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Genetic- and chemical-based resistance to two mammalian herbivores varies across the geographic range of *Eucalyptus globulus*

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ABSTRACT

Background: The lifetime fitness of a plant depends in part on how it responds to interspecific biotic interactions and these interactions can play major roles in shaping the evolution of many phenotypic traits. The interactions between a single plant and its many herbivores may occur independently, or the direction and rate of any evolutionary change in plant defensive traits may be a result of interacting selection pressures.

Questions: Does a single genetically based defensive trait in *Eucalyptus globulus* confer resistance to two mammalian herbivores? Is there a genetically based correlation for resistance to browsing by alternative herbivores? Does the strength of the relationship between plant defensive chemistry and resistance to both herbivores differ across the geographic range of *E. globulus*?

Methods: We offered foliage from a common garden trial (from different genetic hierarchies; race, population, and family) to two herbivores and analysed plants for defensive chemistry. We examined the relationship between relative plant resistance to both herbivores and plant secondary chemistry, both across and within races of *E. globulus*. Furthermore, we examined the genetic-based correlation in preference between herbivores.

Results: Resistance to both herbivores is mediated by genetically based variation in formylated phloroglucinol compounds, particularly sideroxylonal. There was a positive correlation in herbivore resistance among plant populations reared under common conditions and a correlation in resistance within races, thus the preferences and subsequent selection imposed by alternate herbivores are not independent. We discuss these results in light of herbivores as possible agents of selection on plant secondary metabolites in *E. globulus*. In addition, the relationship between chemistry and foliage susceptibility varies across the geographic range of *E. globulus*, suggesting that the extant selection surface is not uniform within the species.

Keywords: eucalypts, genetic basis, marsupials, plant secondary metabolites.

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INTRODUCTION

The lifetime fitness of an organism depends in part on how it responds to interspecific biotic interactions and these interactions can play major roles in shaping the evolution of many phenotypic traits through selection (Futuyma, 1998). The strength and direction of selection on traits for any one species can vary throughout its evolutionary history. Some of the factors that lead to such variation include the presence of multiple selective pressures (Strauss and Irwin, 2004; Agrawal, 2005), the genetic architecture (e.g. the number of genes affecting a given trait and the mode of action of the genes) of the interacting species, and temporal and geographic variation in selection (Thompson, 2005). In addition, traits may undergo change through indirect selection arising from genetic correlations with traits undergoing direct selection (Falconer and Mackay, 1996). These concepts are central to understanding the evolution of species interactions and ultimately the formation, function, and diversification of biotic communities (Whitham *et al.*, 2003, 2006).

Plant–herbivore interactions represent a model approach to investigate the ecological and evolutionary relationships between species. Plants are commonly browsed by a community of herbivores where species may represent the same (e.g. leaf chewers) or different (e.g. leaf chewers and sap suckers) feeding guilds. Consequently, when examining the ecological and evolutionary relationships between plants and their herbivores, it is important not only to consider the single, pair-wise relationships between a plant and a herbivore (e.g. Strong *et al.*, 1993; Adler *et al.*, 1995; Zangerl and Berenbaum, 2004), but also the relationship between plant traits and multiple herbivores (e.g. Gómez, 2008). The interactions between a single plant and its many potential herbivores may occur independently or, conversely, the direction and rate of any evolutionary change in plant defensive traits may be a result of combined herbivory pressures (Stinchcombe and Rausher, 2001; Lankau, 2007; Wise, 2007; Lankau and Strauss, 2008). As such, any evolutionary relationships that exist would do so in a diffuse rather than a pair-wise manner (e.g. Simms and Rausher, 1989; Cronin and Abrahamson, 2001; Agrawal, 2005; Strauss *et al.*, 2005). Investigation of the responses of more than one herbivore to the same or different plant defensive traits provides insight into the type of relationships that exist between a plant species and its herbivores. In addition, analysis of the genetic correlations in preferences by various herbivores also indicates if interactive relationships occur (Leimu and Koricheva, 2006). A lack of correlation implies that the relationships are independent and pair-wise, presumably with herbivores responding to different defensive traits. In contrast, a significant correlation implies that the relationships are not independent and hence that herbivores may be influencing the same defensive traits.

