

# Red leaf margins indicate increased polygodial content and function as visual signals to reduce herbivory in *Pseudowintera colorata*

Luke J. Cooney<sup>1</sup>, John W. van Klink<sup>2</sup>, Nicole M. Hughes<sup>3</sup>, Nigel B. Perry<sup>2</sup>, H. Martin Schaefer<sup>4</sup>, Ignatius J. Menzies<sup>1</sup> and Kevin S. Gould<sup>1</sup>

<sup>1</sup>School of Biological Sciences, Victoria University of Wellington, PO Box 600, Wellington, New Zealand; <sup>2</sup>Department of Chemistry, The New Zealand Institute for Plant & Food Research Limited, University of Otago, PO Box 56, Dunedin, New Zealand; <sup>3</sup>Department of Biology, High Point University, University Station 3591, High Point, NC 27262, USA; <sup>4</sup>Department of Evolutionary Biology and Animal Ecology, Faculty of Biology, University of Freiburg, Hauptstrasse 1, D-79104 Freiburg, Germany

## Summary

Author for correspondence:  
Luke J. Cooney  
Tel: +64 4 4635266  
Email: cooneyluke@myvuw.ac.nz

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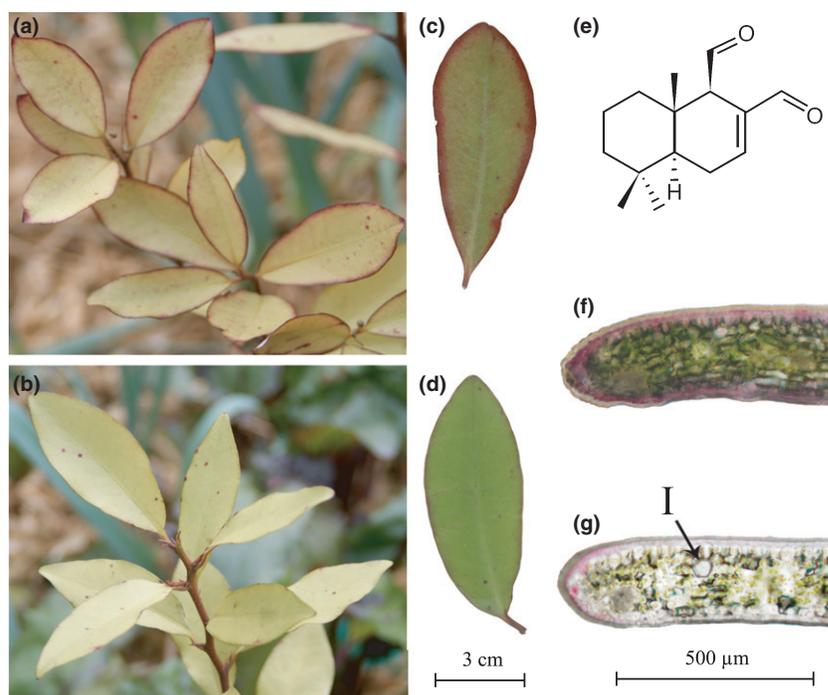
**Key words:** anthocyanin, leaf signalling plant–insect interactions, polygodial, *Pseudowintera colorata*, visual plant aposematism.

- Red-pigmented leaf margins are common, but their functional significance is unknown. We hypothesized that red leaf margins reduce leaf herbivory by signalling to herbivorous insects the presence of increased chemical defences.
- Leaves were collected from a natural population of *Pseudowintera colorata*. Margin size, herbivory damage, anthocyanin content and concentrations of polygodial, a sesquiterpene dialdehyde with antifeedant properties, were quantified. Feeding trials involving larvae of *Ctenopseustis obliquana*, a generalist herbivore, were conducted on red- and green-margined *P. colorata* leaves in darkness, or under white, green or red light.
- Leaves with wider red margins contained higher concentrations of polygodial and anthocyanins, and incurred less natural herbivory. In trials under white light, *C. obliquana* consumed disproportionately more green- than red-margined leaf laminae. Larvae exhibited no feeding preference when light was manipulated such that leaf colour discrimination was impaired.
- Red leaf margins provide a reliable and effective visual signal of chemical defence in *P. colorata*. *Ctenopseustis obliquana* larvae perceive and respond to the colour of the leaf margins, rather than to olfactory signals. Our study provides direct experimental evidence for aposematic coloration in red leaves.

## Introduction

Anthocyanin pigments in plant reproductive organs have long been known to serve as a visual signal to attract animals, promoting pollination and/or seed dispersal (Gould *et al.*, 2009). In vegetative organs, however, anthocyanins might be deployed to repel animals. For example, to explain the autumnal reddening in the leaves of many deciduous trees, Archetti (2000) and Hamilton & Brown (2001) suggested that anthocyanins function as a visual warning to deter browsing by insect herbivores. Accordingly, red leaves would signal enhanced investment in defensive compounds that impair insect fitness. The signal would, in turn, be used by herbivores to select which plant to colonize. Aphids that switch host in autumn would colonize the least defended plants, and aphid infestation would be reduced in the redder, more strongly defended plants (Archetti, 2009). This so-named ‘co-evolution hypothesis’ has received indirect support in recent years from a variety of studies (Hamilton & Brown, 2001; Hagen *et al.*, 2003, 2004; Archetti & Leather, 2005; Karageorgou *et al.*, 2008).

