

Role of Volatile and Non-Volatile Plant Secondary Metabolites in Host Tree Selection by Christmas Beetles

Mamoru Matsuki · William J. Foley · Robert B. Floyd

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Abstract Individual *Eucalyptus* trees in south-eastern Australia vary considerably in susceptibility to herbivores. On the one hand, studies with insect herbivores have suggested that variation in the concentrations of foliar monoterpenes is related to variation in susceptibility. On the other, studies with marsupial folivores have suggested that variation in the concentrations of sideroxylonals (a group of formylated phloroglucinol compounds) is responsible for variation in susceptibility. We examined relative importance of sideroxylonals and 1,8-cineole (a dominant monoterpene) in host tree selection by Christmas beetles (*Anoplognathus* species: Coleoptera: Scarabaeidae) by using no-choice experiments, choice/no-choice experiments, and manipulative experiments in which concentrations of sideroxylonals or 1,8-cineole were altered. We used two species of host *Eucalyptus*, one

species of non-host *Eucalyptus*, and three species of non-host non-*Eucalyptus* trees. Leaf consumption by Christmas beetles was negatively correlated with the concentrations of sideroxylonals and 1,8-cineole. Artificial increases in the concentration of sideroxylonals or 1,8-cineole reduced leaf consumption by Christmas beetles. An artificial reduction in foliar monoterpenes had no effect on leaf consumption by the beetles when leaves contained high or very low concentrations of sideroxylonals. However, when the concentration of sideroxylonals was moderate, a reduction in the foliar monoterpenes increased leaf consumption by the beetles. Therefore, monoterpenes such as 1,8-cineole may be used as a negative cue by Christmas beetles. The pattern of food consumption on non-host *Eucalyptus* species and non-host non-*Eucalyptus* species suggest that both positive and negative cues may be used by Christmas beetles to select host trees.

M. Matsuki · R. B. Floyd
CSIRO Ecosystem Sciences,
GPO Box 1700, Canberra, Australian Capital Territory 2601,
Australia

M. Matsuki · W. J. Foley
Research School of Biology, Australian National University,
Canberra, Australian Capital Territory 0200, Australia

Present Address:

M. Matsuki (✉)
Centre of Excellence in Natural Resource Management, The
University of Western Australia and Department of Agriculture
and Food Western Australia,
444 Albany Highway,
Albany, Western Australia 6330, Australia
e-mail: mamoru.matsuki@agric.wa.gov.au

Present Address:

R. B. Floyd
Australian Safeguards and Non-Proliferation Office,
R. G. Casey Building, John McEwen Crescent,
Barton, Australian Capital Territory 0221, Australia

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Introduction

Plant secondary metabolites play important roles in host selection by herbivores. Some of these compounds may act as positive (attractants) or negative cues (deterrents) that signal the quality of plants as food, while other compounds may act as toxins that reduce performance of insects. At least some specialist insect herbivores choose host plants upon which to oviposit on the basis of plant secondary metabolites (e.g., Steinbauer et al., 2004; Östrand et al., 2008), and many studies have documented negative

relationships between performance of herbivores and concentrations or types of secondary metabolites (e.g., Feeny, 1968; Matsuki and MacLean, 1994 and references therein; Osier et al., 2000). When specialist insect herbivores include a new host species, the new host species is likely to be taxonomically close to, or share at least some secondary metabolites with, the original host species (Futuyma and McCafferty, 1990; Janz and Nylin, 1998; Janz et al., 2001).

The importance of intra-specific variation in secondary metabolites to plant–herbivore interactions is widely accepted (Denno and McClure, 1983; Fritz and Simms, 1992; Hunter, 1997). For feeding or egg-laying, specialist insect herbivores must select not only the right host species but also the right individual of the host species.

Some *Eucalyptus* (Myrtaceae) species show striking intra-specific variation in concentrations of secondary metabolites and susceptibility to herbivores. For example, trees growing in close proximity may show marked variation in susceptibility to Christmas beetles (*Anoplognathus* species: Coleoptera: Scarabaeidae) (Edwards et al., 1993), and occasionally, there are some showing within tree variation in susceptibility. One branch of a particular study tree of *E. melliodora* suffered only 5% defoliation, while the rest of the tree suffered 85% defoliation (Edwards et al., 1990). There were negative correlations between herbivory by Christmas beetles and the proportion of 1,8-cineole (a monoterpene) in six species of *Eucalyptus* (Edwards et al., 1993). 1,8-Cineole and other components of essential oils in leaves of eucalypts are considered to play important defensive roles against other insect herbivores (e.g., Morrow and Fox, 1980; Ohmart and Larsson, 1989; Stone and Bacon, 1994; Southwell et al., 2003).

Studies of arboreal marsupial folivores on *Eucalyptus* have shown that consumption is negatively affected by a group of compounds called formylated phloroglucinol compounds (FPCs) (Pass et al., 1998; Lawler et al., 2000; Wallis et al., 2002; Moore et al., 2004a). Formylated phloroglucinol compounds are found only in some species in Myrtaceae, and leaves of many *Eucalyptus* species contain one to several different FPCs (Ghisalberti, 1996; Eschler et al., 2000).

Sideroxylonals (a class of FPCs) show strong antifeedant activity against marsupial folivores, and these animals can adjust food intake to regulate the amounts of sideroxylonals being ingested (Lawler et al., 1998; Wallis et al., 2002). Sideroxylonals appear to cause emetic responses in animals that have ingested the chemical (Lawler et al., 1998; DeGabriel et al., 2010). However, because sideroxylonals are not volatile, animals have to ingest some leaves to detect concentrations in foliage. It has been hypothesized that volatile monoterpenes might act as pre-ingestive cues to marsupial folivores (Lawler et al., 1999), and subsequently, it has been shown that concentrations of side-

roxytonals and some monoterpenes are positively correlated (Moore et al., 2004b). Following studies with marsupial folivores, it was shown that performance of Christmas beetles and some Lepidopterans are negatively affected by sideroxylonals (Steinbauer and Matsuki, 2004; Andrew et al., 2007; Matsuki et al., 2010).

The purpose of this study was to examine roles of sideroxylonals and 1,8-cineole in host selection and food consumption by Christmas beetles. These beetles are particularly suited to examining roles of the two secondary metabolites. Some of the host species of Christmas beetles (*E. melliodora* and *E. sideroxylon*) show strong within-species variation in concentrations of both sideroxylonals and 1,8-cineole (Watson, 1998; Moore et al., 2004a). As noted, Christmas beetles show strong preference for some individuals of the host species over other individuals (Edwards et al., 1993; Andrew et al., 2007). They chew through leaves, and thus, must ingest sideroxylonals. In this study, laboratory experiments were carried out to examine effects of sideroxylonals and 1,8-cineole on food consumption by Christmas beetles.

Methods and Materials

Study Organisms We examined leaf consumption by 6 species of Christmas beetles that are commonly found in south-eastern Australia (*Anoplognathus chloropyrus*, *A. hirsutus*, *A. montanus*, *A. pallidicollis*, *A. suturalis*, and *A. viriditarsis*). Larvae feed on roots of grasses and crops. Adult beetles preferentially feed on young leaves of *Eucalyptus* that are nearly or completely expanded (Carne et al., 1974). Leaves of less than 3 yr-old also are eaten when young leaves are not available. In south-eastern Australia, Christmas beetles feed on at least 12 species of *Eucalyptus* (Edwards et al., 1993; M. Matsuki, pers. obs.).

We used *E. melliodora*, *E. polyanthemus*, and *E. sideroxylon*. Sideroxylonals (a mixture of three isomers A, B, and C) are the major class of FPCs found in these species (Eschler et al., 2000). *Eucalyptus melliodora* and *E. sideroxylon* are typical hosts of Christmas beetles, while leaves of *E. polyanthemus* trees are rarely eaten (M. Matsuki, pers. obs.; R. Andrew, pers. comm.). Cyanogenic glycosides are in leaves of *E. polyanthemus* but not in leaves of *E. melliodora* or *E. sideroxylon* (Gleadow et al., 2008). The three *Eucalyptus* species grow naturally in woodlands throughout south-eastern Australia and are similar in many aspects of their natural history (Boland et al., 1984). We also used *Betula* sp. (Betulaceae), *Quercus* sp. (Fagaceae), and *Populus tremuloides* (Salicaceae) as non-host species that are only distantly related to the host species. These are exotic ornamental trees in Australia and are not known to contain sideroxylonals or to store monoterpenes.

