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Research

New approaches to tannin analysis of leaves can be used to explain *in vitro* biological activities associated with herbivore defence

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Summary

- Although tannins have been an important focus of studies of plant–animal interactio ditional tannin analyses cannot differentiate between the diversity of structures pre plants. This has limited our understanding of how different mixtures of these widespre ondary metabolites contribute to variation in biological activity.
- We used UPLC-MS/MS to determine the concentration and broad composition of t and polyphenols in 628 eucalypt (*Eucalyptus*, *Corymbia* and *Angophora*) samples, and these to three *in vitro* functional measures believed to influence herbivore defence: precipitation capacity, oxidative activity at high pH and capacity to reduce *in vitro* ni (N) digestibility.
- Protein precipitation capacity was most strongly correlated with concentrations of pr din subunits in proanthocyanidins (PAs), and late-eluting ellagitannins. Capacity to *in vitro* N digestibility was affected most by the subunit composition and mean de polymerisation (mDP) of PAs. Finally, concentrations of ellagitannins and prodelphinid units of PAs were the strongest determinants of oxidative activity.
- The results illustrate why measures of total tannins rarely correlate with animal f responses. However, they also confirm that the analytical techniques utilised here coul researchers to understand how variation in tannins influence the ecology of individu populations of herbivores, and, ultimately, other ecosystem processes.

Introduction

Biologists often assume that a primary role of tannins is to defend plants against herbivory. There are, however, thousands of tannin compounds displaying a diverse array of structures that often occur in complex mixtures of tens to hundreds of compounds. These structures not only influence the response to standard colorimetric assays (Schofield *et al.*, 2001), but they also affect the biological activity of tannins, including their ability to bind proteins (Porter & Woodruffe, 1984; Jones & Palmer, 2000; Karonen *et al.*, 2015), their pro- or antioxidant capacities (Barbehenn *et al.*, 2006; Moilanen & Salminen, 2008; Moilanen *et al.*, 2016), and, ultimately, their effects on herbivores (Ayres *et al.*, 1997; Makkar, 2003; Mueller-Harvey, 2006; Roslin & Salminen, 2008). Consequently, the specific tannins present in a mixture, rather than just the total tannin concentration, are important in understanding biological consequences (Barbehenn *et al.*, 2008; Moilanen & Salminen, 2008). However, it is difficult to characterise complex tannin mixtures, and therefore to at specific biological consequences to tannins. Some ecologis circumvented this problem by relating measures of herbi functional attributes of tannins, such as their capacity to p tate protein (Robbins *et al.*, 1987; e.g. McArt *et al.*, 2009), nitrogen (N) digestibility (e.g. DeGabriel *et al.*, 2009; *et al.*, 2009) or oxidise at high pH (e.g. Appel, 1993; Stei *et al.*, 2016; Marsh *et al.*, 2017b).

Protein binding is the primary mechanism by which t are traditionally thought to affect mammalian herbivores. ecologists measure the capacity of tannins to precipitate dard amount of protein and use this as a functional mea tannin concentrations in forage. For example, Robbin (1987) were able to predict the *in vivo* digestible protein c of plants for mule deer (*Odocoileus hemionus*) and white deer (*O. virginianus*) from the *in vitro* protein precip capacity and total protein concentration of plant extracts. I McArt *et al.* (2009) demonstrated that reduced

availability due to protein precipitation by tannins could explain differences in productivity between moose (*Alces alces*) living in different regions.

DeGabriel *et al.* (2008) developed an alternative method to assess the capacity of tannins to constrain protein digestion in mammalian herbivores. Their method measures the *in vitro* digestibility of plant N in the presence and absence of polyethylene glycol (PEG), a polymer that preferentially binds to tannins and releases protein (Silanikove *et al.*, 1996; Schofield *et al.*, 2001). Using this method, DeGabriel *et al.* (2009) showed that the *in vitro* digestible N concentration of eucalypt foliage influenced the reproductive success of female common brushtail possums (*Trichosurus vulpecula*). As a consequence, *in vitro* digestible N concentrations have been used as indicators of the nutritional quality of habitat for marsupial folivores (DeGabriel *et al.*, 2009; Youngentob *et al.*, 2011; Windley *et al.*, 2016).

In contrast to mammals, there is little evidence that tannins reduce the digestibility of protein in insect herbivores (Barbehenn & Constabel, 2011). The alkaline pH in the midgut of insects can prevent tannins from forming complexes with protein (Martin *et al.*, 1985; Appel, 1993; Barbehenn & Constabel, 2011). This is not to say that tannins are harmless to insects. Instead, the alkaline conditions may promote the oxidation of tannins and other phenolic compounds, leading to the formation of oxygen radicals and, consequently, cell damage (Appel, 1993). For example, the larvae of some tropical lepidopteran species were found to contain the oxidation products of polyphenols in their frass (Vihakas *et al.*, 2015). Likewise, *Lymantria dispar, Orgyia Leucostigma* and *Malacosoma distria* caterpillars that fed on maple leaves with high concentrations of ellagitannins had high levels of semiquinone radicals in their midguts together with increased protein carbonyl contents that suggested increased oxidation of the proteins in the gut (Barbehenn *et al.*, 2005, 2013).

The above examples demonstrate that measuring the activity of polyphenol mixtures has provided a better indication of their probable effects in different biological systems compared with estimates of 'total tannins' or 'total phenolics'. Fortunately, new analytical techniques are now available that allow the broad characterisation of complex polyphenol mixtures in plants. For example, as part of a related study on the phylogeny of tannins in eucalypts, we used ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS, i.e. the Engström method; Engström *et al.*, 2014, 2015; Salminen, 2018b) to measure the concentrations of a range of phenolic subgroups, including four subgroups of tannins, in leaves from 628 eucalypts representing 515 species (Marsh *et al.*, 2017a). This provides an ideal opportunity to examine the relationship between plant polyphenol composition and traditional *in vitro* measures of biological activity. Knowing what groups of tannins are active also improves our ability to identify the genetic architecture of tannin production. For example, Skovmand *et al.* (2018) recently argued that genes responsible for variations in tannins in plants may be 'keystone genes' that are critical to ecosystem function.

