

Leaf colour polymorphisms: a balance between plant defence and photosynthesis

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Summary

1. Whether plants use leaf colour to deter herbivores remains controversial. The warning signal hypothesis predicts that red pigmentation is adaptive by reducing herbivory; plants with predominantly red foliage should have higher fitness than those with green leaves. Despite many discussions, this prediction has rarely been tested, and alternative, non-exclusive hypotheses cannot be ruled out.

2. We have exploited leaf colour polymorphism in *Pseudowintera colorata* to test the warning signal hypothesis and to address possible alternative explanations.

3. Consistent with warning signals, redder foliage contained higher concentrations of polygodial, a sesquiterpene dialdehyde with strong antifeedant properties, and incurred less herbivory than green leaves. Redder plants hosted 22% fewer lepidopteran leafroller larvae than neighbouring green plants.

4. However, contrary to the predictions of the hypothesis, there were no differences in fitness parameters between red and green plants. Overall leaf canopy colour was not a significant predictor of the number of seeds per fruit or of mean seed weight. This may be explained by differences in photosynthesis: green *P. colorata* leaves had 47% higher maximum CO₂ assimilation rates than matched red leaves from neighbouring plants.

5. These results indicate that the benefits of deterring insect herbivores by signalling may be balanced by the higher photosynthetic rate of non-signalling plants. A balance between signalling and photosynthesis is a novel mechanism for the maintenance of leaf colour polymorphisms in nature.

6. Synthesis. Anthocyanin pigments may simultaneously serve multiple functions within leaves, and individuals of the same plant species may use different strategies to cope with insect herbivores. Therefore, investigations into the role of these pigments in plant–insect interactions need to consider plant physiology and the diversity of plant defence mechanisms.

Key-words: anthocyanins, leaf colour signalling, pigments, plant–herbivore interactions, polygodial, *Pseudowintera colorata*, red leaves

Introduction

Most leaves are green, yet red-leafed plants are found in every biome throughout the plant kingdom, from the basal liverworts to the most advanced angiosperms (Lee 2002; Novak & Short 2011). Despite their abundance, the possible functional significance of red foliar pigments remains poorly understood and is hotly debated (Gould 2004; Manetas 2006; Archetti *et al.* 2009; Hughes 2011). The anthocyanins are by far the best-studied class of red pigments (Lee 2007), yet even their function remains controversial. There are a wide range of proposed functions for anthocyanins in leaves; how-

ever, two hypotheses have received the most attention: (i) foliar anthocyanins serve as visual warning signals to deter approaching herbivores, or (ii) they protect leaves against the effects of a variety of abiotic stressors (Karageorgou & Manetas 2006; Rolshausen & Schaefer 2007; Schaefer & Gould 2007; Archetti *et al.* 2009; Landi, Tattini & Gould 2015). Some early papers considered these two hypotheses mutually exclusive, and due to experimental difficulties, most studies have addressed these two hypotheses in isolation from each other. However, it is evident that genes for key enzymes in the biosynthesis of anthocyanins arose independently multiple times in the evolution of plants (Pichersky & Gang 2000), and there is no reason to assume a single, unified function. Indeed, it is entirely possible that foliar anthocyanins serve

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two or more functions simultaneously. Several workers have called for studies that simultaneously address both signalling and physiological hypotheses (Schaefer & Wilkinson 2004; Schaefer & Rolshausen 2006a; Schaefer & Gould 2007; Cooney *et al.* 2015).

The warning signal hypothesis, originally developed to explain the reddening or yellowing of autumn leaves on deciduous plants (Archetti 2000; Hamilton & Brown 2001), posits that red leaf colours are a visual warning to approaching insects that a plant is well defended. This hypothesis makes three testable predictions: (i) red leaf coloration is a reliable signal of a plant's defensive commitment; (ii) insects perceive this signal and thus avoid consuming red leaves; and (iii) warning signals increase the fitness both of plants and of herbivores that respond to the signal (Archetti 2000; Hamilton & Brown 2001). There is strong theoretical support and some empirical evidence for the first two predictions (Hagen, Folstad & Jakobsen 2003; Archetti & Leather 2005; Karageorgou & Manetas 2006; Wong & Srivastava 2011; Cooney *et al.* 2012; Chen & Huang 2013). However, the evidence is equivocal because it does not allow us to distinguish leaf signalling from other functional hypotheses. The third prediction has rarely been examined (Archetti 2009) and has not received strong support (Hagen *et al.* 2004; Markwick *et al.* 2012). No study has yet supported all three predictions; hence, the hypothesis remains controversial.

An adaptive role for anthocyanins in defence is, indeed, difficult to establish experimentally. This is because the phenylpropanoid pathway which leads to the biosynthesis of anthocyanins also delivers other phenolic compounds that have known antifeedant properties (Schaefer & Rolshausen 2006b). Thus, a plant for which phenylpropanoid metabolism has been upregulated to yield anthocyanins is likely to also produce higher concentrations of other phenolic compounds (Gould, McKelvie & Markham 2002). It is entirely possible, therefore, that foliar anthocyanins have evolved to protect physiological processes from the effects of abiotic stressors such as drought or strong light (Gould *et al.* 1995; Gould 2004; Hughes 2011), but because of their upregulated phenylpropanoid pathway, the red leaves are coincidentally more resistant to herbivores. This possibility has been termed the 'defence indication hypothesis' (Schaefer & Rolshausen 2006b). To distinguish between the defence indication hypothesis and leaf signalling hypothesis, we need to first identify and quantify the primary defensive molecule(s) in populations of red- and green-leaved plants growing under similar environmental conditions.