Eucalyptus globulus is a dominant tree in native forests in south-eastern Australia and foliage is consumed by several marsupial herbivores. *Eucalyptus globulus* exhibits heteroblasty during ontogeny (Jordan *et al.*, 1999) and juvenile foliage is accessible to both terrestrial and arboreal marsupials, while adult foliage (present in a taller tree) is only accessible to arboreal marsupials. The arboreal common brushtail possum (*Trichosurus vulpecula*) and the terrestrial red-bellied pademelon (*Thylogale billardierii*) commonly feed on *E. globulus* foliage (Gilbert, 1961). Our previous work shows clear genetic-based intraspecific variation in resistance of *E. globulus* juvenile coppice (regrowth following felling of the adult tree) foliage to these two generalist mammalian herbivores (O'Reilly-Wapstra *et al.*, 2002). We then determined that the genetically based chemical conferring resistance of this foliage to one of these species, *T. vulpecula*, was a specific formylated phloroglucinol compound, sideroxylonal (O'Reilly-Wapstra *et al.*, 2004). In this paper, we revisit these data sets to examine variation in preferences of these two herbivores at two main levels: across broad geographic races and within these populations. More specifically, we wished to determine whether: (1) genetic-based variation in sideroxylonal also confers resistance to *T. billardierii*; (2) there is a genetically based correlation in the preferences of the two herbivores among plant populations within geographic races; and (3) the strength of the relationship between plant defensive chemistry and resistance to both herbivores differs across the geographic range of *E. globulus*.

METHODS

Foliage pedigree

In this study, we used juvenile coppice (regrowth) foliage. Juvenile *E. globulus* coppice foliage can be browsed by both *T. vulpecula* and *T. billardierii* (O'Reilly-Wapstra *et al.*, 2002) and despite coppice foliage representing a recovery mechanism from foliage loss, repeated browsing of more susceptible coppice foliage by mammals incurs fitness consequences such as reduced growth rates and survival compared with more resistant coppiced plants that receive less browsing (J. O'Reilly-Wapstra, unpublished data). While coppice foliage shows variability in vigour (number of coppicing stems) between genotypes in the field (Whittock *et al.*, 2003), when vigour is taken into account, the patterns of genetically based resistance to mammals still holds (O'Reilly-Wapstra *et al.*, 2002). The foliage was sourced from trees classified into genetically distinct hierarchical groupings (race, population within race, and family within population; see below), which were planted in a randomized common environment field trial (Dutkowski and Potts, 1999). The genetic classes of *E. globulus* planted in the field trial were defined as follows: 'race' refers to genetically differentiated broad geographical groupings as defined by Dutkowski and Potts (1999); 'population' refers to a population of trees within a race growing within 10 km of each other as defined by Potts and Jordan (1994); and 'family' refers to progeny derived from open-pollinated seed collected from a single native tree within a population. Foliage used in this study was selected from 54 individual plants from 33 families, from within seven populations (Blue Gum Hill, St. Helens, South Geeveston, Jeeralang North, German Town, Royal George, and South Bruny) and selected from three races (Strzelecki Ranges, North-east Tasmania, and Southern Tasmania). Trees were selected across all replicates in a larger common garden trial in North-west Tasmania, in which 600 open-pollinated families from across the full geographic range of *E. globulus* were initially planted in a fully randomized incomplete block design comprising five replicates (for details, see Dutkowski and Potts, 1999).

Resistance of *E. globulus* to browsing and chemical analysis of foliage

The experimental trials providing data analysed for this paper are covered in full in O'Reilly-Wapstra *et al.* (2002, 2004) and so will only be summarized here. Resistance of the 54 individual coppiced trees to six *T. billardierii* (three males and three females; 4.7–9.0 kg body mass) and *T. vulpecula* (three males and three females; 3.2–4.2 kg body mass) was assessed in a no-choice captive feeding trial (O'Reilly-Wapstra *et al.*, 2002). Foliage was offered to animals as a single bunch each night and the trial was an incomplete block, cross-over trial design that ran for 9 weeks broken up into four equal periods to account for any changes in chemistry of the plants over time [these four time periods were included as a fixed factor in the original analyses (O'Reilly-Wapstra *et al.*, 2002)]. Relative resistance was expressed as the proportion of fresh weight foliage biomass remaining on each coppice sample, after feeding by *T. vulpecula* or *T. billardierii*, divided by the total average proportion of fresh weight foliage biomass remaining for all samples, analogous to estimates of relative fitness (Conner and Hartl, 2004).

