditionally been thought to be of minor importance as prey items to higher trophic levels owing to their poor nutritional content (e.g., Doyle et al., 2007). Due to this low nutritional content, animals known to routinely feed on gelatinous plankton, such as the leatherback turtle (*Dermochelys coriacea*) and the ocean sunfish (*Mola mola*), have a low metabolic rate (Davenport, 1998; Houghton et al., 2006). In contrast, gelatinous plankton alone may not sustain endotherms such as marine mammals and birds that have high metabolic rates. However, despite being an order of magnitude lower in energy density than fish, cephalopods or crustaceans, salps can be equally or even more energy rich than scyphozoans (salps 0.43, scyphozoans 0.41, fish 8.4, squid 5.46 kJ g^{-1} wet mass: Cardona et al., 2012); krill 3.03 kJ g^{-1} wet mass: Gales and Green, 1990), which many seabirds are known to eat (Thiebot et al., 2016, 2017; McInnes et al., 2017b) and on which predation by little penguins was recorded in the wider region (Thiebot et al., 2017). The aforementioned study did not record predation on salps by little penguins, however the authors note that they "seldom, if ever" observed salps in videos, suggesting that there were few recorded opportunities for predation.

Salps are commonly targeted by larval and small pelagic fish (Young et al., 1997; Mianzan et al., 2001), and these are important prey items for many seabirds and marine mammals, including little penguins (Chiaradia et al., 2003). Given that fish occurred in all samples, salp presence could result from penguins eating fish that have recently fed on salps. However, we would expect the RRA to be much lower if their occurrence in the diet was due to secondary predation alone. Around half of the salp-dominated samples contained less than 10% fish sequences, and many of



Prey type	Incubation	Guard	Post guard
Cala	a	a	a
Fish	***a***	***a***	***b***
Harp	a	a	a
Lept	***a***	***b***	***ab***
Mala	a	a	a
Oiko	a	a	a
Salps	***b***	***b***	***a***
Scyph	a	a	a
Siph	a	a	a
Squid	a	a	a

FIGURE 5 | The relative read abundance (RRA) (%) of the 10 most common taxa in fecal samples over breeding stages. The percentages for each taxon over stages are accompanied by a compact letter display indicating significant differences between breeding stages. Breeding stages with the same letter are considered similar to each other, and months with different letters are considered significantly different from one another. Significant differences are also denoted by bold, italic text. Note that January was not incorporated in analyses due to insufficient sample size. Taxa are Cala = Calanoids, Fish, Harp = Harpacticoids, Lept = Leptothecans, Mala = Malacostracans, Oiko = Oikopleurids, Salps, Scyph = Scyphozoans, Siph = Siphonophores, and Squid.

these contained less than 5% fish sequences. Further, there is no information to suggest that the DNA of salps or other ascidians are preferentially recovered by the DNA barcode marker we used. Indeed, the other ascidian in the dataset (*Oikopleuridae*) is recorded only in low abundance and salps are rare in other seabird diet datasets generated with this DNA marker (McInnes et al., 2017b). Finally, because gelatinous tissue degrades so rapidly in comparison to other tissues, it is unlikely that enough salp DNA would persist through two rounds of digestion to create the signal seen in our dataset. Thus, little penguins do appear to directly feed on salps, which are known in the diet of other endotherms (Childerhouse et al., 2001; Cruz et al., 2001; Hedd and Gales, 2001), and even fed to offspring (Cruz et al., 2001).

Small crustaceous copepods and gelatinous oikopleurids, as well as gelatinous Scyphozoa and Hydrozoa, were occasionally found but with very low abundance, each accounting for <2–8% of the reads in the dataset. Our results are in contrast to those by Sutton et al. (2015) who observed with video loggers that 28% of little penguin predation events included scyphozoans from one family. These contrasting results (extended in Thiebot et al., 2017) could be due to temporal and spatial variation in jelly abundance in Bass Strait between the studies, or variation in detection between methods. Given the boom-bust dynamics of scyphozoan populations (Pitt et al., 2014), it is most likely that the disparity indicates differences in local abundance between years and locations. Further, the 16S SSU primer used in this study has previously detected very high predation on Scyphozoa and other jellies by albatross species (McInnes et al., 2017b). The use of cameras and DNA barcoding simultaneously may provide more understanding on predation events and prey occurrence in dietary samples. The presence of very small copepods and oikopleurids (<5 mm) may represent secondary predation events and so patterns observed in these tiny taxa may reflect their changing availability to fish, or predation by penguins on different fish species. Detection of these tiny and cryptic taxa can help us improve the trophic links within the broader little penguin food web.

We detected low presence of cephalopods and malacostraca in the diet of little penguins, although both have been detected previously (Klomp and Wooller, 1988; Flemming et al., 2013; Kowalczyk et al., 2015; Sutton et al., 2015; Chiaradia et al., 2016). While the presence of malacostraca has never been high in little penguin diet analyses, cephalopods can be quite common in their diet (30–60%: Chiaradia et al., 2016). High presence of cephalopods in penguins' diet has been associated with years of low food availability (Cullen et al., 1992; Chiaradia et al., 2003). Given our study season was one of very high breeding success (2.15 chicks per pair), which suggests high food availability (Chiaradia and Nisbet, 2006), this may explain the low presence of cephalopods. Another possibility is that predation on this group has been overestimated in the past, given the tendency for hard parts such as squid beaks to be retained in the stomachs and thereby overrepresented in stomach content analysis (van Heezik and Seddon, 1989). Indeed, no little penguins were observed to prey upon cephalopods during Sutton and colleague's video-logging and only three predation events included krill (Sutton et al., 2015) although we do not know how often opportunities to prey on these sources presented themselves. Finally, it is unlikely but possible that low cephalopod records were the result of technical bias in the metabarcoding (see McInnes et al., 2017b). Further analysis across years should be undertaken to resolve this uncertainty, ideally alongside DNA markers designed for squid and concurrent stable isotope and stomach contents sampling to clarify method-specific biases.

The Role of Salps in a Piscivorous Diet

The high presence and relative abundance of salps shows they may have a role in the diet of this largely piscivorous species for while relative abundance does not necessarily reflect true

mass proportions, it is a useful proxy (Deagle et al., 2018). Salp-dominated samples occurred in all three breeding stages but were restricted to September and October, which coincide with a peak in the incubation. In contrast, fish increased over stages and were in highest proportion in the post-guard stage; a period when a nutrient-rich diet is important for the body condition of chicks (Chiaradia et al., 2003). Avoidance of lower nutrition prey during chick-raising stages by penguins would explain the negative trend we found between salps and breeding stage, but the fact that salp sequences constituted more than a third of the diet sequences during September, with raised sequence proportions in August and October, may signpost an extrinsic influence. Raised salp occurrence and proportions may indicate the presence of a salp bloom during this time within the foraging range of penguins at our study sites. Salp blooms are notorious for blanketing large areas and excluding other planktonic species (Siegel and Harm, 1996; Verity and Smetacek, 1996; Lee et al., 2010) and other consumers have been shown to feed on salps as stomach fillers when other prey is scarce (Mianzan et al., 2001). Furthermore, it is well-known that in SE Australia salp blooms are most prevalent in August, September, and October (Henschke et al., 2015), exactly the time of year when they appeared maximally abundant in the diet of little penguins (**Supplementary Figure S2**). For example, *Thalia democratica* is the most abundant salp in the Tasman Sea with individual zooids, around 1 cm in size, and has been reported to reach densities of up to 1312 individuals/m^3 (Henschke et al., 2014). In such dense blooms the low energy density of salps is presumably offset by the ease with which large numbers can be consumed. So the most parsimonious explanation of our findings is that penguins opportunistically feed on salps when they happen to encounter blooms.

Based on studies of the biochemical and nutritional composition of salps, we do not believe that they contain any limiting resource that penguins would be targeting, but it remains a possibility. The organic content of salps is high in protein and crude fiber (Madin et al., 1981; Amorocho and Reina, 2007), though they are predominantly composed of water (Dubischar et al., 2012), and it has also been suggested that predators may target their visible stomachs, which contain densely packed diatoms and other phytoplankton (Gili et al., 2006), linking primary productivity to higher trophic levels through these direct grazers. Although salps could act as a source of calcium (Caron et al., 1989), by making concentrated diatomaceous calcium available to larger consumers, little penguins are easily able to obtain this resource by swallowing shell grit on the shoreline (Wasiak, 2008).

Shedding Light on Temperate Inshore Food Webs

Regardless of whether taxa uncovered by metabarcoding were obtained through primary or secondary ingestion, this method provides an avenue for capturing food web and population dynamics in gelatinous and otherwise cryptic taxa. Given the method is not taxon-specific, and is non-invasive, a wide variety of range-restricted, central-placed foragers could be used to capture inshore gelatinous patterns of abundance, and resolve trophic links. By pairing a short-range forager with sympatric long-range foragers, such as seals or shearwaters, it would be possible to capture whole-of-ecosystem patterns. Since jellies are expected to flourish under increasingly disturbed conditions while most other taxa are expected to suffer (Richardson et al., 2009; Purcell, 2012) tracking their changing abundance and distribution is of utmost importance if we are to mitigate flow on effects to other taxa (Purcell and Arai, 2001; Pakhomov et al., 2002; Henschke et al., 2016). This method also offers an efficient approach to track cycles of ecologically important taxa, such as small pelagic fish, in locations that are not subject to regular commercial fishing-based surveys, providing important data for ecosystem managers and those managing populations of threatened predators.

DATA ACCESSIBILITY

The datasets generated and analyzed for this study can be found in the FigShare repository:
molecular Operational Taxonomic Units (mOTUs)
https://doi.org/10.26180/5b9614c84773f
SILVA library w FOO and RRA calculation
https://doi.org/10.26180/5b9614661e54e
Frequency of occurrence used in analyses
https://doi.org/10.26180/5b96151bc3f3a
Relative Read Abundance used in analyses
https://doi.org/10.26180/5b961569647e1.

AUTHOR CONTRIBUTIONS

CC, RR, AC, BD and GH, conceived the project. CC and SS performed the fieldwork. CC lead the laboratory analysis. BD and JM assisted with bioinformatics. CC conducted the statistical analysis and led the writing with contributions from all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00381/full#supplementary-material

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Within-colony spatial segregation leads to foraging behaviour variation in a seabird

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ABSTRACT: Central-place foraging in large seabird colonies leads to high levels of intra-specific competition for food resources, often resulting in between-colony spatial segregation. However, little is known about within-colony variation in foraging behaviour that may arise from breeding locations. Using little penguins *Eudyptula minor* from a large colony (ca. 32 000 individuals) on Phillip Island (Australia), we present a novel approach combining GPS, diving, acceleration and bathymetry data. We investigated within-colony variation in 3-dimensional distribution of prey encounters and its consequences for foraging behaviour and breeding success. Over 1 breeding season, we simultaneously tracked 63 little penguins from 2 breeding sites located ~2 km apart and monitored their breeding success. We recorded 58 452 dives, of which 11 992 had prey encounter events associated. Results revealed strong spatial foraging segregation between sites throughout the breeding season and differences between sites in prey encounter depth during chick-rearing (mean ± SE, 11.8 ± 0.2 m vs. 17.3 ± 0.3 m). Birds from one site foraged in deeper waters and apparently experienced higher levels of competition, resulting in lower prey encounter rates and lower foraging efficiency (i.e. body mass gain after foraging trips), hence these birds seemed less successful. However, breeding success was high (>1.5 chicks fledged per pair) at both sites, indicating that food was not a limiting factor that year. Nonetheless, breeding success records over the last 12 yr showed that the site where birds foraged at deeper depths produced fewer chicks. Our findings highlight the importance of understanding small-scale spatial segregation to capture foraging behaviour variation within large seabird colonies.

KEY WORDS: Prey encounter · Diving behaviour · Colonial breeding · Foraging efficiency · Breeding ecology · Bio-logging · Acceleration · *Eudyptula minor*

INTRODUCTION

Colonial breeding, when a number of individuals breed within a central place from which they repeatedly depart in search of food (Wittenberger & Hunt 1985), provides many benefits to diverse taxonomic groups, including reduced predation pressure (Robinson 1985, Danchin & Wagner 1997), exchange of social information (Dall et al. 2005, Robinson et al. 2009, Evans et al. 2016) and increased opportunities for finding a high-quality mate (Dubois et al. 1998). However, individuals also pay fitness costs, such as transmission of parasites and diseases (Møller 1987, Danchin & Wagner 1997), competition for food resources (Furness & Birkhead 1984, Forero et al. 2002) and competition for nest space (Ramos et al. 1997). This trade-off is especially widespread among seabirds, of which 98% of species nest in colonies that can vary from aggregations of few individuals to over 1 million birds (Wittenberger & Hunt 1985).

Seabirds are central-place foragers while breeding, as they must frequently return to the colony to in-

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cubate their eggs or to feed their offspring (Rolland et al. 1998). During brood provisioning, breeding adults need to maintain their body condition while meeting the increasing energetic requirements of their young (Gales & Green 1990, Bevan et al. 2002, Ratcliffe et al. 2018). In order to balance this situation, some seabirds alternate between short trips to feed their offspring and long trips to restore and maintain their own body condition (Weimerskirch et al. 1997, Ropert-Coudert et al. 2004, Saraux et al. 2011), increase foraging effort to meet chick growth requirements (Ratcliffe et al. 2018), switch diet to more energy-dense prey (Booth & McQuaid 2013, Handley et al. 2017) and modify diving behaviour (Booth et al. 2018).

Competition for food resources has been proposed as a regulating mechanism of pelagic seabird populations by prey depletion near the colony ('Ashmole's halo'), forcing individuals to forage further (Storer 1952, Ashmole 1963). Cairns (1989) argued that Ashmole's halo did not consider interactions between neighbouring colonies and, alternatively, predicted complete spatial segregation between adjacent colonies (hinterland model). There is evidence that supports both models (e.g. Lewis et al. 2001, Ainley et al. 2003, Grémillet et al. 2004, Elliott et al. 2009). More recently, Wakefield et al. (2013) found that neighbouring colonies of a wide-ranging seabird, the northern gannet *Morus bassanus*, forage in largely mutually exclusive areas determined by density-dependent competition. The model developed by Wakefield et al. (2013) suggested that spatial segregation could be enhanced by information transfer among individuals at sea and at the colony. In fact, the use of social information can facilitate group foraging (Weimerskirch et al. 2010, Sutton et al. 2017) and optimise foraging efficiency (McInnes et al. 2017) in some seabirds. Hence, indirect competition for food resources and social behaviour could be mechanisms simultaneously underlying spatial foraging segregation.

Most studies investigating foraging segregation between neighbouring colonies are based on wide-ranging seabirds from colonies separated by tens or hundreds of kilometres. Studies at a much finer spatial scale, i.e. within the same colony, using short-ranging seabirds are scarce and often limited to chick-rearing stages. Spatial foraging segregation between colony members can arise from intrinsic individual factors such as sex and age (e.g. Weimerskirch et al. 2006, Pelletier et al. 2014, Cleasby et al. 2015). Often, bird colonies are not defined entities (Jovani et al. 2008) and can be divided into several discrete and isolated sub-colonies or breeding sites (Waggitt et al. 2014), each of which may promote specific foraging behaviours (Masello et al. 2010, Ceia et al. 2015) and differences in breeding success (Hipfner et al. 2007). Yet, such within-colony asymmetries associated with breeding locations have received little attention, although they could have important implications for conservation policies and for individuals' fitness and life history, especially in species with high nest-site fidelity.

Little penguins *Eudyptula minor* are the smallest of all penguin species and have one of shortest foraging ranges (around 20 km) among seabirds during the breeding season (Collins et al. 1999, Preston et al. 2008). Parents alternate nest attendance duties and foraging during incubation and guard (i.e. when chicks are between 1 and 19 d old) reproductive stages, with incubation trips lasting 1–9 d (Kato et al. 2008) and guard trips always lasting 1 d (Chiaradia & Kerry 1999, Preston et al. 2010). During the post-guard period, parents only return at night to feed the young (Chiaradia & Kerry 1999) and alternate between short and long trips (Saraux et al. 2011). Phillip Island (south-eastern Australia) is one of the largest little penguin colonies in the world, a mega-colony by little penguin standards, with an estimated population of 28 000–32 000 individuals (Sutherland & Dann 2014), hence high levels of intra-specific competition are expected. Burrows are located on the coastline of the 3 km long Summerland peninsula (Dann 1991) and penguins come ashore at dusk and leave at sunrise. Penguins come ashore and leave using established pathways, delimited by rocky outcrops, providing a natural division of the whole colony into sub-colonies (hereafter termed 'sites'). This division results in a good model to investigate whether such spatial structure leads to foraging behaviour and breeding success asymmetries within the colony.

Here, we studied the 3-dimensional foraging behaviour and the breeding success of little penguins at 2 neighbouring sites located 2 km apart within the Phillip Island colony. We investigated if penguins from each site shared the same foraging area or segregated in space and compared the bathymetry of their used areas, as it can influence their diving behaviour (Chiaradia et al. 2007, Meyer et al. 2017). We hypothesised that (1) there is a spatial foraging segregation between sites, following the models of Cairns (1989) and Wakefield et al. (2013), (2) there are differences in the foraging activity and efficiency between sites, and (3) breeding success differs between sites as an ultimate consequence of the differences in foraging behaviour.

MATERIALS AND METHODS

Study site and experimental design

Field work was conducted simultaneously at 2 neighbouring little penguin breeding sites, Penguin Parade® and Radio-tracking Bay, located 2 km apart, on Phillip Island, Victoria, Australia (38° 31′ S, 145° 09′ E). Penguin Parade is situated on the eastern margin of the colony and contains both artificial nest boxes and natural burrows, while Radio-tracking Bay is located west of Penguin Parade, sharing its eastern and western margins with other sites, and contains only natural burrows (Fig. 1). We simultaneously monitored foraging behaviour and breeding performance in a subset of 100 nests at each site.

We tested if there were site-specific patterns in the 3-dimensional habitat use of birds by combining horizontal and vertical tracking with acceleration data collected during the 2015/2016 breeding season. To do so, we measured the spatial overlap between sites in the horizontal distribution and depth of prey encounters over the 3 breeding stages: incubation, guard and post-guard. We then compared the bathymetry between sites within the foraging area used at each stage. In order to investigate the consequences of these 3-dimensional patterns, we compared the foraging activity (i.e. number of dives with at least 1 prey encounter event per hour of diving on each foraging trip) and efficiency (i.e. adult body mass change after foraging trips) between sites. We then determined the breeding performance of each site by calculating the peak chick mass (Chiaradia & Nisbet 2006) and breeding success as the number of chicks fledged per pair.