Demonstrating a fitness benefit presents a further challenge. The experimental work carried out thus far has focused largely on the effects of ephemeral coloration in expanding new leaves in Spring or in senescing Autumn foliage. However, any relationship between transient coloration, herbivory and fitness is difficult to evaluate, as fitness is influenced by a variety of factors throughout the entire growing season. While there is evidence in support of the fitness benefits of dull cryptic plant coloration (Klooster, Clark & Culley 2009; Strauss & Cacho 2013; Niu *et al.* 2014), no data have yet

been published on seed set as a function of red plant coloration and natural herbivory pressure.

The putative interplay between plant coloration, herbivory and fitness might be further moderated by simultaneous effects of pigments on leaf physiology. Anthocyanins in red leaves absorb green quanta that could be used in photosynthesis by the lower cell layers, so they may compromise the lifetime net assimilation of a leaf (Karageorgou & Manetas 2006; Hughes & Smith 2007). Yet anthocyanins can also augment the photosynthetic performance of a leaf by reducing the negative impacts of saturating light on photoinhibition and photo-oxidative stress (Gould, Dudle & Neufeld 2010; Nielsen & Simonsen 2011; Zhang *et al.* 2012; Hughes *et al.* 2014). To date, the relative contributions of foliar anthocyanins to herbivory and leaf physiology on plant fitness have not been compared.

The New Zealand endemic plant *Pseudowintera colorata* (Winteraceae) presents an unparalleled opportunity to test the adaptive role of foliar anthocyanins because its leaves vary from entirely green to entirely red (Fig. 1a), because red pigmentation persists throughout the life of the leaf, and because its primary defence compound has been identified as polygodial (Perry & Gould 2010). Polygodial (Fig. 1b) is a sesquiterpene dialdehyde with potent antifeedant properties against a wide variety of phytophagous insects (Moreno-Osorio *et al.* 2008). The terpenoid polygodial and the phenylpropanoid anthocyanins are biosynthesized via different metabolic pathways (Gershenzon 1994; Dewick 2009), which allows us to distinguish between signalling and defence indication. We have already used *P. colorata* to show that red leaf margins provide a reliable and effective visual signal of chemical defence to lepidopteran larvae (Cooney *et al.* 2012).

Here, we report on the relationships between overall colour, polygodial concentrations, herbivory of leaves and reproductive fitness from two natural *P. colorata* populations in distinct ecological conditions. Specifically, we tested whether (i) the proportionate area of red coloration of *P. colorata* leaves is a reliable cue of their chemical defences both in forest understorey and in open scrub; (ii) redder leaves will incur reduced insect feeding damage; (iii) redder plants will experience less herbivory than neighbouring greener plants; and (iv) redder plants will have higher fitness than neighbouring greener plants. Finally, to investigate the possibility that red leaf pigments might affect photosynthesis in addition to herbivory, we compared CO₂ assimilation rates and amounts of pigment in red and green *P. colorata* leaves.

Materials and methods

STUDY SYSTEM AND SAMPLING

In 2007, 15 *P. colorata* (Raoul) Dandy (Winteraceae) plants were randomly selected from a natural population at Waipori Falls Scenic Reserve, Otago, New Zealand (45° 54' 13.8" S, 169° 59' 34.7" E). In 2009, a natural population of *P. colorata* at Belmont Trig, Wellington, New Zealand (41° 11' 0.2" S, 174° 52' 25.9" E), was sampled: 50 plants were randomly selected, stratified over the population (10

