Multi-scale effects of nutrition on an arboreal folivore

Jessie Au

A thesis submitted for the degree of Doctor of Philosophy of
The Australian National University.

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Declaration

This thesis contains published work and work prepared for publication that has been co-authored with collaborating researchers. All the data presented in this work is original research and the contribution of each co-author is stated below. No part of this thesis has been submitted for any previous degree.

Chapter one. Efficient calibration development based on near-infrared spectra in large datasets.

Authors: Jessie Au, Kara N. Youngentob, Ben D. Moore, William J. Foley & Tom Fearn

J. Au conceived the study, conducted field work, collected the nutrient and formylated phloroglucinol compounds data, designed and performed experiments, analysed the data and wrote the first version of the manuscript. K. Youngentob conducted field work and collected nutrient chemical data. B. Moore collected formylated phloroglucinol compounds data. T. Fearn proposed initial experiments and sampling design. W. Foley and K. Youngentob proposed additional experiments. All authors designed the final experiments and read, edited and improved subsequent versions of the manuscript.

This chapter is prepared for future publication in the Journal of Near Infrared Spectroscopy.

Chapter two. Population densities of an arboreal marsupial vary with forage quality.

Authors: Jessie Au, William J. Foley, Robert G. Clark, Karen J. Marsh, Christina. N. Zdenek, Hugh P. Possingham & Kara N. Youngentob

J. Au conducted the field work, collected the most of chemical data, analysed the data and wrote the first version of the manuscript. K. Youngentob, H. Possingham and W. Foley conceived the study. K. Youngentob conducted field work, collected nutrient data and proposed initial experiments. R. Clark developed the sampling design and statistical analyses. K. Marsh collected the unsubstituted B-ring flavanones data. C. Zdenek

assisted with field work and chemical data collection. All authors edited and improved the manuscript.

This chapter is prepared for future publication in Ecology

Chapter three. Changes in forage quality provide a link between disturbance and declines in animal populations.

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J. Au collected the nutrient and unsubstituted B-ring flavanone data, analysed the data and wrote the first version of the manuscript. K. Youngentob collected nutrient data and proposed initial experiments. C. Allen collected the survey data. R. Clark developed the experimental design and statistical analyses. K. Youngentob and W. Foley conceived the study. K. Marsh, I. Sharaf and I.P. Singh collected the unsubstituted B-ring flavanone data. All authors edited and improved the manuscript.

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Chapter four. Bark chewing reveals a nutrient limitation of leaves for a specialist folivore.

Authors: Jessie Au, Kara N. Youngentob, Robert G. Clark, Richard Phillips & William J. Foley

J. Au conducted the field work, collected most of chemical data, analysed the data and wrote the first version of the manuscript. R. Phillips collected most of the chemical data. R. Clark developed the sampling design and statistical analyses. K. Youngentob and W. Foley conceived the study and proposed additional experiments. All authors edited and improved the manuscript.

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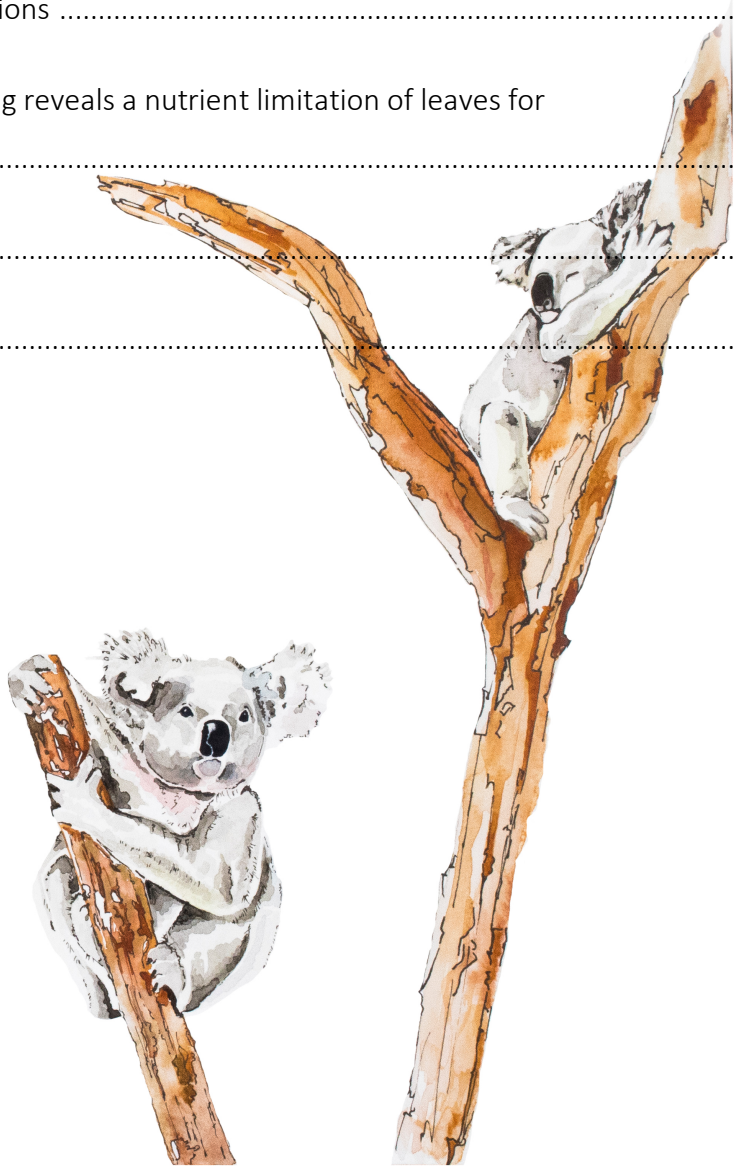


Abstract

Habitat loss is a leading cause of decline in animal populations and identifying suitable habitats are essential for the conservation and management of wildlife. Our ability to identify suitable habitats is reliant on our understanding of the factors that influence the expansion, persistence and loss of animal populations. Nutrition underpins animal growth and reproductive success and is therefore a key factor in animal population dynamics. Both nutrients and anti-feedant secondary metabolites affect the feeding behaviour of folivores such as the koala at local scales. How this translates to whole landscapes remains unknown in any system. I suspect that the availability of plant nutrients and secondary metabolites varies across large Australian forest landscapes, which would explain why there are islands of suitable habitat in a sea of uninhabitable forest.

Our understanding of how nutrition regulates herbivore population density is limited by our ability to collect plant chemistry data across landscapes. This typically involves the collection and analysis of hundreds and thousands of plant samples. Two global near infrared calibration models to predict forage quality across large landscapes in an extensive dataset are introduced in Chapter one. Two biologically-relevant nutritional traits were predicted, available nitrogen (N_A) and formylated phloroglucinol compounds (FPCs). I discuss techniques for developing robust predictive models, facilitating the integration of forage quality into landscape ecology.

In Chapter two, I examined the role of nutrition in explaining the striking differences in koala population densities across its extensive range of habitats. I travelled across the wide distribution of the koala to collect the largest collection of eucalypt leaves for forage quality analysis. I found that forage quality explained variation in transcontinental patterns in herbivore population densities. There was a positive association of nitrogen (as proxies for protein) traits and a negative association of FPCs with koala density. Further, the effect of nutrition remained significant even when other environmental/landscape/climatic variables were accounted for in the model. To my

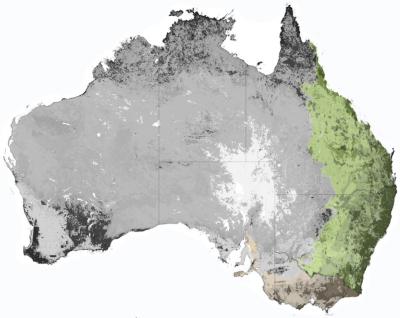
knowledge, this was the first study to show how the effects of nutrition on animal populations can scale up to large landscapes.

In Chapter three, I examined changes in forage quality to describe the cascading negative effects of disturbance on a vulnerable animal population. Following intensive logging and/or wildfires, *Eucalyptus sieberi* dominate the landscape and replace what was once a heterogeneous forest. I combined two large datasets, an large field survey of koala activity, and an extensive forage quality data set. I found that koalas were highly unlikely to be found in these *E. sieberi* dominated (i.e. intensely disturbed) forests. Further, the nutritional quality of forage quality of *E. sieberi* was the poorest of the eucalypt species sampled in the area, with very low concentrations of N_A, and extremely high concentrations of unsubstituted B-ring flavanones (an herbivore-deterrent PSM specific to *Monocalyptus* species). I then demonstrated the process by which disturbance alters the suitability of habitat through changes in forage quality. I simulated the change in tree species composition, from heterogenous forest to *E. sieberi* and showed that forests dominated by *E. sieberi* are nutritionally-poor and unlikely to be suitable habitat for the koala.

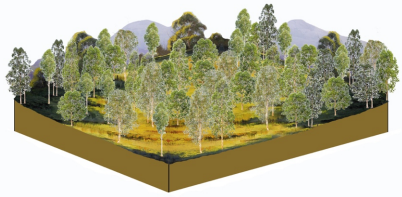
In Chapter four, I described how variation in plant chemistry can inspire baffling bark-eating behaviour in a "fussy leaf eater". Koalas in the Monaro region of New South Wales, Australia eat bark from select individuals of a single species, *E. mannifera*. I revealed that the bark from these chewed trees were significantly higher in sodium and proposed that this remarkable feeding strategy has aided the persistence of a folivore in an otherwise mineral-poor environment. Further, I highlighted that the availability of sodium decreases increasing elevation above sea level and discussed the implications of these findings for the future conservation and management of this iconic animal.

In this thesis, I explore the multi-scale effects of forage quality on an iconic, yet vulnerable specialist folivore, the koala. Each study contributes to our understanding of animal population requirements and we can use this information for effective conservation planning of wildlife populations.

Introduction



Large scale



Medium scale

Small scale

Understanding the factors that influence the distribution and abundance of species is a central goal in ecology. In their classic "why the world is green" paper, Hairston, Smith and Slobodkin (1960) argued that there are "bottom up" and "top-down" processes that regulate species. They proposed that carnivores and decomposers are regulated from the bottom-up, implying that they are resource limited. In contrast, they suggested that herbivores are regulated from the top down, where predators control the number of herbivores and hence why the world is green. This seminal paper provided new insights into how ecosystems might be structured and inspired numerous debates on the strength of these processes on ecosystems. However, it oversimplifies the complexity of interactions between plants and herbivores. One limitation is that the Hairston, Smith and Slobodkin (1960) hypothesis assumed that plants are passive participants in plant-animal interactions and ignored the mechanisms that plants have evolved to avoid being eaten. Not all plants are edible and in recognition of this, alternative hypotheses such as "the world is prickly and tastes bad" have been proposed (Murdoch 1966; Pimm 1991). This introduces a bottom-up constraint on herbivores that can impact foraging decisions and landscape use (Painter 1958; Ehrlich and Raven 1964).

Nutritional ecologists use a "bottom-up" approach to assess how the chemical composition of plants might impact animal foraging strategies and populations (Rhoades and Cates 1976; White 1978; Oksanen et al. 1981; Coley et al. 1985; McNaughton et al. 1989; Hunter and Price 1992). The influence of forage quality and quantity on herbivore feeding behaviour and landscape use has been documented at fine and medium scales. Fine-scale studies include captive feeding studies that investigate how the chemical composition of plant food influences what an animal chooses to eat and how much they can eat (Lisonbee et al. 2009; Spalinger et al. 2010; Marsh et al. 2015). Medium-scale studies look at how chemical composition of forage influences tree and plant choice at a single site (Moore and Foley 2005; Felton et al. 2009; Stolter et al. 2013) or compare home ranges of animals within a population over a limited number of sites (Mammides et al. 2008; Degabriel et al. 2009; McArt et al. 2009). However, there are relatively few studies on the effects of nutrition on animal population dynamics at very large scales (Braithwaite et al. 1983; Grant and Scholes 2006; Wallis et al. 2012). This is partly due to the difficulties associated with landscape-

scale studies, such as collecting animal density data and acquiring meaningful information on the nutritional quality of plants over wide areas and diverse environments (DeGabriel et al. 2014).

Where they do occur, large-scale studies that explore the relationship between animal populations and the quality and/or quantity of forage often use indirect measures of forage quality such as such as soil fertility, rainfall, or crude measures of plant productivity (Coe et al. 1976; Bell 1982; McNaughton et al. 1989; Lawler et al. 1996; Olff et al. 2002; Youngentob et al. 2015). This is due to the relative ease of extending these measures across wide areas. However, simplistic measures of forage quality cannot capture the complex nature of plant foods and the availability of the nutrients within those foods for animals. A deeper understanding of what is forage quality for animals is essential for understanding the effects of food quality on animal populations.

Optimal forage

It is widely believed that animals try to forage optimally, choosing to eat where the food is of the best quality (Schoener 1971; Charnov 1976). Almost a century ago, Elton (1927) argued that finding the right amount and type of food was the primary driving force of all animals. Adequate nutrition is essential for animal growth and reproductive fitness, and therefore should play a key role in population dynamics (White 1983; Hunter and Price 1992; White 1993). However, most investigations linking forage quality and animal populations focus on single simple currencies of food quality and predominantly net energy gain (Thomas et al. 1988; Iason and Murray 1996; Sorensen et al. 2005). In reality, animals must forage to satisfy multiple requirements.

One of the most important aspects of forage quality is protein concentration, because essential amino acids have often been shown to be the limiting nutrient for animal maintenance and growth (McNeil and Southwood 1978; Mattson 1980). Protein supply has numerous implications for animal fitness that have been summarized in several reviews (White 1978; Mattson 1980). Protein is essential for animal growth, tissue repair and reproduction and diets containing more protein are likely to benefit an animal (Simpson et al. 2006; Degabriel et al. 2009). However excessive protein in the diet may lead to difficulties in excreting waste products (Delimaris 2013). If the food quality of a plant to a folivore depends on the protein content of the foliage, then

factors that reduce the net gain of protein to folivores would also need to be considered.

Plant secondary metabolites (PSMs) are ubiquitous in foliage. All animals that eat angiosperms have to contend with PSMs. These secondary metabolites are not essential for the metabolism of the plant but can provide chemical defense against browsing herbivores and influence the feeding behaviour of animals (Painter 1958; Ehrlich and Raven 1964; Feeny 1970; Whittaker and Feeny 1971). There is a diverse array of PSMs that are ingested by browsing herbivores and include compounds such as non-protein amino acids, cyanogenic glycosides, alkaloids, glucosinolates, terpenoids, lectins, flavanoids and tannins (Rosenthal and Berenbaum 1991). Plant secondary metabolites deter herbivory (Freeland and Janzen 1974; Foley et al. 1999) through either exerting toxic effects (e.g. toxicosis, a generalized 'internal malaise' or even death), by interfering with digestion or metabolism of nutrients, or occasionally through non-toxic noxious effects (e.g. taste or contact dermatitis).

The importance of secondary metabolites in mammalian herbivory has been known for a long time and Freeland and Janzen's seminal article in 1974 provided a framework to understand how herbivores might respond to the presence of secondary metabolites in their food (Freeland and Janzen 1974). In brief, the need to detoxify and excrete PSMs limits how much an herbivore can eat. More recent reviews have modified these early syntheses (Foley et al. 1999; Dearing et al. 2005) and shown how the interactions between secondary compounds and mammals work in detail at multiple spatial scales (Foley and Moore 2005; Bryant et al. 2009).

The complex interactions between PSMs and nutrients indicate that measuring "total nutrients" does not necessarily represent the nutritional quality of food. Clearly, secondary metabolites change the relationship between the concentration of nutrients in a plant and the availability of those nutrients. For example, tannins are a chemically diverse group of plant compounds that can bind protein in the plant and thus reduce the digestion of amino acids (Reed 1995). This means that the protein is not available to the animal for digestion. Thus, using integrated measures that assess nutrient availability for digestion are essential. Measures such as available nitrogen (N_A) that take into account for the effect of tannins, provide information on how much nitrogen

(as a proxy for protein) in the plant sample is available to the animal for digestion (DeGabriel et al. 2008). The biological significance of N_A is demonstrated in two influential studies. In Queensland (Qld), variation in N_A, not total nitrogen (N_T) explained the variation in growth of juveniles and fecundity success of females brushtail possums (Degabriel et al. 2009). Similarly, lower availability of N led to lower fecundity and twinning rates in two moose (*Alces alces*) populations in Alaska (McArt et al. 2009). Although tannins affect the digestibility of protein in leaves, it is important include measures of other PSMs that also have a substantial effect on a study system.

The concentrations and types of PSMs can vary across environmental gradients. The carbon-nutrient balance hypothesis (CNB) predicts that PSM concentrations are influenced by the carbon/nutrient ratio in leaves (Bryant et al. 1983; Coley et al. 1985). They proposed that poor-quality soils (low resource availability) lead to a slow growth rate in plants and an increase in the carbon/nutrient ratio in leaves, resulting in increased concentrations of carbon-based PSMs such as phenolic compounds and terpenes. Applications of the CNB hypothesis have helped explain large scale patterns of interactions between boreal plants and snowshoe hares (*Lepus americanus*) in Alaska (Bryant et al. 2009) and browsing patterns of moose (*Alces alces*) in Michigan (Pastor et al. 1993).

Studies on the influence of PSMs on feeding preferences in captive animals have identified important traits that need to be considered when studying foraging behaviour of free ranging browsers. One system that has been well studied is the influence of formylated phloroglucinol compounds (FPCs) on the koala. Formylated phloroglucinol compounds are a group of lipophilic phenolic compounds, which deter herbivory by acting on the emetic (nausea) system (Lawler et al. 1999). These compounds are only present in the *Eucalyptus* subgenus *Symphyomyrtus* (Lawler et al. 2000; Wallis et al. 2010), the subgenus on which the highly specialised koala predominantly feeds (Moore and Foley 2000). Moore et al. (2005) found that increasing concentrations FPCs in eucalypt leaves restricted the food intake of captive koalas. Furthermore, radio tracking studies of wild animals found that FPCs also restricted tree use by free ranging koalas, suggesting that FPCs can influence the distribution of koalas (Moore and Foley 2005; Marsh et al. 2014). However, focussing on *Symphyomyrtus* is likely to provide an

incomplete picture of the nutritional quality of forests because it does not consider of the chemical diversity of *Monocalyptus*, the other dominant eucalypt subgenera.

Monocalyptus co-occurs with *Symphyomyrtus* (Pryor 1959), and recent studies have revealed that it also has its own unique class of PSMs. Almost all monocalypt species contain myriad unsubstituted B-ring flavanones (UBFs), while it appears that symphyomyrtle species do not contain this class of compounds (Tucker et al. 2010). This difference in the presence/absence of UBFs across subgenera is likely to be ecologically relevant for eucalypt specialists. Early feeding studies have shown that UBFs restrict feeding by symphyomyrtle specialists, such as brushtail possums, while having little effect on monocalypt specialists, such as ringtail possums (Marsh et al. 2015, Beale et al. unpublished data, Blyton et al. unpublished data). It is possible that incorporating measures of PSMs such as FPCs and UBFs may provide a more complete picture of forage quality in eucalypt forests, providing an ideal study system to investigate the multi-scale effects of nutrition on arboreal folivores.

Thesis Outline

My thesis builds upon extensive work at fine and medium scales that have revealed some of the key chemical determinants of eucalypt forage quality for the koala and other marsupial folivores. The general aim of my research is to extend our understanding of nutritional ecology from the factors that influence feeding decisions in individual animals to the effects of nutrition on animal populations in the landscape. My thesis is a collection of four original research papers that have been prepared for publication and can be read independently. In these chapters, I explore some of the ways in which both nutrition and PSMs affect free-ranging animals and highlight its importance in habitat assessments.

In 2012, the koala, an Australian icon, was listed vulnerable in (Qld) and New South Wales (NSW; hereafter referred to as the northern koalas). This was due to a series of population declines and crashes across these two Australian states (McAlpine et al. 2015). This happened in stark contrast to the koala population boom experienced by the southern koala populations in Victora (Vic) and South Australia (SA). These

populations boomed to an unsustainable level whereby the koalas stripped the trees bare of leaves, and many koalas began to starve (Duka and Masters 2005; Whisson et al. 2016). Southern koalas are classified as secure native fauna, and not vulnerable. Thus, the conservation status of the koala differs across Australia, and this has captured the attention of ecologists and conservation managers alike.

The Australian federal government provided funding to explore how patterns in forage quality relate to patterns in koala populations densities (NRMMC 2009). The key goals of this research included: (1) Developing a better understanding of koala population requirements and management responses; (2) Using this information to provide policy advice to guide the identification and conservation of high-quality koala habitat.

With the support of my supervisors, advisors, and funding from the Australian federal government, I conduct one of the largest nutritional ecology studies in any study system. Previous work from chemists, nutritionists, ecologists and animal physiologists has increased our knowledge of what forage quality means for arboreal folivores and how this can limit feeding and reproductive success. The major aim of my thesis is to extend this information to answer the question: does population densities of an arboreal folivore vary with forage quality at the large landscape scale? Answering this question requires the collection of nutritional composition data, across forests of a widely distributed animal, and relate this to patterns of population densities. Landscape-scale studies are notoriously difficult to conduct given the scale at which they need to analyse patterns (Wu and Hobbs 2002). Nutritional ecology studies across landscapes require a lot of field and chemical data and demand efficient methods for data collection. Further, a high-level of statistical design and analysis is essential to account for the myriad of factors (for example, geographic, historical, climatic, environmental) that can differ across the landscape. In this thesis, I provide ways to overcome these challenges and highlight the value of large, diverse chemical data sets in ecology.

How can we efficiently assess forage quality across large data sets?

Assessing the forage quality of forests across the koala's range requires the chemical analysis of thousands of samples, which is time-consuming and expensive. I used near infrared spectroscopy (NIRS) to efficiently assess forage quality. In NIRS, leaf samples

are scanned to collect fast and cheap reflectance/absorbance data (spectra), which can be calibrated to expensive and slow chemical trait analyses (traits such as protein). This relationship can then be used to predict the chemical composition from using spectra only, thus significantly reducing future chemical work (Naes et al. 2002).

The efficiency of NIRS therefore relies on a robust model that can be applied to new samples with confidence. Most predictive models in ecology are site-specific and have questionable utility for landscape-data sets that include a diversity of species and cover large geographic areas. In order to efficiently generate the data for forage quality analysis, I identify how the geographic variation within large data sets can help increase the robustness of a model and encourage the sharing of models between research groups. Developing a robust predictive model for N_A is described in Chapter one. Furthermore, I reveal how NIRS can deal with complex chemical traits such as FPCs and explore how the diversity of FPCs can influence the prediction capabilities of a model. Forage quality is complex as it involves myriad nutrients and toxins that can all interconnect and influence the animal (Raubenheimer 1995; Raubenheimer and Simpson 1999). Thus, sufficient measures of nutrition that represent plant nutrients and the complexity of PSMs are essential if we are to investigate the role of nutrition in animal populations (Scrivener et al. 2004; Foley and Moore 2005; DeGabriel et al. 2014).

Does population density of a folivore vary with forage quality across landscapes?

For an animal that is renowned as a "fussy leaf eater", the different environments in which the koala can persist is astonishing. Its distribution covers four states of Australia (Qld, NSW, Vic and SA) and includes a variety of environments such as coastal, swampland, subalpine and semi-arid Australia (Martin and Handasyde 1999). In addition, koala populations are found across various land use types, such as national parks, state forests, urban reserves, and farmland. Identifying the underlying factors that influence koala populations is difficult because it covers vastly different environments and land use (McAlpine et al. 2015).

In Chapter two, I present a leaf sampling design and statistical analysis methods for landscape-scale nutrition studies. In consultation with ecologists, statisticians, nutritional ecologists and experts on koalas, a sampling design was developed that

could be used universally across forests. Using this leaf sampling design, I collected leaf samples for forage quality analysis from most sites with existing koala density data. I collaborated with consultants, academics and government scientists who have extensive knowledge of the koala populations in their research locations and provided estimates on koala densities at their surveyed sites.

I couple this extensive density data set with the largest collection of eucalypt leaves for forage quality analysis. The chemical data set contains nutritional data from four biologically-relevant traits: N_T, N_A, FPCs and UBFs. I have the unique opportunity to explore the relationship between the nutritional traits and how these traits vary across tree species and large landscapes. I use mixed-effect models to show how koala densities vary with forage quality and demonstrate how strong the effect of nutrition is, even when environmental, landscape and climatic differences are accounted for in the model.

Investigating a process that is driving patterns of forage quality across forests

It is clear that the forage quality is important for folivores; however, the processes that drive patterns in forage quality across landscapes can be more difficult to understand. While sampling eucalypt forests across eastern and southern Australia, an interesting case study allowed me to investigate how disturbance can alter forage quality of a forest by changing species composition. *Eucalyptus sieberi* is a fast-growing, monocalypt species that thrives after disturbance. Logging, either intensive or repeated, or intense fire events can result in dense monocultures of *E. sieberi*, replacing what was once a diverse forest (Florence, 1996). To understand the consequences of disturbance on forage quality and further effects on animal populations, I combine an intensive external survey of koala activity in the Bermagui - Mumbulla area of NSW (Allen et al. 2010, 2016) with the extensive forage quality data set from Chapter two. I show how the percentage of *E. sieberi* in a forest can influence the presence of koalas. I also simulate the effects of disturbance by increasing the proportion of *E. sieberi* in modelled forests and show how this change in forest composition alters the forage quality of the landscape.

What micronutrient challenges could animals face?

Developing a better understanding of animal population nutritional requirements typically start from fine-scale studies that investigate the factors that influence diet selection. While travelling for field work in Chapter two, I became aware of an unusual feeding behavior in the Monaro region, NSW. In this area, unusual scarring across the bark of *E. mannifera* remained a mystery until infrared cameras revealed that koalas eat bark. This area includes some of the highest-elevation sites at which koalas are known to occur (900–1,300 m). High elevation environments are renowned to be mineral deficient (Blair-West et al. 1968), and the unusual behavior may represent a way to acquire missing micronutrients.

In Chapter four, I conduct an intensive chemical analysis of the study area and compare the concentrations of minerals in bark and leaf samples. I highlight a unique feeding adaptation of the koala that allows it to persist in an otherwise unsuitable environment. Further, I show how the concentration of micronutrients can vary in the landscape and describe a potential nutritional challenge for animals shifting their distribution to higher altitudes.

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Chapter One

Efficient calibration development based on near infrared spectra in large data sets

Keywords: available nitrogen, forage quality, formylated phloroglucinol compounds, herbivore, large data sets, near infrared, partial least squares regression, sample size.

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Abstract

Large data sets intrinsically contain great spectral and chemical variation that can be used to develop broadly applicable calibration models, increasing the efficiency of near infrared (NIR) spectroscopy. Understanding the causes of this variation and how to use it during calibration development is likely to lead to robust calibration models with realistic estimates of predictive error. In this study, we used partial least squares (PLS) regression to model the relationships between NIR spectra and two chemical traits, available nitrogen (N_A) and total formylated phloroglucinol compounds (FPCs) in *Eucalyptus* leaves. Using an extensive data set of spectra and reference values (RV) from eucalypt leaves, we exploited nested spatial structure in the trait data during calibration development and model validation. Geographic variation amongst samples influenced how well N_A could be predicted. Predictive error of the model was greatest when tested against samples from different Australian states and local government areas to the calibration set. In addition, we found that simply relying on spectral variation (assessed by Mahalanobis distance) may mislead researchers into how many RV samples they really need. The prediction accuracy of the model of N_A differed little whether we included 300 or up to 987 calibration samples and indicated that an excessive number of RVs were obtained. Lastly, we produced a suitable multispecies calibration for FPCs and highlighted how PLS can reveal compounds that covary with NIR spectra. We discuss the difficulties with predicting complex chemical traits such as FPCs in ecological studies where there are likely to be new uncharacterized compounds amongst different species but also within a single species. Directing effort towards broadly applicable models will encourage sharing of calibration models across projects and research groups and facilitate the integration of NIR spectroscopy in many fields.

Introduction

Accumulating spectra and reference values (RV) over many years or across projects and research groups can lead to large data sets with thousands of samples (Berzaghi et al. 2002; Downes et al. 2010; Schimleck et al. 2010). Large data sets (>1000 samples) that are used to predict chemical traits from near infrared spectra present particular challenges in chemometrics. As a project accumulates ever more samples with more diversity, the data set intrinsically contains greater chemical and spectral variation that must be represented in a robust calibration model. If captured successfully however, researchers can develop a broadly applicable "global" calibration that encapsulates chemical biodiversity (Smith and Flinn 1991). Reducing time and cost for NIR calibration development would benefit many fields such as forestry, agriculture, and ecology where large data sets are becoming more common. In saying that, it is still unclear how best to explore, capture, and utilise the variation inherent within a large data set.

Investing in a global calibration involves substantial initial costs, and so approaches to reducing the time and costs of model development are valuable. To tackle a large and diverse data set, one must explore the sources of compositional variation and how it can assist in the selection of samples for calibration and validation of models. Variation derives from differences across measurement days (Kemps et al. 2010), plant parts (Gillon et al. 1999), plant species (Lawler et al. 2006) and laboratories (Berzaghi et al. 2002). Ecological studies present unique challenges because they can extend over a variety of environments, climate, and plant taxonomic groups. Models developed in ecological studies are usually restricted to site-specific analyses and have questionable utility for applications outside the collection sites of the calibration set (Ebbers et al. 2002; Moore and Foley 2005; Degabriel et al. 2009; Youngentob et al. 2011; Marsh et al. 2014; Stalenberg et al. 2014; Windley and Foley 2015). However, there have been some successes in creating global calibration models across wide areas, which open the door to further improvements in model performance (Asner et al. 2011).

Over many years, sharing of samples and NIR spectra in agricultural research groups has facilitated the compilation of very large data sets (Dardenne 2002; Berzaghi et al. 2002).

For example, studies have collated over 25,000 forage samples (e.g. legumes and grasses) from seven countries and compared calibration methods for moisture, crude protein, and neutral detergent fibre (Berzaghi et al. 2002). The accuracy of their models for independent data sets were low and unacceptable for all methods. However, they found that predictive performance of the calibration equations improved with different statistical techniques and standardisation of instruments and procedures. We propose that in addition to these suggestions, incorporating nested spatial structure in large data sets would also improve the performance of global calibrations.

In this study, we compare cross-validation and model validation techniques using a diverse data set of *Eucalyptus* leaves collected as part of a larger landscape study of variations in forage quality for the koala (*Phascolarctos cinereus*) and explore optimal calibration methods for quantitative NIR spectroscopy. A robust global calibration of the nutritional quality of leaves is important for investigations into plant-animal interactions and patterns of animal distribution and abundance (Stolter et al. 2006; Rothman et al. 2009; Wallis et al. 2012), aiding wildlife management and conservation. Our choice of a large ecological data set with samples from many origins will allow us to explore the influence of nested structure and broaden our understanding of different approaches for global calibration development.

An ecological study - eucalypt forage quality

The nutritional quality of eucalypt leaves for vertebrate herbivores has been widely studied and researchers have identified several chemical traits that contribute to foraging behaviour, habitat selection, and reproductive success in populations of wild folivores (DeGabriel et al. 2014). The chemical traits that have been most useful for determining eucalypt forage quality are the foliar concentrations of available protein (Degabriel et al. 2009) and formylated phloroglucinol compounds (FPCs, Marsh et al. 2006).

Available protein or available nitrogen (N_A, with nitrogen being a proxy for protein) is an integrative measure that accounts for the effect of tannins on the amount of total nitrogen available for digestion. Tannins are a ubiquitous class of secondary compounds found in many plants, which can bind to plant protein making it unavailable to the

animal. The variability of N_A in eucalypt leaves across a landscape has been shown to explain the reproductive success of the common brushtail possums (*Trichosurus vulpecula*) and the growth rate of their offspring (Degabriel et al. 2009), as well as food tree choice by wild greater gliders (*Petauroides volans*, Youngentob et al. 2011) and koalas (Stalenberg et al. 2014). In addition, N_A is an influential factor in forage quality for a number of herbivorous species outside of Australia, such as moose in Alaska (McArt et al. 2009) and spider monkeys in Bolivia (Felton et al. 2009).

Formylated phloroglucinol compounds are a diverse group of terpene adducts found largely in the eucalypt subgenus *Symphyomyrtus*. Formylated phloroglucinol compounds have unknown physiological effects in animals but their intake is strongly regulated because they cause nausea and aversions to feeding (Lawler et al. 1998). There are approximately 30 known FPC compounds found in different eucalypts, many as very minor components. Previous studies have focussed on quantifying approximately 17 of the most common of these (Moore et al. 2004) and so quantifying the total FPC concentration in eucalypt leaves is much more difficult using NIR spectra than it is for N_A. Previously, FPC calibration models have only been developed for one to three eucalypt species at a time (Moore and Foley 2005; Youngentob et al. 2011; Marsh et al. 2014) and less than 10 species have been studied (Moore et al. 2004). Developing calibration models for complex chemical traits, like FPCs, across a large number of species with significant spectral and chemical diversity is not simple but is essential to facilitate landscape–scale studies of plant-animal interactions.

Calibration development and model validation

Often, cost and laboratory restrictions dictate compromises during calibration development and users must understand the consequences of these decisions. Some important considerations are how to select calibration samples, how many reference samples are required for calibration, how to validate the model, what is a realistic predictive error for independent samples, how to identify suitable/unsuitable spectra for calibration, and what standardised chemical trait(s) can be used to compare taxonomically-diverse samples. In addition, there are a suite of chemometric options for

developing calibration models and the suitability of these options vary depending on the application and the properties of the data set.

Partial least squares (PLS) regression is widely used for modelling NIR data. It is a powerful multivariate technique that finds latent factors in the data to maximise the covariance between spectra and the chemical trait. As more PLS components are included in the model, more covariance between spectra and the chemical trait is captured. Selecting too many PLS components can capture the random error of the calibration set, can lead to 'over-fitting' of the model and an increase in the error of values predicted by the model with new samples. However, choosing too few PLS components can lead to a model that has not completely captured the underlying relationship between the spectra and the chemical trait (i.e., under-fitting).

The combination of cross-validation of the calibration set and testing the model with an independent set of validation samples are common techniques used for calibration model selection and assessment (Naes et al. 2002). Cross-validation temporarily removes sample(s) from a calibration set (a set of samples with spectral and reference chemistry data), builds a model with the remaining samples and tests the developed model on the excluded sample(s). These steps are repeated until all samples have been tested and the fitted values are compared with the RV. The capability of PLS models with different numbers of latent factors should be assessed; a parsimonious model that balances a low number of PLS components with a low cross-validation error is favoured. To ensure that the model is neither over-fitted nor under-fitted, the spectra of an independent set of samples, the validation set, is then introduced to the newly developed calibration model (Martens and Naes 1989; Naes et al. 2002). In this instance, the predicted values from the model and RV of the independent data set are compared. When an independent test is not available, researchers typically subdivide their RV data into a calibration and validation set. While it is better to have more genuinely independent samples, the nested structure within large data sets may indicate how best to split the RV data (Fearn 2013).

We propose that where there is some structure in large data sets, researchers should exploit this structure during both cross-validation and validation. Using a variety of

subdivision techniques during cross-validation may provide a fairer assessment of the predictive capability of the model for future samples. Three techniques are investigated in this study:

(i) Leave one out (LOO_{CV}): one sample is put aside at each iteration.
(ii) Leave group out (LGO_{CV}): calibration samples are split into groups with similar characteristics such as species or geographic location.
(iii) Random subset (RS_{CV}): K-fold or Monte Carlo groupings: samples are randomly allocated into groups of a set size

We predict that exploiting the nested structure when selecting validation samples provides a tougher, and therefore more convincing, test of the calibration. Introducing tougher challenges during model development should lead to a more robust global calibration which can then be applied with confidence to new sample sets.

Aims

Available nitrogen: exploit nested structure

The first aim of our study was to produce a calibration model for N_A that can accommodate the variation in a multi-tree species, multi-site database, and we estimated its performance on three independent data sets. We hypothesised that there is a nested structure within the diverse collection of leaves from different eucalypt tree species. The spectra of samples may be similar within groups. For example, samples collected from a similar geographic area or across the same or closely-related species (Gillon et al. 1999; Berzaghi et al. 2002; Kemps et al. 2010). Thus, we propose that exploiting this nested structure during cross-validation and validation may provide a more realistic assessment of the sensitivity of the model for new samples.

Available nitrogen: how many calibration samples do you really need?

Our second aim was to explore the effect of the numbers of samples on the relationship between NIR spectra and N_A. Selecting samples for laboratory analysis is typically based on the variability of spectra (as indicated by metrics such as the Mahalanobis distance). However, the variation in the chemical trait of interest may not explain all of the variation in spectra (Kemps et al. 2010). Given that satisfactory calibrations for N_A have

been established in several studies (Wallis et al. 2012; Marsh et al. 2014; Windley and Foley 2015), we hypothesised that selecting samples based on spectral variation alone may lead to an excessive number of RV samples. We aimed to split the RV set and investigate how the number of calibration samples can influence the predictive accuracy of a model.

FPC calibration: do you need every compound?

The third aim was to develop a single calibration model that can predict concentrations of a complex chemical trait, FPCs, across a multi-species database. We will investigate whether the 17 FPC compounds that have served as a relative measure of total foliar FPCs in past studies (Moore et al. 2005; Youngentob et al. 2011) are necessary for robust calibrations of total FPCs or whether the same accuracy can be achieved with a smaller subset of compounds. We hypothesise that a subset of FPCs are likely to be more prominent compounds and have a relatively stronger relationship with NIR spectra. We predict that these prominent compounds can be predicted with higher accuracy, facilitating model calibration and increased prediction accuracy for total FPCs.

Methods and Materials

Sample collection

All data were collected as part of a large landscape project that covered eastern and southern Australia (Fig. 1). We collected mature, fully expanded eucalypt leaf samples over two field seasons. During the first field season (from September 2012 to April 2013), we collected 2,096 eucalypt foliage samples and used this data set to develop the N_A calibration model. In the second field season (from September 2013 to April 2014), we collected 1,576 additional samples from sites different to those in the first field season. Of those, we separated a subset of samples (details on selection process are described below) to use as an independent validation set for the N_A calibration model (referred hereafter as the N_A data set).

The development of an FPC calibration began after both field seasons. Only samples from the eucalypt subgenera *Symphyomyrtus* contained FPCs so this collection was further reduced to 1,605 samples after removing the other subgenera (referred hereafter as the FPC data set). We collected leaf samples using a throw-line launcher (Youngentob et al. 2016). We froze samples immediately in dry ice (CO_2) and upon return to the laboratory, stored them in a freezer at -20°C until they could be further processed (no longer than 2 months from collection).

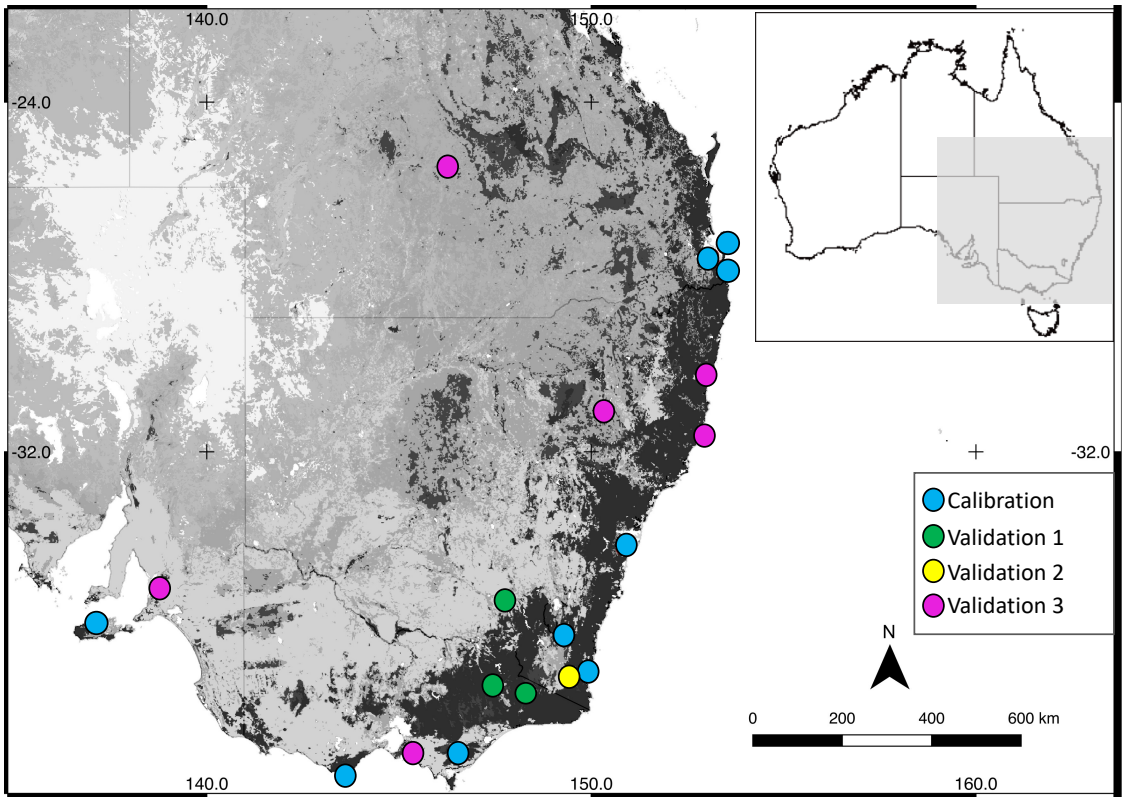

Figure 1. Map of study sites across four Australian states; Queensland, New South Wales, Victoria and South Australia. Land cover is represented by the shading of the map. Sites are coloured to represent the split of samples for calibration and validation.

Sample preparation and spectral collection

We freeze-dried and ground each sample to pass a 0.5 mm screen using a FOSS Cyclotec 1093 cyclone mill (Foss, Hilleroed, Denmark). We stored ground samples in sealed polyethylene containers at 22ºC and away from light. For each sample, we presented two subsamples of ground leaf powder to the instrument. We collected spectra at 2 nm intervals from 400 to 2498 nm using a scanning near infrared

spectrophotometer (Foss-NIRSystems 6500; www.foss.com) with a spinning cup attachment. We averaged these replicates only if the standard error of the difference between the two spectra [expressed as log $(1/R)/10^6$] was less than 150. If the standard error was greater than 150, we re-sampled and re-collected the spectra. We conducted this work in a laboratory at 18 - 22ºC.

Sample selection for chemical analysis

Using the CENTER and SELECT functions in the software WinISI III version 1.50E (Infrasoft International, Port Matilda, PA, USA), we identified subsets of samples for each nutritional trait (N_A and FPCs) that represented the spectral variation of the data set. The CENTER algorithm ranks each spectrum based on its Mahalanobis distance to the average spectra and the SELECT algorithm identifies a suitable calibration set for reference chemistry analyses. The samples in the data sets for calibration development differed for N_A and FPC analysis and these are summarised in Table 1.

Available nitrogen

We selected 987 out of 2,096 samples for N_A analysis and calibration development. We performed principal component analysis (PCA) on all spectral data (*N* = 2,096) and inspected if spectra clustered based on any known variables such as geographic location or species. We then projected spectra from three independent validation sets onto this PCA space and observed where these validation samples lay.

Formylated phloroglucinol compounds

We selected 400 of the 1,605 samples for FPC analysis. We excluded 80 of these samples that showed no evidence of FPCs upon analysis. We included an additional 9 samples to ensure that there were representatives from each transect (FPC data set) and *Symphyomyrtus* species from all data sets. In total, we analysed 329 samples for FPCs.

Table 1. Summary of data used for calibration model development to predict available nitrogen and formylated phloroglucinol compounds. SEL = standard error of laboratory. SEL=√[Σ(y1-y2)^2/2N] where y1 and y2 are duplicate analyses. SD = standard deviation.