Bird instrumentation and tracking

Between September and November of 2015, we tracked 74 little penguins from Penguin Parade and Radio-Tracking Bay using GPS and accelerometer data loggers. We recovered data from 71 birds, but

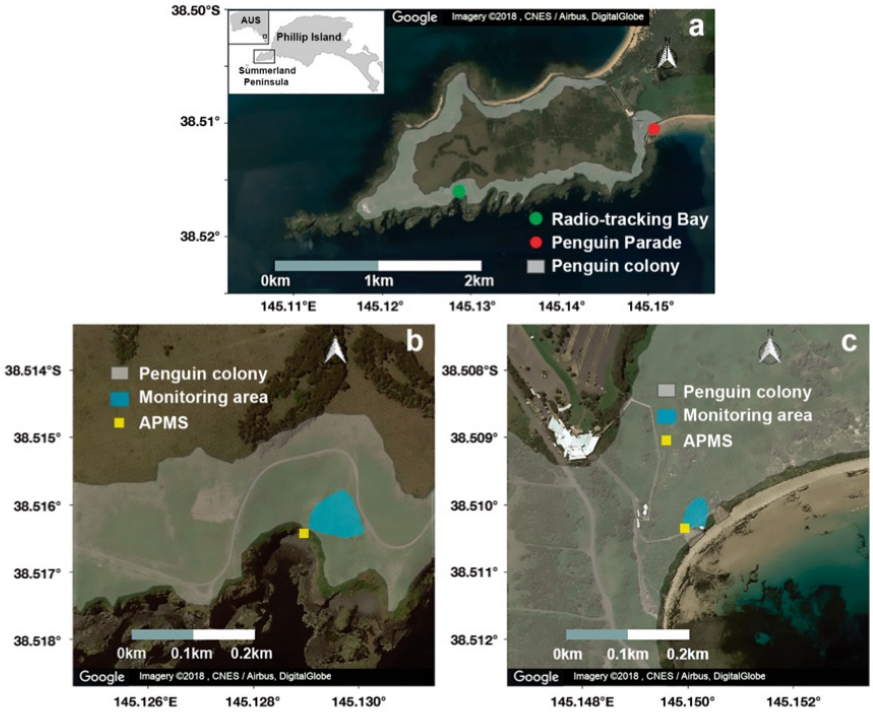

Fig. 1. Satellite images showing the geography of the study area on the Summerland Peninsula, Phillip Island, Victoria, Australia. (a) Distribution of the little penguin *Eudyptula minor* colony on Phillip Island adapted from Sutherland & Dann (2014) and location of the breeding sites monitored in this study. Also shown are the areas containing the monitored burrows (blue) and location of the automated penguin monitoring system (APMS) at each breeding site: (b) Radio-tracking Bay and (c) Penguin Parade

only complete trips with data from both GPS and accelerometer were considered for further analysis. In total, 63 foraging trips over incubation (n = 19), guard (n = 27; chicks between 1 and 19 d old) and post-guard (n = 17; chicks are unattended during the daytime in the colony) were analysed. For each breeding stage, birds from both sites were tracked simultaneously, aiming to maintain the same sample size per site and sex.

Adult birds were captured in their nest boxes or natural burrows and weighed (±10 g) using a spring balance. A GPS (modified as per below; CatTrack 1, 380 mA lithium-ion battery, Catnip Technologies) and a tri-axial accelerometer data logger were attached with Tesa® tape (Beiersdorf) to each bird's middle and lower back, respectively, as per Preston et al. (2008). All accelerometers also had temperature and pressure sensors, and models used were either AXY-Depth (31 × 12 × 11 mm; Technosmart), ORI400-D3GT (45 × 12 mm; Little Leonardo) or Wacu (21 × 13 × 4 mm; IPHC–DEPE). The original packaging of the GPS loggers was removed and the devices were waterproofed inside a heat-shrink tube. Dimensions of customised units were approximately 50 × 20 × 8 mm. The 2 devices together (GPS and accelerometer) weighed ~35 g in air, and the minimum and maximum percentage of body mass for any logger deployment was 2.4 and 4.2%, respectively. The cross-section area of the loggers was ~3% of the bird's frontal area (as calculated by Ropert-Coudert et al. 2007). Birds were immediately returned to their nests after the device attachment and were recaptured in their nests after a single foraging trip to retrieve the loggers. Total handling time was less than 5 min, and the integrity of the plumage was preserved during deployments and retrievals.

GPS loggers recorded at-sea positions from 04:00 to 21:00 h every 120 s for incubation and post-guard trips and every 20 s for guard trips. Accelerometers recorded depth (ORI400-D3GT, range 0–300 m, ±0.1 m) or pressure (AXY-Depth and Wacu, range 0–30 bar, ±0.5 mbar) and temperature (range −10 to 50°C, ±0.1°C) every second, as well as acceleration along the 3 body axes of the penguins: longitudinal (surge), dorso-ventral (heave) and lateral (sway), between −20 and 20 m s^{-2} at 25 Hz for incubation and post-guard trips and 50 Hz for guard trips. The sampling intervals for the different breeding stages were selected as a compromise between the logger battery life and variability in foraging trip duration during incubation and post-guard (Chiaradia & Kerry 1999, Kato et al. 2008, Saraux et al. 2011).

Depth, acceleration and GPS data processing

Dive analysis was conducted on depth and acceleration data downloaded from the accelerometer data loggers and using a purpose-written software in Igor Pro Version 6.37 (Wavemetrics; see Kato et al. 2006). For AXY-Depth and Wacu accelerometers, pressure in millibars was converted to water depth (m). The software considered a dive started when birds descended from the water surface and ended when they returned to it. Dives were analysed sequentially for each foraging trip. According to the resolution of the loggers and waves at the surface (Kato et al. 2006), only dives >1 m were considered for the analysis. Each dive was divided in 3 phases: descent, bottom and ascent phases. The start and end of the bottom phase were defined as the first and last time in a dive when the absolute rate of change of depth was lower than 0.25 m s^{-1} (Ropert-Coudert et al. 2006). The times before and after the bottom phase within the dive were considered the descent and ascent phases, respectively. The deepest point reached during a dive was termed maximum depth.

Prey encounter events were identified using acceleration data. Wingbeats are apparent in acceleration signals as a series of regularly alternating increases and decreases of the heave and surge axes (Watanuki et al. 2006). During prey pursuit, wingbeat signals suddenly increase in frequency and amplitude, resulting in a peak in the overall acceleration (Ropert-Coudert et al. 2006, Zimmer et al. 2011a). The vectorial dynamic body acceleration (VDBA) is the vectorial sum of the 3 axes' dynamic acceleration, being a proxy of the whole body activity (Gleiss et al. 2011):

$$\mathrm{VDBA} = \sqrt{\left(A_x^2 + A_y^2 + A_z^2\right)} \quad (1)$$

where A_x, A_y and A_z are the dynamic acceleration of surge, heave and sway axes, respectively. The dynamic acceleration of each axis was obtained by subtracting the static acceleration (i.e. total acceleration of each axis smoothed over 1 s) from the total acceleration (Shepard et al. 2008). Finally, VDBA values were averaged every second for further analysis. Evaluating the frequency of VDBA during the 3 dive phases, we found an upper inflection point at 0.5 × g common to all birds (see Fig. S1 in Supplement 1 at www.int-res.com/articles/suppl/m606p215_supp1.pdf). We used this upper inflection point as a threshold to detect prey encounter events. When the VDBA became higher than 0.5 × g within a dive, we considered the penguin had encountered and pursued prey, resulting in a peak in the overall acceleration (see Fig. 2 for an illustrative example). The first 5 m of descent

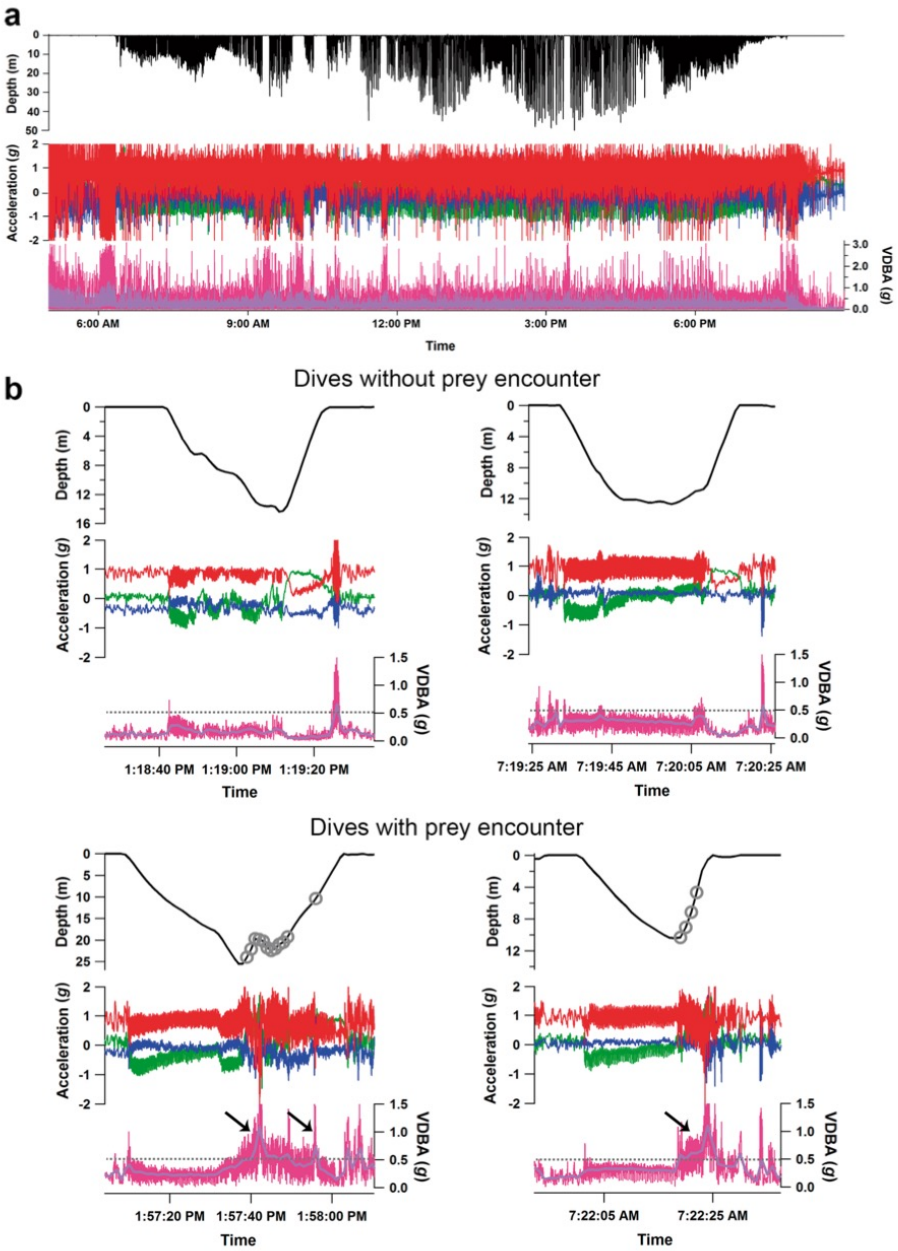

Fig. 2. (a) Depth and acceleration profiles for a complete foraging trip by a little penguin during the guard stage. Diving activity (top), acceleration at 50 Hz (middle) on the surging (green), heaving (red) and swaying (blue) axes and vectorial dynamic body acceleration (VDBA; bottom) at 50 Hz (pink) and smoothed over 1 s (purple). (b) Examples of dives without and with a prey encounter event. Each prey encounter event (1 s) is indicated by the grey circles on the diving profiles and the black arrows on the VDBA graph. Acceleration at 50 Hz is shown on the surging (green), heaving (red) and swaying (blue) axes. VDBA at 50 Hz (pink) and smoothed VDBA (purple). The grey dotted line on the VDBA graph indicates the prey encounter and pursuit threshold at 0.5 × *g* smoothed VDBA

phases were excluded from VDBA threshold detection because birds beat their wings vigorously to overcome buoyancy at the beginning of a dive (Zimmer et al. 2011a,b). Bottom and ascent phases from all dives, regardless of their depth, were kept for prey encounter detection purposes. For each dive with a prey encounter, the depth at which the first VDBA point exceeded 0.5 × *g* was considered as the prey encounter depth (m). For each trip, the foraging activity was measured by calculating a prey encounter rate as the number of dives with prey encounters divided by the sum of all dive durations (h_{div}).

The distance between consecutive GPS locations was calculated using the Haversine algorithm from the 'geosphere' package (Hijmans 2016) within the R statistical environment in order to estimate the swimming speed between points. We excluded GPS locations at the colony and locations with a swimming speed of 0 m s^{-1} (i.e. duplicated points) or greater than 3.3 m s^{-1} (i.e. maximum swimming speed for little penguins; Preston et al. 2008) from the dataset, as they were considered either erroneous locations or did not belong to the foraging phase of the trips. We allocated a location to each dive with a prey encounter by linearly correlating the timestamp at the beginning of each dive event with the closest GPS locations recorded before and after the dive. In some instances, the foraging trip exceeded (by a maximum of 2 h) the battery life of the GPS logger, and the location of dives recorded after the GPS device stopped logging positions could not be determined. However, we included these trips in the analysis, as dives without a location only accounted for 4% of the total dives with prey encounter, indicating that at the end of the trip, birds were mainly travelling back to the colony without actively foraging. In order to assess the accuracy of prey encounter locations, we counted the number of dives with prey encounters that occurred during gaps in the GPS data that exceeded 5 min. As this corresponded to only 3% of the prey encounter dives, we kept these prey encounter locations in the spatial analysis.

Automated penguin monitoring system

At each site, adult birds attending the monitored nests were identified with passive identification transponders (Allflex and Trovan). Two automated penguin monitoring systems (APMS), 1 at each site, located on the main penguin pathway, recorded the transponder number, date/time and individual body mass of adult birds leaving at sunrise and returning ashore at dusk (Kerry et al. 1993, Chiaradia & Kerry 1999). We only considered APMS data recorded during the tracking period and from known breeding adults attending the monitored burrows. In order to eliminate potential erroneous adult mass, we filtered out adult mass lower than the 5th percentile (712 g) and higher than the 95th percentile (1547 g). Two different adult body masses were examined for a given foraging trip: 'departure mass', which was the mass of the bird leaving the colony at sunrise, and the 'return mass', which was the mass of the bird returning to the colony at sunset. These 2 body masses were considered to belong to the same foraging trip when their records were consecutive in date and time for a given transponder number and the trip duration was not longer than 1 d in guard and 21 d in incubation and post-guard. The difference between the 'return mass' and the 'departure mass' was used as an estimate of the mass (in g) of food obtained over the foraging trips, i.e. foraging efficiency (Saraux et al. 2011).

Reproductive performance

During the 2015/2016 breeding season, we monitored 100 penguin nests at each site, 3 times wk^{-1} at Penguin Parade and once weekly at Radio-tracking Bay, to assess laying date, hatching date and fledging success. We identified adults in the nests by scanning their transponder numbers. New birds were injected with transponders as per Chiaradia & Kerry (1999), and sex was determined through measurements of bill morphometrics with a 91% accuracy (Arnould et al. 2004). During each nest visit, chicks were weighed (±10 g), using a spring balance, from hatching until fledging (Saraux et al. 2011). We determined the peak chick mass for each fledged chick (Chiaradia & Nisbet 2006) during the first clutch of the season, i.e. when breeding adults were tracked. Due to the monitoring frequency, this was the most comparable chick parameter between sites.

In order to assess the overall success, breeding success was determined at each site as the number of chicks fledged per breeding pair (Chiaradia & Kerry 1999, Chiaradia & Nisbet 2006) over 12 yr, from 2004 until 2015, following the same monitoring protocol as for the 2015/2016 season.

Data analysis

All statistical analyses were conducted using R 3.4.3 (R Core Team 2017).

Three-dimensional spatial pattern of prey encounter

Geographic coordinates of prey encounter events were projected into a Universal Transverse Mercator projection (zone 55S, WGS 84 Datum). For each stage and site, we used the prey encounter locations to estimate the home range (95% utilisation distribution, UD) and core range (50 and 25% UD) of prey pursuits using kernel density analysis and estimated the areas of each contour with the R package 'adehabitatHR' (Calenge 2006). Following Lascelles et al. (2016), the smoothing factor used in the kernel density analysis was calculated as the average area-restricted search (ARS) behaviour exhibited across all trips within each breeding stage. The ARS was assessed by first passage time analysis with the *fpt* function of the R package 'adehabitatLT' (Calenge 2006). The size of the grid for the kernel density analysis was set at 400 m as a compromise between the resolution of the bathymetry data obtained from Geoscience Australia (250 m × 250 m resolution) and the computation time. We also estimated the 95 and 25% contours of prey pursuits for each individual trip following the above procedure and extracted the median bathymetry within each contour.

The extent of within-stage overlap between Penguin Parade and Radio-tracking Bay home and core ranges was estimated using the UD overlap index (UDOI; Fieberg & Kochanny 2005) with the *kerneloverlaphr* function of the R package 'adehabitatHR'. Using UDOI as our metric, we then used a randomisation procedure (see customised R function in Supplement 2 at www.int-res.com/articles/suppl/m606p215_supp2.txt) to test the null hypothesis that there was no difference in the spatial distribution of prey encounter events between sites within each breeding stage (Breed et al. 2006, Cleasby et al. 2015). For each stage, site was randomly assigned to tracks (i.e. prey encounter locations) using the same site/sex ratio as the observation, and kernel analysis and overlap (i.e. UDOI) estimation were applied. This procedure was iterated 1000 times for the 95, 50 and 25% contours of each breeding stage. The empirical p-value (North et al. 2002) was determined as the proportion of random overlaps that were smaller than the observed overlap (i.e. the probability that spatial segregation was driven by chance). When the empirical p-value was <0.05, we considered the observed overlap to be significantly lower than expected by chance.

