

A global analysis of water and nitrogen relationships between mistletoes and their hosts: broad-scale tests of old and enduring hypotheses

Marina C. Scalon^{*,1,2} and Ian J. Wright¹

¹Department of Biological Sciences, Macquarie University, Sydney 2109, NSW, Australia; and ²Laboratório de Ecofisiologia Vegetal, Departamento de Botânica, Instituto de Ciências Biológicas, Universidade de Brasília, Caixa Postal 04457, 70904-970 Brasília, DF, Brazil

Summary

1. Mistletoes use far more water per unit carbon fixed during photosynthesis than their hosts (i.e. they have lower ‘water use efficiency’, WUE). The widely cited ‘nitrogen-parasitism hypothesis’ posits that N is the most limiting resource for mistletoes and that they use their faster transpiration rates to acquire sufficient N from the host xylem. In a rather different context, the ‘mimicry hypothesis’ arose in the literature suggesting that some mistletoes mimic the morphology of host leaves in order to deploy higher N leaves without suffering higher levels of herbivory. These two non-exclusive hypotheses share the common goal of trying to explain patterns of mistletoe leaf N concentration.

2. We set out to test the generality of both hypotheses at broad geographic scale using data for 168 mistletoes–host pairs, from 39 sites, encompassing all continents except Antarctica. We drew together data from published literature and our own field data on two key plant functional traits, leaf N concentration (N_{mass}) and leaf carbon isotopic composition ($\delta^{13}\text{C}$) (representing long-term WUE and degree of stomatal control over photosynthesis).

3. Key findings included (i) little or no support for the N-parasitism hypothesis: differences in mistletoe and host N_{mass} explained only 3% variation in differences in leaf $\delta^{13}\text{C}$, and mistletoe–host differences in leaf $\delta^{13}\text{C}$ were unrelated to whether or not the hosts were N-fixers (presumed to have higher N concentration in xylem sap); (ii) partial support for the mimicry hypothesis: mimic mistletoes generally had higher N_{mass} when associated with N-fixing hosts (but, on non-N-fixing hosts there was no such pattern); and (iii) more broadly, mistletoes showed similar trait responses as their hosts to environmental drivers; for example, they showed similar-magnitude shifts in N_{mass} and $\delta^{13}\text{C}$ in relation to site aridity.

4. Contrary to current belief, our findings suggest that nitrogen is not the limiting nutrient for mistletoes, at least not the main component driving the faster transpiration rates. Our results also give insight into the evolution of mimicry in mistletoes and show, for the first time, that mistletoes are also constrained by local water availability, exhibiting clear trait adaptations to environmental gradients. By reconsidering these issues at broad geographic scale and across a large number of species, our findings substantially modify current knowledge on the ecology and physiology of mistletoes and their hosts.

Key-words: carbon isotope, Loranthaceae, mimicry, N-parasitism, Santalaceae, Viscaceae, water use efficiency

Introduction

Mistletoes are parasitic angiosperms that connect to the xylem of their host through a modified root system called a

haustorium (Lamont & Southall 1982). Once this connection is established the xylem solution flows from the host to the mistletoe, becoming its only source of water and nutrients (Calder & Bernhardt 1983; Press & Graves 1995). Being hemiparasites, mistletoes produce their own photosynthetically active leaves but, because there is no

*Correspondence author. E-mail: marina.scalon@students.mq.edu.au

connection between the phloem of the two organisms, no photoassimilates are contributed back to the host (Glatzel & Geils 2009). Mistletoes are a widespread group, occurring on every continent in the world except Antarctica (Calder & Bernhardt 1983), and highest species diversity is found in the families Loranthaceae, with 73 genera and over 1500 species, and Santalaceae (formerly treated as the separate family Viscaceae), with seven genera and over 450 species (Nickrent *et al.* 2010; Nickrent 2011).

Because mistletoes do not invest in a complex root system, the acquisition costs for water and nutrients are presumably far lower than those experienced by their hosts. Therefore, mistletoe–host interactions present a unique and intriguing study system to ecophysiologicalists interested in the water and nitrogen costs of photosynthesis (Schulze, Turner & Glatzel 1984; Ehleringer, Cook & Tieszen 1986; Orozco *et al.* 1990; Küppers 1992; Panvini & Eickmeier 1993; Marshall, Dawson & Ehleringer 1994; Bowie & Ward 2004). These costs are sometimes expressed as the ratios ‘water use efficiency’ (WUE; ratio of photosynthetic rate to that of transpirational water loss) and ‘photosynthetic nitrogen use efficiency’ (PNUE; ratio of photosynthesis to leaf N concentration) (Chapin *et al.* 1987; Evans 1989; Farquhar *et al.* 1989; Lambers, Chapin & Pons 1998).

Carbon isotope discrimination ($\delta^{13}\text{C}$) in leaf dry matter, reflecting discrimination against ^{13}C by Rubisco and PEP-carboxylase during photosynthesis, is used as a long-term estimator of $c_i : c_a$ ratio (ratio of leaf-internal to ambient CO_2) (Farquhar *et al.* 1989). Under a given atmospheric humidity, lower $c_i : c_a$ (higher $\delta^{13}\text{C}$) equates to higher WUE. Even under extreme drought conditions, mistletoes generally show faster transpiration rates and far lower $\delta^{13}\text{C}$ than their hosts, and thus far lower WUE (Schulze, Turner & Glatzel 1984; Ullmann *et al.* 1985; Marshall *et al.* 1994; Escher *et al.* 2004, 2008; Glatzel & Geils 2009). Noting this profligate water use, teamed with lower leaf N concentrations than their hosts but very high accumulations of mobile cations such as K^+ and Ca^{2+} (Glatzel 1983; Schulze & Ehleringer 1984), Schulze, Turner & Glatzel (1984) proposed the ‘nitrogen-parasitism hypothesis’, positing that nitrogen limitation is the key driver for rapid transpiration in mistletoes. Evidence in favour of this hypothesis includes enhanced mistletoe performance when growing on hosts with higher N concentration in the xylem, whether due to fertilizer application or to having N-fixing root symbionts; for example, there have been reports of mistletoes showing less negative $\delta^{13}\text{C}$ signatures (Ehleringer, Cook & Tieszen 1986; Bannister & Strong 2001), higher biomass, higher flower production (Schulze & Ehleringer 1984; Gibson & Watkinson 1989; Seel, Cooper & Press 1993) and lower herbivory rates (Adler 2002).