							Calibration				Validation			
Analysis	**Units**	**Dataset name**	**Subgenera**	**Dataset size**	**Reference samples**	**SEL**	**N (samples)**	**Range**	**SD**	**Mean**	**N (samples)**	**Range**	**SD**	**Mean**
Available nitrogen	**%**	N_A	*Monocalyptus Symphymyrtus Angophora Corymbia*	2096	987	0.03	987	0.2 - 2.4	0.33	1.03				
		Validation1	*Monocalyptus Symphymyrtus*		37	0.06					37	-0.02 - 1.81	0.40	0.74
		Validation2			11	0.08					11	-0.02 - 1.81	0.59	0.97
		Validation3			84	0.04					84	0.03 - 2.05	0.40	1.25
$TotalFPCs_{17}$	**mg.g-1**	FPC	*Symphymyrtus*	1605	329	1	200	0 – 53	11	13	129	0 - 59	12	17
$TotalFPCs_8$						1		0 - 46	10	10		0 - 58	10	13

Chemical analyses

Available nitrogen

To determine N_A of each sample, we used an established *in vitro* digestion method (DeGabriel et al. 2008). This analysis estimated the proportion of total N in the leaf sample that is available for digestion by an animal. The indigestible portion is that bound in tannin complexes or present in the cell wall matrix. We weighed 0.8050 ± 0.0050 g of air-dry, ground sample into pre-weighed ANKOM F57 filter bags (Ankom Technology, Macedon, New York) in duplicate. We incubated samples sequentially in three buffers at 37°C; 0.05M Tris–BASE buffer (pH 7.1), pepsin (2 $g.L^{-1}$) and cellulase (6.25 $g.L^{-1}$, pH 4.75). We calculated residual dry matter (DM) of the ground leaf by measuring the average oven DM in 50 random samples. We measured the N concentration of each sample, pre- and post-digestion using a Leco CNS-2000 combustion analyser (Leco, St Joseph, Michigan). We calculated N_A using the proportional difference in N concentration of a sample, before and after the assay.

Formylated phloroglucinol compounds

We determined the concentration of FPCs using high performance liquid chromatography (Moore et al. 2004). We extracted samples by sonication in acetonitrile containing 0.1% trifluoroacetic acid and, 2-ethyl phenol (30 $mg.L^{-1}$) as an internal standard. We weighed 50 ± 2 mg of ground leaf and 4 ml of extraction solvent into glass scintillation vials. We placed the vials in a sonicator for 10 min and then let them stand at room temperature for 60 min. We separated the components of 10 µl of filtered extract on an Agilent HPLC with photo diode array detector using a Wakosil 250×4mm GL 3C18RS (SGE) analytical column. We eluted extracts under gradient conditions with 0.1% TFA in acetonitrile (A) and 0.1% TFA in Milli-Q water (B) as follows: 60% A/40% B for 5 min, linear gradient to 90% A/10% B over 60 min, held for 25 min. Flow rate was 0.75 $ml.min^{-1}$ and the column temperature was 37ºC. We measured the detector response at 275 nm. We used extinction coefficients(Moore et al. 2004) to quantify 17 known FPCs; macrocarpal I (R_t = 13 min), macrocarpal J (R_t = 17 min), eucalyptone (R_t = 28 min), macrocarpal A (R_t = 32 min), macrocarpal (unknown 1, R_t = 34 min), macrocarpal (unknown 2, R_t = 35 min), macrocarpal B (R_t = 37 min), macrocarpal

(unknown 3, R_t = 39 min), macrocarpal (unknown 4, R_t = 42 min), grandinal (R_t = 43 min), macrocarpal (R_t = 48 min), sideroxylonal A (R_t = 44 min), sideroxlyonal C (R_t = 45 min), grandinal (R_t = 53 min), sideroxylonal B (R_t = 55 min), jensenal (R_t = 60 min), macrocarpal G (R_t = 70 min).

Spectral pretreatments

We developed separate calibration models for each nutritional trait (N_A and FPCs) in MATLAB R2016b. Because of the detector shift at 1100 nm in the near infrared spectrophotometer, we cropped spectra to 1102 - 2498 nm. We took Savitzy-Golay derivatives (Savitzky and Golay 1964) to eliminate an additive baseline that arose from non-relevant physical differences such as particle size. As derivatives can introduce some noise to spectra, we applied standard normal variate or multiplicative scatter correction to each spectrum. We tested several combinations of spectral pre-treatments and a summary of these are found in Supplementary Material 1. The best pre-treatment (based on the most parsimonious model with the simplest math treatment) for spectra was first-derivative with a gap window of 7, 2nd order polynomial and standard normal variate (Fig. 2).

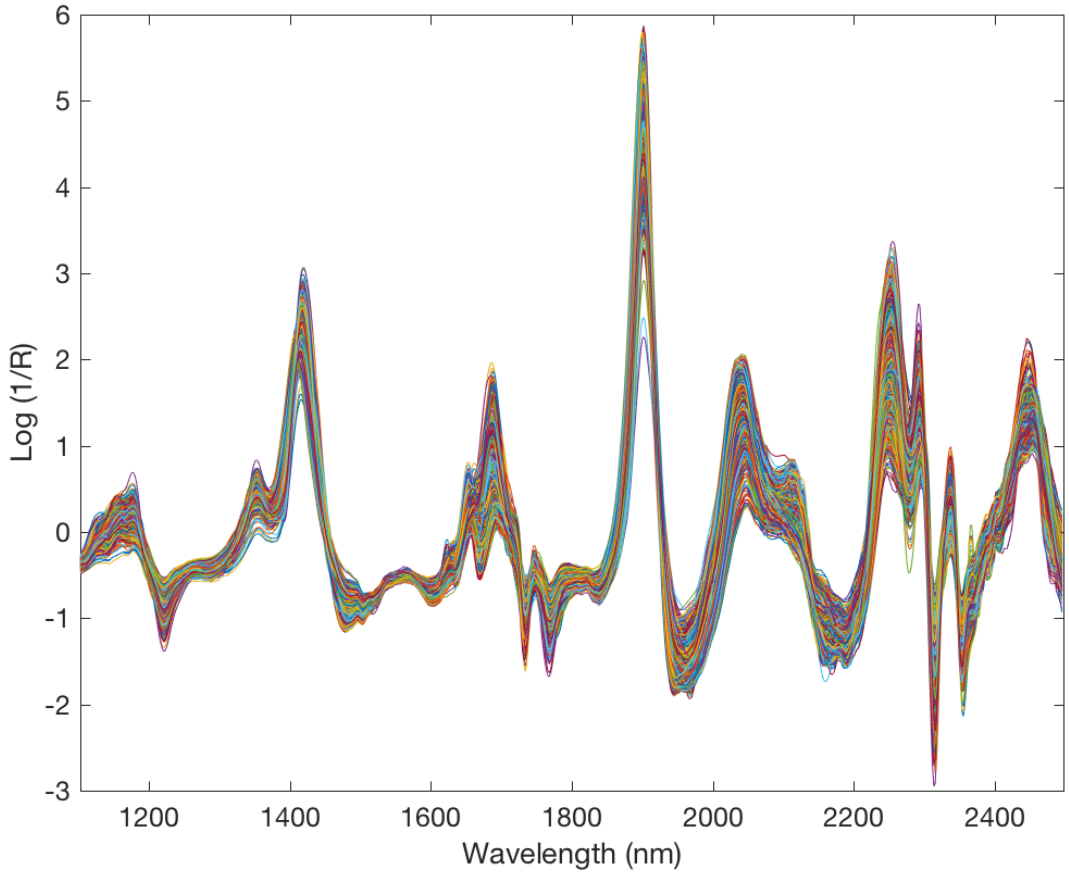

Figure 2. Spectra for all samples (N = 2,096). The pre-treatments applied to spectra were first-derivative with a gap window of 7, 2nd order polynomial and standard normal variate.

Calibration model development

We assessed the robustness of the calibration models by testing the calibration model on samples not included during model development (i.e. validation samples). We compared the root mean squared error of cross-validation (RMSECV) of the models and root mean squared error of prediction (RMSEP) on the validation sets.

Available nitrogen: exploit nested structure

We split the samples used for chemical analysis (referred hereafter as RV data) in four ways and investigated if there was an ideal technique to subdivide data into a calibration and validation set. The RV data were split randomly, by sample site, by local government area and by Australian state (with New South Wales "NSW" or Queensland

"Qld" samples as the split-off validation set). We compared eight different cross-validation techniques using the calibration sets.

- Leave One Out (LOO_{CV}): partition one sample.
- Leave Group Out (LGO_{CV}) – partition samples based on nested structure in the data set
  - $SampleSite_{CV}$: partition data by sample site
  - $LocalArea_{CV}$: partition data by local government area (for example, Brisbane)
  - $State_{CV}$: partition data by Australian state
- Random subset (RS_{CV}): randomly partition RV data into groups with a set number of samples.
  - RS_{10}: partition RV data into 98 groups of 10 samples (98 groups).
  - $RS_{SampleSite}$: partition RV data into 59 groups of 17 samples (59 sample sites)
  - $RS_{LocalArea}$: partition RV data into 17 groups of 61 samples (17 local areas)
  - RS_{State}: partition RV data into 4 groups of 154 samples (4 states)

We then combined all RV samples for a global N_A calibration and compared across eight cross-validation techniques discussed previously. We compared the RMSECV of the calibration model and selected the most parsimonious models to predict the validation sets. We then tested these newly developed PLS models on three independent validation sets with samples analysed by different users;

- Validation1: Eucalypt leaf samples from a separate transect-based koala forage quality data set
- Validation2: Eucalypt leaf samples from Tantawangalo, an area of NSW. We intentionally excluded these samples to test the prediction model as the spectra were unique to most spectra in the transect database.
- Validation3: Eucalypt leaf samples collected from NSW and Victoria (Vic) for an unrelated study

To better understand just how well the eucalypt data set can predict independent data sets, we investigated how the number of PLS components influenced the predictive performance of a model on independent samples. We developed 30 PLS models with 1 to 30 PLS components using all RV data and tested it against the independent data sets, Validation1, Validation2 and Validation3. We compared the three resulting plots of RMSEP values with the eight RMSECV curves. We did not use samples from these validation sets during calibration development.

Available nitrogen: how many samples do you really need?

To reveal if all samples were required to best explain the relationship between NIR spectra and N_A, we developed a series of calibration models and varied the number of samples included during calibration. Using the RV data, we randomly allocated 886 samples to a model development set and 101 samples to a "set –aside" validation set. We developed a series of PLS models, varying both the number of PLS components and number of calibration samples. Using the model development set, we selected 50 samples using the SELECT function in WINISI and developed 30 models with 1 - 30 PLS components. We then tested these models against the split-off 836 samples and the set-aside validation set (N = 101). We repeated this step but increased the number of calibration samples selected in increments of 50. For example, second round = selected 100 calibration samples and 786 split-off validation samples, third round = 150 calibration samples and 736 split-off validation samples. We tested a total of 510 PLS models (17 increments, 30 PLS components) against both the split-off validation sets and the set-aside validation set (N = 101). We then repeated the previous steps, however instead of using the SELECT function to select the various calibration sets, we randomly selected the calibration samples from the model development set.

Formylated phloroglucinol compounds: do you need every compound?

We summed the concentrations of 17 known FPC compounds to get a single index of FPCs for each sample, "$TotalFPCs_{17}$". We randomly chose 200 samples for calibration development and ensured all species and sample sites were represented in calibration set. We set aside the remaining 129 samples for the validation set. We performed LOO_{CV} on the calibration set and selected the most parsimonious model for testing the

validation set. We also performed PLS2 on pre-treated spectra against all 17 FPCs and investigated variations in covariance between compounds and spectra.

Results

Available nitrogen

Spectra

In a PCA of eucalypt data set, the first two principal components explained 71% of the variation in NIR spectra (PC-1 = 55% and PC-2 = 16%). The SELECT function identified 987 samples for N_A analysis that distributed approximately uniformly across the space of the first two principal components (Fig. 3). Samples from Qld, Vic, and NSW separated across PC-1 and PC-2 whereas samples from South Australia (SA) appeared to form compact clusters (Supplementary Material 2a). While samples from the same local government area (e.g.Kangaroo Island) appeared to cluster (Supplementary Material 2b), there were no other obvious clusters with subgenera (Supplementary Material 2c), tree species (Supplementary Material 2d), sample site (Supplementary Material 2e), tree height (Supplementary Material 2f) or DBH (Supplementary Material 2g). Projecting spectra from three independent validation sets onto the PCA space of the eucalypt data set showed much overlap between the data sets (Fig. 3). This suggested that the RV data were mostly appropriate for all data sets. Some samples from Validation1 separated along PC-2, and from the rest of RV data (south east region of Fig 3).

Calibration model development: exploit nested structure

We split the RV data based on nested structure and Table 2 summarises the calibration and validation results. The RMSECV of the calibration sets and the RMSEP of the split-off validation sets ranged from 0.12 - 0.18 % N_A (Table 2). Predicting N_A in samples from a different Australian state provided the weakest predictions (highest error) during calibration development. This was demonstrated in both the $State_{CV}$ (Table 2, RMSECV = 0.15- 0.17% N_A) and when predicting N_A in an Australian state different to those in the calibration (Table 2, RMSEP = 0.15 - 0.18% N_A).

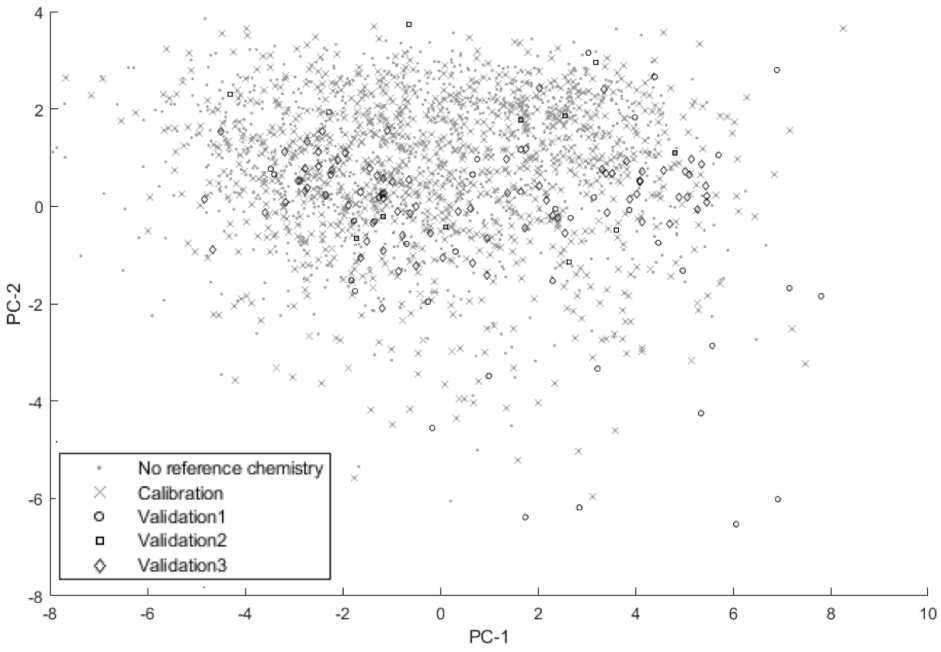

Figure 3. Distribution of scores of the available nitrogen spectral dataset along the first two principal components. Principal component analysis was performed on the landscape eucalypt dataset (N = 2096, samples marked in grey) and 987 samples were selected for calibration development (marked by "x"). Spectral data from three independent validation sets were projected onto the PCA space of the landscape eucalypt data (marked in black).

Table 2. Performance of calibration models with different splits of the reference chemistry set. Samples were split into a calibration and validation set based on known substructure in the dataset. Models are compared against the root mean squared error of cross validation and root mean squared error of prediction on the validation set.

			Calibration			Validation		
Type of split	CV type	PLS components	Local areas	N	RMSECV	Local areas	N	RMSEP
Random	Leave one out	5	All	618	0.13	All	369	0.13
	SiteCode	5			0.14			0.13
	LocalArea	6			0.14			0.13
	State	5			0.15			0.13
	RS_10	6			0.13			0.13
	RS_SiteCode	6			0.13			0.13
	RS_LocalArea	6			0.13			0.13
	RS_state	6			0.13			0.13
Site code	Leave one out	5	All	618	0.12	All	369	0.14
	SiteCode	5			0.14			0.14
	LocalArea	5			0.15			0.14
	State	5			0.16			0.14
	RS_10	6			0.13			0.13
	RS_SiteCode	6			0.13			0.13
	RS_LocalArea	6			0.13			0.13
	RS_state	6			0.13			0.13
Leave NSW out	Leave one out	5	North Stradbroke Island	622	0.12	NSW South Coast	365	0.17
	SiteCode	5	Springsure		0.13	Campbelltown		0.17
	LocalArea	5	Kangaroo Island		0.15			0.17
	State	5	Otways		0.16			0.17
	RS_10	6	Brisbane		0.12			0.17
	RS_SiteCode	6	Gold Coast		0.12			0.17
	RS_LocalArea	6			0.12			0.17
	RS_state	6			0.12			0.17
Leave Qld out	Leave one out	6	NSW South Coast	490	0.13	North Stradbroke Island	497	0.18
	SiteCode	5	Kangaroo Island		0.15	Springsure		0.15
	LocalArea	5	Otways		0.17	Brisbane		0.15
	State	5	Cooma		0.15	Gold Coast		0.15
	RS_10	7	Campbelltown		0.13			0.17
	RS_SiteCode	7			0.13			0.17
	RS_LocalArea	7			0.13			0.17
	RS_state	7			0.13			0.17
All samples	Leave one out	5	All	987	0.13		NA	
	SiteCode	5			0.14			
	LocalArea	5			0.15			
	State	5			0.16			
	RS_10	6			0.13			
	RS_SiteCode	6			0.13			
	RS_LocalArea	6			0.13			
	RS_state	6			0.13			

We then recombined all 987 RV samples to develop a global PLS model. We first tested the previously described eight cross-validation techniques on the large eucalypt data set (Table 2). LOO_{CV} had the lowest RMSECV vs PLS components curve, while $State_{CV}$ had the highest curve, with a difference of about 0.03 % N_A between curves (Fig. 4a, b). We found that RS_{cv} of the data, regardless of the size of the subsets, did not influence the cross-validation error of the model. The shape of the RMSECV curves differed between the cross-validation techniques. In the case of LOO_{cv} and LGO_{cv}, a "bend" in the curve indicated that five latent factors were suitable for building the model. In RS_{cv} however, six latent factors were chosen, and we found that the RMSECV curve of RS_{cv} continued to decrease until 22 latent factors. The RMSECV of all eight cross-validation techniques ranged from 0.12 – 0.17 (Fig. 4).

We compared the performance of two calibration models, one with five and one with six PLS components and tested them against the three independent validation sets (Fig. 5). All independent validation samples were collected from sites in different local government areas to the calibration set. The RMSEP across all three independent validation sets ranged from 0.21 – 0.31 % N_A, approximately 50% greater error than the RMSECV curves (Fig. 4a). We investigated if predictive error of the models correlated with Mahalanobis distance from the mean spectra, but we did not observe any pattern. A large Mahalanobis distance was not necessarily indicative of a large predictive error. Furthermore, the model poorly predicted several samples (Fig 5a, b, d, e.). These samples had relatively small Mahalanobis distances from the mean spectra and were located near many RV samples. We do not know why these samples were unique or different from the rest of the samples in this data set. They were neither unique in collection site, tree species, Australian state, nor chemistry.

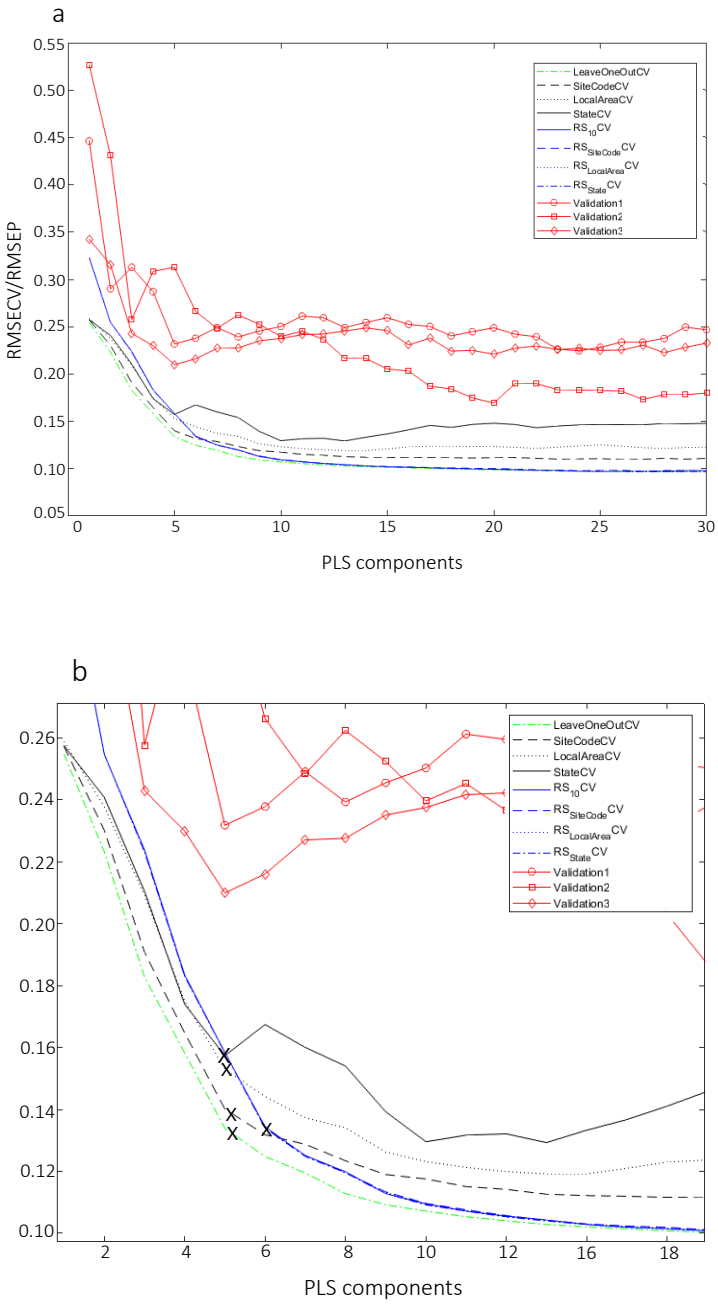

Figure 4. Comparing cross-validation techniques on the koala transect study and testing the calibration set against three independent validation sets. (a) Eight cross-validation techniques were tested for calibration model development and key areas is enlarged in (b). The techniques used were Leave One Out (green line), Leave group out (black lines, splitting data by SiteCode, LocalArea and Australian State), Random subset division (blue lines, RS10 [98 groups], SiteCode [59 groups], LocalArea [10 groups], State [4 groups]. We assessed the predictive capability of the calibration set with three validation sets using the RMSEP of the validation sets (lines with markers). "x" indicates the selection of the number of PLS components selected for the calibration models.

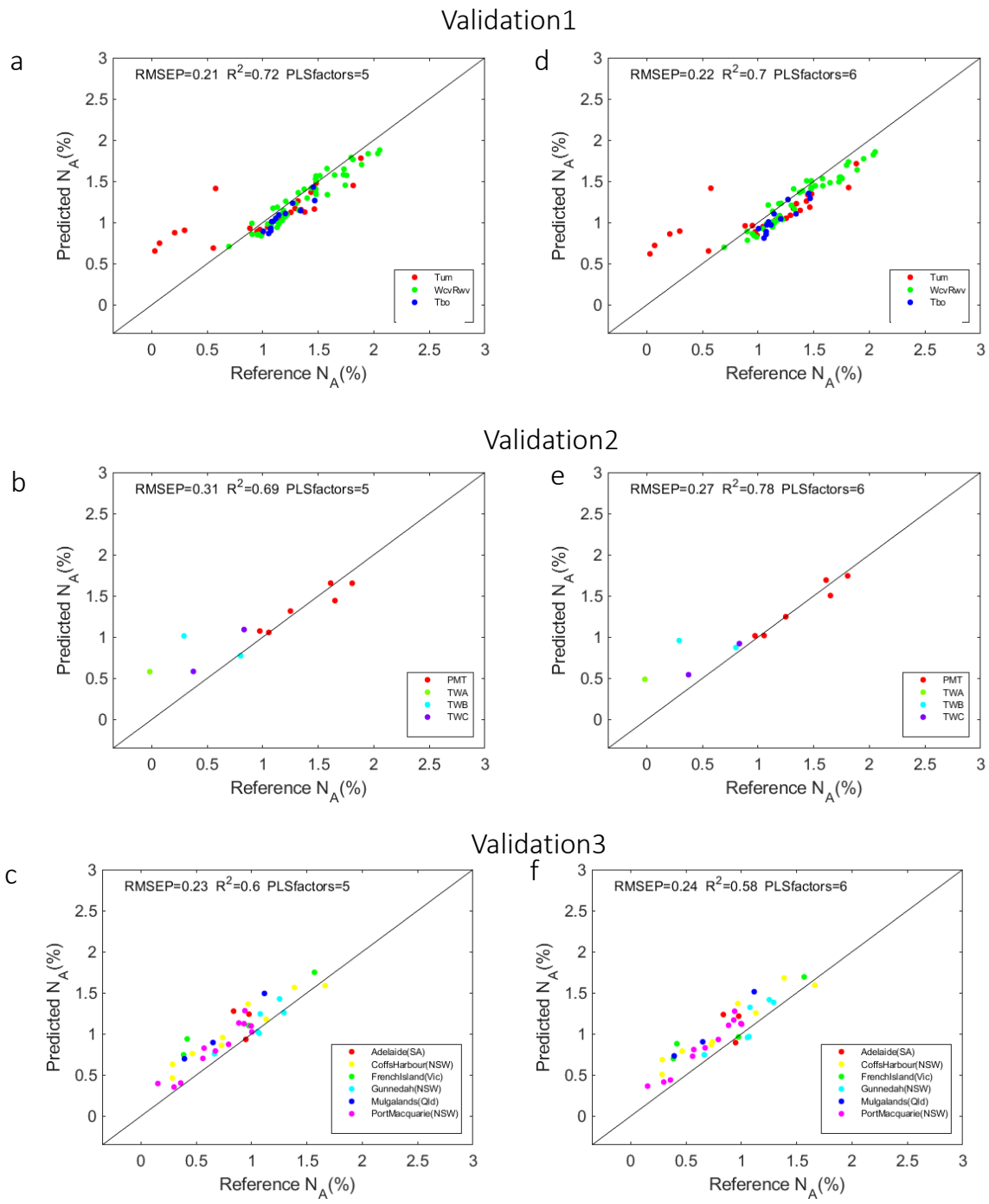

Figure 5. Predicted N_A (%) against reference values for samples in three independent validation sets. Two calibration models were used, (a – c) a model with five PLS components and (d - f) a model with six PLS components, were tested the model on the validation sets. Data were fitted against a 45 degree line. Data points are coloured by different sites within the validation set.

Given that different cross-validation techniques indicated different numbers of "optimal" PLS components, we investigated how changing the number of PLS components influenced the prediction capability of the RV data (Fig. 4a). We generated 30 models, with 1 to 30 PLS components and tested them against all three validation sets. For Validation2 and Validation3, the lowest predictive error was achieved using a model with five PLS components. We found the RMSECV curve for $State_{CV}$ was most useful as it best resembled the predictive error in these two RMSEP curves (Fig. 4a). For Validation3, selecting an appropriate number of components to build the model was difficult because the RMSEP curve continued to decrease until 20 components were included.

Calibration model development: how many samples do we really need?

Throughout the study, we found a strong relationship between N_A and NIR spectra when using a large proportion of RV samples in the model (50 - 60% and 100%). We found similar results whether we selected samples randomly or based on Mahalanobis distance (Fig. 6 a-d) We found that changes to the number of calibration samples and PLS components in the model influenced the prediction performance of the model (Fig 6a-d). Generally, including up to seven PLS components showed smaller prediction errors on both the split-off validation set (the remaining samples in the model development set, Fig. 6a) and the set aside validation set (Fig. 6b, N = 101 samples).

Furthermore, the prediction error of the split-off validation set became smaller as we simultaneously increased the number of calibration samples and decreased the number of split-off validation samples (Fig. 6a, c). We found the lowest prediction error of the split-off validation set when we included 850 samples in the calibration model however, only 37 samples remained in the split-off validation set. Contrastingly, including more samples during calibration did not necessarily improve the prediction performance of the model on the set-aside validation set. We found that the prediction error on the set-aside validation set plateaued after 300 calibration samples were included (Fig. 6b, d).

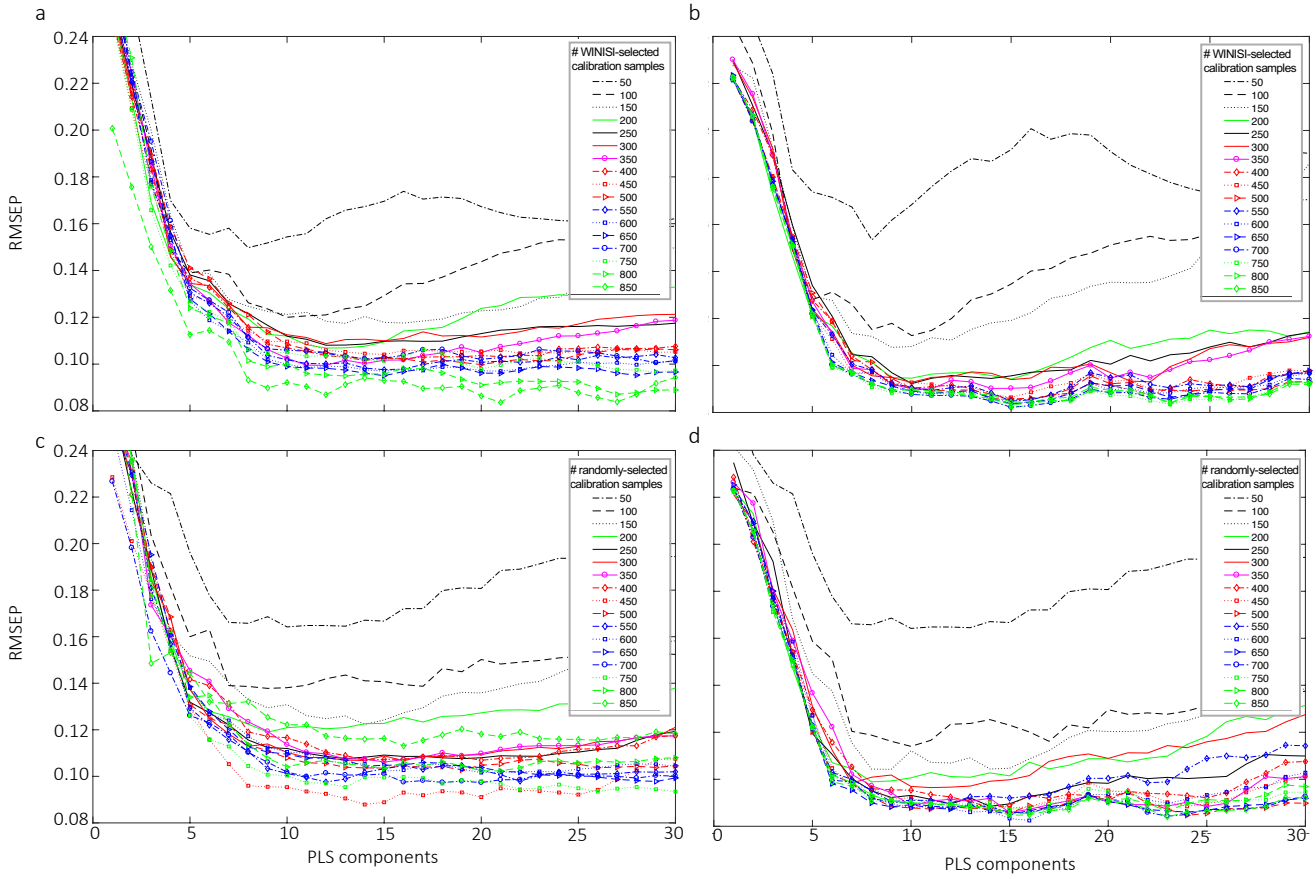

Figure 6. The change in the error prediction (RMSEP) of a model with an increasing number of calibration samples during model development. Using the reference chemistry samples from the koala transect study (N = 987), 886 samples with reference chemistry were allocated to a model development set (MDS) and 101 samples for a separate validation set. Calibration models were developed with an increasing number of samples (in increments of 50 samples) and increasing number of PLS components. Models using WINISI to select calibration samples from the MDS were (a) tested against the remaining samples in MDS and (b) against the set-aside validation set. Models which randomly selected calibration samples from the MDS were (c) tested against the remaining samples in the MDS and (d) against the set-aside validation set.

Formylated phloroglucinol compounds

Spectra

Our data set included 38 species never before analysed for FPCs. In a PCA of *Symphyomyrtus* samples from the FPC Data set, 80% of the spectral variation in NIR spectra were explained by the first two principal components (Fig. 7). The RV samples separated across PC-1 and PC-2. The exception being a few RV samples representing the southern region of the spectral space (Fig. 7).

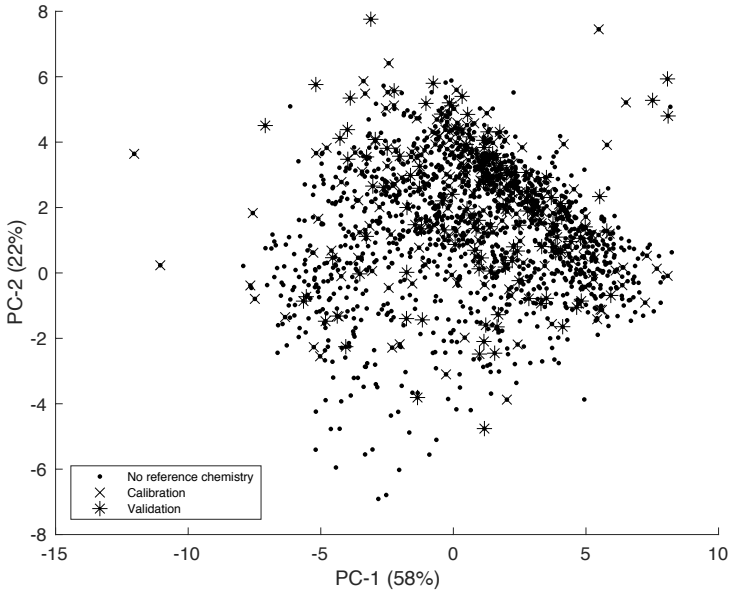

Figure 7. Distribution of scores of formylated phloroglucinol compounds spectral dataset along the first two principal components. Calibration samples are marked by "x" and the validation samples are marked by "*".

Calibration model development: do you need every compound?

We developed a calibration with the sum of the 17 FPCs, TotalFPCs$_{17}$. The LOO$_{CV}$ cross-validation results indicated a PLS model with 14 factors was adequate to explain the variance between TotalFPCs$_{17}$ and NIR spectra (Fig. 8). The RMSECV on the calibration set was 4.4 mg.kg^{-1} and RMSEP on the independent validation set was 4.7 mg.kg^{-1} (Fig. 8a and Fig. 8b).

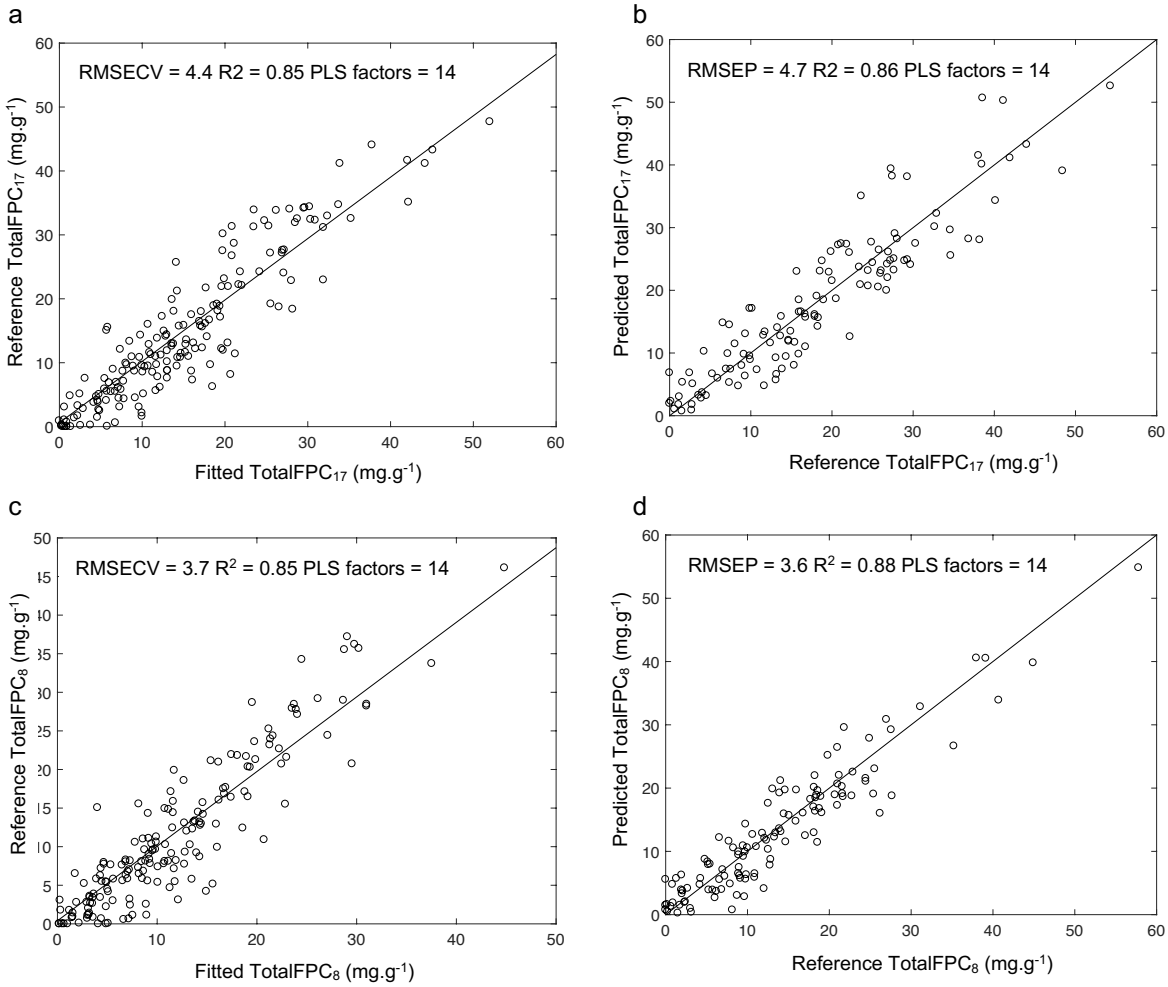

Figure 8. The root mean squared error of cross validation and root mean squared error of prediction results for (a) and (b) TotalFPCs$_{17}$ and (c) and (d) TotalFPCs$_{8}$ in mg.g^{-1}.

We carried out PLS2 with 17 known FPCs to investigate which FPCs co-varied with the NIR spectra. For the first four PLS factors, eight compounds had a high covariance (Y loadings) with the NIR spectra (Fig. 9): eucalyptone, macrocarpal 34, grandinal44, sideroxylonal A, sideroxylonal C, grandinal 54, macrocarpal A, macrocarpal G. We summed across these eight compounds for a new index of FPCs, $TotalFPCs_8$. There was an improvement on the predictive power of the calibration model with 14 PLS factors. The RMSECV on the calibration set was reduced to 3.7 $mg.kg^{-1}$ and RMSEP on the independent validation set was reduced to 3.6 $mg.kg^{-1}$ (Fig. 8c and Fig 8d). The coefficient of variation of the RMSEP between the two FPC indexes was around 30%, indicating that the prediction error across both indexes of FPCs were similar.

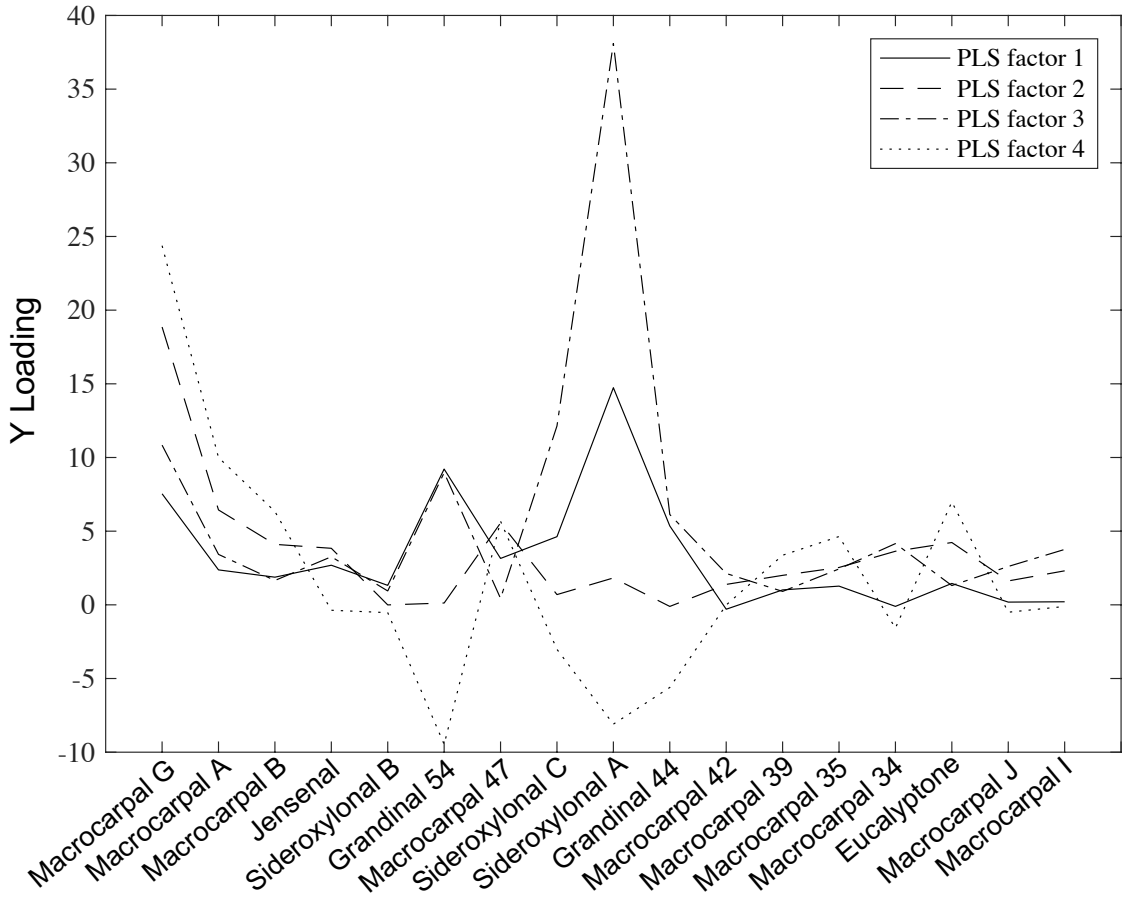

Figure 9. PLS2 on TotalFPCs17 with NIR spectra. The covariance (Y loadings) of each FPC compound with spectral data across the first four PLS factors.

Discussion

This study examined a variety of model development and validation techniques, illustrating different approaches one can use when building broad-based calibrations with large data sets. We explored the spatial variation within a large database of *Eucalyptus* leaf spectra and developed a multi-site and multi-species calibration for predicting N_A. We found that a more conservative approach when building a library of reference values could reduce the initial costs of global calibration development. Furthermore, our work highlights the power of NIR spectroscopy and multivariate analysis to guide analytical work for complex nutritional traits. Using PLS2, we identified eight FPC compounds had a high covariance with NIR spectra of leaves, which helped to understand the sources of error in multi-species calibrations of a trait which is still imperfectly understood.

Available nitrogen: exploit nested structure

The collection sites for all of the leaf samples spanned thousands of kilometres, across eastern and southern Australia (Fig. 1), and contained samples from areas and eucalypt species that have never been assessed for nutritional quality. We explored what types of nested structure could exist in large data sets. The large N_A included 80 eucalypt species from eastern and southern Australia and was highly valuable for building a broad-based N_A calibration. The PCA analysis of the N_A data set revealed that samples from the same local government area and Australian state clustered to varying degrees in spectral space (Supplementary Material 2a, b) and suggested that samples from similar areas may share similar spectral properties. We exploited this spatial structure during cross-validation and validation. Generally, we found that regardless of how we split the eucalypt RV data, the RMSECV and RMSEP values matched well across each test (Table 2). When we exploited local government area or Australian state as part of the structure however, we found the largest differences between RMSECV and RMSEP with the predictive errors being about 0.02 - 0.03 % N_A (about 20%) greater. It is not unusual to find geographically -similar spectra clumping together during PCA (Windley

and Foley 2015). Thus, there are probably spectral properties within different geographic areas that make samples unique.