We compared the bathymetry of the foraging areas between sites for each breeding stage by performing a permutation test using Brunner and Munzel's generalised Wilcoxon test (Neubert & Brunner 2007, Ruxton & Neuhäuser 2013). The null hypothesis tested was that there were no differences in the bathymetry use between sites. For the 95 and 25% contours of each breeding stage, we first estimated the generalised Wilcoxon test statistic between sites of the observed median bathymetry of individual trips. We then ran 10 000 permutations where site was randomly assigned to the median bathymetry of each individual trip, maintaining the sample size for each site, and the generalised Wilcoxon test statistic was estimated for each permutation. The empirical p-value was determined as the proportion of random absolute test statistics larger or equal to the absolute observed test statistic. Empirical p-values <0.05 were considered to show significant differences between sites in the bathymetry within the foraging area used.

The influence of site on prey encounter depth was modelled separately for each breeding stage using linear mixed effects models (LMMs) from the R package 'nlme' (Pinheiro et al. 2016). Site and sex were included as fixed effects and bird ID was included as a random intercept to avoid pseudo-replication and account for any individual differences in dive behaviour of birds. A correlation structure across bird ID was incorporated in the model using the first-order autoregressive to allow for similarities between dives that occurred in temporal clusters. For each LMM, model selection of the fixed effects was conducted by maximum likelihood (ML) estimation, inspection of residual plots and the use of Akaike's information criterion (AIC). The most appropriate random structure was then determined via restricted maximum-likelihood (REML) estimation and selected by a comparison of AIC and residual plots (Zuur et al. 2009). The most parsimonious model was then refitted using REML to obtain parameter estimates and associated p-values (Zuur et al. 2009).

Foraging activity

The influence of site on the prey encounter rate of each foraging trip was modelled separately for each breeding stage using a generalised least square (GLS) approach from the R package 'nlme' (Pinheiro et al. 2016). Site and sex were included as fixed effects. Trip duration was also included for incubation and post-guard models to account for trip duration flexibility in these stages (Kato et al. 2008, Saraux et al. 2011). We detected heterogeneity of variances between sites, which was incorporated in the model via the *weights* argument in the *gls* function. Model selection was conducted using AIC, and the final model was validated inspecting residual plots (Zuur et al. 2009).

Adult mass change and reproductive performance

Differences between sites in the mass change after foraging trips and in the adult body mass at the beginning of the trips were compared using LMMs from the R package 'nlme' (Pinheiro et al. 2016) with bird ID as a random intercept. Each breeding stage was modelled separately, and trip length was incorporated as a fixed effect in the incubation and post-guard models. Model selection and validation were conducted following the same procedure as for the LMMs described above.

Differences in the reproductive performance of the first clutch of the 2015/2016 season between sites were assessed by comparing peak chick mass between sites fitting a linear model.

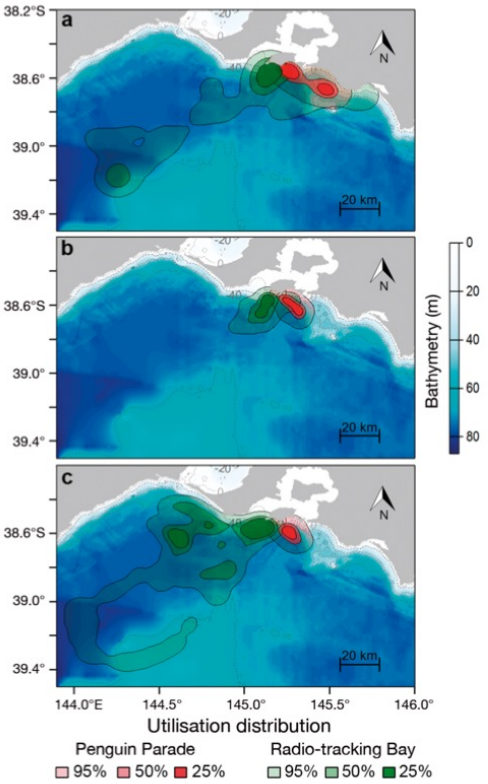

Fig. 3. Kernel density utilisation distribution of prey encounter locations during (a) incubation, (b) guard and (c) post-guard stages of little penguins at Penguin Parade (red) and Radio-tracking Bay (green). Grid size is 400 m, smoothing factor is 4.4 km for incubation, 3.7 km for guard and 2.96 km for post-guard. Smoothing factors were calculated for each stage following Lascelles et al. (2016)

RESULTS

Three-dimensional spatial patterns of prey encounter

Tracking data consisted of 63 complete foraging trips by 63 little penguins over 1 breeding season (Table S1 in Supplement 1). The total number of dives made across all foraging trips at both sites was 58 452 (Penguin Parade: 27 686 dives; Radio-tracking Bay: 30 766), of which 11 992 dives had prey encounter events associated (Penguin Parade: 5241 dives; Radio-tracking Bay: 6751 dives). We were not able to allocate a location for 2487 dives (527 with prey encounter), and therefore they were not included in the spatial analysis.

Birds from Penguin Parade mainly foraged south-east of their breeding site, while birds from Radio-tracking Bay foraged south-west of their site (Fig. 3). Consequently, and based on the randomisation tests of prey encounter spatial overlap, we found that little penguins presented a significant spatial foraging segregation between sites for all breeding stages, except for the incubation home-range (30% overlap, $p = 0.06$, Table 1, Fig. 3). There was no overlap (UDOI = 0) in the core areas of prey encounters between sites during guard and post-guard (Table 1, Fig. 3). Birds from Radio-tracking Bay consistently used larger areas over the breeding season than birds from Penguin Parade (Fig. 3, Table A1 in the Appendix), with the maximum differences observed during incubation (50% core area: 519 vs. 296 km^2, respectively; Table A1) and post-guard (50% core area: 1004 vs. 109 km^2, respectively; Table A1).

Table 1. Estimated utilisation distribution (UD) overlap index (UDOI) in UDs between Parade and Radio-tracking Bay little penguins within incubation, guard and post-guard breeding stages. Empirical p-value (p): the proportion of 1000 randomised overlaps that were smaller than the observed overlap. Randomised overlaps were only calculated if observed overlap was >0

UD (%)	Stage	UDOI	p
95	Incubation	0.3	0.06
	Guard	0.14	0.001
	Post-guard	0.01	0.01
50	Incubation	0.007	0.001
	Guard	0	–
	Post-guard	0	–
25	Incubation	0	–
	Guard	0	–
	Post-guard	0	–

Single long foraging trips occurred in the incubation and post-guard stages with individuals from Radio-tracking Bay, but were rare, occurring in 1 of 9 and 1 of 7 foraging trips, respectively. These long trips resulted in the home-range foraging areas extending further than 100 km to the SW (Fig. 3a,c). However, the finding of a significant spatial foraging segregation between individuals from Radio-tracking Bay versus Penguin Parade was not dependent on these 2 long foraging trips. For example, if these 2 long foraging trips were removed from the analysis, the differences in space use between sites remained (Table A2).

Median bathymetry of the Penguin Parade foraging area during incubation was significantly shallower than that of Radio-tracking Bay for both the home range and core range (Table 2). Median bathymetry of the home-range of prey encounter during guard was shallower at Penguin Parade (Table 2). No significant differences in the bathymetry between sites were found during post-guard (Table 2).

Regarding the use of the water column, prey encounter depths ranged from 2 to 35 m in incubation, from 2 to 61.8 m in guard and from 2 to 55.2 m in post-guard. Sex had no significant effect in the prey encounter depth for any stage (Table 3). There were no significant differences between sites in the prey encounter depth over incubation and post-guard (Table 3, Fig. 4). During the guard stage, birds from Radio-tracking Bay found prey at a significantly greater depth than those from Penguin Parade (6 m deeper, LMM $p < 0.001$; Table 3, Fig. 4).

Table 2. Median (± median absolute deviation) bathymetry (depth in m) within the little penguin foraging areas of individual trips (home range = 95% kernel contour, core area = 25% kernel contour) at Penguin Parade and Radio-tracking Bay during incubation, guard and post-guard. Empirical p-value (p): the proportion of 10 000 random permutations calculating a generalised Wilcoxon test between sites with a test statistic larger or equal to the observed data. Asterisks (*) indicate significant differences between sites ($p < 0.05$)

	Home-range			Core area		
	Penguin Parade	Radio-tracking Bay	p	Penguin Parade	Radio-tracking Bay	p
Incubation	58 ± 2	70 ± 4	0.004*	46 ± 2	64 ± 7	0.01*
Guard	64 ± 5	72 ± 1	0.008*	71 ± 2	72 ± 1	0.12
Post-guard	67 ± 3	74 ± 1	0.7	69 ± 2.5	74 ± 6	0.67

Table 3. Parameter estimates (β), standard errors (SE), lower (2.5%) and upper (97.5%) confidence intervals (CI) and p-values for the linear mixed effect models with a random intercept of little penguin ID and first-order autoregressive structure for prey encounter depth. Models are fitted for incubation, guard and post-guard. Base level of the factor 'sex' is 'female' and base level of the factor 'site' is 'Penguin Parade'

Fixed effect	β + SE	Lower CI	Upper CI	p
Incubation				
Intercept	7.6 ± 1	5.6	9.6	<0.001
Sex(male)	−0.2 ± 1.2	−2.67	2.21	0.8
Site(Radio-tracking Bay)	2.3 ± 1.2	−0.17	4.7	0.07
Guard				
Intercept	10.9 ± 1.3	8.3	13.5	<0.001
Sex(male)	1.7 ± 1.5	−1.3	4.8	0.26
Site(Radio-tracking Bay)	6 ± 1.47	2.9	9	<0.001
Post-guard				
Intercept	13.9 ± 1.7	10.6	17.2	<0.001
Sex(male)	1.5 ± 2	−2.9	5.9	0.48
Site(Radio-tracking Bay)	−1.3 ± 2	−5.7	3.1	0.54

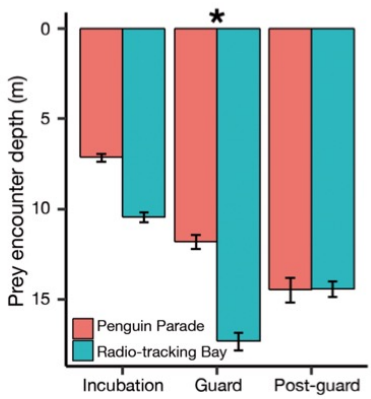

Fig. 4. Mean prey encounter depths and 95% confidence interval over the 2015/2016 breeding season of little penguins at Penguin Parade and Radio-tracking Bay. Asterisks (*) indicate significant differences between sites in guard (LMM: $\beta = 6$, SE = 1.46, $t = 4$, $p < 0.001$)

Prey encounter rate

The prey encounter rate on each foraging trip ranged from 10.4 to 75.0 dives h_{div}^{-1} in incubation, from 10.0 to 44.0 dives h_{div}^{-1} in guard and from 11.0 to 32.5 dives h_{div}^{-1} in post-guard. Sex and trip duration had no significant effect on the prey encounter rate in any stage (Table 4). At guard, birds from

Table 4. Parameter estimates (β), standard errors (SE), lower (2.5%) and upper (97.5%) confidence intervals (CI) and p-values for the general least squared models for prey encounter rate. Models were fitted for incubation, guard and post-guard. Base level of the factor 'sex' is 'female' and base level of the factor 'site' is 'Penguin Parade'

Effect	β + SE	Lower CI	Upper CI	p
Incubation				
Intercept	20.4 ± 7.2	5	35.9	0.01
Sex(male)	13.3 ± 7.2	−2	28.7	0.08
Site(Radio-tracking Bay)	7.1 ± 7.2	−8.3	22.6	0.34
Trip duration	2.8 ± 2	−1.5	7	0.18
Guard				
Intercept	29.5 ± 2.2	24.8	34.2	<0.001
Sex(male)	−1.1 ± 2.5	−6.4	4.1	0.6
Site(Radio-tracking Bay)	−6.1 ± 2.5	−11.3	−0.9	0.02
Post-guard				
Intercept	15.7 ± 3	9.2	22.1	<0.001
Sex(male)	2.5 ± 3.2	−4.5	9.5	0.46
Site(Radio-tracking Bay)	−1.7 ± 4	−10.3	6.8	0.67
Trip duration	1 ± 1.3	−1.7	3.8	0.42

Radio-tracking Bay performed significantly fewer dives with prey encounter per hour diving than birds from Penguin Parade (6 dives fewer per hour, GLS $p < 0.05$; Table 4, Fig. 5a).

Foraging efficiency

Over the 2 mo of the tracking period, the APMS recorded a total of 1091 foraging trips (n) with a known associated mass change for 77 different individuals (N) at Penguin Parade and 831 foraging trips for 61 different individuals at Radio-tracking Bay. The body mass change per trip was significantly higher at Penguin Parade than Radio-tracking Bay in guard trips (mean ± SE, 302 ± 9 g vs. 190 ± 17 g; LMM $p < 0.001$, n = 500, N = 101; Fig. 5b) and in post-guard trips (278 ± 7 g vs. 219 ± 12 g; LMM $p < 0.001$, n = 897, N = 100; Fig. 5b), but no significant differences were observed during incubation (243 ± 10 g for Penguin Parade vs. 209 ± 12 g for Radio-tracking Bay; LMM p = 0.17, n = 525, N = 125; Fig. 5b). There were no significant differences between sites in adult body mass at the beginning of the trips (Table A3).

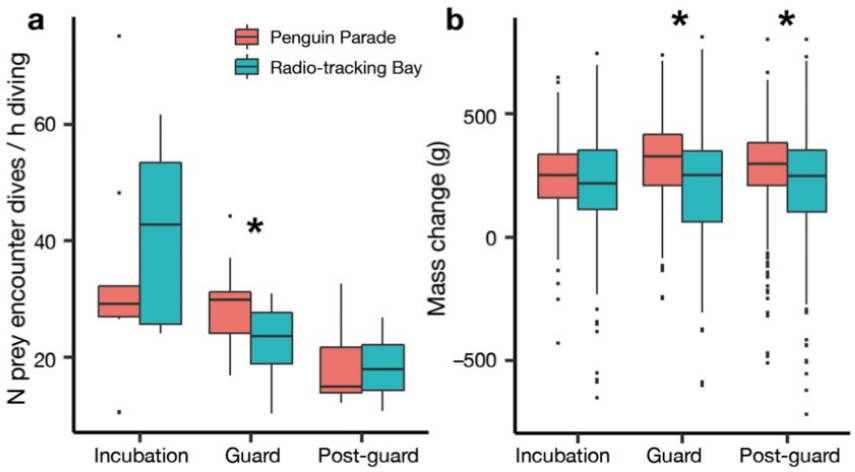

Fig. 5. (a) Prey encounter rate per breeding stage of little penguins as number of dives with prey encounter per hour diving given a foraging trip. Asteriks (*) indicate significant differences between sites in guard (GLS: β = −6.1, SE = 2, $p < 0.05$, *F*-test GLS). (b) Adult mass change per breeding stage associated with foraging trips recorded by the automated penguin monitoring systems at Penguin Parade and Radio-tracking Bay during the first clutch of the 2015/2016 breeding season. *Significant differences in guard (LMM: β = −126, SE = 23, 95% CI = −172, −80, $t = -5.4$, $p < 0.001$) and in post-guard between sites (LMM: β = −61, SE = 17, 95% CI = −95, −26, $t = -3.5$, $p < 0.001$). Lower and upper hinges of boxplots: first and third quartiles, respectively; bar inside box: median; whiskers: largest and smallest values ≤1.5× the interquartile range from the hinges; dots (outliers): data beyond the end of the whiskers

Table 5. Number of little penguin chicks fledged per pair at Penguin Parade and Radio-tracking Bay during 2004–2015. na: data not available

Year	Chicks fledged	
	Penguin Parade	Radio-tracking Bay
2015	2.6	1.7
2014	1.5	na
2013	0.6	0.7
2012	2.1	1.1
2011	1.2	1.0
2010	1.7	1.0
2009	1.7	1.2
2008	0.6	1.0
2007	1.3	1.0
2006	0.9	0.8
2005	1.3	1.1
2004	0.8	0.5

Reproductive performance

Breeding success was consistently 10–30% higher at Penguin Parade than Radio-tracking Bay from 2004/2005 until 2015/2016 (Table 5). The breeding success in 2015/2016 was 2.6 chicks fledged per pair at Penguin Parade and 1.7 chicks fledged per pair at Radio-tracking Bay (Table 5). There were no significant differences in the peak chick mass between sites (1180 ± 9 vs. 1173 ± 11 g; LM: $\beta = -13.59$, SE = 15, $t = -0.87$, $p = 0.4$).

DISCUSSION

Using GPS, depth and accelerometer data loggers simultaneously, we observed a strong spatial segregation between 2 little penguin breeding sites that are only 2 km apart, although their home ranges have a high potential to overlap given that the species' foraging range is 20 km during the breeding season (Collins et al. 1999). Birds preferred areas adjacent to their own breeding site in accordance with Cairns (1989) and Wakefield et al. (2013), suggesting that foraging areas are site-specific within the colony. Several seabirds have shown partial or complete horizontal spatial segregation among colonies (e.g. Grémillet et al. 2004, Wakefield et al. 2013), even at small scale (Masello et al. 2010, Ceia et al. 2015). Other studies, for example, have used 3-dimensional foraging behaviour to test for competition between congeneric penguins (Cimino et al. 2016) and to quantify foraging volumes (Ainley et al. 2015). Here, we used acceleration data loggers in addition to GPS and depth data considered by the aforementioned studies. This approach allowed us to not only identify horizontal segregation between the site-specific foraging areas, but also vertical differences between sites in the spatial distribution of prey encounters over the breeding season, linking birds' movements with their behaviour and prey distribution in 3 dimensions.

Birds from Penguin Parade and Radio-tracking Bay travelled in different directions relative to their breeding sites, leading to a significant within-colony spatial foraging segregation between sites. Central-place foragers such as little penguins can initiate their foraging trips on a bearing consistent with the general orientation of their breeding site (Robson et al. 2004) and return to previous successful foraging grounds (Carroll et al. 2018). Further, foraging aggregations of little penguins have been associated with the departure time from the colony (Sutton et al. 2017). In particular, birds from Phillip Island are known to form non-random groups when departing (Daniel et al. 2007), providing the opportunity for foraging aggregations to form. Thus, the use of site-specific pathways may facilitate information exchange among conspecifics from the same breeding site and their aggregation at sea, mediating the foraging segregation observed here.