A contrasting – but not mutually exclusive – hypothesis suggests that higher transpiration rates in mistletoes may be driven not only by the need of N, but also by the ability to acquire large amounts of carbon via the host xylem (‘heterotrophic’ carbon), in the form of amino acids (Marshall & Ehleringer 1990; Stewart & Press 1990; Schulze

et al. 1991; Marshall *et al.* 1994). Estimates of how important this external source of carbon is to mistletoes vary widely. Early reports suggested that up to 60% of C in the mistletoe *Phoradendron juniper* came via this pathway (Marshall & Ehleringer 1990), and around 50–70% of C in five mistletoe species from Namib Desert (Schulze *et al.* 1991). Subsequent reports – based on more and different species – suggested that heterotrophic carbon gain might be highly variable, ranging from 5% to 21% (Marshall *et al.* 1994) in 11 mistletoes–host pairs from eastern Australia; 50% to 80% (Richter *et al.* 1995) in 10 pairs from Namibia; and from 35% to 78% (Wang *et al.* 2008) in three pairs along the Kalahari Transect. In any case these estimates should be considered somewhat tentative since they are based on differences in $\delta^{13}\text{C}$ between mistletoes and hosts, which presupposes that they are operating at a very different $c_i : c_a$. Certainly, this secondary source of carbon potentially has an impact on the mistletoe carbon isotope signature (Schulze *et al.* 1991).

A rather separate literature has focused on how similar or different mistletoes are from their hosts in terms of their leaf N concentration (N_{mass} hereafter). On the one hand, higher N_{mass} is generally associated with greater photosynthetic capacity; on the other, higher N_{mass} should, all else equal, make leaf tissue more attractive to herbivores (Mattson 1980; Mooney & Gulmon 1982; Marvier 1996). Considering these issues, and the remarkable resemblance between the leaves of many Australian mistletoes and their hosts (especially *Eucalyptus*, *Acacia* and *Casuarina* hosts), Barlow & Wiens (1977) described the ‘mimicry hypothesis’. Barlow and Wiens argued that mistletoes that mimic their hosts (‘mimics’) could get away with having higher N_{mass} than their hosts without suffering serious herbivory, since – for larger herbivores, at least – they will not stand out as being different. By contrast, ‘non-mimic’ species by definition do stand out visually; therefore, the best strategy to avoid serious herbivory in that case would be to have similar or lower N_{mass} than their hosts. This hypothesis has broad-scale empirical support from mistletoe–host pairs measured in both Australia (Ehleringer *et al.* 1986b) and New Zealand (Bannister 1989). One concern with this hypothesis is what type of herbivores could be responsible. For example, in Australia various species of possums are known to eat leaves of both mistletoes and hosts, whereas in New Zealand there are no large vertebrate herbivores to explain this pattern (Bannister 1989). Other concerns include whether mimicry really has any fitness benefit for the mistletoe (Canyon & Hill 1997; Schaefer & Ruxton 2009), or even whether mimicry truly exists (Blick, Burns & Moles 2012).

In this study, we revisited the nitrogen-parasitism and the mimicry hypotheses using a global data set of leaf N and $\delta^{13}\text{C}$ discrimination data compiled from the literature and supplemented with new data from several sites in Australia. We investigated a range of issues related to N and water deployment/use, along the way testing several specific hypotheses. We tested for generality (or otherwise) of trait relationships reported previously in regional analyses,

considering the influence of site climate, and the extent to which observed trends (in N_{mass} and $\delta^{13}\text{C}$) were related to mistletoe taxonomy (family). Specific hypotheses and questions were as follows:

1. In relation to the N-parasitism hypothesis (Schulze, Turner & Glatzel 1984; Ehleringer, Cook & Tieszen 1986):
 - a. Is it globally true that mistletoes have more negative leaf $\delta^{13}\text{C}$ than their hosts, implying that they operate at higher $c_i : c_a$ and have lower WUE?
 - b. Are host and mistletoe leaf $\delta^{13}\text{C}$ positively correlated? This was previously reported by Bannister & Strong (2001), presumably because heterotrophic carbon gain from the host influences $\delta^{13}\text{C}$ signature in the mistletoe (Ziegler 1995), but also because mistletoes and hosts might respond similarly to climatic variations (see also question 3).
 - c. Are host and mistletoe N_{mass} positively correlated? For example because the N concentration in the xylem sap is higher on hosts with higher N_{mass} (Schulze *et al.* 1991; Bannister & Strong 2001; Wang *et al.* 2008).
 - d. Is it generally the case that differences between mistletoes and their hosts in leaf $\delta^{13}\text{C}$ are smaller on N-fixing hosts, or on hosts with higher N_{mass} (Ehleringer *et al.* 1985; Schulze *et al.* 1991; Marshall *et al.* 1994; Richter *et al.* 1995; Bannister & Strong 2001)? These are situations where N concentration in the xylem sap of the host is presumed to be higher, interspecific variation in leaf N being tightly correlated with N concentration of xylem sap (Stewart, Joly & Smirnov 1992; Schmidt *et al.* 1998). This question is the key test of the nitrogen-parasitism hypothesis.
2. In relation to the mimicry hypothesis (Barlow & Wiens 1977; Ehleringer *et al.* 1986b; Bannister 1989):
 - a. Do host-mimic mistletoes have higher N_{mass} than their hosts, while non-mimic mistletoes show similar or lower (i.e. *not* higher) N_{mass} than their hosts?
3. Broader questions in relation to phylogeny and environmental influences:
 - a. Is there patterning in N_{mass} and $\delta^{13}\text{C}$ relationships of mistletoes and hosts in relation to mistletoe family (Loranthaceae vs. Viscaceae)? Differences between families have been suggested by different authors, such as Aukema (2003) and Shaw, Watson & Mathiasen (2004), where Viscaceae mistletoes were suggested to have larger impacts on hosts compared to Loranthaceae mistletoes.
 - b. Does the difference in $\delta^{13}\text{C}$ between mistletoes and hosts vary according to site climate? In particular, is the difference greater at more arid sites? As suggested by Bannister & Strong (2001), in arid sites there should be stronger pressure on hosts to use water efficiently, but somewhat less pressure on mistletoes.