A broad-based calibration model is more robust if it includes samples from a variety of locations and plant species to capture as much spectral variation as possible. To develop a robust N_A calibration model that covered the range of the large Eucalypt Database, we combined all RV data (Table 2). During PCA, we found that most samples overlapped with the RV data and a few samples from Validation1 separated out in the south-east region (Fig. 3). Given that most independent validation samples were within the spectral space of the RV data, we expected that the calibration model would perform well for these samples (Naes et al. 2002). Despite finding strong relationships with N_A and NIR spectra within the RV data set and knowing that this relationship exists in many other previous studies, we still experienced a reduction in prediction accuracy against independent samples. The prediction errors of independent samples were up to 50% greater than that found during calibration development, indicating that the model was somewhat limited when predicting these samples.

Large data sets are invaluable as the heterogeneity of the samples can represent the large natural variability of the study system. However, as we introduce new geographic variables, or species to a calibration model, it is likely that we will encounter unique spectral properties that affect the modelled relationship found between spectra and the chemical trait (non-linear effects). Moreover, it may not be clear which samples are needed to broaden the model. For example, our calibration models performed well for most samples, but we do not know why the models performed poorly for several independent samples (Fig. 5). These poorly-predicted samples were largely within the spectral space of the large eucalypt database and were not unique in terms of chemistry, tree species, or broad geographic location. Thus, it is difficult to assess how well a model has captured the variability of the study system.

Typically, we see a drop in prediction accuracy when we apply the model to independent samples, indicating that not all the natural variability in the study system is accounted for in the model. This has been found when building global calibration models for the freshness of albumen (Kemps et al. 2010), the cellulose content of

eucalypts (Downes et al. 2010), and properties of pine wood (Schimleck et al. 2010) and forage quality (Berzaghi et al. 2002) and digestibility (Coates and Dixon 2011). A general approach is to include some of the independent validation samples to extend the scope of the model, and we caution the use of this method. While this approach makes the current prediction statistics better, we would then need a new independent validation set to test if the predictive performance of the model is indeed better.

Available nitrogen: robust or accurate?

Accurate NIR models that predict complex traits are essential for ecological, silviculture agricultural, and botanical studies, yet deciding if and when a model is accurate enough is difficult without knowing exactly what purpose the data will serve. There is limited information on the predictive error of calibration models in ecological studies. Models are typically described by their cross-validation error which is then extrapolated to predictive error for the remaining samples (Moore and Foley 2005; Degabriel et al. 2009; Marsh et al. 2014; Stalenberg et al. 2014). From our RMSECV results only, we can expect an error between 0.12 – 0.17 % N_A. The upper range of our RMSECV (0.12 – 0.17 % N_A) is considerably higher than the RMSECV previously reported in other NIR calibration statistics for these chemical constituents (ranging from 0.07 – 0.13 % N_A, Moore and Foley 2005; Degabriel et al. 2009; Youngentob et al. 2011; Stalenberg et al. 2014; Windley and Foley 2015). When testing our models on three independent validation sets, the RMSEP ranged from 0.21 - 0.31% N_A, considerably higher than the RMSECV values. Thus, we propose our current N_A models can predict eucalypt samples from independent data sets with a prediction error range of 0.12 - 0.31 % N_A. We suggest that these predictive errors are acceptable as the eucalypt data set had a mean of 1% N_A with a range of 0.2 – 2.7% - considerably wider than that of previous studies (the average N_A in other ecological studies ranges from 0.2 – 0.72 %).

The accuracy tolerance of the calibration model is application dependent. Many important ecological effects depend on relatively small differences between samples, sites, or seasons. For example, subtle differences of 0.1 – 0.2% N_A in the average concentration of N_A across the home ranges of female brushtail possums have a substantial impact on their reproductive success (Degabriel et al. 2009). Although the

prediction error of our model was 0.13 - 0.31 % N_A, this should be able to detect small mean differences when applied over the several hundred trees that made up the home ranges of animals in DeGabriel's (2009) study. It is important to note that a lot of chemistry variability can be explained by tree species (Wallis et al. 2010). Thus, ensuring that data from the plant species of interest is incorporated in the model is also likely to be important in minimizing the predictive error.

In the case of a landscape scale project where the comparisons are between sites that vary over a much larger scale, the accuracy tolerance of a model might not be as strict. To our knowledge, this is yet to be investigated in ecology due to the difficulties associated with collecting chemical data across landscapes. The large Eucalypt Data set will provide an opportunity to explore the accuracy thresholds required to explain differences in animal abundance and distribution. We recognise that the most valid calibration method is dependent on what the user intends to achieve with the calibration model; predicting a single data set accurately or predicting a broad range of data sets adequately.

Available nitrogen: how many samples do you really need?

Accounting for spectral variation is essential for a robust model; however, constructing the initial calibration set for this study based on spectral variation as the major criterion resulted in an excessive number of calibration samples for N_A. Our results comparing split-off and set-aside validation sets suggested that a set-aside validation set is important for determining how many samples are required to efficiently build a calibration model (Fig. 6). When testing the split-off validation set, the prediction error continued to decrease as more samples were included in the calibration. During the split-off validation test, more and more spectrally variable samples were selected for the calibration set. Consequently, those samples left-behind to validate the model were less variable, hence, the decrease in prediction error. While predicting these "left-behind" samples is likely to leave users with great calibration statistics, it is also likely to tempt users to chase a prediction accuracy that may be overly optimistic. With the set-aside validation set, however, prediction error dropped off around 0.1% N_A and we found little improvement in prediction accuracy with 300-837 calibration samples. This

indicated that we could have achieved equivalent calibrations with only 30% of the RV data. This is a concern as we aimed to use NIR spectroscopy to efficiently get N_A values for our data set, and analysing hundreds more samples than required contrasts with that aim.

Simply relying on Mahalanobis distance to select samples may lead to an excess of RV samples as the variation in N_A may not necessarily equal the variation in the spectral space of the data set (Fearn 2011). To our knowledge, this test has been rarely investigated in this field and so we are unsure how frequently this happens in other studies. For future calibrations with chemical traits known to predict well with NIR spectra (such as N_A), we suggest that users should start conservatively. Select a small number of samples for calibration development and then test the predictive performance of the model on independent samples. If required, add additional RV samples unique spectral and/or geographic properties to reduce the prediction error of the model.

Towards a global calibration

The feasibility of a broad-based calibration model requires a common currency that can be compared across studies. N_A is relatively simple to measure and can be compared across all eucalypt samples. In fact, N_A has distinguished some subgenera of *Eucalyptus* and has highlighted the range of inter- and intraspecific variation amongst eucalypt species (Wallis et al. 2010). In contrast, FPCs are a highly diverse group of compounds that include different chemical classes and significant structural diversity although there remain common elements in all known structures. In addition, FPC compounds do not occur in all individuals of a species and the profile can also vary (Wallis et al. 2011). The variability of FPC profiles makes it an intrinsically more complex measurement and limitations in the existing reference method are recognized. It has however, been able to detect many ecologically relevant patterns (Moore et al. 2005; Marsh et al. 2007; Wallis et al. 2011) and some studies have been validated with concurrent studies using isolated FPC compounds (Lawler et al. 2000).

Formylated phloroglucinol compounds: do you need every compound?

We developed a comprehensive calibration model to predict TotalFPC$_{17}$ in 38 different *Symphyomyrtus* species collected over a wide geographic range. Other researchers have used this same reference method (Moore et al. 2004) to quantify the 17 FPC compounds and explain how much an animal can eat and tree use preferences in marsupial folivores (Moore and Foley 2005; Youngentob et al. 2011). In our study, we found that the predictive error of our model with TotalFPC$_{17}$ (RMSECV = 4.5 - 4.7 mg.g^{-1}) was similar to those observed in other ecological studies (with SECV ranging from 2.4 – 5.8 mg.g^{-1}, Moore and Foley 2005; Marsh et al. 2014). We propose that this accuracy is suitable for large-scale landscape studies and we used PLS2 to investigate the sources of error in the calibration.

The covariance between FPC compounds and NIR spectra differed between compounds. Eight compounds had a high covariance with NIR spectra, whereas nine other compounds showed little covariance (Figure 10). We found that a calibration using these eight compounds, TotalFPC$_8$, performed better than the TotalFPC$_{17}$ calibration. The eight compounds with high covariance tend to occur in larger concentrations and most (but not all) have suitable standards for analysis (Moore et al. 2004). The remaining nine compounds occur at lower concentrations or are sparsely distributed and so have proven difficult to isolate for use as standards. In addition, some species which were investigated here for the first time, may contain new FPCs. In particular, *E. tereticornis* which occurred at eight sites showed several late eluting peaks that may be unknown FPCs and indeed a recent paper has reported several new FPCs from this species (Liu et al. 2018).

Although the calibration TotalFPC$_{17}$ and TotalFPC$_8$ is suitable for a broad landscape study, it is clear that it requires ongoing expansion and maintenance. In particular, this work has shown limitations of the reference method and the need to invest significant resources in isolating and purifying additional standards. For example, we highlight the need to investigate prominent, unknown peaks that characterise wide-ranging species such as *E. tereticornis*. Nonetheless, our study provides the first stepping stone towards a broad-based FPC calibration. Further, using a collective "FPC" measure, rather than

focussing on species-specific individual FPCs provides us with a currency that allows us to better compare the nutritional quality of forests across landscapes. Given that the concentration of FPCs and other defensive compounds can restrict how much an herbivore can eat (Lawler et al. 1998; Marsh et al. 2006) and explain animal distribution patterns (Bryant et al. 2009), discovering strategies to better and more efficiently assess these compounds is likely to help support the management and conservation of animals.

Conclusions

Identifying the underlying causes of variation in large data sets can help build broad-based calibration models, facilitating the integration of NIR spectroscopy into many fields. We propose the use of different cross-validation techniques for model fitting and selection. Knowing how to better split RV data and testing calibration models against different independent validation sets can lead to more realistic estimates of model predictive performance. Furthermore, we suggest that assessing model performance while collecting RV data may help researchers avoid analysing an excessive number of RV samples for calibration development. Lastly, we showed that PLS can identify sources of error when building calibration models for complex chemical traits such as FPCs.

We suggest that the application of the model is important when deciding how best to develop calibration models and assess model predictive performance. Those who wish to build a global model that covers large geographic variation and plant diversity are likely to make compromises on the accuracy of their model. Nonetheless, our *Eucalyptus* database could provide a base for a global N_A calibration model. Given the importance of digestible protein in non-Australian study systems (Felton et al. 2009; Spalinger et al. 2010), we propose that our N_A calibration model could be extended to nutritional ecology studies worldwide. Combining leaf samples from multiple projects in a single database would lead to a NIR library that encapsulates the variation of leaf samples for folivores across different countries. If we can capture this variation in a single, global calibration model, we could significantly reduce the number of samples required for chemical analyses in new projects, and thus, facilitate the integration of

forage quality into ecological studies. To do this, future studies will need to investigate how this complexity may affect the development and accuracy of calibration models and our paper has highlighted some of the potential challenges.

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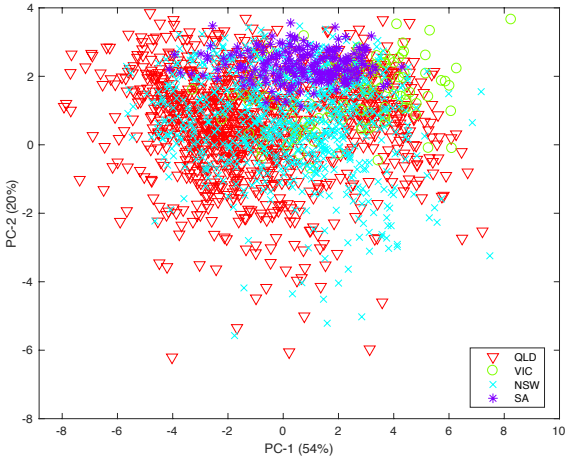
Supplementary material

Supplementary material 1: Table of spectral pretreatments.

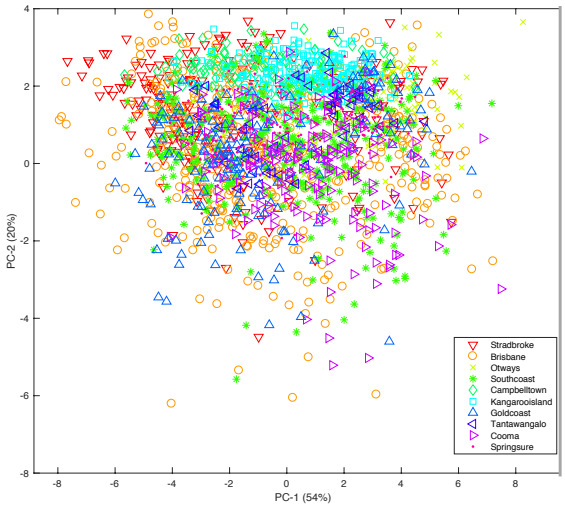
Polynomial	Window	Derivative	Smoothing
-	-	-	-, SNV, MSC
-	-	1, 2	-, SNV, MSC
2	3, 5, 7, 9	-, 1, 2,	-, SNV, MSC
3	5, 7, 9, 11	-, 1, 2,	-, SNV, MSC

Supplementary material 2: Distribution of scores of the spectral dataset along the first two principal components. Points are categorized based on a) Australian state, b) local area, c) eucalypt subgenera, d) eucalypt tree species, e) collection site, f) tree height and g) diameter at breast height.

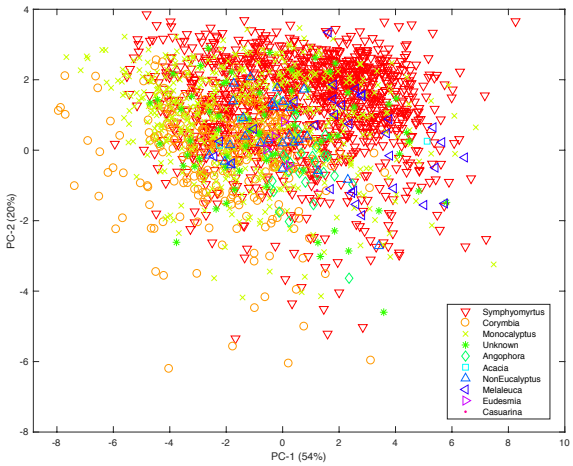
2a. State



2b. Local area



2c. Subgenera



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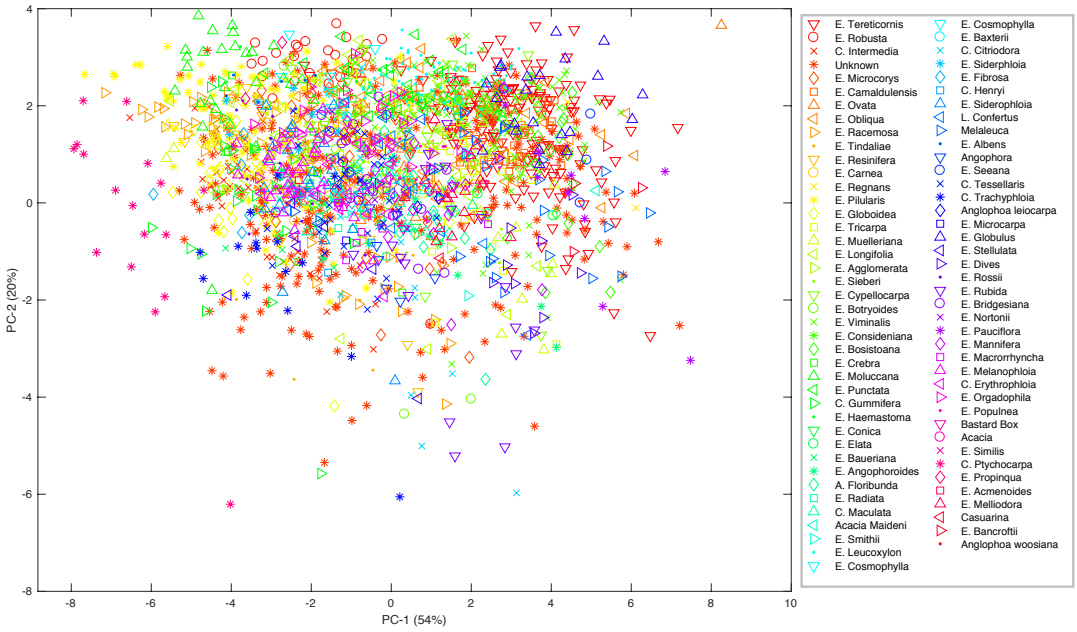
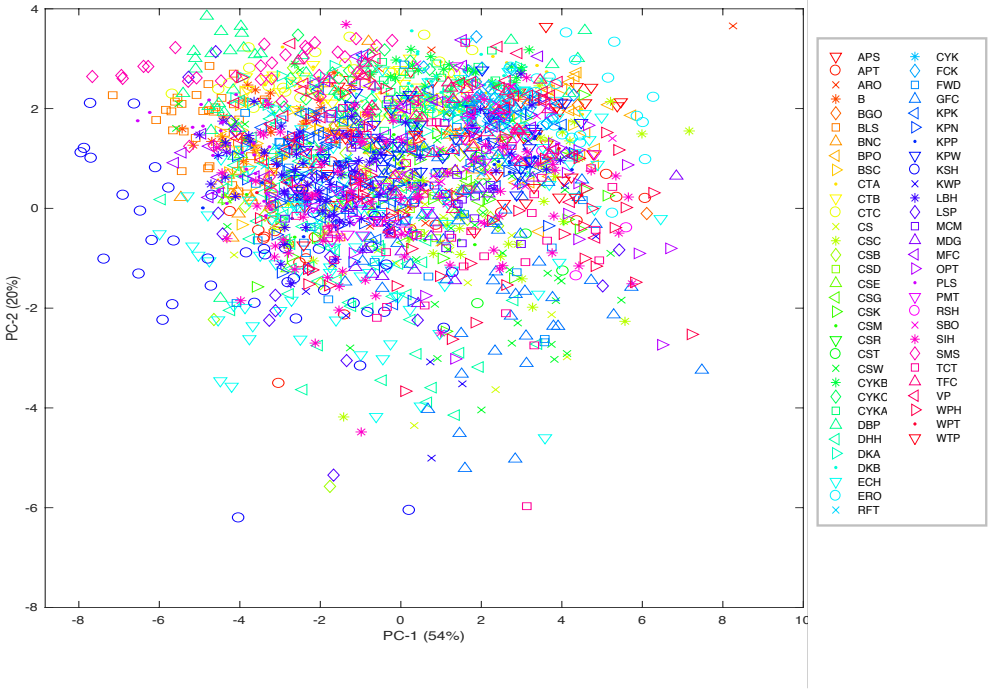
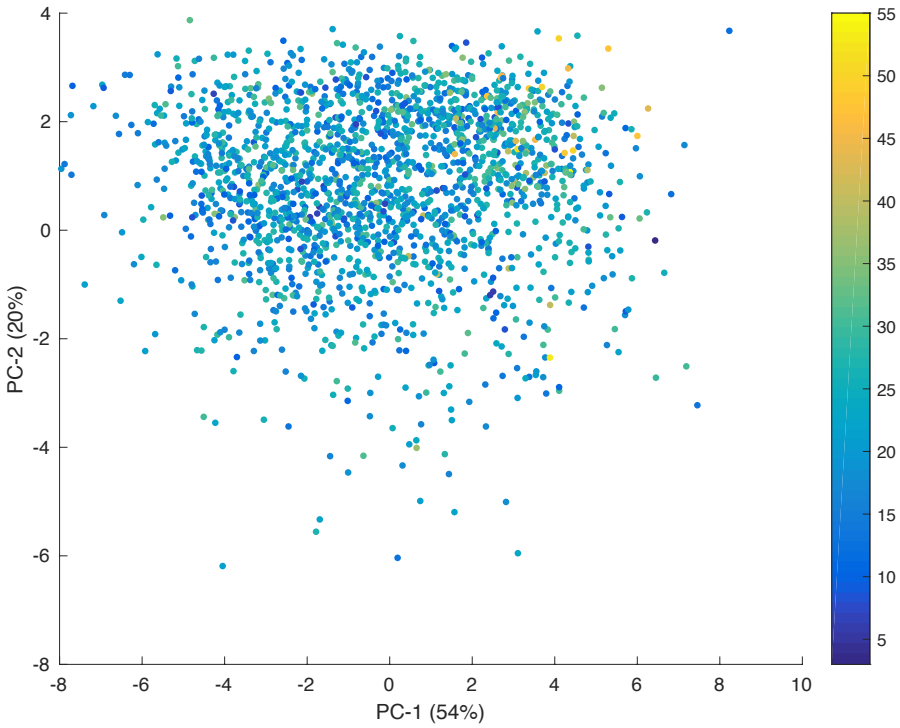
2e. Tree species



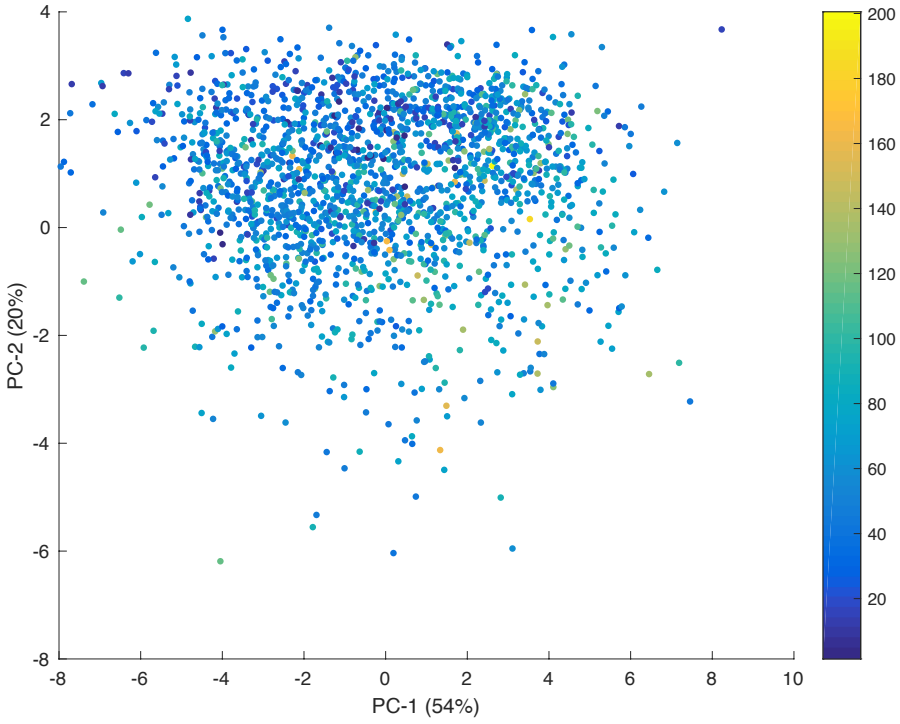
2e. Site code



2f. Tree height



2g. Diameter at breast height



Chapter Two

Population densities of an arboreal marsupial vary with forage quality

Keywords: available nitrogen, forage quality, formylated phloroglucinol compounds, mammalian herbivore, mixed effects linear models, unsubstituted B-ring flavanones.

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Abstract

The concentrations of foliar nutrients and plant secondary metabolites (PSMs) largely regulate the suitability of plant food for herbivores. These chemical constituents can vary within and between plant species and across landscapes, creating a chemically complex environment for animals. At fine scales, we know that the nutritional quality of plants can influence foraging decisions and landscape use. However, extending this understanding to estimate the effect of nutrition on populations remains difficult because it requires extensive plant chemistry and animal population surveys across landscapes. We collected leaf samples across most of the range of a widely distributed mammalian browser, the koala (*Phascolarctos cinereus*), to examine the relationship between variations in forage quality and animal densities. Working closely with many researchers who study koala populations in their local areas, we sampled 2,368 eucalypt trees across 74 sites with known koala population densities in Queensland, New South Wales, Victoria and South Australia. Using a mixed modelling approach, we investigated whether the variation in three meaningful nutritional traits, total nitrogen (N_T), available nitrogen (N_A) and formylated phloroglucinol compounds (FPCs) helped to explain large differences in koala population densities across eastern and southern Australia. There was considerable variation in mean N_T, N_A and FPCs across our sites. Concentrations of N_T and N_A are intrinsically related and thus we compared two nutrition models at the site level, one with mean concentrations of N_T and FPCs, and the second with N_A and FPCs. There was a significant positive effect of N_A, N_T, and a significant negative effect of FPCs on positive koala densities. We identified that both N_T and N_A are significant factors explaining koala density, and the best nutrition model used N_A and FPC data. Moreover, the effect of nutrition remained significant once other climatic and environmental factors known to influence koala presence and abundance were accounted for in the models. By using a very large collection of tree leaf samples for forage quality analyses, this study demonstrates the significant contribution of forage quality to habitat quality and population densities of an arboreal folivore.

Introduction

The nutritional quality of plants can vary both within and between species. This creates a chemical mosaic of forage quality across landscapes, which is likely to influence the distribution and abundance of herbivores (Elton 1927; White 1978). Essential nutrients and secondary metabolites (PSMs) are ubiquitous in plants and affect their nutritional quality by distorting the availability of plant nutrients to animals. We know that variations in PSMs influence the feeding decisions of herbivores, including what plants to eat or avoid (Troyer 1984; DeGabriel et al. 2009a; Ganzhorn et al. 2017), how much to eat (Munn and Dawson 2006; Wiggins et al. 2006; Felton et al. 2009) and where to eat (Pausas et al. 1995; Mduma et al. 1999; Grant and Scholes 2006; Frye et al. 2013). Forage quality can also have substantial impacts on the growth rate and reproductive success of herbivores (Troyer 1984; Munn and Dawson 2006; Degabriel et al. 2009b; McArt et al. 2009) and is therefore a key factor in the expansion, persistence and loss of animal populations. Identifying the mechanisms that regulate animal population densities, whether it be nutrition or other factors, is essential for the management and conservation of our wildlife.

The inadequate environment

Herbivores are patchily distributed across the landscape, suggesting that there are pockets of high-quality habitat amongst large areas of poor or inadequate habitat. There is a myriad of factors that determine habitat quality, including temperature and precipitation (Coe et al. 1976; Okello et al. 2016; Mason-Romo and Farı 2017), access to water (Owen-Smith 2005; Smith et al. 2013), vegetation structure and composition (McNaughton et al. 1989; Cyr and Pace 1993; Lindenmayer et al. 1999; Pettorelli et al. 2011), soil types and fertility (Aumann and Emlen 1965; Braithwaite et al. 1984; Pastor et al. 1993; Ball et al. 2000; Loney et al. 2006), inter and intra-species competition dynamics (Choquenot 1991; Bonenfant et al. 2009; Ripple and Beschta 2012; Kersch-Becker et al. 2017), availability of shelter (Smith and Lindenmayer 1988; Kutt and Gordon 2012), and landscape disturbance history (Chapman et al. 2000; Drapeau et al. 2000; Lesmerises et al. 2012). Although variations in the quality of food across landscapes are also thought to impact habitat quality, particularly for herbivorous

species, these relationships are often overlooked due to the difficulty in identifying and collecting meaningful nutritional data across landscapes (Wu and Hobbs 2002; DeGabriel et al. 2014).

What is forage quality?

The nutritional-limitation hypothesis proposes that food limits the growth and reproduction of an individual, and therefore plays a key role in regulating animal populations (White 1983, 1993). Forage quality relies, in part, on the types and quantities of available plant nutrients to the herbivore. Nitrogenous compounds (protein) are essential for animal growth and are argued to be the single most important factor limiting the abundance of animals (White 1993). Consequently, it is advantageous for plants to reduce the availability of foliar nitrogen to deter herbivory. Plants produce certain PSMs as a way to limit digestible nitrogen and as a form of chemical defense (Fraenkel 1959). For instance, tannins are a common PSM that can bind to protein and make it unavailable for digestion by herbivores (Barbehenn and Constabel 2011). In addition, some toxic plant secondary compounds exert a protein cost through nitrogen required for detoxification, enzyme synthesis, or tissue repair as a result of damage from toxins (Marsh et al. 2006; Au et al. 2013). Thus, the total amount of protein in a plant does not necessarily reflect what the animals can obtain from that plant for maintenance, growth and reproduction. In addition to directly influencing nutrient availability, PSMs can also act as toxins that cause discomfort, pain, nausea or even death in the animal (Lawler et al. 1998; Foley et al. 1999; Marsh et al. 2006).

Simple measures of forage quality that do not account for PSMs may therefore fail to capture the complexity of plant nutritional quality, obscuring the effects of nutrition on animals. Typically, animals prefer plants with relatively high concentrations of protein and low concentrations of herbivore-deterrent PSMs (Farentinos et al. 1981; Schetter et al. 1998; Vourc'h et al. 2001; Moore and Foley 2005; Youngentob et al. 2011; Frye et al. 2013; Ulappa et al. 2014). However, animals make trade-offs between acquiring enough nutrients and regulating PSM intake (Loney et al. 2006). In fact, some herbivores are more willing to eat plants with higher concentrations of PSMs to eat more protein (Marsh et al. 2005; Au et al. 2013), indicating that the relationship between PSMs and nutrient availability is not necessarily constant. Thus, nutrients and PSMs are both likely

to influence the suitability of habitat for herbivores and integrating these effects can be more meaningful for explaining animal distribution and abundance.

Extending the effect of nutrition to a landscape scale.

Plant-animal interactions at small scales (i.e. choice of individual plants) are reasonably well understood in many systems (see reviews in: Foley et al. 1999; Dearing et al. 2005). There is less knowledge about how the same factors might influence the distribution and abundance of animals across landscapes; however, the studies that do exist suggest that forage quality is important in explaining patterns of landscape use and animal densities (Braithwaite et al. 1983; Grant and Scholes 2006). In the south-east coastal forests of Australia, for example, foliar concentrations of nitrogen and phosphorus were the major determinants of the density of folivorous marsupials (Braithwaite et al. 1983). Studies in the Serengeti have shown that foliar concentrations of nitrogen and phosphorus create "nutrient hotspots" that are essential for reproductive success and shape landscape use by large wild herbivores (Grant and Scholes 2006). However, making the link nutrition with population processes remains difficult because secondary compounds are either ignored, assumed to have species-specific concentrations or their mechanisms of action are unknown.

Research into primate populations provides the best evidence for the influence of more complex nutritional traits on animal population densities and biomass. Many separate studies have found strong relationships with primate abundance and protein-to-fibre ratio, a ratio of concentrations of total nitrogen and acid detergent fibre (originally suggested by Milton 1979 , Wasserman and Chapman 2003; Chapman et al. 2004; Hanya et al. 2006; Rode et al. 2006; Fashing et al. 2007). Fibre (e.g. neutral or acid detergent fibre) represents refractory parts of plant foods and often considered an anti-feedant because it requires fermentation by symbiotic bacteria to break down plant cell-walls. Despite showing success in many smaller scale studies, the effect of the protein-to-fibre ratio on broader primate patterns is not clear (reviewed Hanya and Chapman 2013). This is probably due to the ubiquitous presence of tannins in plants that can interfere with the *in vivo* determination of fibre. As mentioned earlier, tannins can form complexes with macromolecules (such as protein), which are insoluble during extraction with acid detergents and consequently lead to an overestimation of fibre

during analysis (Makkar et al. 1995; Wallis et al. 2012). Thus, the presence of PSMs can affect both the nutritional quality of plants and our ability to assess chemical traits during analysis.

Although several studies have linked the distribution and abundance of herbivores to variations in forage quality (Braithwaite et al. 1983; Wasserman and Chapman 2003; Grant and Scholes 2006; Fashing et al. 2007), most of these studies have focused on simple measures of plant nutrient content. For example, single measures of forage quality such as protein (measured as nitrogen, Braithwaite et al. 1983; Grant and Scholes 2006), or ratios that attempt to weight a nutritionally positive trait against a nutritionally negative trait (Wasserman and Chapman 2003; Fashing et al. 2007). These ratios can be misleading because they assume that the relationship between the two traits is linear over the whole range of concentrations (Raubenheimer 1995; Wallis et al. 2012).

In order to more fully disentangle the effects of nutrition on animal populations, it is essential that we use measures of food quality that are meaningful and encompass the complex chemical nature of plants. There are many studies that recognize the negative effects of tannins on grazing ruminants (Robbins et al. 1987; Lisonbee et al. 2009), yet there has been little focus of this prominent class of PSMs in browsers. To account for the cost of tannins on nitrogen availability and provide an integrative measure of the amount of protein available for animal digestion, DeGabriel et al. (2008) developed an *in vitro* assay of available nitrogen (N_A). Available nitrogen, rather than total nitrogen, may provide a better indication of foliar nitrogen for those animals that are sensitive to the effects of protein-binding by tannins. This measure has proven valuable for investigating the relationship between forage quality and reproductive success in another folivore, the brushtail possum eating eucalypt foliage in Australia (Degabriel et al. 2009) and a mixed foliage diet in New Zealand (Windley et al. 2016) and to explore food selection in greater gliders (Youngentob et al. 2011) and koalas (Stalenberg et al. 2014). Further, concentrations of N_A provided a better indication of nutritional quality than the protein-fibre ratio and explained variations in the densities of primates (Wallis et al. 2012).

The koala: a wide-ranging folivore

The koala (*Phascolarctos cinereus*) is a wide-ranging folivore that has a fragmented distribution encompassing around one million square kilometres (Adams-Hosking et al. 2011). Koalas inhabit a variety of ecosystems ranging from coastal to subalpine, including continuous forests, open-woodlands, and even isolated clumps of trees within suburban areas or farmlands (Martin and Handasyde 1999). There are striking variations in population densities across the range of the koala, and these differences have led to distinct regional conservation listings. Northern koala populations in Queensland (Qld) and New South Wales (NSW) are declining markedly in many areas and are listed as vulnerable, while the southern populations in Victoria (Vic) and South Australia (SA) are overabundant in areas and are listed as non-vulnerable. The reasons for differences in population densities across their range are complex and likely have a number of contributing factors, including habitat loss, feral predators, and disease (McAlpine et al. 2015). Variations in forage quality may also play an important role, but this has yet to be explored at scales larger than a koala's home-range.

The koala is an ideal species for studying the relationship between forage quality and animal densities because of its critical dependence on *Eucalyptus* foliage and the strong knowledge that has been developed over the past 20 years on the chemical ecology of diet selection in koalas. Koalas are eucalypt specialists and *Eucalyptus* leaves are a poor-quality food, containing low concentrations of protein and a myriad of PSMs that detract from the nutritional value of leaves. Eucalypt leaves typically contain between 1 and 2% nitrogen (Wallis et al. 2010), as compared to some plants that are eaten by primates in Uganda that can contain as much as 8% foliar nitrogen (Rothman et al. 2011). It is likely that the suitability of habitats for the koala is tightly linked to protein availability as well, given that it has a remarkably low metabolic rate (Hume 1999) and nitrogen requirements (Cork 1986). Consequently, small changes in protein availability can have large effects on animals that live on a fine nitrogen balance (Degabriel et al. 2009b).

Koalas prefer to eat leaves from trees from the eucalypt subgenus *Symphyomyrtus* (Moore et al. 2004a), which tend to have higher concentrations of available nitrogen than the other most prevalent subgenera of eucalypt, *Monocalyptus* (Wallis et al. 2010).

However, many symphyomyrtles also contain FPCs (Eschler et al. 2000), a class of herbivore deterrent lipophilic terpene adducts that act on serotonin receptors and cause nausea and general malaise upon ingestion (Lawler et al. 1998). Concentrations of FPCs vary substantially among eucalypt trees (Lawler et al. 2000; Wallis et al. 2002; Moore et al. 2004b). Captive feeding studies have revealed that koalas decrease their intake of eucalypt leaves when foliar concentrations of FPCs increase beyond 20 $mg.g^{-1}$ (Moore et al. 2005). Free-ranging koalas forage more frequently and longer in trees with lower concentrations of FPCs and higher concentrations of total nitrogen (Moore and Foley 2005; Marsh et al. 2014). While focusing on *Symphyomyrtus* have helped explain feeding preferences of the koala, the other dominant subgenera, *Monocalyptus* appears to have its own suite of herbivore-deterrent PSMs.

A recently discovered class of PSMs, unsubstituted B-ring flavanones (UBFs) are found almost universally in *Monocalyptus*, yet almost completely absent in *Symphyomyrtus* (Tucker et al. 2010). While the physiological mechanisms are yet to be identified, early feeding studies suggest that concentrations of UBFs are likely to also play a role in the nutritional quality of leaves. Studies have shown that UBFs can significantly deter feeding by symphyomyrtle specialists like the brushtail possum (*Trichosurus vulpecula*), while having little effect on monocalypt specialists like the ringtail possum (*Pseudocheirus peregrinus*, Beale et al. unpublished data, Blyton et al. unpublished data, Marsh et al. 2015).

Rationale for the work and objectives

The conservation of koalas depends on understanding how nutrition affects populations at landscape scales. Why are koalas abundant at some sites yet barely present at others? Population densities vary widely and there is extensive dietary research that underpins our understanding of food quality for this species. The primary aim of this study was to investigate how foliar chemistry varies across the range of the koala and examine the relationship between forage quality and koala population densities. In this study, we posed the following questions

(i) What is the range of forage quality within and between sites?

(ii) Does forage quality, in regards to N_T, N_A, UBFs and FPCs, influence the variation in koala densities?

(iii) Controlling for climatic and environmental factors that have been shown to influence koala presence and abundance in other studies, are site-level foliar concentrations of N_T, N_A and FPCs significant factors in explaining the density of koalas?

(iv) How does the threshold of 20 $mg.g^{-1}$ FPCs (threshold of when FPCs become deterrent in captive studies) influence the variation in koala densities?

Materials and methods

Study area

We surveyed eucalypt forests across eastern and southern Australia, across four states, Qld, NSW, Vic and SA and several off-shore islands (Fig. 1). The geographic range was -38 to -25 ° of latitude and 136 – 154 ° of longitude. Our sites included a variety of land-use types such as agricultural, national parks, state forests, reserve land and rehabilitated areas for conservation. Furthermore, our sites covered several different ecosystems including coastal, swampland, subalpine, and semi-arid woodlands. The elevation of our sites ranged from 0 - 1059 m above sea level and distance from the coast ranged from 0 - 700 km.

Koala density data

We identified areas across the koala's range that had existing data on animal density. As a result, this study required significant collaboration with academic, government and industry researchers and independent consultants. A comprehensive list of collaborators can be found in Supplementary material 1. Each researcher surveyed the density of koalas within a local government area (for example, Port Macquarie, or Adelaide Hills) and for the purpose of this study, is referred hereafter as a "region". There were several different methods that researchers used to estimate koala abundance. These methods included counting the number of koalas observed along a transect (Whisson et al. 2016), repeated surveys to identify and capture all individuals in

an area for radio tracking (Close et al. 2017), and surveys for koala faecal pellets (Phillips and Callaghan 2011; Seabrook et al. 2011). In addition, six sites had abundance estimated indirectly through a method developed by Phillips and Callaghan (2000) that modelled the likely abundance of koalas based on the relationship between scat observations and tree species. At these six sites, the abundance of koalas was estimated based on the presence of tree species that were commonly associated with koala scat in the landscape. To standardise across the various methods, the researchers provided an estimate of the number of koalas per hectare based on the data they have collected from the local government area.

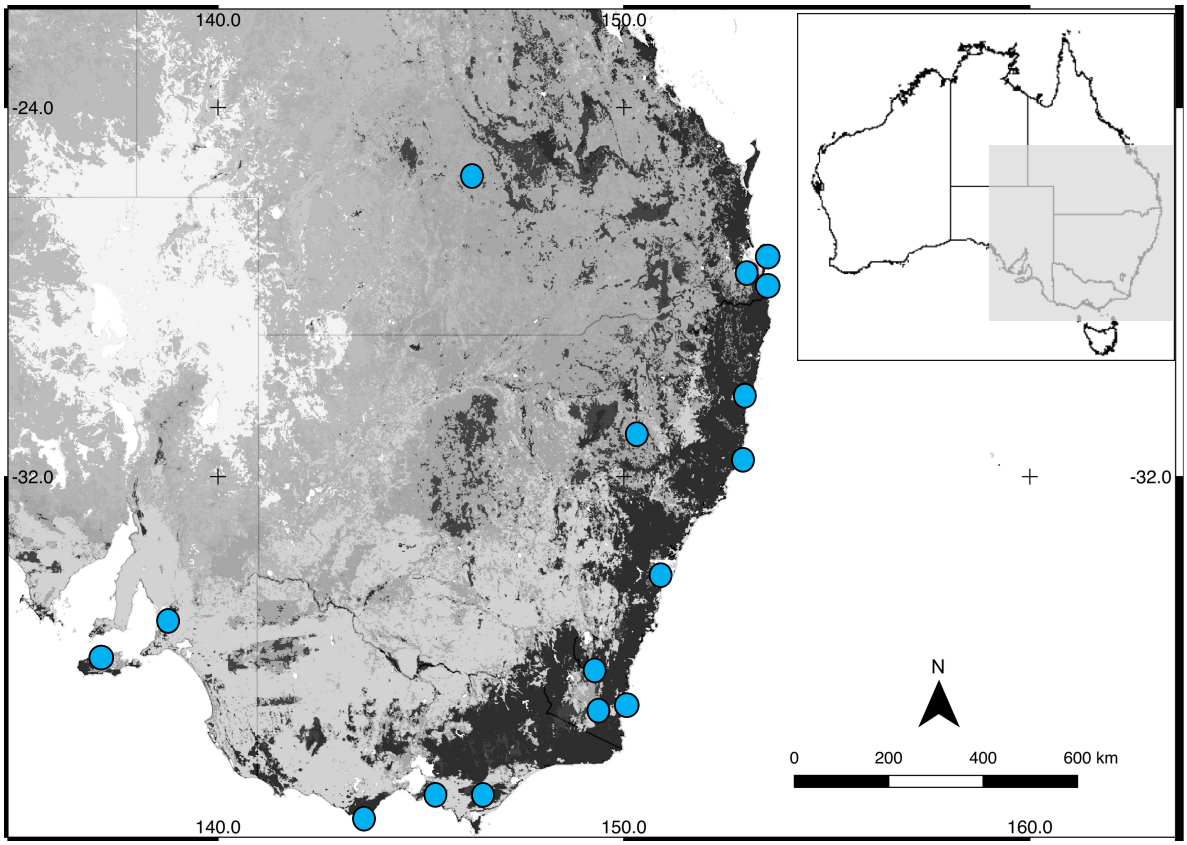

Figure 1. Map of sixteen surveyed regions with koala density data (blue circles) across four Australian states; Queensland, New South Wales, Victoria and South Australia. Land cover is represented by the grey shading of the map.

The home range of a koala is approximately 10 – 135 ha (Ellis et al. 2002; Lassau et al. 2008) and the selected sites were a minimum of 2km distance from each other. The average distance between sites was 20 km and ranged from 2 - 340 km. Where possible for each site, we requested a recent and historical estimate of the number of koalas per hectare. A historical estimate of koala density allowed us to account for site-specific events whereby the recent density estimate may not necessarily reflect the historical population densities. Extreme climatic events such as extended heat waves in Gunnedah, NSW (Lunney et al. 2012) and severe drought in the Mulgalands, Qld (Seabrook et al. 2011) have drastically reduced the koala populations in recent years. Furthermore, the recent low density estimates on Kangaroo Island, SA are a result of fertility suppression and are not reflective of the historical high density populations (Duka and Masters 2005).