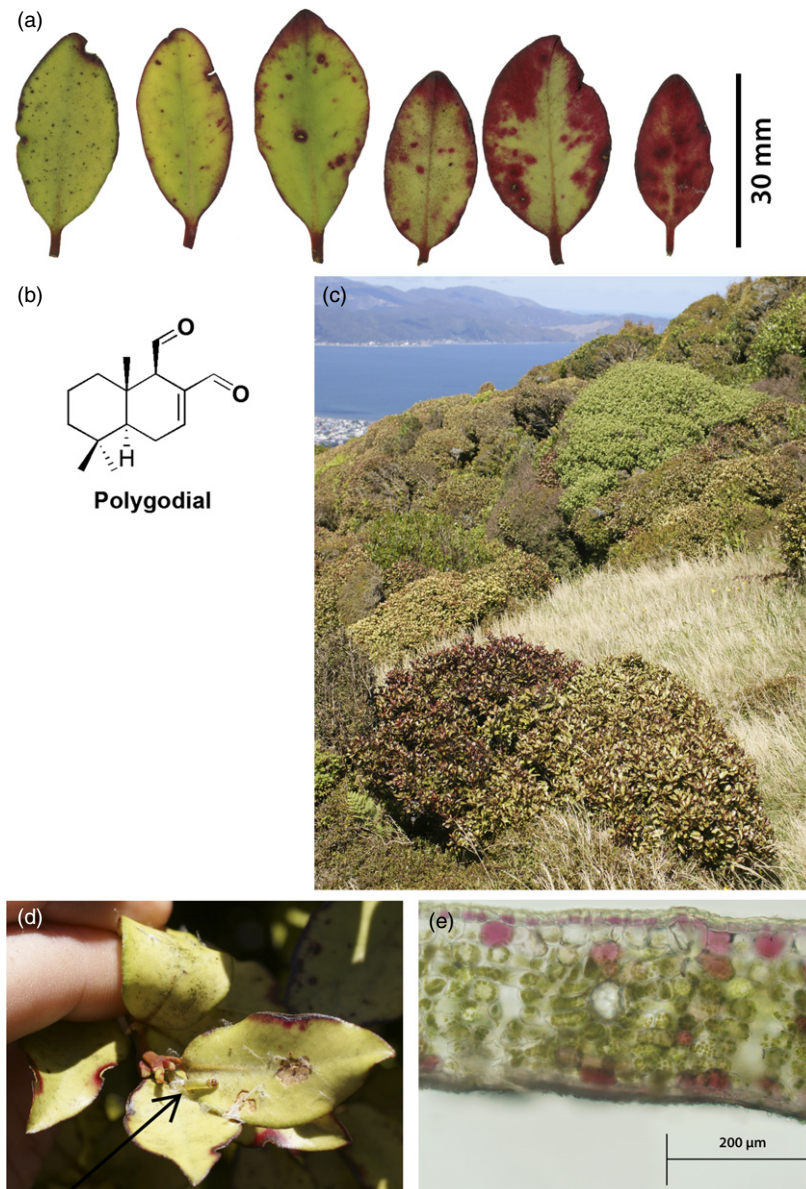


Fig. 1. (a) Range of coloration of *Pseudowintera colorata* leaves; (b) chemical structure of polygodial; (c) a matched pair of *P. colorata* (in foreground, ~1 m tall) contrasting in colour at the scrub site; (d) *P. colorata* leaves (~30 mm long) pulled apart to reveal *Ctenopseustis* sp. leafroller caterpillar (arrowed); and (e) transverse section through a *P. colorata* leaf showing red anthocyanin pigments.

plants from each of five 100-m transects 20 m apart). The two populations represent extremes in the range of typical *P. colorata* habitat: shaded forest understorey at Waipori Falls; and dense scrub on an exposed hilltop, which receives direct sunlight throughout the day, at Belmont Trig (Fig. 1c).

LEAF CHEMISTRY

A subsample of leaves from each population was used for chemical analyses (forest: 46 leaves from four randomly selected branches, each from a separate plant; scrub: 90 leaves from 10 randomly selected plants). Leaves from the forest population were individually freeze-dried, weighed and ground to a powder. Half of each sample was extracted in 3 M HCl:H₂O:MeOH (1:3:16, v/v/v), centrifuged, and absorbances at 522 nm (A_{522}) and 653 nm of the supernatant measured using a Pharmacia Biotech Ultraspec 2000 UV/Visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Anthocyanin concentrations were estimated as $A_{522}-0.24A_{653}$ per unit dry weight. The remaining half of each sample was used for polygodial

quantification. For leaves from the scrub population, both polygodial and anthocyanin concentrations were measured by high-pressure liquid chromatography (HPLC) following the method of Cooney *et al.* (2012).

LEAF COLOUR AND FEEDING DAMAGE

Leaf coloration patterns and feeding damage from chewing insects were quantified from digital images of the harvested leaves following the method of Ramirez, Lavadero & Archetti (2008). As mechanical injury and herbivory can induce anthocyanin biosynthesis in *P. colorata* leaves immediately adjacent to the wound (Gould, McKelvie & Markham 2002), we subtracted the area of any redness associated with such damage from our calculation of total (constitutive) red leaf area. Leaves that incurred no feeding damage were excluded from statistical analyses that involved leaf herbivory, as it was not known if their lack of damage could be attributed to successfully deterring herbivores or instead to not being encountered by an herbivore. If leaves had incurred extensive herbivory, their leaf shape was reconstructed

digitally by comparison with the shapes of adjacent leaves on the branch.

PAIRWISE COMPARISON OF HERBIVORE PRESSURE AND PLANT FITNESS

We selected 30 pairs of *P. colorata* plants at the scrub site. This design allowed us to investigate the effects of leaf colour on herbivory while minimizing the influence of differences in abiotic factors. Each pair contained one plant whose canopy was predominantly green and one whose canopy was much redder (Fig. 1c). Individuals in each pair were located no more than 2 m apart from one another, and closely matched in size, aspect, exposure to direct sunlight and prevailing winds. Canopy surface area of each plant was measured using a quadrat. Very large *P. colorata* plants (>6 m²) were excluded. New Zealand native leafroller caterpillars from the family Tortricidae were the most abundant of the known chewing insect herbivores of *P. colorata* at the scrub site (<http://plant-synz.landcareresearch.co.nz/>; accessed August 2014). From late spring to early summer (November to December) 2010, we counted the caterpillars visible on every leaf on all 60 plants, and recorded evidence of leafroller caterpillar presence (leaves rolled or stuck together with silk, Fig. 1d). Caterpillars were left undisturbed so that we could observe the impact of their feeding on plant fitness at the end of the season.

During late summer to early autumn (February/March 2011, before most fruits were fully ripened), each stem on every plant was inspected and the total numbers of fruits counted. Close to 30 000 fruits from the 30 pairs were counted. For 26 pairs, 20 randomly selected fruits from each plant were dissected and their seeds counted. These seeds were then cleaned, dried, and their mean dry weights determined to test for differences in the number of seeds per fruit and seed weight.

CO₂ ASSIMILATION MEASUREMENTS

We measured photosynthesis in leaves from 10 of the matched pairs of *P. colorata* plants at the scrub site. One stem was selected from the periphery of the north (sunniest) face of the canopy. Within pairs, the stems were closely matched for azimuth, exposure to direct sunlight and prevailing winds. Leaves with significant herbivore or mechanical damage were excluded. Light response curves for CO₂ assimilation were measured for one leaf per stem, (the third to fifth youngest fully expanded leaf) using a LI-6400 photosynthesis system with red and blue LED light sources (LI-COR Biosciences, Lincoln, NE, USA). Sampling took place between 0930 and 1530 h, over 3 days during Autumn 2012. The mean daily temperature of these 3 days ranged from 13 to 17 °C (NIWA National Climate Database, Kelburn Weather Station, <http://cliflo.niwa.co.nz/>; accessed 10 July 2012). The impact of variation in climate within and among sampling days upon photosynthesis was minimized, as both trees of each pair were measured consecutively on the same day. CO₂ concentration in the LiCor 6400 was maintained at 400 µmol CO₂ mol⁻¹ air. The flow rate of air through the leaf chamber was maintained at 500 µmol s⁻¹. The light ramp began at 1500 µmol m⁻² s⁻¹ and decreased progressively until the irradiance was zero, with 1-min pauses between measurements. We used a 2 × 6 cm leaf chamber. As photosynthesis was recorded on a per-unit-leaf-area basis, we corrected for the surface area of any leaves that were smaller than 6 cm². *Pseudowintera colorata* leaves are hypostomatic (Sampson 1980).