Four (one from each period) control bunches of foliage (foliage not offered to the animals) for each of the 54 individual plants offered to the herbivores in the feeding trials were combined, and duplicate random selections of frozen foliage were sub-sampled for each chemical analysis for each individual plant. Plants were assayed for nitrogen, tannins, a particular essential oil (terpene) 1,8-cineole, and all other oils excluding 1,8-cineole (referred to as 'oils') and six formylated phloroglucinol compounds (FPCs): sideroxylonal A, sideroxylonal C, macrocarpal A, macrocarpal B, macrocarpal G, and grandinol. Formylated phloroglucinol compounds in eucalypt foliage have been shown to be important characteristics in influencing intake of foliage by marsupial herbivores (e.g. Lawler *et al.*, 2000; Moore and Foley, 2005). Details of all methods for chemical analysis can be found in O'Reilly-Wapstra *et al.* (2004). In brief, essential oils (terpenes) were extracted using dichloromethane with heptadecane (100 mg per litre of dichloromethane) as an internal standard and extracts were analysed by combined gas chromatography-mass spectrometry (GC-MS). The phenolic fraction referred to as 'tannin' was extracted using acidified (pH 1) methanol (Close *et al.*, 2001). Extracts were analysed by high-pressure liquid chromatography (HPLC). The FPCs were Soxhelt-extracted with light petroleum spirit : acetone (4:1) following Wallis *et al.* (2003). Nitrogen was determined using the semi-micro-Kjeldahl technique following Lawler *et al.* (1998).

Statistics

This analysis was divided into two main levels: across race and within race. First, we investigated the relationship between standardized chemistry (for each trait, subtract the mean from each value and then divide by the standard deviation) and relative resistance of the plants to both herbivore species across races by univariate and multiple linear regression analysis [PROC REG, SAS, version 9.1 (SAS Institute, Inc., 2004)]. The relationships of the univariate analyses were illustrated by the linear regression slope (β), the regression coefficient (R^2), and the level of significance. The partial regression slopes (β_i) and overall model R^2 and significance levels were calculated in the multiple regression analyses. A Bonferroni adjustment was used to take into account the number of individual tests in the univariate analyses ($\mu = 0.005$). For the multiple regression analyses, we ran a full model but first removed chemical constituents from the analysis that were highly inter-correlated [< 0.7 following Tabachnick and Fidell (1989)]. In this case, oils were highly correlated with 1,8-cineole, sideroxylonal A with sideroxylonal C, and macrocarpal G with macrocarpal A. Consequently, our model included sideroxylonal A, macrocarpal G, grandinol, oils, tannin, and nitrogen. As confirmation, we also ran the multiple regression analyses by first fitting a full model, but including all variables whether inter-correlated or not, and then manually backward eliminating chemical variables, starting with the least significant variable (PROC REG). This yielded very similar model statistics to the first model. Hence we have confidence in our multiple analyses output.

Second, we conducted another series of univariate analyses, investigating the relationship between standardized chemistry and relative resistance of the plants to both herbivore species within races. We did this by first accounting for racial variation (race as a class variable), then including the chemical variables as covariates and also adding the race × chemical variable interaction term in the model (PROC GLM, SAS). This relationship for each chemical variable is represented by an overall model R^2. The slope (β) for each chemical variable was also calculated (without the interaction term in the model) and the F and P values were also calculated (Type I sums of squares) for each term in the model (race, chemical, race × chemical interaction). Again, a Bonferroni adjustment was used to take into account the number of individual tests in the univariate analyses ($\mu = 0.005$). If the interaction term was significant, indicating that the slopes of each race were significantly different, then slopes for the relationship between the chemical variable and each race were calculated separately. We also ran a multiple regression analysis by accounting for race first, then adding all chemical covariates to the model. Again highly inter-correlated variables were excluded. These relationships were illustrated by calculating an overall model R^2 and P value. The partial regression slopes and P value for each chemical variable were also calculated.

We also examined differences between racial means for each chemical variable using a general linear model (PROC GLM, SAS) in which race was a fixed effect. Data for macrocarpal B were log transformed, while data for the three macrocarpal compounds and grandinol were square root transformed to meet assumptions of heterogeneity and normality.

At the among-population level (the level at which spatial genetic divergence in this species has been shown and the level at which sampling is robust enough for analysis), the relationship between concentration of sideroxylonal and resistance of foliage to *T. vulpecula* and *T. billardierii* was determined by univariate regression analysis (PROC REG, SAS). The genetically based correlation between resistance of foliage to the two herbivore species at the among-population level (foliage was sourced from a common garden trial and so differences in resistance reflect a genetic component) was assessed by Pearson's correlation coefficient (PROC CORR, SAS). The phenotypic correlation (PROC CORR) of resistance of foliage to both herbivores within races was also determined by standardizing each value (by race) to a mean of 0 and a standard deviation of 1.