- c. Do mistletoes show the same trend in leaf $\delta^{13}\text{C}$ and N_{mass} in relation to site aridity as do their hosts (and other species)? Or, do mistletoes show a dampened trend? for example because of weaker selective pressure to be efficient in their photosynthetic water use.

Materials and methods

Leaf N concentration and carbon isotope signature data from mistletoes and their hosts ('M-H pairs' hereafter) were compiled from the literature (135 different M-H pairs from 23 published papers), to which we added data from our own sites in Australia (33 M-H pairs), yielding a data set comprising 168 M-H pairs from 39 sites (Table S1, Supporting information). When a given mistletoe species was reported growing on several different host species, each instance was considered a different M-H pair. The majority of the pairs were sampled in Australia (43.5%), New Zealand (17.8%) and United States (8.9%). Eleven countries contributed the remaining 30% of data (Fig. 1). Loranthaceae was the best represented mistletoe family (141 pairs), Viscaceae contributing the other 27. The best represented host family was Fabaceae, accounting for 45 pairs. We only had C isotope data for 93 of the 168 M-H pairs, of which 84% included a Loranthaceous mistletoe and 16% a Viscaceous mistletoe.

We also recorded site latitude and longitude, biome type, whether the host was a N-fixing species, and whether mistletoes were considered host-mimics by the authors or by Barlow & Wiens (1977). An approximate latitude and longitude were derived from the written description of site location in cases when precise information on the geographical coordinates was not available in the original paper. Geographical coordinates were used to retrieve the mean annual temperature (MAT) and mean annual precipitation (MAP) from the CRU CL2.0 global climate data set (New *et al.* 2002). Potential evapotranspiration (PET) was estimated following Wang, Prentice & Ni (2012), and moisture index was calculated as the ratio between MAP and PET (Table S2, Supporting information).

To the literature data, we added information on M-H pairs that were sampled at four Australia locations between 2011 and 2013 (Table S1). Three fully expanded sun leaves were collected from at least three different individuals per species, oven-dried at 60 °C for 72 h and finely ground in preparation for chemical analyses. For the $\delta^{13}\text{C}$ determination, leaves from the same species were bulked and analysed at the Mass Spectrometry Facility at the Australian National University, Canberra. For nitrogen analysis, individual samples were analysed by LECO TruSpec CHN combustion technique at the Analytical Service Unit from the School of Agriculture and Food Science at The University of Queensland.



Fig. 1. The distribution of the mistletoe–host pairs data globally.

DATA ANALYSES

Data for leaf N concentration (N_{mass} ; mg of N per g dry leaf mass) and climate variables were log-transformed to meet assumptions of normality (Shapiro–Wilk’s test of normality, $P > 0.1$). While paired t -tests are an appropriate statistic for quantifying mean differences between mistletoes and their hosts (which are intrinsically paired), this approach is limited to testing for differences in just one factor at a time. Therefore, we also used linear mixed effect analysis to compare the different aspects of our data set simultaneously and to assess the relative importance of potential predictors of the difference in N concentration between mistletoes and hosts, and the difference in $\delta^{13}\text{C}$ isotope composition between mistletoes and hosts. Arithmetic differences in these properties were calculated in all cases as trait (mistletoe) – trait (host). As fixed effects, we considered the environmental factors (MAP and temperature), the family of the mistletoe (Viscaceae or Loranthaceae), mimicry (yes or no) and nitrogen-fixing host (yes or no). Study location and the family of the host were treated as random effects.

Standardized major axis (SMA) slopes (Warton *et al.* 2006) were used to compare the best fit proportional relationship of traits between mistletoes and hosts. Pearson correlation and ordinary least square (OLS) regression were used for quantifying relationships between N and $\delta^{13}\text{C}$ with climate (climate being the independent variables).

All statistical analyses were performed using R software v. 2.13 (R Core Team, Vienna, Austria). The package LME4 (Bates, Maechler & Bolker, 2013) was used for the linear mixed model analyses, and SMATR v. 3 package (Warton *et al.* 2012) was used to test for differences between SMA slopes.

Results

Mistletoes typically showed lower $\delta^{13}\text{C}$ than their hosts (host mean $\pm$ SD: $-27.5 \pm 2.7\text{‰}$; mistletoe mean $\pm$ SD: $-29.5 \pm 2.2\text{‰}$; paired t -test: $P < 0.001$, $n = 93$), implying that mistletoes typically operate at higher c_i/c_a ratios (i.e. they are less water use efficient). Mistletoe and host carbon isotope signatures were positively correlated ($r^2 = 0.39$, $P < 0.001$), with a fitted slope not significantly different from 1 (SMA slope = 1.01, 95% CIs = 0.86–1.19; Fig. 2a).