After the CO₂ assimilation measurements, the leaves were excised, scanned at 300 dpi using a CanoScan LiDE 20 desktop scanner (Canon,

Tokyo, Japan) and sealed in plastic 20-mL vials in a -80 °C freezer until pigment extraction. Leaf area was measured from the digital images using IMAGEJ v1.41 (National Institutes of Health, Bethesda, MD, USA) following the methods of Cooney *et al.* (2012). Unfortunately, the leaves from three of the pairs were not scanned and frozen until 48 h after being excised. Noticeable drying of these leaves occurred as well as changes in their colour. The data from these three pairs of leaves were excluded from the pigment extraction analysis.

PIGMENT ANALYSIS

A ~1-cm² section of leaf lamina was excised from a point normal to the centre of the midrib (~0.02 g fresh weight). After weighing, leaf material was placed in pre-cooled 2.0-mL microcentrifuge tubes (Biotix, Inc., San Diego, CA, USA) containing 1.5 mL of 80% (v/v) acetone and a 5-mm stainless steel ball bearing (Qiagen N.V., Venlo, The Netherlands). The samples were agitated in a pre-cooled bead mill (Tissuelyser LT; Qiagen N.V.) for 6 min at 50 oscillations s⁻¹ and centrifuged at 15 625 g for 5 min in a microcentrifuge (5415 D, Eppendorf AG, Hamburg, Germany). Absorbance of the supernatant was measured at 470, 647 and 663 nm using a UV-2550 UV-vis spectrophotometer (Shimadzu Corp., Kyoto, Japan) and the concentrations of chlorophyll and carotenoid pigments calculated following the methods of Lichtenthaler (1987).

STATISTICAL ANALYSES

Leaf colour, chemistry and herbivory

General linear-mixed models were used to assess the relationships between leaf pigment and leaf redness, polygodial and leaf pigment, polygodial and leaf redness, and between the proportion of leaf area removed by chewing insects (herbivory) and leaf redness, using the 'lme' command of the NLME package in R, with maximum likelihood estimation (R Core Team 2013). Following a Shapiro-Wilk test, the response variables for each model were transformed to improve normality (anthocyanin: ln; polygodial: ln; herbivory: arcsine square root). The models included nested random factors (scrub: individual nested within transect; forest: branch nested within individual) to reflect the different sampling methodologies at the two sites and to account for the lack of independence of leaves collected from the same plant. The Fligner-Killeen test in R was used to compare variability of herbivory for different amounts of leaf redness.

HERBIVORE PRESSURE AND PLANT FITNESS

General linear-mixed models were used to assess the relationships between plant colour, caterpillar numbers and various measures of fitness. Plant surface area was included as a covariate to assess its influence on herbivore pressure and plant fitness. Following a Shapiro-Wilk test, the response variables for each model were ln transformed to improve normality. The models included a random factor to reflect the matched-pair sampling technique.

Photosynthesis

The parameters of light response curves are frequently analysed using repeated-measures ANOVA. We treated each pair of matched red and green *P. colorata* trees as a subject, and used repeated-measures ANOVA to test whether the mean response differed among the different light

intensities (PAR, a factor with 10 or 15 levels depending on the light curve), and to assess whether the relationship between the response variable and light intensity differed within-subjects (i.e. within-pairs of neighbouring matched red and green *P. colorata* trees).

We carried out multiple tests; one for each of the various parameters of the CO₂ assimilation and chlorophyll fluorescence light response curves: *A*, Φ_{PSII} , *qP*, and *NPQ*. Normality within different light intensities and tree colours was assessed using Shapiro–Wilk tests. The amount of variance explained by each factor was manually calculated from the sum of squares (SS) of each test (Levine & Hullett 2002).

Differences in light curve parameters and pigment concentrations between red- and green-matched *P. colorata* leaves were assessed using either paired Student's *t*-test or Wilcoxon signed-rank tests, depending on the outcome of a Shapiro–Wilk test. All statistical analyses were carried out using PSAW/SPSS Version 18.0 (SPSS, Chicago, IL, USA).

Results

In both the forest and the scrub population, the proportion of red coloration varied considerably among *P. colorata* leaves. Digital image analysis showed that red leaf area ranged from a barely visible 0.03% to almost 100% in both populations. For most leaves, red coloration was concentrated at leaf margins, veins and small irregular blotches over the adaxial surface of the lamina (Fig. 1). Anthocyanin concentrations were greater in those leaves that had a larger proportion of lamina area coloured red (Table 1a).

REDDER LEAVES CONTAIN HIGHER CONCENTRATIONS OF CHEMICAL DEFENCES

Polygodial concentrations ranged from 8.0 to 91.2 (mean = 37.9) mg g⁻¹ DW as measured by HPLC. Polygodial concentrations tended to be greater in those leaves that held the higher concentrations of anthocyanins, both in forest and open scrub (Table 1b). In green leaves, polygodial concentrations were highly variable, but in increasingly redder

leaves, polygodial concentrations tended to be higher. Accordingly, the proportion of red-coloured leaf area reliably indicated polygodial concentrations, and hence the degree of chemical defence (Table 1c and Fig. 2).