Although originally proposed to explain the reddening or yellowing of the entire lamina in senescing leaves, Archetti’s (2000) and Hamilton & Brown’s (2001) hypotheses might similarly apply to more localized patterns of anthocyanin accumulation in nonsenescing leaves (Lev-Yadun *et al.*, 2002). In many plants, anthocyanic pigmentation is restricted to the stipules, petiole, major veins, trichomes, marginal teeth and/or leaf apex (Wheldale, 1916; Hatier & Gould, 2009). Of particular interest in relation to animal communication are the anthocyanins in the epidermal and/or subepidermal cells at leaf margins. These can provide a prominent red border that presents a sharp chromatic contrast against the green lamina (Fig. 1a–d). Although there has been no published systematic survey of species bearing leaves with red margins, they are evidently very common. Red margins are often used by taxonomists as a character for species identification (Bayly & Kellow, 2006; Versieux & Wanderley, 2007; Redden, 2008), and as a marker to study genetic linkages in crop species as diverse as maize (Flint-Garcia *et al.*, 2005), banana (Jarret *et al.*, 1993), lettuce (Sabharwal & Doležel, 1993), mustard (Nick *et al.*, 1993) and rice (Hadagal *et al.*, 1981). There are also numerous



**Fig. 1** Red- (a, c, f) and green-margined (b, d, g) leaves of *Pseudowintera colorata*: (a, b) photographs of individual branches; (c, d) photographs of leaf laminae; (e) polygodial chemical structure; (f, g) photomicrographs of transverse sections. I, idioblast.

reports of anthocyanic leaf margins being induced in response to mineral nutrient deficiencies (Walker, 1956; Johanson & Walker, 1963; Nyborg & Hoyt, 1970; Haque & Walmsley, 1973; Balo *et al.*, 1975; Hassouna, 1977; Raese, 2002).

The function (if any) of anthocyanins at the leaf margin is unknown, but their extremely restricted histological distribution within the leaf argues against many of the physiological hypotheses for foliar anthocyanins, such as photoprotection and antioxidant activities (Gould, 2004; Hatier & Gould, 2009). However, because many herbivores initiate feeding at the leaf edges, it is at least possible that the marginal anthocyanins function as a visual signal to indicate that the leaves contain unpalatable compounds. Edge-feeding is prominent among Orthopteran, Coleopteran and Lepidopteran larvae (Bernays, 1998), and the ability to detect red hues is well established among members of Lepidoptera and Coleoptera (Briscoe & Chittka, 2001).

During the process of edge-feeding, a plant loses not only those resources contained within the leaf portion eaten, but also the future photosynthetic potential of that portion. A damaged margin enhances evaporative water loss and could place water stress on the adjacent tissues, as well as increase the risk of infection by pathogens. Thus, edge-feeding is costly, and can be more detrimental than other types of herbivory, such as phloem feeding, wherein the leaves are left relatively intact. Relative growth rates of *Solidago altissima*, for example, have been shown to be reduced significantly by edge-feeding beetles, but are unaffected by sap-feeding aphids (Meyer, 1993). It is not surprising, therefore, that secondary metabolites which are considered to be involved in defence against insect herbivores are often more concentrated at the periphery than in the interior regions of a leaf lamina (Gutterman & Chausser-Volfson, 2000; Kester *et al.*, 2002; Shroff *et al.*, 2008; Hughes *et al.*, 2010). Anthocyanins may

provide the means for a plant to advertise defensive compounds in areas in which herbivores prefer to initiate feeding.

To our knowledge, only one previous study has thus far addressed a possible antiherbivory role for red leaf margins. Hughes *et al.* (2010) compared insect damage and leaf phenolic content across 11 *Veronica* species which differed in leaf margin colour. Contrary to the authors' hypothesis, the presence of anthocyanins did not correspond to increased phenolic content at the leaf margins. However, the authors acknowledged limitations to their dataset as all plants had been growing together in a common garden and were not, therefore, subjected to natural herbivory pressure, and the study did not account for interspecific differences in leaf structure and biochemistry. Moreover, measurements of total phenolic content may not be the best estimate of defensive strength; the primary role of many phenolics appears to be to protect leaves from photodamage rather than from herbivory (Close & McArthur, 2002), and the phenolics which are known to be involved in defence constitute only a small fraction of the total phenolic pool (Lawler *et al.*, 1998, 1999). Hughes *et al.* (2010) therefore recommended that future studies focus on specific defensive compounds, rather than general phenolic pools, to explore a possible defensive role of anthocyanic leaf margins, and to utilize intraspecific systems for better control of variation in leaf structure and chemistry. Thus, the hypothesis that red leaf margins reduce insect herbivory remains a possibility that warrants further investigation.

The New Zealand pepper tree, *Pseudowintera colorata* (Winteraceae), is, for several reasons, a particularly useful model to test for defensive functions of red leaf margins (Perry & Gould, 2010). First, populations exhibit pronounced variation in leaf margin colour and size among individuals (Fig. 1; *P. colorata* leaves also develop red margins around areas of mechanical

damage (Gould *et al.*, 2002), but this induced coloration was not a factor in the present study). Second, the primary defence compound in *P. colorata* leaves is known; it is the sesquiterpene dialdehyde polygodial (Fig. 1e), which imparts a pungent taste and has potent insect antifeedant properties (Barnes & Loder, 1962; Asakawa *et al.*, 1988). *In vitro* studies have shown polygodial to be an effective antifeedant at concentrations of 3 mg g<sup>-1</sup> (Gerard *et al.*, 1993), whereas *P. colorata* leaves contain at least 10 mg g<sup>-1</sup> (Wayman *et al.*, 2010). Finally, *P. colorata* herbivores have been documented (<http://plant-synz.landcareresearch.co.nz/>; accessed November 2011). Thus, *P. colorata* presents an opportunity both to collect correlative data on margin colour, herbivory damage and polygodial content, and to conduct feeding trials under controlled laboratory conditions using natural herbivores.