Site selection

We sampled sites that had data on the density of koalas over two field seasons, between the months of August and April, 2012-2013 and 2013-2014. We travelled to each of the four states across both field seasons. Nearly every region that had reliable data on the density of koalas was included in our study, and this mostly covered the distribution of the koala. For logistic reasons, we were unable to include some well-known koala populations in tropical Australia including Magnetic Island and the broader Townsville region in Qld. For each region, the number of sites sampled for analysis of food quality approximately reflected the range in koala population densities and thus, determined the number of sites we sampled. In many regions, there were both relatively high and low-density sites available for sampling. In order to target differences in foliage chemistry between high and low-density sites, where possible, we aimed to capture all of the local environmental variation across both high and low-density sites. This includes differences in elevation, slope of area, distance to water, forest type (tree species composition) and land use type (for example, protected area, agricultural, suburban).

Leaf sampling design for estimating forage quality at a site

To investigate the role of nutrition across the landscape, it was essential to establish a sampling design that could be used across all ecosystems, and efficiently captured the chemical variation of trees at each site. We confined our leaf sampling to include only *Eucalyptus* species that the koala is known to eat. A complete list of these eucalypt species can be found in the Supplementary material 2. For most of the sites, we focussed our sampling on two eucalypt subgenera only, *Symphyomyrtus* and *Monocalyptus*. In three sites, however, *Corymbia intermedia* and *Corymbia gummifera* were included during sampling. This is unlikely to cause an issue with comparing sites given that *Corymbia* is known to occur in koala habitat, has been included in previous surveys on habitat use by koalas (Jurskis and Potter 1997; Ellis et al. 2009), and *Corymbia* was only recently split from *Eucalyptus* based on cladistic analyses of recent molecular data (Hill and Johnson 1995; Udovicic et al. 1995).

To sample chemical variation in a site, we set up a single transect in each site. Where possible, a transect started in the middle of an area that had been previously surveyed for koala density by the collaborating researcher(s) and from there, we selected the nearest eucalypt tree (the centre tree) to sample and its three nearest eucalypt neighbours (referred hereafter as a "clump" of trees). The distance between the neighbours and the centre tree within a clump provided information about the density of the trees at each transect. Trees were marked with an aluminium tag and a galvanised iron nail. For each tree, we identified the tree species and recorded its location (as GPS co-ordinates), diameter at breast height and approximate tree height. Once all the information was recorded, we returned to the centre tree and a random bearing was chosen for the direction of the transect.

Studies have shown that the genetic relationships of a tree strongly influences the chemical composition of its leaves, and that trees are less likely to be related to each other if they are at least 60 m apart (Andrew et al. 2007). Thus, distance between sampled clumps was an important consideration for the sampling design of our study. We walked along the direction of the transect for 420 m, sampling at 60 m intervals. At each 60 m interval, we repeated the sampling steps and sampled the nearest eucalypt

tree and its three nearest neighbours. Thus, there were a total of 8 clumps (with four trees per clump) sampled along a 420 m transect, resulting in 32 trees sampled per site.

Sample collection and preparation

Employing a sharp-shooter to collect leaves would have been cost prohibitive and potentially restricted in some areas, so we developed an alternative method to collect leaves from tall trees using a throwline-launcher (Youngentob et al. 2016). We used this approach to obtain a sunlit branch from each selected tree. We recorded the direction in which the branch was growing from the trunk and its location in the canopy (for example, top, middle or bottom third of the canopy.

The leaves were stripped into two different bags, one for leaf moisture analysis and one for chemistry analysis. Care was taken to avoid collecting leaves that had active insect infestations like lerps or gals. For leaf moisture analysis, 10 g of fully expanded, mature leaves were placed into a polythene bag with some moist paper towel. Leaf moisture was determined by the difference in the weight of fresh wet weight of leaves and then the dry weight. Fresh wet weight was recorded upon collection in the field and these leaves were placed in paper bags to air dry until placed in a forced draft oven at the Australian National University. Leaves were then dried for 48 h at 40°C to a constant mass and the dry weight of the leaves was recorded.

For chemical analysis, 50 g of fully expanded, mature leaves were placed into paper bags and immediately frozen at -80°C on a bed of solid CO_2 pellets for chemical analysis. Upon arrival at the ANU, samples were stored at -20°C pending analysis. To prepare samples for analysis, samples were then freeze-dried and ground to pass a 0.5mm screen using a Tecator Cyclotec Mill (Foss, Hillerød, Denmark). The resulting leaf powder was stored in clear polythene 50 mL specimen containers in a dark room at 18- 20°C.

Near infrared spectroscopy

To efficiently determine leaf chemistry data across thousands of samples, we used a combination of wet chemical analyses and near infrared (NIR) spectroscopy. This technology is non-destructive, rapid, and reliable and used broadly in industry and ecological and agricultural research globally (Batten 1998; Vance et al. 2016). Light reflected in the near infrared region depends on the nature of the C-H, N-H and O-H

bonds in the sample. Thus, we can develop a predictive statistical model from a training set of foliage samples that models the relationship between features in NIR spectra and the known chemical composition (Naes et al. 2002). Using this model, we can then predict the chemical composition of new (unknown) samples solely from the NIR spectra. Expanding the use of NIR spectroscopy has helped answer many ecological questions about animal feeding behavior and distribution patterns, including in the koala (reviewed Vance et al. 2016).

Near infrared spectroscopy: sample selection

We collected the NIR spectra of each sample at 2 nm interval from 400 to 2498 nm using a FOSS - NIRSystems 6500 scanning spectrophotometer with spinning cup attachment (Foss - NIRSystems, Silver Spring, Maryland, USA). Using the CENTER and SELECT functions in the software WinISI III version 1.50E (Infrasoft International, Port Matilda, PA, USA), a subset of leaf samples which represented the spectral variation of the dataset was identified for each chemical trait. The number of samples selected for analysis of each nutritional trait varied because the PSMs investigated are subgenera specific. The CENTER algorithm ranks spectra based on their Mahalanobis distance to the average spectra and the SELECT algorithm identifies a suitable calibration set for wet chemistry analyses.

Chemical analysis

Total nitrogen and available nitrogen

For our study, we needed to develop comprehensive calibration models that embraced the variation in foliage chemistry on a continental scale. Thus, we also combined a dataset of 1,262 eucalypt trees from a related project that was undertaken in the same time period and collected using the same methods. This is the largest known database of *Eucalyptus* leaf samples with foliage from 3,630 individual trees. We chemically analysed two nutritional traits in 1,024 out of 3,630 samples: total nitrogen (N_T) and available nitrogen (N_A). N_T is the concentration of N in a plant sample. We analysed the N content of each sample using a Leco TruSpec carbon/nitrogen instrument (Leco Corporation, Saint Joseph, Missouri, USA). To determine N_A and dry matter digestibility (DMD) of each sample, digestion and fermentation in a mammalian herbivore was

emulated in the N_A method described in DeGabriel et al. (2008). To summarise, 0.8050 ± 0.0050 g of air-dry, ground sample was weighed into each of two dry, preweighed ANKOM F57 filter bags (Ankom Technology, Macedon, New York). These samples underwent the N_A assay which included a 24 h buffer stage (0.05M Tris–BASE buffer, pH 7.1), 24 h digestion stage (pepsin 2 $g.L^{-1}$) and a 48 h fermentation stage (cellulase 6.25 $g.L^{-1}$, pH 4.75) at 37°C. Dry matter (DM) was calculated by taking the average oven DM in fifty random samples. Dry matter digestibility is calculated by the proportional difference in the weight of the initial sample and the residue after digestion in the enzyme solutions. N_A is calculated by the proportional difference in N % of a sample and the residue, before and after digestion in the enzyme solutions.

Formylated phloroglucinol compounds: extraction and quantification

Formylated phloroglucinol compounds are present in *Symphyomyrtus* only, and thus we truncated our eucalypt dataset to include only *Symphyomyrtus* samples. There were 1600 *Symphyomyrtus* samples out of 2,368 leaf samples. We selected 400 out of 1600 *Symphyomyrtus* samples to represent the spectral variation of the *Symphyomyrtus* collected in our survey. We determined the concentration of FPCs using high performance liquid chromatography using the methods described in Moore et al. (2004b). To summarise, we weighed 50 ± 2 mg of ground leaf and 4 ml of extraction solvent into glass scintillation vials. The extraction solvent contained acetonitrile with 0.1% trifluoroacetic acid and, 2-ethyl phenol (30 $mg.L^{-1}$) as an internal standard. We extracted samples in a sonicating water bath for 10 min and then let them stand at room temperature for 60 min. To separate the components in the extract, we used an Agilent HPLC with photo diode array detector and a Wakosil 250 × 4 mm GL 3C18RS (SGE) analytical column. We injected 10 µl of filtered extract and measured the detector response at 275 nm. We set the column temperature to 37°C and eluted extracts under gradient conditions with 0.1% TFA in acetonitrile (A) and 0.1% TFA in Milli-Q water (B) as follows: 60% A/40% B for 5 min, linear gradient to 90% A/10% B over 60 min, held for 25 min. Flow rate was 0.75 $ml.min^{-1}$. We used extinction coefficients from Moore et al. (2004b) to quantify 17 FPCs; macrocarpal I (R_t = 13 min), macrocarpal J (R_t = 17 min), eucalyptone (R_t = 28 min), macrocarpal A (R_t = 32 min), macrocarpal (unknown 1, R_t = 34 min), macrocarpal (unknown 2, R_t = 35 min), macrocarpal B (R_t = 37 min), macrocarpal

(unknown 3, R_t = 39 min), macrocarpal (unknown 4, R_t = 42 min), grandinal (R_t = 43 min), macrocarpal (R_t = 48 min), sideroxylonal A (R_t = 44 min), sideroxlyonal C (R_t = 45 min), grandinal (R_t = 53 min), sideroxylonal B (R_t = 55 min), jensenal (R_t = 60 min), macrocarpal G (R_t = 70 min). For each sample, the quantities of 17 compounds were summed to get a single measure of total FPCs.

Unsubstituted B-ring flavanones: extraction procedure

Unsubstituted B-ring flavanones are present in *Monocalyptus* only, and thus we truncated our eucalypt dataset to only *Monocalyptus* samples. There were 771 *Monocalyptus* out of 2,368 samples collected. We selected 250 spectrally-representative samples from these 771 *Monocalyptus* for UBF analysis. While the initial developments to analyse UBFs have been published (Saraf et al. 2017), this study provides the first description of a thorough method for quantification of UBFs in eucalypt leaves.

We extracted dried, ground leaves (20.5 ± 0.5 mg) for 10 mins in 1 mL methanol containing 0.05 $mg.mL^{-1}$ naringenin as an internal standard in a sonicating water bath. We then centrifuged the solution at 2500 rpm for 10 min and removed the supernatant. The extraction procedure was repeated, and the two extracts were combined and filtered through a 0.2 µm syringe filter. Preliminary analysis showed that two extractions were sufficient to extract all UBFs from the ground leaf material (i.e. UBFs were absent from a third extract; data not shown).

Unsubstituted B-ring flavanones: identification by LCMS-MS

Extracts from a single individual from each of 22 species, and a solution containing a mixture of 10 standards (100 µL each of the stock solutions of pinocembrin (5,7-dihydroxy flavanone), pinostrobin (5-hydroxy-7-methoxy flavanone), dimethylpinocembrin (5,7-dimethoxy flavanone), alpinetin (7-hydroxy-5-methoxy flavanone), cryptostrobin (5,7-dihydroxy-8-methyl flavanone), strobopinin (5,7-dihydroxy-6-methyl flavanone), desmethoxymatteucinol (5,7-dihydroxy-6,8-dimethyl flavanone), 5-hydroxy-7-methoxy-8-methyl flavanone, 5-hydroxy-7-methoxy-6,8-dimethyl flavanone and the 17.2min UBF. The 17.2min UBF is yet to be characterised, however we know it is a UBF based on its mass spectral fragmentation patterns and we

know that it has a molecular weight of 298. We analysed the extracts by LCMS-MS in positive ion mode using a reverse phase Sunfire C-18, 5 µm, 4.6 x 250 mm column (Waters, Sydney, NSW, Australia), fitted with a Sunfire VanGuard C-18, 5 µm, 3.9 x 5 mm guard column (Waters, Sydney, NSW, Australia). We set the oven to 40 °C and the injection volume was 20 µL. The solvents were Milli-Q water (A) and methanol (B), both containing 0.1% formic acid, and the flow rate was 1 $mL.min^{-1}$. The running conditions were isocratic 60% B for 0-2 min, followed by a gradient to 70% B at 10 min, and then to 100% B at 25 min. These conditions were held until 35 min, and then returned to the starting conditions over the following 2 min. The starting conditions were held for a further 8 min before the next injection.

Unsubstituted B-ring flavanones: quantification by HPLC

We analysed all sample extracts on a Shimadzu VP HPLC fitted with an autosampler and diode array detector. The HPLC was controlled using Class-VP v 7.3 software (Shimadzu, Kyoto, Japan). Running conditions were the same as those described for the LC-MS experiments, and the same column and guard column were used. Compounds in eucalypt samples were identified based on their retention time and absorbance spectra (λ max) relative to the authentic standards (where available), or to their retention time and absorbance spectra relative to compounds identified during LC-MS in the absence of standards. Standard curves were prepared by serially diluting stock solutions in methanol, and peak areas were quantified at 330 nm. We summed concentrations of these 10 UBFs to estimate total UBFs which was the trait used in all analyses.

Near infrared spectroscopy: calibration model development and validation

Separate calibration models for each nutritional trait with NIR spectra were developed in Matlab R2017b. Details on the calibration methods can be found in Chapter One. We used partial least squares (PLS) regression to develop separate models relating each nutritional trait and NIR spectra. The root mean squared error of prediction determined the best prediction model for each nutritional trait. Calibration and validation statistics for N_T, N_A, FPCs and UBFs can be found in Supplementary material 3. We used the predicted nutritional data for all of the subsequent statistical analyses.

Climate and environmental variables

A summary of the climatic and environmental variables included in the analysis can be found in Table 1. We extracted solar radiation, precipitation and temperature data from the WorldClim database) at 30 arc seconds (~1 km) resolution (Fick and Hijmans 2017). We collected soil and landscape attribute data at 3 arc seconds (~90 m) for slope, aspect, soil nitrogen, soil phosphorus and soil available water capacity using the Soil Landscape Grid Database (Grundy et al. 2015). In addition, we collected humidity and normalized difference vegetation index (NDVI) data from the Atlas of Living Australia (anon. 2015).

Statistical analysis

We performed all analyses in the R statistical environment (R Core Team 2017). To summarise the N_A and N_T data at the site level, we calculated the average value across the 32 trees in a site. Given that FPCs only occur in the subgenus *Symphyomyrtus*, we calculated the average FPC value across the number of *Symphyomyrtus* trees in the site. In addition, we calculated the percentage of *Symphyomyrtus* trees in a site. We repeated these steps for the UBF data. We calculated the average UBF value across the number of *Monocalyptus* trees and calculated the percentage of *Monocalyptus* trees in a site.

The methods for collecting UBF data were developed only recently and additional statistical methods needed to be explored to incorporate this data into the nutrition model. Koalas prefer symphyomyrtle foliage and in our data set there were many sites with no *Monocalyptus* trees sampled. For these reasons, the UBF data were used for observational purposes only and not included in our statistical analyses.

We assessed the influence of foliar nutritional chemistry on the likelihood of presence and density of koalas across the study area using mixed effect models in the R package *lme4* (Bates et al. 2015). Because the dependent variable (density) was equal to zero in thirteen sites and was continuous in the remaining sites, it was appropriate to fit separate models: one with the dependent variable as presence/absence and the other as density (as a continuous variable where we excluded sites where density was zero, Martin et al. 2005).

Table 1. Nutritional, climatic, environmental and landscape variables measured at study sites and their definitions.

Variable	Abbreviation	Description
Nutrition attributes		
Foliar total nitrogen (%)	N_T	Mean foliar total nitrogen across all trees in a site
Foliar available nitrogen (%)	N_A	Mean foliar available nitrogen across all trees in a site
Total formylated phluoroglucinol compounds ($mg.g^{-1}$)	FPCs	Mean formylated phluoroglucinol compounds across *Symphyomyrtus* trees in a site
Total unsubstituted B-ring flavanones ($mg.g^{-1}$)	UBFs	Mean unsubstituted B-ring flavanones across *Monocalyptus* trees in a site
Habitat attributes		
Symphyomyrtus proportion (%)	SymphProp	Proportion of *Symphyomyrtus* (preferred subgenera of koalas) trees in a site
Monocalyptus proportion (%)	MonoProp	Proportion of *Monocalyptus* trees in a site
Density of trees (m)	DensT	Mean distance between centre tree and neighbour trees in a site
Degree of heterogeneity (%)	DegHet	Percentage of different tree species within transect: for example, 1 tree species / 32 trees in a site = 0.03%
Normalised difference vegetation index (NDVI x 100)	NDVI	Mean annual normalised difference vegetation index
Distance to water (m)	DistWat	Distance to water body
Elevation (m)	Elevation	Elevation above sea level
Topography	Topog	Topography of transect in categories: gully, ridgeline, open flatland, hillside
Land use (categories)	LandUse	Land use of site in categories: national park, other leasehold, private land, road reserve, reserve, state forest,
Vegetation cover (categories)	Vcov	The vegetation cover of the transect: continuous, discrete
Vegetation space (categories)	Vspace	The layout of the transect: patch or strip of forest
Slope (%)	SlopeCont	The median slope within a 300 m radius representing the typical slope in the local landscape
Aspect (°)	Aspect	Aspect measures the direction in which a land surface slope faces. The direction is expressed in degrees from north
Soil attributes		
Soil total nitrogen (%)	SoilN	Mass fraction of total nitrogen in the soil by weight at 0-5cm
Soil total phosphorus (%)	SoilP	Mass fraction of total phosphorus in the soil by weight at 0-5cm
Available water capacity (%)	SoilAWC	Available water capacity computed for each of the specified depth increments at 100-200cm
Climate attributes		
Annual mean relative humidity (%)	Humidity	The mean of 24 monthly values at 9am and 3pm
Solar radiation of warmest quarter ($W.m^{-2}$)	Radiation	WorldClim variable 26
Mean temperature of warmest quarter (°C)	Temp	BiolClim variable 10
Precipitation of warmest quarter (mm)	Prec	BiolClim variable 18

Foliar nutrition and likelihood of koala presence

We converted the data on koala density to presence (1) or absence (0) and performed binomial generalised linear mixed modelling using the 'glmer' function. The models included the random effect of region to account for regional differences in climate, environment and the methods used to survey koala density. Given that concentrations of N_A and N_T are intrinsically related, high correlations were found between mean N_A and N_T (Wallis et al. 2010) and therefore it is not appropriate to include both traits in the same model. The fixed effects were mean N_A or mean N_T and mean FPCs (when the proportion of *Symphyomyrtus* was greater than zero), and an indicator set up for when the percentage of *Symphyomyrtus* was zero. This allowed us to include all sites (ranging from *Symphyomyrtus*-dominant to *Monocalyptus*-dominant), while modelling that effect of FPCs separately when (i) proportion of *Symphyomyrtus* is greater than zero and when (ii) proportion of *Symphyomyrtus* equals zero. The proposed mixed effect logistic model to investigate if nutrition influenced the likelihood of koala presence is:

$$\text{Presence/Absence} \sim N_{trait} + \text{FPCs} + (\text{proportion of } \textit{Symphyomyrtus} > 0) + (1|\text{Region})$$

Foliar nutrition and koala density

We proposed that where an animal population is present, nutrition is likely to regulate the density of the population. We looked at the distribution of the positive density data using histograms and chose a log transformation for normality. We considered the use of linear mixed effects models (using the 'lmer' function) as appropriate based on the linear response of density against the nutritional traits during data inspection. We fitted a linear mixed effect model using restricted maximum likelihood (REML) to determine how much variation in koala density can be explained by food quality when density > 0. The proposed mixed effect linear model to investigate the effect of nutrition on density of koalas per hectare is:

$$\text{Density} \sim N_{trait} + \text{FPCs} + (\text{proportion of } \textit{Symphyomyrtus} > 0) + (1|\text{Region})$$

A REML estimation in linear mixed models accounts for the degrees of freedom lost during estimation and adjusts for the uncertainty about the fixed parameters. Thus, we cannot compare REML models with different fixed effects (i.e. different forage quality

traits) because differences in the fixed parameters and contrasts can invalidate the comparison. To adequately compare across the various nutrition models, we also fitted these linear mixed effect models by maximum likelihood (ML). We assessed the variance explained (R^2) by the fixed (conditional R^2) and fixed + random (marginal R^2) effects (Nakagawa and Schielzeth 2013). To do this, we used the 'r.squaredGLMM' function in the R package *MuMin* (Bartoń 2009). Furthermore, we also calculated the Akaike Information Criterion corrected for sample size (AICc) for these ML models using the R package *MuMin* (Bartoń 2009). We used the threshold of 2 AICc units to determine if a model was clearly better (Burnham and Anderson 2002).

How strong is the effect of nutrition?

The aim of our study was to investigate the effect of nutrition on the density of koalas. We designed our sampling strategy to investigate how plant chemistry varied with koala population densities. However, we acknowledged that other environmental/climatic/landscape (ELC) variables have been shown to affect koala distribution and abundance of other folivores (Table 1). Therefore, for exploratory purposes only, we performed multiple linear mixed modelling to detect the effects of ecologically-relevant ELC variables against the nutritional traits. Alongside this, this set-up allowed also us to account for differences across sites as well and effectively hold them constant to see the effects of nutrition once they were considered. Using the same REML nutrition model described earlier, we also separately added each of the known ELC variables into the model. We investigated one variable at a time due to the limited number of sites with density data greater than zero (N = 61 sites). We assessed if the ELC variable had a significant effect on koala density using *P*-values in the REML model. For models that had categorical fixed effects, the *P*-value for each ELC variable was calculated using chi-square likelihood ratio test. To adequately compare across the various ELC models in Table 2, we fitted these linear mixed effect models by ML and compared models using the marginal and conditional R^2 and AICc. The proposed model to investigate how strong the effect of nutrition is on the density of koalas, accounting for ELC variables:

Density ~ N_{trait} + FPCs + (proportion of *Symphyomyrtus* > 0) + ELC variable

Many studies have alluded to abiotic influences on foliar chemistry (Braithwaite et al. 1983; Fashing et al. 2007; Okello et al. 2016), and thus ELC variables may be highly correlated with our nutrition data. To determine if there was multicollinearity, we tested the variance inflation factor (VIF) for each REML model developed in Table 2 using the 'vif' function in the R package *car* (Fox and Weisberg 2011). The VIF estimated how much of the variance of each fixed effect was inflated due to multicollinearity in the REML model. High correlations between variables were indicated by a VIF > 2.

Results

How does forage quality vary?

We collected leaf samples from 2,368 trees, across 63 eucalypt tree species. We mostly collected from two subgenera, *Monocalyptus, Symphyomyrtus* with a further 29 leaf samples from *Corymbia*. Boxplots of the variation in nutritional chemistry across tree species are shown in Figure 2. At the tree level, we found a strong correlation between N_A and N_T (Pearson's correlation coefficient = 0.89, Spearman's correlation coefficient = 0.86). We found no correlations between FPCs and N_A (Pearson's correlation coefficient = -0.009 and Spearman's correlation coefficient = -0.004) or between FPCs and N_T (Pearson's correlation coefficient = -0.06 and Spearman's correlation coefficient = -0.05). At the site level, we again found a strong correlation between N_A and N_T (Pearson's correlation coefficient = 0.88, Spearman's correlation coefficient = 0.84) and thus, only one N variable was included at a time in the nutrition models. Further, we found a weak correlation between N_A and FPCs (Pearson's correlation coefficient = -0.07 or Spearman's correlation coefficient = -0.09) and a moderate correlation between FPCs and N_T (Pearson's correlation coefficient = -0.21 or Spearman's correlation coefficient = -0.22).

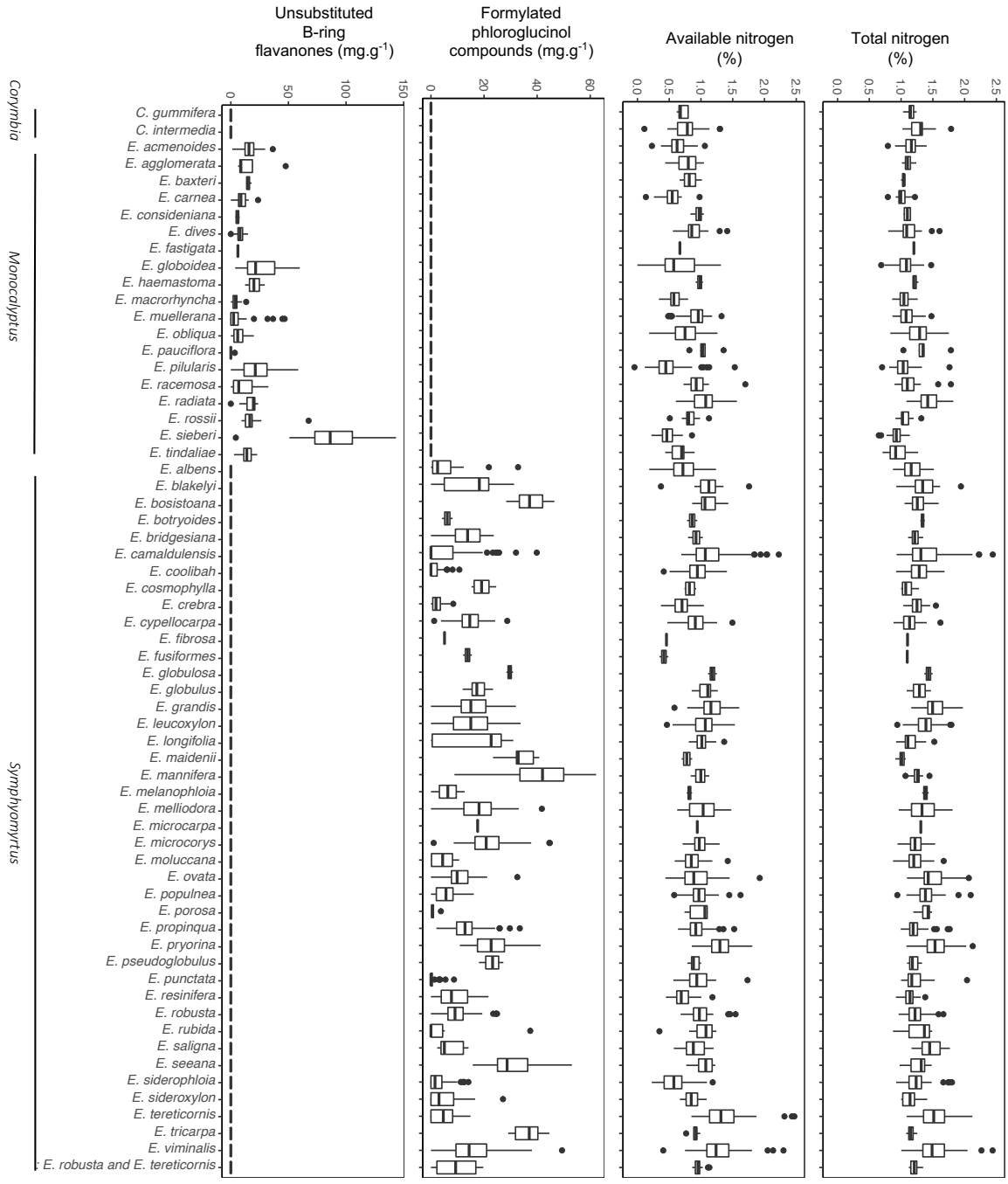

Figure 2. Box and whisker plots showing the concentrations of (a) total nitrogen, (b) available nitrogen, (c) total formylated phloroglucinol compounds and (d) unsubstituted B-ring flavanones in *Eucalyptus* species. The boxes incorporate the 25–75% quartiles and the median (solid line) while the whiskers cover the range of values.

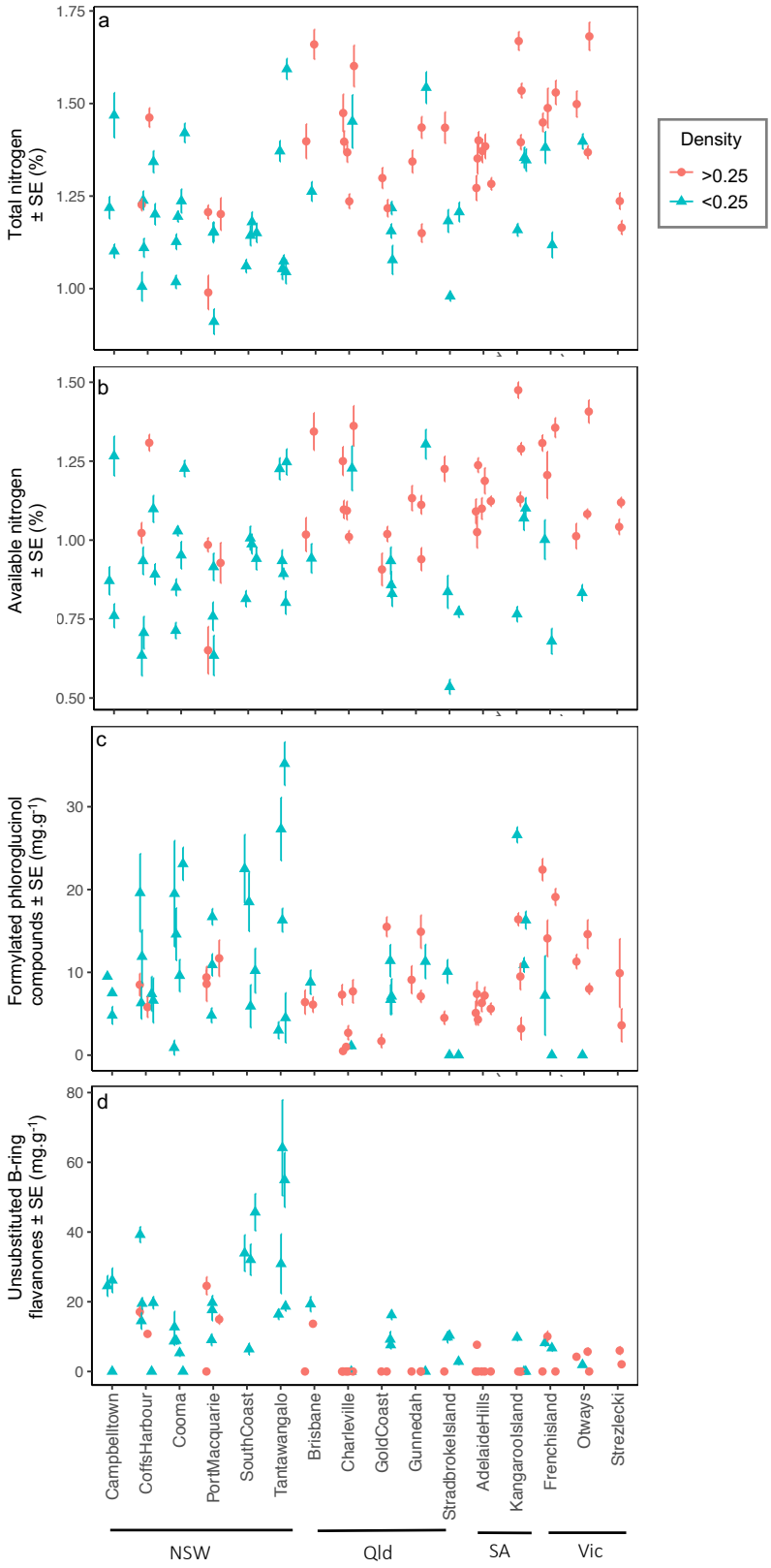

Figure 3. The mean concentrations ± standard error of a) total nitrogen, b) available nitrogen, c) total formylated phloroglucinol compounds and d) unsubstituted B-ring flavanones at 74 sites across 16 regions.

We found variations in forage quality both across and within tree species, subgenera, sites and regions (Fig. 2, 3). Many sites were dominated by *Symphyomyrtus*, with 30 sites containing 100% symphyomyrtles. Fourty-four sites contained at least one *Monocalyptus* tree. In general, we observed that *Symphyomyrtus* species tended to have higher concentrations of N_A whereas there was no clear pattern with N_T (Fig. 2). Sites with higher densities of koalas (density > 0.25.ha^{-1}) tended to have higher concentrations of N_A and N_T (Fig. 3). In contrast, sites with higher densities of koalas tended to also have lower concentrations of UBFs and FPCs. There was no clear pattern with concentrations of N_A and N_T at the regional level or state level.

Nutritional chemistry and koala density

Foliar nutrition and likelihood of koala presence

Our binomial mixed models showed that neither N_A + FPCs or N_T + FPCs in a site influenced the probability of koalas being present (R^2 marginal = 0.030, R^2 marginal = 0.028 respectively). The random effect of region, however, appeared to explain a considerable amount of variance in the presence/absence data (R^2 conditional = 0.333, R^2 conditional = 0.329 respectively).

Koala density data

Our study included 74 sites with separate koala density data. Thirteen sites had zero koalas observed and sixty-one sites had at least one koala present. Of these 61 sites, 50 sites had between 0.001 to 1 koala.ha^{-1} (Fig. 4a). In addition, 11 sites had densities of 1 – 17 koala.ha^{-1}. The southern koala populations had the highest densities of koalas. The koala densities in Vic ranged from 0 – 17, and in SA ranged from 0 – 7 koala.ha^{-1}. The northern koalas had much lower densities. Sites in Qld sites had a range of densities between 0 – 1 koala.ha^{-1} and NSW had the lowest density range from 0 – 0.63. The koala density data was negatively skewed and we transformed the data to normality using a log (density + 0.05) transformation (Fig. 4b).

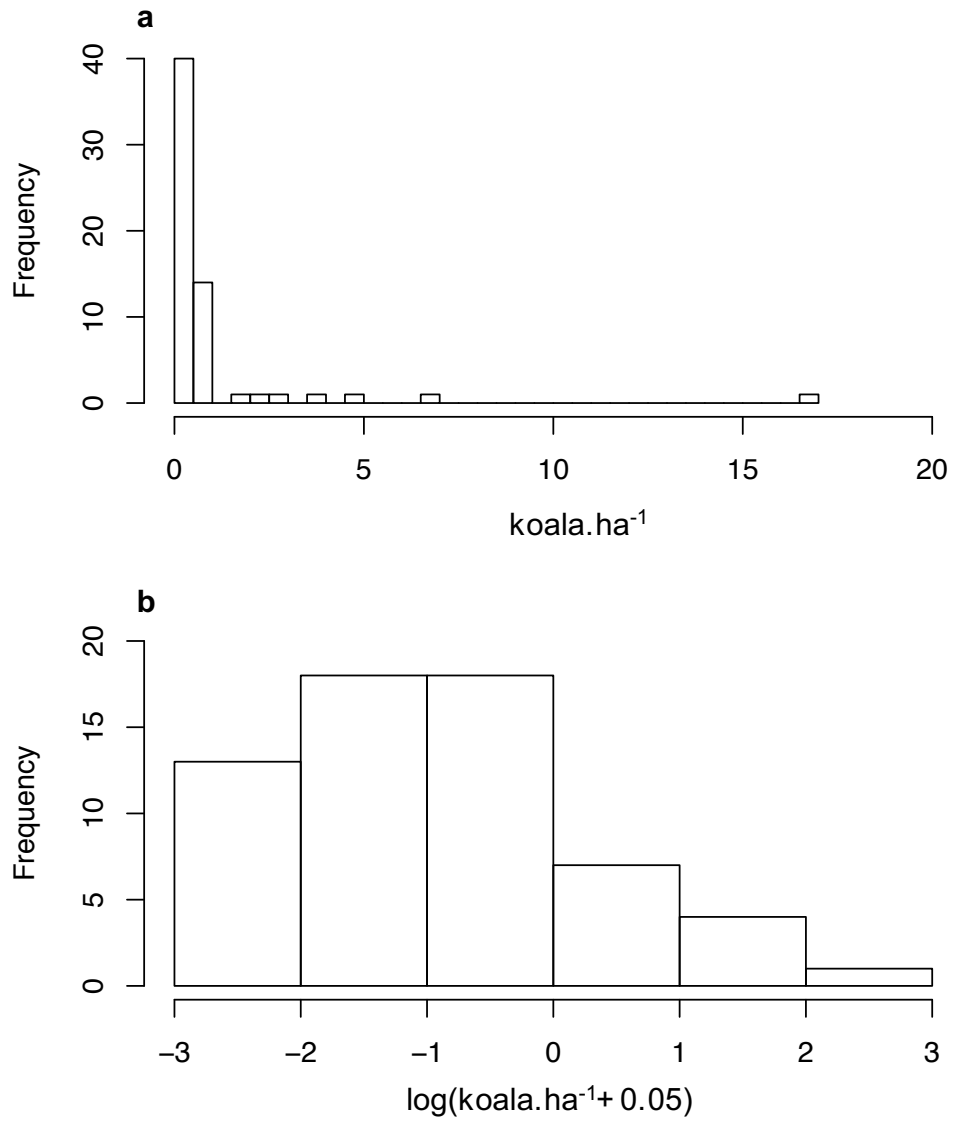

Figure 4. The distribution of koala density data as (a) number of koalas per hectare and (b) log(koala.ha^{-1} + 0.05) transformation.

Foliar nutrition and koala density

We investigated the role of nutrition in explaining the variation in positive koala densities. As described earlier, the model included mean nitrogen (either total or available), mean FPC (when there were some *Symphyomyrtus* trees) and an indicator for presence of symphyomyrtle. The final nutrition models were:

Model N_T: $\log(\text{koala.ha}^{-1} + 0.05) \sim N_T + \text{FPCs} + (\text{proportion of } \textit{Symphyomyrtus} > 0) + (1|\text{Region})$

Model N_A: $\log(\text{koala.ha}^{-1} + 0.05) \sim N_A + \text{FPCs} + (\text{proportion of } \textit{Symphyomyrtus} > 0) + (1|\text{Region})$

Using univariate plots, we showed apparent relationships between koala density and the four chemical traits at the site level (Fig. 5). Investigating the effect of UBFs on koala density was not possible for the reasons explained previously. We confirmed that there were significant effects of N_T + FPCs (model N_T) and N_A + FPCs (model N_A) on koala density (Table 2a, b). Mean concentrations of N_T and N_A showed significant positive associations (estimate = 2.859, SE = 0.75, $P < 0.001$; estimate = 2.388, SE = 0.57, $P < 0.001$ respectively). Mean concentrations of FPCs showed a significant negative association with koala densities in the nutrition model with N_A (estimate = -0.038, SE = 0.02, $P = 0.05$, Table 2b). In contrast, FPCs was not significant in the model with N_T (estimate = -0.031, SE = 0.02, $P = 0.11$, Table 2a). The proportion of *Symphyomyrtus* greater than zero had a significant positive effect on koala density in nutrition model N_T (estimate = 2.183, SE = 0.54, $P < 0.001$) and model N_A (estimate = 1.911, SE = 0.54, $P < 0.001$). The model with N_A was clearly better than the model with N_T (AICc = 164.0; AICc =167.4 respectively) and explained more of the variation in koala density (R^2 conditional = 0.74; R^2 conditional = 0.68 respectively).

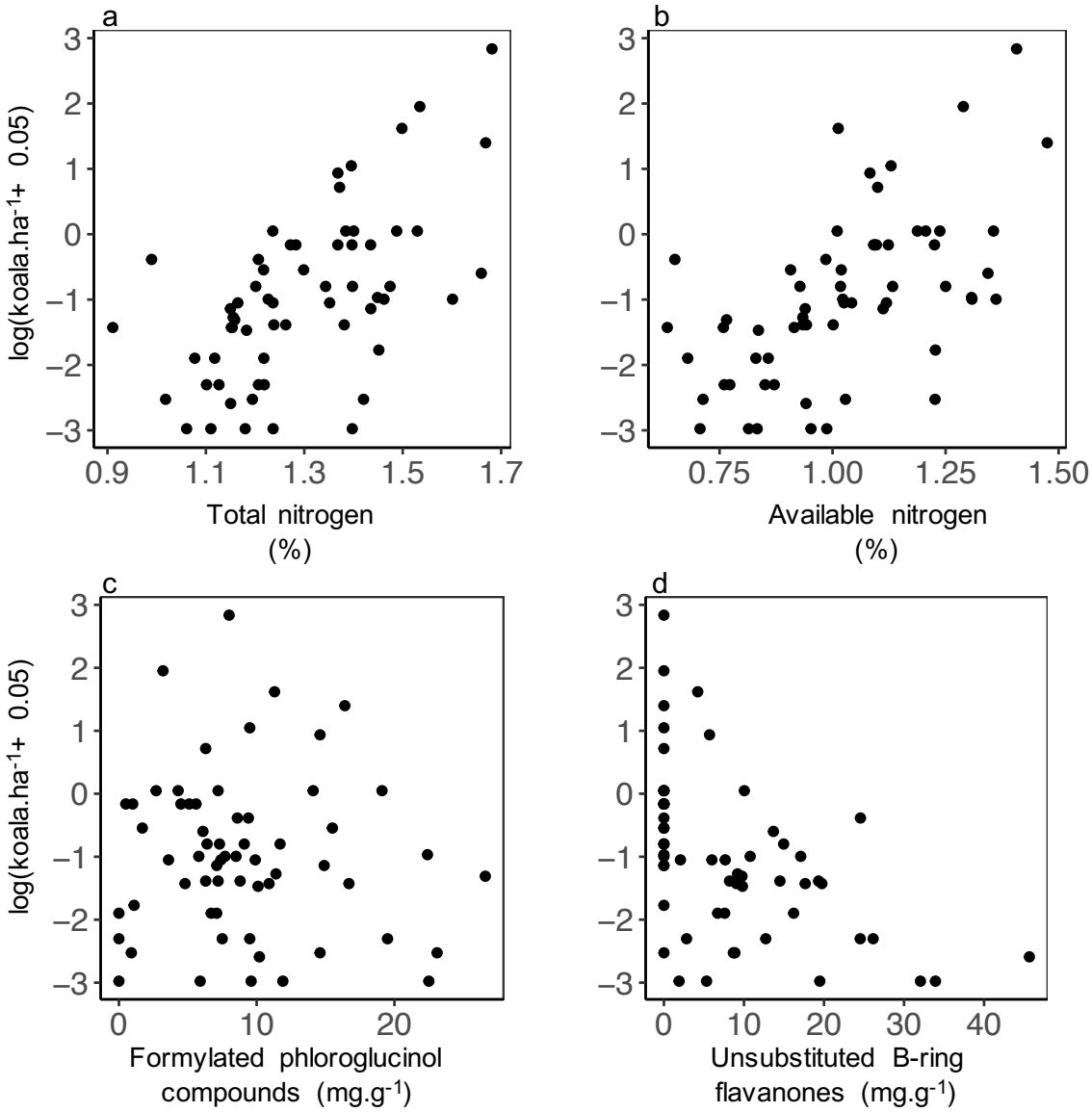

Figure 5. Scatter plots showing the univariate relationship between log(koala.ha^{-1} + 0.05) with concentrations of (a) total nitrogen, (b) available nitrogen, (c) formylated phloroglucinol compounds and (d) unsubstituted B-ring flavanones.