Another sesquiterpene dialdehyde, 9-deoxymuzigadial, was present in the leaves. However, the amounts of this compound did not correlate with leaf colour or anthocyanin concentrations in leaves from either population.

REDDER LEAVES INCUR LESS INSECT FEEDING DAMAGE

Redder *P. colorata* leaves tended to incur less feeding damage from chewing insects than green leaves at both locations

Table 1. General linear-mixed models showed that leaf redness predicted plant defences (polygodial) and herbivory on *Pseudowintera colorata* trees in two populations. Models included random factors to reflect sampling methods (scrub: individual nested within transect; forest: branch nested within individual). Following a Shapiro–Wilk test, the response variables for each model were transformed to improve normality (anthocyanin: ln; polygodial: ln; herbivory: arcsine square root). Bold indicates *P* < 0.05

| Model | Estimate | SE | d.f.NUM | d.f.DENOM | F-value | <i>P</i> |
|------------------------------|----------|--------|---------|-----------|---------|-------------------|
| (a) Anthocyanin ~ redness | | | | | | |
| Scrub | 0.09 | 0.011 | 1 | 79 | 67.7 | <0.0001 |
| Forest | 0.02 | 0.003 | 1 | 41 | 27.1 | <0.0001 |
| (b) Polygodial ~ anthocyanin | | | | | | |
| Scrub | 0.004 | 0.001 | 1 | 79 | 17.9 | 0.0001 |
| Forest | 1.42 | 0.34 | 1 | 41 | 17.3 | 0.0002 |
| (c) Polygodial ~ redness | | | | | | |
| Scrub | 0.03 | 0.008 | 1 | 79 | 14.0 | 0.0003 |
| Forest | 0.03 | 0.006 | 1 | 41 | 25.8 | <0.0001 |
| (d) Herbivory ~ redness | | | | | | |
| Scrub | -0.001 | 0.0005 | 1 | 329 | 5.5 | 0.0195 |
| Forest | -0.003 | 0.001 | 1 | 378 | 8.3 | 0.0042 |

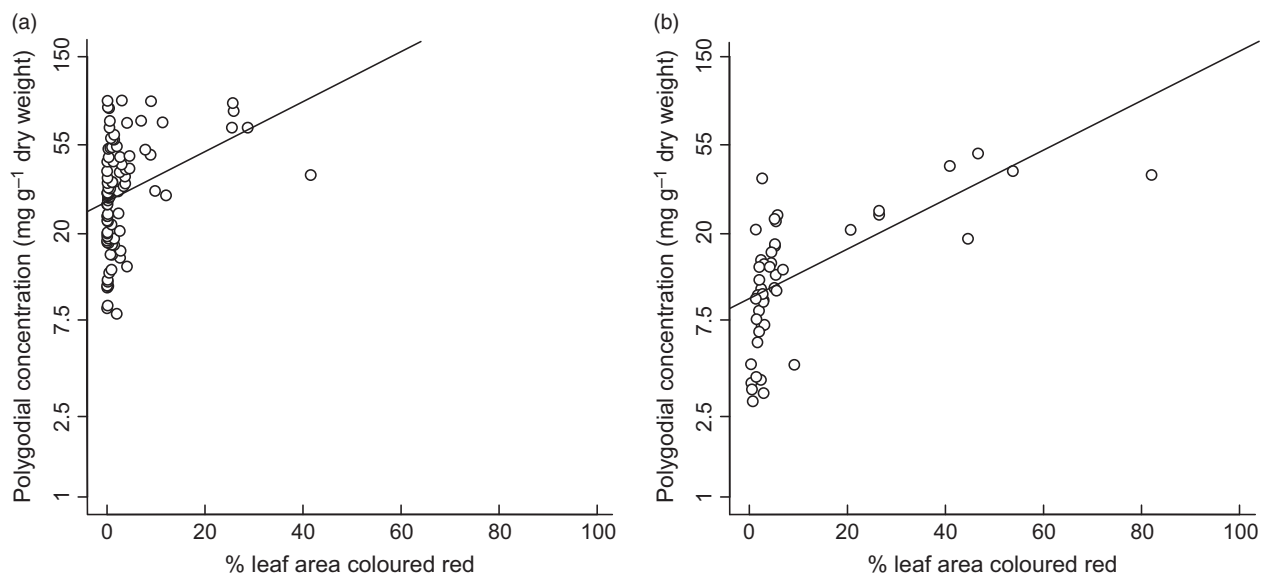


Fig. 2. Proportionate leaf redness reliably indicated polygodial concentrations in leaves of *Pseudowintera colorata* from (a) scrub (*n* = 90 leaves from 10 plants) and (b) forest populations (*n* = 46 leaves from four plants).

(Fig. 3 and Table 1d). The extent of leaf area removed by insect feeding was far less variable in the redder leaves; there was a critical proportionate red leaf area above which variance in leaf damage was significantly reduced; this was 4.25% for leaves from the scrub habitat (Fligner–Killeen, d.f. = 1, $P < 0.001$) and 4% for those from the forest (d.f. = 1, $P = 0.013$). No leaf with >30% red area incurred more than 15% damage in either population.

REDDER PLANTS INCUR LESS INSECT PRESSURE THAN DO NEIGHBOURING GREENER PLANTS

General linear-mixed models, including plant size as a covariate to assess its influence on caterpillar numbers, showed that the redder plants hosted significantly fewer leafroller caterpillars than did neighbouring green plants (Table 2a and

Table 3). Leafroller caterpillar densities upon *P. colorata* plants varied substantially, ranging from 1.4 to 130.3 m⁻². On average, the red-leaved plants had five (22%) fewer caterpillars per m² canopy area than did the green plants.