Overall, there was no difference between mistletoe and host N_{mass} (host mean $\pm$ SD: $16.1 \pm 6.6 \text{ mg g}^{-1}$, mistle-

toe mean $\pm$ SD: $16.3 \pm 8.8 \text{ mg g}^{-1}$; paired t -test: $P = 0.298$, $n = 168$); and leaf N concentration of mistletoes strongly reflected that of their hosts ($r^2 = 0.35$, $P < 0.001$; Fig. 2b). This relationship had a slope slightly steeper than 1 (SMA slope = 1.25, 95% CIs = 1.10–1.41; $P < 0.001$, Fig. 2b).

According to the N-parasitism hypothesis, the lower the N concentration in the host xylem, the more water mistletoes will need to transpire in order to fulfil their nitrogen requirements. By extension, a higher N_{mass} in the host leaves (indicating higher N in xylem) is expected to be associated with smaller difference between mistletoe and host $\delta^{13}\text{C}$. As it turned out, we found only a weak, marginally significant relationship between M-H differences in $\delta^{13}\text{C}$ and M-H differences in N_{mass} ($r^2 = 0.03$, $P = 0.08$, Fig. 3a). In addition, there was no patterning in M-H differences in carbon isotope signature in relation to whether the hosts were nitrogen fixers or not (Fig. 3b; $P = 0.49$). That is, the N-parasitism hypothesis was not supported.

There was a positive relationship between $\delta^{13}\text{C}$ and N_{mass} both in mistletoes ($r^2 = 0.24$, $P < 0.001$) and in hosts ($r^2 = 0.08$, $P < 0.01$; Fig. 4), meaning the lower the N_{mass} the lower the WUE (more negative $\delta^{13}\text{C}$). The mistletoe-specific and host-specific relationships did not differ in slope ($P = 0.256$) but they were significantly offset ($P < 0.001$) such that, at a given N_{mass} , mistletoes had c. 1.5‰ more negative $\delta^{13}\text{C}$ than their hosts.

Next, we tested predictions from the mimicry hypothesis. Overall, there was no difference in N_{mass} between mimic and non-mimic mistletoes (mimics, mean $\pm$ SD = $15.47 \pm 8.35 \text{ mg g}^{-1}$, $n = 50$; non-mimics, mean $\pm$ SD = $16.68 \pm 8.98 \text{ mg g}^{-1}$, $n = 118$; $P = 0.40$). However, the family of the mistletoe together with the interaction between mimicry and N-fixing status of the host accounted for 28% of the variance found in M-H differences in leaf N concentration ($r^2 = 0.28$, all $P < 0.001$, Table 1). The positive interaction between mimicry and N-fixing host showed that mistletoes considered mimics and parasitizing N-fixing hosts did indeed have higher N_{mass}

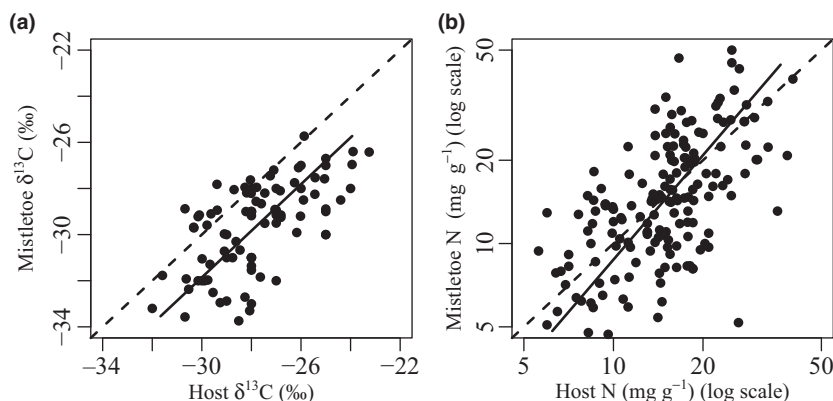


Fig. 2. Positive relationship between mistletoes and hosts across different mistletoes-pair species reported on the literature and our own data for (a) carbon isotope discrimination (slope (95% confidence intervals) = 1.01 (0.86, 1.19); $r^2 = 0.39$, $P < 0.0001$); and (b) leaf N concentration (slope (95% confidence intervals) = 1.25 (1.10, 1.41); $r^2 = 0.35$, $P < 0.0001$). The dashed line corresponds to the 1 : 1 relationship, and the solid line represents the fitted line based on the Standardized major axis values.

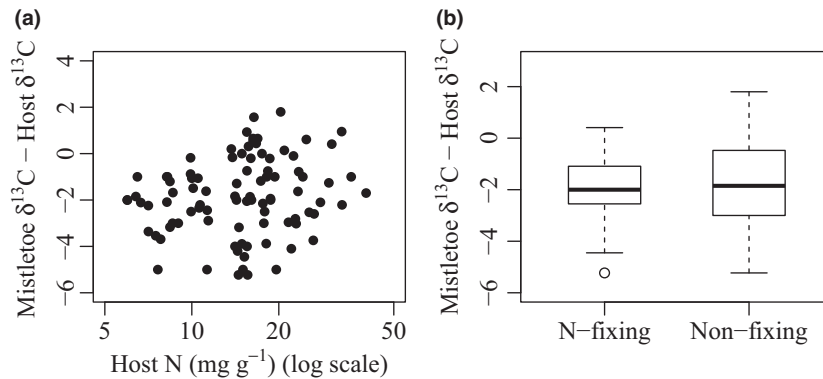


Fig. 3. (a) Mistletoe to host difference in $\delta^{13}\text{C}$ in relation to host leaf N concentration ($r^2 = 0.03$, $P = 0.08$); and (b) pairwise comparison between mistletoe to host difference in $\delta^{13}\text{C}$ in N-fixing ($n = 19$) and non-fixing hosts ($n = 71$; $t = -0.69$, $P = 0.49$).