Choice of Trees Trees (m1–m3, s1–s3, & p1–p3) were chosen on the basis of variation in leaf consumption by marsupial folivores. Among 8 *E. melliodora* trees tested, leaf consumption by koala (*Phascolarctos cinereus*: Moore et al., 2004a) and common brushtail possum (*Trichosurus vulpecula*: Watson, 1998) was highest on m3, intermediate on m2, and lowest on m1. Among 12 *E. sideroxylon* trees tested, leaf consumption by common brushtail possum (Watson, 1998) and common ringtail possum (*Pseudocheirus peregrinus*: Lawler et al., 2000) was highest on s3, intermediate on s2, and lowest on s1. Among 36 *E. polyanthemos* trees tested, leaf consumption by common ringtail possum (Lawler et al., 2000) was highest on p3, intermediate on p2, and lowest on p1. We also chose some susceptible (m6, m7, s6, and s7) and resistant (m4, m5, s4, and s5) trees of *E. melliodora* (m4–m7) and *E. sideroxylon* (s4–s7) on the basis of the records of herbivory by Christmas beetles (mostly *A. montanus*) in the field (P. B. Edwards and W. J. Wanjura, unpubl.). These were some of the trees studied by Edwards et al. (1993). Trees s8–s11 (*E. sideroxylon*) had never been used in studies with vertebrates or insects. During the study period, the trees used in this study suffered only minor defoliation by herbivores. Trees m4–m7 were located in Yeoval, New South Wales (32°44'S, 148°39'E), and s4–s7 were located in Cumnock, New South Wales (32°55'S, 148°44'E). All other trees were located in Canberra, Australian Capital Territory (35°16'S, 149°7'E). The tree numbers indicate unique trees. For example, m1 indicates the same tree throughout this paper.

Physical and Chemical Characteristics of Leaves We measured leaf water content and specific leaf weight. *Eucalyptus* leaves contain volatile terpenes, and leaves were dried at 40°C for 2 d so as not to lose these compounds. Specific leaf weight was measured by weighing a small disk of a known area cut from the leaf blade.

In 1997–99, concentrations of monoterpenes were determined by gas chromatography (GC) using the method described in Edwards et al. (1993). Two leaves per tree were analyzed for monoterpenes. The concentration of sideroxylonals was determined by high performance liquid chromatography (HPLC) using the method described in Lawler et al. (2000). A mixture of the three isomers of sideroxylonal was extracted and purified as described elsewhere (Eschler and Foley, 1999). Concentrations of leaf nitrogen in 1997–98 and those of 1,8-cineole and sideroxylonals in 1999–2000 were estimated by using near-infrared reflectance spectroscopy, NIRS (Lawler et al., 2000).

The calibration equation for the concentration of leaf nitrogen was developed with a set of NIRS spectra and measured nitrogen content of 226 pre-existing samples of various *Eucalyptus* species by using a modified partial least square regression (ASTM 1995). We validated the calibra-

tion equation with 3 samples of *E. melliodora* and 6 samples of *E. sideroxylon* that were not used in the calibration equation. The calibration equations for sideroxylonals and 1,8-cineole were developed from a set of NIRS spectra and measured sideroxylonals and 1,8-cineole concentrations of 49 *E. melliodora* samples by using partial least square regressions. We used a cross-validation procedure (Infrasoft International, 1996; Brown, 1993) to validate calibration equations for total sideroxylonals and 1,8-cineole.

We classified leaves into three broad categories of concentrations of sideroxylonals or 1,8-cineole: low concentrations (<5 mg sideroxylonals or 1,8-cineole g⁻¹ leaf dry matter); intermediate concentrations (5–20 mg sideroxylonals g⁻¹ leaf dry matter or 5–10 mg 1,8-cineole g⁻¹ leaf dry matter); and high concentrations (>20 mg sideroxylonals g⁻¹ leaf dry matter or >10 mg 1,8-cineole g⁻¹ leaf dry matter).

Consumption Trials We conducted five types of experiments in the three consecutive Austral summers between 1997 and 2000: (1) no-choice experiments; (2) choice/no-choice experiments; (3) experiments where the foliar concentration of sideroxylonals was enhanced; (4) an experiment where the foliar concentration of cineole was reduced via steaming; and (5) experiments where the foliar concentration of cineole was enhanced (see below for details).

For each experiment, several small branches were taken from each experimental tree so that leaves used were representative of leaves on experimental trees. Size and age of leaves in a given season were standardized visually in order to minimize these effects on leaf consumption. In the summer of 1997–1998, we used leaves of 1.5–2.5 year old because leaves less than 1 yr-old were rare. However, the study trees produced new leaves in the autumn–spring of 1998, and by late December 1998, almost all old leaves had abscised. Therefore, we used leaves of 6- to 8-mo-old in the summer of 1998–1999. We used leaves that were approximately 1.5 yr-old in the summer of 1999–2000.

Adult beetles were collected in the field around Canberra. *Anoplognathus chloropyrus* also was collected from three other locations: Ginninderra, (35°09'S, 149°03'E); Gunning (34°47'S, 149°16'E); and Boorowa (34°26'S, 148°43'E), New South Wales.

To estimate the dry weight of leaf consumed by each beetle, we used two leaves for each insect. Each leaf was weighed and placed individually in clear plastic screw-top vials (250 ml) with moist plaster of Paris to maintain leaf turgor. At the beginning of an experiment, each beetle was weighed and placed in one of the two vials. Vials for each beetle were kept together during an experiment. Experiments were terminated after 5–15 h when some leaves were

nearly completely consumed. All experiments were started in the morning and terminated in the evening of the same day. Each beetle was weighed again at the end of each experiment. Each leaf was placed in a small envelope and dried at 40°C for 2 day. The water content of the leaf that had not been offered to insects (control leaf) was used to estimate the water content of the leaf that had been offered to an insect (trial leaf). The dry weight of leaf consumed by each insect was estimated as described elsewhere (Ayres and MacLean, 1987; Matsuki et al., 1994). There was no difference in relative consumption rates between male and female beetles for a given tree, and we used the pooled data for analyses.

No-choice and Choice/no-choice Experiments For no-choice and choice/no-choice tests, separate experiments were carried out for each *Eucalyptus* species, each leaf age, and each *Anoplognathus* species. In each no-choice experiment, the experimental design was 1 *Eucalyptus* species × 1 leaf age × 1 *Anoplognathus* species × 5 blocks × 3–4 treatments (trees) × 3 replicates. In choice/no-choice experiments, the experimental design was 1 *Eucalyptus* species × 1 leaf age × 1 *Anoplognathus* species × 5 blocks × 6 treatments (tree combinations) × 3 replicates. Blocks in the experiments represented rooms in three different buildings; however, the same five rooms were used in all experiments.

In no-choice experiments, one fresh leaf was placed in each vial. In choice/no-choice experiments, two fresh leaves were placed in each vial. For each *Eucalyptus* species, three trees were used in each choice/no-choice experiment: the susceptible, the resistant, and an intermediate tree (as defined above). We used all six pair-wise combinations of trees, i.e., susceptible-susceptible, susceptible-intermediate, susceptible-resistant, intermediate-intermediate, intermediate-resistant, and resistant-resistant. For example using *E. melliodora*, six pair-wise combinations of trees were m3-m3, m3-m2, m3-m1, m2-m2, m2-m1, and m1-m1, respectively.

We calculated relative consumption rates of leaves by Christmas beetles using a dry matter based method (Waldbauer, 1968). Using a subset of beetles, dry mass of beetles was estimated from the ratio of wet mass to dry mass for each beetle species/sex combination.