The spatial segregation between sites intensified throughout the breeding season, and prey encounter depth increased from incubation to chick-rearing. Chick-rearing is a highly demanding period due to the increasing energetic requirements of the young combined with the central-place foraging constraints (Gales & Green 1990, Bevan et al. 2002). Thus, indirect competition for food resources is expected to increase from incubation to chick-rearing, and parents can meet this high energetic demand by increasing foraging effort, either by diving deeper (e.g. gentoo penguins *Pygoscelis papua*, Ratcliffe et al. 2018; northern rockhopper penguins *Eudyptes moseleyi*, Booth et al. 2018), switching diet to more energetic prey (e.g. gentoo penguins, Handley et al. 2017) or expanding the foraging area (e.g. Adélie penguins *P. adeliae*, Ainley et al. 2004). Expanding the foraging area during the guard stage compared to incubation is not an option for little penguins, and at-sea density of penguins is expected to be higher, as guard trips are always 1 d long (Chiaradia & Kerry 1999, Chiaradia & Nisbet 2006), whereas incubation trips can last several days (Kato et al. 2008). Instead, birds seemed to mitigate within-site competition by diving deeper (i.e. deeper prey encounters) and between-site competition by increasing segregation level. Wakefield et al. (2013) were only able to reproduce the spatial

segregation between northern gannet *Morus bassanus* colonies if models incorporated density-dependence constraints (Ashmole's halo), interactions with neighbouring colonies (hinterland model), information transfer and memory. Our results suggest similar underpinning mechanisms for the within-colony spatial segregation observed here.

Inter- and intra-specific competition and changes in environmental conditions have the potential to reduce the abundance and availability of prey and enlarge the foraging area (Lewis et al. 2001, Pichegru et al. 2013). Areas off Phillip Island support a high biomass and diversity of seabirds (Norman 1991, Dann et al. 2003) and a large Australian fur seal *Arctocephalus pusillus doriferus* colony (Kirkwood et al. 2005). Little penguins in our study area are at the top of the food chain (Chiaradia et al. 2016). Small pelagic fish are their key prey, and other common prey include barracouta *Thyrsites atun*, red cod *Pseudophysis bachus* and arrow squid *Nototodarus gouldi* (Chiaradia et al. 2003, 2010). These are also significant prey for other predators within the area, such as short-tailed shearwaters *Puffinus tenuirostris* (Montague et al. 1986), crested terns *Thalasseus bergii* (Chiaradia et al. 2002) and Australian fur seals (Deagle et al. 2009). Little penguins may compete with crested terns for food resources or be affected by the similar prey availability variation, as low numbers of breeding crested terns have coincided with low breeding success of little penguins in the past (Chiaradia et al. 2002). However, knowledge is scarce about inter-specific competition interactions among marine predators off Phillip Island and how they might affect penguins' foraging behaviour.

Bathymetry is another important factor influencing prey availability and at-sea distribution of many marine predators, including marine mammals and seabirds (e.g. Guinet et al. 2001, Yen et al. 2004, Weimerskirch et al. 2007). Colonies with shallower waters are favourable to little penguins, as birds show a lower diving effort (i.e. total diving duration per hour) compared to conspecifics from colonies with deeper waters (Chiaradia et al. 2007, Meyer et al. 2017). Here, the kernel UDs of prey encounters showed that the Radio-tracking Bay birds used a deeper and larger foraging area than birds from Penguin Parade throughout the breeding season. Therefore, individuals from Radio-tracking Bay were not only exposed to less favourable bathymetry conditions, but might have also experienced higher levels of intra-specific competition within their area, reducing prey availability in the site vicinity, which forced birds to forage further in accordance with Ashmole's halo theory (Ashmole 1963). Deeper prey encounters in the water column could be a mechanism by birds from Radio-tracking Bay to expand their niche width and reduce within-site competition, as has been observed in gentoo penguins (Ratcliffe et al. 2018). At the same time, generalist marine predators, such as little penguins (Chiaradia et al. 2010), modify their foraging behaviour according to prey type (Elliott et al. 2008) and match the 3-dimensional distribution of their prey (Carroll et al. 2017). Hence, differences in prey encounter depths could also be a response to availability of different prey types at each site or different prey vertical distribution. However, diet composition in the year of this study did not differ between sites, with birds foraging mainly on sardines (C. Cavallo unpubl. data). Therefore, rather than differences in diet, we suggest that bathymetry and higher competition within Radio-tracking Bay foraging grounds could explain the higher diving effort (i.e. deeper prey encounters) and the lower prey encounter rate at this site during chick-rearing, leading to a lower foraging efficiency (i.e. lower mass gain after foraging trips).

Seabirds with a small foraging range are highly sensitive to variations in local food availability and abundance (Chiaradia et al. 2010, Kowalczyk et al. 2014, 2015). All of the foraging parameters analysed here (i.e. size of foraging area, prey encounter depth, prey encounter rate and body mass gain) suggest that Penguin Parade birds foraged more efficiently in more profitable foraging grounds. Higher body mass gain after foraging trips mean larger chick meals (Saraux et al. 2011, 2016). From albatrosses to little penguins, when chicks receive large and frequent meals they have a higher probability of fledging (Huin et al. 2000, Saraux et al. 2016). Hence, the higher body mass gain by Penguin Parade adults during the 2015/2016 season could result in larger meals for their chicks and a higher breeding success. The breeding records for the last 12 yr indicated that Penguin Parade has consistently been more productive than Radio-tracking Bay, suggesting that this site is overall more successful. Nonetheless, both sites were highly productive (>1.5 chicks fledged pair^{-1}) in the 2015/2016 season, which is indicative of no food limitations (Chiaradia & Nisbet 2006), and raised chicks with similar peak chick mass. These findings suggest that when food resources are not limiting, the different foraging strategies from each site and their costs result in chicks with similar condition, and other factors, such as nest predation (Ekanayake et al. 2015), might be partially responsible for the differences in breeding success. Hence, the link between foraging behaviour and breeding success remains

unknown. Yet, birds from Penguin Parade could have an advantage by foraging more efficiently and in more profitable areas than those from Radio-tracking Bay. Future studies should investigate how the differences in foraging efficiency between sites influence chick fitness in years of food shortage. Overall, our findings show that differences in foraging behaviour of birds from the same colony were associated with breeding sites, and the site with the highest foraging efficiency produced more fledglings.

CONCLUSIONS

Bio-logging data have been used worldwide in numerous studies to identify key areas for marine megafauna (e.g. Bailey & Thompson 2009, Lascelles et al. 2016, Augé et al. 2018), and seabird biodiversity hotspots in the oceans coincide with areas in serious need of conservation (Ramírez et al. 2017). However, incorporation of these data into conservation strategies remains underutilised (Hays et al. 2016). Our findings highlight the importance of understanding fine-scale spatial segregation and intra-specific competition mechanisms within large colonies and their consequences for seabird foraging and breeding ecology. Within-colony segregation may be the norm when large seabird colonies are spatially structured in breeding sites, leading to site-specific foraging behaviours. Therefore, we argue that assessing more than 1 breeding site, over different breeding stages, is necessary to capture the foraging behaviour and breeding success variability within a colony, providing meaningful knowledge on marine spatial usage to obtain accurate population assessments and to incorporate into conservation strategies.

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Appendix

Table A1. Total areas (km^2) of kernel utilisation distribution (UD) for the locations of prey encounters at Penguin Parade and Radio-tracking Bay during the 2015/2016 little penguin breeding season

Stage	% UD	Penguin Parade	Radio-tracking Bay
Incubation	95	1038	4099
	50	296	519
	25	122	152
Guard	95	438	716
	50	119	205
	25	53	84
Post-guard	95	442	4562
	50	109	1004
	25	45	289

Table A2. Estimated utilisation distribution (UD) overlap index (UDOI) in UDs between Penguin Parade and Radio-tracking Bay little penguins within incubation and post-guard breeding stages after dropping the long trips. Empirical p-value (p): the proportion of 1000 randomised overlaps that were smaller than the observed overlap. Randomised overlaps were only calculated if observed overlap was > 0

UD(%)	Stage	UDOI	p
95	Incubation	0.45	0.037
	Post-guard	0.11	0.001
50	Incubation	0.003	0.001
	Post-guard	0	–
25	Incubation	0	–
	Post-guard	0	–

Table A3. Parameter estimates (β), standard errors (SE), lower (2.5%) and upper (97.5%) confidence intervals (CI) and p-values for the linear mixed effect models with a random intercept of bird ID for little penguin adult body mass at the beginning of foraging trips. Models were fitted for incubation, guard and post-guard. Base level of the factor 'site' is 'Penguin Parade'

Fixed effect	β + SE	Lower CI	Upper CI	p
Incubation				
Intercept	1097 ± 13.5	1071	1124	<0.001
Site(Radio-tracking Bay)	−24.7 ± 19	−62	13	0.2
Guard				
Intercept	1067 ± 12	1044	1091	<0.001
Site(Radio-tracking Bay)	−30.8 ± 20	−71	9	0.13
Post-guard				
Intercept	1075 ± 11	1055	1096	<0.001
Site(Radio-tracking Bay)	−20 ± 18	−55	14	0.25

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Appendix C: Scat Collection Protocol created for (and in use by) Phillip Island Nature Parks

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Scat collection protocol: Phillip Island

Equipment

- Cardboard
- U-shaped turf pegs
- Small tubes half filled with 70-80% ethanol (EtOh)
- Stainless steel spatula (5-6mm wide) and stainless steel tweezers (flat-ended, no more than 6mm wide)
- Waterproof marker pen to mark tubes.
- Kimwipes or tissues
- Sturdy canvas (coles/woolies type) shopping bags
- Hammer
- Pencil and waterproof/robust notebook
- Bag for rubbish

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Sample size

Aim to contribute to the final dataset 20-30 scats per timepoint, 50-100 per stage. There is a fair bit of attrition between stages of collection and analysis with this method, so you should aim to collect more samples than needed for analysis. Best to sample 50 nests per time-point.
Preparation

Prepare sterile collection tubes with 70-80% ethanol. It is important to fill tubes to half-full only so that you can shake them to mix thoroughly with scat sample.
Cut cardboard to place in the entrance of nests
Natural nest card dimensions: roughly 25*40 cm
Wooden boxes: 30*30 cm
Prepare notebook with nests to be sampled, e.g.:

Site: Area 3 Date: 17/9/2017 Collected: 25/50

Nest number	Breeding stage I=incubation G= guard PG= postguard	Cardboard deployed	Sample collected	Sample notes W= Wet (collect) M=Moist (collect) Dry=Too dry to collect Dirty= too dirty to collect
3090	G	✔	✔	M
3091	I	✔	✗	Dirty
3100	PG	✗	NA	Chicks fledged

Site: RTB Date: 17/9/2017 Collected: 30/50

Nest number	Breeding stage I=incubation G= guard PG= postguard	Cardboard deployed	Sample collected	Sample notes W= Wet (collect) M=Moist (collect) Dry=Too dry to collect Dirty= too dirty to collect
43119	I	✔	✗	Nest predated
43020	I	✔	✔	W
43001	G	✔	✗	Penguins destroyed cardboard

These columns get filled in in the field Day 1
These columns get filled in in the field Day 2

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Deployment (Day 1)

Wooden nest-boxes

1. Fold sides of cardboard up and slide into doorway so that the "hallway" of the nest is covered and the cardboard protrudes out the front.

2. Hammer a U-shaped turf peg into the front third of the cardboard to secure into nest.

Natural nests

1. Fold one side of the cardboard up and insert into the "hallway" of the nest with the folded side resting against the wall. It is important to determine whether the burrow turns left or right, and only cover the back wall (i.e. left hand wall if burrow bends right). This is to ensure you do not block the entrance to the burrow.

2. Secure the cardboard with a peg in the doorway.

With these nests, a large portion of the card should protrude from the nest. The cardboard should NOT intrude into the nest chamber itself.

Every nest is different, so care needs to be taken to set up the cardboard in the best way for each nest. If there is no easy wall to set cardboard against, just use a flat piece, as shown above.

It is very important that we don't damage nests while sampling. This includes allowing the cardboard to displace the vegetation shielding the burrow. Anything that makes it easier for ravens to get in must be avoided.

Collection (Day 2)

1. Return to field site with a couple of bags to collect cardboard in.

2. Assess cardboard and determine whether scat is present AND worth collecting. See images at end doc.

3. Avoid any scat that is contaminated with dirt, these samples will not amplify. A tiny amount of sand is ok, but not soil.

4. Collect only wet or moist samples. Dry samples are generally too degraded. Collect only if desperate and make a note.

5. It is important to minimize the collection of vegetation and other debris.

6. Starving (incubating) birds often leave goopy, green or yellow scats. These can be collected, but contain very little prey DNA.

7. Collect a pea-sized portion of the dark part of the scat (not the white part, which is mainly urea).

8. Transfer scat to tube and mix with spatula. Close cap and shake until scat is completely mixed with ethanol (grey, sparkly solution).

9. Write the nest number on the cap of the tube and return to collection box.

10. Wipe spatula thoroughly to remove traces of scat and avoid contaminating the next sample.

In the office (Day 2)

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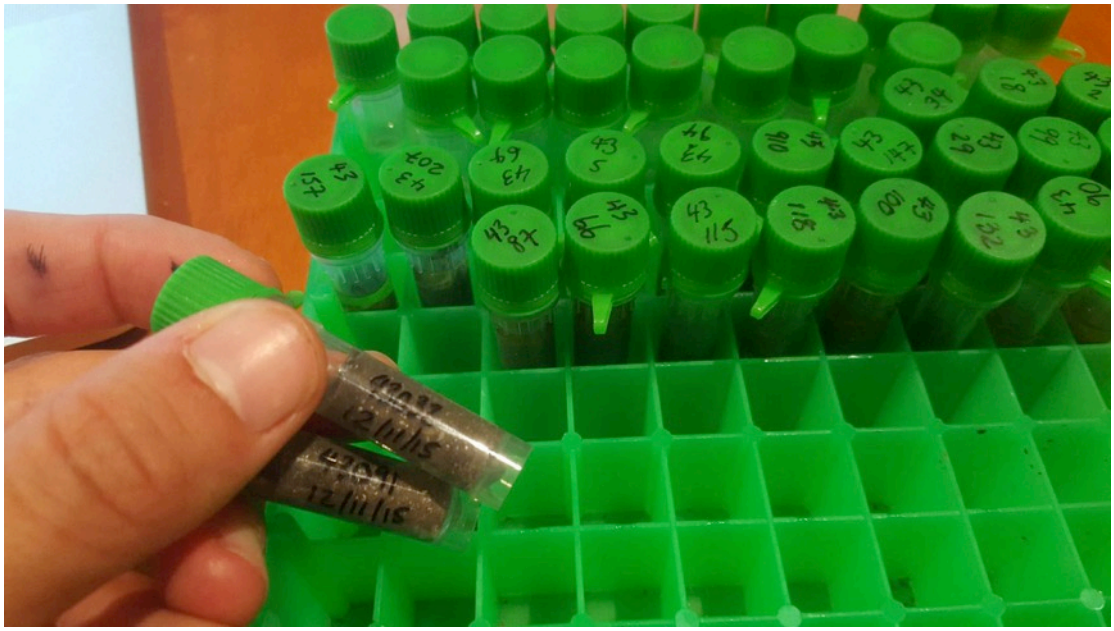

1. Label tubes with full nest number and date on the tube with waterproof pen.
2. Sticky tape over labels to prevent them from rubbing off. Almost no pen is ethanol proof. Sticky tape seems to be the only thing that protects the label.
3. Put in the -20°C freezer straight away.
4. Update spreadsheet.
5. Cut cardboard for next week

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Scat notes

In the lab, the DNA is extracted from the scats and then amplified via the Polymerase Chain Reaction. PCR amplification is a competitive process, so DNA that is more abundant in a scat gets amplified to a greater extent than trace amounts of DNA. This is why it is so important to avoid contamination between scats and from the environment to the scats. Unfortunately, it is impossible to determine from a stored sample whether it will amplify well or not. So the best time to make these decisions is when collecting in the field.

Any shade of grey is a good sample. Light grey likely to be chicks, dark grey likely to be adults. Different prey affects the shade and lustre of the scat. I collect any grey scats and try and minimize the amount of white urea collected. Yellow or green scats contain very little food. These scats are typical of birds that have been sitting on eggs for several days, and so are common during incubation. Because we need to collect scats from each stage, do collect green or yellow scats, but make a note of their colour in the field-book.

Soil contains humic substances that inhibit the PCR amplification process. Therefore, if a scat is covered in soil it is not worth collecting. Additionally, soil is full of contaminants such as fungi and insects, whose DNA we don't want in the sample. If you can pick lumps of soil off the sample and avoid getting them in the tube then you can collect this sample, you just don't want soil getting into the tube or mixed in with the sample. Sand contains less of these humic substances, but will still contain contaminants. Where you can, only collect clean scats, but a few grains of sand will be ok.

Avoid letting vegetation get into the tube. This includes sticks and leaves etc. living or dead.

If there are lots of parasites present (worms or fleas or elsewise crawling in the sample) try and collect the scat with the least parasites. This comes back to PCR being a competitive process. With lots of fresh, living parasite DNA in the sample, the more degraded prey DNA will get less of a chance to amplify.

From the second a scat leaves the bird and is exposed to air and light, the DNA begins to degrade. This is why it is important to collect samples the next morning. I try to collect only wet and moist samples, because dry samples amplify much less DNA. Additionally, as time wears on the number of contaminant and parasite DNA rises, while the amount of prey DNA decreases.

Some notes on scat appearance and quality

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Some beautiful samples here

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Tiny baby chick poo. If both chick and adult poo is present, prioritise the adult poo. Unless it is green… then prioritise grey poo.

Prey DNA in chick poo will be more degraded because of the time spent in the parent's gullet.

Nothing to collect here. One is flooded, one the penguins have destroyed.

