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A continental-scale assessment of variability in leaf traits: within species, across sites and between seasons

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44 **Running headline:** Inter- and intraspecific variation in leaf traits

45 Summary

- 46 1. Plant species show considerable leaf trait variability that should be accounted for in
47 dynamic global vegetation models (DGVMs). In particular, differences in the
48 acclimation of leaf traits during periods more and less favourable to growth have rarely
49 been examined.
- 50 2. We conducted a field study of leaf trait variation at seven sites spanning a range of
51 climates and latitudes across the Australian continent. 80 native plant species were
52 included. We measured key traits associated with leaf structure, chemistry and
53 metabolism during the favourable and unfavourable growing seasons.
- 54 3. Leaf traits differed widely in the degree of seasonal variation displayed. Leaf mass per
55 unit area (M_a) showed none. At the other extreme, seasonal variation accounted for
56 nearly a third of total variability in dark respiration (R_{dark}).

- 57 4. At the non-tropical sites, carboxylation capacity (V_{cmax}) at the prevailing growth
58 temperature was typically higher in summer than in winter. When V_{cmax} was normalised
59 to a common reference temperature (25°C), however, the opposite pattern was observed
60 for about 30% of the species. This suggests that metabolic acclimation is possible, but
61 far from universal.
- 62 5. Intraspecific variation – combining measurements of individual plants repeated at
63 contrasting seasons, different leaves from the same individual, and multiple conspecific
64 plants at a given site – dominated total variation for leaf metabolic traits V_{cmax} and R_{dark} .
65 By contrast, site location was the major source of variation (53%) for M_a . Inter-specific
66 trait variation ranged from only 13% of total variation for V_{cmax} up to 43% for nitrogen
67 content per unit leaf area.
- 68 6. These findings do not support a common practice in DGVMs of assigning fixed leaf trait
69 values to plant functional types. Trait-based models should allow for inter-specific
70 differences, together with spatial and temporal plasticity in leaf structural, chemical and
71 metabolic traits.

72 **Key-words:** aridity, dynamic global vegetation models, intraspecific variation, leaf traits,
73 nitrogen, phosphorus, photosynthesis, respiration

74 Introduction

75 Leaves play a vital role in the exchange of carbon between the atmosphere and land. Dynamic
76 global vegetation models (DGVMs) therefore need to characterise leaf traits accurately if they are
77 to fulfil the twin objectives of developing our understanding of vegetation distribution and
78 providing parent Earth System models with a dynamic representation of carbon, water and
79 energy budgets (Scheiter, Langan & Higgins 2013). To date, there have been two main
80 approaches to describing leaf traits in DGVMs (Prentice & Cowling 2013). The first organises
81 plant species into a small number of distinct groups, plant functional types (PFTs), and assigns a
82 single coefficient or model parameter to all members of the group. The second approach uses
83 leaf trait-trait relationships and trade-offs to predict variations in biogeochemical fluxes (e.g.
84 Pavlick *et al.* 2013).

85 All healthy leaves fix carbon and under the standard biochemical model of C_3
86 photosynthesis (Farquhar, von Caemmerer & Berry 1980), rates of carboxylation are limited by
87 the amount of activated Ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), which sets
88 the maximal rate of the reaction at any given temperature (V_{cmax}). Because of the high nitrogen

89 (N) content of key proteins in the leaf's photosynthetic apparatus, notably Rubisco, many
90 DGVMs treat V_{cmax} as a function of leaf N content. The relationship with N (also applied to
91 respiration) is based on empirical observations (e.g. Kattge *et al.* 2009). Like all enzymes,
92 Rubisco's activity is temperature-dependent and many DGVMs therefore employ the Arrhenius
93 function to predict metabolic rates at growing temperatures above and below a reference
94 temperature (often 25°C). This approach is based on the enzyme's observed short-term
95 responses to temperature, but assumes that the leaf's metabolism does not acclimate to sustained
96 changes in growth temperature; that simplifying assumption has potentially important
97 implications for modelled fluxes (Kattge & Knorr 2007).

98 Thermal acclimation in plants is often studied by making comparisons between
99 contrasting groups at a standardised measurement temperature; due to a range of processes,
100 which might include lowered capacity or demand, acclimation can lead to warm-grown plants
101 having a slower metabolic rate (at standard temperature) than those that experience cooler
102 growing conditions (Atkin, Holly & Ball 2000). Acclimation may also result in changes to trait-
103 trait relationships linking leaf metabolism with traits such as N content per unit leaf area (N_{area})
104 and leaf mass per unit leaf area (M_{a}) (Atkin *et al.* 2008) (see Fig. S1 in Supporting Information).
105 Field-based evidence of seasonal thermal acclimation of leaf dark respiration (R_{dark}) has been
106 shown in deciduous and evergreen species growing in a variety of ecosystems (e.g. Zaragoza-
107 Castells *et al.* 2008; Reich *et al.* 2016). For photosynthesis (A), however, there is less consensus
108 on whether thermal acclimation is common, with sometimes contradictory results (Way &
109 Yamori 2014). Importantly, the degree of photosynthetic thermal acclimation operating in
110 natural settings, where there are often large seasonal variations in growth temperature (and other
111 abiotic variables such as rainfall and irradiance), remains uncertain (but see Lin *et al.* 2013).

112 A major objection to the prevalent approach within DGVMs of assigning a single trait
113 value to all members of a PFT is that possible sources of variation, such as climate seasonality,
114 are ignored. Whilst plant trait initiatives such as the TRY database (Kattge *et al.* 2011) and
115 Glopnet (Wright *et al.* 2004) have provided insights into how trait values vary among species and
116 environmentally contrasting sites, many plant trait studies have focused on species' mean values
117 for measurements made during a single season. The degree of intraspecific variability (ITV) is
118 often (implicitly) assumed to be of minor importance compared to the variation among species
119 (Violle *et al.* 2012). Although some studies have compared leaf trait values at different times of
120 year (e.g. Misson *et al.* 2006), few have considered possible seasonal variation in trait-trait
121 relationships. Considering traits as mean values per species, and ignoring ITV, also

122 underestimates niche and trait overlap between species so that changes in species composition
123 under climate change projections may be exaggerated (Jung *et al.* 2014).