RESULTS

Across all plants, the univariate analysis in Table 1 shows that greater relative resistance of individual *E. globulus* plants to browsing by *T. vulpecula* was associated with increases in concentrations of sideroxylonals and macrocarpals. Furthermore, relative resistance of *E. globulus* to browsing by *T. billardierii* was also influenced by sideroxylonal A; consequently, sideroxylonals appear consistent in influencing choice of *E. globulus* plants by the two browsing herbivores. Further investigation in the multiple regression analysis confirmed these patterns (Table 2; note that sideroxylonal A is correlated with sideroxylonal C and to a degree represents their combined effects; similarly, macrocarpal G is correlated with the other two macrocarpal compounds). There was also a significant negative relationship between nitrogen and relative resistance to *T. vulpecula*, indicating animals were choosing plants with higher nitrogen concentrations (Table 2).

These patterns most likely reflect among-population effects where, for example, there was a significant relationship between relative resistance to *T. vulpecula* and *T. billardierii* and sideroxylonal A among populations (*T. vulpecula*: $R^2 = 0.64$, $n = 7$, $P = 0.03$; *T. billardierii*:

Table 1. Results of the univariate regression analyses of browsing responses of *Trichosurus vulpecula* and *Thylogale billardierii* against each chemical variable across all races ($n = 54$)

	Univariate slope β	R^2	P
T. vulpecula			
Sideroxylonal A	0.076	0.24	**0.0002**
Sideroxylonal C	0.079	0.26	<**0.0001**
Macrocarpal G	0.078	0.25	<**0.0001**
Macrocarpal B	0.064	0.17	**0.002**
Macrocarpal A	0.083	0.28	<**0.0001**
Grandinol	0.052	0.11	0.014
Oils	0.052	0.11	0.014
1,8-cineole	0.058	0.14	0.006
Tannin	−0.012	0.01	0.581
Nitrogen	−0.033	0.05	0.120
T. billardierii			
Sideroxylonal A	0.086	0.15	**0.004**
Sideroxylonal C	0.081	0.13	0.007
Macrocarpal G	0.016	0.01	0.602
Macrocarpal B	0.015	0.005	0.618
Macrocarpal A	0.029	0.02	0.338
Grandinol	0.017	0.006	0.580
Oils	−0.006	0.001	0.856
1,8-cineole	0.014	0.004	0.639
Tannin	0.062	0.08	0.042
Nitrogen	−0.062	0.08	0.039

Note: **Bold** indicates significance at the 0.005 level after Bonferroni adjustment for the multiple univariate analyses.

$R^2 = 0.58$, $n = 7$, $P = 0.04$). There was also a significant genetically based correlation among populations between relative resistance of *E. globulus* to both herbivores (Fig. 1).

When plant race variation was removed and chemistry was analysed as a covariate, the two sideroxylonals and three macrocarpals were still important in explaining patterns of choice by *T. vulpecula* (Table 3). However, while there was a weak effect, sideroxylonals no longer influenced relative resistance to *T. billardierii* after Bonferroni adjustment ($P = 0.04$ for sideroxylonal A and sideroxylonal C respectively; Table 3). These patterns for *T. vulpecula* and *T. billardierii* were also supported in the multiple regression analysis when variation attributed to race was taken into account first (Table 4; the effect of race was non-significant for both *T. vulpecula* and *T. billardierii*: $P > 0.05$). At this within-race level, there was a significant correlation in the relative resistance of *E. globulus* populations to the two herbivores (Pearson's correlation, $r = 0.31$, $n = 54$, $P = 0.02$).

There were clear differences in slope estimates for the interactions between relative resistance to *T. vulpecula* and the three macrocarpals as evidenced by significant race × macrocarpal interactions (Table 3). Relative resistance was significantly, positively correlated with macrocarpal levels within the North-east Tasmania and the Southern Tasmania races, but not within the Strzelecki Ranges race (Table 5). These patterns are

Table 2. Results of the multiple regression analyses of browsing responses of *Trichosurus vulpecula* and *Thylogale billardierii* against chemicals across all races ($n = 54$)

	Partial regression slope β_i	P
T. vulpecula		
Sideroxylonal A	0.06	0.0006
Macrocarpal G	0.08	0.0002
Grandinol	0.02	0.404
Oils	−0.03	0.214
Tannin	−0.03	0.141
Nitrogen	−0.05	0.009
T. billardierii		
Sideroxylonal A	0.10	0.002
Macrocarpal G	0.04	0.272
Grandinol	0.02	0.445
Oils	−0.06	0.089
Tannin	0.05	0.182
Nitrogen	−0.03	0.381

Note: Overall model statistics are: *T. vulpecula*, $R^2 = 0.51$, $P < 0.001$; *T. billardierii*, $R^2 = 0.30$, $P = 0.01$. Chemical constituents that were highly inter-correlated (<0.7) were removed from the analyses.

consistent with the idea that ecological and evolutionary relationships between plants and herbivores may vary depending upon geographic variation in populations. Table 6 shows racial means and standard deviations for all chemical variables. It is worth noting that macrocarpal concentrations were generally significantly higher for the North-east Tasmania and Southern Tasmania races than the Strzelecki Ranges race, and the variance in these traits was less than for the Strzelecki Ranges race.