NEIGHBOURING RED AND GREEN PLANTS DO NOT DIFFER IN FITNESS

Canopy surface area was a significant predictor of the number of fruits produced by *P. colorata* plants (mean ± SE = 482 ± 79.8), but canopy colour was not (Table 2b). Neither canopy colour nor canopy surface area was significant predictors of the number of seeds per fruit (mean ± SE = 4.06 ± 0.10; Table 2c) or of mean seed weight (mean ± SE = 2.00 ± 0.09 mg; Table 2d) of different *P. colorata* plants.

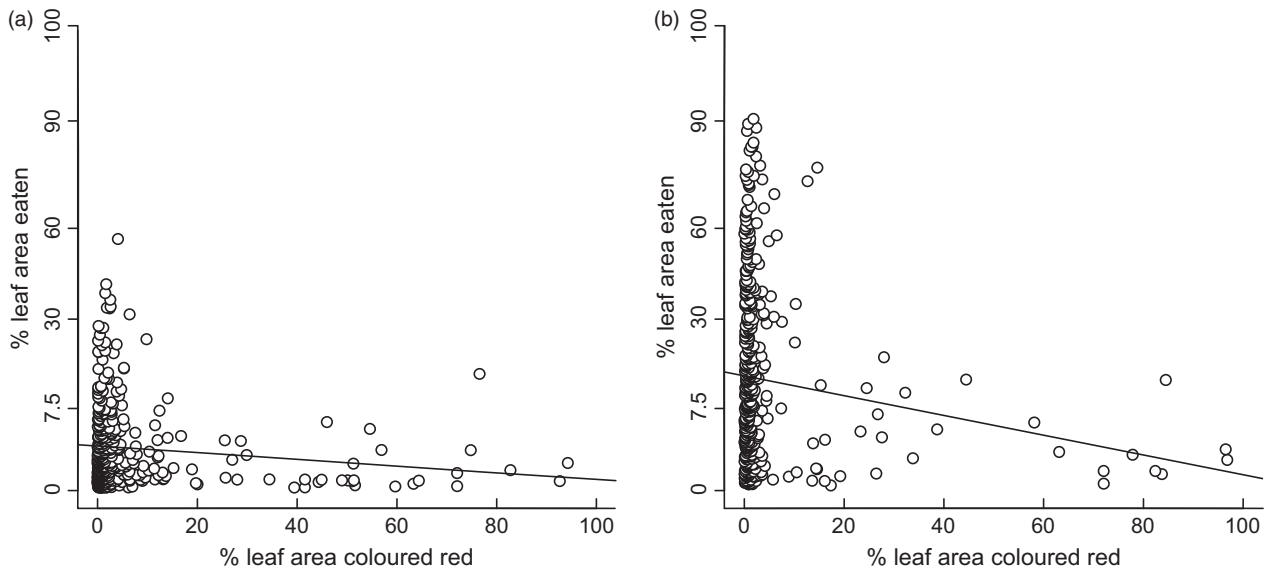


Fig. 3. Redder *Pseudowintera colorata* leaves incurred less feeding damage than did green leaves from (a) scrub ($n = 380$ leaves from 50 plants) and (b) forest ($n = 408$ leaves from 15 plants) habitats.

Table 2. General linear-mixed models showed that although red- and green-leaved *Pseudowintera colorata* trees had different numbers of herbivores, the morphs did not differ in fitness parameters (number of fruits per tree, seeds per fruit or seed mass). Models included a random factor (Pair) to reflect the matched-pair sampling design, and a covariate factor (surface area) to assess the influence of plant size on herbivore numbers and plant fitness. Bold indicates $P < 0.05$

| Model | Estimate | SE | d.f.NUM | d.f.DENOM | F-value | P |
|--|----------|---------|---------|-----------|---------|---------------|
| (a) Number of caterpillars per tree ~ leaf colour + surface area | | | | | | |
| Colour (red) | -0.44 | 0.18 | 1 | 28 | 7.1 | 0.0125 |
| Surface area | 0.23 | 0.10 | 1 | 28 | 4.8 | 0.0362 |
| (b) Number of fruits per tree ~ leaf colour + surface area | | | | | | |
| Colour (red) | 0.17 | 0.29 | 1 | 28 | 0.08 | 0.7847 |
| Surface area | 0.51 | 0.15 | 1 | 28 | 11.0 | 0.0025 |
| (c) Mean number of seeds per fruit ~ leaf colour + surface area | | | | | | |
| Colour (red) | 0.06 | 0.05 | 1 | 24 | 1.4 | 0.2432 |
| Surface area | -0.006 | 0.02 | 1 | 24 | 0.08 | 0.7834 |
| (d) Mean seed mass ~ leaf colour + surface area | | | | | | |
| Colour (red) | 0.0001 | 0.00009 | 1 | 24 | 1.7 | 0.2074 |
| Surface area | -0.00007 | 0.00006 | 1 | 24 | 1.5 | 0.2352 |

GREEN LEAVES HAVE SIGNIFICANTLY HIGHER LIGHT-SATURATED CO₂ ASSIMILATION RATES THAN RED LEAVES

There were no statistically significant differences between matched red and green *P. colorata* leaves in net CO₂ assimilation rates (*A*) at low irradiances. The dark respiration rate, light compensation point, apparent maximum quantum yield/photo-synthetic efficiency and light saturation point of matched red and green *P. colorata* leaves also did not differ significantly (Table 4). However, when irradiated with saturating (>500 μmol m⁻² s⁻¹) red and blue light, *A*_{sat} values were on average 47% higher in the green than the red leaves (repeated-measures ANOVA, $F_{1,7} = 5.7$, $P = 0.049$, $\eta^2 = 0.0035$, Fig. 4).