Table 2. Summary of two restricted maximum likelihood models explaining among-site variation in log(koala.ha^{-1} + 0.05). The fixed effects were a) total nitrogen or b) mean available nitrogen, and an effect of mean formylated phloroglucinol compounds when the proportion of *Symphyomyrtus* trees greater than zero. The random term included in the model was region.

a log(koala.ha^{-1} + 0.05) ~ N_T + FPCs + (proportion of *Symphyomyrtus* > 0) + (1|Region)

Variable	Estimate	SE	df	*t* value	*P*
Intercept	-6.527	0.983	54.945	-6.64	<0.001
Total nitrogen	2.859	0.750	56.034	3.81	<0.001
Formylated phloroglucinol compounds	-0.031	0.019	48.981	-1.62	0.112
Proportion of *Symphyomyrtus* > 0	2.183	0.537	50.555	4.06	<0.001
	Variance	Standard deviation			
Random term (region)	0.605	0.778			
Random term (residual)	0.511	0.715			

b log(koala.ha^{-1} + 0.05) ~ N_A + FPCs + (proportion of *Symphyomyrtus* > 0) + (1|Region)

Variable	Estimate	SE	df	*t* value	*P*
Intercept	-4.953	0.617	55.943	-8.03	<0.001
Available nitrogen	2.388	0.572	50.002	4.18	<0.001
Formylated phloroglucinol compounds	-0.038	0.018	47.706	-2.06	0.045
Proportion of *Symphyomyrtus* > 0	1.911	0.535	48.715	3.57	<0.001
	Variance	Standard deviation			
Random term (region)	0.749	0.866			
Random term (residual)	0.451	0.672			

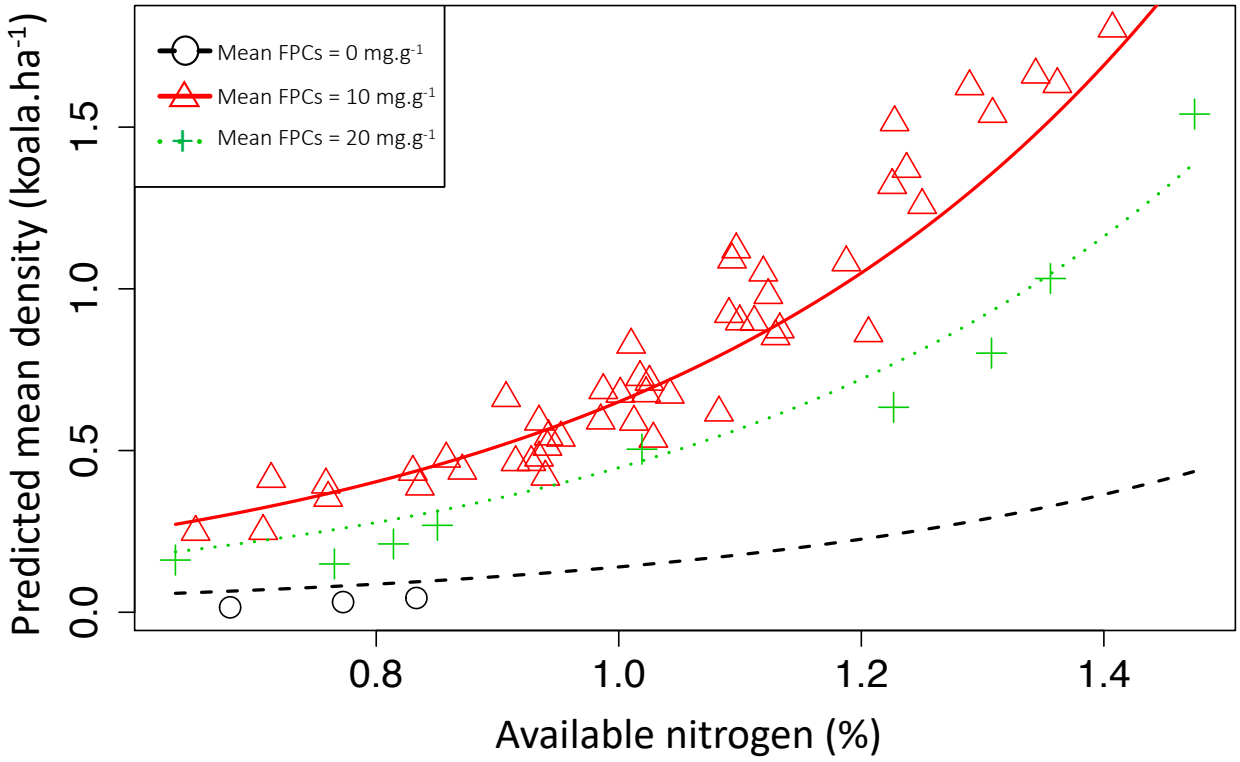

Figure 6. Predicted density of koalas (number of koalas per hectare) in response to available nitrogen (N_A) and formylated phloroglucinol compounds (FPCs) at three concentrations of FPCs. The *dashed line* represents when FPCs equals zero, indicating no *Symphyomyrtus* trees found in the site, and thus, likely unsuitable koala habitat. The *solid line* represents sites with mean concentrations of FPCs = 10 $mg.g^{-1}$, which is below the anti-feedant FPC threshold. The *dotted line* represents sites with mean concentrations of FPCs = 20 $mg.g^{-1}$, which is at the FPC threshold where feeding from koalas is deterred.

Figure 6 demonstrates the predicted effect of nutrition on koala densities using model N_A. As mean concentrations of N_A in a site increased from 0.6 – 1.4 % N_A, the model predicted an increase from 0 to 1.6 koala.ha^{-1}. Categorising the sites to three concentrations of FPCs revealed the negative effects of FPCs on koala densities. As mean concentrations of FPCs in a site increased to 20 mg.g-^{1}, there was a 0.1 - 0.5 reduction in koala.ha^{-1}. For sites with zero concentrations of FPCs (indicating sites with no *Symphyomyrtus*), koala densities only increased from 0 – 0.5 koala.ha^{-1}, the lowest range of koala densities in the three concentrations of FPCs.

How strong is the effect of nutrition?

The N_A model explained 35% (marginal R^2 =0.35) and 74% (conditional R^2=0.74) of the deviance in koala density among study sites (Table 2). It was the best nutrition model out of the models considered in terms of its AICc value. Thus, we used the following model to compare the effect of nutrition against the ELC variables.

> log(koala.ha^{-1} + 0.05) ~ N_A + FPCs + (proportion of *Symphyomyrtus* > 0) + ELC variable + (1|Region)

The ELC variables showed little collinearity with the nutritional traits (Table 3). The VIF was less than two for all ELC variables had a VIF. Adding an ELC variable did not improve the nutrition model's capacity to explain the variance in koala density nor explain the variation attributed to the random effect of Region (Table 3). Furthermore, the positive effect of N_A and negative effect of FPCs remained significant regardless of the addition of any ELC variables (data not shown). Nutrition models that included ELC variables such as DensT, Elevation, Humidity, DegHet and SoilP performed similar to model N_A. In contrast, the addition of Aspect, Prec, DistWat, Vcov, Temp, SoilAWC, Vspace, Slope, NDVI, Radiation, SoilN, LandUse to the nutrition model lead to poorer performing models.

Table 3. Summary of model comparison results for log(koala.ha^{-1} + 0.05). We compared the nutrition model (with random effect of region and fixed effects as available nitrogen and an interaction between formylated phloroglucinol compounds when the proportion of *Symphyomyrtus* greater than zero) against the same nutrition models with an added environmental, landscape or climate variable.

Variable Added	AICc	ΔAICc	R^2 marginal	R^2 conditional	*P*	Variance inflation factor
Nutrition model (no variable added)	163.5	0.0	0.35	0.74		
+ Density of trees	164.4	0.9	0.37	0.75	0.22	1.03
+ Elevation	164.4	0.9	0.37	0.73	0.17	1.01
+ Annual mean relative humidity	165.2	1.7	0.35	0.73	0.40	1.05
+ Degree of heterogeneity	165.3	1.8	0.38	0.73	0.40	1.07
+ Soil total phosphorus	165.4	1.9	0.35	0.75	0.41	1.06
+ Aspect	165.5	2.0	0.35	0.74	0.48	1.05
+ Precipitation of warmest quarter	165.5	2.0	0.40	0.74	0.49	1.03
+ Distance to water	165.6	2.1	0.36	0.75	0.55	1.09
+ Vegetation cover	165.8	2.3	0.33	0.74	0.41	*1.06
+ Mean temperature of warmest quarter	165.9	2.4	0.35	0.74	0.70	1.03
+ Available water capacity	165.9	2.4	0.35	0.75	0.72	1.05
+ Vegetation space	166.0	2.5	0.33	0.74	0.47	*1.01
+ Slope	166.1	2.6	0.31	0.75	0.49	1.13
+ Normalised difference vegetation index	166.1	2.6	0.33	0.74	0.51	1.01
+ Radiation warmest quarter	166.5	3.0	0.30	0.74	0.75	1.01
+ Soil total nitrogen	168.1	4.6	0.37	0.76	0.63	1.03
+ Land use	171.9	8.4	0.43	0.80	0.11	*1.02

*Generalised VIF corrected for degrees of freedom in models containing categorical variables.

Discussion

We found highly significant relationships between traits related to the nutritional quality of eucalypt leaves across sites and the density of koalas. Two statistical models that captured the variation in foliar nutritional quality, one with N_T and FPCs and one with N_A and FPCs, demonstrated that population densities vary with food quality across the range of koalas. Both traits that were proxies for foliar protein (N_T and N_A) showed positive correlations with density of koalas whereas the foliar concentration of the most prevalent anti-feedant compounds, FPCs, showed a negative association with koala densities. We found that the best model included measures of N_A and FPCs but the alternative model that included N_T and FPCs was also able to explain significant variation in density. The effect of variations in foliar nutritional quality remained significant, even when other environmental/landscape/climatic (ELC) variables that have been predicted to influence koala distribution and abundance across landscapes were accounted for in the statistical models. Food quality is thus a good predictor of the abundance of koalas (and we would also suggest for other folivores) and our approach shows how nutritional quality can be integrated into decisions about the conservation and management of vulnerable species.

Population densities vary with forage quality

Our study revealed that the nutritional quality of 74 forest sites varied across the wide distribution of the koala and explained patterns in koala densities. These findings support the nutritional-limitation hypothesis and demonstrate that it is possible to extend the effect of nutrition to landscape-scale studies. Recall that prior to this study, there had been a range of laboratory and field studies that had ranged from detailed studies of chemical ecology in captive koalas to small-scale studies of diet choice using a variety of approaches (Lawler et al. 1998; Moore and Foley 2005; Moore et al. 2005; Marsh et al. 2014). These fine-scale studies suggested very significant effects of FPCs on diet choice with protein and digestibility being important modifiers of the negative effects of FPCs and other secondary metabolites. Few other studies of the nutritional limitation hypothesis have been built on such a strong foundation. Nonetheless even using less specific measures of nutrients and PSMs, many studies support the

hypothesis at finer scales; e.g. primates (Milton 1979; Wasserman and Chapman 2003; Chapman et al. 2004; Hanya et al. 2006; Rode et al. 2006; Fashing et al. 2007), large herbivores (Grant and Scholes 2006), and arboreal marsupials (Braithwaite et al. 1983, Kavanagh and Lambert 1990). However, there are also many studies that have rejected this hypothesis and our study proposes a possible explanation for the conflicting evidence.

Studies that have rejected the nutritional-limitation hypothesis have often used crude measures of forage quality that probably fail to capture the chemical complexity of plants (Sinclair et al. 1988; Boonstra and Krebs 2006). Researchers may be less likely to see the effect of nutrition on animal populations if the chemical traits that are measured provide an incomplete picture of the nutritional value to animals. The whole rationale for measuring the concentration of protein, fibre or secondary metabolites assume that they directly affect an animal. In most studies, however there are few data linking what is being measured to animal performance. Crude measures such as total protein, protein-to-fibre ratio or easy but misleading indices of hugely complex secondary metabolites do not account for the effect of PSMs and thus, are unlikely to show a realistic nutritional value of plants to animals (Degabriel et al. 2009b; Wallis et al. 2012). Further, PSMs have an extraordinary diversity of chemical structures and in a single *Eucalyptus* leaf there may be as many 60 different terpenes (Külheim et al. 2015), and several hundred phenolic compounds (Marsh et al. 2017). Crude measures such as "total phenolics" may include PSM compounds that do not influence feeding and can create "noise" that mask the variation of meaningful PSM compounds (Lawler et al. 2000). In contrast, this research provides a detailed understanding of what positively and negatively influences nutritional quality for a koala.

Meaningful nutritional traits are essential

Knowing how plants and animals interact can provide specific hypotheses for how nutrition influences population densities. We know the protein requirements of the koala feeding on *E. punctata* foliage (Cork 1986) and how FPCs can interact with the physiology of the koala and restrict feeding behaviour (Lawler et al. 1998; Moore et al. 2005; Marsh et al. 2007, 2014). Studies have extended the information from these fine-scale studies to medium-scales and shown that variation in protein and FPCs can guide

tree choice and habitat suitability (Moore and Foley 2005). In our study, we found significant positive associations with both measures of foliar protein (N_T and N_A) traits and the density of koalas and a significant negative association with FPCs. This study further extends the effect of nutrition and demonstrates that concentrations of N_T, N_A and FPCs can explain variation in population densities at a landscape scale.

The findings of this study showed that including measures of N_A, a meaningful trait which integrates the effects of protein concentration, active tannins and digestibility, lead to a better model for explaining koala densities in terms of the composition of their food. Despite the high correlation between N_A and N_T in our data set, it is apparent that N_A data can provide more information during model development. We know that concentrations of N_A, not N_T, explained variation in reproductive success in female brushtail possums and moose (Degabriel et al. 2009b; McArt et al. 2009). N_A also explained variable browsing of brushtail possums in New Zealand (Windley et al. 2016). Similarly, concentrations of N_A were a better predictor of primate abundance than the protein-to-fibre ratio (Wallis et al. 2012). Thus, we suggest that integrated measures of nutritional quality that focus on estimates of the effects of fibre and tannins on protein availability, rather than simple measures of N_T, are likely to be more valuable for understanding animal density patterns. Even so, there are of course other factors that affect how much of this protein can be used by animals for maintenance and growth. The detoxification of ingested PSMs imposes a significant protein cost on animals (Au et al. 2013) which indicates that even N_A may not tell us the whole picture of how much N is available. Thus, while it currently provides the most realistic estimation of protein availability, relying on N_A measures alone could overestimate the nutritional quality of forage for animals.

We acknowledge that collecting N_A (or similar measures that estimate the amount of protein available for digestion) data might not always be possible. Chemical analyses that mimic digestion in animals are both expensive and time-consuming. It may be more feasible/attractive to use a single, easily measured trait, such a N_T. We found that our statistical model using N_T could also explain a significant proportion of the variation in koala density (Table 2). However, our results also show that simple measures of forage quality such as N_T provide an incomplete picture of nutritional effects on populations in

systems where plants are rich in PSMs. Where possible, more complex considerations of forage quality that incorporate both nutrients and PSMs should be included in forage quality analysis. A way to overcome the barriers associated with measuring complex chemical constituents across wide areas would be to develop more efficient methods for assessing forage quality. One method could be to extend the NIR methods described in Chapter One and build robust forage quality models that can predict forage quality across a variety of study systems. Another solution could be to include remote sensing techniques to collect nutritional data at large scales. While the second option has been demonstrated in theory (Ustin et al. 2004; Youngentob et al. 2012), several key limitations in sensor accessibility, data processing, and ground truthing must be overcome before it can be widely utilized (personal communications Kara Youngentob). Both options would reduce future analytical costs and help integrate meaningful nutritional traits in ecological studies. It is possible that meaningful measures of nutrition can help us answer fundamental questions in ecology about how organisms interact with their environment, the degree to which plants can impact populations at higher trophic levels, what are the ecological mechanisms that govern species' range and margins, how processes at the level of individuals impact patterns in populations, what governs the movement decisions of organisms in relation to dispersal and migration, how local is adaptation, what traits determine the resilience of populations to disturbance and perturbation, and more (Sutherland et al. 2013). The answers to any of these questions provide a better understanding of the many complex contingencies on which the existence of each species depends (Darwin 1859) and help us identify key features of suitable habitat for assessing and managing habitat quality for animals.

Using forage quality as a currency to assess habitat quality

Forage quality directly affects the animal and provides a currency to assess habitat quality. It removes the use of indicators of forage quality, such as vegetation composition, primary productivity or soil fertility (McNaughton et al. 1989; Maier et al. 2005; Augustine and McNaughton 2006; Riginos and Grace 2008; McGregor et al. 2013). This is particularly useful for wide-ranging animals such as the koala, where its distribution covers many different forests types and environments (Martin and Handasyde 1999; Adams-Hosking et al. 2011). We found that measures of two nitrogen

traits and FPCs can be used to assess habitat quality across the range of the koala. From our observation results, concentrations of UBFs may have a negative effect on koala density; however, this needs to be further investigated. Having a currency to rank habitat suitability will provide better systematic approaches for the conservation and management of vulnerable wildlife (McAlpine et al. 2015). Further, it would allow us to prioritise those areas that would remain suitable under a range of climate change scenarios (Moilanen et al. 2009).

Climate change is occurring at an alarming rate (IPCC 2014) and understanding how habitat suitability will be affected by climate change is essential for predicting the persistence of animal populations. The overall nutritional quality of forests is predicted to decrease in response to climate change (Robinson et al. 2012; Rothman et al. 2015). Increases in atmospheric CO_2 affect the carbon-nitrogen ratio in plants, and typically lead to poorer-quality plants with more carbon and less nitrogen (Lawler et al. 1996; Hovenden and Williams 2010). This is likely to increase concentrations of fibre and PSMs such as tannins and flavonoids, and also reduce the amount of plant protein available to herbivores (Bidart-Bouzat and Imeh-Nathaniel 2008). This has particularly serious consequences for species that live in fine nutrient balance, such as arboreal marsupials (Hume 1999). In fact, changes in food quality was one factor that determined the IUCN's listing of the koala as highly vulnerable to climate change (Johnson et al. 2009).

Rising global temperatures are predicted to exacerbate the effects of poor forage-quality on herbivores (Dearing 2013; Beale et al. 2018). Animals that consume PSM-rich diets need to metabolise and excrete PSMs to manage their potentially toxic effects. Several studies (Marsh et al. 2005; Shipley et al. 2006; Ulappa et al. 2014) have shown that animals regulate their feeding to avoid toxicity. Processes of detoxification are typically exothermic, and some PSMS can affect thermoregulation (Beale et al 2018). Further, the rate of detoxification is tightly linked to how much an animal can eat (Marsh et al. 2005; Au et al. 2013). If ambient temperatures rise, we can expect a negative effect on an animal's ability to dissipate heat, leading to a reduced detoxification rate, and this will limit how much an animal can eat (Kurnath and Dearing 2013; Kurnath et al. 2016; Beale et al. 2018). Restricting the detoxification rate will

further compound the negative effects of climate change on herbivores because animals cannot simply eat more of the poorer-quality food to meet nutrient requirements. Given the potentially serious consequences of climate change on herbivores, we urge researchers to include measures of forage quality when investigating how animal populations will persist over time.

Habitat quality is multifaceted

Habitat quality is multifaceted, and we do not expect nutrition to be only the influence on the distribution and abundance of free-ranging animals. Using mixed-effect logistic regression, we found that food quality did not explain the likelihood of koala presence at a site. An important caveat is that the sampling strategy was not designed to investigate the relationship between forage quality and the presence or absence of koalas, hence the data was heavily skewed towards sites with at least some koalas. Understanding what other factors regulate animal populations is difficult because as the geographic range of investigation increases, the more site-specific factors can differ between populations. Apart from nutrition, the persistence of animals could also be affected by the presence of predators and/or disease, vehicle collisions, extreme heat events and disturbance/hunting history (Kavanagh 1988; Scott 1988; Chapman et al. 2000; Trombulak and Frissell 2000; Seabrook et al. 2011). There are contrasting regional trends in koala densities (McAlpine et al. 2015) and the Australian Government proposed separate management strategies between the north and the south koala populations (NRMMC 2009). The aim for this strategy was to conserve koalas using a multidisciplinary approach that investigates the influence of urbanisation, disease, habitat loss and nutrition on koalas.

It was not surprising that the random effect of region in our models was also important for explaining variation in koala densities. We found that 33% of the variation in koala presence and 39% of the variation in koala densities above zero was attributed to the random effect of region. We suggest that the effect of region in our models reflects the striking variation in population densities of the koala in our data set and allowed us to account for the absolute differences in densities across regions. Broadly speaking, the northern koalas suffer from population declines whereas the southern koalas (particularly in Victoria and South Australia) are overabundant. By accounting for the

regional differences as a random effect, we highlighted the variation in koala densities between sites, which best reflects the level at which animals select habitat (Pulliam and Danielson 1991).

Landscape-scale studies are challenging because it is impossible to account for the many site-specific differences in historical, climatic, environmental and social factors across large landscapes. Although it was for strictly exploratory purposes only, we attempted to account for this regional variation using ELC variables that are likely to vary at the regional level and known to influence koala distribution and habitat use. However, none of the ELC variables helped explain the random effect of region (Table 3). There are many other factors that could influence regional variation in koala densities.

The koala covers a wide-distribution and has had a varied history with the environment and with humans. From the northern to the southern end of their range, there are contrasting regional trends in their susceptibility to disease, morphology, preferred food tree species, disturbance history, and whether they were introduced, translocated or natively occurred in the area (McAlpine et al. 2015). It is possible that the effect of disease could also help us explain regional trends in koala densities (Polkinghorne et al. 2013). Whereas most mainland koalas have chlamydia, only the northern koalas show symptoms. Chlamydiosis reduces female fertility, which in turn would prevent populations of koalas from reaching high densities (Polkinghorne et al. 2013). It is unclear why susceptibility of disease differs across the range of the koala and warrants further investigation if we are to use measures of disease to explain animal population patterns. It is possible that there are interacting effects between disease and nutrition as well given that nutritional status of an animal can influence its immune system and susceptibility to disease (Keusch 2003). Thus, it may be difficult to disentangle the effects disease and nutrition and future studies should consider this potentially confounding relationship.

Understanding animal population patterns requires accurate population data. While much of the focus of this study was to scrutinise the effect of nutrition, it is possible that there is noise in our density data. Estimating animal population size is notoriously difficult and there is low-confidence in many survey methods (Lindenmayer et al. 2001;

Pollock et al. 2006; Keiter et al. 2017). We collected data on koala densities from collaborators who were experts in their region. Studies suggest that expert knowledge is valuable when estimating population densities (Adams-Hosking et al. 2016). There is however much scope to improve the accuracy of population estimates (Marques et al. 2013). Audio surveys reveal that there can be more koalas in forests than previously estimated using traditional koala survey methods (DPI 2018). Methods such as this could provide more accurate population density estimates, which could result in tighter relationships between nutrition and koala densities. It is remarkable that the effect of nutrition remains significant at a large landscape scale, even though so many other factors could affect population densities, and that there is likely to be noise in our density data. It is possible that there are systematic biases that differ between survey methods. In future studies, it would be valuable to investigate how these biases may affect our ability to directly compare density data and what methods could help to reduce their effects.

Statistical challenges arise with landscape studies

The success of our research relied on a high level of statistical design and analyses. In order to understand the role of nutrition on animal populations, we required a sampling design that could effectively and efficiently compare the nutritional composition of sites. We dedicated many months to discussing the sampling design, speaking with numerous statisticians, landscape ecologists, nutritional ecologists and experts on koalas. As a result, we used the same sampling design to collect 2,378 eucalypt leaf samples from 74 sites that covered a variety of ecosystems and tree species compositions.

When summarising the chemical data for each site, we had to ensure that the chemical data accurately reflected what was available to the koala. Because we recorded the tree species for every tree, we could break up the proportions of *Symphyomyrtus* and *Monocalyptus* within a site to get a better idea of the concentrations of UBFs and FPCs in the site. From this, we became aware that many of sites did not follow Pryor's rule (Pryor 1959) as thirty sites were completely dominated by *Symphyomyrtus*. As a result, we had limited sites to assess the effects of UBFs and our results were observational only. Nonetheless, UBFs appear to have a negative effect on koala densities and

incorporating this data into future nutrition models would be valuable. Given that *Monocalyptus* and *Symphyomyrtus* tend to co-occur (Pryor's rule, Pryor 1959), landscape-scale studies should include measures of FPCs and UBFs to encompass the dominant chemical variation of PSMs. We acknowledge that there are many more ways to characterise a site nutritionally than just taking the mean of chemical constituents across trees. Some research suggests that the carrying capacity of a landscape depends on a relatively small number of high-quality forage trees or "nutrient hot spots" (Grant and Scholes 2006; Youngentob et al. 2011), indicating that researchers should consider these extremely high-quality trees when summarising their chemical data. Thus, how best to summarise the chemical variation of sites warrants further investigation.

Detecting nutritional relationships in the field is difficult because the use of a tree/environment can be affected by factors other than nutrition, for example, plant size or foraging risk (Scrivener et al. 2004; DeGabriel et al. 2014). Consequently, it is common to see data in a "wedge-shape scatter" where the non-linear relationships with nutrition can arise. This pattern was also prominent in our results (Fig. 5). Studies have investigated potential ways to analyse this type of data, for example using quantile regression (Cade et al. 1999; VanDerWal et al. 2009) or generalised additive modelling/generalised additive mixed modelling (Moore et al. 2010). In this study, we used mixed effect linear models and found strong effects of nutrition. By accounting for broad regional differences in landscape studies that may mask the effect of nutrition, we demonstrated that it is possible to find a linear relationship between nutrition and animal populations at a landscape scale (Fig. 6). We acknowledge that a limitation of our methods is that the effect of nutrition was only evident when we removed sites with no koalas. Thus, we can provide explanations for why folivore populations are present in the landscape but have limited ability to explain why animals are absent.

Conclusions

To our knowledge, this is the first time that transcontinental variation in forage quality has been used to explain patterns in folivore density, particularly as measured by N_A and FPCs. Specific hypotheses on how N_A and FPCs effect folivores were essential for extending the effect of nutrition to large scales. We argue that forage quality is essential for the expansion and persistence of a population and should be integrated with other

measures during habitat assessment. Forage quality provides a currency for assessing habitat quality and can help guide management and conservation efforts for vulnerable wildlife.

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Supplementary material

Supplementary material 1: List of collaborators with data on koala densities.

Region	Collaborators
Gold Coast	John Callaghan
Brisbane	Deidre de Villiers
	Cathryn Dexter
Charleville	Nicole Davies
	Clive McAlpine
North Stradbroke Island	Romane Cristescu
Port Macquarie	Steve Phillips
Gunnedah	Dan Lunney
	Mathew Crowther
	John Lemon
Coffs Harbour	John Turbill
	Mark Fisher
	Martin Smith
Campbelltown	Robert Close
Bermagui – Mumbulla, Strezlecki, Tantawangalo, Cooma,	Chris Allen
	Rod Pietsch
	Rob Summers
	Dean Turner
	Yuri Wiedenhofer
French Island	Scott Coutts
The Otways	Desley Whisson
Adelaide Hills	Jason Van Weenan
Kangaroo Island	Robyn Molsher
	Andrew Schoefield

Supplementary material 2: List of eucalypt species from which koalas are known to feed.

Scientific Name
Eucalyptus acaciiformis
E. acmenoides
E. agglomerata
E. albens
E. amplifolia
E. bancroftii
E. banksii
E. baueriana
E. baxteri
E. bensonii
E. bicostata
E. biturbinata
E. blakelyi
E. blaxlandii
E. bosistoana
E. botryoides
E. bridgesiana
E. caliginosa
E. camaldulensis
E. cameronii
E. camfieldii
E. camphora
E. canaliculata
E. cannonii
E. capitellata
E. carnea
E. cephalocarpa
E. chloroclada
E. cinera
E. conica
E. conjuncta
E. consideniana
E. coolabah
E. crebra
E. cypellocarpa
E. dalrympleana
E. dealbata
E. dives
E. dunnii

E. dwyeri
E. eugenioides
E. eximia
E. exserta
E. fastigata
E. fibrosa
E. glaucina
E. globoidea
E. globulus
E. goniocalyx
E. grandis
E. gummifera
E. haemastoma
E. henryi
E. imitans
E. interstans
E. laevopinea
E. largeana
E. largiflorens
E. leucoxylon
E. ligustrina
E. longifolia
E. macrocarpa
E. macrohyncha
E. maculata
E. maidenii
E. major
E. malacoxylon
E. mannifera
E. mckieana
E. melliodora
E. michaeliana
E. microcorys
E. moluccana
E. muelleriana
E. nandewarica
E. nicholii
E. nortonii
E. notabilis
E. nova-anglica
E. obliqua
E. oblonga
E. ochrophloia

E. odorata
E. ovata
E. paniculata
E. parramattensis
E. pauciflora
E. pellita
E. pilularis
E. piperita
E. polyanthemos
E. populnea
E. praecox
E. prava
E. prominula
E. propinqua
E. pseudoglobulus
E. pulligaensis
E. punctata
E. quadrangulata
E. racemosa (previously *E. signata*)
E. radiata
E. ralla
E. regnans
E. resinifera
E. retines
E. robusta
E. rossii
E. rubida
E. rudderi
E. rummeryi
E. saligna
E. Scias
E. scoparia
E. seeana
E. siderophloia (previously *E. drepanophylla*)
E. sideroxylon
E. sparsifolia
E. stannicola
E. subtilior
E. tenella
E. tereticornis
E. tindaliae
E. vicina
E. viminalis

E. volcanica
E. williamsiana
E. yangoura
E. youmanii

Supplementary material 3: Calibration and validation statistics for predicting total nitrogen, available nitrogen, formylated phloroglucinol compounds and unsubstituted B-ring flavanones. Partial least squares regression was used to develop the models and assessed using root mean square error of cross validation and root mean square of prediction.

Nutritional trait	Units	Range	Mean	*N* calibration	PLS factors	RMSECV	*N* validation	RMSEP
Total nitrogen	%	0.67 - 2.92	1.34	987	9	0.08	37	0.13
Available nitrogen	%	0.20 - 2.40	1.03	987	5	0.15	37	0.23
Formylated phloroglucinol compounds	$mg.g^{-1}$	0 - 59	13	200	14	4.4	129	4.7
Unsubstituted B-ring flavanones	$mg.g^{-1}$	0 - 146	24	220	16	4.2	30	7.0

Chapter Three

Changes in forage quality provide a link between disturbance and declines in animal populations

Keywords: available nitrogen, forage quality, hurdle models, landscape disturbance, mammalian herbivore, unsubstituted B-ring flavanones.

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Abstract

Landscape disturbance can cause shifts in vegetation communities, which can impact habitat quality for animals. However, the processes that are responsible for the cascading effects between plants and animals are often poorly understood. We demonstrate a mechanism by which landscape disturbance can degrade habitat quality by altering the nutritional composition of a forest using a case-study of koalas (*Phascolarctos cinereus*) in Australian eucalypt forests. We used two large datasets, an intensive survey of koala presence across 26,000 hectares of forests on the south coast of New South Wales and an extensive eucalypt forage quality study, to investigate the relationship between changes in tree species composition and changes in forage quality. The forests in the Bermagui-Mumbulla area of NSW, Australia, have a long and extensive history of logging and wildfires. *Eucalyptus sieberi,* a species native to this region, responds particularly well to disturbance and can rapidly replace formerly diverse eucalypt forests with near monocultures following intensive or repeated landscape disturbance. Using hurdle models, we found that observations of koala scat decreased as the proportion of *E. sieberi* in an area increased beyond zero. We found that *E. sieberi* had significantly lower available nitrogen and total nitrogen than most other eucalypt species in the area (mean = 0.70% and 0.94 respectively). *Eucalyptus sieberi* also had the highest foliar concentrations of herbivore deterrent plant secondary metabolites, unsubstituted B-ring flavanones (mean = 75 $mg.g^{-1}$). Based on existing knowledge of koala forage requirements and a decrease in koala presence as the proportion of *E. sieberi* increases, it is unlikely that forests dominated by *E. sieberi* can support koala populations. Active management approaches should be considered to mitigate the proliferation of *E. sieberi* following disturbance in areas where koalas and other eucalypt folivores require habitat protection. This study reveals a critical but often overlooked relationship connecting landscape disturbance to changes in tree species composition, which in turn impacts forage quality for folivores.

Introduction

Landscape disturbance can impact habitat quality and alter community composition in both plants and animals (Drapeau et al. 2000). Although the patterns of landscape change are relatively easy to observe, the processes that drive the relationships between changes in vegetation and changes in animal populations are often more difficult to identify and can be interconnected (Mouillot et al. 2013). For example, structural and compositional changes in vegetation can result in the loss of shelter for some species (Lindenmayer et al. 1997; Chapman et al. 2000), provide more shelter for other species (Kutt and Gordon 2012; Vanthomme et al. 2013), affect local climate and habitat suitability due to changes in temperature and humidity (Hogg and Norris 1991), impact the availability or quality of forage (Hanley et al. 1989; Chapman et al. 2000; Greenberg et al. 2011), alter competition dynamics (Youngentob et al. 2013), and affect predator-prey interactions (Shapira et al. 2008; Muhly et al. 2011). Identifying the underlying factors that shape the cascade of interactions between plants and animals is important for wildlife conservation (Lindenmayer et al. 2016). Without this understanding, it is difficult to determine the specific management activities that could mitigate the impacts of landscape change on threatened species (Lindenmayer et al. 2012; Reside et al. 2017). We present a case study using data from two landscape-scale studies to illustrate how changes in tree species composition can influence forage quality and the occurrence of an iconic folivore, the koala (*Phascolarctos cinereus*). Based on our findings, we propose targeted management actions to reduce the negative consequences of forest disturbance on this threatened species.

Disturbance related changes in forest composition and the proliferation of *E. sieberi*

The management of forest resources in Australia, like many other countries, involves a range of timber harvesting practices that include intensive (i.e., clear-felling), selective, and salvage logging in both native and plantation forests (Evans 2016). Logging can cause changes in plant species communities as it can either remove essential tree species (Lindenmayer et al. 1997), affect understory plant species that influence microclimate (Ough and Murphy 2004) or can have long term effects on forest structure and

composition (Osazuwa-Peters et al. 2015). Fire, whether natural or man-made, also plays an important role in shaping forest landscapes (Bradstock et al. 2012). The interaction between fire and logging can have further impacts on the composition, function, future fire intensity, and sustainability of forest ecosystems (Siegert et al. 2001; Lindenmayer et al. 2009, 2016; Bowd et al. 2018). Shifts in vegetation communities following landscape disturbance are common and well-documented across many ecosystems, although the long-term resilience and regenerative capabilities of ecosystems vary widely (Sousa 1984; Keenan, Rodney and Kimmins 1993; Ellison et al. 2005; Osazuwa-Peters et al. 2015). Both logging and fire can alter vegetation competition dynamics, favouring those species that respond well to the resulting landscape change or that have adapted specifically to take advantage of these disturbance events.

Eucalyptus sieberi is found across south-eastern Australian and responds particularly well to disturbance. Intensive logging, repeated logging, or intense fires can all result in dense monocultures of *E. sieberi* (Fig. 1), replacing what was once a more diverse eucalypt forest (Florence 1996). Its fast regeneration and proliferation are widely recognised and viewed favourably by foresters due to its suitability as pulp wood (Williams and Potts 1996). This species also occurs naturally, as a non-dominant species, in mixed species forest in NSW and Victoria. It is considered a food species for the koala based primarily on records of koala scat located near the base of *E. sieberi* (Allen et al. 2010, 2016), although few previous studies have investigated its suitability as a food resource (Stalenberg et al. 2014). Due to declining populations, the koala has recently been listed as vulnerable in NSW and QLD (NRMMC 2009; McAlpine et al. 2015), increasing the need to obtain a better understanding of habitat requirements for maintaining koala populations. The proliferation of *E. sieberi* across some areas of NSW and Victoria and concern over vulnerable koala populations has prompted a more careful examination of the relationship between forest species composition and the presence of koalas.

Figure 1. Image of *Eucalyptus sieberi* regrowth following logging. Photographer Tony Rodd.

An iconic eucalypt folivore, the koala

The koala is a eucalypt folivore with a wide distribution that spans most of the eastern half of Australia (Martin and Handasyde 1999). This arboreal marsupial can persist in a variety of eucalypt forest types and is thought to eat leaves from more than 100 different eucalypt species (Phillips 1990). Like many other species that have a wide distribution, it can be difficult to pinpoint why some forested landscapes are suitable while others are not. However, we do know that variations in forage quality can impact landscape use by many species, including the koala (de Vries and Schippers 1994; Moore and Foley 2005; Bryant et al. 2009). The palatability and nutritional quality of forage varies both within and between plant species (Moore et al. 2010; Wallis et al. 2010; Hanley and Barnard 2014), and this creates a patchy distribution of forage quality across landscapes (Braithwaite et al. 1983). As a result, forest composition can impact its nutritional quality, even for animals that consume leaves from a wide range of tree species. Relatively small changes in the amount of available nutrients in plant forage can have significant impacts on animal reproductive

success (Degabriel et al. 2009; McArt et al. 2009) and population densities (Rode et al. 2006).

The koala generally prefers to forage from trees of the *Eucalyptus* subgenera, *Symphyomyrtus* (Moore et al. 2004a). Symphyomyrtle species tend to have higher concentrations of available nitrogen (N_A) than the other most common subgenera, *Eucalyptus* (common name monocalypt). Foliar protein (measured as total or available nitrogen) is considered a limiting nutrient for many herbivores, and the higher N_A content in symphomyrtles may explain why the koala and several other eucalypt folivores favour this subgenus of eucalypt trees despite the fact that monocalypts and symphyomyrtles generally co-occur (i.e., Pryor's Rule, Pryor 1959). Each of these subgenera of *Eucalyptus* also contain specific herbivore deterrent plant secondary metabolites (PSMs). Formulated phloroglucinol compounds (FPCs) are a group of terpene adduct chemicals found in most symphyomyrtle foliage that act as antifeedants in mammals (Lawler et al. 1998). As concentrations of FPCs increase, foliage consumption in all marsupial folivores decreases, but the sensitivity to FPCs is much more pronounced in monocalypt specialists like the ringtail possum (*Pseudocheirus peregrinus*, Jensen et al., 2014). Monocalypts do not synthesise FPCs but instead make a class of PSMs called unsubstituted B-ring flavanones (UBF, Tucker et al. 2010). A specific anti-feedant UBF, pinocembrin, has recently been isolated from several monocalypt species (Marsh *et al.* 2015). Early feeding studies suggest that increasing concentrations of this compound and related UBFs may deter feeding by symphyomyrtle specialists like the common brushtail possums and the koala but should have little or no effect on monocalypt specialists like the ringtail possum (Marsh *et al.* 2015, Beale *et al.* unpublished data, Blyton *et al.* unpublished data).

Aims

To investigate how forest composition influences forage quality and the presence of koalas, we utilized data from two landscape-scale studies. The first dataset comprised a detailed survey of forest species composition and koala occurrence in the Bermagui-Mumbulla area of NSW Australia. We also incorporated data on eucalypt forage quality from a large-scale survey of the nutritional quality of eucalypt leaves collected from sites across most of the

koala's range, including forests in Bermagui-Mumbulla. We used these data to answer the following questions:

1) How does the nutritional quality of eucalypt leaves from *E. sieberi* in the Bermagui-Mumbulla area compare to other eucalypt species sampled in the same region in regards to total nitrogen (N_T), $N_{A,}$ and UBFs.
2) Does the probability of observing koala scat in a site decrease as the proportion of *E. sieberi* increases, relative to other eucalypt species?
3) Based on modelled eucalypt forage quality data, how does the amount of available nutrients and subgenera specific PSMs change in a forest area as the percentage of *E. sieberi* trees increases?

For the first time, we examine how disturbance related changes in forest tree species composition can alter habitat suitability for an arboreal folivore through changes in forage quality.

Methods

Study area

The Bermagui–Mumbulla area is 380 km southwest of Sydney on the south-east coast of New South Wales, Australia. The study area covered approximately 26,000 hectares of closed-canopy coastal, schlerophyll, and wet-temperate eucalypt forests across State Forest and National Park land tenures (Fig. 2). The composition of forest tree species varies depending on topographical and edaphic features, and disturbance history of the landscape (Turner et al. 1978; Austin et al. 1996). The most common canopy tree species in the study area include *E. muelleriana*, *E. sieberi*, *E. globoidea, E. longifolia* and *Angophora floribunda*, and common understory species include *Allocasuarina littoralis*, *Acacia falciformis* and *Acacia mearnsii* (see Supplementary material 1 for a list of all tree species and understory species found in the study area). The geology is primarily a mix of Ordivician sediments (sandstone, mudstone, shale) and Devonian granite-like rocks (Kelly and Turner 1978), and the elevation ranges from 35 m – 200 m. Rainfall in the summer months ranges

from 80 mm to 100 mm (mean max and min) and 50 mm to 85 mm (mean max and min) in the winter. The mean maximum and minimum temperatures are 21°C to 29°C in the summer and 15°C to 21°C in the winter (Bureau of Meteorology, 2017).

A number of arboreal marsupial folivore are native to the area including greater gliders (*Petauroides volans*), sugar gliders (*Petaurus breviceps*), common ringtail possums (*Pseudocheirus peregrinus*), and common brushtail possums (*Trichosurus vulpecula*). Historical records show that koalas were once abundant in areas dominated by *E. tereticornis* (forest red gums. From the mid-1800s until the mid-1900s, large areas of fertile land dominated by *E. tereticornis* were cleared for pasture, leading to habitat loss for the koala and many other native animals (Bradshaw 2012). The remaining native forests in the study area support low densities of koalas ranging from 0.001 to 0.025 animals per hectare. These forests are managed for mixed-use recreation, conservation, and logging. Native hardwood trees in state forests are selectively logged for pulp wood and timber. Clearfall-logging was once widely practiced in the study area but ceased in 1999 (Regional Forestry Agreement 1999). Fire is used extensively by landscape managers to reduce understory fuel load in an attempt to decrease the occurrence of severe, uncontrolled fires. Nevertheless, severe fires involving the crowns of trees still happen occasionally. The most recent occurred in 2004 and previously in 2002.

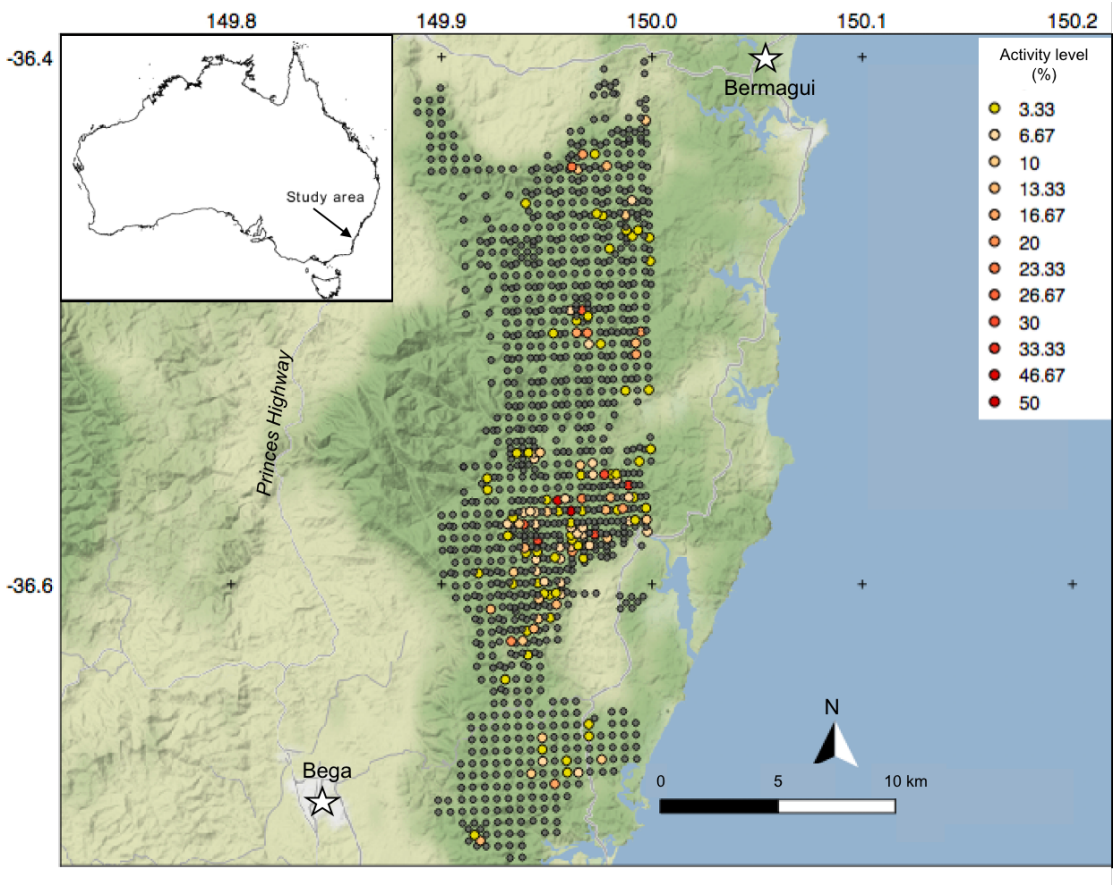

Figure 2. Map of koala and forest survey sites in the Bermagui – Mumbulla area, New South Wales, Australia. Activity level of koalas (%, the proportion of trees with koala scats at each search site) is marked by colour (key on right).