DISCUSSION

A common plant trait deters two herbivores

Our results show that resistance of *E. globulus* juvenile coppice foliage to both mammalian herbivore species is mediated predominantly by variation in the concentrations of formylated phloroglucinol compounds, particularly the highly heritable (Andrew *et al.*, 2005) compound, sideroxylonal. The relationship between relative resistance to *Trichosurus vulpecula* and chemistry was mediated by both sideroxylonals and macrocarpals, although it was sideroxylonal that affected resistance to both herbivores. This pattern was evident both across and within races, although the strength of the relationship between relative resistance to *T. billardierii* and the FPCs weakened within races. While the relationships between these compounds and *T. vulpecula* have been illustrated in previous work (O'Reilly-Wapstra *et al.*, 2004), we have shown here the consistency in deterrent effects of these compounds in *E. globulus* to a sympatric mammalian herbivore, *T. billardierii*. This finding, coupled with a significant positive genetic correlation between relative resistance of *E. globulus* to the two herbivores,

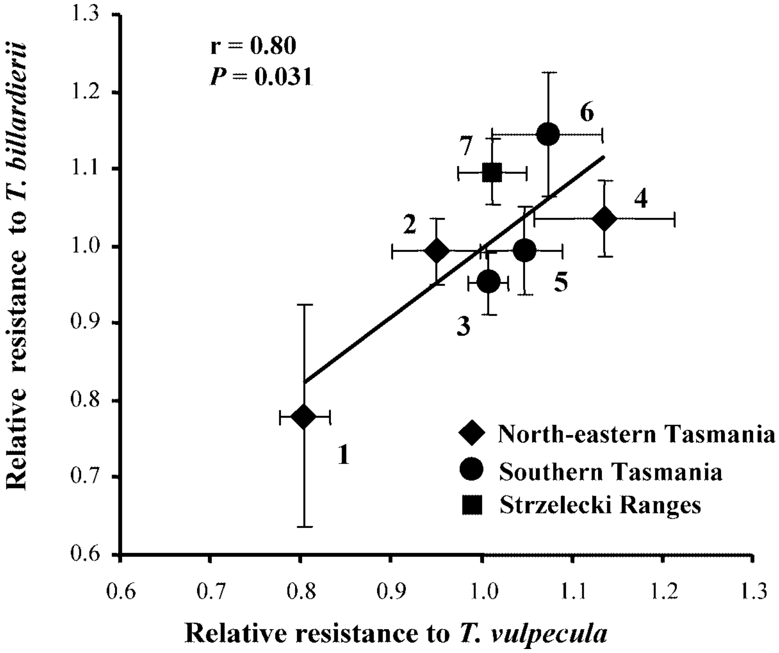

Fig. 1. Correlation between relative resistance of the seven populations after browsing by *Trichosurus vulpecula* and *Thylogale billardierii*. Numbers refer to the seven populations sourced from the three races. 1, St. Helens; 2, German Town; 3, South Geeveston; 4, Royal George; 5, South Bruny; 6, Blue Gum Hill; 7, Jeeralang North. St. Helens, German Town, and Royal George are from the North-east Tasmania race; South Geeveston, Blue Gum Hill, and South Bruny are from the Southern Tasmania race; Jeeralang North is from the Strzelecki Ranges race.

both among and within populations, indicates that the relationship between these herbivores, and any subsequent selection on the plant resistance traits, does not appear to be independent (Andrew *et al.*, 2007).

Does evidence support marsupial herbivores as selective agents?