Eucalypts are the dominant forest and woodland trees in Australia, with *c.* 900 species belonging to the genera *Eucalyptus, Angophora* and *Corymbia* (Bayly *et al.*, 2013). Eucalypt foliage makes up a large proportion of the diet of four species of pials – the koala (*Phascolarctos cinereus*), greater glider roides volans*), common ringtail possum (*Pseudocheirus pere* and common brushtail possum (*Trichosurus vulpecula*) (*et al.*, 2004) – and a wide variety of insect herbivores (Morrow, 1981; Paine *et al.*, 2011). Importantly, eucalyp tain variable concentrations and types of tannins and polyphenols, suggesting that there may also be significan tion in biological activity associated with herbivore c (Marsh *et al.*, 2017a).

We had several expectations about how specific tanni groups would influence traditional measures of *in vitro* bic activity (protein precipitation capacity, oxidative activi capacity to reduce digestible N) of eucalypt samples (Ta However, we also understood that our hypotheses are c cated by the fact that individual tannins within the sam group can differ over six-fold in both their protein precip (Karonen *et al.*, 2015) and their oxidative activities (Moila Salminen, 2008). Thus, differences in the types and con tions of individual tannins between eucalypt species could ence the degree of the response to the standard Nevertheless, we expected to reveal the major patterns b the tannin groups and their bioactivities, and hoped to se of the more detailed patterns within the tannin subgroups.

We anticipated that protein precipitation capacity wo positively correlated with the concentrations of proanthocy (PAs; also known as condensed tannins), their mean de

Table 1 The polyphenol constituents measured in this study, and th expected effects on biological activity.

Constituent	Polyphenol class	Expected eff biological act	
		PPC	OA
Small procyanidin	PA	+	
Medium procyanidin	PA	+	
Large procyanidin	PA	++	
Small prodelphinidin	PA	+	+
Medium prodelphinidin	PA	++	+
Large prodelphinidin	PA	+++	+
% prodelphinidin	PA	+	+
mDP	PA	++	
Early-eluting HHDP derivatives	HT		+++
Late-eluting HHDP derivatives	HT	+	++
Early-eluting galloyl derivatives	HT		++
Late-eluting galloyl derivatives	HT	++	+
Kaempferol derivatives	Flavonol		
Quercetin derivatives	Flavonol		
Myricetin derivatives	Flavonol		+
Quinic acid derivatives	Flavonoid		+

A '+' indicates an expected positive relationship, with '++' and '+++ indicating the constituents hypothesised to have the strongest effec biological activity.
PA, proanthocyanidin; HT, hydrolysable tannin; PPC, protein precipitation capacity (mg g^{-1} DM pentagalloyl glucose equivalent OA, oxidative activity (mg g^{-1} DM gallic acid equivalents); CND, capacity to reduce N digestibility (percentage units); mDP, m degree of polymerisation of proanthocyanidins; HHDP, hexahydroxydiphenoyl.

polymerisation (mDP) and the proportion of prodelphinidin subunits in PAs (Jones *et al.*, 1976; Porter & Woodruffe, 1984; McManus *et al.*, 1985; Aerts *et al.*, 1999). At a finer scale, we used the Engström method (Engström *et al.*, 2014) to measure PA concentrations at three cone voltages (see Salminen, 2018b), which provides additional information about the size distribution of PAs. We expected that large prodelphinidin-rich PAs in particular would contribute positively to the protein precipitation capacity of samples, assuming that these types of PAs were in sufficient concentrations in the extracts. Because most of the galloyl groups in our samples originate from monomeric ellagitannins or simple galloyl glucoses, rather than gallotannins (Marsh *et al.*, 2017a), and because gallotannins have a better protein precipitation capacity compared with monomeric ellagitannins or simple galloyl glucoses (Haslam, 1988; Kawamoto *et al.*, 1996; Kilkowski & Gross, 1999; Salminen & Karonen, 2011), we did not expect a strong correlation between protein precipitation capacity and galloyl-derivative concentrations as such, unless the galloyl derivatives were the main tannins of the species.

We hypothesised that the properties of tannins that affect protein precipitation capacity would also influence their capacity to reduce *in vitro* N digestibility. The gut contains both exogenous protein from the diet and endogenous proteins, such as enzymes, sloughed mucosal cells and microbial protein. Tannins that precipitate any of these proteins will reduce the apparent digestibility of N. We therefore expected that the concentration, size and composition of PA molecules would strongly influence the capacity to reduce *in vitro* digestible N, and that there would be a positive correlation between protein precipitation capacity and capacity to reduce *in vitro* N digestibility in eucalypts.

In contrast to the major role that PAs may play in influencing protein precipitation capacity and capacity to reduce N digestibility, we predicted that the concentration of hexahydroxydiphenoyl (HHDP) derivatives (i.e. ellagitannins), particularly those that elute earlier during UPLC separation, would drive the oxidative activity of eucalypt samples, if they are present at sufficient concentrations compared to other oxidatively active compounds in the samples. This is because ellagitannins appear to be the class of tannins that are most oxidatively active at high pH (Barbehenn *et al.*, 2006; Moilanen & Salminen, 2008), and those with shorter UPLC retention times tend to oxidise more readily than those with longer retention times (Salminen *et al.*, 2011; Moilanen *et al.*, 2013). Other polyphenol constituents, including prodelphinidin subunits of PAs and myricetin derivatives, with pyrogallol-type substitution of the flavonoid B-ring, may also contribute to oxidative activity to a lesser extent (Vihakas *et al.*, 2014).