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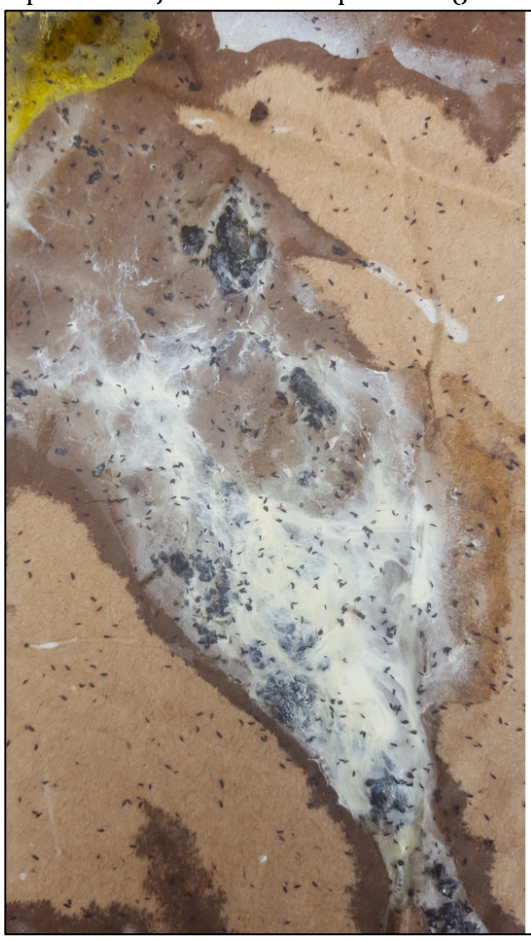

Try to collect a sample without (or with very few) flies on it but they may have already contaminated the sample.

Lots of clumps of brown dirt here that look like poo. There is poo somewhere on this board, but it will be difficult to distinguish it from the soil in this picture. If contaminated by soil, don't collect.

Lots of clumps of brown dirt here that look like poo. Lots of grey poo too. Avoid contaminated samples

This is really tricky because the scat is a light brown-grey colour. It is difficult to distinguish it from the soil.

Have a look for physical differences to separate them. If really unsure, don't bother collecting.

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Work around the dirt here.
Samples in bottom right
most workable

<< Green poo

<< Mixed poo

<< Good poo sample

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This is penguin vomit. It's very uncommon, but don't accidentally collect it.

Check out the cool parasites!

Bioinformatics Pipeline-18s

Cathy Cavallo

6/24/2018

Next Generation Sequencing Bioinformatics Pipeline
Broad spectrum primer example -- 18s_SSU

This code will allow the user to clean and organise their DNA sample library and then calculate measures of taxon abundance for analysis. Important steps include:

- Removing
  - samples with less than 100 target reads
  - Non-target sequences
  - Specific samples (e.g. those that did not meet thresholds for inclusion, like CT)
- Producing group totals for higher taxonomic levels
- Calculating the frequency of occurrence (FOO) and relative read abundance (RRA) of items in the dataset
- Matching samples with sample metadata for analysis

Preparation

Load packages

```
library(dplyr)

##
## Attaching package: 'dplyr'

## The following objects are masked from 'package:stats':
##
##  filter, lag

## The following objects are masked from 'package:base':
##
##  intersect, setdiff, setequal, union

library(tidyverse)

## Loading tidyverse: ggplot2
## Loading tidyverse: tibble
## Loading tidyverse: tidyr
## Loading tidyverse: readr
## Loading tidyverse: purrr
```

```
## Conflicts with tidy packages ----------------------------------------------

## filter(): dplyr, stats
## lag(): dplyr, stats

library(data.table)

##
## Attaching package: 'data.table'

## The following object is masked from 'package:purrr':
##
##  transpose

## The following objects are masked from 'package:dplyr':
##
##  between, first, last

library(tidyr)
```

Load identified sample library

```
Metazoa<-read.csv("CCavallo_metazoa_2016_2018_20180709.csv",stringsAsFactors =
FALSE)
```

Inspect

```
head(Metazoa)
```

Clean

Sum all reads in each sample and food reads in each sample

```
Total<-colSums(Metazoa[,6:765])#Totals for numeric columns (from first to last
sample columns)
PreyOnly<-Metazoa[Metazoa$Type == "Prey",]
TotalFood<-colSums(PreyOnly[,6:765])#remember to change 6:744 to the column
numbers for your library
```

Create a vector to retain only samples w > 100 food reads

```
ColumnIndex<-which(as.vector(TotalFood >= 100))
ColumnIndex<-ColumnIndex+5 #Add 5 to all values in this vector because we
skipped the first five columns when calculating the column sums. i.e. what was
column 1 will become column 6, column 3 becomes column 8 etc..
```

Drop non-food taxa and drop samples with < 100 food reads

```
ncol(Metazoa)

## [1] 765
```

```
MetazoaNoLowreads<-PreyOnly[,c(1:5, ColumnIndex)]
ncol(MetazoaNoLowreads)

## [1] 447
```

Organise

Create rows with group totals

You will need to define the names of your groups in a column called "Group" in your library prior to importing into R.

```
MetazoaGrpTotals<-MetazoaNoLowreads%>%
 group_by(Group)%>%
 summarise_at(-c(1:4),funs(sum))
```

Add the group totals to your library

```
ColsToAppend<-data.frame(matrix(rep(NA, (nrow(MetazoaGrpTotals)*4)), ncol = 4))
names(ColsToAppend)<-names(MetazoaNoLowreads[,1:4])
MetazoaWGrpTotals<-cbind(ColsToAppend, MetazoaGrpTotals)
MetazoaTotalData<-rbind( MetazoaNoLowreads, MetazoaWGrpTotals)
```

Drop redundant columns

Remember that the column numbers you choose to drop will depend on what is in your library.You may not need to drop any.

```
MetazoaForFilters<-MetazoaTotalData[,c(3,5:ncol(MetazoaTotalData))]
```

Calculate

Now that your library is organised and includes only the samples and sequences necessary for your analysis, you can start calculating some measures of presence and abundance!

Relative read abundance (RRA)

Get sample totals again

```
MetazoaForFilters$Class[is.na(MetazoaForFilters$Class)]<-"GroupTotal"
SampleTotals<-colSums(MetazoaForFilters[which(MetazoaForFilters$Class !=
"GroupTotal"),3:ncol(MetazoaForFilters)])
```

Check that the totals for groups and totals for Metazoa are the same.

```
GROUPTotals<-colSums(MetazoaForFilters[which(MetazoaForFilters$Class ==
"GroupTotal"),3:ncol(MetazoaForFilters)])
SampleTotals<-c(NA, NA, SampleTotals)
```

Bind them

```
MetazoaForFilters<-rbind( MetazoaForFilters, SampleTotals)
MetazoaForFilters$Group[nrow(MetazoaForFilters)]<-"Total"
```

Convert each cell or group total to a percentage of the sample total
This converts the library into a sheet of percentages.

```
class(MetazoaForFilters$Group)

## [1] "character"

MetazoaRRA<-MetazoaForFilters
MetazoaRRA[, 3:ncol(MetazoaRRA)]<-NaN #replace values with NaN (not a number)
for(i in 3:ncol(MetazoaForFilters)){#Loop from column 3 til the end (3:ncol)
 ActiveTotal<-MetazoaForFilters[MetazoaForFilters$Group == "Total", i]#subset
the data so that only the total is left for the column we are working in
 for(j in 1:nrow(MetazoaForFilters)){#and then from the first row to the last
row
 ActiveRRA<-(MetazoaForFilters[j,i]/ActiveTotal)*100 #calculate RRA Column i,
row j.
 MetazoaRRA[j, i]<-ActiveRRA #in the Metazoa RRA data frame update the exact
corresponding cell with the RRA value you have just calculated
 }
}
```

Frequency of occurrence (FOO)

Frequency of occurrence describes the percentage of samples in the dataset in which a given taxon is present, but, like all presence–absence records, can overestimate the importance of common prey items that are ingested in very small amounts, including in the digestive tract of targeted prey (secondary predation).

Convert all cells where RRA is >1% to "1", cells <1% get "0".

This converts the library to presence/absence type data.

```
MetazoaFOO<-MetazoaRRA
MetazoaFOO[, 3:ncol(MetazoaFOO)]<-NaN #replace values with NaN (not a number)
for(i in 3:ncol(MetazoaRRA)){#Loop from column 3 til the end (3:ncol)
 for(j in 1:nrow(MetazoaRRA)){#and then from the first row to the last row
 if(MetazoaRRA[j,i]>=1){
  MetazoaFOO[j,i]<-1#present
 }else{
  MetazoaFOO[j,i]<-0#absent
 } #calculate RRA Column i, row j.
 }
}
```

Rename group totals

```
for(i in which(MetazoaRRA$Class=="GroupTotal")){ MetazoaRRA$Class[i]<-
paste(MetazoaRRA$Group[i],"Total")
}
for(i in which(MetazoaFOO$Class=="GroupTotal")){ MetazoaFOO$Class[i]<-
paste(MetazoaFOO$Group[i],"Total")
}
for(i in
which(MetazoaForFilters$Class=="GroupTotal")){ MetazoaForFilters$Class[i]<-
paste(MetazoaForFilters$Group[i],"Total")
}
```

Clean away some stuff you don't need

```
rm(ColsToAppend,MetazoaWGrpTotals,MetazoaGrpTotals,MetazoaTotalData,Metazoa,Che
ck,MetazoaNoLowreads,PreyOnly,MetazoaRRAt,MetazoaFOOt,MetazoaGrpTotals)

## Warning in rm(ColsToAppend, MetazoaWGrpTotals, MetazoaGrpTotals,
## MetazoaTotalData, : object 'Check' not found

## Warning in rm(ColsToAppend, MetazoaWGrpTotals, MetazoaGrpTotals,
## MetazoaTotalData, : object 'MetazoaRRAt' not found

## Warning in rm(ColsToAppend, MetazoaWGrpTotals, MetazoaGrpTotals,
## MetazoaTotalData, : object 'MetazoaFOOt' not found

## Warning in rm(ColsToAppend, MetazoaWGrpTotals, MetazoaGrpTotals,
## MetazoaTotalData, : object 'MetazoaGrpTotals' not found
```

Combine with sample metadata

Transpose data back into a format that makes more sense

```
names<-MetazoaRRA$Class
MetazoaRRAt<-as.data.frame(t(MetazoaRRA[,-c(1:2)]))#transpose all but the first
column (Sample)
colnames(MetazoaRRAt)<-names#set column names
MetazoaRRAt<-cbind(rownames(MetazoaRRAt),data.frame(MetazoaRRAt))
names(MetazoaRRAt)[1]<-"Sample"

MetazoaRRAt$Sample <- substr(x = MetazoaRRAt$Sample, start = 2,stop=100) #If
the sample name starts with 'X' this should work. If it starts with 'X.' you
will need to change the start = to 3

names<-MetazoaFOO$Class
MetazoaFOOt<-as.data.frame(t(MetazoaFOO[,-c(1:2)]))
colnames(MetazoaFOOt)<-names
MetazoaFOOt<-cbind(rownames(MetazoaFOOt),data.frame(MetazoaFOOt))
names(MetazoaFOOt)[1]<-"Sample"

names<-MetazoaForFilters$Class
MetazoaForFilterst<-as.data.frame(t(MetazoaForFilters[,-c(1:2)]))
colnames(MetazoaForFilterst)<-names
```

```
MetazoaForFilterst<-
cbind(rownames(MetazoaForFilterst),data.frame(MetazoaForFilterst))
names(MetazoaForFilterst)[1]<-"Sample"
```

Drop leading X's if you have them

```
MetazoaFOOt$Sample <- substr(x = MetazoaFOOt$Sample, start =2,stop=100)

MetazoaForFilterst$Sample <- substr(x = MetazoaForFilterst$Sample, start
=2,stop=100)
```

Bring in the sample field data (tied to sample number)

```
fielddata1<-read.csv("metadataIDs_metazoa_20180710.csv",stringsAsFactors =
FALSE)

fielddata<-fielddata1[c(1:10)]
```

Rename sample in the metadata/field data database to match sample name in sequence library

```
colnames(fielddata)[colnames(fielddata)=="X20180215.labels"]<-"SampleField"
```

Then bind sample field data to sample diet data for both RRA and FOO sheets

You may have samples in either your library or field database that you want to drop. You can drop these here.

```
todrop<-
c("X2016a_CRC321_s376","X2018_CRC177_s034","X2018_CRC323_s226","X2018_CRC165_s2
30","X2018_CRC321_s267","X2018_CRC183_s279","X2018_CRC264_s170","X2018_CRC264_s
171","X2018_CRC422_s216","X2018_CRC16095_s096","X2018_CRC122_s210","X2018_CRC12
2_s209","X2018_CRC321_s267","X2018_CRC323_s227","X2018_CRC099_s095",
"X2016a_CRC376_s327")

todrop2<-
c("X2016a_CRC321_s376","X2018_CRC177_s034","X2018_CRC323_s226","X2018_CRC165_s2
30","X2018_CRC321_s267","X2018_CRC183_s279","X2018_CRC264_s170","X2018_CRC264_s
171","X2018_CRC422_s216","X2018_CRC16095_s096","X2018_CRC122_s210","X2018_CRC12
2_s209","X2018_CRC321_s267","X2018_CRC323_s227","X2018_CRC099_s095",
"X2016a_CRC376_s327","X2016a_CRC376_s327")

MetazoaRRAtX<-MetazoaRRAt[!rownames(MetazoaRRAt)%in%todrop,]
MetazoaFOOtX<-MetazoaFOOt[!rownames(MetazoaFOOt)%in%todrop2,]
MetazoaForFilterst<-
MetazoaForFilterst[!rownames(MetazoaForFilterst)%in%todrop,]
write.csv(MetazoaFOOtX,"Metazoasamplesfiltered.csv")
```

Now bring in the data

```
fielddatanames<-names(fielddata)
MetazoaRRAnames<-names(MetazoaRRAtX)
```

Clear away some extraneous data frames and things

```
rm(ActiveSample,fielddataRow,RRARow,RowsBound1,MetazoaRRAcomplet,ActiveTotal,Ac
tiveRRA)

## Warning in rm(ActiveSample, fielddataRow, RRARow, RowsBound1,
## MetazoaRRAcomplet, : object 'ActiveSample' not found

## Warning in rm(ActiveSample, fielddataRow, RRARow, RowsBound1,
## MetazoaRRAcomplet, : object 'fielddataRow' not found

## Warning in rm(ActiveSample, fielddataRow, RRARow, RowsBound1,
## MetazoaRRAcomplet, : object 'RRARow' not found

## Warning in rm(ActiveSample, fielddataRow, RRARow, RowsBound1,
## MetazoaRRAcomplet, : object 'RowsBound1' not found

## Warning in rm(ActiveSample, fielddataRow, RRARow, RowsBound1,
## MetazoaRRAcomplet, : object 'MetazoaRRAcomplet' not found
```

Match up your field data with your RRA data

```
MetazoaRRAcomplet<-data.frame()
i=1
for(i in 1:nrow(MetazoaRRAtX)){
 ActiveSample<-MetazoaRRAtX$Sample[i]# isolating that one cell (sample in row
i)
 fielddataRow<-fielddata[fielddata$SampleField == ActiveSample,]#subset to a
single row in RRA. Works for pulling the small sheet into the big sheet.
 RRARow<-MetazoaRRAtX[i,] #i is always the active row
 RowsBound1<-cbind(fielddataRow,RRARow)
 MetazoaRRAcomplet<-rbind(MetazoaRRAcomplet,RowsBound1)
}

MetazoaRRAcomplet<-data.frame(MetazoaRRAcomplet)
```

Match up your field data with your FOO data

```
i=2
MetazoaFOOnames<-names(MetazoaFOOtX)
MetazoaFOOcomplet<-data.frame()
for(i in 1:nrow(MetazoaFOOtX)){
 ActiveSample<-MetazoaFOOtX$Sample[i]
 fielddataRow<-fielddata[fielddata$SampleField == ActiveSample,]
 FOORow<-MetazoaFOOtX[i,]
 RowsBound<-cbind(fielddataRow,FOORow)
 MetazoaFOOcomplet<-rbind(MetazoaFOOcomplet,RowsBound)
}

MetazoaFOOcomplet<-data.frame(MetazoaFOOcomplet)
```

Match up your field data with MetazoaForFilters

```
MetazoaForFilterstcomplet<-data.frame()
i=187
for(i in 1:nrow(MetazoaForFilterst)){
 ActiveSample<-MetazoaForFilterst$Sample[i]
 fielddataRow<-fielddata[fielddata$SampleField == ActiveSample,]
 RRARow<-MetazoaForFilterst[i,]
 RowsBound1<-cbind(fielddataRow,RRARow)
 MetazoaForFilterstcomplet<-rbind(MetazoaForFilterstcomplet,RowsBound1)
}
```

Drop extra dataframes and objects

```
rm(FOORow,RRARow,RowsBound,RowsBound1,MetazoaFOO,MetazoaRRA,MetazoaForFilters,M
etazoaFOOt,MetazoaRRAt,MetazoaForFilterst,MetazoaFOOtX,MetazoaRRAtX,fielddata,f
ielddata1,fielddataRow)
```

Drop columns you aren't interested in

There is also an opportunity to rename some columns here if you want.

```
drop<-c("SampleFinal","Sample","labels","Sample.1")
MetazoaRRAcomplete<-MetazoaRRAcomplet[,!(names(MetazoaRRAcomplet)%in%drop)]
setnames(MetazoaRRAcomplete, old=c("Date", "Month", "Site","Stage",
"Burrow","Year"), new=c("DATE", "MONTH","SITE", "STAGE","BURROW","SEASON"))

drop<-c("SampleFinal","Sample","labels","Sample.1")
MetazoaFOOcomplete<-MetazoaFOOcomplet[,!(names(MetazoaFOOcomplet)%in%drop)]
setnames(MetazoaFOOcomplete, old=c("Date", "Month", "Site","Stage",
"Burrow","Year"), new=c("DATE", "MONTH","SITE", "STAGE","BURROW","SEASON"))

drop<-c("SampleFinal","Sample","labels","Sample")
MetazoaForFilterstcomplete<-
MetazoaForFilterstcomplet[,!(names(MetazoaForFilterstcomplet)%in%drop)]
setnames(MetazoaForFilterstcomplete, old=c("Date", "Month", "Site","Stage",
"Burrow","Year"), new=c("DATE", "MONTH","SITE", "STAGE","BURROW","SEASON"))
```