124 Intraspecific trait variation has two potentially complementary sources: genetic
125 differences (adaptation) and the interaction of a single genotype with altered environments
126 (plasticity) (Albert *et al.* 2010). Total ITV can theoretically be partitioned among populations,
127 among individuals and within individuals; but the spatial scale under consideration is important
128 and Albert *et al.* (2011) have proposed that the extent of ITV saturates asymptotically as the scale
129 widens to eventually encompass a species' entire environmental range. A number of recent
130 studies strengthen the case for DGVMs to incorporate leaf trait variation: ITV has been shown
131 to match or even exceed inter-specific variation for key traits such as M_a (e.g. Messier, McGill &
132 Lechowicz 2010) and can be more important than species turnover in plant community
133 responses to environmental change (Jung *et al.* 2014). In regard to trait-environment
134 relationships, a study of specific leaf area (the inverse of M_a) responses to light gradient across
135 forest patches in Brazil found that the relationship between SLA and canopy openness was
136 stronger when the analysis included both inter- and intra-specific variation (Carlucci *et al.* 2015).
137 Most recently, a review of four state-of-the-art DGVMs, Restrepo-Coupe *et al.* (2017) found that
138 the models performed poorly in simulating seasonal carbon fluxes in the Amazon forest. Such
139 difficulties are potentially greater still for ecosystems at higher latitudes where seasonal variation
140 in climate conditions is more pronounced.

141 Edaphic conditions in Australia - typified over large areas by highly weathered, low-P
142 soils - have played a major role in differentiating the flora, and plant performance is often
143 considered more constrained by the supply of P than of N (Beadle 1962). Under such
144 conditions, co-limitation of photosynthetic capacity by N and P may alter the form of a putative
145 \mathcal{A} -N relationship (Kattge *et al.* 2009; Reich, Oleksyn & Wright 2009). A number of
146 ecophysiological studies in Australia have sought to exploit the steep natural gradients in rainfall
147 and nutrient availability across the continent. Wright *et al.* (2001) measured 79 plant species
148 across four sites in eastern New South Wales to describe a suite of water conservation traits:
149 higher levels of leaf N and phosphorus (P) were associated with faster rates of \mathcal{A} for a given
150 level of stomatal conductance (g_s), but at the cost of higher R_{dark} . In a related study, Prentice *et al.*
151 (2014) confirmed shallower slopes for the positive $V_{\text{cmax}} - N_{\text{area}}$ relationship at drier sites, with
152 greater N investment in photosynthetic apparatus producing faster rates of carboxylation for a
153 given g_s (see also Schulze *et al.* 2006). Australian studies looking at seasonal variation in leaf traits

154 have focused primarily on plant water use efficiency, but with contrasting results (Prior, Eamus
155 & Duff 1997; Eamus *et al.* 1999).

156 In this study, our goal was to assess spatial and seasonal variability in leaf traits and the
157 trait-trait relationships that underpin modern DGVMs. Such attempts face the challenge of how
158 to contend with biodiversity linked to large environmental differences; one consequence of
159 Australia's diverse environments and flora is that we found only four species in sufficient
160 numbers at more than one site to permit inter-site comparisons between populations. We were
161 interested, however, in genetic influences on trait variability at a range of scales rather than how
162 individual species respond to environmental change. In seeking to explore sources of variation
163 within leaf functional traits we hypothesised that:

- 164 1. For key traits related to carbon uptake and release and nutrient content (V_{cmax} , R_{dark} ,
165 N_{area}), the nature of trait-trait relationships would vary with season;
- 166 2. For those sites with a pronounced seasonal shift in growth temperature, temperature-
167 standardised metabolic rates (i.e. V_{cmax} and R_{dark} at 25°C) would be faster in the cooler
168 season. We also considered evidence of seasonal variation in metabolic rates at those
169 tropical sites where seasonality is characterised by rainfall rather than temperature;
- 170 3. Given the wide spatial scale within our dataset, inter-specific variation would outweigh
171 intra-specific variation.

172 **Materials and methods**

173 **Study sites and climate data**

174 Our seven study sites are a subset of the TERN SuperSites network (www.supersites.net.au),
175 which is a facility of the Terrestrial Ecosystem Research Network (TERN) (Karan *et al.* 2016).
176 The site locations and key descriptors of dominant vegetation and soil type are presented in
177 Table 1. The sites were chosen, from the existing network, to provide a wide range in vegetation
178 and environmental conditions, with an emphasis on rainfall and temperature variability (Fig. S2).

179 Depending on the type of analysis attempted, we made use of climate data from a variety
180 of sources. Each SuperSite is equipped with a flux tower (TERN OzFlux network) that records
181 a common suite of meteorological data (Beringer *et al.* 2016). Our initial visits preceded the
182 installation of the standard OzFlux system at three sites; in those instances we used the
183 ANUClimate model (Hutchinson *et al.* 2009) and data from the Australian Bureau of
184 Meteorology's nearest weather station. Long-term climate data for each site were obtained from

185 interpolated values (covering the period 1970–2012) produced at 0.01° spatial resolution by the
186 TERN eMAST facility (Ecosystem Modelling and Scaling Infrastructure; www.emast.org.au).

187 We performed spatial analyses to assemble the seven sites across a matrix of bioclimatic
188 indices that emphasised patterns of seasonality in rainfall and temperature (data not shown). The
189 degree of similarity among the sites was interpreted using an un-rooted (phylogenetic-type) tree
190 (Fig. S3). Based on the branching observed, we have assigned the sites to three clusters: ‘Arid’
191 comprising Alice Mulga, Calperum Mallee and Great Western Woodlands; ‘Temperate’
192 comprising Cumberland Plain and Warra; ‘Tropical’ comprising Daintree and Robson Creek.