Our final prediction was that there would be a negative correlation between oxidative activity and protein precipitation capacity. This prediction was made for two reasons. First, hydrolysable tannins (HTs) with high protein precipitation capacity tend to have lower oxidative activity (Moilanen *et al.*, 2013). And second, there is an inverse relationship between the concentrations of PAs and HTs in eucalypt leaves, probably because they compete for biosynthetic pathways (Marsh *et al.*, 2017a). Given the expected reciprocal effects of PAs and HTs on oxidative activity and protein precipitation capacity, protein precipitation capaci be higher in those samples dominated by PAs, while s dominated by HTs may be more oxidatively active.

Materials and Methods

The collection of 628 leaf samples (515 eucalypt species fr allied genera *Eucalyptus* L'Her., *Corymbia* Hill & Johnsc *Angophora* Cav. – duplicates of species were predominantly ent subspecies) from Currency Creek Arboretum, South Au and the measurement of the phenolic composition of these s by UPLC-MS/MS is described in detail in Marsh *et al.* (2 Hydrolysable tannins (HHDP and galloyl derivatives), hc were re-integrated to determine concentrations of early-eluti late-eluting derivatives for each group. We used the dimeric tannin, oenothein B, as a marker to distinguish the dif between these two groups. The early-eluting HHDP and derivatives eluted before oenothein B (0.5–2.9 min), and th eluting ones were those from oenothein B onwards (2.9–(Likewise, we re-analysed the previously collected data to det the concentration of procyanidins and prodelphinidins in (the three size classes explained in Salminen (2018b). The procyanidin and prodelphinidin oligomers were detected b voltages of 75 and 55 V, the medium procyanidin and prode din oligomers and polymers by cone voltages of 85 and 80 the large procyanidin and prodelphinidin polymers by con ages of 140 and 130 V, respectively. A summary of the pl constituents that were measured is given in Table 1, alon their expected effects on biological activity.

Protein precipitation capacity

The protein precipitation capacity of eucalypt extracts was tified by the radial diffusion assay (Hagerman, 1987) usin as the protein and pentagalloylglucose as the quantificatio dard. Briefly, the original, nondiluted eucalypt extract (*et al.*, 2017a) was concentrated two-fold via freeze-dryin redissolving into Milli-Q water. A 24 μl aliquot of this c trated extract was applied to three wells punched onto a Pe filled with BSA-agar gel. The Petri dishes were covere parafilm and incubated at 30°C for 72 h to form reproc rings with tannins and BSA. The ring area was documente a camera on a tripod and measured by the IMAGEJ software.

Oxidative activity

The portion of total phenolics that was easily auto-oxid pH 10 was measured as both mg g^{-1} dry weight and as a p age of total phenolics using the method of Salminen & K (2011), calibrated with gallic acid. In short, the total pl content of 15× dilutions (Milli-Q water) of the original eu extracts (Marsh *et al.*, 2017a) and the pH 10-oxidised e were measured with a well-plate reader at 730 nm. The diffe in the total phenolic concentrations between these measure revealed the level of easily oxidised phenolics in the sampl the oxidative activity in mg g^{-1} or in % of total phenolics).

Capacity to reduce *in vitro* N digestibility

A subset of leaves from each tree were freeze dried and then ground in a Foss Cyclotec 1093 mill (Foss, Höganäs, Sweden) until they passed through a 1 mm sieve. The capacity to reduce *in vitro* N digestibility was determined using a modified version of the method of DeGabriel *et al.* (2008). The method involves sequential digestion of samples of ground foliage in acid pepsin and cellulase in the presence and absence of polyethylene glycol 4000 (PEG), which binds to both HTs and PAs (Silanikove *et al.*, 1996; Schofield *et al.*, 2001).

For each sample, 0.8050 ± 0.0050 g leaf powder was weighed into each of four Ankom F57 fibre filter bags (Ankom Technology, Macedon, NY, USA). Two bags per sample were placed into beakers (100 bags per beaker) containing 25 ml Tris-base buffer solution (pH 7.1) per bag with 33.33 g l^{-1} PEG. The remaining bags were placed into the buffer solution without PEG. Samples were incubated at 37°C for 24 h, after which they were washed thoroughly with water and dried to constant mass at 40°C. Bags were then placed into 25 ml per bag of 0.1 M HCl containing 2 g l^{-1} pepsin for 48 h at 37°C. Samples were removed from the pepsin solution and washed briefly, before a final incubation at 37°C for 24 h in 25 ml per bag of 100 mmol acetic acid buffer (pH 4.75) containing 6.25 g l^{-1} cellulase. Samples were washed thoroughly, dried at 40°C to constant mass and weighed.

After the digestion process, the N concentration was determined in 120 ± 20 mg of residue from each bag, as well as in the original ground leaf samples, using a Leco Truspec C/N analyser (Leco Corporation, Sydney, NSW, Australia). These values were used to calculate the *in vitro* digestibility of N in the presence and absence of PEG. Any samples with a coefficient of variation > 5% between duplicate analyses were repeated. The capacity to reduce *in vitro* N digestibility was calculated as the difference in N digestibility between samples incubated with and without PEG. The digestibility with PEG was used rather than total N, because the total N of a plant will never be completely digested, due to some of the N being bound in complex cell-wall polymers.

Statistical methods

We compared the mean biological activity (protein precipitation capacity, oxidative activity and capacity to reduce *in vitro* N digestibility) of eucalypt phylogenetic clades (see Marsh *et al.*, 2017a for the allocation of species to clades) using the gls function in package NLME for R (Pinheiro *et al.*, 2017) and Pagel's λ covariance structure (R package APE; Paradis *et al.*, 2004) to account for the phylogenetic nonindependence of samples. For all analyses, the dataset and analyses were at the individual tree level, with multiple subspecies and trees observed for some species, while other species were observed only once. Pagel's λ model is equivalent to including a within-species error term in addition to phylogenetic between-species term, and so is suitable for data where there are multiple observations per species. We used Tukey contrasts to determine which clades differed significantly ($P < 0.05$) from one another. Residuals were checked for normality and variance homogeneity in all models.