In this study, we tested the fundamental requirement of any visual signal – that red leaf margins in *P. colorata* provide a reliable indication of the defensive status of a plant. We hypothesized that: polygodial concentrations would be highest around the leaf margin, polygodial concentrations in leaves with red margins would be greater than those in leaves with green margins, and leaves with the larger red margins would incur less herbivory. We also tested whether a natural edge-feeding generalist herbivore of *P. colorata*, the brownheaded leafroller moth (*Ctenopseustis obliquana*), responds to the red leaf margins as a visual signal, and avoids the more strongly defended plants. We hypothesized that *C. obliquana* larvae would prefer to eat, and adults to oviposit, on leaves with green margins, but only under light conditions in which colour discrimination was possible.

## Materials and Methods

### Plant material

*Pseudowintera colorata* (Raoul) Dandy leaves were collected on 10 March 2010 in the Otaki Forks region of the Tararua Forest Park (40°54'27.8"S, 175°15'21.1"E) in New Zealand, an area of montane forest (altitude, 860 m) dominated by *Nothofagus* spp. In this population, *P. colorata* is a prominent understorey tree and a polygodial-rich chemotype (Wayman *et al.*, 2010). Random sampling was achieved using six parallel transect lines, 10 m apart and running northeast to southwest. Each transect was 50 m long and spanned a range in elevation not > 20 m. *Pseudowintera colorata* plants within 2 m of each transect were sampled if the plants were at least 10 m from the nearest walking track, between 1.25 and 1.75 m tall and at least 3 m away from any previously sampled plants. A lateral branch bearing at least seven fully expanded leaves was chosen at random and excised from each of 98 plants. These branches were refrigerated within 4 h of collection.

### Margin width and herbivory measurement

Digital images of the abaxial and adaxial surfaces of leaves from nodes 1, 3 and 7 were captured using a Canon CanoScan 8400F flatbed scanner (Tokyo, Japan). Nodes were numbered basipetally from the youngest fully expanded leaf. Images were

processed using GIMP v2.6.7 (<http://www.gimp.org/>) to highlight all red pixels with values between 240 and 255 R units in the RGB colour model. The images were exported into ImageJ v1.41 (National Institutes of Health, Bethesda, MD, USA) and used to measure the width of a red margin at a point normal to the centre of the midrib. If that point had been removed by herbivory, the nearest portion of remaining margin was measured. Because the width of the red margin was relatively uniform around each leaf, a single measurement of margin width was adequate for each leaf.

To quantify herbivory damage, the residual surface area of each leaf lamina was measured using ImageJ. Adobe Photoshop 5.0 (San Jose, CA, USA) was used to re-create the original leaf shape by filling in areas lost to herbivory. If the herbivory damage was too extensive for a confident reconstruction, the original leaf shape was estimated from comparison with that of the adjacent leaf along the branch from which it had been removed. Once the pre-herbivory leaf shape had been reconstructed, its lamina area was again measured using ImageJ. The amount of herbivory was calculated as the difference between pre- and post-herbivory surface areas.

### Anthocyanin and polygodial quantification

A further 20 *P. colorata* individuals were sampled on the same date as above, 10 of which bore leaves with red margins and 10 with green margins. Because of constraints on the numbers of leaves that could be sampled, we restricted the chemical analysis to those leaves for which the margin size was at the upper and lower extremes of the range. A leaf margin was designated as red if its anthocyanic pigmentation at the leaf's widest point extended beyond 10% of the lamina width, and was designated as green if anthocyanins extended to < 2% of the lamina width. The samples excluded leaves that had previously incurred herbivory. Fully expanded leaves from node 1 were removed, the margins were excised from the interiors, and both margin and interior were frozen at - 80°C. Margins were dissected from the most peripheral 5 mm of the leaf's entire circumference. Samples were freeze-dried for 24 h, ground to a powder using a mortar and pestle, and polygodial and anthocyanin concentrations were measured by high-performance liquid chromatography (HPLC) using a procedure modified after Wayman *et al.* (2010). Leaf subsamples (*c.* 10 mg) were extracted with rectified spirits (1 ml) containing C<sub>10</sub> anilide (200 µg) as an internal standard (Perry *et al.*, 1996), briefly sonicated (*c.* 30 s), stirred overnight and then filtered through a 0.45-µm polytetrafluoroethylene (PTFE) filter. Analyses were performed on an Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA, USA) fitted with a diode array detector, using a Luna (II) 250 × 3 mm RP-18 (5 µm) column (Phenomenex, Torrance, CA, USA) with a SecurityGuard™ 4 × 2 mm C18 guard column (Phenomenex) at 30°C. Peaks were monitored at 206, 230 280, 330 and 530 nm. The mobile phase was methyl cyanide (MeCN) in H<sub>2</sub>O, both containing formic acid (0.1%): 5% MeCN at 0 min, 100% at 30 min, 5% at 35 min, 5% at 40 min. The flow rate was 1.0 ml min<sup>-1</sup> with injection volumes of 5 µl. The quantification of polygodial

concentrations was based on calibration with an isolated reference sample of polygodial against the internal standard, with detection at 230 nm. The quantification of anthocyanins was achieved by calibrating with a commercial source of cyanidin-3-*O*-glucoside (Extrasynthese, Genay, France) at 530 nm vs the internal standard at 206 nm. Total anthocyanins were reported as cyanidin-3-*O*-glucoside equivalents.

### Leaf microscopy and reflectance

Transverse hand sections through the interior and margin of fresh leaf material were examined in an Olympus AX70 photomicroscope (Olympus Optical Co., Hamburg, Germany) and photographed using an Olympus DP70 digital camera (Olympus Optical Co.).