GREEN LEAVES HAVE SIGNIFICANTLY HIGHER CHLOROPHYLL CONCENTRATIONS THAN RED LEAVES

Chlorophyll *a* and total chlorophyll (Chl_{*a+b*}) concentrations (per FW) were significantly higher in the green than the red leaves (Table 5), although the magnitude of these differences varied considerably across the seven matched pairs (Fig. 5). When one outlier pair was removed the difference in Chl *a* between green and red *P. colorata* leaves remained statistically significant ($n = 6$ pairs, $Z = -2.0$, $P = 0.028$). Chl *b* concentrations, and Chl *a:b* ratios did not differ significantly between the two leaf colours. Neither the total carotenoid concentrations (Car_{*x+c*}), nor the ratios of Car_{*x+c*}: Chl_{*a+b*} differed significantly between matched red and green leaves (Table 5).

Discussion

We found substantial support for the warning signal hypothesis. Specifically, red *P. colorata* leaves were generally better

Table 3. Red-leafed *Pseudowintera colorata* trees had fewer herbivores than green *P. colorata* trees

| | Mean | Median | SD | <i>N</i> |
|--|------|--------|------|----------|
| Number of leafroller caterpillars per m ² canopy area | | | | |
| Red | 20.3 | 11.2 | 27.1 | 30 |
| Green | 26.0 | 16.1 | 25.5 | |

Table 4. Parameters of the initial phase of CO₂ assimilation light curves from matched green and red *Pseudowintera colorata* leaves. Means of $n = 9 \pm$ SE. No statistically significant differences were found

| Photosynthetic parameter | Green | Red |
|---|--------------|--------------|
| Dark respiration rate (μmol CO ₂ m ⁻² s ⁻¹) | -0.64 ± 0.2 | -0.58 ± 0.3 |
| Light compensation point (μmol m ⁻² s ⁻¹) | 15 ± 3.0 | 18 ± 5.0 |
| Apparent max. quantum yield (Φ _a) | 0.03 ± 0.004 | 0.02 ± 0.003 |
| Light saturation point (μmol m ⁻² s ⁻¹) | 622 ± 70 | 456 ± 60 |

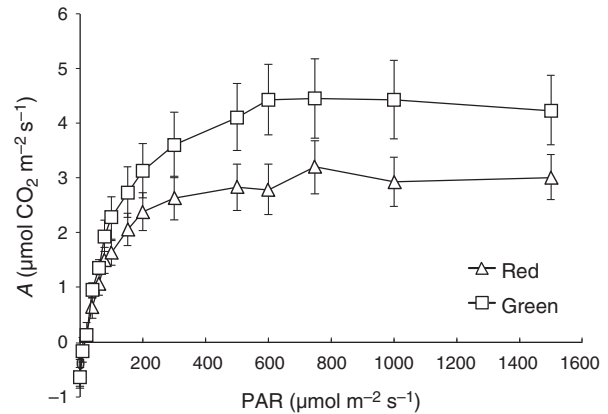


Fig. 4. Light response curves for CO₂ assimilation (*A*) of green (□) and red (Δ) *Pseudowintera colorata* leaves in the field. Means ± SE, $n = 9$.

Table 5. Concentrations of chlorophylls (Chl) *a* and *b* and carotenoid (Car_{*x+c*}) pigments in matched pairs of red and green leaves of *Pseudowintera colorata* (Mean ± SE, $n = 7$ pairs)

| Pigment | Green | Red |
|---|-------------|-------------|
| Chl <i>a</i> (μg g ⁻¹ FW) | 190 ± 53 | 121 ± 15* |
| Chl <i>b</i> (μg g ⁻¹ FW) | 203 ± 65 | 117 ± 18 |
| Chl _{<i>a+b</i>} (μg g ⁻¹ FW) | 390 ± 120 | 240 ± 32* |
| Chl <i>a:b</i> | 1.0 ± 0.08 | 1.1 ± 0.07 |
| Car _{<i>x+c</i>} (μg g ⁻¹ FW) | 10 ± 4.0 | 13 ± 2.4 |
| Car _{<i>x+c</i>} : Chl _{<i>a+b</i>} | 0.04 ± 0.02 | 0.06 ± 0.01 |

Asterisk indicates statistical difference (Wilcoxon signed-ranks test, $P < 0.05$).

chemically defended, they incurred less herbivory, and plants with redder canopies hosted fewer Lepidoptera. However, green *P. colorata* leaves had higher maximum rates of CO₂ assimilation than red leaves, and green- and red-coloured *P. colorata* plants produced similar numbers and sizes of seeds. The similar seed production of red and green individuals suggests that the benefits of reduced herbivory from signalling may be balanced by the higher photosynthetic rate of non-signalling plants.

Pseudowintera colorata individuals with redder foliage experienced reduced herbivore pressure, hosting fewer caterpillars than neighbouring greener plants. Given that leafroller caterpillars can consume over 0.30 cm² day⁻¹ of *P. colorata* foliage (Menzies 2013), this difference is likely to exert considerable selective pressure as the loss of foliage can substantially influence the overall photosynthetic capacity (Zangerl *et al.* 2002). Our data are consistent with previous studies, which documented a herbivore preference for individual plants with green over red foliage (Hagen, Folstad & Jakobsen 2003; Archetti & Leather 2005; Markwick *et al.* 2012; Maskato *et al.* 2014). However, unlike these studies, we can eliminate the 'Defence Indication' hypothesis because the primary mechanism of defence in *P. colorata* is known to be