To examine preference of a tree *i* in choice/no-choice experiments, we used the index *D* (Jacobs, 1974):

$$D_i = \frac{DR_i - RA_i}{RA_i + DR_i + 2RA_iDR_i}$$

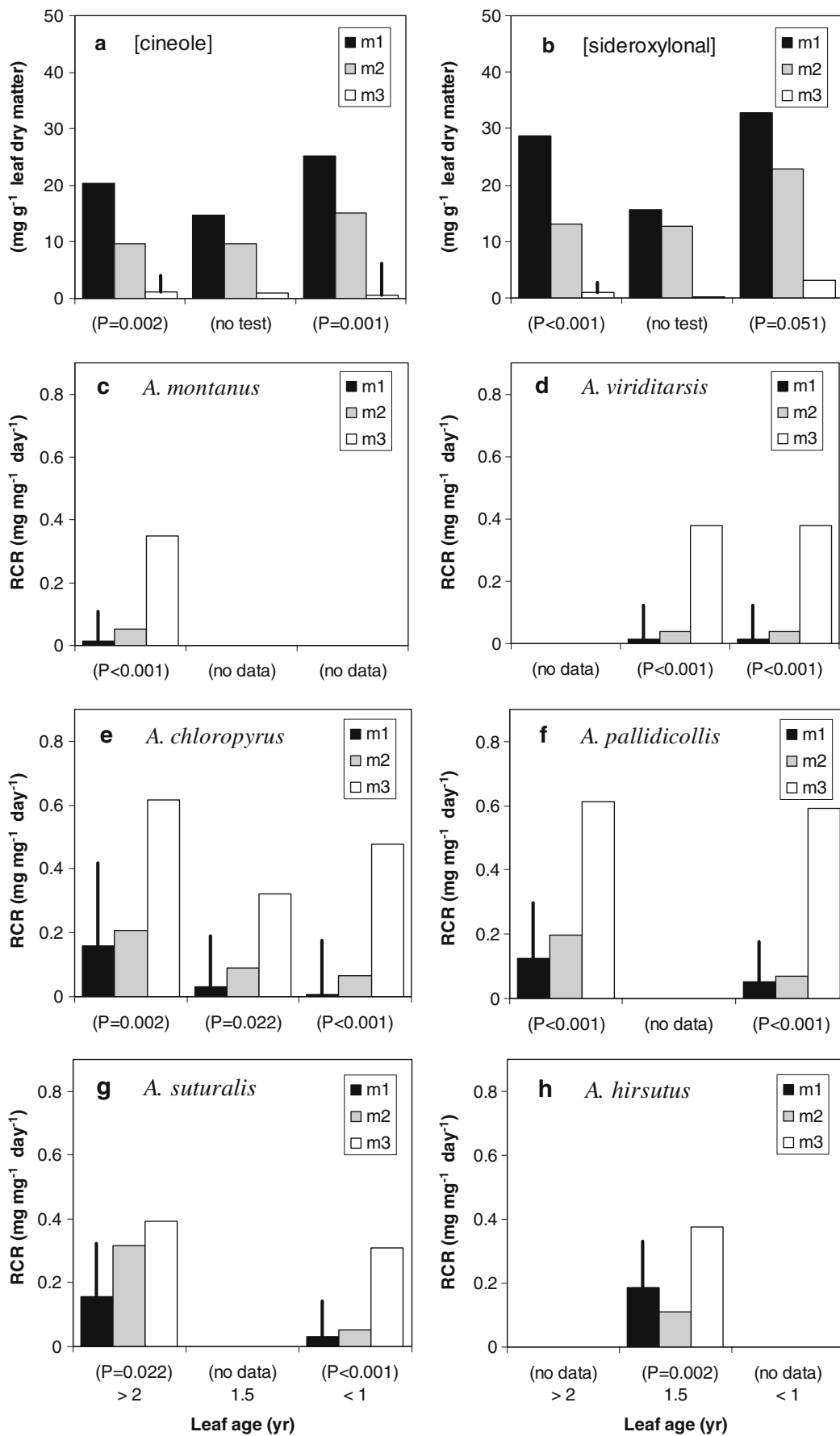
Where DR_i is the dietary ratio, defined as the ratio between dry matter consumption of a leaf of tree *i* (DMC_i) and the total dry matter consumption of leaves from tree *i* and tree *j* (DMC_j): $DR_i = DMC_i / DMC_j$. RA_i is the relative amounts of leaf matter of tree *i* offered to a beetle during an experiment

and is defined as the ratio between dry matter of a leaf of tree *i* offered (DMO_i) and the total dry matter of leaves of tree *i* and tree *j* (DMO_j): $RA_i = DMO_i / DMO_j$. Negative values of the index *D* indicate avoidance, while positive values indicate preference. For each experiment, the mean D_i s were calculated, and 95% confidence intervals were used to detect whether the mean preference was significantly positive or negative. We used the index *D* because it met all three criteria for a good index of diet preference proposed by Cock (1978) and is sensitive to small changes in preference (Kam et al., 1997).

Manipulating the Foliar Concentration of Sideroxylonals We manipulated the foliar concentration of sideroxylonals by painting leaves with solutions of purified compounds. We used leaves of an *E. melliodora* tree that was most susceptible to marsupial folivores (m3) and another that was most resistant to marsupial folivores (m1). Purified sideroxylonals were dissolved in 10% methanol: 90% dichloromethane. We painted solutions of sideroxylonals onto leaves of m3 so that the treatment leaves would have approximately 20, 40, or 80 mg sideroxylonals per g leaf dry matter. In *E. sideroxylon* and *E. melliodora* in the field, only a small number of trees have greater than 40 mg sideroxylonals per g leaf dry matter (Watson, 1998; Wallis et al., 2002). We also painted leaves with solvent only as controls. Solutions of sideroxylonals were prepared in 3 different concentrations so that approximately the same amount of the solutions was applied to each leaf. The experimental design of the sideroxylonal manipulation experiments was 1 *Anoplognathus* species × 5 blocks × 7 treatments (2 trees × no solvent or solvent control + 3 concentrations of sideroxylonals) × 3 replicates. Blocks were as in no-choice and choice/no-choice experiments. Separate experiments were carried out for four *Anoplognathus* species.

Cineole Addition Experiments We also artificially increased the concentration of 1,8-cineole by painting leaves with purified compound. Approximately 0.1 ml of purified 1,8-cineole was applied to leaves of the most susceptible *E.*

Fig. 1 Mean concentrations of sideroxylonals and 1,8-cineole and mean relative consumption rates (RCR) of Christmas beetles. **a–h** *Eucalyptus melliodora* trees (m1–m3) and **i–p** *Eucalyptus sideroxylon* trees (s1–s3) with records of susceptibility to marsupial folivores. *N*=15 beetles per tree. Grey bars and open bars represent intermediate and susceptible trees, respectively, to marsupial folivores. Solid bars represent a resistant tree to marsupial folivores. The *P*-value for the differences among three trees for each leaf age is shown in parentheses. When *P*<0.05, the LSD for comparing the means is shown as the vertical line. No statistical test was carried out for concentrations of sideroxylonal and 1,8-cineole in 1.5 yr-old leaves because the concentration for each tree was estimated from ground material of multiple leaves per estimate using NIRS