Leaf reflectance was measured using an Ocean Optics (Dunedin, FL, USA) USB2000 spectrometer fitted with a PX-2 pulsed xenon light source. Five red- and five green-margined leaves, all from node 1 of different plants, were detached and their reflectances were measured at the leaf margins. The criteria used to categorize leaf margin colour were the same as those used in the polygodial quantification. Light was provided at 45° to the leaf's adaxial surface and diffuse reflectance was measured at 0.4-nm intervals from 400 to 700 nm, and referenced to an Ocean Optics WS-1 diffuse reflectance standard.

### Feeding and oviposition trials

*Ctenopseustis obliquana* (Walker) larvae and pupae were purchased from The New Zealand Institute for Plant & Food Research, Auckland. All had been raised in captivity on a general purpose diet (Singh, 1983). A third-instar larva, 8–10 mm in length, was placed into each of 136 Petri dishes (15 cm in diameter) containing one green- and one red-margined *P. colorata* leaf. The criteria used to categorize leaf margin colour were the same as those used in the polygodial quantification. Leaves were harvested using the sampling strategy described above and stored overnight at 4°C in a plastic bag on a moist paper towel. Both leaves in each dish were similar in size and shape, free of edge-feeding herbivory damage and originated from node 1. Leaves were scanned to measure their surface areas using ImageJ, and placed 3 cm apart within the Petri dish. One larva per dish was placed equidistant from the two leaves. Feeding trials were conducted at 18°C in darkness, or under 25  $\mu\text{mol m}^{-2} \text{s}^{-1}$  white, green or red light, using 34 replicates per treatment. Light was provided by Phillips 36 W cool white fluorescent tubes, with the colour and intensity manipulated using Rosco Supergel (Stamford, CT, USA) plastic filters #398 (neutral density), #389 (green; maximum transmittance 500–520 nm) and #19 (red; maximum transmittance > 620 nm). The dark treatment was obtained by shielding the dishes with opaque polyvinyl. After 72 h, the leaves were re-scanned and the leaf area eaten was calculated using ImageJ to measure the post-herbivory surface area.

Approximately 24 h after pupation, two male and two female adult *C. obliquana* were introduced into each of 80 plastic pots, 18 cm tall  $\times$  20 cm in diameter, which contained two cut stems

of *P. colorata* held in water. The stems had been defoliated with the exception of one mature leaf, and were arranged such that each pot held one red- and one green-margined leaf. All leaves were confirmed to be free of Lepidopteran eggs. To discourage oviposition on the pots themselves, their sides were lined with sandpaper, the bottoms filled with vermiculite and the tops covered with white nylon mesh. Half of the replicates were placed under natural light in a glasshouse, and the remainder held in darkness, with both areas maintained at 18°C. After 72 h, the leaves were removed and the total numbers of eggs were counted.

### Statistical analysis

All statistical analyses were performed using SPSS 16.0 (Chicago, IL, USA). Margin widths and herbivory damage were  $\log_{10}$  transformed to satisfy normality, and a general linear model (GLM) was used to evaluate their relationship, treating individuals as a random factor, and nodes as a fixed factor. Nonlinear regressions using unlogged margin size and herbivory values were performed for each node. Fligner–Killeen tests were used to compare the variability of area eaten for green- and red-margined leaves. To evaluate the correlation between margin width and area eaten for a whole plant, margin widths were ranked for each node from smallest to largest. The ranking of margin width removed the effect of absolute margin width differences between nodes. Nonlinear regressions were performed using the mean margin rank and mean area lost for each plant.

Differences in margin size between nodes, and in anthocyanin and polygodial concentrations between margin and leaf interior, were compared using paired, two-tailed *t*-tests. Anthocyanin and polygodial concentrations between red- and green-margined leaves were compared using an independent, two-tailed *t*-test. A Wilcoxon signed rank test was used to compare the amounts of leaf area eaten between red- and green-margined leaves following feeding trials, and the numbers of eggs deposited following oviposition trials.

## Results

### *Pseudowintera colorata* margin pigments

The width of red leaf margins varied substantially among individuals from the forest population of *P. colorata* (Fig. 1a–d). The margin size ranged from a barely discernible sliver to an expansive band covering up to 28% of the leaf width. The anthocyanins were predominantly located in the vacuoles of a contiguous band of epidermal cells (Fig. 1f), which extended > 200 cells across in some leaves. Leaves for which the margin was not visibly discernible were also found to hold small clusters of anthocyanic cells (Fig. 1g); these, however, extended only a few cells inwards from the margin, too small to be detected by the unaided human eye. Both the red and green regions of every leaf contained large, spherical idioblast oil cells, the likely sites of polygodial biosynthesis and/or storage, within the palisade and spongy mesophyll cells (Fig. 1g).

Green leaf margins reflected substantially more light across almost the entire visible spectrum than did the red margins

(Supporting Information Fig. S1). However, the ratio of red (600–700 nm) to green (500–600 nm) reflected light was three-fold higher in the red margins. Similarly, the ratio of blue (400–500 nm) to green reflected light was almost twice as high in the red margins.

The red margin width varied less among leaves within an individual than among individuals. Plants that had wider red margins at node 1 also had wider red margins at nodes 3 and 7. The margin width at node 1 correlated positively with that at nodes 3 ( $r^2 = 0.22$ ,  $P < 0.001$ ) and 7 ( $r^2 = 0.24$ ,  $P = 0.016$ ) for leaves on the same plant. Similarly, margin widths at node 3 correlated positively with margin width at node 7 ( $r^2 = 0.46$ ,  $P < 0.001$ ). For all plants, margins were progressively narrower at consecutively older nodes ( $F_{2,291} = 40.7$ ,  $P < 0.001$ ; Fig. 2a).