You may need to correct some column classes

```
MetazoaRRAcomplete$MONTH<-as.character(MetazoaRRAcomplete$MONTH)
MetazoaRRAcomplete$BURROW<-as.character(MetazoaRRAcomplete$BURROW)
MetazoaRRAcomplete$SEASON<-as.character(MetazoaRRAcomplete$SEASON)
MetazoaRRAcomplete$DATE<-as.Date(MetazoaRRAcomplete$DATE,format="%d/%m/%Y")


MetazoaFOOcomplete$MONTH<-as.character(MetazoaFOOcomplete$MONTH)
MetazoaFOOcomplete$BURROW<-as.character(MetazoaFOOcomplete$BURROW)
MetazoaFOOcomplete$SEASON<-as.character(MetazoaFOOcomplete$SEASON)
MetazoaFOOcomplete$DATE<-as.Date(MetazoaFOOcomplete$DATE,format="%d/%m/%Y")

MetazoaForFilterstcomplete$MONTH<-
as.character(MetazoaForFilterstcomplete$MONTH)
MetazoaForFilterstcomplete$BURROW<-
as.character(MetazoaForFilterstcomplete$BURROW)
MetazoaForFilterstcomplete$SEASON<-
```

```
as.character(MetazoaForFilterstcomplete$SEASON)
MetazoaForFilterstcomplete$DATE<-
as.Date(MetazoaForFilterstcomplete$DATE,format="%d/%m/%Y")
```

Export your data

I use this version to record a new version each time and avoid overwriting previous versions.

```
currentDate <- Sys.Date()
csvFileName1 <- paste("MetazoaFOO_",currentDate,".csv",sep="")
csvFileName2 <- paste("MetazoaRRA_",currentDate,".csv",sep="")
write.csv(MetazoaFOOcomplete, file=csvFileName1)
write.csv(MetazoaRRAcomplete, file=csvFileName2)
csvFileName3 <- paste("MetazoaForFilters_",currentDate,".csv",sep="")
write.csv(MetazoaForFilterstcomplete, file=csvFileName3)
```

NGS Bioinformatics Pipeline 16s

Cathy Cavallo

6/22/2018

Next Generation Sequencing Bioinformatics Pipeline
Group-specific primer example -- 16s_Fish

This code will allow the user to clean and organise their DNA sample library and then calculate measures of taxon abundance for analysis. Important steps include:

- Removing:
  - samples with less than 100 target reads
  - Non-target sequences
  - Specific samples (e.g. those that did not meet thresholds for inclusion, like CT)
- Producing group totals for higher taxonomic levels
- Calculating the frequency of occurrence (FOO) and relative read abundance (RRA) of items in the dataset
- Matching samples with sample metadata for analysis

Preparation

Load packages

```
library(dplyr)

##
## Attaching package: 'dplyr'

## The following objects are masked from 'package:stats':
##
##  filter, lag

## The following objects are masked from 'package:base':
##
##  intersect, setdiff, setequal, union

library(tidyverse)

## Loading tidyverse: ggplot2
## Loading tidyverse: tibble
## Loading tidyverse: tidyr
## Loading tidyverse: readr
## Loading tidyverse: purrr

## Conflicts with tidy packages ----------------------------------------------
```

```
## filter(): dplyr, stats
## lag(): dplyr, stats

library(data.table)

##
## Attaching package: 'data.table'

## The following object is masked from 'package:purrr':
##
##  transpose

## The following objects are masked from 'package:dplyr':
##
##  between, first, last

library(tidyr)
```

Load identified sample library

```
Fish<-read.csv("CCavallo_Fish16S_2016_2018_20180913nodups.csv",stringsAsFactors
= FALSE)
```

Inspect

```
tail(Fish)
```

Clean

Sum all reads in each sample and food reads in each sample

```
#Totals for numeric columns (samples)
Total<-colSums(Fish[,6:733])
#Total
#total within columns all cells that correspond to a row with "Prey" in the
"Group" column.
#TotalFood<- Fish %>%
PreyOnly<-Fish[Fish$Type == "Prey",]
TotalFood<-colSums(PreyOnly[,6:733])
```

Create a vector to retain only samples w > 100 food reads

```
ColumnIndex<-which(as.vector(TotalFood >= 100))
ColumnIndex<-ColumnIndex+5 #Add 5 to all values in this vector because we
skipped the first five columns when calculating the column sums. i.e. what was
column 1 will become column 6, column 3 becomes column 8 etc..
```

Drop non-food taxa and drop samples with < 100 food reads

```
FishNoLowreads<-PreyOnly[,c(1:5, ColumnIndex)]
```

Organise

Create rows with group totals

You will need to define the names of your groups in a column called "Group" in your library prior to importing into R.

```
FishGrpTotals<-FishNoLowreads%>%
 group_by(Group)%>%
 summarise_at(-c(1:4),funs(sum))
```

Add the group totals to your library

```
#rm(ColsToAppend)
#rm(FishWGrpTotals)
ColsToAppend<-data.frame(matrix(rep(NA, (nrow(FishGrpTotals)*4)), ncol = 4))
names(ColsToAppend)<-names(FishNoLowreads[,1:4])#has the correct columns (no
Group)
FishWGrpTotals<-cbind(ColsToAppend, FishGrpTotals)
FishTotalData<-rbind( FishNoLowreads, FishWGrpTotals)
```

Drop redundant columns

Remember that the column numbers you choose to drop will depend on what is in your library.You may not need to drop any.

```
FishForFilters<-FishTotalData[,c(3,5:ncol(FishTotalData))]
```

Calculate

Now that your library is organised and includes only the samples and sequences necessary for your analysis, you can start calculating some measures of presence and abundance!

Relative read abundance (RRA)

Get sample totals again

```
FishForFilters$Revised[is.na(FishForFilters$Revised)]<-"GroupTotal"
SampleTotals<-colSums(FishForFilters[which(FishForFilters$Revised !=
"GroupTotal"),3:ncol(FishForFilters)])
```

Check that the totals for groups and totals for Metazoa are the same.

```
GROUPTotals<-colSums(FishForFilters[which(FishForFilters$Revised ==
"GroupTotal"),3:ncol(FishForFilters)])
SampleTotals<-c(NA, NA, SampleTotals)
```

Bind them and inspect

```
FishForFilters<-rbind( FishForFilters, SampleTotals)
FishForFilters$Group[nrow(FishForFilters)]<-"Total"
```

Convert each cell or group total to a percentage of the sample total

This converts the library into a sheet of percentages.

```
class(FishForFilters$Group)

## [1] "character"

FishRRA<-FishForFilters #FishRRA is a dataframe that exactly matchesd
fishforfilters
FishRRA[, 3:ncol(FishRRA)]<-NaN #replace values with NaN (not a number)
for(i in 3:ncol(FishForFilters)){#Loop from column 3 til the end (3:ncol)
 ActiveTotal<-FishForFilters[FishForFilters$Group == "Total", i]#subset the
data so that only the total is left for the column we are working in
 for(j in 1:nrow(FishForFilters)){#and then from the first row to the last row
 ActiveRRA<-(FishForFilters[j,i]/ActiveTotal)*100 #calculate RRA Column i, row
j.
 FishRRA[j, i]<-ActiveRRA #in the fish RRA data frame update the exact
corresponding cell with the RRA value you have just calculated
 }
}
```

Frequency of occurrence (FOO)

Frequency of occurrence describes the percentage of samples in the dataset in which a given taxon is present, but, like all presence–absence records, can overestimate the importance of common prey items that are ingested in very small amounts, including in the digestive tract of targeted prey (secondary predation).

Convert all cells where RRA is >1% to "1", cells <1% get "0".

This converts the library to presence/absence type data.

```
FishFOO<-FishRRA
FishFOO[, 3:ncol(FishFOO)]<-NaN #replace values with NaN (not a number)
for(i in 3:ncol(FishRRA)){#Loop from column 3 til the end (3:ncol)
 for(j in 1:nrow(FishRRA)){#and then from the first row to the last row
 if(FishRRA[j,i]>=1){
  FishFOO[j,i]<-1#present
 }else{
  FishFOO[j,i]<-0#absent
 } #calculate RRA Column i, row j.
 }
}
```

Rename group totals

```
for(i in which(FishRRA$Revised=="GroupTotal")){ FishRRA$Revised[i]<-
paste(FishRRA$Group[i],"Total")
}
for(i in which(FishFOO$Revised=="GroupTotal")){ FishFOO$Revised[i]<-
paste(FishFOO$Group[i],"Total")
}
for(i in
which(FishForFilters$Revised=="GroupTotal")){ FishForFilters$Revised[i]<-
```

```
paste(FishForFilters$Group[i],"Total")
}
```

Clean away some stuff you don't need

```
rm(PreyOnly,FishWGrpTotals,FishTotalData,FishNoLowreads,ColsToAppend,FishGrpTot
als,Fish)
```

Combine with sample metadata

Transpose data back into a format that makes more sense

```
names<-FishRRA$Revised
#transpose all but the first column (Sample)
FishRRAt<-as.data.frame(t(FishRRA[,-c(1:2)]))
colnames(FishRRAt)<-names#set column names
FishRRAt<-cbind(rownames(FishRRAt),data.frame(FishRRAt))
names(FishRRAt)[1]<-"Sample"
FishRRAt$Sample <- substr(x = FishRRAt$Sample, start = 2,stop=100) #If the
sample name starts with 'X' this should work. If it starts with 'X.' you will
need to change the start = to 3

names<-FishFOO$Revised

FishFOOt<-as.data.frame(t(FishFOO[,-c(1:2)]))
colnames(FishFOOt)<-names
FishFOOt<-cbind(rownames(FishFOOt),data.frame(FishFOOt))
names(FishFOOt)[1]<-"Sample"

names<-FishForFilters$Revised
FishForFilterst<-as.data.frame(t(FishForFilters[,-c(1:2)]))
colnames(FishForFilterst)<-names
FishForFilterst<-cbind(rownames(FishForFilterst),data.frame(FishForFilterst))
names(FishForFilterst)[1]<-"Sample"
```

Drop leading X's if you have them

```
FishFOOt$Sample <- substr(x = FishFOOt$Sample, start =2,stop=100)
FishForFilterst$Sample <- substr(x = FishForFilterst$Sample, start =2,stop=100)
```

Bring in the sample field data (tied to sample number)

```
fielddata<-read.csv("metadataIDs_20180601.csv",stringsAsFactors = FALSE)
```

Rename sample in the metadata/field data database to match sample name in sequence library

```
colnames(fielddata)[colnames(fielddata)=="X20180215.labels"]<-"SampleField"
```

Then bind sample field data to sample diet data for both RRA and FOO sheets

You may have samples in either your library or field database that you want to drop. You can drop these here.

```
todrop<-
c("X2016a_CRC321_s376","X2018_CRC177_s034","X2018_CRC323_s226","X2018_CRC165_s2
30","X2018_CRC321_s267","X2018_CRC183_s279")
FishRRAtX<-FishRRAt[!rownames(FishRRAt)%in%todrop,]
FishFOOtX<-FishFOOt[!rownames(FishFOOt)%in%todrop,]
FishForFilterstX<-FishForFilterst[!rownames(FishForFilterst)%in%todrop,]
write.csv(FishForFilterst,"Fishsamplesfiltered.csv")
```

Now bring in the data

```
fielddatanames<-names(fielddata)
FishRRAnames<-names(FishRRAtX)

fielddatanames<-names(fielddata)
FishForFilterstnames<-names(FishForFilterst)
```

Match up your field data with your RRA data

```
FishRRAcomplet<-data.frame()
#i=401
for(i in 1:nrow(FishRRAtX)){
 ActiveSample<-FishRRAtX$Sample[i]# isolating that one cell (sample in row i)
 fielddataRow<-fielddata[fielddata$SampleField == ActiveSample,]#subset to a
single row in RRA. Works for pulling the small sheet into the big sheet.
 RRARow<-FishRRAtX[i,] #i is always the active row
 RowsBound<-cbind(fielddataRow,RRARow)
 FishRRAcomplet<-rbind(FishRRAcomplet,RowsBound)
}
```

Match up your field data with your FOO data

```
FishFOOnames<-names(FishFOOtX)
FishFOOcomplet<-data.frame()
for(i in 1:nrow(FishFOOtX)){
 ActiveSample<-FishFOOtX$Sample[i]# isolating that one cell (sample in row i)
 fielddataRow<-fielddata[fielddata$SampleField == ActiveSample,]#subset to a
single row in FOO Works for pulling the small sheet into the big sheet. If I
need the reverse, ask Rowan.
 FOORow<-FishFOOtX[i,] #i is always the active row
 RowsBound<-cbind(fielddataRow,FOORow)
 FishFOOcomplet<-rbind(FishFOOcomplet,RowsBound)
}#ends function
```

Match up your field data with FishForFilters

```
FishForFilterstcomplet<-data.frame()
for(i in 1:nrow(FishForFilterstX)){
 ActiveSample<-FishForFilterstX$Sample[i]
 fielddataRow<-fielddata[fielddata$SampleField == ActiveSample,]
 RRARow<-FishForFilterstX[i,]
 RowsBound<-cbind(fielddataRow,RRARow)
```

```
 FishForFilterstcomplet<-rbind(FishForFilterstcomplet,RowsBound)
}
```

Drop columns you aren't interested in

There is also an opportunity to rename some columns here if you want.

```
drop<-c("SampleFinal","CRC","labels")
FishRRAcomplete<-FishRRAcomplet[,!(names(FishRRAcomplet)%in%drop)]
setnames(FishRRAcomplete, old=c("Date", "Month", "Site","Stage",
"Burrow","Year"), new=c("DATE", "MONTH","SITE", "STAGE","BURROW","SEASON"))

drop<-c("SampleFinal","CRC","labels")
FishFOOcomplete<-FishFOOcomplet[,!(names(FishFOOcomplet)%in%drop)]
setnames(FishFOOcomplete, old=c("Date", "Month", "Site","Stage",
"Burrow","Year"), new=c("DATE", "MONTH","SITE", "STAGE","BURROW","SEASON"))

drop<-c("SampleFinal","CRC","labels")
FishForFilterstcomplete<-
FishForFilterstcomplet[,!(names(FishForFilterstcomplet)%in%drop)]
setnames(FishForFilterstcomplete, old=c("Date", "Month", "Site","Stage",
"Burrow","Year"), new=c("DATE", "MONTH","SITE", "STAGE","BURROW","SEASON"))
```

You may need to correct some column classes

```
FishRRAcomplete$MONTH<-as.character(FishRRAcomplete$MONTH)
FishRRAcomplete$BURROW<-as.character(FishRRAcomplete$BURROW)
FishRRAcomplete$SEASON<-as.character(FishRRAcomplete$SEASON)
FishRRAcomplete$DATE<-as.Date(FishRRAcomplete$DATE,format="%d/%m/%Y")


FishFOOcomplete$MONTH<-as.character(FishFOOcomplete$MONTH)
FishFOOcomplete$BURROW<-as.character(FishFOOcomplete$BURROW)
FishFOOcomplete$SEASON<-as.character(FishFOOcomplete$SEASON)
FishFOOcomplete$DATE<-as.Date(FishFOOcomplete$DATE,format="%d/%m/%Y")

FishForFilterstcomplete$MONTH<-as.character(FishForFilterstcomplete$MONTH)
FishForFilterstcomplete$BURROW<-as.character(FishForFilterstcomplete$BURROW)
FishForFilterstcomplete$SEASON<-as.character(FishForFilterstcomplete$SEASON)
FishForFilterstcomplete$DATE<-
as.Date(FishForFilterstcomplete$DATE,format="%d/%m/%Y")
```

Export your data

I use this version to record a new version each time and avoid overwriting previous versions.

```
currentDate <- Sys.Date()
csvFileName1 <- paste("FishFOO_",currentDate,".csv",sep="")
csvFileName2 <- paste("FishRRA_",currentDate,".csv",sep="")
csvFileName3 <- paste("FishForFilters_",currentDate,".csv",sep="")
write.csv(FishFOOcomplete, file=csvFileName1)
write.csv(FishRRAcomplete, file=csvFileName2)
write.csv(FishForFilterstcomplete, file=csvFileName3)
```

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Adapted from Julie McInnes' protocol (Julie.mcinnes@aad.gov.au)

Appendix E: pCPUE and CPUE RMD

Calculating predator-derived measures of prey availability (pCPUE)

Cathy Cavallo

8/01/2019

Calculating predator-derived measures of prey availability (pCPUE)

This code will allow the user to subset and filter a weighbridge dataset, calculate measures of foraging success and effort and combine with diet information to calculate prey-specific catch per unit effort (pCPUE) and overall catch per unit effort (CPUE).