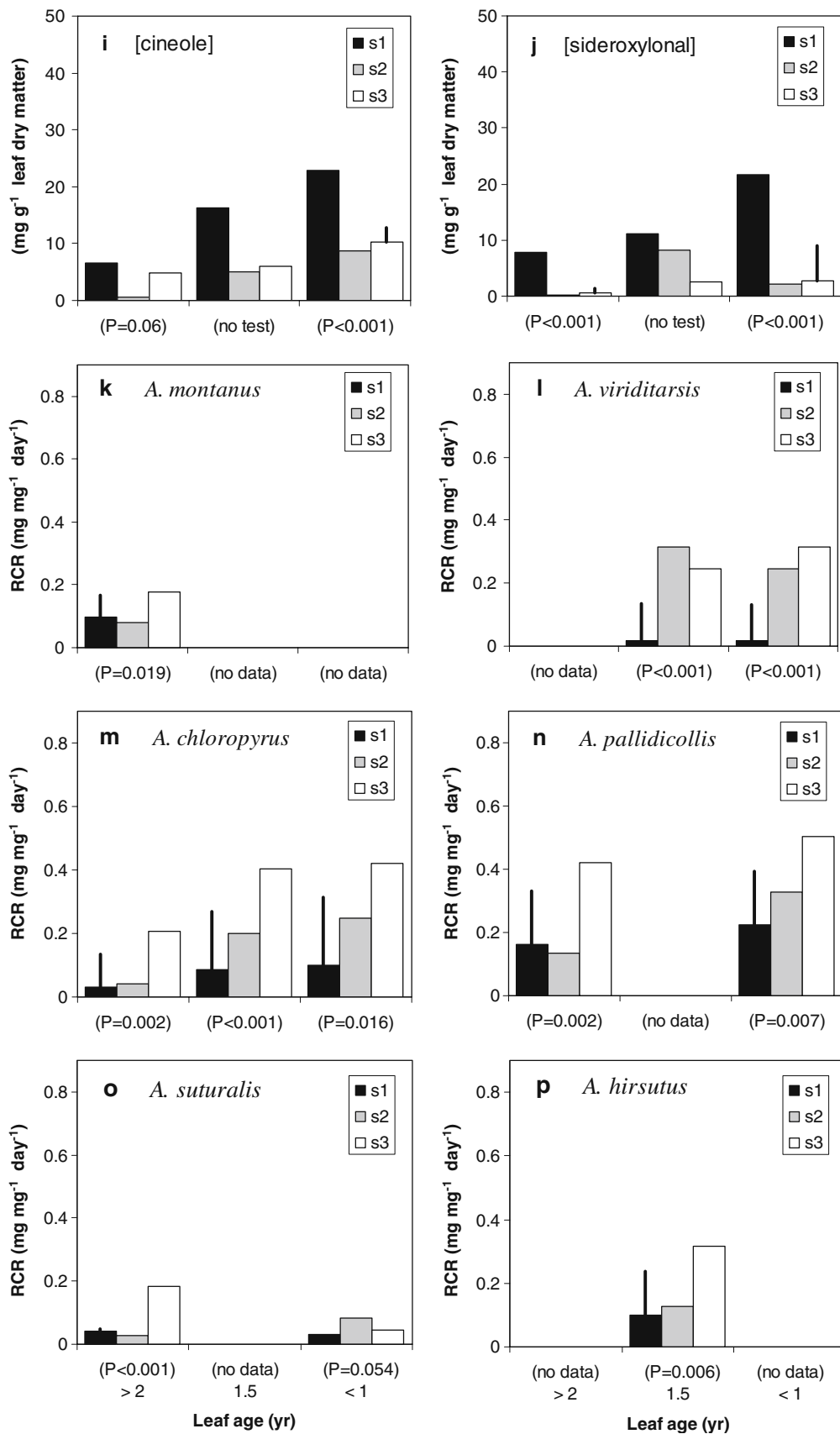


Fig. 1 (continued)

meliiodora tree (m3). We used meshed screw-caps to avoid the build-up of vapor pressure inside the vials due to evaporation of 1,8-cineole. The design of the cineole addition experiments was 1 *Anoplognathus* species \times 1 block \times 2 treatments (cineole addition or control) \times 1 tree \times 10 replicates. Separate experiments were carried out for five *Anoplognathus* species.

Manipulating the Foliar Concentration of 1,8-Cineole by Steaming We reduced the foliar concentration of 1,8-cineole by placing leaves in a stream of steam. In this experiment, we used leaves of the two *E. meliiodora* trees used in the sideroxylonal manipulation experiments (i.e., m1 and m3) and an *E. sideroxylon* tree (s9) with an intermediate concentration of sideroxylonals and a high concentration of 1,8-cineole. Leaves were steamed for 1 min. Fresh (i.e., unsteamed) leaves of the 3 trees were used as controls. Examination of the steamed leaves by GC revealed that steaming resulted in approximately 50% reduction in all monoterpenes, including 1,8-cineole (data not shown). The experimental design of the cineole reduction experiment was 5 blocks \times 2 treatments (steaming or control) \times 3 trees \times 3 replicates. Blocks were as in no-choice and choice/no-choice experiments. This experiment was carried out with *A. chlorophyrus* only.

Experiment with Exotic Species To examine leaf consumption of species without sideroxylonals or monoterpenes, we used 3 species of exotic ornamental trees (*Betula* sp., *Quercus* sp., and *Populus tremuloides*) in a no-choice experiment. Leaves of the exotic species were collected from ornamental trees around the laboratory at CSIRO in Canberra. We included the most susceptible tree of *E. meliiodora* (m3) as the standard in the experiment. The experimental design of the exotic species experiment was 5 blocks \times 4 treatments (tree species) \times 3 replicates. Blocks were as in no-choice and choice/no-choice experiments. This experiment was carried out with *A. chlorophyrus* only.

Statistical Analyses We analyzed data on leaf physical and chemical characteristics and consumption using ANOVAs (when the data set was balanced) or restricted maximum likelihood analyses (when the data set was unbalanced). When we examined differences among species, populations, and individual trees in leaf physical and chemical traits, we treated species, populations, and trees as fixed factors in nested ANOVAs. Since we used only 3 individuals of *E. polyanthemus* from 1 population in this study, we excluded *E. polyanthemus* from the analysis of differences between species.

In this study, we did not statistically examine effects of host plant population/beetle species on leaf consumption by beetles. The reasons for this are: (1) separate experiments

were conducted for each beetle/host population combination; and (2) leaf consumption by beetles decreased gradually as the season progressed. Therefore, if a particular species of Christmas beetle shows lower consumption in an experiment conducted late in the season with a host population when compared with its consumption in an experiment conducted early in the season with another host population, the difference in consumption is likely due to combined effects of season and differences in host plant leaf chemistry.

However, we were able to examine relationships between the reduction in the mean relative consumption rates (RCRs) and changes in leaf traits, (specific leaf weight and concentrations of sideroxylonals and 1,8-cineole) in *E. meliiodora* and *E. sideroxylon*. For each no-choice experiment, we calculated differences in the mean RCRs and the three leaf traits between a pair of trees: (1) resistant – susceptible; (2) resistant – intermediate; and (3) intermediate – susceptible. For each beetle species, the changes in the mean RCRs and leaf traits between pairs of trees from different experiments were pooled. *Anoplognathus montanus* and *A. chlorophyrus* were the only species with more than five comparisons per tree pair, and other beetle species were not used in this analysis. For each beetle species and each of the three pairs of trees, we then examined the slope of the regressions between the changes in the mean RCRs (response variable) and the changes in each of the tree leaf traits (explanatory variable). Because the three leaf traits sometimes showed significant correlations (i.e., multicollinearity), we were not able to compare relative strength of the three leaf traits by using multiple regressions. We used GENSTAT (Genstat 5 Committee, 1993) and SAS (SAS Institute, 1989) for the statistical analyses.

Results

Host Eucalyptus Species Under no-choice situations, leaf consumption by all six species of Christmas beetles showed marked variation among three trees of *E. meliiodora* (m1–m3) and three trees of *E. sideroxylon* (s1–s3), and all six species of beetles showed qualitatively consistent pattern of leaf consumption (Fig. 1). The Christmas beetles consumed more leaves from trees with markedly lower concentrations of sideroxylonals and 1,8-cineole than trees with high concentrations of sideroxylonals and 1,8-cineole. Leaves of the resistant trees (with high concentrations of sideroxylonals and 1,8-cineole) tended to contain higher concentrations of some other monoterpenes such as α -pinene, limonene, and terpineol but lower concentrations of *p*-cymene and α -phellandrene than leaves of susceptible trees (with low concentrations of sideroxylonals and 1,8-cineole) (Table 1).