### Polygodial and anthocyanin analysis

All leaves, regardless of margin colour, held significantly higher polygodial concentrations at the leaf margin than in the interior of the lamina (red  $t_9 = 12$ ,  $P < 0.001$ ; green  $t_9 = 7.2$ ,  $P < 0.001$ ). Compared with green leaves, those with red margins had higher polygodial concentrations in both the margin ( $t_{18} = 3.5$ ,  $P = 0.003$ ) and interior ( $t_{18} = 3.1$ ,  $P = 0.006$ ; Fig. 3a).

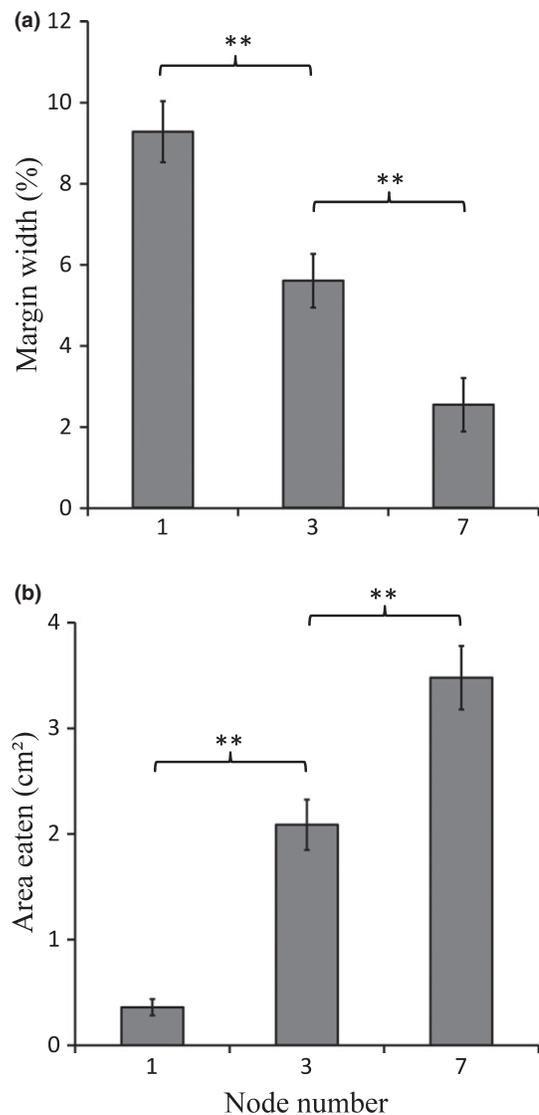
Anthocyanin concentrations in the leaf margins exceeded those of the lamina interior for both red- ( $t_9 = 9.3$ ,  $P < 0.001$ ) and green-margined ( $t_9 = 4.6$ ,  $P = 0.001$ ) leaves. Compared with green leaves, those with red margins had higher anthocyanin concentrations in both the margin ( $t_{18} = 6.4$ ,  $P < 0.001$ ) and interior ( $t_{18} = 3.8$ ,  $P = 0.001$ ; Fig. 3b).

Another sesquiterpene dialdehyde, 9-deoxymuzigadial, was present in trace amounts ( $3 \pm 0.5 \text{ mg g}^{-1}$ ) in both sets of leaves. Although 9-deoxymuzigadial has previously been shown to possess antifeedant properties (Gerard *et al.*, 1993), its concentration was, on average, 17-fold lower than that of polygodial, and therefore it was not considered further in this study.

### Herbivory

Of the 294 leaves sampled in the field, all but 17 showed evidence of edge-feeding herbivory. The proportion of lamina consumed varied between 0.01% and 70%. Although the leaves at node 1 were significantly larger ( $17 \pm 0.5 \text{ cm}^2$ ) than those at nodes 3 ( $15 \pm 0.7 \text{ cm}^2$ ) and 7 ( $15 \pm 0.6 \text{ cm}^2$ ), they had incurred the least edge-feeding herbivory ( $F_{2,291} = 47.8$ ,  $P < 0.001$ ; Fig. 2b). Only leaves at node 1 showed any recent herbivory, as evidenced by the lack of necrotic tissue and anthocyanin pigmentation around wounded areas (Gould *et al.*, 2002).

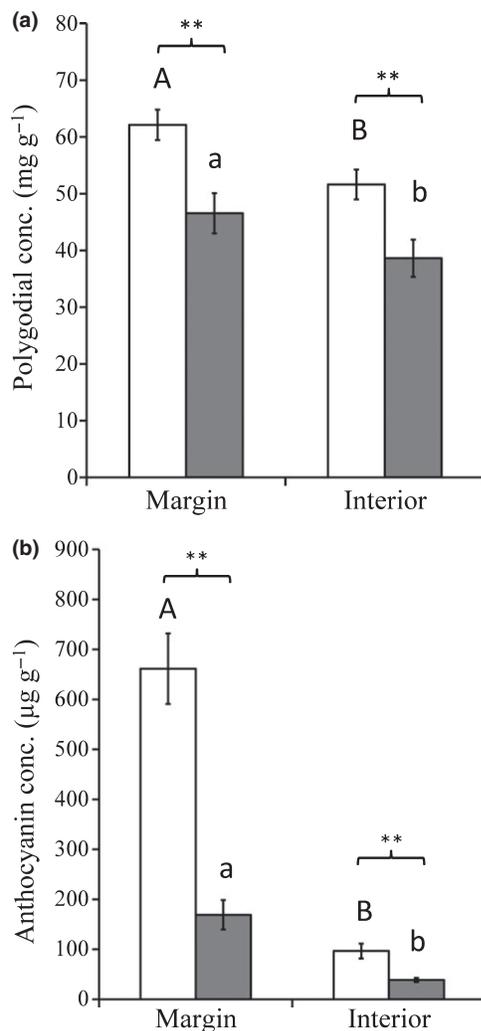
Overall, leaves with the widest red margins had incurred the least edge herbivory (GLM,  $F_{1,191} = 5.2$ ,  $P = 0.02$ ). However, the relationship between herbivory damage and percentage margin width (Fig. 4a–c) differed significantly across the three nodes examined ( $F_{2,191} = 3.491$ ,  $P = 0.03$ ). The consumed lamina area correlated negatively with the percentage margin width at nodes 3 ( $r^2 = 0.21$ ,  $P < 0.001$ ) and 7 ( $r^2 = 0.06$ ,  $P = 0.015$ ), but not at node 1 ( $r^2 = 0.01$ ,  $P = 0.3$ ). The relationships were nonlinear, best approximated by negative logarithmic curves. In all instances,