Install necessary packages

```
library(dplyr)

##
## Attaching package: 'dplyr'

## The following objects are masked from 'package:stats':
##
##  filter, lag

## The following objects are masked from 'package:base':
##
##  intersect, setdiff, setequal, union

library(tidyverse)

## Loading tidyverse: ggplot2
## Loading tidyverse: tibble
## Loading tidyverse: tidyr
## Loading tidyverse: readr
## Loading tidyverse: purrr

## Conflicts with tidy packages ----------------------------------------------

## filter(): dplyr, stats
## lag(): dplyr, stats

library(data.table)

##
## Attaching package: 'data.table'

## The following object is masked from 'package:purrr':
##
##  transpose
```

```
## The following objects are masked from 'package:dplyr':
##
##  between, first, last

library(tidyr)
library(ggplot2) #for plotting nice figures
library(reshape2) #use this to convert dataframes from wideform to longform

##
## Attaching package: 'reshape2'

## The following objects are masked from 'package:data.table':
##
##  dcast, melt

## The following object is masked from 'package:tidyr':
##
##  smiths

library(lubridate) #for use with dates

##
## Attaching package: 'lubridate'

## The following objects are masked from 'package:data.table':
##
##  hour, isoweek, mday, minute, month, quarter, second, wday,
##  week, yday, year

## The following object is masked from 'package:base':
##
##  date
```

Load scat sampling date data to use to subset APMS (weighbridge) data.

```
SampleDates<-read.csv("SampleDatesWDummy.csv",stringsAsFactors = FALSE)
SampleDates$Poo.collection.date<-
as.Date(as.character(SampleDates$Poo.collection.date,format="%d/%m/%Y"))
#if the above doesn't give the right day format, use the other one (both
options below)
#as.POSIXct( SampleDates$Poo.collection.date, format = "%d/%m/%Y)
#as.Date(as.character(SampleDates$Poo.collection.date,format="%d/%m/%Y"))
```

Subset the data to 1 week before scat sampling

add two columns to create date range

Note that if you have brought Poo.collection.date in as POSIXct then these columns won't work. Make sure it is as.Date(as.character())
This is because the following function will try and knock 1, 4 and 7 seconds off, not days if you have it in the wrong format!

```
SampleDates$Poo.deposited<-SampleDates$Poo.collection.date-1
SampleDates$Week.before<-SampleDates$Poo.collection.date-7
```

Bring in APMS (weighbridge data)

```
APMS<-read.csv('APMS_20180516.csv',stringsAsFactors = FALSE)

APMS$Burrow<-as.factor(APMS$burrow.apms)
APMS$weight<-as.numeric(APMS$weight)

## Warning: NAs introduced by coercion

APMS$datetime<-as.POSIXct( APMS$datetime, format = "%d/%m/%Y %H:%M",tz="UTC")
APMS$date<-as.Date(APMS$date, format = "%d/%m/%Y")



APMS$Localdatetime<-format(APMS$datetime, tz="Australia/Sydney",usetz=TRUE)
APMS$Localdatetime<-as.POSIXct(APMS$Localdatetime,tz="Australia/Sydney")
APMS$Localdate<-date(APMS$Localdatetime)
```

Subset APMS data to only trips within your sampling dates

```
MyTrips<-data.frame()
class(APMS)

## [1] "data.frame"

#i=9
for (i in 1:length(SampleDates$Poo.collection.date)){
 #We first subsample the data from the weighbridge we want matching our dates
from CPUFERRA data.
 NewAPMS<-APMS[which(APMS$Localdate<=SampleDates$Poo.deposited[i]&
     APMS$Localdate>=SampleDates$Week.before[i]),]

 #We then create the columns we need in our weighbridge data subsample
 NewAPMS$Poo.collection.date<-SampleDates$Poo.collection.date[i] #but this
stage stuffs it up
 NewAPMS$Poo.deposited<-SampleDates$Poo.deposited[i]
 NewAPMS$Week.before<-SampleDates$Week.before[i]

 #We rowbind the weighbridge data subsample to our Final data set
 MyTrips<-rbind(MyTrips,NewAPMS)
 }


 #rm(NewAPMS,i)
currentDate <- Sys.Date()
csvFileName <- paste("My Trips APMS",currentDate,".csv",sep="")
write.csv(MyTrips, file=csvFileName)
```

Calculate trip duration & mass gain

Create functions to calculate trip duration and mass gain

First, make sure that the APMS data is sorted by the datetime column.

```
MyTrips<-MyTrips[order(MyTrips$datetime, decreasing = FALSE),]
```

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Adapted from Julie McInnes' protocol (Julie.mcinnes@aad.gov.au)

Trip Duration

```
data<-MyTrips
tag<-"tag"
datetime<-"Localdatetime"
direction<-"direction"

tripDurationdf<-function(data, tag, datetime, direction){
 DateTimeColumn<-which(names(data)== datetime)
 TagColumn<-which(names(data)== tag)
 DirectionColumn<-which(names(data)== direction)
 data$TripDuration<-NA

 data$sortorder<-1:nrow(data) #To preserve the original order of the data

 data<-data[order(data[,DateTimeColumn]),] #Sort by time so that ins that
should follow outs are consecutive
 DataHold<-data[1,]
 DataHold[1,]<-NA

 #i<-unique(data[,TagColumn])[1]
 for(i in unique(data[,TagColumn])){
 ActiveTagRows<-data[which(data[,TagColumn]==i),]

 for(j in 1:nrow(ActiveTagRows)){
  if(j==nrow(ActiveTagRows)){
  break
  }
  if(ActiveTagRows[j,DirectionColumn] == "out" &
ActiveTagRows[j+1,DirectionColumn]=="in"){
  TripDuration<-difftime(time1 = ActiveTagRows[j+1,DateTimeColumn], time2 =
ActiveTagRows[j,DateTimeColumn], units = "hours")
  ActiveTagRows$TripDuration[(j+1)]<-as.numeric(TripDuration) #insert it into
the TripDuration column of the "in" row
  }
 }
 DataHold<-rbind(DataHold,ActiveTagRows)
 }
 data<-DataHold[-1,]
 data<-data[order(data$sortorder),]
 return(data)
}
```

Mass Change

```
data<-MyTrips
tag<-"tag"
datetime<-"Localdatetime"
direction<-"direction"
mass<-"weight"
MassChangedf<-function(data, tag, datetime, direction,mass){
 DateTimeColumn<-which(names(data)== datetime)
 TagColumn<-which(names(data)== tag)
```

```
 DirectionColumn<-which(names(data)== direction)
 MassColumn<-which(names(data)==mass)
 data$MassChange<-NA

 data$sortorder<-1:nrow(data) #To preserve the original order of the data

 data<-data[order(data[,DateTimeColumn]),] #Sort by time so that ins that
should follow outs are consecutive
 DataHold<-data[1,]
 DataHold[1,]<-NA
 i<-unique(data[,TagColumn])[2]
 for(i in unique(data[,TagColumn])){
 ActiveTagRows<-data[which(data[,TagColumn]==i),]

 for(j in 1:nrow(ActiveTagRows)){
  if(j==nrow(ActiveTagRows)){
  break
  }
  if(ActiveTagRows[j,DirectionColumn] == "out" &
ActiveTagRows[j+1,DirectionColumn]=="in"){
  if(!is.na(ActiveTagRows[j+1,MassColumn])
& !is.na(ActiveTagRows[j,MassColumn])){
   MassChange<-(ActiveTagRows[j+1,MassColumn]-ActiveTagRows[j,MassColumn])
   ActiveTagRows$MassChange[j+1]<-MassChange #insert it into the TripDuration
column of the "in" row
  }
  }
 }
 DataHold<-rbind(DataHold,ActiveTagRows)
 }
 data<-DataHold[-1,]
 data<-data[order(data$sortorder),]
 DropColumn<-which(names(data)== "sortorder")
 data<-data[,-DropColumn]
 return(data)
 }
```

Run these functions on your trip data

```
MyTrips<-tripDurationdf(data=MyTrips,tag = "tag",datetime =
"Localdatetime",direction = "direction")
MyTrips<-
MassChangedf(data=MyTrips,tag="tag",datetime="Localdatetime",direction="directi
on",mass="weight")
```

Filtering

Filter to retain only trips that returned on the day poo was deposited

```
MyTrips$DateOfReturn<-as.Date(NA)
MyTrips$DateOfReturn[MyTrips$direction == "in"]<-
MyTrips$Localdate[MyTrips$direction == "in"]
```

```
FilterReturns<-
which(MyTrips$direction=='in'&MyTrips$DateOfReturn==MyTrips$Poo.deposited)

MyTrips<-MyTrips[FilterReturns,]
class(MyTrips$date)

## [1] "Date"

class(MyTrips$direction)

## [1] "character"

class(MyTrips$DateOfReturn)

## [1] "Date"

class(MyTrips$Localdatetime)

## [1] "POSIXct" "POSIXt"
```

My Trips

When checking these (and the one before) remember that the first "in" direction rows will have NA for all calculated trip data!

Convert trip duration hours to days

```
MyTrips$TripDurationDays<-ceiling(MyTrips$TripDuration/24)
```

Limits

Limit trips to =<7 days
Limit mass gain to between 0 & 420 g

```
MyTripsAC<-MyTrips
#Mass gain from published papers
FilterMassAC<-which(MyTripsAC$MassChange>(0) & MyTripsAC$MassChange<420
&MyTripsAC$TripDurationDays<=7)

MyTripsAC$ValidMassChangeAC<-NA
MyTripsAC$ValidMassChangeAC[FilterMassAC]<-MyTripsAC$MassChange[FilterMassAC]
MyTripsAC$ValidMassChangeAC[!FilterMassAC]<-NA


#Filter to only trips that would match diet data
FilterIndices<-which(MyTripsAC$TripDurationDays<=7)

MyTripsAC$trip.duration.daysValid<-NA
MyTripsAC$trip.duration.daysValid[FilterIndices]<-
MyTrips$TripDurationDays[FilterIndices]
MyTripsAC$trip.duration.daysValid[!FilterIndices]<-NA

currentDate <- Sys.Date()
csvFileNameAC <- paste("My Trips APMS Valid trip data_AC
```

```
",currentDate,".csv",sep="")
write.csv(MyTripsAC, file=csvFileNameAC)
```

Stage Summary Get tripdata number of stages per date

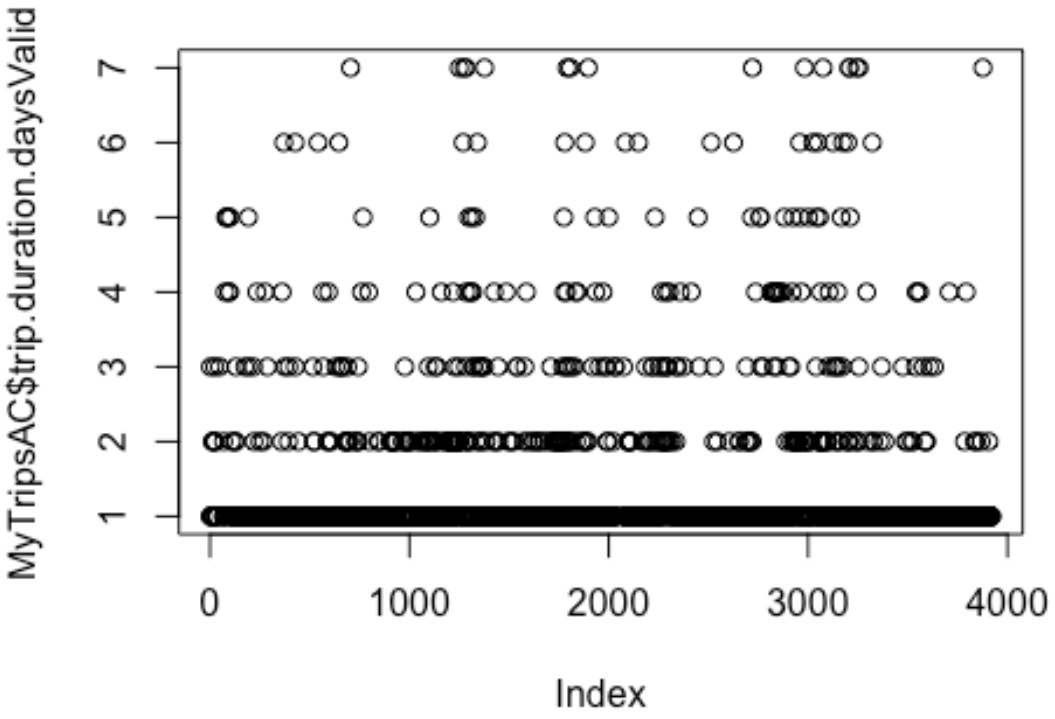
This isn't needed but it is good for checking whether you have balanced breeding stages per each date. You probably won't unless your species/colony nests asynchronously.

```
MyTrips2<-MyTrips
MyTrips2$MONTH<-month(MyTrips2$datetime)
TripStageSummary<-MyTrips2%>%
 group_by(date,MONTH,stage)%>%
 summarise(Nstage=n())

TripStageSummary$stage<-as.character(TripStageSummary$stage)
TripStageSummary<-TripStageSummary[TripStageSummary$stage ==
"I"|TripStageSummary$stage == "G"| TripStageSummary$stage == "P",]

#make stage a factor again
TripStageSummary$fStage<-as.factor(TripStageSummary$stage)
class(TripStageSummary$date)

## [1] "Date"

write.csv(TripStageSummary,"TripStageSummary_20181102.csv")
```

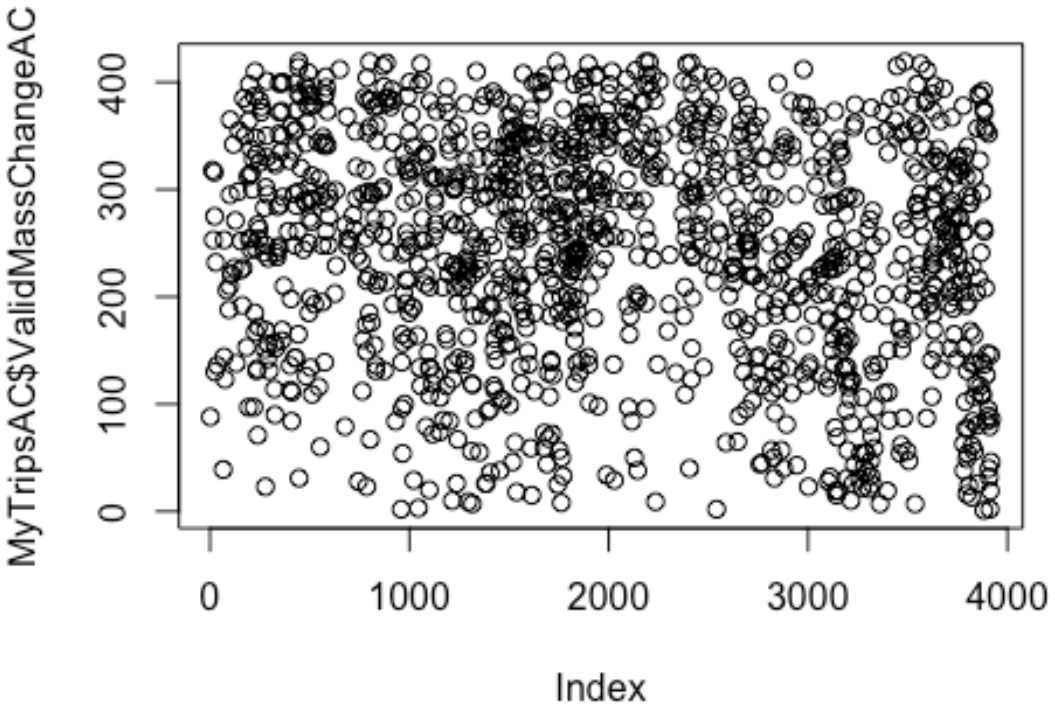
Which average to use? Mean, median or mode?

Test visually and statistically.

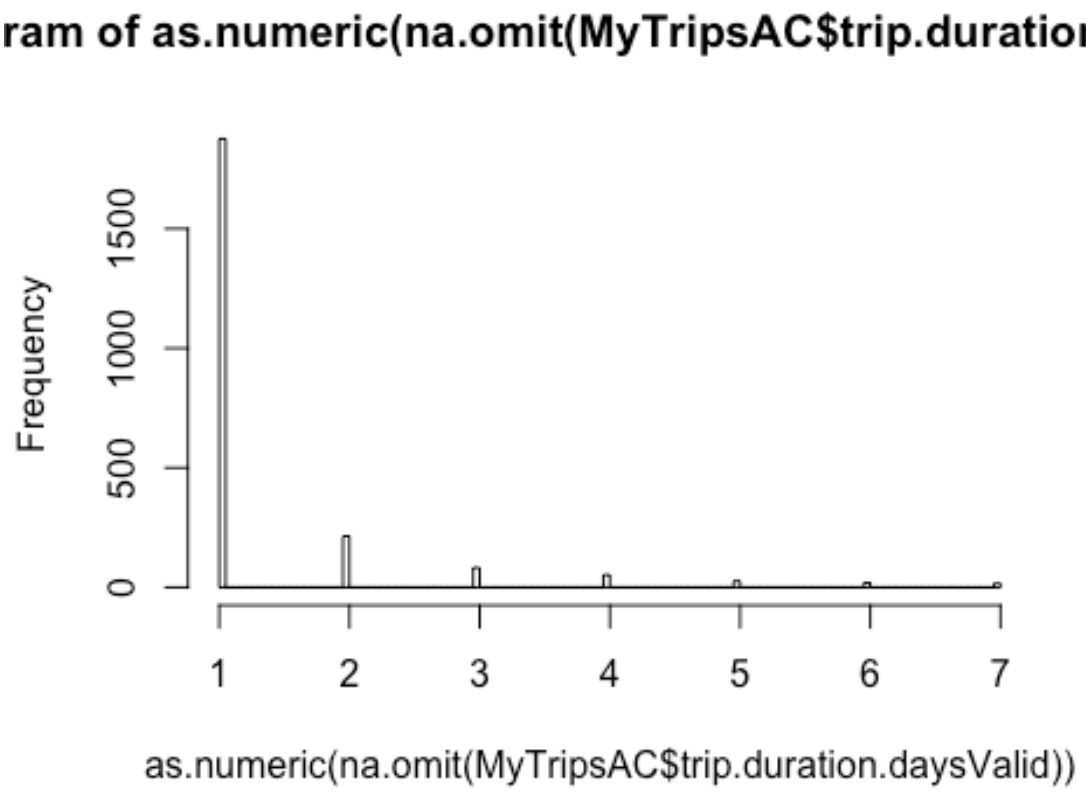
```
plot(MyTripsAC$trip.duration.daysValid)
```