Surveys of the presence of koalas

We estimated the presence of koalas across the study area using a Regularised Grid-Based Spot Assessment Technique (RGBSAT, Phillips and Callaghan 2000). Survey teams conducted searches using a predetermined grid pattern across the study area. Initially, searches were conducted at sites at 500m intervals. After 14 months of fieldwork, we found that indicated that koala activity could also be detected at 1000 m intervals and adopted this coarser sampling interval for the remainder of the survey to increase the geographic coverage of the koala activity survey.

The searches began at the center of each search site. We selected the closest 30 live trees over 15 cm in diameter at breast height (DBH), with tree 1 as the closest to the center point and tree 30 as the furthest. At the base of each tree, we searched for koala scats within a

meter from the trunk. We recorded the presence/absence of koala scats and the species of each tree.

Collection of *Eucalyptus* leaf samples

We subsetted 504 trees from 18 species, of which 50 were *E. sieberi* and 454 were non-*E. sieberi*, across the study area as part of a separate, larger study to investigate how eucalypt forage quality varies across the entire range of the koala (Chapter Two). Trees were sampled from both transect lines established in areas with known koala population densities as described in Chapter Two and some additional point sampling within the study area for ground-truthing a separate, remote sensing forage quality component of the larger nutritional study as described in Youngentob 2015. The eucalypt leaves were obtained from the top half of the tree canopies using a throw-line launcher and following the methods described in Youngentob et al. (2016). Leaf samples were then immediately frozen in solid CO_2 (dry ice) for at least 24 hours and then stored in a -20°C freezer until further analysis that took place within two months of collection.

Analysis of chemical composition of leaves

We determined foliar concentrations of N_T, N_A, *in vitro* dry matter digestibility (DMD), and UBFs from the eucalypt leaf samples (units are in %DM) using near infrared reflectance spectroscopy (NIRS). The frozen leaf samples were freeze-dried and then ground to pass a 0.5mm screen using a FOSS Cyclotec 1093 cyclone mill (Foss, Hilleroed, Denmark). Near-infrared reflectance spectra were collected following the methods described in Chapter One. We selected 987 samples from the larger forage quality study (n = 3600) to conduct wet chemical analysis for N_T, N_A, and DMD. We determined the concentration of N_T using Leco CNS-2000 combustion analyser (Leco, St Joseph, Michigan). We then determined the concentration of N_A and DMD using an *in vitro* digestion procedure described in Degabriel *et al.* (2009). In short, we sequentially incubated leaf samples at 37°C with a buffer (24 h, 0.05M Tris–BASE buffer, pH 7.1), pepsin (24 h, 2 $g.L^{-1}$) and cellulase (48 h, 6.25 $g.L^{-1}$, pH 4.75). This assay mimics digestion in a hindgut fermenter.

We selected 250 out of 771 monocalypt samples from the larger foliar composition study for UBF analysis that covered the range of monocalypt species collected. We used high

performance liquid chromatography (HPLC) to quantify UBFs in leaf samples and detailed methods are described in Chapter Two. In short, we extracted dried, ground leaves (20.5 ± 0.5 mg) in 1 mL methanol containing 0.05 $mg.mL^{-1}$ of naringenin as an internal standard. We centrifuged the solutions at 2500 rpm for 10 min, removed the supernatant and then repeated the extraction procedure. We filtered extracts filtered through a 0.2 µm syringe filter. We analysed all sample extracts on a Shimadzu VP HPLC fitted with an autosampler and diode array detector. The HPLC was controlled using Class-VP v 7.3 software (Shimadzu, Kyoto, Japan). We separated the components using a reversed phase Sunfire C-18, 5 µm, 4.6 x 250 mm column (Waters, Sydney, NSW, Australia), fitted with a Sunfire VanGuard C-18, 5 µm, 3.9 x 5 mm guard column (Waters, Sydney, NSW, Australia). The oven was set to 40 °C and the injection volume was 20 µL. The solvents were Milli-Q water (A) and methanol (B), both containing 0.1% formic acid, and the flow rate was 1 $mL.min^{-1}$. The running conditions were isocratic 60% B for 0-2 min, followed by a gradient to 70% B at 10 min, and then to 100% B at 25 min. These conditions were held until 35 min, and then returned to the starting conditions over the following 2 min. The starting conditions were held for a further 8 min before the next injection. We used extinction coefficients from Chapter Two to quantify 10 known UBFs: pinocembrin (5,7-dihydroxy flavanone), pinostrobin (5-hydroxy-7-methoxy flavanone), dimethylpinocembrin (5,7-dimethoxy flavanone), alpinetin (7-hydroxy-5-methoxy flavanone), cryptostrobin (5,7-dihydroxy-8-methyl flavanone), strobopinin (5,7-dihydroxy-6-methyl flavanone), desmethoxymatteucinol (5,7-dihydroxy-6,8-dimethyl flavanone), 5-hydroxy-7-methoxy-8-methyl flavanone, 5-hydroxy-7-methoxy-6,8-dimethyl flavanone and the 17.2min UBF. The 17.2min UBF is yet to be characterised, however we know it is a UBF based on its mass spectral fragmentation patterns and we know that it has a mass of 298. We summed concentrations of these 10 UBFs to estimate total UBFs which was the trait used in all analyses.

Following the methods in Chapter One, we developed NIRS models based on the chemical values for these samples (training dataset). A predictive model was developed for each nutritional trait and then used to predict the same chemical values in our remaining

samples (see Supplementary 2 for results from the best performing NIRS models for N_T, N_A, DMD and UBF).

Statistical analysis

Koala presence and the percentage of E. sieberi

We explored the distribution of koala activity across the search sites. Koala activity is measured by the sum of the total number of scat trees (trees found with koala scats) at each search site; hereafter referred as scat count. We determined the percentage of *E. sieberi* trees against the total number of eucalypts recorded at each site.

Zero-inflated count data is common in animal abundance data (Cunningham and Lindenmayer 2005). In some cases, this type of data can be modelled by over dispersed count distributions such as the negative binomial. In other cases, hurdle models model separate processes for the probability of seeing a non-zero value and for the distribution of counts given that they are non-zero (Martin et al. 2005).

We performed all analysis in the R statistical environment (R Core Team 2017). We fitted Poisson, negative binomial, hurdle Poisson and hurdle negative binomial models. The hurdle models were fitted using the R package *pscl* (Zeileis et al. 2008). We compared Poisson, negative binomial, hurdle Poisson and hurdle negative binomial models using the Akaike Information and Bayesian Information Criteria (AIC and BIC) to find the best fitting model. Although scat counts were at most 30, the proportion out of 30 was universally small (never above one-third). Thus, it was appropriate to consider unbounded count models, rather than the binomial distribution. In this case, binomial distributions were very well approximated by Poisson distributions, and negative-binomial models represent over-dispersed Poisson. All models contained two covariates, one for the proportion of *E. sieberi* (between 0 and 1), and an indicator variable set to 1 when *E. sieberi* was exactly equal to zero. The latter was included because there were zero *E. sieberi* at 55% of sites and these appeared to be different in terms of the distribution of scat count compared to sites where the proportion of *E. sieberi* was low but not zero. These two covariates were the only ones considered as the proportion of *E. sieberi* was the only explanatory variable for the full set of 1,074 sites.

In the study area, presence of koalas clustered in patches and indicated that sites closer together may be more similar than those further apart (Fig. 2). To ensure correct standard errors and p-values, we estimated standard errors of model coefficients using a block jack-knife estimator, where blocks were spatial groupings of sites. The blocks were constructed using the R package *geosphere* (Hijmans 2017). We first created a distance matrix using the 'geo.dist' function on the coordinates of all search sites. We then performed hierarchical clustering analysis using the 'hclust' function so that sites in similar locations clustered together. Finally, we cut the dendrogram into 35 blocks using the 'cutree' function, with each block corresponding to a separate cluster. We combined two blocks, each with very small number of search sites, to form a single block and thus, 34 blocks were used for the block jack-knife.

Forage quality across eucalypt species sampled from the study area

We plotted the distribution of the four nutritional compositional traits against the eucalypt species using boxplots. We calculated the estimated marginal mean of each trait, for each species, using the 'emmeans' function in the package *Emmeans* (Lenth 2018). We compared the nutritional quality between different eucalypt species using the Bonferroni method and pairwise comparisons.

Simulating the effects of increasing the proportion of E. sieberi *on forage quality*

We then simulated the change in concentrations of N_A in a forest with an increasing proportion of *E. sieberi* trees. We used all leaf composition data collected from the Bermagui–Mumbulla region ($N_{E.\ sieberi}$ = 50 and $N_{\text{non } E.\ sieberi}$ = 454 samples). For each forest simulation, we sampled a total of 504 trees and varied the percentage of *E. sieberi* in a forest from 0 to 100%, with intervals of 10%. Thus, we simulated 10 mixed forests. To mimic the non-*E. sieberi* tree component, we randomly sampled N_A from the 454 non-*E. sieberi* samples. To mimic the *E. sieberi* component, we resampled the N_A data from the 50 *E. sieberi* samples with replacement as needed. We plotted the distribution in N_A in the simulated forests using side-by-side boxplots.

Results

Surveys of the presence of koalas

There were 1,074 search sites and 32,250 tree bases searched for koala scat. We recorded koala scats in 133 of the 1,074 search sites, indicating that the data were substantially zero inflated. Koala scat was found under 349 number of trees, the most common species with associated koala scat were (*E. longifolia* – 102 scats, *E. globoidea* – 58 scats and *E. sieberi* – 49 scats). The study area was dominated by *Eucalyptus* (22,862 trees, 18 different species). The dominant eucalypt species were *E. muelleriana* (25%), *E. longifolia* (16%), *E. sieberi* (16%), *E. globoidea* (12%), *E. agglomerata* (7%) and *E. cypellocarpa* (6%). There were 9 567 non-eucalypts which mostly consisted of *Casuarina* or *Allocasuarina* species (45%) and *Acacia* species (20%). Additional summaries of these data can be found in Allen *et al.* (2010, 2016).

Koala presence and the percentage of *E. sieberi*

Figure 3 shows that koala presence data were zero inflated whether *E. sieberi* was absent, low, medium or high in the site. We compared hurdle and non-hurdle negative binomial and Poisson models and found that the negative binomial model was the best fit: it had the equal lowest AIC (equal to the hurdle negative binomial model to two decimal places) and the lowest BIC (by a margin of about 14). Even though there was a high proportion of zero values in the data, the AIC and BIC values show that the negative binomial model adequately modelled this phenomenon without the need for a hurdle model. Table 1 shows the model coefficients of the negative binomial models. The exponents of the coefficients are interpreted as relative risks, that is, the factor by which the expected proportion of trees with scats increases when the covariate increases by 1. Both covariates are significant at the 0.01 significance level. An increase of one percentage point in the *E. sieberi* proportion is associated with a 1.9% decrease in the proportion of trees with scat (RR = 0.981). Figure 4 shows the predicted proportion of trees with scat as a function of the percentage of *E. sieberi* trees. At 0% *E. sieberi*, the predicted proportion with scat is 0.7%. For small non-zero proportions of *E. sieberi*, the scat proportion is 2.4%. The predicted scat

proportion then decreases smoothly to 0.3% as the proportion of *E. sieberi* increases to 100%. However, when the *E. sieberi* is exactly zero, the proportion of trees with scat drops by 71% (RR=0.29).

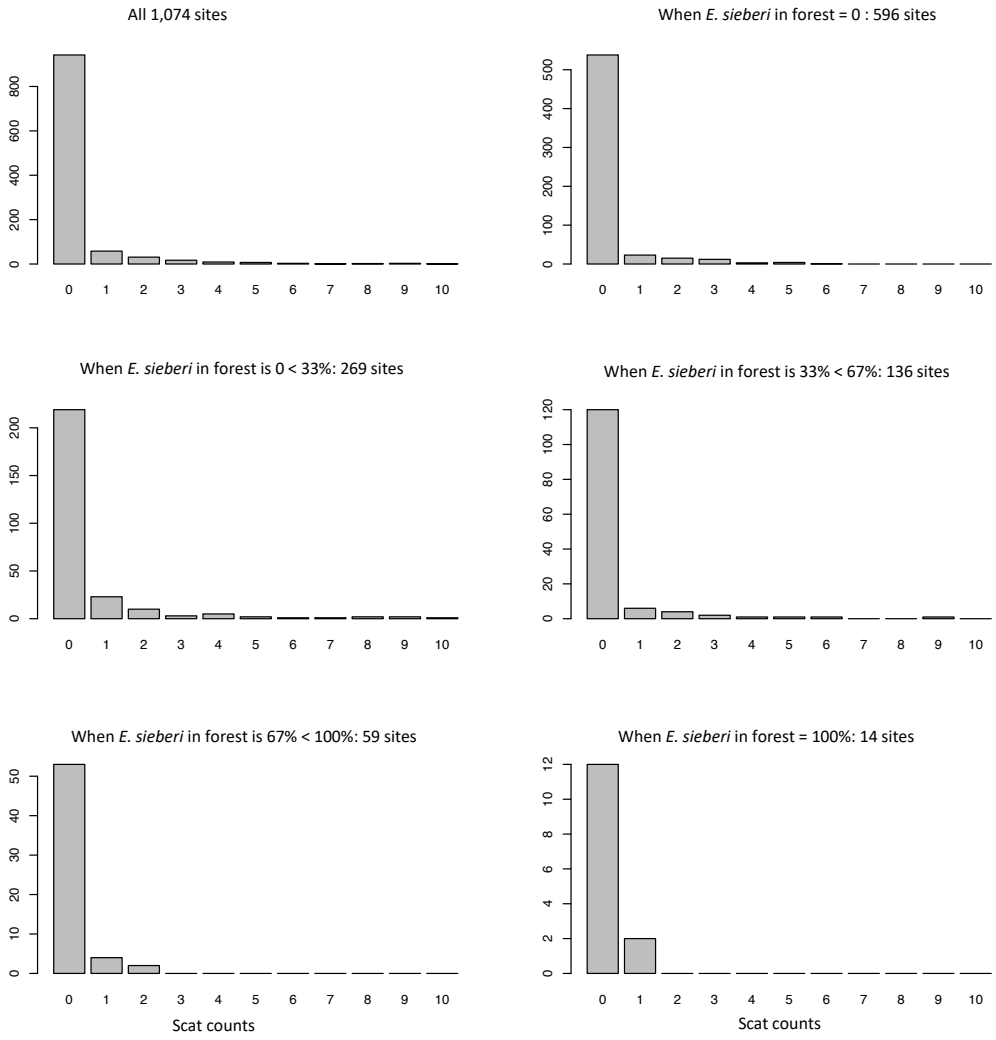

Figure 3: Distribution of scat counts by area (out of a maximum of area) both for all areas, and for subsets of areas based on the proportion of their trees that were *E. sieberi*.

Table 1. Results of fitted negative binomial model. All models contained two covariates, one for the proportion of E. sieberi (between 0 and 1), and an indicator variable set to 1 when E. sieberi was exactly equal to zero.

Effect	Estimate	Standard Error	Relative Risk	*P*-value
Intercept	-0.3133	0.2638		0.235
E. sieberi absent from site	-1.2328	0.3070	0.291	<0.001
Percentage of *E. sieberi*	-0.0196	0.0064	0.981	0.002

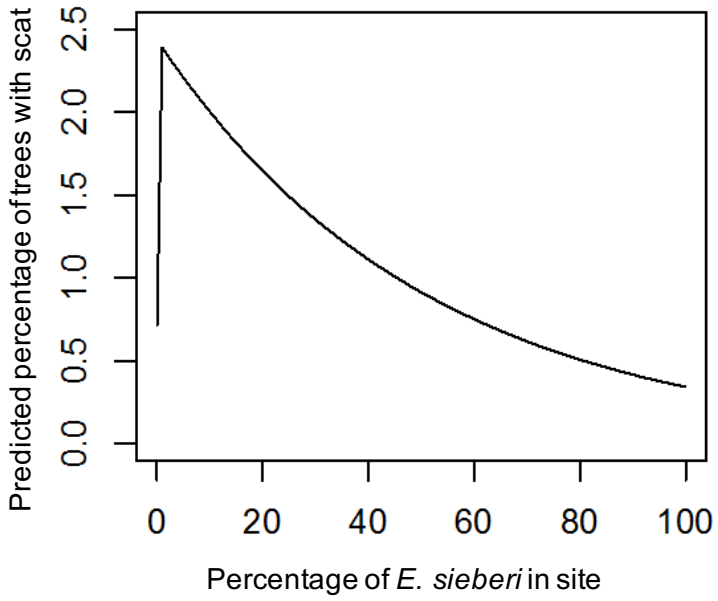

Figure 4. Model predicted values of the percentage of trees with scat present given the proportion of *E. sieberi* trees in a site

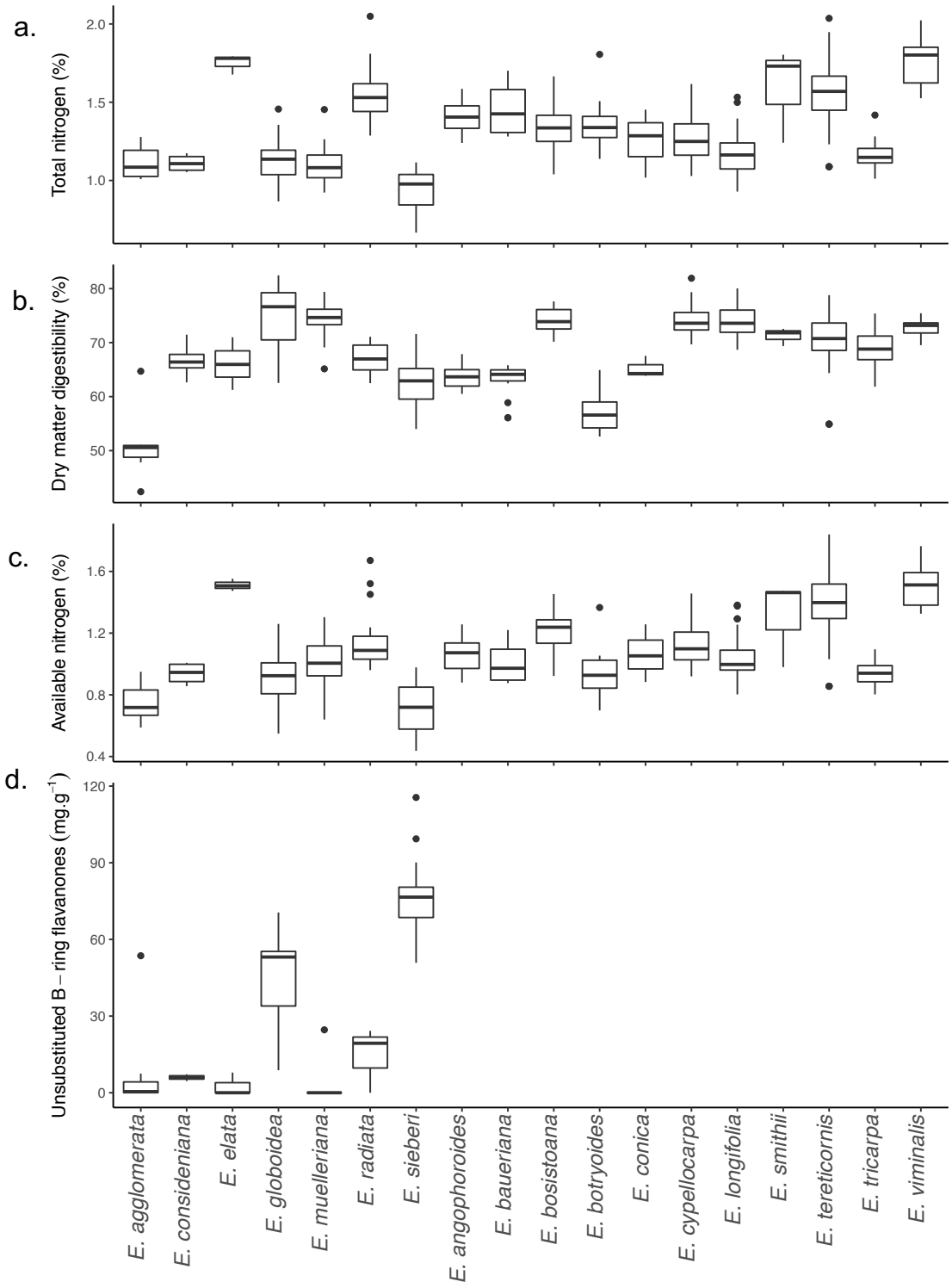

Figure 5. Box and whisker plots showing the concentrations of a) available nitrogen, b) dry matter digestibility, c) total nitrogen, and d) unsubstituted B-ring flavanones in eucalypt species in the Bermagui - Mumbulla area

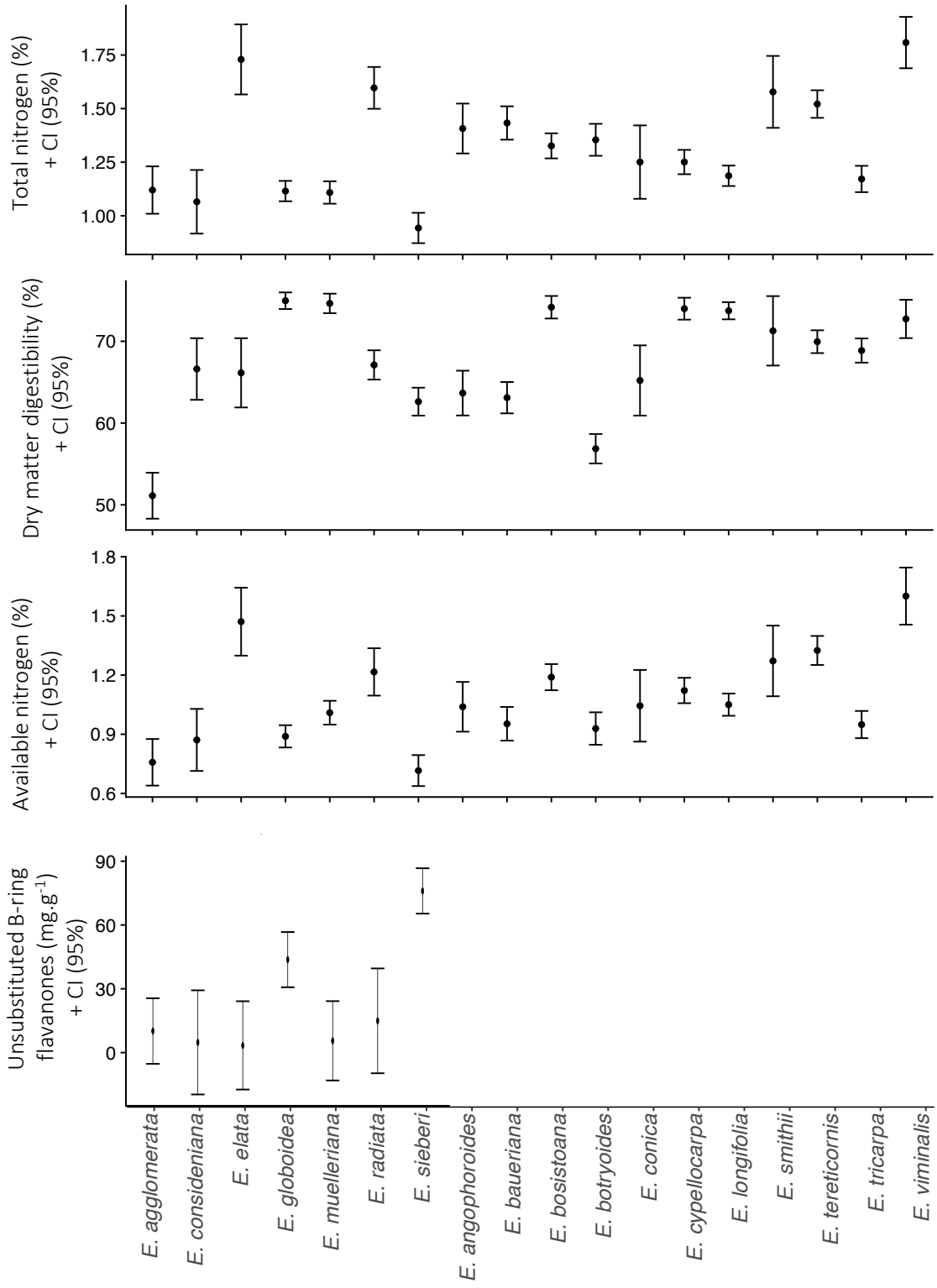

Figure 6. Estimated marginal means of a) total nitrogen, b) dry matter digestibility, c) available nitrogen and d) unsubstituted B-ring flavanones. Error bars indicate 95% confidence interval.

Forage quality across eucalypt species sampled from the study area

We observed clear differences in forage quality across eucalypt species in the study area (Fig. 5, Fig 6). We found that *E. sieberi* and *E. agglomerata* to be the poorest quality species as they contained the lowest mean concentrations of N_A, N_T and DMD (Fig. 5, Fig 6). While the mean N_T for *E. agglomerata* was moderate amongst the tree species (N_T = 1.05 %), it had the poorest digestibility and consequently a low mean N_A (N_A = 0.72 %). We found the lowest mean concentrations of N_T and N_A in *E. sieberi* (mean = 0.70% and 0.94 respectively). Furthermore, *E. sieberi* was the least nutritious eucalypt species as it had a much smaller range and lowest minimum concentration of N_A and N_T than *E. agglomerata* (range in N_T = 0.7 - 1.1%, range in N_A =0.4 - 0.9%). In addition, we found that *E. sieberi* had the highest concentrations of UBF (range = 50 – 120, mean = 75 $mg.g^{-1}$). Pairwise comparisons showed that *E. sieberi* had significantly lower concentrations of N_T, N_A and DMD than all other eucalypt species, except for *E. agglomerata* and *E. condensiana* (Table 2). Furthermore, we found that *E. sieberi* had significantly higher concentrations of UBFs than any eucalypt species in the study area (Table 2).

Table 2. Results of pairwise comparisons of eucalypt species. P values were calculated using the Bonferroni method for total nitrogen, dry matter digestibility and available nitrogen (17 comparisons), and unsubstituted B-ring flavanones (7 comparisons).

Contrast	Nutritional trait	estimate	SE	df	t.ratio	*P* value adjusted^
E. sieberi - E. agglomerata	Total nitrogen	-0.18	0.07	368.88	-2.70	0.124
E. sieberi - E. consideniana		-0.12	0.08	366.07	-1.48	2.383
E. sieberi - E. elata		-0.79	0.09	376.99	-8.77	***
E. sieberi - E. globoidea		-0.17	0.04	311.91	-4.25	***
E. sieberi - E. muelleriana		-0.17	0.04	342.32	-3.97	**
E. sieberi - E. radiata		-0.65	0.06	49.63	-11.09	***
E. sieberi - E. angophoroides		-0.46	0.07	278.07	-6.90	***
E. sieberi - E. baueriana		-0.49	0.05	338.44	-9.44	***
E. sieberi - E. bosistoana		-0.38	0.04	315.24	-8.53	***
E. sieberi - E. botryoides		-0.41	0.05	376.96	-8.58	***
E. sieberi - E. conica		-0.31	0.09	360.18	-3.31	*
E. sieberi - E. cypellocarpa		-0.31	0.04	363.79	-7.21	***
E. sieberi - E. longifolia		-0.24	0.04	343.53	-6.06	***
E. sieberi - E. smithii		-0.63	0.09	350.71	-6.91	***
E. sieberi - E. tereticornis		-0.58	0.05	207.16	-12.29	***
E. sieberi - E. tricarpa		-0.23	0.04	362.95	-5.16	***
E. sieberi - E. viminalis		-0.87	0.07	53.00	-12.56	***
E. sieberi - E. agglomerata	Dry matter digestibility	-0.04	0.07	376.31	-0.61	9.201
E. sieberi - E. consideniana		-0.16	0.09	376.58	-1.79	1.259
E. sieberi - E. elata		-0.76	0.09	375.52	-8.04	***
E. sieberi - E. globoidea		-0.17	0.04	348.72	-4.03	**
E. sieberi - E. muelleriana		-0.29	0.04	362.92	-6.63	***
E. sieberi - E. radiata		-0.50	0.07	58.88	-7.18	***
E. sieberi - E. angophoroides		-0.32	0.07	341.58	-4.51	***
E. sieberi - E. baueriana		-0.24	0.05	365.66	-4.32	***
E. sieberi - E. bosistoana		-0.47	0.05	347.11	-9.90	***
E. sieberi - E. botryoides		-0.21	0.05	376.00	-4.24	***
E. sieberi - E. conica		-0.33	0.10	374.66	-3.36	*
E. sieberi - E. cypellocarpa		-0.41	0.04	374.29	-9.03	***
E. sieberi - E. longifolia		-0.33	0.04	367.59	-7.87	***
E. sieberi - E. smithii		-0.56	0.10	363.64	-5.70	***
E. sieberi - E. tereticornis		-0.61	0.05	291.25	-12.02	***
E. sieberi - E. tricarpa		-0.23	0.05	374.00	-5.00	***
E. sieberi - E. viminalis		-0.88	0.08	61.96	-10.91	***

^ *P* value adjusted for multiple comparisons using the Bonferroni method

* *P* <.05; ** *P* <.01; *** *P* <.001

Contrast	Nutritional trait	estimate	SE	df	t.ratio	*P* value adjusted^
E. sieberi - E. agglomerata	Available nitrogen	-0.04	0.07	376.31	-0.61	3.789
E. sieberi - E. consideniana		-0.16	0.09	376.58	-1.79	0.519
E. sieberi - E. elata		-0.76	0.09	375.52	-8.04	***
E. sieberi - E. globoidea		-0.17	0.04	348.72	-4.03	**
E. sieberi - E. muelleriana		-0.29	0.04	362.92	-6.63	***
E. sieberi - E. radiata		-0.50	0.07	58.88	-7.18	***
E. sieberi - E. angophoroides		-0.32	0.07	341.58	-4.51	***
E. sieberi - E. baueriana		-0.24	0.05	365.66	-4.32	***
E. sieberi - E. bosistoana		-0.47	0.05	347.11	-9.90	***
E. sieberi - E. botryoides		-0.21	0.05	376.00	-4.24	***
E. sieberi - E. conica		-0.33	0.10	374.66	-3.36	*
E. sieberi - E. cypellocarpa		-0.41	0.04	374.29	-9.03	***
E. sieberi - E. longifolia		-0.33	0.04	367.59	-7.87	***
E. sieberi - E. smithii		-0.56	0.10	363.64	-5.70	***
E. sieberi - E. tereticornis		-0.61	0.05	291.25	-12.02	***
E. sieberi - E. tricarpa		-0.23	0.05	374.00	-5.00	***
E. sieberi - E. viminalis		-0.88	0.08	61.96	-10.91	***
E. sieberi - E. agglomerata	Unsubstituted B-ring flavanone	-0.04	0.07	376.31	-0.61	***
E. sieberi - E. consideniana		-0.16	0.09	376.58	-1.79	***
E. sieberi - E. elata		-0.76	0.09	375.52	-8.04	***
E. sieberi - E. globoidea		-0.17	0.04	348.72	-4.03	***
E. sieberi - E. muelleriana		-0.29	0.04	362.92	-6.63	***
E. sieberi - E. radiata		-0.50	0.07	58.88	-7.18	***

^ *P* value adjusted for multiple comparisons using the Bonferroni method

* *P* <.05; ** *P* <.01; *** *P* <.001

Simulating the effects of increasing the proportion of *E. sieberi* on forage quality

Simulations of forests with increasing proportions of *E. sieberi* demonstrated the negative influence of *E. sieberi* on the N_A of the forest (Fig. 7). With increasing percentage of *E. sieberi*, we found a decrease in N_A of the forest. With 0 to 10% *E. sieberi*, the mean N_A of the forest hovered around 1%, ranging from 0.6 – 1.8 % N_A. As we increased the percentage of *E. sieberi*, the mean N_A and range of N_A dropped. Forests with 80 – 90% *E. sieberi* had a mean around 0.8 – 0.9% and range of 0.4 – 1.6 % N_A. Finally, as we increased the percentage of *E sieberi* to 100%, the mean and range decreased to 0.7; 0.3 – 1.2% N_A. We found that forests dominated by *E. sieberi* had the poorest food quality for koalas.

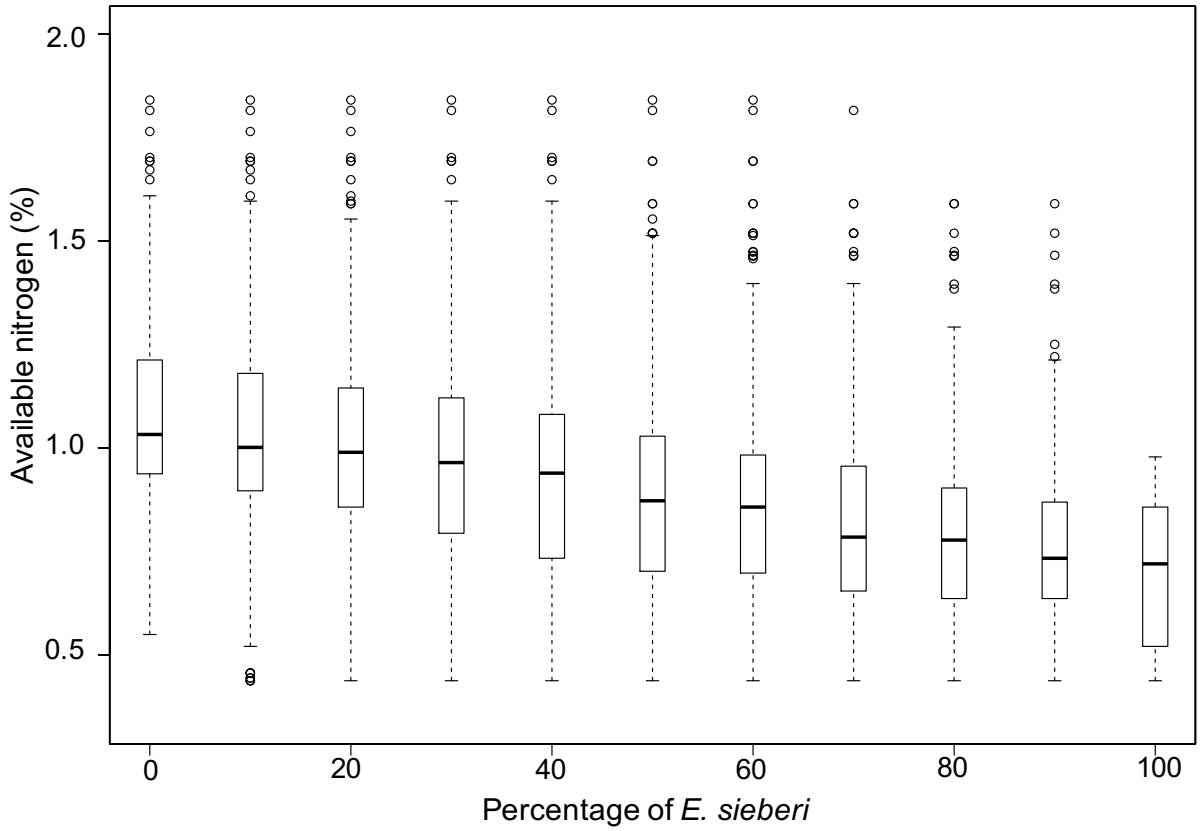

Figure 7. Results of the simulated distribution of available nitrogen with increasing percentage of *E. sieberi* in a forest.

Discussion

Anthropogenic landscape change can alter the composition of forest tree species, this study demonstrated that this can impact forage quality for browsing folivores. *Eucalyptus sieberi* is a disturbance-adapted species that can increase in abundance relative to other sympatric eucalypt species in disturbed forests. We found that *E. sieberi* had substantially poorer nutritional quality for koalas reflected in protein availability (N_A) and foliar concentrations of (UBFs) than any of the other eucalypt species that we sampled in the study area. The probability of koalas occurring decreased significantly as the proportion of *E. sieberi* in a site increased beyond zero. Models that simulated the impacts of increasing the proportion of *E. sieberi* relative to other eucalypt species demonstrated a mechanism by which changes in the composition of tree species can decrease food quality and therefore habitat quality for an arboreal folivore.

Changing individual plant species in a landscape can alter forage quality, because forage quality has a large component of species specificity. While there is much intraspecific chemical variation, researchers have shown that interspecific differences can describe feeding preferences of plants to herbivores. The chemical differences between eucalypt subgenera have helped explain how the co-existence of arboreal marsupials in Australian landscapes (Jensen et al. 2014). Further, in Bolivia almost 50% of the daily macronutrient intake of spider monkeys was derived from timber tree species (largely from figs) that were of harvestable size (Felton et al. 2010). Given the importance of specific forage tree species for animals, changes in forest composition from disturbance might reduce the overall nutritional return from the residual community.

Despite the paucity of research on the impacts of tree species composition on forage quality for arboreal folivores, several studies have compared forage quality between early successional forests and old growth forests for browsing ungulates (Happe et al. 1990; Ford et al. 1994), and small mammals (Carey and Johnson 1995). Happe et al. (1990) found lower concentrations of crude protein and higher concentrations of tannins in early successional forests than old growth forests, indicating that mature forests had greater

nutritional quality for elk and deer (Happe et al. 1990). In contrast, Ford et al. (1994) found no difference in the concentration of tannins between early successional and old-growth forest browse; however, the quantity of browse was greater in early successional forests, and deer tended to forage more in these areas.

The increase in browse availability in early successional forests is reported in several studies and is partially a result of increased light penetration that promotes more vigorous understory vegetation growth (Hart et al. 2006). In some cases, this can also increase the diversity of forage species, resulting in more available food resources for a larger variety of browsers (Isabirye-basuta and Kasenene 1987; Nummelin 1990; Johnson et al. 1995; Mammides et al. 2008; Greenberg et al. 2011). Early successional species also tend to dedicate more energy to fast growth than to chemical defenses (Reich et al. 1995). As a result, early successional plants tend to be more palatable to herbivores once they become established (Cates and Orians 1975).

Eucalyptus sieberi is relatively fast growing and proliferates after disturbance, often suppressing tree regeneration through direct competition with seedlings and saplings, which is a quality of many early successional species (Kasenene 1987). However, in other ways, *E. sieberi* does not fit the definition of an early successional species either physiologically or functionally. Although, we do not know how foliar nutrition and chemical defenses in *E. sieberi* may differ with age, we found that *E. sieberi* leaves from trees with a DBH of 15cm or greater had high foliar concentrations of UBFs and low N_A compared to the other eucalypt species in the study area (Fig. 5). *Eucalyptus sieberi* is also a relatively long-lived tree (up to 160 years) and a common canopy species in mature, mixed-species forests (Keith and Sanders 1990). In some parts of the landscape that have experienced severe and/or frequent disturbance, *E. sieberi* can also comprise a near monoculture of mature forest by outcompeting other canopy species (Florence 1996).

Eucalyptus sieberi has lignotubers that promote its ability to coppice (i.e., regenerate from a remaining portion of trunk). Coppice growth has the advantage of using an existing root system, which usually results in faster growth than new seedlings and allows coppice growth to outcompete seedlings for nutrient and light resources (Smith 1962). Logging can lead to exposure, drying, and erosion of soil, and in these poor site conditions, *E. sieberi* has

been reported to develop especially vigorous coppice shoots (Forrester et al. 2003). In addition, *E. sieberi* seeds are particularly resilient to periods of drying and exposure (Gibson and Bachelard 1986). The propensity for coppice growth and a resilient seedbank, particularly in exposed and poor soil environments, can give *E. sieberi* a competitive advantage in disturbed forest landscapes. Once mature *E. sieberi* dominated forests have become established, it is not clear whether the other eucalypt species that once co-occurred in those areas can ever return in similar proportions without human intervention. Given *E. sieberi's* poor nutritional quality, a forest dominated by *E. sieberi* can have potentially severe consequences for many arboreal folivores.

The proliferation of *E. sieberi* dominate forests is likely to lead to an increase in unsuitable habitat for folivorous species that are sensitive to UBFs and require higher foliar concentrations of protein than *E. sieberi* can provide. The koala maintains nitrogen balance on foliage containing approximately 1% N_T (Cork 1986). Given that *E. sieberi* has the lowest mean N_T (mean = 0.93% N_T) of any eucalypt species in the study area, koalas are unlikely to meet daily nitrogen requirements living in *E. sieberi* - dominated environments. Moreover, we simulated the effects of increasing the proportion of *E. sieberi* in a forest and found a reduction in mean N_A from 1.0% to 0.7% as the percentage of *E. sieberi* increased from 0 to 100% of total eucalypt species. This can be seen as a 30% reduction in food quality, which is likely to have a substantial negative effect on the survival of eucalypt specialists who live in a fine-nutrient balance (Hume 1999). For example, Degabriel et al. (2009) found that a difference of just 0.20% N_A in eucalypt foliage decreased breeding success from 100% to 20% in female brushtail possums. The high concentrations of UBFs further exacerbate the poor nutritional quality of *E. sieberi* for symphyomyrtle specialists, like the koala (Fig. 5). The mean concentration of UBFs in *E. sieberi* from our study area exceeded those known to deter other symphyomyrtle specialists, such as the brushtail possum (Marsh et al. 2015). The presence of UBFs may help explain why some folivores prefer symphyomyrtle species over monocalypts (Marsh et al. 2015). However, further research is needed to obtain a more complete understanding of how UBFs affect the feeding and physiology of other symphyomyrtle specialists, like the koala.

A reduction in forage quality is particularly alarming for vulnerable animal populations. Koalas were once common across the Bermagui – Mumbulla area, but they now only occur in this area at very low densities, with an estimated 50-60 koalas across 26,000 hectares (Allen et al. 2010, 2016). Previous studies found that the nutritional quality of the remaining forests in our study area were much lower than other parts of the koala's range, and this has likely played a role in limiting population densities (Stalenberg et al. 2014). It is notable that even in a low-density area (only 133 of 1074 sites in our study area had koala scat), the negative impact of *E. sieberi* on koala presence was still significant. We would expect an even larger observable effect of decreasing forage quality in areas with higher koala population densities.

Although forage quality for the koala decreased with increasing proportion of *E. sieberi*, we also found that forests without any *E. sieberi* were less likely to be occupied by koalas. It is not clear why this was the case. *Eucalyptus sieberi* is a naturally occurring species in mixed-species forest throughout this area and an absence of *E. sieberi* might be indicative of some other environmental condition that is not favorable to the koala. We looked at potential differences between forests with no *E. sieberi* present and forests with at least some *E. sieberi* in terms of elevation, slope, and underlying geology, and found no obvious differences between areas. Given *E. sieberi*'s proliferation after disturbance, a lack of *E. sieberi* might be indicative of long-undisturbed forests. However, it is not clear if this is actually the case or if it is, why such landscapes might not be suitable for koalas. Further research in this area is required.

Another important caveat is that we did not sample *E. sieberi* across its entire range, and like all eucalypt species, it may show variability in foliar concentrations of nutrients and PSMs (Eschler et al. 2000; Moore et al. 2004b; Bustos-Segura et al. 2017). There could be some populations of *E. sieberi* that are more palatable and provide a better diet for koalas than the *E. sieberi* that we sampled for this study. However, Marsh et al. (unpublished data) investigated the range of UBFs in eucalypts and found that *E. sieberi* collected from other areas of NSW had the highest concentrations of UBFs than any of the other 20 monocalypt species. They also found relatively low concentrations of N_A in their *E. sieberi* samples (Marsh et al. unpublished data). Moreover, a vegetation survey in the Eden area

demonstrated that *E. sieberi* had the lowest concentrations of N_T among 14 eucalypt species (Lambert and Turner 1983). In addition, anecdotal reports of forests dominated by *E. sieberi* appearing to be "biological wastelands" or strangely devoid of activity have been widely reported by other field ecologists in regard to the presence and abundance of brushtail possums, greater gliders, and koalas (personal communications. David Scott, David Lindenmayer, Philip Gibbons, and Chris Allen).