193 With the exception of Alice Mulga, each site was visited on two occasions. The timing of
194 the visits was designed, within logistical constraints, to provide the widest possible seasonal
195 contrast. The prevailing climate conditions leading up to each campaign are provided in Table
196 S1 (Supporting Information). Each visit to a site was designated as either the ‘Favourable’ or
197 ‘Unfavourable’ season based on a local assessment of growing conditions. Our study focused on
198 the impact of seasonal contrasts (i.e. sub-annual) on leaf metabolic traits and so the monthly
199 scale has been adopted here to present corresponding time-averaged climate conditions, on the
200 basis that 30 days is likely to provide sufficient time for acclimation to occur within pre-existing
201 leaves of long-lived broadleaved species (Zaragoza-Castells *et al.* 2008).

202 Leaf gas exchange

203 The plants measured (407 individuals of 80 species, Table S6), were selected to include locally
204 dominant species and to provide a wide range of leaf morphology. At each visit, we chose
205 young, fully developed leaves from two sun exposed branches. Leaf gas exchange measurements
206 were concentrated in the morning and performed using portable photosynthesis systems (Li-Cor
207 6400, Li-Cor, Lincoln, NE, USA), using a 6 cm² chamber fitted with a red-blue light source (Li-
208 Cor 6400-02B LED, Li-Cor, Lincoln, NE, USA). Upper canopy branches were excised using
209 forestry shears on telescopic poles and the cut end of the branches immediately placed in a
210 bucket and then recut under water to re-establish the xylem water column (Domingues *et al.*
211 2010). Performing gas exchange measurements on excised branches can affect subsequent
212 calculations where stomatal conductance is heavily depressed; our initial data exploration was
213 designed to identify any such outliers. For each leaf, approximately light-saturated (1500 μmol
214 photons m⁻² s⁻¹) measurements of net photosynthesis were taken at ambient CO₂ concentration,
215 400 μmol mol⁻¹ (ppm) (A_{400}). The leaf was next wrapped¹ in aluminium foil for 30 minutes
216 before R_{dark} was measured, still at 400 ppm CO₂. Air flow was held constant and a constant
217 chamber block temperature (T_{Block}) was adopted for all measurements at a given site and season,

218 set marginally (*ca.* 1°C) higher than expected morning air temperatures to counter the effect of
219 transpirational cooling and to ensure leaf and ambient air temperatures were similar. T_{Block}
220 settings ranged from 10°C for the winter visit to Warra to 32°C for the summer visit to
221 Calperum, reflecting the wide range of air temperatures experienced. The Li-Cor 6400 system is
222 capable of maintaining T_{Block} values $\pm 6^\circ\text{C}$ from ambient and this operating constraint precluded
223 measuring gas exchange at a common temperature across all sites and seasons. With a constant
224 flow rate, chamber humidity conditions varied and mean vapour pressure deficits within the
225 chamber ranged from 0.51 kPa for the winter visit to Warra to 3.16 kPa for the summer visit to
226 Calperum, mirroring differences in ambient conditions across sites and seasons.

227 When assessing whether photosynthesis shows seasonal acclimation, concurrent seasonal
228 changes in g_s might confound analysis - an important consideration in semi-arid zones. Unlike
229 net photosynthesis, V_{cmax} (classically estimated as the slope of the $A-C_i$ response curve in the
230 initial CO_2 limited region) is theoretically independent of g_s (Farquhar & Sharkey 1982). In the
231 absence of $A-C_i$ response curves, we estimated V_{cmax} based on our light-saturated A_{400} values
232 using the 'one point method', whose accuracy was established by de Kauwe *et al.* (2016).
233 Mitochondrial respiration in the light (R_{day}) was here assumed to be equivalent to R_{dark} (but this
234 simplifying assumption has only a very minor effect on the estimation of V_{cmax}). To allow
235 comparisons of metabolic rates across sites and seasons, we calculated rates at both a standard
236 temperature (25°C) and at the prevailing air temperature for the 30-day period leading up to the
237 end of each field campaign (Table S1). From the flux recorded at a given measurement
238 temperature, normalised R_{dark} was calculated by applying a temperature-dependent Q_{10} function
239 (Tjoelker, Oleksyn & Reich 2001). Normalised V_{cmax} was calculated from the original estimate
240 by applying the Arrhenius function (Medlyn *et al.* 2002) assuming an activation energy of 64.8 kJ
241 mol^{-1} (Badger & Collatz 1977).

242 Leaf morphology and nutrient determination

243 On completion of the gas exchange measurements leaves were weighed for fresh mass and
244 scanned for leaf area before being dried in an oven at 70 °C for a minimum of 48 hours, after
245 which their dry mass was recorded. The ratio of leaf dried mass to surface area (M_a , gm^{-2}) allows
246 interconversion between area- and mass-based leaf parameters. Oven-dried leaf material was
247 used for determination of total concentrations of leaf N and P: dried ground leaf material was
248 hot-digested in acid-peroxide before colorimetric analysis using a flow injection system
249 (QuikChem 8500, Lachat Instruments, Loveland, Colorado, USA).

250 Plasticity index and statistical analyses

251 A plasticity index (PI) ranging from 0 to 1 was calculated for each leaf trait and site as the
252 absolute difference between the favourable and unfavourable seasonal means divided by the
253 maximum trait value observed across all species (Zunzunegui *et al.* 2011). This index permits
254 plasticity comparisons among traits recorded in different units and with variable ranges.