We estimated correlations between protein precip
capacity and oxidative activity, protein precipitation capac
capacity to reduce *in vitro* N digestibility, and oxidative a
and capacity to reduce *in vitro* N digestibility using the co
function in the APE package in R to take the phylogenetic n
ness of species into account. Standard errors and *t*-statist
the estimated correlations were calculated using a para
bootstrap with 30 replicates under the null hypothesis o
pendence, using simple phylogenetic regression models
using the gls function in the APE package in R.

We also used the gls function to fit four regression moc
each of the square root of protein precipitation capacity,
tive activity and the square root of the capacity to red
digestibility, while taking the phylogeny into account. The
root transformation was applied to two of these three dep
variables in order to better satisfy the assumption of norma
tributed errors with equal variances. The first model fo
dependent variable contained the intercept only. The
model contained the total polyphenol concentration (the s
all measured constituents). The third model contained two
total tannins (the sum of all constituents in the PA ar
classes; Table 1) and total flavonols (the sum of all constitu
the flavonol class; Table 1). The fourth model contained a
vidual constituents listed in Table 1. We tested the significa
the fourth model using likelihood ratio tests relative to the
and third models, which are both submodels of it.

To explore which constituents were most important in p
ing biological activity, we performed a backward selectior
of the covariates in the fourth model outlined in the pr
paragraph for each of the three transformed activities ba
the significance of omitting variables as calculated using
squared likelihood ratio test with a cutoff of 0.05 for signif
So that models made biological sense, either the total con
tion of prodelphinidin (sum of the concentrations of p
phinidin from small, medium and large PAs; Table 1
allowed in the model or one or more of the separate
medium or large concentrations of prodelphinidin. Co
tions of total prodelphinidin with prodelphinidin subgroup
not allowed. The total procyanidin (or small, medium and
total HHDP derivatives (or early and late) and total gall
derivatives (or early and late) variables were treated sir
Residuals were checked for normality and homogeneity o
ances, and six outliers with high leverage were removed.
included two samples with oxidative activity and three s
with protein precipitation capacity almost double that of th
highest samples, and a sample which turned out to be an
in regression models of *in vitro* N digestibility.

Results

In vitro polyphenol-derived biological activity

We found wide variation in the protein precipitation ca
(0–229 mg g^{-1} dry matter (DM) pentagalloyl glucose e
lents), oxidative activity (2–94 mg g^{-1} DM gallic acid e
lents, or 3–79% of total phenolics) and capacity to

in vitro N digestibility (0–89 percentage units) between eucalypt species (Table 2). Mean biological activity did not differ between phylogenetic clades when the relatedness of species was taken into account (Table 2).

The capacity to reduce *in vitro* N digestibility was not correlated with either the oxidative activity ($t=-0.95$, $P=0.341$) or protein precipitation capacity of eucalypt leaves ($t=0.36$, $P=0.719$). However, there was a positive correlation between the protein precipitation capacity and oxidative activity of samples ($r=0.54$, $t=3.90$, $P<0.001$).

Polyphenol composition and protein precipitation capacity

The model containing all of the polyphenol constituents in Table 1 explained significantly more of the variation in the square root of protein precipitation capacity compared with models that contained only the total polyphenol concentration or a combination of the total tannin and total flavonol concentrations ($P<0.001$ for both model comparisons).

There were strong positive relationships between protein precipitation capacity and the concentrations of late-eluting HHDP derivatives (Fig. 1a), procyanidin subunits from polymeric PAs (Fig. 1b), galloyl derivatives and prodelphinidin subunits from medium-sized PAs (Table 3). Although the relationships were not as strong, the concentration of early-eluting HHDP derivatives and the mDP of PAs were negatively correlated with protein precipitation capacity (Table 3). Pagel's lambda for the model was 0.31 (likelihood ratio $\chi^2=17.3$ on 1 degree of freedom, $P<0.001$).

Polyphenol composition and oxidative activity

The full model containing all measured polyphenol constituents explained significantly more variation in oxidative activity compared with either the total polyphenol concentration alone, or a combination of the total tannin and total flavonol con tions, with $P<0.001$ for both model comparisons.

The total concentration of HHDP derivatives had strong effect on the oxidative activity of samples (Fig. 2a) there were also strong positive relationships between the ox activity of samples and the concentrations of prodelphinid units from large PAs (Fig. 2b), and early-eluting galloyl tives (Table 4). The mDP of PAs and the concentrati quercetin and quinic acid derivatives were also positively lated with oxidative activity, while the concentration of phinidin subunits from medium-sized PAs was neg correlated (Table 4). Pagel's lambda for the model was 0.5 lihood ratio $\chi^2=21.9$ on 1 degree of freedom, $P<0.001$).

Table 2 The mean (range) biological activity of species belonging to different eucalypt clades.

Phylogenetic clade	*n*	PPC[1]	OA[2]	CND[3]
Angophora	5	25 (9–59)	21 (6–40)	19 (8–44)
Corymbia I	19	18 (0–40)	16 (5–30)	29 (11–45)
Corymbia II	13	28 (5–53)	19 (6–34)	24 (13–38)
Eudesmia	10	42 (6–92)	29 (10–50)	40 (1–89)
Monocalyptus	79	36 (0–122)	19 (4–56)	25 (0–70)
Symphyomyrtus I	32	25 (0–89)	12 (2–51)	10 (0–22)
Symphyomyrtus II	97	26 (0–98)	19 (3–48)	11 (0–68)
Symphyomyrtus III	125	43 (0–229)	22 (2–86)	9 (0–38)
Symphyomyrtus IV	76	30 (0–113)	22 (2–94)	28 (0–67)
Symphyomyrtus V	166	40 (0–201)	20 (3–50)	10 (0–58)
Pagel's λ		0.46	0.77	0.82
F-statistic		0.99	1.65	1.60
P-value		0.453	0.091	0.103

[1]Protein precipitation capacity (mg g^{-1} DM pentagalloyl glucose equivalents).
[2]Oxidative activity (mg g^{-1} DM gallic acid equivalents).
[3]Capacity to reduce N digestibility (percentage units).