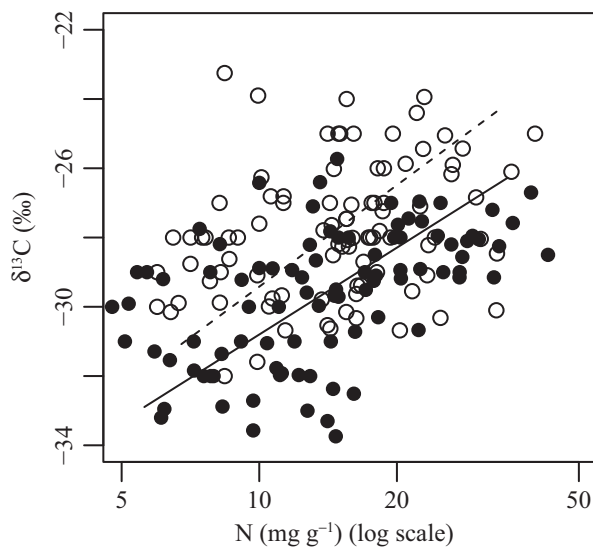


Fig. 4. Standardized major axis (SMA) relationship between $\delta^{13}\text{C}$ and leaf N concentration for mistletoes [filled symbols, solid line; slope (95% confidence intervals) = 0.83 (0.79, 1.00); $r^2 = 0.24$, $P < 0.001$] and hosts [empty symbols, dashed line; slope (95% confidence intervals) = 0.97 (0.80, 1.19); $r^2 = 0.08$, $P = 0.004$]. Common slope (95% confidence intervals) $\beta = 0.89$ (0.71, 1.02).

than their hosts (ANOVA, $F_{1,163} = 7.86$, $P = 0.005$; in support of the mimicry hypothesis), whereas this was not the case for mimics growing on non-N-fixing hosts (Fig. 5). Consequently, neither mimicry (yes/no) nor host N-fixing status (yes/no) alone explained significant variation in M-H differences in N_{mass} . By contrast, mistletoe family did explain significant variation in M-H differences in N_{mass} : on average Loranthaceae mistletoes showed similar N_{mass} than their hosts (paired t -test, $P = 0.061$), while Viscaceae mistletoes had higher N_{mass} than their hosts (paired t -test, $P < 0.01$).

ENVIRONMENTAL EFFECTS

As expected, host plants showed less negative $\delta^{13}\text{C}$ (higher WUE) at drier sites but, interestingly, the same was clearly

true of mistletoes (trends in relation to precipitation shown in Fig. 6a, and in relation to site moisture index in Fig. 6b). Because of the similarity in mistletoe and host relationship slopes, site aridity did not explain significant variation in M-H differences in $\delta^{13}\text{C}$ (e.g. see mixed model results incorporating all effects in Table 1). Both species groups showed a weak but significant tendency for higher N_{mass} at drier sites (mistletoes: $r^2 = 0.07$; host: $r^2 = 0.13$; both $P < 0.01$, Fig. 6c).

Unexpectedly, we found that MAT explained 24% of variation M-H differences in $\delta^{13}\text{C}$ (Fig. 7a), and this effect was still highly significant when all other effects were accounted for (mixed model results; Table 1). Specifically, mistletoes and hosts did not on average differ in $\delta^{13}\text{C}$ (and thus WUE) at cold sites, while at warmer sites mistletoes were increasingly more profligate in water use than their hosts (they had lower WUE). Figure 7b illustrates that this result was caused mainly by a response to MAT in mistletoes and not in hosts: mistletoes $\delta^{13}\text{C}$ decreased while $\delta^{13}\text{C}$ in hosts was relatively constant across the temperature gradient.

Discussion

To summarize the main results, we found support for lower $\delta^{13}\text{C}$ in mistletoes relative to their hosts, suggesting that they operate at lower $c_i : c_a$ (and are therefore less water use efficient), except at colder sites. We also showed that N_{mass} in mistletoe and host is positively correlated, and the same was found in relation to $\delta^{13}\text{C}$, suggesting coupled carbon and N metabolisms. Our results provided little or no support for the N-parasitism hypothesis: more nitrogen in hosts (i.e. N-fixing hosts and higher N_{mass}) was not related to more similar WUE between hosts and mistletoes (as indexed by differences in leaf $\delta^{13}\text{C}$). However, we found clear support for the mimicry hypothesis considering N-fixing hosts: mimic mistletoes had higher N_{mass} than their hosts, whereas non-mimic species did not differ. In contrast, no support for the mimicry hypothesis was found when considering non-N-fixing hosts (or, indeed, when considering all

Table 1. Results of linear mixed effects models estimating effects of mimicry, nitrogen-fixing host, environmental aspects and family between mistletoe and host on (i) nitrogen leaf concentration difference; and (ii) carbon isotopic composition difference. For each model, the sum of squares and *F*-values are shown. Significant non-zero slope estimates are highlighted ($P < 0.001$)

Variable	Predictor	Coefficient	<i>P</i>	<i>F</i>	d.f.	<i>R</i> ²
N_{mass} difference	General model		<0.001	8.72	6, 134	0.28
	Intercept	-3.92	0.03			
	Mimicry	1.35	0.34			
	N-fixing host	1.77	0.24			
	Mimicry*N-fixing host	6.58	<0.001			
	Mean annual temperature (MAT)	0.05	0.61			
	Precipitation	0.00	0.62			
$\delta^{13}\text{C}$ difference	Family	10.10	<0.001			
	General model		<0.001	5.93	7, 84	0.33
	Intercept	0.20	0.73			
	N_{mass} difference	0.01	0.81			
	Mimicry	0.43	0.32			
	N-fixing host	0.64	0.22			
	Mimicry*N-fixing host	0.38	0.68			
	MAT	-0.18	<0.001			
	Precipitation	0.00	0.15			
	Family	0.59	0.28			