255 Our study design included repeat measurements of the same plants and so a mixed
256 effects modelling approach was adopted to infer trait differences between seasons or among sites
257 and site clusters (Pinheiro *et al.* 2012). The random intercept term adopted for such models was
258 plant identity nested within species. Post-hoc differences were assigned using Tukey's Honest
259 Significant Differences (HSD). To draw inferences from trait-trait plots, standardised major axis
260 (SMA) analysis was used to determine the best-fitting lines ($\alpha = 0.05$) (Warton *et al.* 2012). We
261 also used linear mixed effects models to partition trait variation by adopting a structure that
262 reflected the combination of crossed and partially nested factors in our sampling design. The
263 dataset provided five hierarchical terms: season, site, site:season, species:site:season and
264 plant_number:species:site:season (here an interaction is denoted term1:term2). Mixed models
265 adopting this structure as the random intercept term were fitted using restricted maximum
266 likelihood estimation where the response variable was the trait in question and no predictor
267 (fixed) variables were included. Any residual variation in the model was assigned between
268 branches within a given plant. All statistical analysis and modelling was performed using the
269 open-source statistical environment R (R Development Core Team 2017) employing the
270 packages: *dplyr*, *ggplot2*, *lme4* and *smatr*.

271 Results

272 Spatial differences at the plant community level

273 Differences between the site clusters were observed for traits of both leaf structure and
274 chemistry. M_a was largest and most variable in the arid sites (Fig. 1) while the high leaf N:P
275 ratios were all indicative of plant communities growing on relatively P-impooverished soils
276 (Güsewell 2004). Overall, we found that trait values relating to leaf chemistry and construction
277 were consistent with global patterns reported in the worldwide leaf economic spectrum (Wright
278 *et al.* 2004) (Fig. S4).

279 **Metabolic acclimation to seasonal changes in temperature**

280 Marked seasonal differences in mean leaf metabolic traits were observed for certain species, but
281 the direction of change depended on whether rates were normalised to the prevailing
282 temperature or to a common reference of 25°C (Fig. 2, Table S7). For species at non-tropical
283 sites, V_{cmax} at the prevailing temperature was most frequently higher in summer than in winter.
284 However, our expectation of faster metabolic rates at a common reference temperature in winter
285 versus summer was not consistently borne out. At GWW, for example, only one species showed
286 a significantly faster $V_{\text{cmax},25}$ in winter. At Warra, where differences existed, the $R_{\text{dark},25}$ rates were
287 generally faster in summer (three of four cases; Fig. S5). Nonetheless, where seasonal mean
288 values were statistically distinguishable (13 of 41 species), the trend at the non-tropical sites was
289 for V_{cmax} at 25°C to be faster in winter than in summer, which is opposite to the pattern for
290 V_{cmax} at the prevailing growth temperature. Clear seasonal patterns in leaf metabolic traits were
291 not detectable at the tropical sites.

292 **Seasonal plasticity**

293 The degree of seasonal plasticity observed across the sites was highly trait-specific. Seasonal
294 plasticity in M_a and total N_{area} were broadly similar across all sites, but P contents per unit leaf
295 area (P_{area}) were much more plastic for the tropical compared with the arid sites (Fig. 3). For the
296 leaf metabolic traits, normalised to 25°C, plasticity scores for individual species varied widely at
297 many sites and this was especially true at Warra. Our plasticity index, by grouping species, is
298 essentially a site measure that combines inter- and intraspecific variability. For a given trait, we
299 can attempt to unravel this by partitioning variation among successive hierarchical terms (see
300 below).

301 **Seasonal and spatial flexibility in trait-trait relationships**

302 We found extensive flexibility in the scale and direction of seasonal mean $V_{\text{cmax},25}$ and $R_{\text{dark},25}$
303 relationships with total N_{area} (Fig. 4). Whilst overall positive trends were observed for the
304 tropical and temperate sites, there was wide variation among the species. Few consistent trends
305 emerged: faster metabolic rates in a given season were not associated with concurrent changes in
306 levels of N_{area} . Importantly, the patterns here did not support either of the hypothesised models
307 of thermal acclimation (Fig. S1); i.e. separate relationships for the two seasons, or a shift along a
308 perennial relationship driven by seasonal changes in N_{area} .

309 Our dataset included four species measured at two different sites (Fig. 5). These data were used
310 to explore whether leaf trait-trait relationships altered under different environments. Here again

311 the anticipated $V_{\text{cmax},25} - N_{\text{area}}$ relationships proved weak, even when combining both sites and
312 seasons for a given species (e.g. *Cryptocarya mackinnoniana*: $r = 0.44$, $p = 0.030$). Only in the case
313 of *Cardwellia sublimis* did SMA analysis confirm different $V_{\text{cmax},25} - N_{\text{area}}$ slopes at the two sites:
314 steeper at Robson Creek (Likelihood ratio = 7.826, $p = 0.005$); however, the small sample sizes
315 meant that the regression line fits for the two sites lacked predictive power (e.g. $r^2 = 0.27$, $n = 12$,
316 $p = 0.083$ at Daintree). For *Acacia aneura*, faster levels of V_{cmax} for a given N_{area} at GWW
317 compared to Alice Mulga suggest differences between the two sites in patterns of either leaf N
318 allocation or the Rubisco activation state.

319 Given such wide variability in $V_{\text{cmax},25} - N_{\text{area}}$ relationships for individual sites and species, we
320 next attempted a multiple regression type model designed to predict values of $V_{\text{cmax},25}$ at the site
321 cluster level. Model performance was improved by inclusion of P_{area} as well as N_{area} , but not in
322 interaction with site clusters i.e. the common positive relationship between foliar P and $V_{\text{cmax},25}$
323 did not vary at these broad spatial scales (Table 2). There was limited support for retaining
324 different $V_{\text{cmax},25} - N_{\text{area}}$ slopes for the different clusters (steeper for the moist tropical forests),
325 but in selecting a parsimonious model knowledge of P_{area} was a more valuable predictive term
326 (compare models 6 and 7, Table 2). At this clustered site scale, differentiating between
327 favourable and unfavourable seasons did not improve model performance.