To understand the evolution of traits, we must understand the selective processes acting on those traits. While there have been a plethora of studies investigating different selective pressures on traits in numerous systems (Hoekstra *et al.*, 2001), including selection by herbivores on plant defences in plant/invertebrate systems (Rausher, 2001; Zangerl *et al.*, 2008), there has been a paucity of studies in plant/mammal systems investigating the evolutionary processes behind intraspecific variation in plant resistance (e.g. Snyder, 1993; Stinchombe and Rausher, 2001; Vourc'h *et al.*, 2002; Bailey *et al.*, 2004; Laitinen *et al.*, 2004; Wise, 2007). If there is a significant impact of browsing on fitness in our system, our results indicate that both herbivore species have the potential to play a role in the evolution of plant defences in this tree species. Due to the longevity of *E. globulus* (several hundred years) and the length of time to reproductive maturity (4–5 years), it is essentially impossible to quantify a true fitness consequence of browsing (and hence estimate natural selection) by mammalian herbivores. However, we do know that foliage biomass lost due to browsing on *E. globulus* and other eucalypt species (*E. morrisbyi*)

Table 3. Results of the univariate analyses of browsing responses of *Trichosurus vulpecula* and *Thylogale billardierii* against each chemical variable within races (variation attributed to race is accounted for first)

Chemical	*Trichosurus vulpecula*			*Thylogale billardierii*		
Sideroxylonal A	$F = 4.79$, $P = 0.001$, $R^2 = 0.33$			$F = 2.23$, $P = 0.07$, $R^2 = 0.19$		
	β	F	P	β	F	P
Race	—	1.99	0.15	—	2.78	0.07
Sideroxylonal A	0.08	15.11	**0.0003**	0.07	4.38	0.04
Race × Sideroxylonal A	—	2.42	0.10	—	0.61	0.55
Sideroxylonal C	$F = 5.05$, $P = 0.001$, $R^2 = 0.34$			$F = 2.77$, $P = 0.03$, $R^2 = 0.22$		
	β	F	P	β	F	P
Race	—	2.03	0.14	—	2.91	0.06
Sideroxylonal C	0.08	15.69	**0.0002**	0.07	4.28	0.04
Race × Sideroxylonal C	—	2.75	0.07	—	1.88	0.16
Macrocarpal G	$F = 8.08$, $P < 0.0001$, $R^2 = 0.46$			$F = 1.75$, $P = 0.14$, $R^2 = 0.15$		
	β	F	P	β	F	P
Race	—	2.45	0.10	—	2.67	0.08
Macrocarpal G	See Table 5	23.78	**< 0.0001**	0.04	1.29	0.26
Race × Macrocarpal G	—	5.85	0.005	—	1.07	0.35
Macrocarpal B	$F = 5.27$, $P = 0.001$, $R^2 = 0.36$			$F = 2.39$, $P = 0.05$, $R^2 = 0.20$		
	β	F	P	β	F	P
Race	—	2.06	0.14	—	2.82	0.07
Macrocarpal B	See Table 5	12.02	**0.001**	0.03	0.71	0.40
Race × Macrocarpal B	—	5.11	0.01	—	2.80	0.07
Macrocarpal A	$F = 7.24$, $P < 0.0001$, $R^2 = 0.43$			$F = 2.19$, $P = 0.07$, $R^2 = 0.19$		
	β	F	P	β	F	P
Race	—	2.33	0.11	—	2.77	0.07
Macrocarpal A	See Table 5	24.48	**< 0.0001**	0.05	2.40	0.13
Race × Macrocarpal A	—	3.52	0.04	—	1.51	0.23
Grandinol	$F = 2.31$, $P = 0.06$, $R^2 = 0.19$			$F = 1.75$, $P = 0.14$, $R^2 = 0.15$		
	β	F	P	β	F	P
Race	—	1.65	0.20	—	2.67	0.08
Grandinol	0.05	5.57	0.02	0.03	0.69	0.41
Race × Grandinol	—	1.35	0.27	—	1.36	0.27

Table 3.—*continued*

Chemical	*Trichosurus vulpecula*			*Thylogale billardierii*		
Oils	$F = 1.50$, $P = 0.21$, $R^2 = 0.14$			$F = 1.20$, $P = 0.33$, $R^2 = 0.11$		
	β	F	P	β	F	P
Race	—	1.54	0.14	—	2.54	0.09
Oils	0.05	4.19	0.05	−0.01	0.18	0.67
Race × Oils	—	0.11	0.90	—	0.36	0.70
1,8-cineole	$F = 2.03$, $P = 0.09$, $R^2 = 0.18$			$F = 1.23$, $P = 0.31$, $R^2 = 0.11$		
	β	F	P	β	F	P
Race	—	1.61	0.21	—	2.55	0.09
1,8-cineole	0.06	5.70	0.02	0.01	0.06	0.80
Race × 1,8-cineole	—	0.62	0.54	—	0.50	0.61
Tannin	$F = 1.19$, $P = 0.33$, $R^2 = 0.11$			$F = 3.35$, $P = 0.01$, $R^2 = 0.26$		
	β	F	P	β	F	P
Race	—	1.49	0.23	—	3.04	0.06
Tannin	−0.01	0.18	0.67	0.06	5.10	0.29
Race × Tannin	—	1.38	0.26	—	2.79	0.07
Nitrogen	$F = 1.05$, $P = 0.40$, $R^2 = 0.10$			$F = 2.84$, $P = 0.03$, $R^2 = 0.23$		
	β	F	P	β	F	P
Race	—	1.48	0.24	—	2.92	0.06
Nitrogen	−0.03	2.26	0.14	−0.07	5.47	0.02
Race × Nitrogen	—	0.03	0.97	—	1.43	0.25