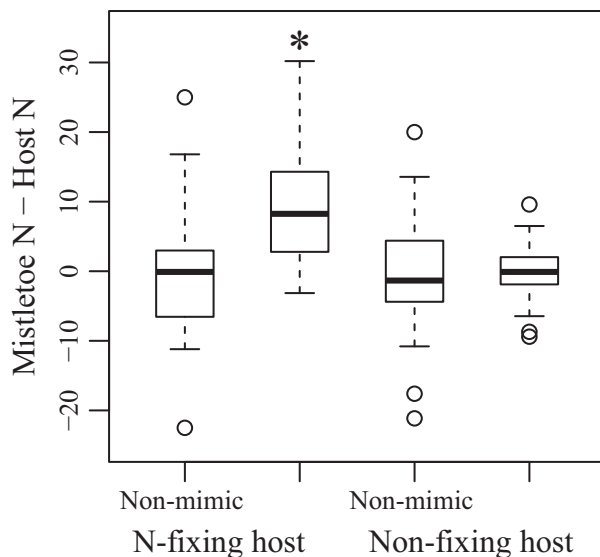


Fig. 5. Pairwise comparisons between mistletoe to host differences in N concentration in mimic ($n = 50$) and non-mimic ($n = 118$) mistletoes growing on N-fixing ($n = 48$) and non-fixing hosts ($n = 119$). The continuous line within the box shows the median, error bars show 10th and 90th percentiles, and open circles represent outliers. Mimic mistletoes growing on N-fixing hosts showed higher difference values than the other groups (ANOVA, $F_{1,163} = 7.86$, $P = 0.005$).

species together). More broadly, we could see significant differences between the two mistletoe families (Loranthaceae showed marginally lower N_{mass} , while Viscaceae mistletoes showed higher N_{mass} , compared to their hosts). We also found similar shifts of N_{mass} and $\delta^{13}\text{C}$ to site aridity in mistletoes and hosts, but differences in the patterning of $\delta^{13}\text{C}$ to site temperature.

WATER USE EFFICIENCY AND THE N-PARASITISM HYPOTHESIS

Ehleringer *et al.* (1985) showed that, across species sampled from three continents, M-H differences in $\delta^{13}\text{C}$ were smaller on hosts with higher leaf N concentration (N_{mass}). These authors argued that this constituted strong evidence in support of the N-parasitism hypothesis, reasoning that, given sufficient access to host N in the xylem stream, there would be less advantage to mistletoes having markedly lower WUE. Here, we took a different approach to testing the hypothesis, considering individual pairs of mistletoe and host rather than using an average value for each continent, and we did not find the same strong pattern (Fig. 3a). Moreover, we showed that M-H differences in $\delta^{13}\text{C}$ are no lower on N-fixing hosts than on non-fixing hosts, suggesting that higher N in the host xylem does not seemingly influence WUE in mistletoes (Fig. 3b). At best, we found very weak support for this contention, with host N_{mass} explaining just 3% of variation in M-H differences in $\delta^{13}\text{C}$ (Fig. 3a).

One could interpret the positive relationship between N concentration and $\delta^{13}\text{C}$ in mistletoes (Fig. 4) as the outcome of a strategy to extract more nitrogen from hosts by maintaining a steep differential in xylem water pressure (via keeping the stomata open), which would support the nitrogen-parasitism hypothesis. However, a similarly positive slope was found for the relationship in hosts, suggesting that there is little difference between mistletoe and host water and N use behaviour in this regard. For instance, N_{mass} is known to be positively correlated with $\delta^{13}\text{C}$ (Högberg, Johannisson & Hällgren 1993; Guehl, Fort & Ferhi 1995; Sparks & Ehleringer 1997) because of the strong influence of nitrogen on photosynthetic capacity (Evans 1989), and the negative correlation (all else being

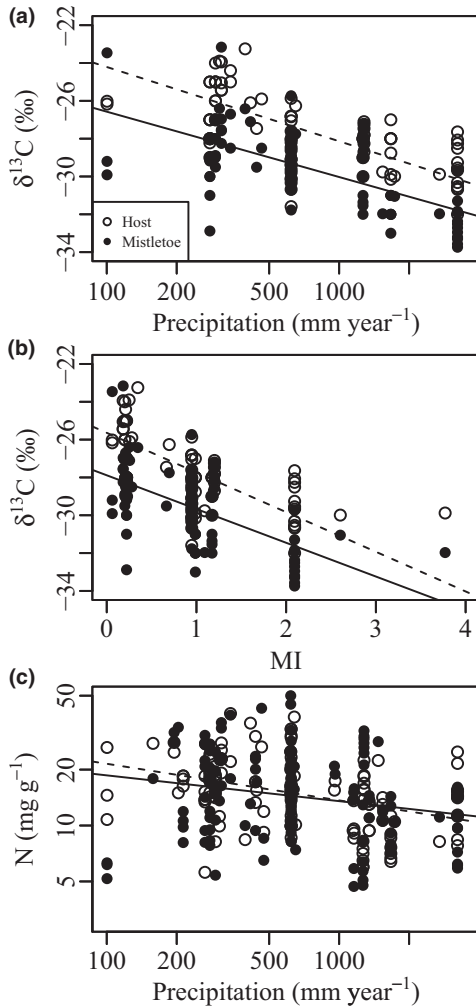


Fig. 6. Relationship between $\delta^{13}\text{C}$ and (a) precipitation (mistletoes: $r^2 = 0.41$; host: $r^2 = 0.35$; $P < 0.001$); and (b) moisture index (mistletoes: $r^2 = 0.35$, $P < 0.001$; hosts: $r^2 = 0.12$, $P < 0.001$). (c) Relationship between N concentration and precipitation (mistletoes: $r^2 = 0.07$; host: $r^2 = 0.13$; $P < 0.01$) for mistletoes (empty symbols) and hosts (filled symbols) across a precipitation gradient.

equal) between photosynthesis with intercellular CO_2 concentration (Farquhar, Ehleringer & Hubick 1989; Sparks & Ehleringer 1997). In summary, we found little support for the N-parasitism hypothesis, although it was indeed true that mistletoes operated with lower WUE.