Polyphenol composition and capacity to reduce *in viti* digestibility

The full model containing all measured polyphenol const explained significantly more of the variation of the square the capacity to reduce *in vitro* N digestibility compare models containing only the total polyphenol concentratio combination of the total tannin and total flavonol concent ($P<0.001$ for both model comparisons).

There were strong positive relationships between the c to reduce *in vitro* N digestibility and the proportion of phinidin in PAs (Fig. 3a), and the mDP of PAs (F Table 5). The concentration of PD in small PAs had a negative effect on the capacity to reduce *in vitro* N diges (Table 5). There was a weaker negative relationship betw capacity to reduce *in vitro* N digestibility and the concen of quercetin derivatives (Table 5). Pagel's lambda for the was 0.66 (likelihood ratio $\chi^2=72.0$ on 1 degree of fr $P<0.001$).

Discussion

The current study demonstrates that quantifying tanni groups in complex polyphenol mixtures provides valuable mation about the biological activity of plant sample subgroup composition of tannins in eucalypt extracts ex significantly more of the variation in oxidative activity, precipitation capacity and capacity to reduce *in vi* digestibility compared with the sum of the concentrations measured constituents. This is not necessarily surprising, b eucalypt species with the same total polyphenol concen can have vastly different polyphenol profiles (Marsh 2017a). Nevertheless, researchers frequently (and often cessfully) attempt to relate total tannin concentrations example, various measures of herbivory (Ayres *et al.*, 1997; *et al.*, 2014; Masette *et al.*, 2015; Volf *et al.*, 2015; Felto 2018).

Different tannin subgroups influenced different types logical activity, although not all of these correlations match expectations. For example, concentrations of late-eluting H derivatives (ellagitannins) were more strongly correlate protein precipitation capacity compared with concentrati

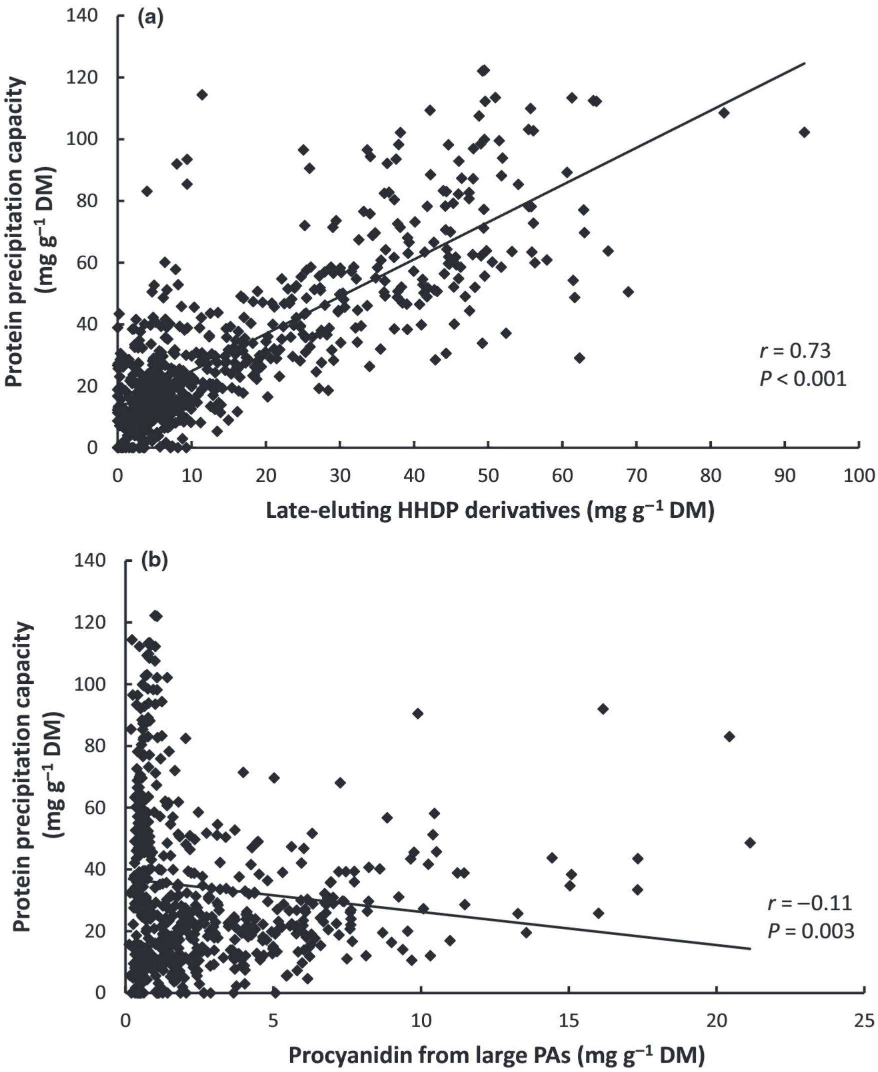

Fig. 1 The relationship between the p precipitation capacity of eucalypt leav the two polyphenol constituents that l strongest correlation with this measur ($n = 628$): (a) late-eluting hexahydroxydiphenoyl (HHDP) deriva and (b) procyanidin subunits from pol proanthocyanidins (PAs). Note that th relationships are indicative only, becau they do not take into account other covariates or phylogenetic correlation the statistical model.

PA subunits. It is likely, however, that high concentrations of ellagitannins relative to PAs drove this association. Likewise, the size and composition of PAs, rather than their concentration, correlated most strongly with the capacity to reduce *in vitro* N

Table 3 Final statistical model showing the phenolic constituents that had a significant effect on the square root of protein precipitation capacity.