Management implications

Although research that investigates the relationship between tree species composition and forage quality for arboreal folivores is rare, that does not mean that this pattern is not more widespread. Where it occurs, changes in tree species composition that negatively impact forage quality should be considered a threatening process. Unfortunately, considerations of forage quality in evaluations of habitat quality are often overlooked, particularly for arboreal folivores. As a result, actions that could target the specific issues that are negatively impacting habitat quality, such as mitigating the proliferation of a poor-quality forage species, are not undertaken. We recommend that landscape managers take steps to reduce the proliferation of *E. sieberi* in forest landscape where koala conservation is a desired outcome. Further, the effects of changes in tree species composition on forage quality should be a research priority in other areas given the fast rate of landscape change and disturbance as a result of anthropogenic activities and climate change (Lindenmayer et al. 2017).

Assessing forage quality over wide areas is difficult, but becoming increasingly feasible with advances in near-infrared spectrophotometry and remote sensing technology (Youngentob et al. 2012). Near-infrared spectrophotometry allows for the rapid assessment of leaf chemical composition where we can predict the nutritional quality of plants in large datasets based on near-infrared spectra (Foley et al. 1998). In addition, airborne imaging spectrometers have made it possible to measure plant chemical composition and plant productivity across landscapes remotely (Youngentob et al. 2015). However, imaging spectrometry, and particularly higher resolution airborne data, is still limited in by its costs, accessibility, and large data processing requirements. Additional resources and research should focus on making these technologies more widely available.

It is important to note that *E. sieberi* has been previously documented as a food species for the koala based primarily on evidence of koala scat under *E. sieberi* trees (Phillips and Callaghan 2000). Our findings suggest that *E. sieberi* could not make up a large or even necessary component of the koala's diet, and areas dominated by this tree species are unable to support koala populations. This highlights the need to be cautious about assigning food status to a tree species based on associations with animal scats or even observations of an animal in a tree without detailed data on actual feeding and forage quality.

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Supplementary material

Supplementary material 1. Summary of tree species in Bermagui - Mumbulla

Tree species	Frequency
Acacia	1916
Acmeni smithii	79
Angophora floribunda	1559
Backhousia myrtlefolia	199
Baeckea virgata	1
Banksia	1
Bedfordia arborescens	1
Brachychiton populneus	6
Casuarina	4284
Corymbia gummifera	266
Corymbia maculata	721
Doryphora sassafras	15
Elaeocarpus reticulatus	3
Eucalyptus agglomerata	1675
Eucalyptus angopheroides	2
Eucalyptus baueriana	97
Eucalyptus bosistoana	803
Eucalyptus botryoides	655
Eucalyptus bridgesiana	7
Eucalyptus consideniana	67
Eucalyptus cypellocarpa	1326
Eucalyptus elata	341
Eucalyptus eugenoides	5
Eucalyptus fastigata	3
Eucalyptus globoidea	2656
Eucalyptus longifolia	3739
Eucalyptus macroryncha	9
Eucalyptus muelleriana	5803
Eucalyptus paniculata	140
Eucalyptus pilularis	2
Eucalyptus radiata	92
Eucalyptus sideroxylon subspecies *tricarpa*	949
Eucalyptus sieberi	3694
Eucalyptus smithii	526
Eucalyptus species unidentified	5
Eucalyptus tereticornis	27
Eucalyptus viminalis	1
Exocarpus cuppressiformis	154
Hakea eriantha	2
Hakea macraeana	76
Hakia species	1
Melaleuca armillaris	8
Melia azedarach	3
Notalea venosa	4
Unidentified	6
Pittosporum	36
Polyscias murrayi	4
Pomaderris	4
Prosanthera	2
Rainforest species	26
Rapanea howittiana	8
Tristaniopsis laurina	28
Unidentified stringybark	197

Supplementary material 2. Summary of calibration and validation statistics for predicting available nitrogen, dry matter digestibility, total nitrogen and formylated phloroglucinol compounds. Partial least squares regression was used to develop the calibration models and models were assessed using the root mean square error of cross validation (RMSECV) and root mean square error of prediction (RMSEP).

Nutritional trait	Units	Range	Mean	*N* calibration	PLS factors	RMSECV	*N* validation	RMSEP
Total nitrogen	%	0.67 - 2.92	1.34	987	9	0.08	37	0.13
Dry matter digestibility	%	34 - 85	63	987	11	5.2	37	20.3
Available nitrogen	%	0.20 - 2.40	1.03	987	5	0.15	37	0.23
Unsubstituted B-ring flavanones	mg,g^{-1}	0 - 146	24	220	16	4.2	30	7.0

Chapter Four

Bark chewing reveals a nutrient limitation of leaves for a specialist folivore

AU, J. [1], YOUNGENTOB, K. N. [1], CLARK, R. G. [2], PHILLIPS, R. [3], FOLEY, W. J. [1], 2017. Bark chewing reveals a nutrient limitation of leaves for a specialist folivore. Journal of Mammalogy. 98:1185-92.

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Keywords: *Eucalyptus*, mammalian herbivore, matched case-control, micronutrients, sodium.

Abstract

Mineral micronutrients are critical for basic physiological function, and variable availability of minerals over the landscape can influence foraging decisions. Sodium is essential for nerve function and osmotic balance, however, it can be limiting in some environments, such as those at high elevations. Koalas (*Phascolarctos cinereus*) living in subalpine regions have been observed eating *Eucalyptus mannifera* bark, an unusual food choice for a folivore. We hypothesized that sodium may be deficient in leaves at high elevation sites, and that the bark from trees could be a potential sodium source. We compared the mineral content of eucalypt bark and leaves in three areas where koalas chew bark and in leaves of a preferred food tree of koalas, *E. viminalis*, across a range of elevations. Individual chewed trees were rare compared to non-chewed conspecifics and patchily distributed. Bark from chewed *E. mannifera* trees had significantly higher concentrations of sodium than plant parts from non-chewed neighboring conspecifics and other *Eucalyptus* species trees. We also found that *E. viminalis* foliage had significantly less sodium at higher elevations than lowland populations. We propose that koalas have developed the unusual bark-eating behavior to meet sodium requirements in an otherwise sodium-poor landscape. Given the physiological importance of sodium, behavioral adaptations such as these may be critical to the ability of mammalian herbivores to survive in nutrient-deficient landscapes.

Introduction

The concept of food quality for herbivores often focuses on key nutrients, such as protein, and plant secondary metabolites (PSMs) that are known to influence the feeding decisions of herbivorous species (Dearing et al. 2005; Villalba and Provenza 2009). The patchy distribution of nutrients and plant toxins across the landscape has helped describe patterns of animal movement and reproductive success (Wallis et al. 2012). For example, the abundance of herbivores has been linked to "nutrient hot spots" where vegetation contains relatively high concentrations of protein or other macronutrients (Braithwaite et al. 1983; Wasserman and Chapman 2003). Radio-tracking studies have shown that folivores make decisions about which trees to feed on that are linked to integrated measures of forage quality that consider both foliar nitrogen (as a proxy for protein) and the widespread class of PSMs, tannins (Youngentob et al. 2011). While the importance of macronutrients and PSMs in explaining foraging decisions in herbivores is well studied, the role of micronutrients (e.g., minerals) is less understood.

Plants can be imbalanced as a source of minerals for animals. Indeed, herbivores are known to seek minerals from non-vegetative sources, such as soil, to meet important nutritional requirements (Tracy and McNaughton 1995; Best et al. 2013). Less commonly, some herbivores are able to exploit higher concentrations of minerals in refractory plant parts, such as wood or bark. In Uganda, unusual behaviors such as gorillas eating decayed wood (Rothman et al. 2006) and colobus monkeys chewing the bark of some *Eucalyptus* species (Rode et al. 2003) were linked to their richness as sodium sources. Masaki et al. (2004) reported that sika deer strip bark from coniferous trees in summer to meet elevated calcium demands during lactation. It is likely that many generalist herbivores meet nutritional demands by consuming unusual items, such as bark or soil (Stephens et al. 2006; Best et al. 2013). However, this behavior has rarely been observed in specialist folivores that survive almost exclusively on a diet of plant leaves.

The koala (*Phascolarctos cinereus*) is a specialist folivore that feeds primarily on leaves from

the genus *Eucalyptus* (Martin and Handasyde 1999). This animal has an extensive distribution across Australia, ranging from Townsville in northern Queensland to the Adelaide ranges in South Australia and various off-shore islands. Research into foraging behavior of koalas has focused on the effects of a few key foliar nutrients and a group of toxic PSMs called formylated phloroglucinol compounds (FPCs; Lawler et al. 1998; Marsh et al. 2013). Both captive and free-ranging koalas prefer to eat leaves containing high concentrations of available nitrogen and low concentrations of FPCs (Lawler et al. 1998; Marsh et al. 2013). In contrast, the role of micronutrients for koalas has been largely ignored (but see McOrist and Thomas 1984).

Recently, unusual scarring has been observed on *E. mannifera* trees in the subalpine Monaro region of New South Wales (NSW; Fig. 1a-c). These remained a mystery until infrared video captured images of koalas scraping bark from the bole of the tree with their teeth (video provided in Supplementary Data S1). These unique chew marks appear on the bark as paired parallel scars, 3-6 mm wide, 1-3 mm thick, 4-6 cm in length and often angled at 40 degrees to the horizontal (Fig. 1b). Since the marks do not penetrate the cambium or the sapwood, we assume that they are made for bark consumption and not for sap-feeding. In addition, no bark chips were found at the base of scarred trees and thus it is likely the bark is consumed by the animal and not just stripped from the tree. Bark chewing has never been observed in other populations of koalas despite extensive and long-term research on this species by numerous researchers across the animal's range (for a list of researchers with whom we have spoken, see Youngentob 2014).

Figure 1. Images of chew scars on Eucalyptus mannifera with (a) fresh chew scars (b) a tree with a few older chew scars and (c) a heavily scarred tree with old chew marks.

The Monaro region includes some of the highest-elevation sites at which koalas are known to occur (900 m – 1,300 m). This area also is well known to be a nutrient-deficient environment in terms of phosphorus, potassium, and sulfur (Costin 1954). Given its elevation and that it lies in a rain shadow of the Australian Alps, it is likely that sodium also would be poorly available in the Monaro (Jenkins and Morand 2002). Previous research in alpine and subalpine environments of southeastern Australia has demonstrated that herbivores are sodium-deficient in these landscapes (Blair-West et al. 1968; Myers 1970). Several experimental studies showed that a wide range of native and introduced herbivores have a pronounced appetite for sodium and will avidly chew on wooden stakes that have been soaked in sodium chloride and sodium bicarbonate in spring and early summer (Blair-West et al. 1968). Similar stakes soaked in potassium or magnesium chloride were largely ignored. Myers (1970) observed histological changes in the adrenal gland of rabbits at these sites that result in increased plasma aldosterone that would help resorb sodium in the kidney.

We hypothesize that eucalypt bark provides koalas in the Monaro with a source of sodium. Eucalypt bark also may provide other essential micronutrients such as calcium, which has been shown to be an important component of bark forage for other mammalian herbivores (Masaki et al. 2004). To test this hypothesis, we conducted a survey of mineral concentrations (calcium [Ca], copper [Cu], iron [Fe], potassium [K], magnesium [Mg], manganese [Mn], sodium [Na], phosphorus [P], zinc [Zn]) in the bark and leaves of chewed trees and their unchewed neighbors in 3 sites across the Monaro. We analyzed the mineral content of both leaves and bark to understand if bark provides a mineral source that may otherwise be limited in the koala's typical diet of foliage. We also investigated whether there were differences in the foliar sodium concentrations of a favored food species, *E. viminalis* (Marsh et al. 2013), collected from a variety of elevations in NSW, Victoria, and South Australia. Analyzing the sodium content in leaves from a variety of elevations, including the Monaro, would help identify whether these subalpine trees have less foliar sodium than their lowland conspecifics, as the existing literature suggests (Blair-West et al. 1968). This is the first time that sodium and other micronutrients have been investigated on a landscape scale in relation to the foraging behavior of folivores in Australia.

Materials and Methods

Study site

We investigated bark and leaves of eucalypt trees in 3 forest sites in the Monaro region of New South Wales, Australia: Black Ridge, Mount Dowling, and Hammershill Wildlife Trust (Fig. 2). The elevation at each of the 3 sites ranged from 800 m – 1,000 m, 1,000 m – 1,100 m, and 800 m – 1,100 m, respectively. Large-scale tree clearing over the past 100 years has converted much of the native woodland to pasture, but remnants and large patches of native dry sclerophyll forest remain. The dominant tree species include *E. mannifera*, *E. dives*, *E. rossii*, and *E. macrorhyncha* with an understory of *Acacia* species (Martin and Phillips 2015).

The sites lie on yellow podsolic and grey-brown podsolic soils (Costin 1954; Martin and Phillips 2015) and these soil types are poorly aerated and typically low in fertility (Stace 1968). The temperatures in summer range from 9°C to 27°C, and in winter from -3°C to 13°C (mean max and mean min, Bureau of Meteorology 2016). The average annual rainfall is about 550 mm with a pronounced peak in summer (December to February, Bureau of Meteorology 2016).

Sample collection

Chew marks are not found on every *E. mannifera* tree but in clusters ranging from 2 to 10 trees within close proximity. From here on, these clusters will be referred to as a "chew patch." We visited each site to locate chew patches. Some chew patches were known to property owners and managers prior to the study and others were found during extensive foot traverses of the sites at the start of the project. We aimed to sample bark at the time of year that it was chewed. Anecdotally, this occurred in spring and so we checked chew patches at each of the study sites from July to October 2015 until fresh chew marks were observed early October 2015. Fresh chew marks have a distinct yellow color with a pale green edge (Fig. 1a) and over time they darken to a red-brown color (Fig. 1b, c).

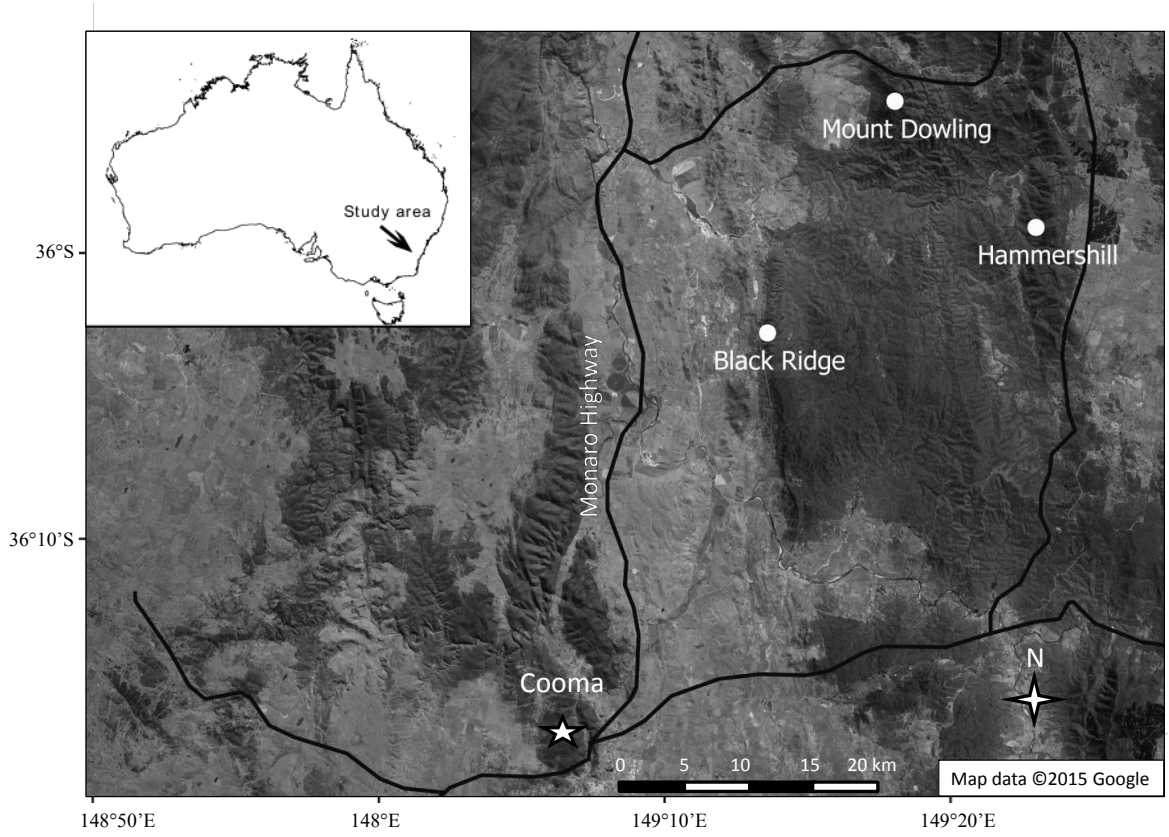

Figure 2. Map of study sites in the Monaro region, New South Wales.

Approximately 1 g of bark and 5 g of mature leaf were collected from each tree for analysis of minerals. Chew marks occur on the outermost layer of the bark, so samples of bark (1 mm to 3 mm deep) and between 50 and 100 cm above ground were collected with a knife adjacent to existing chew marks. Samples from unchewed trees were collected at the same height above the ground as their chewed neighbor but on a randomly selected side of the tree. Leaf samples were collected using either a telescopic pruning pole or an arborist throw-line launcher. Samples were then air-dried upon arrival at the Australian National University before being dried to a constant mass in a forced draft oven at 60°C. Samples were then ground to a fine powder using a GyralGrinder® Puck and Ring Grinder (Chemplex Industries, Palm City, Florida).

Additional eucalypt leaf samples across a range of elevations

The dominant eucalypt tree species in our study area do not occur at low elevations; however, one of the less prevalent species at our sites, *E. viminalis*, does occur across a large gradient of elevations through parts of South Australia, Victoria, and New South Wales. *Eucalyptus viminalis* is also a favored food tree of the koala (Marsh et al. 2013). We used *E. viminalis* leaf samples that were collected for 2 separate, landscape-scale studies of forage quality for marsupial folivores (Youngentob et al. 2011; Youngentob 2014). The *E. viminalis* samples were collected during the spring and summer months of the southern hemisphere (September - March). This timing also coincides with the period when nutritional demands on koalas are higher due to reproduction and lactation (Martin and Handasyde 1999). We selected 60 *E. viminalis* samples using a stratified approach to incorporate several trees from each of the sites and across the range of elevations included in the studies (Fig. 4, 21 m to 1,000 m). Although these leaf samples were not collected specifically for this study, they provided an acceptable, preliminarily investigation into the relationship between sodium concentrations in eucalypt foliage and elevation. These leaf samples were obtained in the same manner as the leaf samples from our study but were frozen immediately upon collection at -80^{o} C on a bed of solid CO_2 pellets. Samples were then freeze-dried and ground to pass a 1 mm screen using a Tecator Cyclotec Mill (Foss, Hillerød, Denmark). Given how the leaf samples were stored, it is unlikely that there would

have been substantial changes in the foliar concentrations of the measured chemical constituents between the time that the leaf samples were collected for the earlier studies and their additional analysis for sodium and other micronutrients in 2016 (Ratti 2001).

Chemical analyses

The dried, ground samples of bark and leaf for mineral analysis were weighed (100 ± 5 mg of each sample) and placed into individual vessels. We added 8 mL 65% nitric acid and 2 mL 36% hydrochloric acid and digested each sample in a Milestone S.r.l. - Start D - Microwave Digestion System (Milestone Inc, Shelton, Connecticut) using US EPA Method 3051. Mineral concentrations (Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn) were determined using a Varian Vista-Pro Inductively Coupled Plasma-Optical Emission Spectrometer. These 9 minerals were chosen because of their biological relevance to mammals (Morris 1991).

Statistical analyses

We observed different amounts of bark chewing from individual *E. mannifera* trees (e.g., Fig. 1a-c). However, this was difficult to quantify because chew marks were not evenly spread across the trunk. Some trees had chew markings that were heavily concentrated in one area of the trunk, whereas other trees had chew marks spread across the trunk. Thus, we avoided giving trees a relative ranking based on our perceptions of the intensity of use and assigned all trees with at least 1 chew mark to the category, “chewed.” Their unchewed neighbors were assigned to a separate category, “unchewed.”

We developed a logistic regression model to investigate if bark chewing is associated with variations in concentration of minerals or in traits of trees such as DBH and tree height. The design was matched case-control, with each chewed tree paired to the nearest non-chewed tree. To reflect this, the logistic regression model was fitted using the conditional likelihood method described in Heinze and Puhr. (2010), implemented with the CLOGIT function in the Survival package (Therneau 2015) in the R statistical environment (R Core Team 2015). See Gibbons et al. (2008) for an application of matched case-control and the conditional logit method to ecology.

Every possible subset of the 11 covariates (Ca, Cu, Fe, K, Mg, Mn, Na, P, Zn, DBH, and tree height) was fitted and models were ranked using the Akaike Information Criterion (AIC). The significance of the covariates in the final model was assessed using likelihood ratio and permutation tests.

Given the large differences in concentrations of minerals, we plotted the concentrations of sodium in bark of chewed against unchewed *E. mannifera* trees. In addition, we summarized the distributions of the 9 minerals across all 4 eucalypt species in our Monaro study. We used Spearman correlations and associated permutation significance tests to quantify and test the relationship between elevation and foliar sodium concentration in *E. viminalis*, calculated using the cor.test function in the R Statistical Environment. In addition, we used a Mann–Whitney *U*-test to determine if foliar sodium concentrations of sampled trees in the Monaro region were different to those of *E. viminalis* at lower elevations. Data are presented as mean ± 1 standard deviation and range.

Results

Chewed trees

We identified 17 locations (chew patches) with chewed trees across the 3 study sites and traversed approximately 9 km^2 at each site in our search for these patches. Four chew patches were identified at Black Ridge, 6 at Mount Dowling, and 7 at Hammershill. The distance between chew patches ranged from 0.5 km to 9 km. Each chew patch contained 1 to 5 trees with chew marks on the trunk. We found chew scars of various ages on 34 *E. mannifera* trees in total and no chew scars were found on the other eucalypt species in the area. The distance between the chewed *E. mannifera* and their unchewed neighbors ranged from 0.5 m to 3 m.

Relationship between chewed trees and bark mineral concentrations in *Eucalyptus mannifera*

All subset selections from a logistic regression of the trait "chewed or unchewed" resulted in Na as the only covariate in the final model, using the AIC as the criterion. The final model had AIC at least 2.0 lower than all other models. The Na covariate was statistically significant ($P < 0.001$) according to both a likelihood ratio and a permutation test, however the covariate was so powerful that the coefficient estimate was essentially infinite. This can happen in conditional logistic regression, when the distributions of the covariates for the cases (chewed trees) and for the controls (unchewed trees) do not overlap (Heinze and Puhr 2010). The Na concentration for every chewed tree was higher than the corresponding unchewed tree (Fig. 3), with a median ratio for Na for chewed/unchewed trees of 5.2. Effectively, Na is statistically significant but so influential that a finite estimate of its coefficient cannot be calculated.

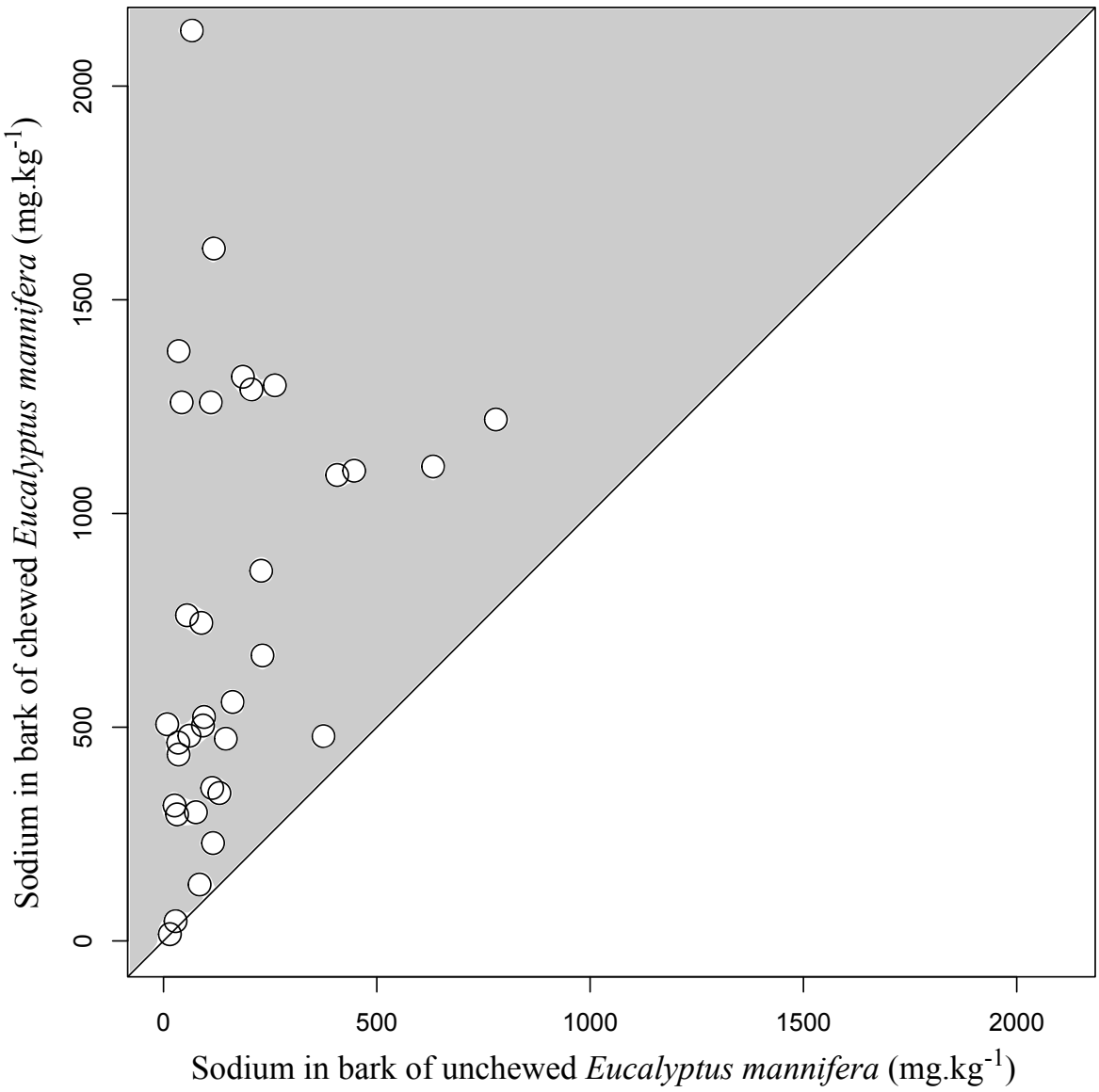

Figure 3. The concentration of sodium in the bark of chewed trees is plotted against corresponding concentrations in unchewed trees for all 34 *Eucalyptus mannifera* pairs. The shaded region represents pairs which have higher sodium concentrations in the bark of chewed than unchewed trees. All 34 pairs reside within this region.

Concentrations of minerals in bark and leaves of all sampled eucalypt species in chew patches

We observed significant variation in concentrations of measured minerals across species and between the bark and leaves (Table 1; also see Supplementary Data S2). Bark from chewed *E. mannifera* trees was higher in Na than the bark or leaves from any of the other sampled eucalypt species in the Monaro (Table 1).

Comparison of minerals in *Eucalyptus viminalis* leaves collected across a range of elevations

We found that concentrations of Na in foliage decreased with increasing elevation (Spearman correlation = -0.77, non-parametric rank-based test, $P < 0.001$, Fig. 4). The lowest Na concentrations were found in *E. viminalis* foliage from the Monaro region (mean 119 ± 114 $mg.kg^{-1}$, range = 16 – 347, $n = 11$) and from the Tumut area of New South Wales (mean 23 ± 16 $mg.kg^{-1}$, range = 5 – 53, $n = 7$), which also were the highest-elevation sites sampled. *Eucalyptus viminalis* foliage at lower elevations (less than 800 m, $n = 42$) had significantly higher concentrations of foliar Na than leaves from any sampled species in the Monaro (Mann – Whitney *U*-test, $P < 0.001$, $n = 102$). Leaves from *E. viminalis* sampled at elevations below 800 m had similar ranges of foliar sodium concentrations (300 $mg.kg^{-1}$ – 2,300 $mg.kg^{-1}$) to that of chewed *E. mannifera* bark in the Monaro region (100 $mg.kg^{-1}$ – 2,130 $mg.kg^{-1}$).

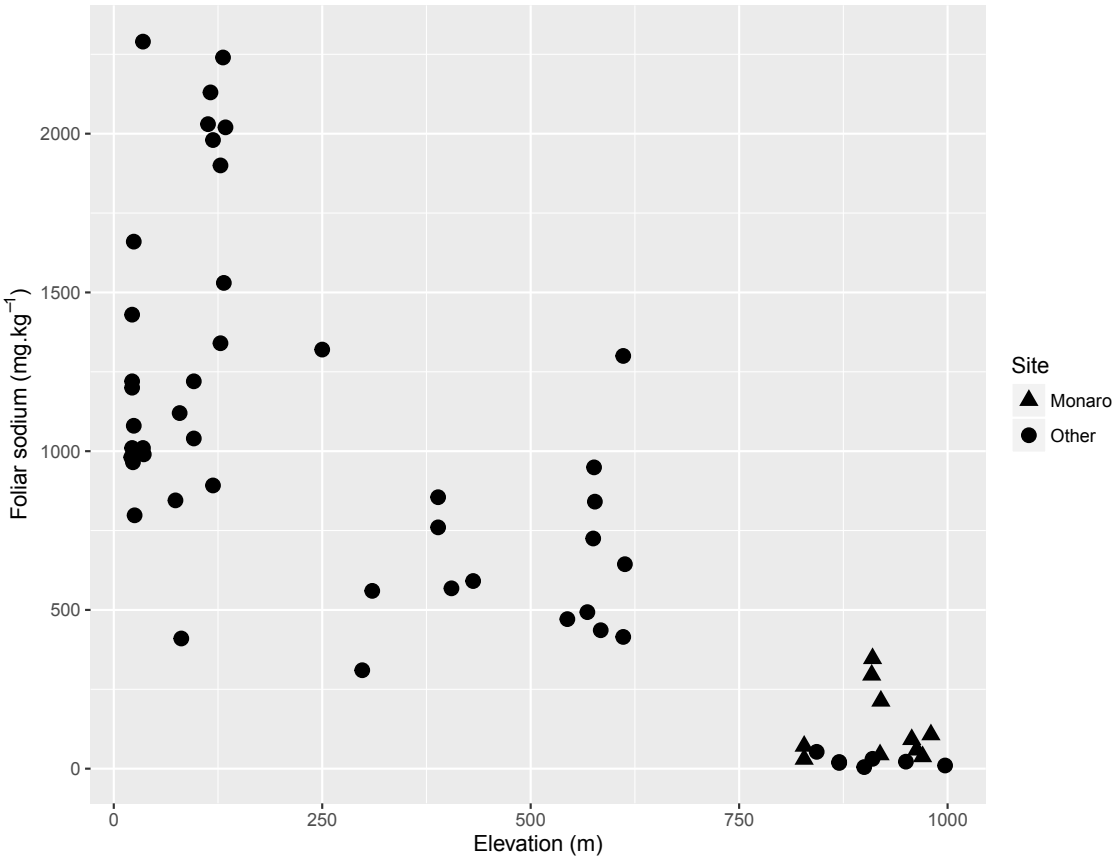

Figure 4. The concentration of sodium in Eucalyptus viminalis foliage (mg.kg-1) at various elevations (m).

Table 1. The concentrations of calcium, copper, iron, potassium, magnesium, manganese, sodium, phosphorus, zinc (mg.kg^{-1}) in eucalypt bark and leaf samples from study sites in the Monaro region of New South Wales. *Eucalyptus mannifera* trees were separated into two groups, one group where the bark of the tree was chewed and one where the bark was unchewed. * indicates *Eucalyptus viminalis* leaf samples from the landscape elevation study.

		Ca (mg.kg-1)		Cu		Fe		K		Mg		Mn		Na		P		Zn	
Plant part	*n*	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median
Eucalyptus dives bark	10	1749 ± 1250	1525	2.7 ± 1.3	2.4	190 ± 93	187	369 ± 92	375	567 ± 347	524	78 ± 62	60	25 ± 17	19	177 ± 76	197	11.1 ± 8.8	8.4
E. macrorhyncha bark	12	315 ± 161	318	1.6 ± 0.9	1.4	176 ± 149	132	238 ± 117	250	114 ± 57	110	17 ± 11	17	29 ± 23	18	72 ± 38	60	5.2 ± 2.5	4.8
E. mannifera bark from chewed trees	34	6748 ± 5931	5120	1.9 ± 0.63	1.9	59 ± 96	32	3678 ± 1300	3340	3653 ± 1017	3570	531 ± 602	377	753 ± 502	542	211 ± 46	213	10.5 ± 5.4	9.2
E. mannifera bark from unchewed trees	34	6001 ± 4850	3975	1.4 ± 0.4	1.3	36 ± 18	31	3907 ± 1440	3680	3721 ± 1399	3450	479 ± 359	337	163 ± 178	103	197 ± 55	190	9.8 ± 5.6	9.6
E. rossii bark	9	2868 ± 1721	3040	1.1 ± 0.7	0.9	27 ± 17	21	1406 ± 637	1470	2456 ± 970	2730	161 ± 86	166	77 ± 98	32	77 ± 35	81	5.6 ± 4.3	5.1
E. dives leaf	10	2815 ± 886	2534	3.3 ± 0.6	3.1	78 ± 44	73	3975 ± 1892	3717	1575 ± 513	1469	375 ± 264	280	12 ± 10	11	546 ± 144	561	11.4 ± 4.8	10.5
E. macrorhyncha leaf	12	3263 ± 557	3179	2.9 ± 0.6	2.7	39 ± 5	38	4239 ± 349	4388	1943 ± 247	1958	301 ± 65	279	19 ± 9	21	636 ± 176	607	7.8 ± 1.5	7.7
E. mannifera leaf from chewed trees	34	5834 ± 2263	5230	3.8 ± 1.2	3.5	66 ± 28	57	5366 ± 1092	5195	1580 ± 397	1515	650 ± 296	656	92 ± 135	25	703 ± 128	688	12.8 ± 6.8	11.4
E. mannifera leaf from unchewed trees	34	6976 ± 2647	6740	4.0 ± 1.1	3.8	67 ± 28	57	4929 ± 911	4875	1667 ± 424	1715	796 ± 379	721	34 ± 50	20	747 ± 170	712	12.5 ± 3.4	12.2
E. rossii leaf	9	2807 ± 880	2903	2.2 ± 0.6	2.3	38 ± 15	31	3246 ± 902	2890	1978 ± 651	2170	348 ± 201	230	20 ± 8	21	328 ± 133	303	8.7 ± 3.9	6.9
E. viminalis (elevation < 800m) *	42	7446 ± 2483	6990	7.8 ± 4.5	6.4	52 ± 17	50	4525 ± 989	4465	419 ± 306	328	1109 ± 544	1000	1363 ± 378	1365	746 ± 197	712	13.4 ± 4.6	12
E. viminalis (elevation > 800m) *	18	7291 ± 3146	6330	4.0 ± 1.2	3.9	50 ± 27	40	4921 ± 1127	4700	478 ± 335	490	82 ± 100	41	1391 ± 545	1370	593 ± 161	601	8.2 ± 2.2	7.3

Discussion

A sodium source: *Eucalyptus mannifera* bark

The high sodium concentrations of bark strongly suggest that koalas in the Monaro region consume this plant part as a sodium source. Sodium was significantly higher in chewed *E. mannifera* bark compared to bark and leaf samples from the other eucalypt species sampled at the 3 chew sites (Table 1). Moreover, we found striking differences in the concentration of sodium within bark and leaf samples of *E. mannifera* (Fig. 3). Sodium was heavily concentrated in the bark of *E. mannifera* but almost absent from the leaves of all sampled species in the area (Table 1). Chewed trees always had higher concentrations of sodium in the bark than their unchewed neighbor.

Sodium is the principal cation for electrolyte balance and nerve function in animals and sodium deficiency can affect growth rate, muscle strength, and reproduction (Morris 1991). Sodium is the only mineral for which animals have an appetite in order to avoid deficiency, which highlights sodium's critical role in maintaining animal health (Denton 1982). The bark chewing behavior of koalas in the Monaro region has parallels with other unusual feeding behaviors in animals that have been linked to sodium acquisition (Belovsky and Jordan 1981; Rothman et al. 2006; Best et al. 2013)

The daily sodium requirement of koalas is unknown. Ullrey et al. (1981) suggest that foliar sodium concentrations of only 300 $mg.kg^{-1}$ in foliage was sufficient to meet requirements of captive zoo animals but actual sodium balance was not measured. Daily intake of leaf by koalas is approximately 200 g $DM.d^{-1}$ for a 7- 8 kg animal (Cork et al. 1983; Marsh et al. 2013). Based on Ullrey's data, koalas may need only 60 $mg.d^{-1}$ of sodium. Although this is low compared to estimates for some other herbivores, animals can maintain balance on sodium-depleted diets at a much lower intake through reabsorbing sodium in the distal nephron via enhanced secretion of aldosterone. Koalas eating 10 g (DM) of sodium-rich bark per day (2,000 $mg.kg^{-1}$) would obtain about the same amount of sodium as they would from consuming 200 g (DM) of foliage with 100 $mg.kg^{-1}$ Na. Therefore, the use of bark as a source of sodium in the Monaro is likely to be critical in these animals meeting their daily sodium requirements.

A range of factors including the capacity of the gut and slow rates of digestion (Cork and Warner 1983), plus the limiting effects of potentially toxic secondary compounds (Lawler et al. 1998), limit the amount of leaf that koalas can eat. Therefore, consuming sufficient sodium from leaf alone would not be possible in a landscape like the Monaro where foliar sodium levels are very low (35 $mg.kg^{-1}$ average). In addition, animals eating diets that include high concentrations of toxic PSMs, like koalas, may have additional sodium needs for the excretion of these toxins (Foley et al. 1995). Diets rich in PSMs are known to disrupt sodium balance in lagomorphs (Iason and Palo 1991) and so consuming more leaf to try to meet requirements may exacerbate the potential risks of sodium deficiency.

Our finding that sodium concentrations in subalpine foliage were lower than that in foliage from trees at lower elevation is supported by other studies involving non-eucalypt plant species (Blair-West et al. 1968). However, these findings are based on a few sites and further studies are needed to determine how foliar sodium in both eucalypt leaf and bark may vary across seasons, other tree species, and landscapes. Lowland koala populations may not need an additional sodium source given the high sodium concentrations present in foliage there, which is similar in concentration to that found in *E. mannifera* bark in the Monaro (Table 1). We propose that bark chewing in koala populations in the Monaro region developed as a unique adaptation to low concentrations of foliar sodium in that area.

Sodium variation within and between eucalypt species and plant parts.— We do not know why sodium concentrations in *E. mannifera* vary so widely, even amongst close neighbours. For example, one unchewed *E. mannifera* tree had almost 10-fold lower sodium concentrations in its bark than a chewed neighbour 2 metres away. It is possible that the roots of these chewed trees are accessing a saline water source that is not available to their neighbours. Limnological studies on the Monaro have found that sodium concentrations of peneplain lakes vary dramatically over short distances (69 – 21,255 $mg.kg^{-1}$; Williams et al. 1970). This suggests that historically there have been geomorphological processes that can lead to local variation in sodium in the Monaro environment.

The relatively high concentration of sodium in bark compared to leaves in the Monaro region may be explained by how certain plants process excess sodium. Excess sodium can be devastating to plants, and an accumulation of sodium can lead to plant dehydration, tissue damage, and reduced photosynthesis (Munns 2002). Plants can limit the accumulation of sodium in leaves by excluding most of the sodium entry via the roots, and also limiting the accumulation of sodium in the leaves, particularly in the cytoplasm of the leaf cells (reviewed in Munns and Tester 2008). Plants can limit sodium in the cytoplasm by transporting excess sodium to vacuoles and exporting it to the phloem (Munns and Tester 2008), which can ultimately be sequestered and shed in the bark (Borger 1973). Like many other eucalypts, *E. mannifera* sheds its bark annually and so it is likely that excess sodium is stored in the bark for ultimate disposal. This hypothesis could be tested by measuring sodium concentrations in different layers of the bark with the expectation that sodium would be most heavily concentrated in the older, outer bark.

Notably, lowland populations of *E. viminalis* can tolerate foliar concentrations of sodium at higher levels than we observed in *E. mannifera* bark. We only measured foliar concentrations of sodium across a range of elevations in *E. viminalis*, so it is not yet clear whether these foliar sodium concentrations are also found in other eucalypt species at lower elevations. However, we would expect a similar relationship between elevation and foliar sodium concentrations for other species of eucalypts given that this is the pattern observed in other plant taxa as well (Blair-West et al. 1968).

Conservation and management implications

There has been much progress in identifying and recognizing the importance of mineral resources for herbivores (Fahrig 2001; Warne 2014). The patchy distribution of sodium sources can have profound effects on animal distributions and interactions. For example, variations in the spatial distribution of elephants in an African National Park was closely correlated with sodium in waterholes (Weir 1972). Crowding at natural mineral licks highlights how essential sodium is to animals and that it can be a rare resource in some landscapes (Weir 1972; Rothman et al. 2006). Future behavioural studies on koalas in the Monaro region should focus on how critical, patchily distributed resources, such as sodium-rich trees, may impact social interactions and population

dynamics in an otherwise solitary animal. The prevalence of bark consumption by koalas in the spring compared to other seasons also warrants further investigation, since this could relate to increased sodium requirements during lactation.

Sodium supplementation has been used to positively influence reproductive success and increase population densities in some species (Batzli 1986; McBurnie et al. 1999) and is often used as part of the nutritional management of livestock (Dove and Kelman 2015), particularly in sodium-deficient landscapes. Our preliminary efforts to supplement koalas with sodium have not been successful. We attached blocks of wood impregnated with sodium chloride to *E. mannifera* trees in areas where koalas ate bark. These failed to attract any interest from koalas. However, this was not entirely surprising given that other studies have had difficulty in creating artificial diets for koalas to replace fresh eucalypt foliage (Pahl and Hume 1990). It may be equally difficult to replicate other parts of a koala's diet. The mechanism that attracts a koala to the bark of salty trees is not yet understood. Given our inability to manufacture a sodium source that appealed to koalas, sodium supplementation as a conservation measure to support koala populations in sodium-deficient landscapes may not be feasible. Therefore, the conservation of chewed trees should be a priority as they are likely essential for maintaining the sodium balance of Monaro koala populations. Further research into the distribution of chewed trees is needed to ensure that enough and appropriate habitat is preserved for koalas in these sodium-deficient landscapes.