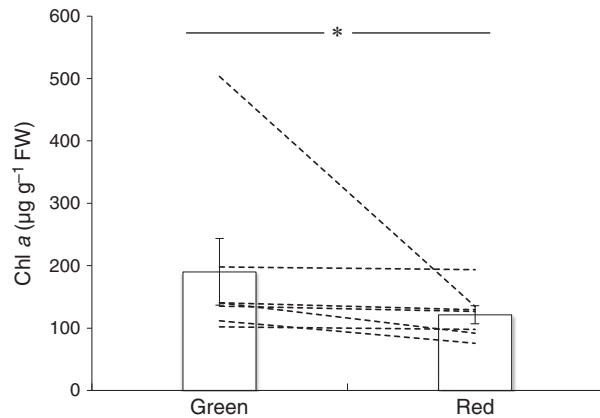


Fig. 5. Concentrations of chlorophyll (Chl) *a* pigments in matched red and green leaves of *Pseudowintera colorata* (Means \pm SE, $n = 7$ pairs). Dashed lines connect the concentrations in matched leaves. Asterisk indicates statistical differences within pairs (Wilcoxon signed-rank test, $P < 0.05$).

polygodial, in a distinct metabolic pathway from the anthocyanins (Perry & Gould 2010).

In two wild populations with distinct abiotic regimes, redder leaves incurred less leaf damage, and less variability in leaf damage, than greener leaves. These differences in leaf colour correlated with differences in leaf defence chemistry. Cooney *et al.* (2012) demonstrated that the differences in feeding preference of leafroller larvae on *P. colorata* leaves are only realized when light conditions are such that the green: red chromatic contrast might be perceived. Although carried out at the leaf-scale rather than individual-scale, that study adds further evidence that the inter-individual patterns in herbivore density described above are the result of signalling by foliar anthocyanins.

Why does the reduction in herbivory not confer fitness benefits to the red-leafed plants? Net assimilation rates of leaves from matched red and green individuals indicated that there are other physiological differences between red and green plants beyond those predicted by the visual signalling hypothesis. While redder *P. colorata* individuals hosted on average 22% fewer lepidopteran larvae, leaves from greener *P. colorata* individuals had 47% higher mean A_{sat} values than matched leaves from redder individuals (Fig. 4). The benefits of signalling apparently did not outweigh the benefits of the higher photosynthetic rate of leaves from the greener plants.

Differences in photosynthetic rates between red and green leaves have been reported to result from shading by a light-screening anthocyanin filter (Karageorgou & Manetas 2006; Hughes & Smith 2007). However, the pigment profiles of red *P. colorata* leaves were not consistent with those of shade-adapted leaves; there was no difference in the Chl *a*: *b* ratio within matched pairs of red and green leaves, and total Chl was actually greater in the green leaves (Table 5). Rather, the higher A_{sat} values of greener leaves are likely a product of the higher Chl levels of leaves from greener *P. colorata* individuals (Table 5).

To date, discussions about the evolution of plant–animal signalling have focused on chemical defences that provide resistance to herbivores. However, in recent years there has been greater recognition of the role of plant tolerance to herbivory (Strauss & Agrawal 1999; Fornoni 2011). Diverse mechanisms of tolerance allow plants to mitigate the negative effects of herbivory on fitness, such as increased photosynthetic rate, increased shoot growth rate and reallocation of carbon stores from roots to shoots after damage (Stowe *et al.* 2000). In some species, an individual may employ both resistance and tolerance mechanisms (Salgado-Luarte & Gianoli 2010) and there can be inter-individual differences in patterns of allocation to these two defence strategies (Leimu & Koricheva 2006; Núñez-Farfán, Fornoni & Valverde 2007). It is plausible that, once a warning signalling system evolves, the increased herbivory experienced by non-signalling individuals may lead to increased selection for tolerance mechanisms.

We therefore hypothesize that leaf colour reveals whether a plant has invested in tolerance or resistance as a defence strategy. Accordingly, redder-leafed plants would employ a strategy of resistance by investing in high concentrations of defence compounds and in non-photosynthetic leaf pigments for warning signals; the greener individuals, as indicated by their higher Chl concentrations, would employ a strategy of tolerance, incurring more herbivory but investing more in the ability to photosynthesize in order to mitigate the negative fitness consequences of herbivore damage (Strauss & Agrawal 1999; Núñez-Farfán, Fornoni & Valverde 2007; Carmona & Fornoni 2013). Although not directly testing for tolerance mechanisms, Nikiforou *et al.* (2010) found higher numbers of leaves per unit shoot length in green as compared with red *Cistus creticus* plants. However, in the same study, red plants showed higher stem elongation rate. We suggest that experimental and theoretical studies of red leaf signalling will benefit from including tolerance in their discussions of plant defence.

We predict that inter-individual differences in tolerance and resistance are common in leaf colour polymorphisms but have been hitherto overlooked. Thus, the contemporary dichotomy between ecological studies focussing on warning signals and physiological studies focussing on photosynthetic abilities is misleading (Schaefer & Gould 2007). Studies on flower colour polymorphisms have yielded important insight into evolutionary biology such as drift, the role of mutations and how adaptive change constrains future evolutionary potential (Wright 1943; Bradshaw & Schemske 2003; Zufall & Rausher 2004). In comparison, colour polymorphisms in leaves are seldom studied but can significantly advance our understanding of plant defence theory and of the interplay between plant physiology and ecology throughout plant evolution.

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Data accessibility

Data are deposited at Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.p211h>.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Further details of sampling and experimental methods.

Table S1. Median and interquartile range [IQR = Q_1 – Q_3] for air temperature (T_{air}), relative humidity (RH), and wind speed during sampling days at Belmont Trig scrub site.

Figure S1. Transmittance spectra from Li-Cor 6400-02B LED source (from Figure 8-3 of Li-6400 Instruction Manual).