Note: **Bold** indicates significance at the 0.005 level after Bonferroni adjustment for the multiple univariate analyses. Overall model statistics for each chemical variable is written on the first line of each variable (degrees of freedom for the overall model *F* values are all 5,53).

in the field significantly lowers reproductive output at age 5–10 years and repeated browsing of seedlings and coppice can result in tree death (J. O'Reilly-Wapstra *et al.*, unpublished data; C. Borzak *et al.*, unpublished data). It is plausible, therefore, that both the genetic-based variation in resistance of *E. globulus* and in the defensive traits (FPCs) conferring this resistance is under selection by the mammals in this system. The extent to which these common environment field and captive feeding trial results can be extrapolated into the wild is unclear. However, genetic-based differences in sideroxylonal have been demonstrated in native stands of other eucalypt species (Andrew *et al.*, 2005). In addition, while feeding choices in the wild are much more complex, Moore and Foley (2005) have shown that high concentrations of total FPCs in native *E. globulus* and *E. viminalis* trees reduced use of those trees by koalas.

Table 4. Results of the multiple regression analyses of browsing responses of *Trichosurus vulpecula* and *Thylogale billardierii* against chemicals within races (variation attributed to race is accounted for first; see text)

	Partial regression slope β_i	P
T. vulpecula		
Sideroxylonal A	0.05	0.04
Macrocarpal G	0.09	<0.0001
Grandinol	0.02	0.27
Oils	−0.03	0.23
Tannin	−0.03	0.98
Nitrogen	−0.06	0.004
T. billardierii		
Sideroxylonal A	0.06	0.09
Macrocarpal G	0.06	0.10
Grandinol	0.03	0.34
Oils	0.07	0.07
Tannin	0.04	0.23
Nitrogen	−0.04	0.22

Note: Overall model statistics are: *T. vulpecula*, $R^2 = 0.54$, $P < 0.001$; *T. billardierii*, $R^2 = 0.34$, $P = 0.01$. Chemical constituents that were highly inter-correlated (<0.7) were removed from the analyses.

Table 5. Slopes β (S.E.) of the regression between browsing responses of *Trichosurus vulpecula* and each macrocarpal for each race

Race	Macrocarpal G	Macrocarpal B	Macrocarpal A
North-east Tasmania	0.22 (0.47)**	0.18 (0.05)*	0.18 (0.04)*
Southern Tasmania	0.06 (0.02)*	0.08 (0.03)*	0.08 (0.02)*
Strzelecki Ranges	0.06 (0.03)	0.02 (0.03)	0.06 (0.03)

*$P < 0.05$; **$P < 0.001$.

Although we are confident in this and other (e.g. Loney *et al.*, 2006) studies that variation in sideroxylonal influences feeding choices by *T. billardierii*, we suspect that *T. vulpecula* may exert stronger selection on the key defensive compound sideroxylonal than *T. billardierii*. First, across races the relationship between *T. billardierii* and sideroxylonal was weak or not significant. Second, *T. vulpecula* consumes significantly more eucalypt foliage biomass when feeding [up to ten times more (O'Reilly-Wapstra *et al.*, 2002; Close *et al.*, 2003)] than *T. billardierii*, so consequent fitness effects of individuals on *E. globulus* may be greater. Third, *T. vulpecula* is arboreal but comes to ground regularly and can feed on all life stages of the plant (seedling, sapling, adult, and coppice regrowth), while *T. billardierii* is a ground-dwelling macropod that can only feed on juvenile foliage. Absolute sideroxylonal concentrations are higher in juvenile coppice *E. globulus* foliage than adult foliage (perhaps reflecting greater browsing pressure at this ontogenetic stage), but there is a strong genetic correlation across life stages

Table 6. Racial means and standard deviations (in parentheses) for all chemical variables