Model term	Parameter estimate	SE	*t* statistic	*P*-value	Standardised coefficient
(Intercept)	2.393	0.439	5.45	< 0.001	
Late HHDP	0.120	0.006	18.94	< 0.001	0.882
Large procyanidin	0.219	0.018	11.89	< 0.001	0.298
Total galloyl	0.056	0.006	8.75	< 0.001	0.235
Medium prodelphinidin	0.178	0.022	8.17	< 0.001	0.209
Early HHDP	−0.027	0.010	−2.73	0.007	−0.112
mDP of PAs	−0.047	0.018	−2.59	0.010	−0.070

Degrees of freedom for all *t*-statistics is 563 ($n = 625$).
HHDP, hexahydroxydiphenoyl derivatives; mDP of PAs, mean degree of polymerisation of proanthocyanidins.

digestibility. As expected, however, the concentration of tannins had the greatest effect on the oxidative activity c ples. This study significantly advances our understand structure–function relationships in natural plant tannin mi and demonstrates that modern analytical techniques sho incorporated into studies examining relationships betwee nins and herbivory. Below, we discuss the relationships b phenolic composition and biological activity in greater and the implications for herbivores consuming foliage.

Protein precipitation by tannins has been advocated as a anism by which plants can defend themselves against a vai mammalian herbivores, including against those that feed oi lypt foliage (Marsh *et al.*, 2003; DeGabriel *et al.*, 2009). A concentrations, in a broad sense, PAs have a greater capaci gallotannins or ellagitannins to precipitate protein, alt accurate compound-specific studies with PAs are scarce (H 1988; Kilkowski & Gross, 1999; Salminen & Karonen, The tannin profiles of many of the eucalypts that we ai were dominated by ellagitannins, with some having con tions > 100 mg g^{-1} (Fig. 2a; Marsh *et al.*, 2017a). In this tion, higher concentrations may compensate for

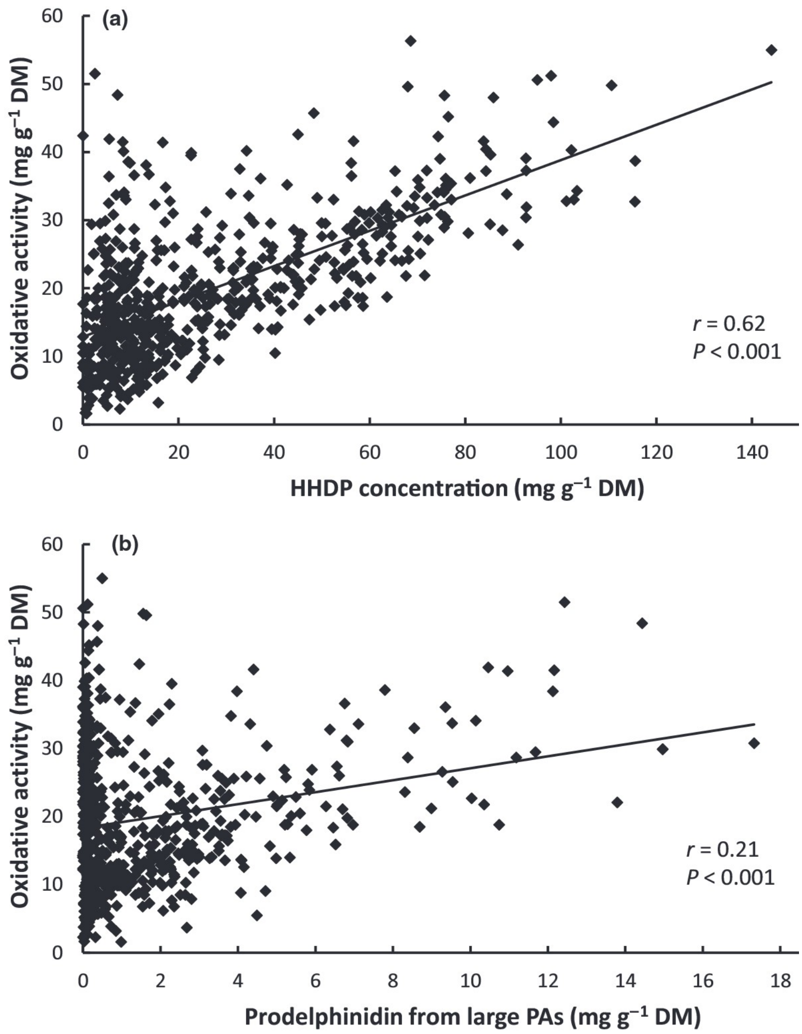

Fig. 2 The relationship between the oxidative activity of eucalypt leaves and the two polyphenol constituents that had the strongest correlation with this measurement ($n = 628$): (a) hexahydroxydiphenoyl (HHDP) derivatives, and (b) prodelphinidin subunits from large proanthocyanidins (PAs). Note that these relationships are indicative only, because they do not take into account other covariates or phylogenetic correlations from the statistical model.

bioactivity, and can make a significant contribution to protein precipitation capacity (Johnson *et al.*, 2014). This probably explains why the protein precipitation capacity of eucalypt extracts was most strongly correlated with the concentration of late-eluting HHDP derivatives, and then secondarily with the concentrations of procyanidin subunits in large PAs and prodelphinidin subunits in medium-sized PAs.