328 Partitioning trait variation

329 Under our schema (Fig. 6), ITV is composed of variation among branches, conspecific trees and
330 between seasons (Branches + Trees + Seasons). After controlling for site and taxonomic effects,
331 seasonal variation accounted for less than 14% of total variation for traits related to leaf structure
332 and chemistry, but 31% of variation in R_{dark} normalised to the prevailing air temperature. For
333 M_a and N_{area} , a greater proportion of total variation was explained by inter-specific variation than
334 ITV. For the metabolic traits and P_{area} the reverse was true and ITV was highest for $R_{\text{dark},25}$
335 accounting for 69% of total variation. Variation partitioning for the metabolic traits depended,
336 to some extent, on the temperature normalisation adopted: variation between seasons was much
337 less pronounced when rates were normalised to a common reference of 25°C. Combined ITV,
338 however, was broadly similar under the two approaches: e.g. 51% of total for $V_{\text{cmax, Prevailing}}$ versus
339 46% for $V_{\text{cmax},25}$.

340 Discussion

341 *The relative importance of intraspecific trait variation*

342 The timing of our repeat field visits was designed to provide pronounced seasonal contrasts in
343 growing conditions. Our leaf trait seasonal plasticity (PI) scores appeared low, however, against
344 a comparable index reported for seedlings of 16 congeneric shrubs native to the Panamanian
345 rainforest: mean PI scores of 0.41 for M_a , 0.59 for photosynthetic capacity and 0.61 for R_{dark}
346 (Valladares *et al.* 2000). Our combined PI (mean of the five individual leaf traits, Fig. 3a) ran
347 from a low (0.09-0.10) at the arid sites of GWW and Calperum to a high (0.23) at Warra; a
348 ranking that broadly coincides with gradients of increasing LAI and canopy stature, suggesting
349 that leaf seasonal plasticity for these communities may be driven by radiation differences
350 mediated through LAI and the more diverse light environments created by taller stands
351 (Rozendaal, Hurtado & Poorter 2006). For a given trait, our site PI scores reflected underlying
352 diversity (the number of species included ranged from 8 at GWW to 16 at Daintree) and
353 emphasised community composition rather than the characteristics of dominant species. We
354 next consider ITV whilst controlling for species biodiversity.

355 The design of our variance partitioning model (although unable to account fully for
356 variation between populations) allowed us to attribute trait variation to discrete ITV components
357 whilst controlling for spatial and taxonomic terms. Consistent with earlier studies (e.g. Albert *et al.*
358 *et al.* 2010), we found that the pattern of variance partitioning changed from trait to trait. To our
359 surprise, seasonal differences (greatest for $R_{\text{dark Prevailing}}$ at 31%) had little explanatory power for
360 three of the five traits considered here: M_a , N_{area} and P_{area} . In a recent study of leaf trait variation
361 in 12 woody species in Chile, Fajardo & Siefert (2016) also found that the partitioning of
362 variation was highly trait specific and that interspecific variation was much higher than temporal
363 ITV for M_a . For species with long-lived leaves that must still contend with seasonal changes in
364 the growing environment, relatively fixed traits of morphology and chemistry may necessitate
365 greater variability within the metabolic traits like photosynthesis and respiration.

366 Across our sites, inter-specific variation was highest for N_{area} (43% of total) and this may
367 reflect the range of N fixation capabilities exhibited by Australian flora (Sprent, Ardley & James
368 2017) – a trait influenced in turn by soil P conditions (Houlton *et al.* 2008). The dominance of
369 phylogenetic variation (32.8%) over site (26.4%) for P_{area} is surprising given the closed nature of
370 the P-cycle, and converse findings in the Amazon (Fyllas *et al.* 2009). It may be that plant-
371 available soil P is so low across much of Australia (Kooyman, Laffan & Westoby 2017) that

372 genetic adaptations have been necessary for the viability of a great many species (e.g. Sulpice *et al.*
373 2014).

374 In a meta-analysis of variation in plant communities, Siefert *et al.* (2015) reported typical
375 ITV proportions at 25% of total: with higher ITV for chemical than morphological traits, but
376 with ITV less than interspecific variation in all cases. Whilst there are important differences
377 between our analysis and the Siefert *et al.* study (which did not include leaf metabolic traits,
378 seasonal contrasts, or attempt to distinguish the sources of ITV), our results provide partial
379 confirmation with ITV greater for N_{area} (18%) than for M_a (12%) and ITV less than interspecific
380 variation for both those traits. Refuting our hypothesis, we found that ITV outweighed inter-
381 specific variation for R_{dark} , V_{cmax} and P_{area} ; this was true whether metabolic rates were normalised
382 to the prevailing temperature or to a common reference temperature. For the metabolic traits,
383 levels of variation within individual plants (18% for $V_{\text{cmax Prevailing}}$ and 20% for $R_{\text{dark Prevailing}}$) were
384 lower than reported in a recent study in coffee crops which found that variation among leaves
385 within an individual branch accounted for approximately 25% of total variation in light saturated
386 photosynthesis (Martin *et al.* 2016). Overall, our results indicate that ITV can be an important,
387 even dominant, component of leaf trait variation, especially for metabolic processes. Our field
388 study of mature plants provided an opportunity to assess how far leaves that experience both
389 favourable and unfavourable growing conditions acclimate their physiology to seasonal changes
390 in the environment.

391 ***Thermal acclimation of leaf metabolism***

392 Compelling evidence for seasonal acclimation in leaf metabolic traits will require modification to
393 the standard temperature responses implemented in DGVMs (Smith & Dukes 2013;
394 Huntingford *et al.* 2017). In a study of juvenile trees of ten species native to temperate and
395 boreal forests in North America, Reich *et al.* (2016) found that for both forest types seasonal
396 acclimation (late spring vs. summer vs. early autumn) offset anticipated increases in leaf R_{dark} of
397 non-acclimated plants by as much as 80%. Plant or leaf developmental stage might be an
398 important consideration here since previous studies have demonstrated that thermal acclimation
399 is more fully achieved by newly developed (e.g. ‘cold grown’) rather than pre-existing plant tissue
400 (e.g. Hurry *et al.* 1995). It has been shown, however, that fully-expanded, long-lived leaves can
401 show a high degree of thermal acclimation over timescales of weeks to months (Campbell *et al.*
402 2007). Indeed, the ability to acclimate might be particularly important at sites dominated by
403 species with long-lived leaves where there may be little benefit in producing leaves tailored to
404 transient seasonal climatic conditions (Kitajima, Mulkey & Wright 1997).