Table 1 Differences in physical and chemical characteristics of leaves (>2 yr-old) among species, populations, and trees of *Eucalyptus melliodora* and *Eucalyptus sideroxylon* and differences between resistant and susceptible trees of the two *Eucalyptus* species

	Differences among			Resistant vs. susceptible		
	Species <i>P</i> ^a	Populations <i>P</i> ^a	trees <i>P</i> ^a	<i>P</i> ^b	Means	Unit
water content	0.20	0.41	< 0.001	0.40	44.4 vs. 46.0	%
SLW ^c	0.23	0.87	< 0.001	0.08	0.18 vs. 0.16	mg mm ⁻²
[nitrogen]	0.55	0.02	–	0.20	135 vs. 147	mg g ⁻¹
[sideroxylonals]	0.25	0.25	< 0.001	0.07	16.1 vs. 5.67	mg g ⁻¹
[α-pinene]	0.07	0.04	< 0.001	0.24	1.53 vs. 0.59	mg g ⁻¹
[α-phellandrene]	0.92	0.49	< 0.001	0.02	0.16 vs. 1.09	mg g ⁻¹
[limonene]	0.14	0.96	< 0.001	0.007	0.91 vs. 0.12	mg g ⁻¹
[1,8-cineole]	0.73	0.72	< 0.001	0.003	15.2 vs. 2.1	mg g ⁻¹
[<i>p</i> -cymene]	0.44	0.96	< 0.001	0.007	0.06 vs. 0.77	mg g ⁻¹
[terpineol]	0.15	0.69	< 0.001	0.06	0.88 vs. 0.05	mg g ⁻¹

^a from ANOVAs. *P*<0.05 are shown in bold

^b from planned contrasts. *P*<0.05 are shown in bold

^c SLW=Specific Leaf weight

However, 1,8-cineole was the dominant component of the *Eucalyptus* oils. The concentration of 1,8-cineole ranged from 0 to 27 mg g⁻¹ leaf dry matter, while the maximum concentration of any one monoterpene other than 1,8-cineole was 3.5 mg g⁻¹ leaf dry matter. Leaf nitrogen concentration, leaf water content, and specific leaf weight did not differ significantly between the resistant and susceptible trees (Table 1). When we examined relationships between changes in leaf consumption and changes in leaf traits between pairs of trees (resistant – susceptible, resistant – intermediate, and intermediate – susceptible), the decreases in relative consumption rates in *A. montanus* and *A. chloropyrus* tended to be more strongly associated with the increases in concentrations of sideroxylonals and 1,8-cineole than the changes in specific leaf weights (Table 2).

The same qualitative pattern of difference in leaf consumption of the same six trees (m1–m3 and s1–s3) was observed between years and between young (<1 yr-

old) and old leaves (>2 yr-old). The same qualitative pattern also was observed in three different populations of *A. chloropyrus* (the only species examined) in the same year (Fig. 2). Moreover, for at least two species of Christmas beetles (*A. montanus* and *A. chloropyrus*) the same qualitative pattern of tree discrimination associated with the same pattern of leaf chemical composition was observed in trees from Yeoval (m4–m7) and Cumnock (s4–s7) (Fig. 3). For these trees, leaf consumption in the laboratory was correlated directly with the historical record of defoliation by Christmas beetles (mostly *A. montanus*).

There was a striking difference in the behavior of Christmas beetles depending on the leaves offered. The entire leaf, except mid vein and leaf margins, was consumed when leaves with low concentrations of sideroxylonals and 1,8-cineole were offered. In contrast, when leaves with high concentrations were offered, beetles either did not feed at all or took one to several

Table 2 Relationships between reduction in relative consumption rates (ΔRCRs) by *Anoplognathus montanus* and *Anoplognathus chloropyrus* and increases in concentrations of sideroxylonals (Δsideroxylonals) or 1,8-cineole (Δ1,8-cineole), or changes in specific leaf weight (ΔSLW)

Response variable	<i>N</i> ^a	Explanatory variable		
		Δsideroxylonals	Δ1,8-cineole	ΔSLW
<i>A. montanus</i>				
resistant – susceptible	8	-0.80±0.24 ^b (0.02 ^c)	-0.66±0.31 (0.08)	-0.57±0.41 (0.22)
resistant – intermediate	6	0.55±0.43 (0.25)	-0.88±0.23 (0.02)	-0.12±0.50 (0.83)
intermediate - susceptible	6	-0.64±0.38 (0.17)	-0.97±0.13 (0.002)	-0.89±0.23 (0.02)
<i>A. chloropyrus</i>				
resistant – susceptible	7	-0.76±0.29 (0.05)	-0.92±0.17 (0.003)	-0.29±0.31 (0.54)
resistant – intermediate	8	0.10±0.14 (0.82)	-0.97±0.10 (< 0.001)	-0.11±0.41 (0.79)
intermediate - susceptible	8	-0.66±0.31 (0.08)	-0.87±0.20 (0.005)	-0.58±0.33 (0.13)

^a *N*=number of comparisons

^b Standardized regression coefficient (= slope)±SE

^c *P*-value for whether the slope=0. *P*<0.05 are shown in bold

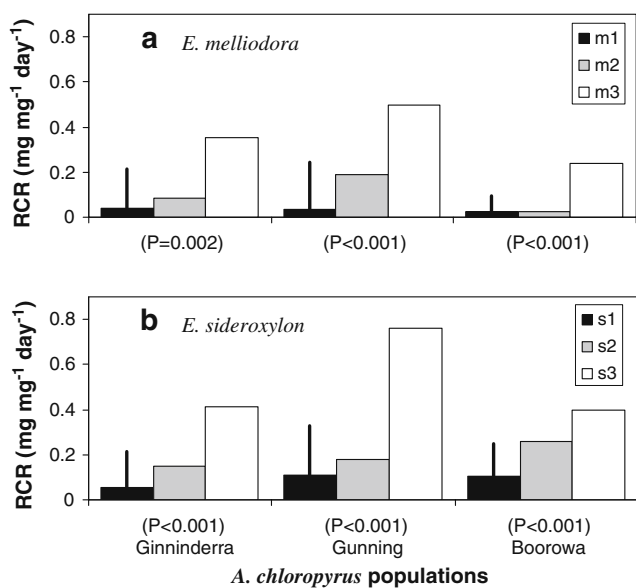


Fig. 2 Mean relative consumption rates (RCR) of three different populations of *Anoplognathus chloropyrus* on **a** *Eucalyptus melliodora* and on **b** *Eucalyptus sideroxylon* trees. $N=12-15$ beetles per tree. Ginninderra, Gunning, and Boorowa are locations from which the three populations have been collected. The P -value for the differences among three trees for each population is shown in parentheses. When $P<0.05$, the LSD for comparing the means is shown as the vertical line. 1.5 yr-old leaves were used in all experiments, and the concentrations of sideroxylonals and 1,8-cineole were shown in Fig. 1

bites at a number of locations along the leaf margins but never continued feeding. Leaves of some trees (e.g., s9) either were consumed nearly entirely (as leaves of the susceptible trees) or not touched at all. Leaves of s9 contained an intermediate concentration of sideroxylonals and a high concentration of 1,8-cineole (Fig. 3). Some leaves of s9 were never chewed. These observations lead to the hypothesis that trees with low to moderate concentrations of sideroxylonals might be avoided by Christmas beetles due to presence of strong negative cues (e.g., high concentrations of 1,8-cineole). This hypothesis was tested by artificially manipulating the concentrations of sideroxylonals and 1,8-cineole (see below).

When beetles were given a choice between leaves from two different trees of *E. melliodora*, they always preferred the susceptible to the resistant or the intermediate tree (Table 3). The intermediate tree always was preferred to the resistant tree. When leaves from two trees of *E. sideroxylon* were offered together, beetles also always preferred the susceptible to the resistant or the intermediate tree (Table 3). The intermediate tree was preferred to the resistant tree by three species of Christmas beetles, but not by *A. chloropyrus*. Differences in preference among the three *E. sideroxylon* trees were not as large as those among the three *E. melliodora* trees.