Our finding that the concentration of late-eluting HHDP derivatives had a greater impact on protein precipitation capacity compared with early-eluting derivatives supports previous work that individual ellagitannins can differ greatly in their capacity to precipitate protein (Salminen *et al.*, 2011; Moilanen *et al.*, 2013). Purified ellagitannins with greater protein precipitation capacity tend to elute later during reversed-phase LC analyses due to their greater structural flexibility, lower water solubility and higher molecular mass (Salminen *et al.*, 2011; Moilanen *et al.*, 2013; Karonen *et al.*, 2015; Engström *et al.*, 2019). The following four structural features primarily increase the protein precipitation capacity and retention time of ellagitannins: the number of galloyl, HHDP and other functional groups attached to the central glucose core; the presence of two galloyls instead of one HHDP that is formed by C–C coupling of the two galloyls; the pr of a central glucopyranose unit instead of acyclic glucose; a oligomerisation degree of the ellagitannin (Engström 2019). Thus, separately integrating early- and late-eluting tannins in complex mixtures of unidentified ellagitannins provide useful information about the proportional bio activity of these compounds. In addition, their elution p could give a hint of their structures that could be then veri UV and MS spectra (Moilanen *et al.*, 2013).

Quantifying PAs within different size classes (i.e. the En method; Engström *et al.*, 2014; Salminen, 2018b) coul improve our understanding of how different mixtures affect biological activity. The statistical models for all thr logical activities identified correlations between biological and specific size classes of PA subunits, rather than total c trations. This suggests that we need a better understanding biological activities of individual PAs, and again illustrat understanding the relationship between biological activi tannins is so difficult in plant samples; variation in tannin position, even within subgroups of tannins, influences bio activity.

Table 4 Final statistical model showing the phenolic constituents that had a significant effect on oxidative activity.

Model term	Parameter estimate	SE	*t* statistic	*P*-value	Standardised co
(Intercept)	6.656	3.344	1.99	0.047	
Total HHDP	0.303	0.012	24.78	< 0.001	0.763
Large prodelphinidin	2.801	0.420	6.67	< 0.001	0.737
Early galloyl	0.329	0.086	3.84	< 0.001	0.113
mDP of PAs	0.235	0.092	2.56	0.011	0.079
Medium prodelphinidin	−1.015	0.411	−2.47	0.014	−0.271
Quercetin	0.259	0.111	2.33	0.020	0.063
Quinic acid	0.273	0.125	2.19	0.029	0.067

Degrees of freedom for all *t*-statistics is 563 ($n = 626$).
HHDP, hexahydroxydiphenoyl derivatives; mDP of PAs, mean degree of polymerisation of proanthocyanidins.

Some of the eucalypt samples in our study possessed very high oxidative activity (up to 94 mg g^{-1} DM gallic acid equivalents). This is at the higher end of values that have been reported in other plant species (Vihakas *et al.*, 2014, 2015). In theory, the oxidative capacity of polyphenols could affect plant resistance to insect herbivory (Appel, 1993), but this has yet to be demonstrated conclusively. Oxidative polyphenols may a more effective against some insect species than against othe example, in two species of lepidopteran larvae, *A aurantiaria* was much more efficient than *Epirrita autum* metabolising pentagalloylglucose, a model hydrolysable (Salminen, 2018a). Interestingly, even though *A. aura*

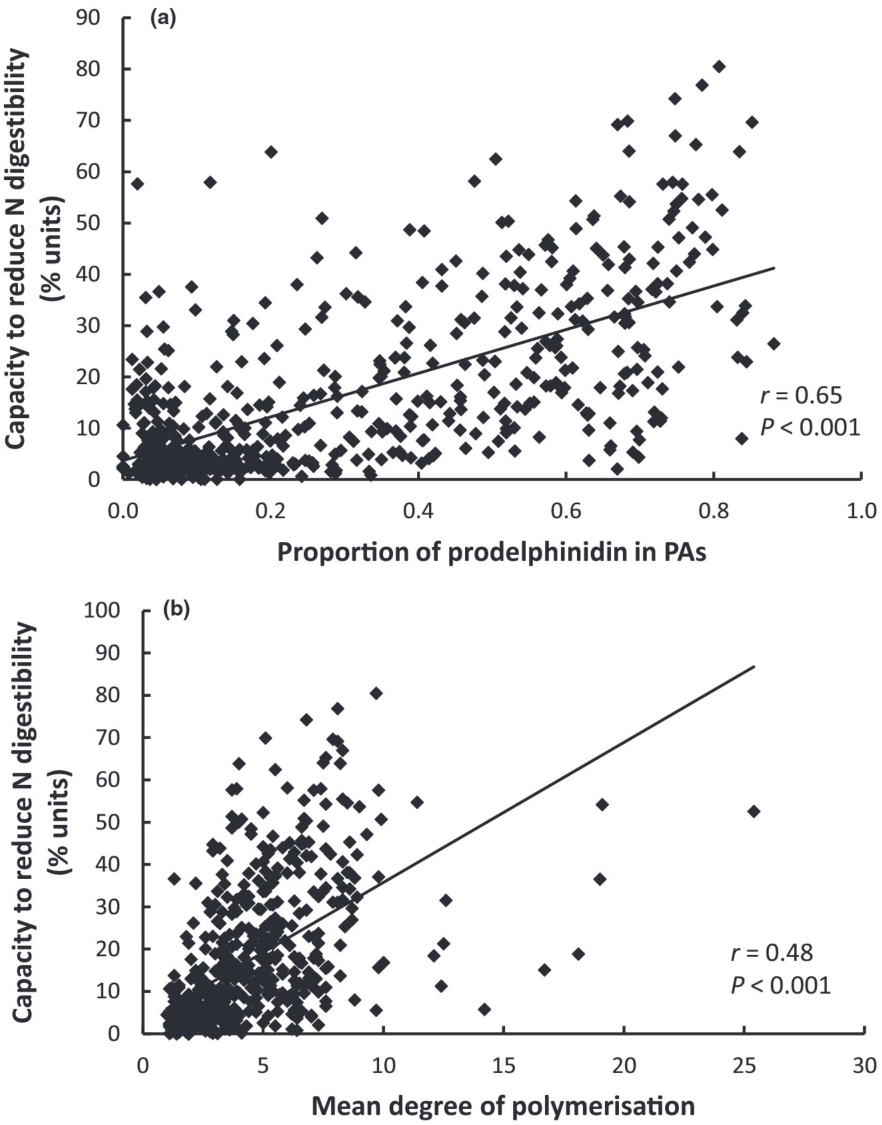

Fig. 3 The relationship between the c to reduce the *in vitro* nitrogen (N) digestibility of eucalypt leaves and (a) proportion of proanthocyanidins (PAs comprising prodelphinidin, and (b) the degree of polymerisation (mDP) of proanthocyanidins ($n = 628$). Note tha relationships are indicative only, becau they do not take into account other covariates or phylogenetic correlation the statistical model.