405 At sites other than the tropical forests, we found that metabolic rates normalised to the
406 prevailing growing temperature were not homeostatic and, where seasonal differences emerged
407 (the majority of species), rates were always faster in summer than in winter. A different picture
408 emerged when those same rates were normalised to a common reference temperature of 25°C; in
409 this case many fewer species exhibited seasonal differences (only 13 of the 41 species), but for
410 those that did (with a single exception) rates of $V_{\text{cmax},25}$ were faster in winter than summer. Our
411 hypothesis of faster 25°C-standardised rates in the cooler season was not universally supported,
412 however; for species at Warra, for example, normalised rates of R_{dark} were either unchanged or
413 faster in summer than in winter, suggesting that seasonality in metabolic rates can respond to
414 signals other than temperature.

415 In a recent study that explored leaf trait coordination and optimisation, Dong *et al.* (2017)
416 tested the predictability of N_{area} across sites and species using explanatory factors that included
417 M_a , the ratio of intercellular to ambient CO₂ and environmental variables of irradiance and
418 MAT. The authors argue that whereas both $V_{\text{cmax},25}$ and N_{area} allocated to photosynthetic
419 machinery should decrease with increasing growth temperature, V_{cmax} assessed at growth
420 temperature should increase with increasing growth temperature (albeit less steeply than would
421 be predicted simply from enzyme kinetics). The study of Dong *et al.* also found that major
422 patterns of variation observed in N_{area} could be accounted for if both an optimised metabolic
423 component and a structural component proportional to M_a were combined. Our results extend
424 the analysis of Dong *et al.* to seasonal variations and support the general idea, embodied in the
425 widely used Lund-Potsdam-Jena (LPJ) DGVM (e.g. Sitch *et al.* 2003), that variation in V_{cmax}
426 across species and sites may be adaptive.

427 ***Seasonal effects on trait-trait relationships***

428 Our results showed that the seasonal effect on individual leaf traits was species-dependent, with
429 site (or community) based trait-trait relationships showing no consistent seasonal patterns.
430 Anticipated relationships between key metabolic traits and total N_{area} were unconvincing (Fig. 4)
431 while the lack of a uniform seasonal signal appears consistent with earlier studies that have
432 reported a relatively minor role for climate in influencing leaf trait relationships. Wright *et al.*
433 (2004) and Reich *et al.* (2007), for example, found that including climate variables within multiple
434 regression models improved trait prediction by less than 15%.

435 Why were the leaf trait-trait relationships observed in our study so weak? One possibility
436 is that our site-based analyses (e.g. Fig. 4) are at too fine a spatial scale to conform to a
437 worldwide leaf economic spectrum (Messier *et al.* 2017). Yet the site with the broadest range in

438 species-averaged values of N_{area} in our dataset (Alice Mulga: 2.7 to 7.2 g m⁻²) provided
439 approximately half the coverage reported in Glopnet (0.3 to 9.1 g m⁻²) (Wright *et al.* 2004); and
440 extending our analysis to include all seven sites did not reveal an underlying relationship for
441 species-averaged values of $V_{\text{cmax},25} - N_{\text{area}}$ ($r = 0.20, p=0.07$; data not shown). Broad variability in
442 $A-N$ relationships has been widely reported (e.g. Evans 1989) and interspecific variability in
443 photosynthetic N use efficiency will be influenced by environmental conditions such as light and
444 nutrient availability manifested through physiological mechanisms including CO₂ diffusion, light
445 interception and enzyme kinetics (Hikosaka 2004). Allocation of N within the leaf is likely to
446 play a crucial role and whilst available data lend support to an hypothesised trade-off between
447 photosynthetic capacity and longevity, up to half of leaf N may be invested in pools other than
448 the proteins associated with photosynthesis and cell walls (Onoda *et al.* 2017). We infer that the
449 premise of a universal relationship linking leaf N content to photosynthetic capacity is
450 questionable. For tropical systems in particular, P has been invoked as an important co-limiting
451 factor for rates of photosynthesis (Domingues *et al.* 2010) and the same constraint is likely to
452 apply over much of Australia. But despite uniformly high foliar N:P ratios across our study sites,
453 we did not find support for the notion that foliar P content influences the slope of the $A-N$
454 relationship (cf Kattge *et al.* 2009; Reich, Oleksyn & Wright 2009). The N:P ratio is itself a
455 plastic trait and provides only an approximate measure of relative ‘limitation’ – due, for example,
456 to storage of P in the vacuole. Irrespective of stoichiometry, leaf metabolism is heavily
457 dependent on N-rich proteins, but equations that ignore environmentally or phylogenetically
458 mediated variations in trait-trait relationships may have limited predictive power (Adams *et al.*
459 2016).

460 A more fruitful approach for DGVMs would be to avoid prescribing the values of
461 photosynthetic traits for a given PFT or biome and instead allow these traits to vary adaptively in
462 time and space. This alternative approach has a long history since Haxeltine & Prentice (1996)
463 and Dewar (1996) first noted the existence of an optimal photosynthetic capacity for any given
464 growth temperature and light environment (see also Hirose & Werger 1987). This optimality
465 assumption underlies the LPJ model and its successors. Further empirical research is required to
466 quantitatively evaluate such optimality hypotheses across different biomes and climates.

467 Conclusion

468 The aim of our study was to explore seasonal and spatial drivers of intraspecific leaf trait
469 variation and how seasonal changes across environmental gradients might affect trait-trait
470 relationships that are incorporated in many current DGVMs. Most species at the non-tropical

471 sites showed faster metabolic rates in summer than in winter, but variable responses amongst co-
472 occurring species and differing degrees of plasticity for selected traits frustrated attempts to find
473 clear seasonal differentiation in leaf trait-trait relationships. These results question the common
474 practice in DGVMs of assigning constant trait values to PFTs or assuming fixed trait-trait
475 relationships (e.g. between V_{cmax} and N_{area}). Instead, our results support the idea that metabolic
476 traits vary within species through acclimation in both space and time. This is a key point for the
477 design of trait-based DGVMs, with important implications for modelling species' responses to a
478 changing environment.