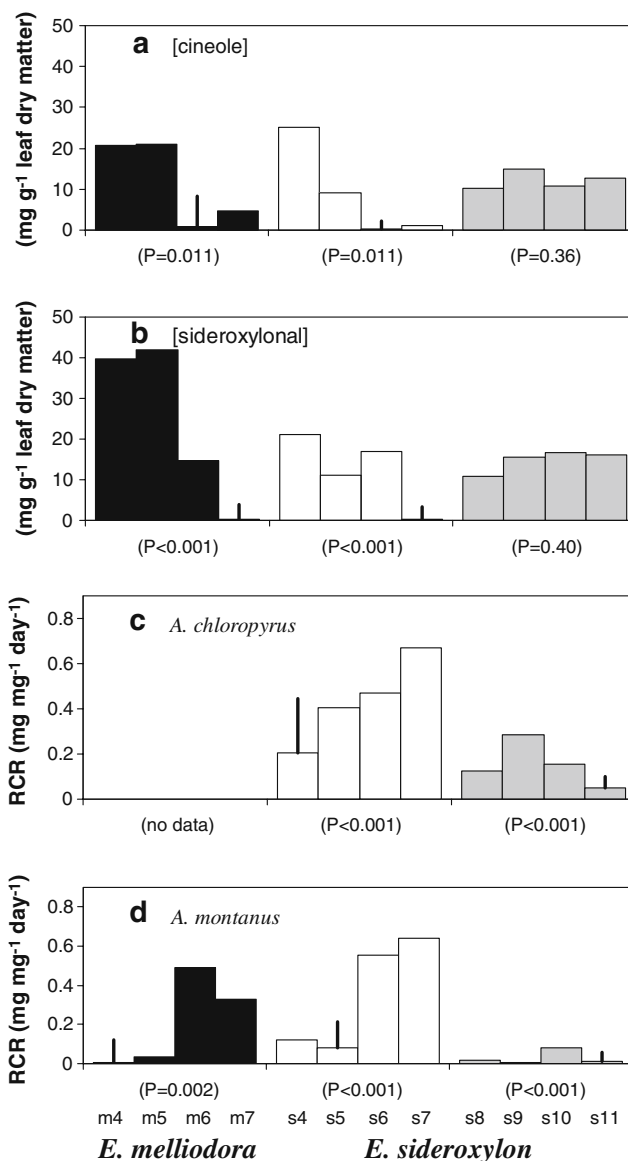


Fig. 3 Mean concentrations of **a** 1,8-cineole and **b** sideroxylonals of *Eucalyptus melliodora* trees from Yeoval (solid bars) and *Eucalyptus sideroxylon* trees from Cumnock (open bars) with records of herbivory by Christmas beetles and *Eucalyptus sideroxylon* trees from Canberra (grey bars) without records of herbivory and mean relative consumption rates (RCR) of **c** *Anoplognathus chloropyrus* and **d** *Anoplognathus montanus*. $N=15$ beetles per tree. The P -value for the differences among four trees for each population is shown in parentheses. When $P<0.05$, the LSD for comparing the means is shown as the vertical line. 1.5–2 yr-old leaves were used in all experiments

Non-host Eucalyptus Species Leaf water content and specific leaf weight of *E. polyanthemos*, which was not a preferred host of Christmas beetles, did not differ from those of either *E. melliodora* or *E. sideroxylon* (data not shown). In terms of leaf chemistry, *E. polyanthemos* differed from the other two species by having generally low values and little between-individual variation in

Table 3 Between-tree feeding preference by *Anoplognathus* species with three species of *Eucalyptus* species

Trees compared	<i>E. melliodora</i> ^a	<i>E. sideroxylon</i> ^a	<i>E. polyanthemus</i> ^b
susceptible over intermediate	0.43 ^{c,d} (15 ^c) – 0.96 (15)	0.43 (13) – 0.77 (13)	0.34 (10) – 0.56 (5)
susceptible over resistant	0.41 (14) – 0.94 (14)	0.26 (15) – 0.49 (12)	–0.05 (10) – 1.04 (5)
intermediate over resistant	0.42 (15) – 0.63 (14)	–0.81 (3) – 0.96 (5)	0.52 (10) – 0.99 (6)

All leaves used in choice/no-choice experiments were >2 yr-old

^a experiments using *Anoplognathus montanus*, *Anoplognathus chloropyrus*, *Anoplognathus pallidicollis*, and *Anoplognathus suturalis*

^b experiments using *Anoplognathus montanus* and *Anoplognathus chloropyrus* only

^c Ranges of mean values of the index D. When D>0, preference of trees as in the “Trees compared” column. When D<0, reversal in preference of trees from that in the “Trees compared” column

^d Bold-faced numbers indicate values significantly greater than 0 at $\alpha=0.05$

^e Sample size

concentrations of α -phellandrene (0.19–0.53 mg g⁻¹ leaf dry matter) and *p*-cymene (0 mg g⁻¹ leaf dry matter). In contrast, like *E. melliodora* and *E. sideroxylon*, concentrations of sideroxylonals and 1,8-cineole in the three *E. polyanthemus* trees differed markedly (Fig. 4). Leaf consumption by Christmas beetles, however, did not show corresponding differences (Fig. 4). *Anoplognathus suturalis* consumed as much leaf material of *E. polyanthemus* as that of *E. melliodora* and *E. sideroxylon*, while five other species of Christmas beetles consumed much less leaf material of *E. polyanthemus* compared with the other *Eucalyptus* species.

Effects of Sideroxylonals and 1,8-Cineole on Leaf Consumption There was a negative dosage-dependent effect of sideroxylonals on leaf consumption in all four species of Christmas beetles tested (Fig. 5). Relative consumption rates (RCR) were reduced to approximately one third of those in the control when concentrations of sideroxylonals were high (40 and 80 mg g⁻¹ leaf dry matter). In *A. chloropyrus* and *A. pallidicollis*, there was no effect of increased concentrations of sideroxylonals at least up to one half of the maximum concentration found naturally in leaves of *E. sideroxylon* and *E. melliodora* (i.e., 20 mg g⁻¹ leaf dry matter).

When pure 1,8-cineole was painted onto leaves of *Eucalyptus* branches, Christmas beetles immediately ceased feeding and quickly moved far away from the painted 1,8-cineole. An artificial increase in the concentration of 1,8-cineole reduced leaf consumption by all five species of Christmas beetles tested (Fig. 5). The beetles did not feed on leaves painted with 1,8-cineole until the 1,8-cineole had evaporated. Once that happened, the treatment leaves were consumed just as the control leaves. Therefore, the observed reduction in consumption is due to effective reduction in feeding time caused by evaporating 1,8-cineole.

An artificial 50% reduction in the concentration of 1,8-cineole by steaming leaves increased leaf consumption by *A. chloropyrus* on s9 with the moderate natural concentration of sideroxylonals and high natural concentration of 1,8-cineole (Fig. 6: see s9 in Fig. 3a and b for natural concentrations of sideroxylonal and 1,8-cineole). However, steaming leaves did not affect the consumption of leaves when the natural concentrations of both sideroxylonals and 1,8-cineole were high (m1) or low (m3).