		Race		
Chemical	Units	North-east Tasmania	Southern Tasmania	Strzelecki Ranges
Sideroxylonal A	$\text{mg} \cdot \text{g}^{-1}$ dry mass	3.57 (1.95)^a	5.06 (2.03)^b	6.14 (1.35)^b
Sideroxylonal C	$\text{mg} \cdot \text{g}^{-1}$ dry mass	1.20 (0.63)^a	1.70 (0.67)^b	1.84 (0.42)^b
Macrocarpal G	$\text{mg} \cdot \text{g}^{-1}$ dry mass	5.61 (1.60)^a	6.66 (2.40)^a	4.05 (2.82)^b
Macrocarpal B	$\text{mg} \cdot \text{g}^{-1}$ dry mass	1.46 (0.51)^{ab}	1.74 (0.58)^a	1.18 (1.12)^b
Macrocarpal A	$\text{mg} \cdot \text{g}^{-1}$ dry mass	2.09 (0.74)^a	2.70 (0.85)^a	1.56 (1.22)^b
Grandinol	$\text{mg} \cdot \text{g}^{-1}$ dry mass	0.73 (0.46)^{ab}	1.00 (0.50)^a	0.57 (0.29)^b
Oils	1,8-cineole equivalents $\text{mg} \cdot \text{g}^{-1}$ dry mass	29.69 (10.24)^a	40.20 (12.09)^b	30.92 (12.40)^{ab}
1,8-cineole	$\text{mg} \cdot \text{g}^{-1}$ dry mass	15.27 (5.07)^a	21.70 (6.78)^b	16.22 (6.56)^a
Tannin	TeGG equivalents $\text{mg} \cdot \text{g}^{-1}$ dry mass	157.32 (67.06)^a	144.78 (42.01)^a	159.26 (53.76)^a
Nitrogen	% dry mass	2.32 (0.25)^a	2.28 (00.21)^a	2.35 (0.31)^a

Note: Letters that differ indicate significant differences ($P < 0.05$) between races following Tukey-Kramer adjustment for multiple comparisons. TeGG = tetragalloglucose.

[between adult and juvenile coppice foliage (O'Reilly-Wapstra *et al.*, 2007)]. *Trichosurus vulpecula* may exert selective pressure for higher sideroxylonal concentrations in all life stages of the plant. Alternatively, if genes controlling the expression of this quantitative trait are little modified with life stage, as suggested by co-locating QTL for sideroxylonal in adult *E. nitens* (Henery *et al.*, 2007) and juvenile *E. globulus* (Freeman *et al.*, 2008), then selection through browsing at either life phase would elicit a correlated response in the other life phase. Furthermore, if *T. vulpecula* provides the stronger selective pressure on *E. globulus* defensive chemistry, then *T. billardierii* may be caught up via an indirect ecological and evolutionary interaction (Bailey and Whitham, 2003), influencing its feeding choices through evolution of resistance in a shared food source.

Geographic variation in the relationship between defensive chemistry and resistance

We were able to investigate variation within specific geographically distinct races of *E. globulus*, in the relationship between key defensive chemistry and resistance to these two mammalian herbivores, and the relationships for one group of compounds were not uniform between races for *T. vulpecula*. The effects of sideroxylonal on plant resistance to *T. vulpecula* appear to be consistent regardless of whether we investigate this relationship across or within races. However, the strength of the relationship between macrocarpals and relative resistance to this herbivore varied within races, as evidenced by a significant race × macrocarpal interaction within races (Table 3). The relationship between relative resistance and the three macrocarpal compounds was significant within the North-east Tasmania race and the Southern Tasmania race, but not the Strzelecki Ranges race (Table 5). If past selection had acted on macrocarpals in the Strzelecki Ranges race (thus showing no current relationship between resistance and the compounds) but not in the other two races, then we would expect high macrocarpal concentrations and low variance in the Strzelecki Ranges race compared with the other two races. This is not the case. Mean macrocarpal concentrations are higher in the North-east and Southern Tasmanian races and thus there may be a threshold response for these compounds. That is, herbivores need a certain threshold in concentration of the compound before a response is elicited (Eaton and Gilbert, 2008) and this is not reached in samples from the Strzelecki Ranges race. However, regardless of the mechanism, the selection surface on which herbivores can act on in this system is not uniform across the range of *E. globulus*.

This study has demonstrated the responses of more than one mammalian herbivore to eucalypt plant defensive traits, thus providing insight into the type of relationships that exist between a plant species and its multiple herbivores. The next step is to explore why selective impacts and/or variance in defensive compounds differ between races, and what influence other selective biotic agents, such as invertebrate herbivores, have on the system.

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