479

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491 **Authors' contributions**

492 ICP, OKA and KJB conceived the ideas and designed methodology; KJB, JJGE, LSH, HFT and
493 LZ collected the data; KJB analysed the data and led the writing of the manuscript. All authors
494 contributed critically to the drafts and gave final approval for publication.

495 **Data accessibility**

496 The combined dataset can be found here:

497 <http://supersites.tern.org.au/knb/metacat/supersite.949/html>

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742 **Supporting information**

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

744 Fig. S1. Conceptual diagram for a leaf trait-trait relationship of two possible thermal acclimation
745 responses to seasonal variation

746 Fig. S2. Australian map with location and key climate indicators for each of the study sites

747 Fig. S3. Unrooted (phylogenetic-type) tree of the study sites based on spatial analysis using
748 bioclimatic indices

749 Fig. S4. Three-way scatterplot showing the associations between total leaf phosphorus
750 concentration, leaf mass per unit area and total leaf nitrogen concentration.

751 Fig. S5 Two-way interaction plots (Season : Species) contrasting dark respiration per unit leaf
752 area normalised to the prevailing air temperature and to a common reference temperature of
753 25°C.

754

755 Table S1. Climate conditions for each fieldwork campaign

756 Table S2. Leaf traits by site, averaged across both seasons and all species

757 Table S3. Photosynthetic parameters by site, averaged across both seasons and all species

758 Table S4. Leaf traits by site and season, averaged across all species

759 Table S5. Photosynthetic parameters by site and season, averaged across all species

760 Table S6. Leaf traits by site and season and species

761 Table S7. Photosynthetic parameters by site and season and species

762 Figure captions

763 Fig. 1 Box plots by site cluster for leaf mass per unit area (M_a , plot a) and the ratio of total leaf nitrogen to phosphorus (plot b).
764 Boxes indicate the interquartile range and median values. Whiskers extend to the largest or smallest observations that fall within
765 1.5 times the box size; any observations outside these values are shown as individual points. Boxes which share the same letter
766 correspond to cluster mean trait values that were not significantly different (Tukey's HSD). The dashed horizontal line in Fig. 1b
767 shows the N:P ratio of 20 and indicates a threshold above which conditions may be considered phosphorus limited (Güsewell
768 2004).

769 Fig. 2 Two-way interaction plots (Season : Species) for species' mean estimates of maximal carboxylation capacity per unit leaf
770 area normalised to: the prevailing air temperature at the time of the measurement campaign (Table S1) ($V_{\text{cmax, Prevailing}}$, top panel)
771 and to a common reference temperature of 25°C ($V_{\text{cmax, 25}}$, bottom panel). Paired plots are shown for each of the sites (Alice
772 Mulga is not included because only a single visit was made to that site). Individual species for a given site, ranked in alphabetical
773 order, are labelled numerically (0 through 9) and then with letters (a, b, c etc.). Trace lines connecting the two seasonal means for
774 a given species are only shown where the seasonal means were significantly different. Site_Season_Species mean trait values are
775 shown in Table S7. Notice that axes ranges vary to accommodate seasonal temperature variability from site to site.