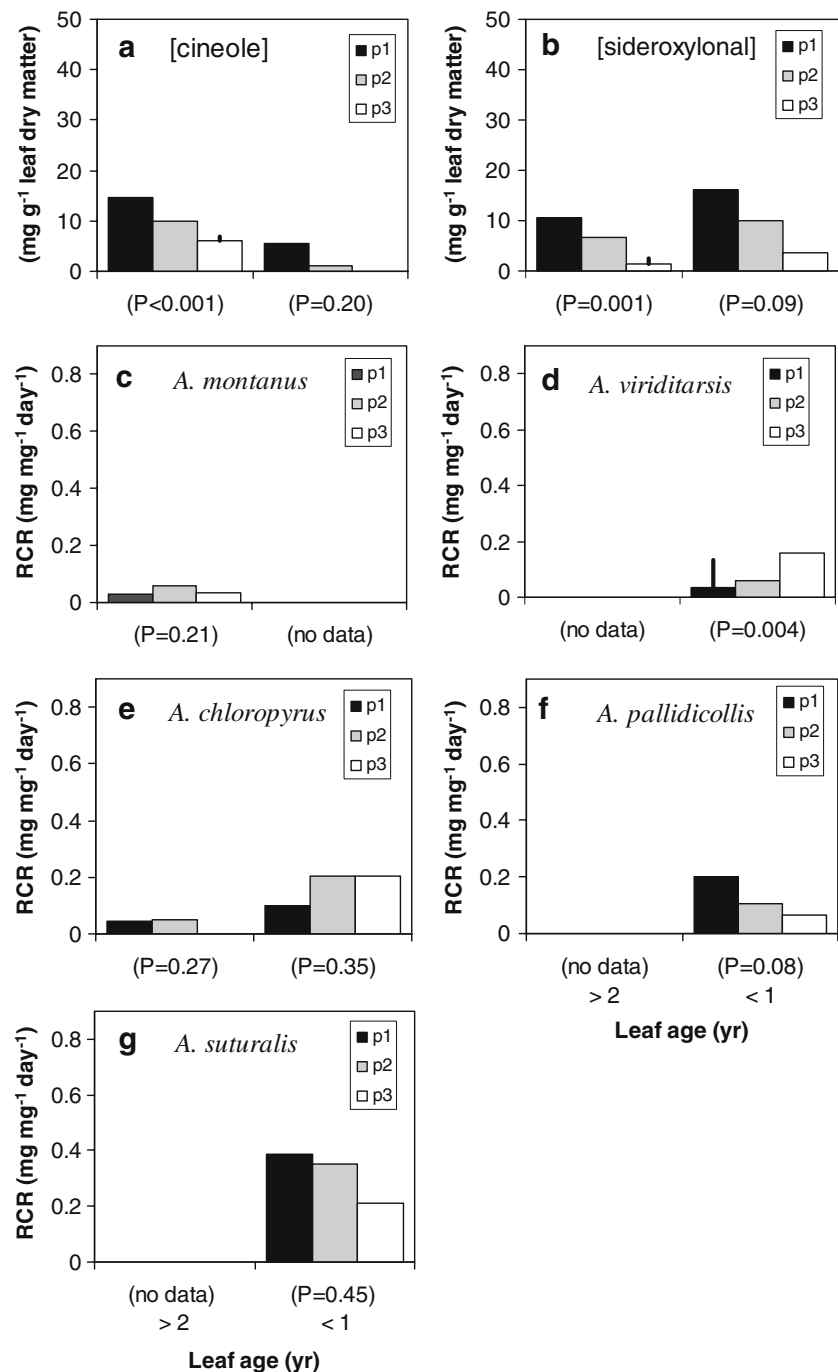
Exotic Species The mean relative consumption rates (RCRs) by *A. chloropyrus* varied among species of exotic ornamental trees: very low in *Populus tremuloides* (0.014 mg mg⁻¹ day⁻¹) and moderately high in *Betula* sp. (0.20 mg mg⁻¹ day⁻¹) and *Quercus* sp. (0.32 mg mg⁻¹ day⁻¹). The mean RCR on *Quercus* sp. was not different from the mean RCR on m3 (0.45 mg mg⁻¹ day⁻¹) (LSD=0.14 for comparing the means). Monoterpenes were not detected in leaves of these species.

Discussion

New findings from this study are: (i) high concentrations of sideroxylonals or 1,8-cineole alone reduce leaf consumption by Christmas beetles, and (ii) sideroxylonals may act as post-ingestive deterrents, while 1,8-cineole may act as a pre-ingestive deterrent in host selection by Christmas beetles. Consistent with previous studies, we also found that: (i) there were positive correlations between concentrations of 1,8-cineole and sideroxylonals in *Eucalyptus melliodora* (as in Moore et al., 2004a), *E. sideroxylon*, and *E. polyanthemus*, and (ii) trees with low concentrations of sideroxylonals (as in Andrew et al., 2007) and 1,8-cineole (as in Edwards et al., 1990, 1993) suffered greater leaf consumption by beetles than trees with high concentrations

Fig. 4 Mean concentrations of **a** 1,8-cineole and **b** sideroxytonals of *Eucalyptus polyanthemos* trees and **c–g** mean relative consumption rates (RCR) of Christmas beetles.

$N=15$ beetles per tree. The P -value for the differences among three trees for each leaf age is shown in parentheses. When $P<0.05$, the LSD for comparing the means is shown as the vertical line. *Solid bars* represent a resistant tree to common ringtail possum. *Grey bars* and *open bars* represent intermediate and susceptible trees, respectively, to common ringtail possums

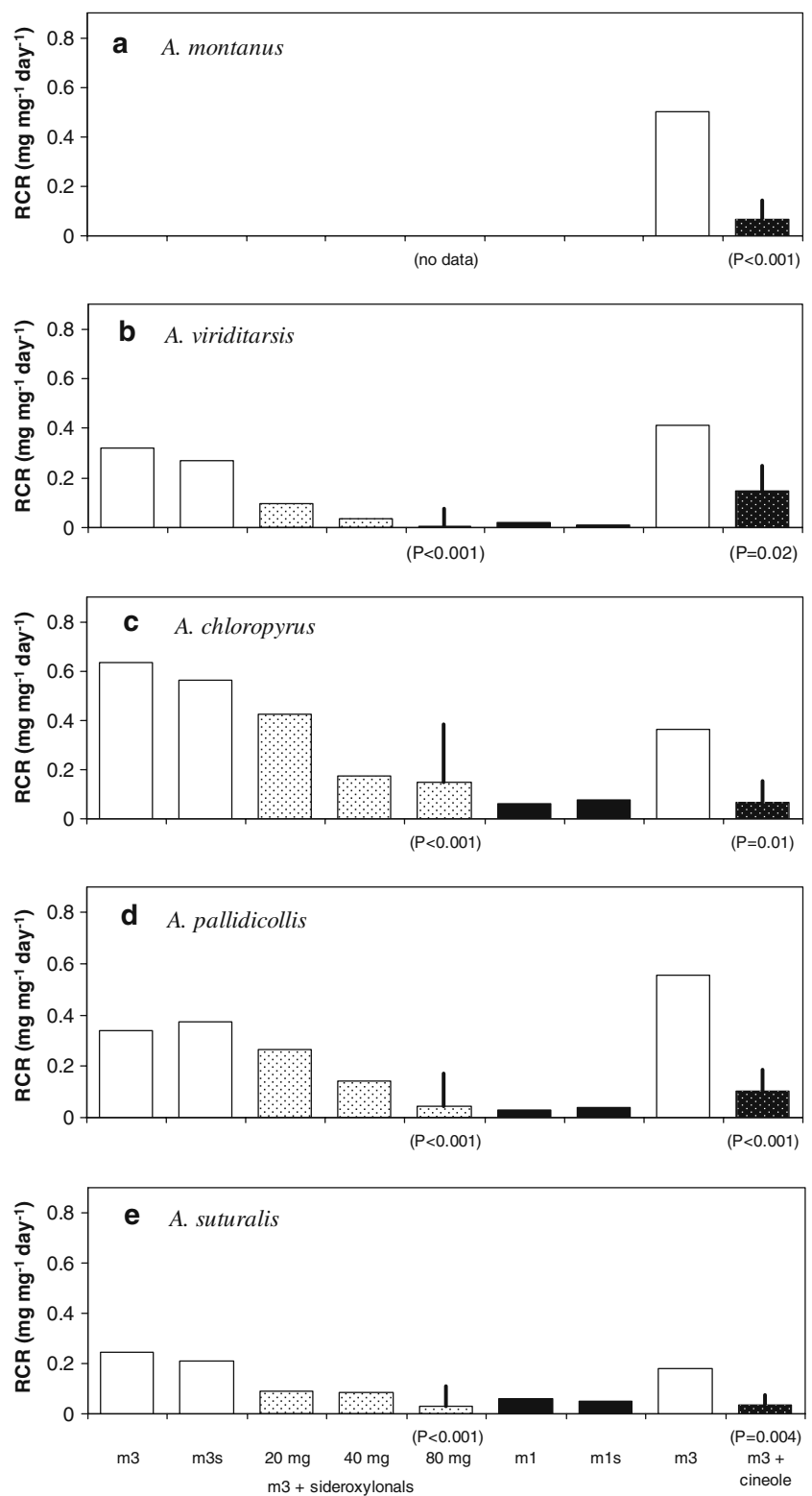


of the two metabolites. Andrew et al. (2007) suggested that high concentrations of sideroxytonals conferred resistance of some individual trees of *E. melliodora* to Christmas beetles. However, by examining and manipulating multiple compounds in host and non-host species, we found that beetles might select host trees by detecting more than one plant secondary metabolite.

When leaves with high concentrations of sideroxytonals were offered, Christmas beetles took only one to several bites that were spread along the margin of leaves (M. Matsuki, pers.

obs.). Thus, they appeared to detect the presence of sideroxytonals using sensory organs in their mouthparts. However, when leaves with moderate concentrations were offered, beetles continued feeding for some time but never consumed entire leaves. Thus, it appears that, even when sideroxytonals are ingested at low concentrations, there is another mechanism which stops Christmas beetles from ingesting large amounts of sideroxytonals. Marsupial folivores also regulate intake of sideroxytonals and some other FPCs (Lawler et al., 1998; Stapley et al., 2000; Wallis et al., 2002).