**Fig. 2** Positional differences in red margin width as a percentage of lamina width (a) and area eaten of *Pseudowintera colorata* leaves (b). Means ( $\pm$  SE),  $n = 98$  leaves per node. Statistically significant difference between groups: \*\*,  $P < 0.001$ .

edge herbivory better correlated with the proportionate margin widths than the absolute margin widths. When all three leaves were averaged per plant, those individuals with the largest average margin width had experienced the least herbivory. The mean rank of margin width correlated negatively with the mean area lost ( $r^2 = 0.098$ ,  $P = 0.002$ ). When only nodes 3 and 7 were averaged, where most herbivory had occurred (Fig. 2b), the relationship between rank of margin width and area lost was stronger ( $r^2 = 0.17$ ,  $P < 0.001$ ).

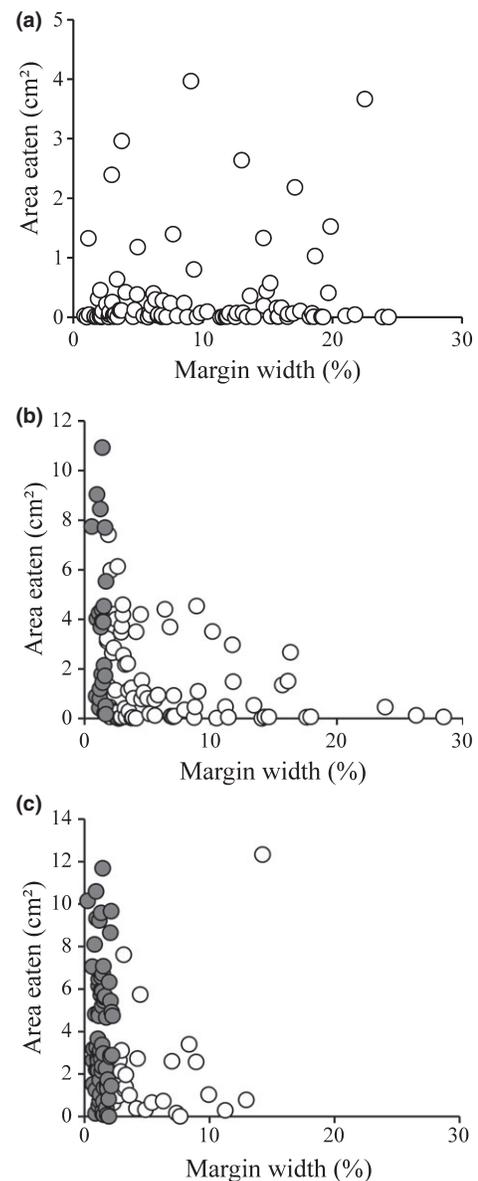
For the leaves at nodes 3 and 7, wider red margins were also associated with a reduced variance in lamina area consumed (Fig. 4b,c). There were critical margin widths above which variance in herbivory damage was diminished significantly. These were 1.75% for the leaves at node 3 (Fligner–Killeen,  $df = 1$ ,  $P < 0.01$ ) and 2.25% for those at node 7 ( $df = 1$ ,  $P = 0.03$ ).



**Fig. 3** Concentrations of polygodial (a) and anthocyanins (b) in the leaf margin and interior of red- (open bars) and green-margined (closed bars) *Pseudowintera colorata* leaves. Means ( $\pm$  SE),  $n = 10$ . Letters above bars indicate significant difference between margin and interior ( $P < 0.001$ ). Statistically significant difference between leaf types: \*\*,  $P < 0.01$ .

### Feeding and oviposition trials

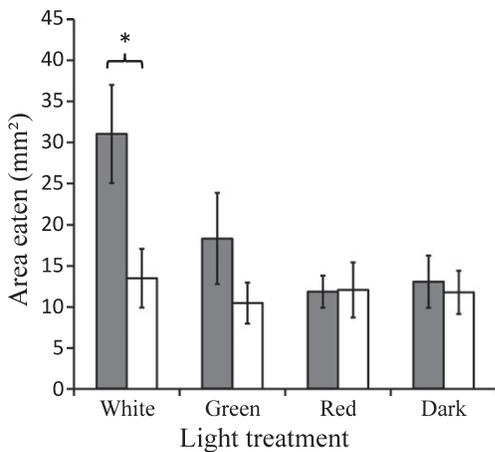
Herbivory rates were high in the laboratory feeding trials, with only one *C. obliquana* larva of 136 not consuming any leaf material. A further three larvae died, which reduced the numbers of replicates for white light, green light and dark treatments to 33 each. Under white light, the leaf area consumed was significantly greater for the green- than for the red-margined leaves (Wilcoxon signed rank test,  $n = 33$ ,  $P = 0.045$ ; Fig. 5). No difference was found under green light ( $n = 33$ ,  $P = 0.3$ ), red light ( $n = 33$ ,  $P = 0.5$ ) or in the dark ( $n = 33$ ,  $P = 0.6$ ). Most larvae (72% of all replicates) had consumed portions of both the red- and green-margined leaves supplied to them. For those larvae that had sampled both leaves under white light, significantly more leaf area was consumed on green- than red-margined leaves (Wilcoxon signed rank test,  $n = 20$ ,  $P = 0.03$ ). For the remaining replicates under white light, for which only one leaf type was chosen, no