One explanation for this lower intercept value (lower WUE) in the regression between $\delta^{13}\text{C}$ and N_{mass} in mistletoes (Fig. 4) could be that mistletoes usually develop inside the canopy of the hosts, so that the average light availability and the microclimate they experience are different (Watson 2001; Cooney, Watson & Young 2006), in turn influencing intercellular CO_2 concentration ($c_i : c_a$). Indeed, several studies indicate that leaf $\delta^{13}\text{C}$ can vary with canopy position, becoming more negative as leaves become more shaded (Medina & Minchin 1980; Francey *et al.* 1985; Ehleringer *et al.* 1986a).

The carbon-parasitism hypothesis (Marshall & Ehleringer 1990; Schulze *et al.* 1991; Marshall *et al.* 1994) also

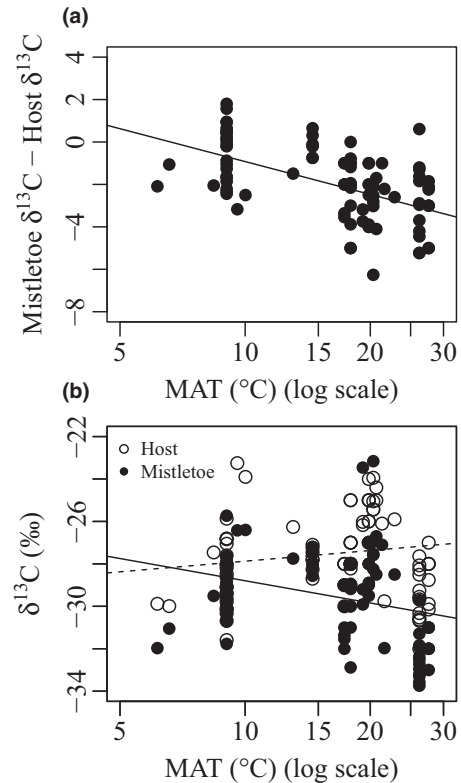


Fig. 7. (a) Mistletoe to host $\delta^{13}\text{C}$ difference becomes higher in warmer sites. The more negative the difference, the greater the difference between mistletoe and host $\delta^{13}\text{C}$ ($r^2 = 0.24$, $P < 0.0001$, $n = 93$). (b) Relationship between $\delta^{13}\text{C}$ and mean annual temperature for mistletoes (empty symbols, dashed line; $r^2 = 0.08$, $P = 0.003$) and hosts (filled symbols, solid line; $P = 0.26$).

does not help to explain why mistletoes exhibit such a low $\delta^{13}\text{C}$ signal compared to their hosts. The carbon retrieved from the host xylem is expected to be less negative than the $\delta^{13}\text{C}$ measured in the host leaves, because structural carbon from dry matter in leaves shows higher discrimination compared to the xylem sap (Evans *et al.* 1986; Cernusak, Pate & Farquhar 2002; Keitel *et al.* 2003), and heterotrophic tissues are ^{13}C -enriched compared to leaves (Cernusak *et al.* 2009). Therefore, the higher assimilation of amino acids from the host xylem should result in more enriched $\delta^{13}\text{C}$ signal (less negative) in the mistletoe leaf (Cernusak, Pate & Farquhar 2004). For example, holoparasitic plants, which derive all their carbon from the host, exhibit a $\delta^{13}\text{C}$ signal 1.0–1.5‰ less negative than their hosts (Cernusak, Pate & Farquhar 2004). Therefore, for mistletoes, if it were possible to measure the $\delta^{13}\text{C}$ of photosynthetic carbon only (i.e. not including any carbon from the host), then these values should be even more negative than the $\delta^{13}\text{C}$ signatures of the observed (combined heterotrophic and autotrophic) carbon. In addition, the current models used to calculate heterotrophy in mistletoes are still rather untrustworthy, yielding unrealistic values when mistletoes have similar or higher $\delta^{13}\text{C}$ compared to their hosts (Bannister & Strong 2001; Tennakoon, Chak &

Bolin 2011). In order to fully understand all the mechanisms underlying mistletoe carbon balance and water use, we need better models or approaches to verifying the extent to which heterotrophic carbon gain helps to explain mistletoe carbon isotopic signature.

MIMICRY HYPOTHESIS

Overall, host-mimic mistletoes in this study did not show higher N_{mass} in relation to their hosts, compared to differences seen for non-mimic mistletoes. However, when mimicry was considered together with nitrogen-fixing ability of the host, we found a significant effect on the host–mistletoe N_{mass} difference. N-fixing host alone was not a significant factor, nor the mimicry, but only the interaction between the two factors (Table 1, Fig. 5), suggesting that the positive interaction between mimicry and N-fixing hosts might be a result of a combined additive effect. Host-mimic mistletoes parasitizing non-N-fixing hosts do not show the same trend, perhaps due to limiting N concentration in the host xylem (Fig. 5). Considering optimal defence theory (McKey 1974; Rosenthal, Janzen & Applebaum 1979), it is reasonable to assume that higher N concentration in mistletoe leaves relative to the surrounding vegetation will increase their attractiveness to herbivores, leading to a greater selective advantage for investing on herbivore avoidance strategies. Coincidentally, N-fixing plants usually do have higher amounts of N-based toxic defences, such as alkaloids, cyanogenic glycosides, metal-binding factors and protease inhibitors (McKey 1974; Mattson 1980; Johnson, Liu & Bentley 1987; Møller 2010). There is the possibility that mistletoes on N-fixing hosts could also accumulate N-based defences from the hosts, and the evolution of mimicry could be favoured in these situations where the presence of an N-fixing host affords the luxury of having higher N concentration compared to the hosts.