Fig. 5 Effects of artificially increased concentration of sideroxylonals and 1,8-cineole on relative consumption rate (RCR) of *Eucalyptus melliodora* leaves by five species of Christmas beetles. Data present means of $N=11-15$ beetles per tree. The P -value for the differences among the treatment is shown in parentheses. When $P<0.05$, the LSD for comparing the means is shown as the vertical line. Separate P -values and LSDs are shown for the sideroxylonal addition experiment (seven bars on the left) and the cineole addition experiment (two bars on the right). Open bars represent the susceptible tree (m3), and closed bars represent the resistant tree (m1). Solvent controls are represented as m1s and m3s. Trees m1 and m3 were also shown in Figs. 1, 2 and 6. > 2 yr-old leaves were used with *Anoplognathus chloropyrus* and *Anoplognathus pallidicollis*, while <1 yr-old leaves were used with *Anoplognathus montanus*, *Anoplognathus viriditarsis*, and *Anoplognathus suturalis*



Monoterpenes (e.g., 1,8-cineole) also can have harmful effects on insects post-ingestively (Rose, 1985). However, *A. montanus* and *Paropsis atomaria* (Coleoptera: Chrysomelidae) feeding on *Eucalyptus* leaves may simply

“tolerate” (Morrow and Fox, 1980) or “avoid” (Edwards et al., 1993) monoterpenes.

Results of the experiment with steamed leaves suggest that Christmas beetles may use 1,8-cineole, or other volatile

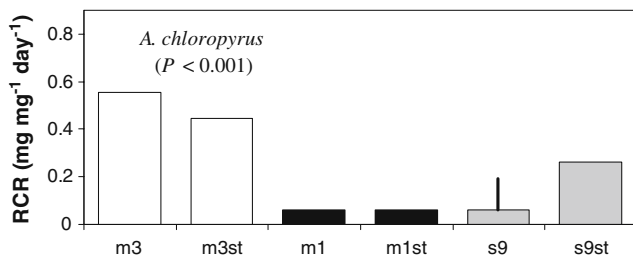


Fig. 6 Effects of artificially decreased concentration of 1,8-cineole by steaming leaves on relative consumption rate (RCR) of *Eucalyptus* leaves by *Anoplognathus chloropyrus*. Data present means of $N=15$ beetles per tree. The P -value for the differences among the treatment is shown in parentheses. The LSD for comparing the means is shown as the vertical line. Open bars represent the susceptible tree (m3), and the closed bars represent the resistant tree (m1). Trees m1 and m3 were also shown in Figs. 1, 2 and 5. Leaves of tree s9 (grey bars) contain high concentration of 1,8-cineole but only moderate concentration of sideroxylonals (cf. Fig. 3a & b). Leaf consumption on steamed leaves is indicated as m3st, m1st and s9st. >2 yr-old leaves were used in the experiment

compounds in *Eucalyptus* oils, as a pre-ingestive deterrent for selecting host trees. In the field, Christmas beetles fly around the canopy at close range. If a tree had high concentrations of sideroxylonals and 1,8-cineole, many beetles flew away without landing on a leaf or landed on a leaf but took off without feeding (M. Matsuki, pers. obs.). Since Christmas beetles seem to detect high concentrations of sideroxylonals with sensory organs in their mouthparts, the observed host selection behavior in the field is not likely driven by sideroxylonals in leaves. Christmas beetles may detect high concentrations of volatile terpenes in the atmosphere immediately surrounding resistant trees.

Guilford and Cuthill (1991) have suggested that evolution of unpalatability is not a likely result of selection on individuals that share the same phenotype (i.e., synergistic selection) because, unlike aposematic coloration in animals, palatable and unpalatable plants appear identical except for the difference in palatability. However, volatile compounds such as 1,8-cineole may act as a signal for the presence and the concentration of compounds such as sideroxylonals (this study; Lawler et al., 1999: for the debate on whether one plant secondary metabolite can serve as a signal for another, see Dethier and Post, 1979; Eisner and Grant, 1981; Launchbaugh and Provenza, 1993; Augner, 1994; Augner and Bernays, 1998).

Tuomi and Augner (1993) and Leimar and Tuomi (1998) showed that evolution of plant unpalatability might result from synergistic selection, even without a cue, if herbivores took samples from a population of plants and made generalizations about the population. *Eucalyptus melliodora* and *E. sideroxylon* showed striking variation in leaf chemistry among neighboring individuals (Edwards et al. 1993). Therefore, if synergistic selection has been involved in evolution of chemical defenses using sideroxylonals, the

presence of volatile compounds such as 1,8-cineole, which can be used as a cue (Guilford and Cuthill 1991), is a more likely mechanism than leaf sampling and subsequent generalization by herbivores (Tuomi and Augner 1993; Leimar and Tuomi 1998).

An interesting twist in the *Eucalyptus*—herbivore system is that some trees of *E. sideroxylon* seem to have only low to intermediate concentrations of sideroxylonals but high concentrations of 1,8-cineole (e.g., s8–s11 in this study). If these trees suffer low levels of herbivory in the field, as is observed in the laboratory experiments, then it may be possible that high concentrations of 1,8-cineole in these trees act as a false signal.

So far, we have focused on the role of sideroxylonals and 1,8-cineole as negative cues in host selection, but host selection by Christmas beetles may involve negative and positive cues. Under natural conditions, Christmas beetles encounter four types of plants: (1) host *Eucalyptus* species that contain sideroxylonals and monoterpenes; (2) non-host *Eucalyptus* species that also contain sideroxylonals and monoterpenes; (3) non-host species that contain monoterpenes (but no sideroxylonals); and (4) non-host species that do not contain sideroxylonals or monoterpenes. Moreover, within the host *Eucalyptus* species, only a small proportion of individuals contain low concentrations of sideroxylonals (Lawler et al., 2000; Wallis et al., 2002). If negative cues (i.e., high concentrations of sideroxylonals and 1,8-cineole) alone were used to locate a small number of suitable host trees in the field, Christmas beetles may have to spend great amounts of time and energy before starting to feed.

Christmas beetles readily consumed leaves of some exotic species in captivity. Yet, they rarely consume leaves of these exotic plant species in the field. Leaves of the exotic species do not contain sideroxylonals or 1,8-cineole. Therefore, lack of feeding by beetles on those exotic species under natural conditions cannot be explained by high concentrations of sideroxylonals or 1,8-cineole in the leaves. Christmas beetles did not feed on *Populus tremuloides*. This is possibly because *P. tremuloides* have phenolic glycosides in their leaves (e.g., Osier et al., 2000).

Steinbauer and Wanjura (2002) suggested that Christmas beetles mistook *Schinus molle* (Anacardiaceae) for eucalypts because leaves of this exotic species contained monoterpenes. Other specialist insect herbivores also are attracted to non-host species that have similar secondary metabolites to the hosts (e.g., Murphy and Freeland, 2006; Östrand et al., 2008). However, presence of monoterpenes in general does not seem to signal the suitability as host plants because many *Eucalyptus* species are non-hosts for Christmas beetles. For example, leaves of most trees of *E. polyanthemos* are not consumed by Christmas beetles in the field even when beetles consume leaves on trees of *E.*

meliiodora and *E. sideroxylon* in the same area (M. Matsuki, pers. obs.). Since three species of Christmas beetles consumed moderate amounts of *E. polyanthemos* leaves in captivity, it is more likely that *E. polyanthemos* leaves lack positive cues than that the leaves have negative cues. Concentrations of α -phellandrene and *p*-cymene generally were low and showed little between-individual variation in *E. polyanthemos*. In contrast, concentrations of these monoterpenes are negatively correlated with concentrations of 1,8-cineole in *E. sideroxylon* and *E. meliiodora*. Thus, Christmas beetles may be using α -phellandrene or *p*-cymene as a positive cue for host plants. In noctuid moth species, positive and negative cues in a blend of odor are distinguished and processed separately in the brain (Vickers et al., 1998). Thus, to select trees, Christmas beetles may use both positive and negative cues. Alternatively, the ratio of various monoterpenes may be used to select host species by Christmas beetles (Edwards et al., 1993).

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