Table 5 Final statistical model showing the phenolic constituents that significantly influenced the square root of the capacity to reduce *in vitro* N digestibility.

Model term	Parameter estimate	SE	*t* statistic	*P*-value	Standardised co
(Intercept)	5.883	0.831	7.08	< 0.001	
% prodelphinidin	3.244	0.308	10.54	< 0.001	0.420
mDP of PAs	0.294	0.028	10.37	< 0.001	0.503
Small prodelphinidin	−0.217	0.023	−9.35	< 0.001	−0.376
Quercetin	−0.061	0.025	−2.48	0.014	−0.075

Degrees of freedom for all *t*-statistics is 566 ($n = 627$).
mDP of PAs = mean degree of polymerisation of proanthocyanidins.

appears to have a higher gut pH than *E. autumnata* (Kim *et al.*, 2018), pentagalloylglucose was not more harmful to *A. aurantiaria*. While we learn more of plant chemistry, we should also examine the effects on herbivores, because plant chemistry alone cannot reveal the fate of the compounds in herbivores.

Eucalypts could be an ideal system in which to test hypotheses that relate oxidative activity to insect herbivory because there is wide variation in oxidative activity between eucalypt species (this study) and between individuals within species (Marsh *et al.*, 2017b), a variety of insect herbivores feed on eucalypt foliage (Fox & Morrow, 1981; Paine *et al.*, 2011), and herbivory by insects differs between eucalypt species and individuals (Fox & Macauley, 1977; Paine *et al.*, 2011; Marsh *et al.*, 2017b). If oxidative activity does deter herbivory by some insects, our results suggest that the most likely eucalypts to benefit would be those containing high concentrations of ellagitannins, as well as prodelphinidin subunits in large PAs. Caffeic acid derivatives, such as caffeoyl quinic acids, are also efficiently oxidised at high pH, and by plant oxidative enzymes (Kim *et al.*, 2018). These compounds could be important in eucalypts as well, because quinic acid derivatives partially determined the oxidative activity of eucalypt extracts.

The fact that ellagitannin concentrations had strong effects on both protein precipitation capacity and oxidative activity probably explains why there was a positive correlation between the two biological activities. On the surface, this suggests that plants containing high concentrations of ellagitannins might be somewhat protected against both mammalian (through protein binding) and insect (through oxidation) herbivores. However, before making these sorts of assumptions, we need a better understanding of the relationship between specific *in vitro* activity and the *in vivo* effects of tannins on herbivores. This is particularly pertinent given that ellagitannin concentrations did not affect the capacity to reduce *in vitro* N digestibility, even though they affected protein precipitation capacity.

This is the first study to investigate the particular structural features of tannins that correlate with changes in *in vitro* N digestibility, which can affect habitat quality and the reproductive success of marsupial folivores (DeGabriel *et al.*, 2009; Youngentob *et al.*, 2011). The results suggest that PAs might be particularly important in influencing *in vitro* N digestibility. Despite our expectations, different tannin subgroups affected the capacity to reduce *in vitro* N digestibility relative to *in vitro* protein precipitation capacity. The capacity to *in vitro* N digestibility was strongly positively correlate the proportion of prodelphinidin subunits in PAs, ar mDP of PAs. Both of these factors have previously been to influence protein precipitation capacity generally (e.g. *et al.*, 1976; Porter & Woodruffe, 1984; Kumar & Hor 1986; Osborne & McNeill, 2001; Lokvam & Kursar, Huang *et al.*, 2011; Saminathan *et al.*, 2014), which m surprising that there was a negative relationship betwe protein precipitation capacity of eucalypt samples ar mDP of PAs. Nevertheless, this could be due to a negati relation between the mDP of PAs and the concentrat late-eluting HHDP derivatives (data not shown). It wo useful to know what happens to the hydrolysable tanr samples during the digestible N assay, such as whethe dissociate from protein or hydrolyse in response to th conditions, because they clearly precipitate protein in th tein precipitation capacity assay.

The results of our study demonstrate that the biologica ity of tannin mixtures in plants is a complex trait relying eral classes of compounds and, probably, many ind structures, as well as the specific conditions and proteins e tered after ingestion by a herbivore. Despite recent breakth in identifying genes underlying some aspects of tannin st (e.g. Liu *et al.*, 2016), there are unlikely to be genes of larg that explain this biological activity (Kulheim *et al.*, 2011). mand *et al.* (2018) argue that the genes responsible for synthesis could act as keystone genes influencing many eco processes, but the complexity of tannin composition in f tion trees, such as eucalypts, suggests that genes of large ef not likely.

Conclusions

Our study shows that the tannin composition of plant e affects their biological activity. In particular, it is possible cidate the broad structural features that contribute to bio activity, even when each individual compound in a comple ture has not been identified. This is important, because firms that the new analytical techniques utilised here coul valuable tool allowing researchers to understand how the c sition of a widespread group of plant secondary metabolit as the tannins influences the ecology of both individuals an ulations of herbivores. It also suggests that future studi

characterise the individual tannins in specific subgroups could provide more detailed insight into the major patterns revealed in the current work.

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Author contributions

WJF, IRW and J-PS conceived the idea. DN and IRW collected the samples, and KJM and J-PS conducted the chemical assays. KJM, CK and RC analysed the data. KJM led the writing of the manuscript, with contributions from all other authors.

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