It is important to recognize that there is some confusion in the literature regarding the application of the terms ‘mimicry’ and ‘crypsis’ in cases of mistletoe and host leaf resemblance (Vane-Wright 1980). If herbivores are searching exclusively for mistletoe leaves but are deceived because they are indistinguishable from host leaves, it is a case of protective crypsis (Endler 1981). Protective crypsis implies that the mistletoe should have traits that otherwise would make their leaves more attractive to herbivores, such as higher N_{mass} (as a proxy for higher leaf palatability). However, if herbivores already actively avoid leaves from a specific host, mistletoes would benefit from being morphologically similar to the host leaves, and it would consist an example of Batesian mimicry (Vane-Wright 1980). In this case, mistletoes would not necessarily have higher N_{mass} , but their hosts should have lower palatability traits (or higher chemical and physical defences) compared to the surrounding vegetation. Further investigation into herbivory rates and investment in chemical and physical

defences are needed to determine if there is support for this hypothesis, which would help to explain the evolution of leaf morphological resemblance in mistletoes and hosts.

FAMILY DIFFERENCES BETWEEN MISTLETOE TRAITS

Differences in N concentration between mistletoes and host leaves could also be explained by inherent differences between the two distinct families (Loranthaceae and Viscaceae, Table 1). For instance, Loranthaceae species in this study tended to show lower N_{mass} compared to Viscaceae species. Loranthaceae family has a Gondwanan origin, and is mainly distributed in the tropical region (Geils, Cibrián Tovar & Moody 2002) whereas Viscaceae is thought to have originated in East Asia and radiated through Laurasia, occurring mostly in tropical and temperate zones of the Northern Hemisphere (Geils, Cibrián Tovar & Moody 2002). Although Loranthaceae and Viscaceae were considered closely related families and even classified as a single family in the past (Engler & Krause 1935), there are significant differences between them (Kuijt 1969; Polhill & Wiens 1998). More recently, the two families are considered to have evolved parasitism independently and are classified as non-sister taxa (Nickrent *et al.* 2010; Nickrent 2011).

Loranthaceae mistletoes almost exclusively have large, colourful flowers, can develop multiple connections by epicortical roots and are highly varied in leaf colour, while all Viscaceae mistletoes have small flowers, are connected by a single haustorium and have pale-green leaves (Nickrent 2011). Implicit differences in the evolutionary history between the two families might be reflected not only in these morphological traits, but also in their physiology and the resource exploitation strategy of their hosts. The higher N_{mass} in Viscaceae suggests that this family might indeed have a greater impact on the host, as suggested in previous studies (Aukema 2003; Shaw, Watson & Mathiasen 2004), though the specific mechanism remains to be clarified.

ENVIRONMENTAL FACTORS AFFECTING HOST–MISTLETOE LEAF TRAITS

Increasing aridity was correlated with higher $\delta^{13}\text{C}$ signature (Fig. 6a,b) and higher leaf N_{mass} (Fig. 6c) for both mistletoes and hosts. The tendency for higher aridity to be associated with less negative $\delta^{13}\text{C}$ in non-parasitic, C_3 plants has been demonstrated in many studies, both regionally and globally (Stewart *et al.* 1995; Weiguo *et al.* 2005; Diefendorf *et al.* 2010; Hartman & Danin 2010; Prentice *et al.* 2011; Ma *et al.* 2012). Plants from more arid climates also tend to have higher leaf N per unit area (Wright, Reich & Westoby 2003; Wright *et al.* 2005; Prentice *et al.* 2011). Mistletoes also become more conservative in their water use as aridity increases (Fig. 6b), suggesting that they are not only capable of adjusting some of the physiological traits to couple with their hosts’

characteristics (Fig. 1a,b), but also respond similarly to environment differences in water availability (Fig. 6).

Unexpectedly, M–H differences in leaf $\delta^{13}\text{C}$ were negatively correlated with MAT (Table 1; Fig. 7a) with a mean difference of *c.* 3‰ at sites with MAT of 25 °C but no mean difference at sites with MAT of *c.* 5 °C. Neither of the underlying trends (i.e. in mistletoes or hosts) was as consistent as the combined trend; still, it was clear that the trend in M–H differences was largely driven by that in mistletoes, there being no relationship between leaf $\delta^{13}\text{C}$ and MAT in host plants (Fig. 7b). The trend in mistletoes, indicating lower average $c_i : c_a$ at colder sites, is consistent with the predicted and then observed trend seen in non-mistletoe species along a temperature gradient in eastern Australia (Prentice *et al.* 2014; but see Diefendorf *et al.* 2010), where the prediction of lower $c_i : c_a$ at colder sites was mainly due to the effect of temperature on Rubisco kinetics. Why this was seen here in mistletoes but not hosts is unknown, as is the overall significance of this trend in M–H differences in $\delta^{13}\text{C}$ with respect to site temperature. What we can say is that this result was still clearly observed when variation in a wide variety of other factors (of both hosts and mistletoes) was simultaneously accounted for (Table 1).

Conclusion

We found little support for the N-parasitism hypothesis and partial support for the mimicry hypothesis in a global context. Mistletoes considered to be mimics and occurring on N-fixing host had higher N concentrations compared to the host, suggesting that the evolution of mimicry in mistletoes could be associated with higher N availability in the hosts. We also found that N_{mass} is patterned with respect to different taxonomic groups, with Viscaceae showing higher N_{mass} than hosts compared to the tropical Loranthaceae mistletoes. Our study shows, for the first time, that mistletoes and hosts have similar responses to precipitation and moisture index gradients considering water and nitrogen use in a global context, but also respond differently in terms of a temperature gradient.

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Data accessibility

All data used in this manuscript are present in the manuscript and its supporting information.

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Supporting Information

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

Table S1. Mistletoe–hosts pairs species considered in this study.

Table S2. Climatic variables of sites from which mistletoes–hosts pair data were collected.