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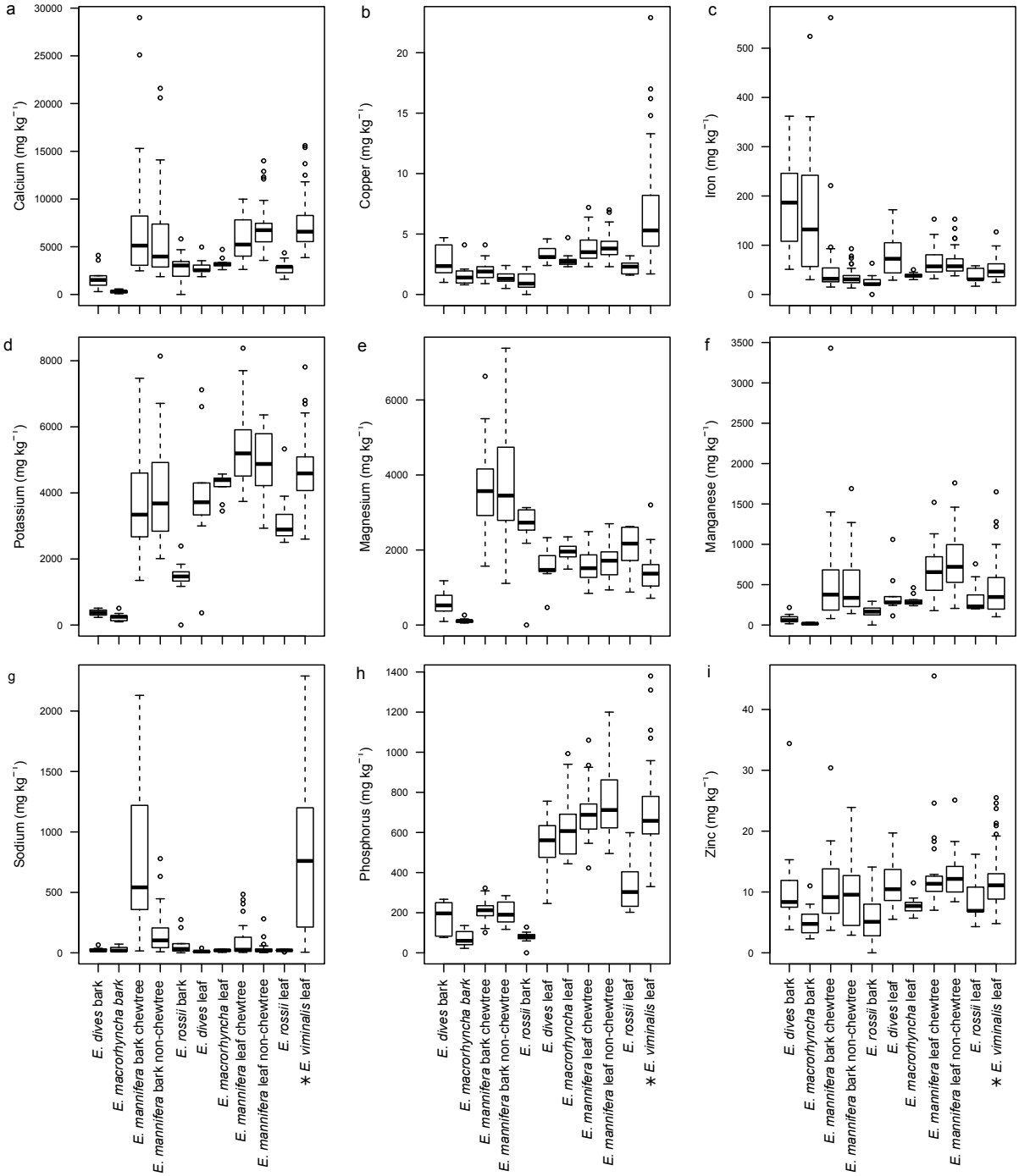
Supplementary Data

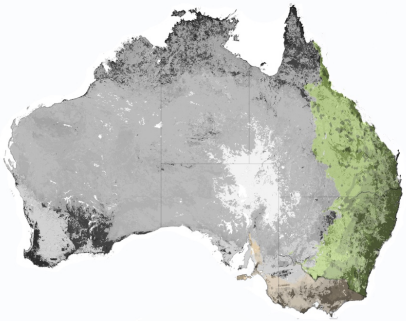
Supplementary Data S1. Video footage of a koala chewing *Eucalyptus mannifera* bark. Footage courtesy of Dr. Andrew W. Claridge, NSW National Parks and Wildlife Service, Queanbeyan NSW, Australia.

Please see online version.

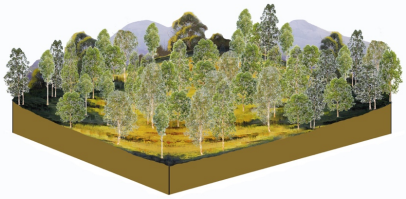
Supplementary Data S2. Summary of the concentration of minerals in bark and leaf samples collected from *Eucalyptus* trees in the study area.



Synthesis



Large scale



Medium scale

Small scale

Habitat loss is a leading cause of animal population declines worldwide. Global biodiversity is declining as humans increasingly modify the landscape and overexploit its natural resources (Butchart et al. 2010). Further, at least one fifth of the world's vertebrate species are classified as threatened (Hoffmann et al. 2010). Identifying suitable habitat is therefore crucial for the conservation and management of vulnerable wildlife. Protected areas, such as national parks, can help provide suitable habitats for our animals, but it is clear that the evidence for what is "suitable" habitat is still lacking for many species (Geldmann et al. 2013). We should approach our research using metrics with variance that best encapsulates the environment, interconnect a range of factors and provide a currency in which to measure habitat suitability.

The ability for animals to obtain essential nutrients is a key habitat requirement. We know that nutrition underpins animal growth and reproduction (White 1983, 1993), and can be correlated with, or affected by genetic, climatic, environmental, and historical factors (Braithwaite et al. 1983; Chapman et al. 2000; Augustine and McNaughton 2006). I investigated the importance of nutrition for koalas at multiple scales from individual tree use to the patterns of population density across extensive landscapes. I found that variation in nutrients and plant secondary metabolites (PSMs) explained a large proportion of the variation in the abundance of koalas. In addition, differences in nutritional quality between individual trees and tree species helped explain landscape use of this vulnerable folivore.

How can we efficiently assess forage quality across large data sets?

Previous studies have identified key foliar chemical traits to assess forage quality for koalas. However, investigating the relationship between foliar chemistry and animal population densities still required the collection of thousands of leaf samples across multiple locations throughout the range of the koala. Chapter One described methods in near infrared (NIR) reflectance spectroscopy for efficiently collecting compositional data in large data sets. Developing robust calibration models for large data sets requires considerable investment. However, if successful, the calibration model and/or compositional database can be used for new projects, reducing future costs of laboratory analyses in nutritional ecology. These robust calibration models and more realistic estimates of the prediction errors are likely to help facilitate the integration of nutritional data in ecological studies.

It is a challenge to know what samples and how many samples to analyse in the laboratory in order to develop robust calibrations. Selecting samples based on spectral variation alone can result in a surplus of leaf samples analysed that do not improve the predictive capabilities of the calibration model. In Chapter One, I found the predictive capacity of my available nitrogen (N_A) model was similar whether I used all of the reference chemistry samples or just 30% of the samples. Thus, collecting a wide representation of the spectral variation in the calibration set might not be that useful for traits that have a well-established relationship with NIR spectra. This coincides with another challenging decision; do we sacrifice precision in the analysis of laboratory samples (for example, by not analysing samples in duplicate) and instead include more samples to obtain a greater representation of the spectral variation? Windley et al. (2015) chose to analyse only single samples to predict concentrations of N_A in tree foliage from New Zealand, whereas I chose to analyse all of my samples in duplicate. There is not a general approach when deciding between precision versus spectral coverage, but rather it is dependent on the application of the model and the chemical traits to be predicted. Understanding which approach is better, and in what circumstances, is likely to further increase the efficiency of NIRS for predicting chemical traits and would be valuable for studies that aim to develop broader calibration models.

Broadening calibrations from Chapter One could help eliminate costs of laboratory analyses for future studies of the nutritional ecology of other folivores that eat eucalypts. If we can encourage the sharing of compositional data and spectra across research groups and projects worldwide, we could catalogue this variation and use it in a global food quality calibration that could predict the forage quality of many different plant samples. Of course, there are many challenges ahead in order for this to be a reality, such as standardising the spectral differences between plant parts, NIR spectrophotometers and particularly compositional analyses. For example, a recent study (Quentin et al. 2015) showed that estimates of the concentration of non-structural carbohydrates in *E. globulus* leaves varied up to 100 - fold between different laboratories. One way to address this would be to incorporate this variation during calibration development for example, “leave-instrument-out” or “leave-laboratory-out” cross- validation. This is similar to the approach I used in Chapter One to investigate how sources of geographic variation influenced the capacity of the calibration model’s to predict samples during the cross-validation process. Furthermore, we could use a

data set from a separate instrument as an independent test to validate the calibration model. While this is an exciting direction to broaden the future use of NIR spectroscopy in ecology, it is clear from Chapter One that issues remain during calibration development that need to be addressed in order to efficiently develop these global calibrations.

The challenges of collecting samples by hand and then developing efficient models to predict the chemical composition of future samples are significant. Therefore, there has been significant effort to collect spectral data by remote sensing. Remote sensing technology aims to estimate the chemical composition of forests using air-borne visible/NIR spectrometers on planes, drones or satellites (Curran 1989). It has been used successfully to estimate N and FPCs in eucalypt tree canopies (Huang et al. 2004, 2007). Although the errors remained significant, the approach has been applied to identifying key habitat for arboreal folivores in Australia (Youngentob et al. 2012) and grazing ungulates in Africa (Knox et al. 2011). However, some limitations of remote sensing data are that the identification of individual tree crowns can be difficult in closed canopy forests and there is much more noise with air-borne spectrometers than with laboratory spectrometers. Consequently, this can lead to less accurate estimates of chemical composition. Whether this accuracy is sufficient to draw out meaningful variation in the chemical composition of forests for wildlife remains to be answered by broader ecological studies. We know from Chapter One that increasing the robustness of a model requires some sacrifice in the accuracy of the predictive model and we gain some insight on whether this provides meaningful forage quality data in Chapter Two. Nonetheless recent studies that aim to predict N concentration of tree crowns and other vegetation in the absence of large numbers of hand-collected plant samples are very promising and may allow broader more rapid landscape assessment of some plant traits (Mahajan et al. 2014; Pellissier et al. 2015; Schweiger et al. 2016; Wang et al. 2018).

Do population densities of a folivore vary with forage quality across landscapes?

The environment is rich in patterns and it is difficult to disentangle the factors that drive variation in animal population densities from those that appear to correlate with them. There are many potential factors that can impact the distribution and abundance of animal populations including climate (Coe et al. 1976; Okello et al. 2016; Mason-Romo

and Farı 2017), soil fertility (Aumann and Emlen 1965; Braithwaite et al. 1984; Pastor et al. 1993; Ball et al. 2000; Loney et al. 2006), landscape disturbance history (Chapman et al. 2000; Drapeau et al. 2000; Lesmerises et al. 2012), competition (Choquenot 1991; Bonenfant et al. 2009), predation (Ripple and Beschta 2012; Kersch-Becker et al. 2017), vegetation composition (McNaughton et al. 1989; Cyr and Pace 1993; Lindenmayer et al. 1999; Pettorelli et al. 2011), and others. To answer fundamental questions in ecology, such as what limits animal populations, we need studies that span the distribution of species and encompass the variation between and within the environments that these animal populations cover. In Chapter Two, I collected leaf samples for forage quality analyses across a large extent of the koala's range and revealed the role of nutrition in explaining transcontinental variation in koala population densities.

In consultation with statisticians and ecologists, I developed a universal sampling design that could efficiently capture the chemical variation of forests and high-level statistical methods to showcase the effect of nutrition. Capturing the variation between and within environments across koala populations in a data set and incorporating this during statistical analysis was key to finding this result. I travelled to most areas with available koala density data, which meant that I met many of the prominent koala researchers in Australia. Sampling leaves across a large extent of the koala's range allowed me to capture a lot of the environmental variation within the koala's distribution. It was essential to use mixed effects statistical models, so I could account for the broad ecological differences between various regions (such as climate, landscape history, and forest type) and investigate how forage quality might influence the variation in densities between sites. It was also important to acknowledge the "inconsistent" effect of formylated phloroglucinol compounds (FPCs). Although sites with low foliar concentrations of FPCs are generally preferred by koalas, there are some subgenera of eucalypts that do not produce FPCs and instead have other secondary compounds, such as UBFs and have low concentrations of N_A. Thus, integrating nutritional quality with PSMs is essential. I highlighted this conflicting relationship of PSMs in Chapter Two and demonstrate the need to capture the complexity of the foliar PSMs of the forest.

In Australia, two eucalypt subgenera, *Monocalyptus* and *Symphyomyrtus* dominate the landscape and they each have their own specific classes of PSMs, which may contribute

to defence against diverse herbivores (Eschler et al. 2000; Tucker et al. 2010). Although these subgenera tend to co-occur, (Pryor's rule, Pryor 1959), it is evident from my data set that eucalypt forests can contain a mix of the subgenera or be dominated by a single subgenus. An obvious future direction would be to include measures of unsubstituted B-ring flavanones (UBFs, a specific class of PSMs for monocalypts) to better represent the nutritional composition of the forest to folivores. From the observational results in Chapter Two, there may be a negative relationship between UBFs and koala densities and I urge future studies to test if this effect is significant. How to test the effects of nutrition, using measures of N_A, FPCs and UBFs combined is not clear. From preliminary analyses, incorporating site-level averages for all three nutritional traits did not improve model performance; therefore, considerations of subgenera composition and/or other site-level measures beyond simple averages (i.e., number of trees over or under a certain value) may be required. Nonetheless, this is the first time that it has been possible to obtain nutritional data that encompasses the dominant chemical variation in eucalypt forests.

Nutrition is complex, and animals often have to make trade-offs between acquiring nutrients to meet their requirements and contending with PSMs. Thus, simply measuring the individual nutritional traits and then incorporating them in a model may not best represent these subtle, yet significant trade-offs. For example, we know that FPCs deter feeding by koalas, but studies have shown that this deterrence can be greater in some eucalypt species than others. Moore et al. (2005) found feeding was significantly more restricted when koalas ate *E. globulus* leaves than when eating *E. viminalis*, even though the concentrations of FPCs were similar across the two species. The geometric framework is probably the best tool for assessing these trade-offs as it models the key relationships among relevant variables in nutritional ecology (Simpson and Raubenheimer 1993; Raubenheimer and Simpson 1999; Raubenheimer et al. 2009). Two relevant studies using locusts and brushtail possums have demonstrated that the effectiveness of a PSM as a deterrent was strongly linked to the protein : carbohydrate composition of the food (Behmer et al. 2002; Nersesian et al. 2012). In particular, concentrations of plant protein proved to be the largest influence on how well the animals could tolerate PSMs. My own previous work on common brushtail possums (Au et al. 2013) extended the simple observations of Neresian et al. (2012) and showed that the mechanisms of this interactions is (at least in part) due to increased whole body

protein turnover associated with the ingestion of PSMS. Thus, there is a mechanistic basis for expecting foliar protein to be a modulator of tolerance to PSMs.

An exciting future study would be to use the forage quality models developed in my thesis to predict concentrations of N_A, UBFs and FPCs in samples with known palatability estimates (a measure of how much an animal is willing to eat from a plant as described by McIlwee et al. 2001 and Moore et al. 2010) or in forest tree samples with data on the habitat use of eucalypt specialists. This would allow us to investigate how palatability and habitat use of different eucalypt specialists separate on a geometric framework with N_A, UBFs and FPCs. We already know that the spectra of leaves predicts intake much better than any combination of chemical traits and exploring the inclusion of whole spectra into statistical models of koala density could be informative (Moore et al. 2010). The direct analysis of meaningful traits allows us to model the subtle trade-offs that animals make during feeding (Raubenheimer 1995) and has been used to address many nutritional ecology questions in primates and cervids (Raubenheimer et al. 2015; Felton et al. 2018). The process for predicting concentrations of N_A across multiple projects is relatively simple because we can use the N_A calibration for any eucalypt plant sample, irrespective of subgenera. However, the process for predicting FPCs and UBFs is not so straightforward.

One challenge to overcome is that FPCs and UBFs predictive models are specific to their subgenus, and currently we need to ensure that UBFs models are used to predict only *Monocalyptus* samples and FPCs models are used to predict only *Symphyomyrtus* samples. For example, during FPC calibration, I separated a subset of the *Symphyomyrtus* spectra from the broader eucalypt data set and then developed a calibration to predict FPCs in this subset of *Symphyomyrtus*. I found that if I used my FPC calibration on *Monocalyptus* spectra, I observed a range of "predicted FPCs values" for the *Monocalyptus* samples, from negative to positive values. Given the extensive research to show the monocalypts do not contain FPCs (and I also performed some checks during my analysis), I simply assigned a zero value for FPCs in *Monocalyptus* samples. All of this relies on knowing the subgenus and/or species of the sample collected, and this is not always known or the species correctly identified. Recent advances in using whole genome marker sets (e.g. DART-Seq) appears promising for unequivocally identifying closely related eucalypt species (B. D. Moore personal

communication). If we can develop methods to more efficiently acquire both nutrients and PSM data and use measures of forage quality to identify suitable habitat for folivores, we can then also track how anthropogenic landscape change can alter it.

Investigating a process that is driving patterns of forage quality across forests

Conservation biology is known as a "crisis-orientated" or "mission-driven" discipline where it aims to conserve nature through identifying and mitigating its threatening processes (Soule 1985; Mace 2014). Wide-scale anthropogenic landscape change is negatively impacting the biodiversity and persistence of our ecosystems and animal populations (Lindenmayer et al. 2017). Conservation biologists are developing tools to identify and track these negative effects and it is contingent on understanding the patterns and processes responsible for the distribution and abundance of organisms (Krebs et al. 1995; Fischer and Lindenmayer 2006). In Chapter One, I successfully produced a large chemical database for eucalypts, and in Chapter Two, I demonstrated that these nutritional traits can act as a currency to rank habitat quality for a vulnerable folivore. In Chapter Three, I used these nutritional traits to demonstrate one process by which disturbance can negatively impact animal populations by reducing forage quality.

Many heterogenous forests in the south east of NSW and in particular in the Bermagui – Mumbulla area were replaced by dense monocultures of *E. sieberi* following intensive logging and/or intense wildfires (Florence 1996; Bradshaw 2012). I combined an external survey of koala presence across this region (Allen et al. 2010, 2016) with an extensive foliar chemical data set (Chapter Two) to show that disturbance related changes in forest composition can alter the forage quality of forests. I found that the probability of koalas occurring in the area decreased significantly with an increasing proportion of *E. sieberi* in forests. Using simulations, I showed that forests dominated by *E. sieberi* are strikingly nutritionally-poor, making it unsuitable habitat for koalas and other marsupials that rely on symphyomyrtle species for their food. These findings provide avenues for targeted action to reduce the effects of disturbance on animal populations by mitigating the proliferation of poor-quality forage.

In the first instance, koala presence and absence based on pellet surveys may mistakenly identify species such as *E. sieberi* as important food trees. Koalas rest in many species of trees from which they do not eat (Marsh et al. 2013) and common species in the forest such as *E. sieberi* are likely to be used this way. However, if this

results in *E. sieberi* being listed as a food tree species as has been proposed, then forest managers might argue that their disturbances are improving forest quality for koalas whereas my nutritional data suggests the exact opposite. It is clear that conservation efforts based on simple lists of "food tree species" are not sufficient to ensure the successful management and restoration of koalas (Ellis et al. 2013).

I recommend that where possible, land managers reduce or eliminate intensive logging regimes to avoid the proliferation of poor-quality, *E. sieberi*-dominant forests. If the landscape can be restored following disturbance, I suggest that land managers take active steps to restore a heterogenous eucalypt forest. For example, they could modify the number of newly established *E. sieberi* trees by post germination thinning and/or ensure the establishment of non-*E. sieberi* species in the landscape. Importantly though, for unknown reasons, the presence of some, but not many, *E. sieberi* was positively associated with koala presence and it may be that the ability for *E. sieberi* to occur at low densities is indicative of certain forest conditions or landscape histories that are beneficial to koala populations. Future research should focus on investigating this pattern and its potential underlying causes more closely. It is apparent that disturbance can have long-term consequences on ecosystems and animal populations (Chapman et al. 2000; Haddad et al. 2015; Lindenmayer et al. 2017), and typically the success of active restoration is greater than natural regeneration in disturbed landscapes (Crouzeilles et al. 2017). The need to protect suitable habitat and restore disturbed environments is even more pressing given the compounding negative impacts of landscape change and climate change on animals.

What other micronutrient challenges could animals face?

Anthropogenic climate change is shifting the distribution of many terrestrial organisms towards the poles and up higher in elevation (Parmesan and Yohe 2003; Chen et al. 2011) and is likely to introduce new nutritional obstacles for animals. Chapter Four reveals an unusual bark-eating behaviour that could allow koalas to persist in an otherwise unsuitable environment. I showed that koalas living in subalpine forests in the Monaro region, NSW could only meet their sodium requirements by eating the bark of *E. mannifera* trees. It was clear that koalas living in the Monaro would be severely sodium-deficient if they were to rely only on their typical diet of eucalypt leaves for food. Further, I showed that the availability of foliar sodium significantly decreased with

increasing elevation. This was expected and reflects a global pattern in sodium availability (Blair-West et al. 1968; Denton 1982). Thus, it is possible that the predicted uphill migration of animals may be restricted by an animal's ability to acquire essential nutrients in mineral-poor landscapes. Fortunately, there are many examples in the literature of herbivores adopting unusual feeding strategies in order to meet sodium requirements, such as moose, elephants, gorillas and colobus monkeys (Weir 1972; Belovsky and Jordan 1981; Bowell et al. 1996; Rode et al. 2003; Rothman et al. 2006). However, given the speed at which the climate is changing, animals may not have the capacity to adapt new feeding strategies within such a short time-frame.

Identifying and protecting essential micronutrient hotspots is likely to increase the success of protected areas for animals. However, we do not know how common or rare these hotspots are in the landscape. I suggest that conservation managers should assess the availability of micronutrients in the landscape and include it in measures of forage quality when assessing habitat quality for animals. Further, future studies could investigate if it is possible to supplement the diet of folivores, for example, do koalas use salt licks in the landscape? It would be valuable to know how we can directly encourage the persistence of animals in a particular area, especially given that anthropogenic climate change is also reducing the nutritional quality of forage (Lawler et al. 1997; Hovenden and Williams 2010; Robinson et al. 2012; Rothman et al. 2015). Artificially supplementing limiting resources through the provision of things like salt licks, or through targeted replanting of trees with higher forage quality, may be important future strategies for protecting threatened wildlife populations.

Final conclusions

My thesis demonstrates the multi-scale effect of nutrition on arboreal folivores. I have extended our knowledge from nutritional ecology studies at fine and medium scales to develop a better understanding of how forage quality impacts populations. I have used a combination of transcontinental, field-based sampling, nutritional trait analyses and high-level statistical techniques to understand how nutrition can drive the variation in folivore populations across landscapes. I highlight the need to integrate measures of forage quality in habitat quality assessments and provide advice and methods to researchers, conservation managers and policy makers. Improving the assessment and identification of suitable habitat is essential to combat the global declines in wildlife

populations in the face of rapid anthropogenic landscape alteration and a changing climate.

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Appendix One

MARSH, K. J, B. YIN, I. P. SINGH, I. SARAF, A. CHOUDHARY, J. AU, D. J. TUCKER, W. J. FOLEY. 2015. From leaf metabolome to *in vivo* testing: identifying antifeedant compounds for ecological studies of marsupial diets. Journal of Chemical Ecology 41:513–519.

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From Leaf Metabolome to *In Vivo* Testing: Identifying Antifeedant Compounds for Ecological Studies of Marsupial Diets

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Abstract Identifying specific plant secondary metabolites that influence feeding behavior can be challenging, but a solid understanding of animal preferences can guide efforts. Common brushtail possums (*Trichosurus vulpecula*) predominantly eat *Eucalyptus* species belonging to the subgenus *Symphyomyrtus*, and avoid eating those belonging to the *Monocalyptus* subgenus (also called subgenus *Eucalyptus*). Using an unbiased ^{1}H NMR metabolomics approach, a previous study identified unsubstituted B ring flavanones in most species of monocalypts examined, whereas these compounds were absent from symphyomyrtles. We hypothesised that unsubstituted B ring flavanones act as feeding deterrents for common brushtail possums. In the current study, we tested this hypothesis by comparing how much possums ate of a basal diet, with diets containing one of four structurally related compounds; pinocembrin, flavanone (unsubstituted B ring flavanones), chrysin (the flavone analogue of pinocembrin), and naringenin (a flavanone with B ring substitution). We found that pinocembrin and flavanone deterred feeding relative to the basal diet, but that chrysin and naringenin did not at equivalent concentrations. Thus, unsubstituted B-ring flavanones may explain why brushtail possums avoid eating monocalypt species. Furthermore, small differences in the structure of secondary compounds can have a large impact on antifeedant properties. These results demonstrate that metabolomics can be a valuable tool for ecologists seeking to understand herbivore feeding preferences.

Keywords Pinocembrin · Flavanone · *Eucalyptus* · Plant secondary metabolites · Herbivore · Metabolomics

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Introduction

Although plant secondary metabolites (PSMs) are widely recognized as influencing food selection and the nutritional quality of foods for vertebrate herbivores, there are few examples where specific characterized compounds have been linked to diet quality in the laboratory, and even fewer under field conditions. Many studies still focus on broad groups of uncharacterized compounds, such as tannins and essential oils (Simmen *et al*. 2014; Villalba *et al*. 2014; Vourc'h *et al*. 2002). When specific compounds have been identified, it opens up opportunities for understanding how animals recognize them and regulate their intake (Lawler *et al*. 1998), their spatial distribution and heritability (Andrew 2005; Andrew *et al*. 2007; Moore *et al*. 2010), and ultimately the role that herbivores might play in selecting for these compounds in natural populations (Andrew *et al*. 2007).

Variations in the tolerance of marsupial folivores to specific secondary compounds explain, in part, their food choices, and we have argued that these sorts of differences facilitate the coexistence of different species of folivores in *Eucalyptus* forests (Jensen *et al*. 2014; Moore *et al*. 2004). For example, common ringtail possums (*Pseudocheirus peregrinus*) are highly

sensitive to the effects of formylated phloroglucinol compounds (FPCs) that are found only among species belonging to the *Eucalyptus* subgenus, *Symphyomyrtus*. As a consequence, ringtail possum feeding is focused on species belonging to the other major *Eucalyptus* subgenus, *Monocalyptus* (also known as subgenus *Eucalyptus*) (Moore *et al*. 2004). In contrast, common brushtail possums (*Trichosurus vulpecula*) are more tolerant of FPCs (Jensen *et al*. 2014) and tend to eat symphyomyrtle species. They are rarely reported eating *Monocalyptus* leaves, and ate little of the monocalypt species, *E. rossii* and *E. consideniana*, even when offered no alternative food (Marsh *et al*. 2003b). Although brushtail possums are more sensitive to the effects of tannins than are ringtail possums (DeGabriel *et al*. 2009; Marsh *et al*. 2003a, b), the inactivation of monocalypt tannins with polyethylene glycol does little to remove the deterrent effect of these leaves. Instead, monocalypt species likely contain additional plant secondary metabolites that deter feeding by brushtail possums (Marsh *et al*. 2003b).

It has always been a challenge to identify the specific compounds that deter feeding from the large mix of potential compounds present, but careful observation of animal preferences can help to focus on the plant material most suited to chemical investigation. Combining animal observations with bioassay-guided fractionation and chemical characterization has identified PSMs with strong antifeedant properties in arctic, temperate and marine systems (Bryant *et al*. 1983; Diaz Napal *et al*. 2009; Enge *et al*. 2012; Pass *et al*. 1998; Reichardt *et al*. 1990; Taylor *et al*. 2003). Recently, we used a new approach based on unbiased ^{1}H NMR metabolomics in our investigations of *Eucalyptus* (Tucker *et al*. 2010). This approach does not make prior assumptions about the types of compounds present, and the results suggested that an important difference between species of *Sympyomyrtus* and *Monocalyptus* was the presence of free flavanones without B ring substitution in monocalypts. These compounds appeared to be absent from *Symphyomyrtus* species, but almost universally present in the monocalypt species examined (Tucker *et al*. 2010). We hypothesized that this group of compounds could be responsible for the reported unwillingness of brushtail possums to feed on leaves from species of *Monocalyptus*. Furthermore, given that related compounds, including flavones, flavonols, and flavanones with B ring substitution occur in eucalypts of both subgenera (Conde *et al*. 1995; Hillis 1966, 1967; Tucker *et al*. 2010; Wollenweber and Kohorst 1981), we expected that the flavanone structure and the lack of substitution on the B ring would be important features conferring deterrent activity against marsupial folivores.

In order to test this, pinocembrin (Fig. 1a) was chosen as a representative unsubstituted B ring flavanone that is known to occur in monocalypt foliage (Bick *et al*. 1972; Saraf *et al*. 2015; Tucker *et al*. 2010). Three other compounds, flavanone, naringenin, and chrysin, were chosen due to their structural similarities to pinocembrin. Flavanone is an unsubstituted B ring flavanone that also lacks substitution on the A ring (Fig. 1b). Naringenin has the same A ring substitution pattern as pinocembrin, but has a hydroxy substitution at the 4′ position on the B ring (Fig. 1c). Chrysin is the flavone analog of pinocembrin (Fig. 1d). We added these compounds individually to an artificial diet prepared for common brushtail possums in order to test three predictions. First, that the two flavanones lacking B ring substitution (pinocembrin and flavanone) would deter feeding, second, that the flavone (chrysin) would not deter feeding, and finally, that the flavanone with B ring substitution (naringenin) would not deter feeding.

Methods and Materials

Chemicals Leaves from five *Eucalyptus sieberi* trees were collected from Forbes Creek, NSW, Australia, using an extendable pruning pole. The leaves from all trees were pooled, freeze-dried, and then crushed coarsely. Pinocembrin was extracted according to the accelerated solvent extraction methods of Saraf *et al*. (2015) at 80 °C. Briefly, dried coarse leaf powder was extracted in a stainless steel cell with methanol for two cycles of 20 min, at 100 bar. Following this, the extract was concentrated to dryness. It was suspended in water and partitioned with hexane and diethyl ether. Pinocembrin was purified from the diethyl ether fraction by column chromatography and finally by crystallization from methanol as described earlier (Saraf *et al*. 2015). The final purity of pinocembrin was 98 % (HPLC). Naringenin, chrysin, and flavanone were purchased from Sigma-Aldrich (Sydney, Australia).

Animals and Diets Six male common brushtail possums (*Trichosurus vulpecula*) were captured in wire cage traps, baited with apple, on the campus of the Australian National University, Canberra, Australia. They were housed individually in pens measuring ~2×2×4 m, which contained two nesting boxes, branches for climbing and fresh water. The pens were inside a shed, protected from wind and rain, but were otherwise subject to natural lighting and temperature fluctuations. During acclimation to captivity, possums were fed a variety of leaves and chopped fruit. This was gradually replaced by a basal diet consisting of 42.5 % apples, 28 % bananas, 10 % carrots, 6 % rolled oats, 4.5 % ground rice hulls (Coprice, Leeton, Australia), 4 % ground lucerne chaff, 1.67 % acid casein, 1.5 % Solka-Floc® (powdered cellulose; International Fiber Corporation, NY, USA), 1.25 % vegetable oil, 0.3 % NaCl, 0.25 % dicalcium phosphate, and 0.03 % Nutrequin vitamin and mineral mix (Kentucky Equine Research, Mulgrave, Vic., Australia), all on a wet matter basis. The basal diet was 31.6 % dry matter (DM).

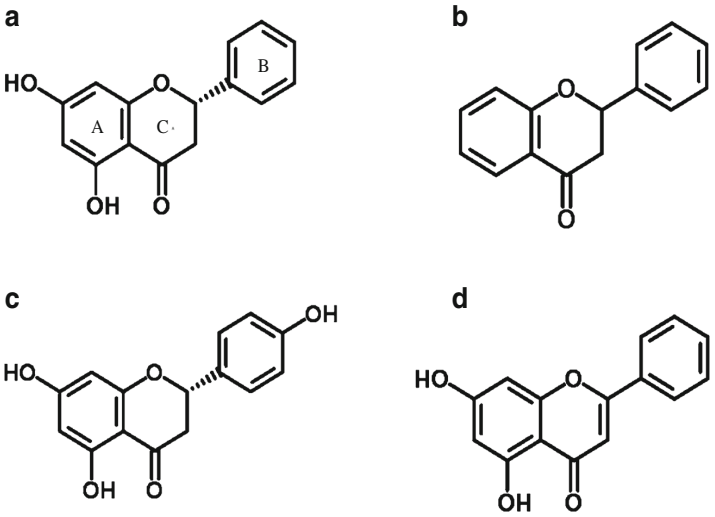

Fig. 1 The structures of compounds used in the study: **a** pinocembrin, **b** flavanone, **c** naringenin, and **d** chrysin. The *A*, *B* and *C* rings are labelled on pinocembrin

There were nine experimental diet treatments: the basal diet, and the basal diet containing 0.8, 1.6, or 4.7 % (DM) flavanone, 1.6 or 4.7 % (DM) chrysin or naringenin, or 1.6 % (DM) pinocembrin. When compounds were added, an equivalent amount of Solka-Floc was removed in order to keep the DM content of the diets consistent. The range of concentrations used (0.8 to 4.7 % DM) mimic known concentrations of pinocembrin in eucalypt foliage (Saraf *et al.* 2015). It was not possible to feed all compounds at all concentrations due to limited availability of some of the compounds.

The experiment took place during May 2014 (autumn). Diet treatments were evaluated over six nights, with four of the six possums receiving each diet. The number of possums offered each diet was limited by the amount of each compound that was available. In other words, we used the minimum number of animals required to produce statistically meaningful results, in order to allow the maximum number of concentrations of each compound to be tested. A crossover design was generated using CycDesigN5.1 (VSN International Ltd, Hertfordshire, UK) to ensure that the same diet was never given on the same night, and no animal received the same diet twice. In addition, diet assignment was balanced to account for possible carryover effects. Diets were prepared immediately before feeding, and possums were offered a preweighed amount at 1700 hr. A sample of each diet was dried at 60 °C to constant mass and used to calculate the amount of DM offered to each possum. Uneaten food was removed at 1900 hr and dried to constant mass to determine the amount of DM refused. Dry matter intake (DMI) for each possum was calculated as DM offered minus DM refused. For the remainder of the night (1900 to 0900 hr), possums were provided with the basal diet without flavanones, but DMI was not recorded over this period. The 2 hr experimental period was chosen to reduce the amount of compound required for the experiment. Preliminary work showed that possums ate approximately half of their nightly intake in the first 2 hr after presentation of food (both with and without PSMs), and that this behavior occurred even when diets containing PSMs were replaced with the basal diet after 2 hr (data not shown). In other words, they did not refuse to eat PSM-containing food in anticipation of the food being replaced with the basal diet 2 hr later.

Statistical Analysis The residual maximum likelihood mixed model in Genstat v17.1 (VSN International Ltd, Hertfordshire, UK) was used to test for differences in DMI between diet treatments. The individual possum identity and the day on which diets were offered were included as random effects to account for the experimental design. A least significant difference ($LSD_{0.05}$) test was used to determine whether treatment means were significantly different from one another.

Results

Possums ate different amounts depending on the diet that was offered ($F_{8,23}$=4.66, P=0.002; Fig. 2). The amount that possums ate of diets containing either chrysin (the flavone) or naringenin (the flavanone with a substituted B ring) did not differ significantly from the amount that they ate of the basal diet (Fig. 2). In contrast, pinocembrin and flavanone, the two unsubstituted B ring flavanones, deterred feeding relative to the basal diet at all concentrations offered (Fig. 2).

The mean DMI of possums offered 1.6 % (DM) pinocembrin was very similar to that of possums offered 1.6 % (DM) flavanone (16.7 and 15.7 g DM, respectively; Fig. 2). This was significantly less than possums ate of diets containing 1.6 % (DM) chrysin or naringenin. Likewise,

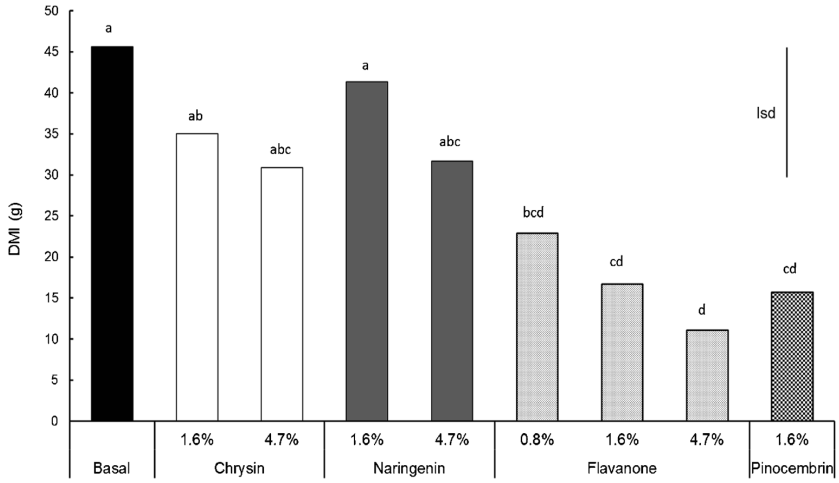

Fig. 2 The mean dry matter intake (DMI) of common brushtail possums (N=4 per treatment) offered a basal diet containing chrysin, naringenin, flavanone or pinocembrin at different concentrations (given as % (DM) of the diet). Treatments sharing the same letter are not significantly different from one another

possums ate less of the flavanone diet at a concentration of 4.7 % (DM) than of the chrysin and naringenin diets (there was not enough pinocembrin to feed at this concentration; Fig. 2).

Possums appeared to respond to compounds in a dose-dependent manner. In other words, they ate most of the basal diet (0 % compound), and least at the highest concentration of each compound offered. However, there was no significant difference in DMI between the lowest and highest concentrations fed within any compound (Fig. 2).

Discussion

This study validates the use of ^{1}H NMR metabolomics as a tool to identify potential antifeedant compounds in plants. As hypothesized, the two flavanones with an unsubstituted B ring (flavanone and pinocembrin; Fig. 1a and b) deterred feeding by common brushtail possums, whereas naringenin, which possesses B ring substitution (Fig. 1c), did not. Likewise, the flavone, chrysin (Fig. 1d), had little effect on feeding, which was expected due to the presence of a variety of flavones, including chrysin, eucalyptin, and 8-demethyleucalyptin, in eucalypt species from both *Monocalyptus* and *Symphyomyrtus* (Tucker *et al*. 2010; Wollenweber and Kohorst 1981).

Small differences in the structure of flavonoids can have a large impact on their activity (Pannala *et al*. 2001). Naringenin differs from pinocembrin only by the presence of a 4′-hydroxy substitution on ring B. Likewise, chrysin is the flavone analogue of pinocembrin. These small structural variations resulted in dramatic differences in the feeding responses of possums. The amount of food eaten by brushtail possums offered diets containing up to 4.7 % (DM) naringenin or chrysin did not differ significantly from the amount that they ate of the basal diet. In contrast, relative to the basal diet, possums offered a diet containing 1.6 % (DM) pinocembrin ate almost two thirds less. The responses of possums to diets containing flavanone mirrored that of pinocembrin, although we were able to feed a wider range of flavanone concentrations because more of this compound was available. Together, the results suggest that an unsubstituted B ring on flavanones, but not on flavones, is an important feature mediating their deterrence to common brushtail possums.

This is not the first demonstration that the structure of flavonoids influences their antifeedant effects. Morimoto *et al*. (2003) demonstrated large variation in the antifeedant activity of flavones with minor structural differences against the common cutworm (*Spodoptera litura*). Likewise, in their search for natural insecticides, Diaz Napal *et al*. (2009) demonstrated that pinocembrin was a more effective antifeedant than a variety of other flavonoids, including naringenin, against three herbivorous insect species, *Epilachna paenulata*, *Xanthogaleruca luteola,* and *Spodoptera frugiperda*. Pannala *et al*. (2001) also showed that the B ring structure was the most important determinant of antioxidant activity in flavonoids, with those with an unsubstituted B ring possessing low antioxidant activity. Other classes of PSMs, such as diterpenoids, triterpenoids, cinnamic acid derivatives, benzoates, and acetophenones, also demonstrate structure-specific activity against herbivores (Clark and Shah 1994; Lang *et al*. 2013; Li *et al*. 2014; Nolte *et al*. 1993; Watkins *et al*. 1999), but there are few examples from mammals, probably because it is difficult to obtain relevant PSMs in large enough quantities to conduct feeding studies. One example, however, comes from marsupials. Lawler *et al*. (1999) demonstrated that the presence of formyl groups on an aromatic ring were more important in determining anti-feedant activity against common ringtail possums than were phenol groups. Hence, jensenone, a formylated phloroglucinol compound (FPC) has strong anti-feedant activity, while torquatone and demethyl-torquatone, which are

structually similar but lack formyl groups, are less active (Lawler *et al*. 1999).

Although the amount of food eaten by possums offered diets containing chrysin and naringenin did not differ from the basal diet at any of the concentrations offered, the pattern of response did appear to be dose-dependent, suggesting that possums may be deterred by higher concentrations of these compounds than were used in the current study. It was not possible to explore this due to restrictions on the amount of each compound that was available, but it is unlikely that chrysin contributes to the rejection of monocalypt foliage by brushtail possums, given that it has also been reported in symphyomyrtles (Wollenweber and Kohorst 1981). Nevertheless, it would be useful to ascertain the concentration at which chrysin occurs in eucalypt foliage in order to determine whether higher concentrations should be considered in future feeding studies.

Although artificial diets were used in the current study, the concentrations of flavanone and pinocembrin that deterred feeding (0.8 to 4.7 % (DM)) reflect concentrations of pinocembrin that have been measured in monocalypt leaves. Saraf *et al*. (2015) found pinocembrin at concentrations of 3.4 % (DM) in *E. sieberi*, 2.4 % (DM) in *E. fraxinoides,* and 0.3 % (DM) in *E. pauciflora*. This suggests that unsubstituted B ring flavanones are either the cause of, or strongly contribute to, the unwillingness of common brushtail possums to eat leaves from species belonging to the monocalypt subgenus. In contrast, the fact that monocalypt species form a major part of the diet of common ringtail possums (Moore *et al*. 2004) suggests that these possums should be more tolerant of high concentrations of unsubstituted B ring flavanones, just as brushtail possums are more tolerant of the FPCs found in symphyomyrtle species that they commonly eat (Jensen *et al*. 2014). This remains to be tested, but could be a contributing factor to dietary niche partitioning between the marsupial folivores (Moore et al. 2004). One potential reason that the two marsupial folivore species tolerate different classes of PSMs is that they metabolize xenobiotics in different ways. For example, ringtail possums produce more extensively oxidized metabolites than do brushtail possums for the naturally occurring terpene, *p*-cymene (Boyle *et al*. 1999), and also produce different metabolites for the non-steroidal anti-inflammatory drug, meloxicam (Kimble *et al*. 2014). These alternative strategies may give the two species different advantages when confronted with contrasting classes of eucalypt PSMs.

As well as using artificial diets, another way to confirm whether unsubstituted B ring flavanones are more deterrent to brushtail possums than to ringtail possums, would be to offer them leaves from a variety of trees that vary naturally in these compounds. However, in order to do this we need a better understanding of the distribution of unsubstituted B ring flavanones in *Monocalyptus* species. Little is known, but several unsubstituted B ring flavanones can occur in the same plant, and different species can contain dissimilar flavanone profiles. For example, Bick *et al*. (1972) identified three flavanones lacking B ring substitution in *E. sieberi*; pinocembrin, O,O-dimethyl pinocembrin and alpinetin. Tucker *et al*. (2010) likewise found at least two co-occurring unsubstituted B ring flavanones in each of *E. agglomerata* and *E. rossii*, and the *E. rossii* flavanone structures differed from those observed in the other species investigated. These observations raise two questions that need to be answered. First, are different unsubstituted B ring flavanones equally deterrent? The similar responses of possums to flavanone and pinocembrin suggest that they could be, although it is difficult to be sure when only two compounds, with a single concentration of pinocembrin, were used. Second, when several unsubstituted B ring flavanones are present, do their combined concentrations determine the level of deterrence? Individual FPCs in symphyomyrtles have a similar effect on feeding, and, when mixtures are present, the combined concentration drives their anti-herbivore effect (Moore *et al*. 2005). In order to better understand how variation in unsubstituted B ring flavanone concentrations in natural foliage diets correlate with the feeding behavior of different eucalypt folivores, we are currently in the process of developing methods to identify and quantify these compounds in a range of *Monocalyptus* species.

Identifying compounds that deter feeding in potential food items is a vital step towards understanding how animals perceive habitat quality, and how habitat quality in turn influences factors such as animal distributions and abundance. In previous studies, we have shown that FPCs influence the selection of symphyomyrtles by free-ranging folivores (Marsh *et al*. 2014). However, most eucalypt stands contain a mix of subgenera (Austin *et al*. 1983), and until the current study, we were not able to explain why some folivores select symphyomyrtles over monocalypts. In this study, we demonstrated that unsubstituted B ring flavanones deter feeding by common brushtail possums, and thus have taken the first step towards being able to integrate the antifeedant effects of monocalypt PSMs with the nutritional quality of mixed *Eucalyptus* forests for marsupial folivores.

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