**Fig. 4** Area eaten and relative margin width of *Pseudowintera colorata* leaves from nodes 1 (a), 3 (b) and 7 (c). Closed symbols show points beneath the critical margin width at which the variance in area consumed is diminished significantly ( $P < 0.05$ ).  $n = 98$ .

difference was found in the area eaten between red- and green-margined leaves ( $n = 13$ ,  $P = 0.6$ ). No difference was found under green light ( $n = 24$ ,  $P = 0.6$ ), red light ( $n = 25$ ,  $P = 0.5$ ) or in the dark ( $n = 27$ ,  $P = 0.2$ ) for larvae that had sampled both leaf types.

Rates of successful egg-laying in the oviposition trials were generally low. Eggs were found in only 50% of the replicates in which moths oviposited in natural light and 70% of the replicates in which oviposition was completed in the dark. There was no statistically significant difference between the oviposition rates on green- or red-margined leaves in either the light ( $n = 20$ ,  $P = 0.3$ ) or dark ( $n = 28$ ,  $P = 0.2$ ) environments (Table 1). There was also no statistically significant difference between the oviposition rates on the adaxial and abaxial surfaces.



**Fig. 5** Area of red- (open bars) and green-margined (closed bars) *Pseudowintera colorata* leaves eaten by *Ctenopseustis obliquana* larvae under white, green or red light, or in darkness. Means ( $\pm$  SE),  $n = 33$ . Statistically significant differences between groups: \*,  $P < 0.05$ .

**Table 1** Mean ( $\pm$  SE) numbers of *Ctenopseustis obliquana* eggs oviposited on green- and red-margined *Pseudowintera colorata* leaves

| Treatment | Position | Green-margined leaf | Red-margined leaf |
|-----------|----------|---------------------|-------------------|
| Light     | Adaxial  | 6 (2)               | 12 (4)            |
|           | Abaxial  | 2 (2)               | 10 (7)            |
|           | Total    | 8 (3)               | 22 (9)            |
| Dark      | Adaxial  | 9 (3)               | 12 (6)            |
|           | Abaxial  | 5 (3)               | 2 (1)             |
|           | Total    | 14 (4)              | 13 (6)            |

## Discussion

Three sets of evidence from our analysis of *P. colorata* indicate that red leaf margins may function as a visual signal to deter insect herbivores. First, relative to the green leaves, those leaves that bore a wide red margin were significantly richer in polygodial, a potent insect antifeedant (Fig. 3a). The red margins are therefore a reliable signal of increased investment in this defensive compound. Second, the presence of red margins was associated with a reduced propensity for herbivory damage in a natural *P. colorata* population (Fig. 4b,c). Third, in laboratory trials, the larvae of *C. obliquana*, a natural herbivore of *P. colorata*, preferred to feed on green- rather than red-margined leaves, but only when the experiment was conducted under white light which enabled colour vision. The larvae evidently respond to a visual rather than an olfactory signal. Collectively, these data present a compelling case for a new functional role of anthocyanins in leaf margins, and they add empirical support to the co-evolution hypothesis (Archetti, 2000; Hamilton & Brown, 2001) and aposomatic coloration in red leaves (Lev-Yadun, 2006, 2009; Lev-Yadun & Gould, 2007, 2009; Lev-Yadun & Holopainen, 2009).

Irrespective of colour, levels of polygodial were consistently greater at the leaf margins than in the leaf interior (Fig. 3a). Previous studies have similarly shown higher concentrations of phenolic compounds (Gutterman & Chauser-Volfson, 2000; Hughes *et al.*, 2010), glucosinolates (Shroff *et al.*, 2008),

nicotine (Kester *et al.*, 2002) and trichome density (Rautio *et al.*, 2002), all implicated in herbivory defence, at the leaf margin. By concentrating defensive compounds around the leaf margin, plants may be selectively targeting edge-feeding herbivores. As the interiors of red-margined leaves were also richer in polygodial than those of green leaves (Fig. 3a), anthocyanins may indicate not only that the leaf periphery is especially rich in polygodial, but also the overall differences in investment in defences among individuals.

A conceptual problem with the co-evolution hypothesis is that many insects are incapable of perceiving red hues (Döring & Chittka, 2007; Archetti, 2009). Although the receptors needed to perceive red light have evolved multiple times in Lepidoptera (Briscoe & Chittka, 2001), it is not known whether they are present in *C. obliquana* larvae. Nonetheless, insects lacking red receptors have been shown to distinguish red from green by using the ratio of green to blue light reflected from a leaf (Doring *et al.*, 2009), and, for *P. colorata*, the ratio of reflected green to blue light was almost two-fold lower in the green margins (Fig. S1). Aphids lack red receptors, yet apparently discriminate between red and green leaves (Doring *et al.*, 2009). Lepidopteran larvae, including one species within Tortricidae, the family containing *C. obliquana*, have similarly been shown to discriminate between red and green stimuli (Harris *et al.*, 1995; Singh & Saxena, 2004).