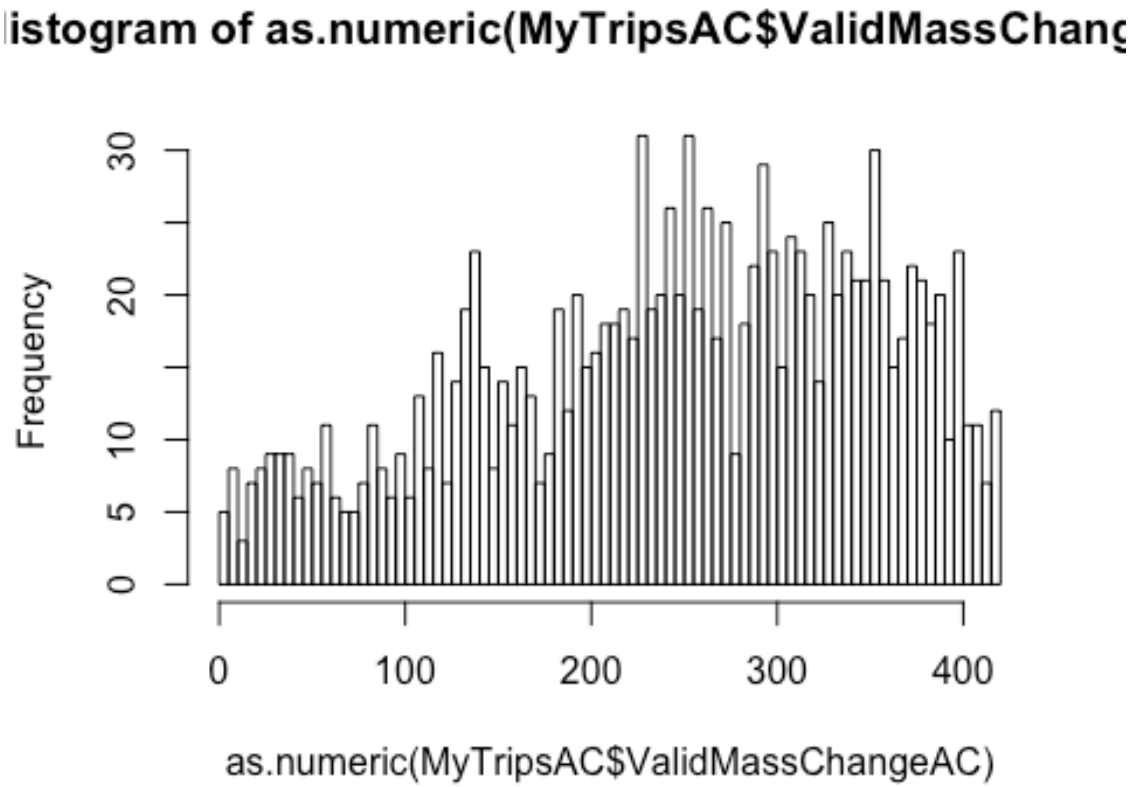

```
plot(MyTripsAC$ValidMassChangeAC)
```



```
hist(as.numeric(na.omit(MyTripsAC$trip.duration.daysValid)),breaks=100)
```




```
hist(as.numeric(MyTripsAC$ValidMassChangeAC),breaks=100)
```




```
library(moments)
agostino.test(MyTripsAC$trip.duration.daysValid)

##
## D'Agostino skewness test
##
## data: MyTripsAC$trip.duration.daysValid
## skew = 3.4331, z = 34.3520, p-value < 2.2e-16
## alternative hypothesis: data have a skewness

agostino.test(MyTripsAC$ValidMassChangeAC)

##
## D'Agostino skewness test
##
## data: MyTripsAC$ValidMassChangeAC
## skew = -0.40237, z = -5.69360, p-value = 1.244e-08
## alternative hypothesis: data have a skewness
```

Create a function that calculates mode

Remember that the mode is the value that occurs most frequently

```
getmode<-function(d){
 uniqd<-na.omit(unique(d))
 uniqd[which.max(tabulate(match(d,uniqd)))]
}
```

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Adapted from Julie McInnes' protocol (Julie.mcinnes@aad.gov.au)

Average foraging trip duration and mass gain by stage and date

I have left in the ability for people to choose median, mean or mode as their average because it may differ between groups. However, judging by the fact that foragers will most likely try and fill up before returning from a foraging trip I expect the frequency distribution of mass change will always be negatively skewed.

```
i=5
#rm(i)
HoldingFrame<-data.frame()

 for (i in 1:length(SampleDates$Poo.collection.date)){#for each unique
Poo.collection.date

ActiveTripsStage<-
MyTripsAC[which(MyTripsAC$Poo.collection.date==SampleDates$Poo.collection.date[
i]),] #Subset from MyTripsAC to just the rows for which the date is between
Week.Before and Poo.deposited.


NewColumnsStage<-ActiveTripsStage%>% #In ActiveTrips, average all the trip
durations for that Poo.collection.date that arent NAs.
#Return averaged values in columns with suffix _mean, _median, _mode.
 group_by(stage)%>%

summarise(trip.duration.daysValid_count=sum(!is.na(trip.duration.daysValid)),tr
ip.duration.daysValid_mean=mean(trip.duration.daysValid,
na.rm=TRUE),trip.duration.daysValid_median=median(trip.duration.daysValid,na.rm
=TRUE),trip.duration.daysValid_mode=getmode(trip.duration.daysValid),ValidMassC
hangeAC_count=sum(!is.na(ValidMassChangeAC)),
ValidMassChangeAC_mean=mean(ValidMassChangeAC,na.rm=TRUE),ValidMassChangeAC_med
ian=median(ValidMassChangeAC,na.rm=TRUE),ValidMassChangeAC_mode=getmode(ValidMa
ssChangeAC))
NewColumnsStage$Poo.collection.date<-SampleDates$Poo.collection.date[i]
HoldingFrame<-rbind(HoldingFrame,NewColumnsStage)
 }
#now rename holding frame
AveragedTripsACStage_all<-HoldingFrame
class(AveragedTripsACStage_all$stage)

## [1] "character"

AveragedTripsACStage_all$StageTrips<-
as.factor((AveragedTripsACStage_all$stage))
```

Load the diet (scat sample) data

```
RRA16<-read.csv("FishRRA_2018-10-07.csv",stringsAsFactors = TRUE)
RRA16$Weekbeginspoo<-floor_date(as.Date(RRA16$DATE, "%Y-%m-%d"), unit="week")
RRA16$Poo.collection.date<-as.Date(RRA16$DATE, "%Y-%m-%d")
RRA16$SampleField<-as.character(RRA16$SampleField)
RRA16$SEASON<-as.factor(RRA16$SEASON)
RRA16$StageDiet<-RRA16$STAGE
```

Subset

```
RRA16en<-
RRA16[,c("SampleField","Poo.collection.date","BURROW","SITE","SEASON","StageDie
t","MONTH","BARRACOUTA.Total","RED.COD.Total" ,"JACKMACKERAL.Total","SARDINE.To
tal","WAREHOU.Total" ,"Bluefin.leatherjacket","Velvet.leatherjacket","Spiny.gur
nard" ,"Weedfish.sp.","Acanthaluteres.sp." , "ANCHOVY.Total" ,
"Upeneichythys.sp." )]
```

Check for interactions with no values because these will need to be removed from the analysis

```
table(with(RRA16en, interaction(MONTH,StageDiet,SEASON)))

##
## 1.0.2015 2.0.2015 3.0.2015 8.0.2015 9.0.2015 10.0.2015 11.0.2015
##   0   0   0   0   0   0   0
## 12.0.2015 1.E.2015 2.E.2015 3.E.2015 8.E.2015 9.E.2015 10.E.2015
##   1   0   0   0   4  29   6
## 11.E.2015 12.E.2015 1.G.2015 2.G.2015 3.G.2015 8.G.2015 9.G.2015
##   7   7   3   5   0   0  18
## 10.G.2015 11.G.2015 12.G.2015 1.P.2015 2.P.2015 3.P.2015 8.P.2015
##  30  12  16   2  26   0   1
## 9.P.2015 10.P.2015 11.P.2015 12.P.2015 1.0.2016 2.0.2016 3.0.2016
##   7  14  29  35   0   0   0
## 8.0.2016 9.0.2016 10.0.2016 11.0.2016 12.0.2016 1.E.2016 2.E.2016
##   0   0   0   0   0   1   0
## 3.E.2016 8.E.2016 9.E.2016 10.E.2016 11.E.2016 12.E.2016 1.G.2016
##   0   0   8   6   4   1  11
## 2.G.2016 3.G.2016 8.G.2016 9.G.2016 10.G.2016 11.G.2016 12.G.2016
##   3   0   0  12  51  22   5
## 1.P.2016 2.P.2016 3.P.2016 8.P.2016 9.P.2016 10.P.2016 11.P.2016
##   8   7   6   0   5  22  40
## 12.P.2016
##  11

table(with(RRA16en, interaction(MONTH,StageDiet)))

##
## 1.0 2.0 3.0 8.0 9.0 10.0 11.0 12.0 1.E 2.E 3.E 8.E 9.E 10.E 11.E
## 0 0 0 0 0 0 0 1 1 0 0 4 37 12 11
## 12.E 1.G 2.G 3.G 8.G 9.G 10.G 11.G 12.G 1.P 2.P 3.P 8.P 9.P 10.P
## 8 14 8 0 0 30 81 34 21 10 33 6 1 12 36
## 11.P 12.P
## 69 46

table(with(RRA16en, interaction(MONTH,SEASON)))

##
## 1.2015 2.2015 3.2015 8.2015 9.2015 10.2015 11.2015 12.2015 1.2016
##  5  31  0  5  54  50  48  59  20
## 2.2016 3.2016 8.2016 9.2016 10.2016 11.2016 12.2016
##  10  6  0  25  79  66  17

table(with(RRA16en, interaction(StageDiet,SEASON)))
```

```
##
## 0.2015 E.2015 G.2015 P.2015 0.2016 E.2016 G.2016 P.2016
##   1   53   84 114   0   20 104   99
```

```
table(with(RRA16en, interaction(SITE,SEASON)))
```

```
##
## PP.2015 RTB.2015 PP.2016 RTB.2016
##   126   126   95   128
```

```
table(with(RRA16en, interaction(SEASON)))
```

```
##
## 2015 2016
## 252 223
```

CPUFE preparation

Create date range in diet

Note that if you have brought Poo.collection.date in as POSIXct then these columns won't work. Make sure it is as.Date(as.character()) This is because the following function will try and knock 1, 4 and 7 seconds off, not days if you have it in the wrong format!

```
RRA16noNA<-RRA16en[complete.cases(RRA16en),]
class(RRA16noNA$Poo.collection.date)
```

```
## [1] "Date"
```

```
RRA16noNA$Poo.deposited<-RRA16noNA$Poo.collection.date-1
RRA16noNA$Week.before<-RRA16noNA$Poo.collection.date-7
```

Replace E's with I's

Use this to clean up data when you have used different names in different datasets. The breeding stages recorded during fieldwork are always assigned "I" for incubation, because it is part of a pre-defined long-term monitoring set up. Unfortunately, I used "E" for eggs in my scat sampling data.

```
class(RRA16noNA$StageDiet)
```

```
## [1] "factor"
```

```
levels(RRA16noNA$StageDiet)[levels(RRA16noNA$StageDiet)=="E"] <- "I"
RRA16ForCPUFE<-RRA16noNA
names(RRA16noNA)
```

```
## [1] "SampleField"   "Poo.collection.date"
## [3] "BURROW"    "SITE"
## [5] "SEASON"    "StageDiet"
## [7] "MONTH"     "BARRACOUTA.Total"
## [9] "RED.COD.Total"   "JACKMACKERAL.Total"
## [11] "SARDINE.Total"   "WAREHOU.Total"
## [13] "Bluefin.leatherjacket" "Velvet.leatherjacket"
## [15] "Spiny.gurnard"   "Weedfish.sp."
## [17] "Acanthaluteres.sp." "ANCHOVY.Total"
```

```
## [19] "Upeneichythys.sp."  "Poo.deposited"
## [21] "Week.before"

class(RRA16ForCPUFE$StageDiet)

## [1] "factor"

levels(RRA16ForCPUFE$StageDiet)

## [1] "0" "I" "G" "P"

RRA16ForCPUFE$StageDiet<-as.character(RRA16ForCPUFE$StageDiet)
RRA16ForCPUFE<-RRA16ForCPUFE[RRA16ForCPUFE$StageDiet != "0",]
RRA16ForCPUFE$fStageDiet<-as.factor(RRA16ForCPUFE$StageDiet)
```

Drop NA/0 levels in APMS (weighbridge) data

Sometimes we don't have reproductive information about the birds crossing the weighbridge. These get dropped because we care about breeding-stage effects in this species.

```
#drop levels
AveragedTripsACStage_all$StageTrips<-
as.character(AveragedTripsACStage_all$StageTrips)
AveragedTripsACStage_all<-
AveragedTripsACStage_all[AveragedTripsACStage_all$StageTrips ==
"I"|AveragedTripsACStage_all$StageTrips == "G"|
AveragedTripsACStage_all$StageTrips == "P",]

#make stage a factor again
AveragedTripsACStage_all$fStageTrips<-
as.factor(AveragedTripsACStage_all$StageTrips)
```

Merge APMS & diet data

```
ForCPUFE_ACStageAll<-data.frame()
i=2
for(i in 1:nrow(RRA16ForCPUFE)){
 ActiveSample<-RRA16ForCPUFE[i,c("Poo.collection.date","fStageDiet")]#
isolating that one cell (date in row i)
 TripsRow<-
AveragedTripsACStage_all[AveragedTripsACStage_all$Poo.collection.date ==
ActiveSample$Poo.collection.date &
        AveragedTripsACStage_all$fStageTrips ==
ActiveSample$fStageDiet,]#subset to a single row in CPUFERRA Works for pulling
the small sheet into the big sheet. If I need the reverse, ask Rowan.
 BurrowRow<-RRA16ForCPUFE[i,] #i is always the active row
 if( length(TripsRow$fStageTrips)>0){
 RowsBound<-cbind(BurrowRow,TripsRow)
 }
 ForCPUFE_ACStageAll<-rbind(ForCPUFE_ACStageAll,RowsBound)

}#ends function
```

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Adapted from Julie McInnes' protocol (Julie.mcinnes@aad.gov.au)
Insert the seasonal relative read abundance of fish (Actinopterygii) from each year for pCPUE calculations

```
ForCPUFE_ACStageAll$FishRRA<-ifelse(test =
ForCPUFE_ACStageAll$SEASON=="2015",62,64)
```

CPUFE calculation with stage

Define the fish you are interested in as "My Species".
Here I have left it so that the user can decide whether to use the median or mean values for foraging trip duration and mass change.

```
MySpecies<-
c("BARRACOUTA.Total","RED.COD.Total","JACKMACKERAL.Total","SARDINE.Total","WARE
HOU.Total","Bluefin.leatherjacket","Velvet.leatherjacket","Spiny.gurnard","Weed
fish.sp.","Acanthaluteres.sp." , "ANCHOVY.Total" , "Upeneichythys.sp.")
i="BARRACOUTA.Total"
#i="RED.COD.Total"
k=1
ForCPUFE_ACStageAll<-as.data.frame(ForCPUFE_ACStageAll)
CPUFE_ACStageAll<-as.data.frame(ForCPUFE_ACStageAll)
for (i in MySpecies){
 print(i)#this will print after each loop so you can see progress
 ColumnIndexStage<-which(names(ForCPUFE_ACStageAll) == i) #Which column number
is the current fish in
 ColumnSubsetStage<-ForCPUFE_ACStageAll[,(ColumnIndexStage)]#subset to all
rows, for the single column of the current fish

CPUFE_meanStage<-vector()
CPUFE_medianStage<-vector()#create a empty vector (a vector because we want to
bind a single vector to the datasheet. I don't 100% understand yet)
 for(k in 1:nrow(ForCPUFE_ACStageAll)){#for each row get the DeltaMass and FTD.
 ActiveMass_meanStage<-ForCPUFE_ACStageAll$ValidMassChangeAC_mean[k]
 ActiveMass_medianStage<-ForCPUFE_ACStageAll$ValidMassChangeAC_median[k]
 ActiveFTD_meanStage<-
as.numeric(ForCPUFE_ACStageAll$trip.duration.daysValid_mean[k])
 ActiveFishRRA<-ForCPUFE_ACStageAll$FishRRA[k]
  ActiveFTD_medianStage<-
as.numeric(ForCPUFE_ACStageAll$trip.duration.daysValid_median[k])
 ActiveRRAStage<-ColumnSubsetStage[k]#then use the same row index to select our
fish column of interest (i). k is a single row within column i (i.e. one cell)
 ActiveCPUFEmeanStage<-
(((ActiveRRAStage/100)*(ActiveFishRRA/100))*ActiveMass_meanStage)/as.numeric(Ac
tiveFTD_meanStage) #compute CPUFE for one row of the fish column of interest at
a timeas.double(ActiveFTD_mean,units='days'
 CPUFE_meanStage<-c(CPUFE_meanStage,ActiveCPUFEmeanStage)#bind them.
  ActiveCPUFEmedianStage<-
(((ActiveRRAStage/100)*(ActiveFishRRA/100))*ActiveMass_medianStage)/as.double(A
ctiveFTD_medianStage,units='secs')
  #compute CPUFE for one row of the fish column of interest at a
timeas.double(ActiveFTD_median,units='secs')
 CPUFE_medianStage<-c(CPUFE_medianStage,ActiveCPUFEmedianStage)#bind them.
 ColumnSeedStage<-data.frame(CPUFE_meanStage,CPUFE_medianStage)
 }#closes the row loop. At this bracket we have computed all of the rows for a
```

```
single fish column
 CPUFEcolumnsStage<-ColumnSeedStage#rename vector seed as CPUFEcolumn
CPUFE_ACStageAll<-cbind(CPUFE_ACStageAll,CPUFEcolumnsStage) #bind the new
column to the existing burrows complete
 names(CPUFE_ACStageAll)[names(CPUFE_ACStageAll) == 'CPUFE_meanStage'] <-
paste(i,"_CPUFE_mean", sep = '')#Assign the name of that fish and append CPUFE
to the newly created column
names(CPUFE_ACStageAll)[names(CPUFE_ACStageAll) == 'CPUFE_medianStage'] <-
paste(i,"_CPUFE_median", sep = '')
}
```

```
## [1] "BARRACOUTA.Total"
## [1] "RED.COD.Total"
## [1] "JACKMACKERAL.Total"
## [1] "SARDINE.Total"
## [1] "WAREHOU.Total"
## [1] "Bluefin.leatherjacket"
## [1] "Velvet.leatherjacket"
## [1] "Spiny.gurnard"
## [1] "Weedfish.sp."
## [1] "Acanthaluteres.sp."
## [1] "ANCHOVY.Total"
## [1] "Upeneichythys.sp."
```

Calculate total CPUFE

This is a simple measure of foraging success divided by foraging effort. It allows us to see whether predators are having to expend more effort to catch ANY prey. This is useful for generalist predators that are able to switch prey sources.

```
CPUFE_ACStageAll<-CPUFE_ACStageAll[,-c(28)]
CPUFE_ACStageAll<-CPUFE_ACStageAll%>%
 group_by(Poo.collection.date)%>%

mutate(CPUFEtotal=ValidMassChangeAC_median/as.double(trip.duration.daysValid_me
dian,units='secs'))
```

Export your data

I use this version to record a new version each time and avoid overwriting previous versions.

```
currentDate <- Sys.Date()
csvFileName <- paste("CPUFE_ALL_ACStage_FishRRA_",currentDate,".csv",sep="")
write.csv(CPUFE_ACStageAll, file=csvFileName)
```