Compared with chemoreception, the importance of visual detection in locating insect host plants has been largely neglected (Reeves, 2011). In our study, *C. obliquana* larvae did not appear to distinguish between leaves with different margin colours when illuminated under monochromatic green or red light, or in the absence of light. However, under white light, green-margined leaves were clearly preferred (Fig. 5). Under the monochromatic light, the colour contrast between red margin and leaf interior disappears, although an achromatic contrast remains between the part reflecting light (e.g. red margin under red light) and the leaf part not reflecting light (e.g. green interior). Our data indicate that the larvae used chromatic rather than achromatic contrast to distinguish between the two leaf types.

The feeding preference for green leaves under white light was evident only when both leaf types had been sampled. The avoidance of red-margined leaves appears not to be innate but, rather, relies on gustatory or post-ingestive feedbacks. Importantly, *C. obliquana* larvae did not use olfactory cues or the position of leaves within an enclosure to discriminate between the food sources. Although previous studies have demonstrated an innate preference by some Lepidopteran larvae towards green stimuli (Singh & Saxena, 2004; Hora *et al.*, 2005; Yasui *et al.*, 2006), our study suggests that learning based on colour contrasts may play an important role in distinguishing host quality.

Red leaf margins would be a more effective herbivore deterrent if the signal were perceived by gravid females as well as their larvae. Lepidopteran adults are generally more mobile than larvae (Hagstrum & Subramanyam, 2010), and *C. obliquana* females deposit multiple eggs during oviposition. Contrary to our hypothesis, the frequency of oviposition by gravid *C. obliquana* was similar on green- and red-margined leaves, under both white

light and darkness (Table 1). Qiu *et al.* (1998) found that polygodial inhibited oviposition by the diamondback moth, although this involved an assay in which polygodial solution had been applied to the surface of test filter paper discs. Our result may be attributable to the life history of the animals used for oviposition trials, for which the adults had been raised on a general purpose diet (Singh, 1983) and had no previous experience of *P. colorata*. The oviposition preference of some Lepidoptera has been shown to be influenced by their diet during larval stages (Anderson *et al.*, 1995; Akhtar & Isman, 2003; Chow *et al.*, 2005; Hora *et al.*, 2005; Olsson *et al.*, 2006). As the feeding preference of *C. obliquana* larvae appears to be a learned response, it is reasonable to hypothesize that adult oviposition preference, too, requires previous experience with *P. colorata*.

For convenience, leaf margins were categorized as red or green. In reality, however, red margin size represented a continuum (Fig. 4); clusters of anthocyanic cells were present in all *P. colorata* leaves examined, including those which, to the human eye, appeared green (Fig. 1g). As leaves with the larger margins incurred proportionately less damage by herbivores (Fig. 4b,c) it is of interest to ask whether there is a critical size beneath which a red leaf margin ceases to be effective as a visual cue. We observed a sharp decline in the variance of herbivory damage when the red margin was *c.* > 2% of the lamina width. Thus, only an extremely small proportion of the lamina is required to be red for it to act as an effective deterrent. *Pseudowintera colorata* leaves can have red margins that extend 25% or more across the leaf lamina (Fig. 4), but the potential additional antiherbivory benefit of these large margins appears to be slight.

Herbivory damage correlated more strongly with the relative rather than absolute widths of leaf margins. Insect preference is evidently determined by the ratio of red to green leaf areas, rather than by the margin size *per se*. Relative size may be easier for an insect to evaluate against a concept of the maximally acceptable margin size. Alternatively, given that margin polygodial concentrations exceeded the interior for all leaves, the size of red margins may represent the proportion of lamina that is highly defended, that is, the amount of highly defended lamina relative to less defended lamina.

Unlike older leaves, the margin width at node 1 did not correlate with the extent of herbivory (Fig. 4a). Leaves at this position possessed the broadest margins (Fig. 2a) and, by virtue of their location at the periphery of the canopy, were the most prominent to any approaching herbivore. However, the levels of herbivory on leaves at node 1 were significantly lower than those at lower nodes (Fig. 2b), most probably because these younger leaves had been exposed to herbivores for a shorter duration. The benefits of red margins would be increasingly apparent as the leaves aged.

Although we have referred to red margins as visual 'signals', the term has an inherent implication which is yet to be confirmed. 'Signal' implies that red margins evolved in response to herbivore pressure (Otte, 1974). The alternative, 'cue', implies that the association between red margins and polygodial evolved for another purpose, and insects have subsequently altered their behaviour to make use of it. Because neither scenario can, as yet, be confirmed, we maintain the use of 'signal' whilst

acknowledging its implications. Despite this limitation, one criterion for a signalling relationship was satisfied. The effect of a red leaf margin appears to translate to an overall benefit for an individual plant. When averaging all three nodes for a single plant, those with the largest mean rank in margin width incurred the least overall herbivory. Evidently, the benefits received by a single leaf from the deterrence of herbivores can be scaled up to benefit an entire plant, and is consistent with red leaf margins in *P. colorata* having evolved in response to edge-feeding herbivores.

## Conclusions

Red leaf margins in *P. colorata* signal increased polygodial concentrations and correlate with reduced herbivory by edge-feeding insects. In laboratory feeding trials, a preference for green-margined leaves by *C. obliquana* was dependent on ambient light quality. When the conditions necessary to perceive margin colour were removed, no difference was found in the consumption of red- vs green-margined leaves. Although there has been increasing theoretical support for aposematic coloration in red leaves (Lev-Yadun, 2009; Schaefer & Ruxton, 2011), direct experimental evidence has thus far been lacking. Our study is the first to demonstrate a possible adaptive function for this common pattern in leaf coloration.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Mean ( $\pm$  SE) reflectance spectra for red (white) and green (grey) *Pseudowintera colorata* leaf margins.

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