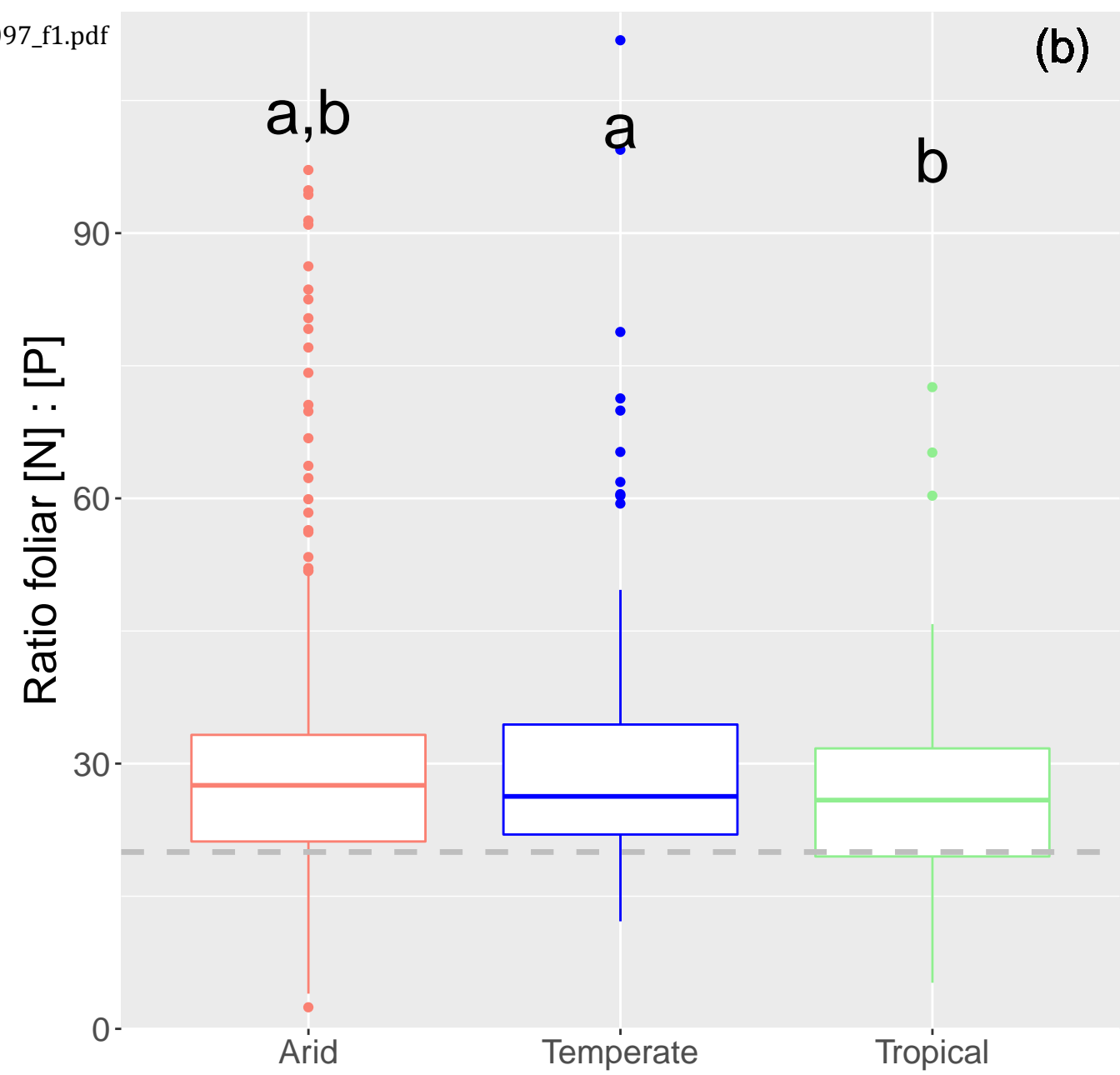
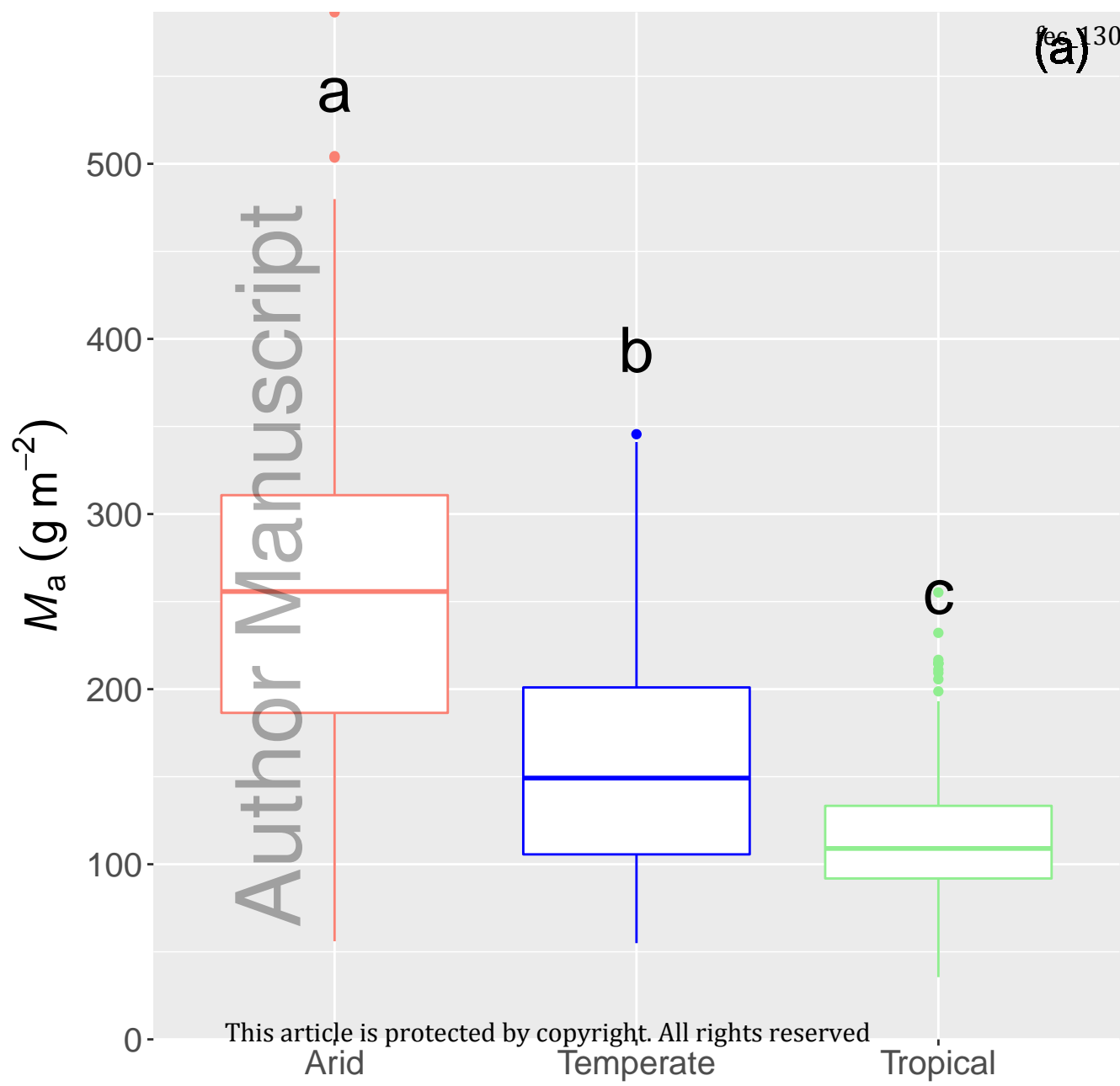
776 Fig. 3 Community-level leaf trait plasticity by site (Calperum CAL; Great Western Woodlands GWW; Cumberland Plain CBLP;
777 Warra WAR; Robson Creek RCR; Daintree DRO; Alice Mulga is not included because only a single visit was made to that site).
778 For a given trait, the Site_Species Plasticity Index (PI) is calculated as the absolute index of the difference between favourable
779 and unfavourable seasonal means divided by the maximum trait value observed. The box and whiskers plots show the range of
780 individual species' PIs at a given site; the number of species included for each site is indicated above the boxes in panel (a).
781 Separate panels are shown for (b) leaf mass per unit area (M_a); (c) total nitrogen content per unit leaf area (N_{area}); (d) total
782 phosphorus content per unit leaf area (P_{area}); (e) maximal rate of carboxylation per unit leaf area and normalised to a common
783 reference temperature of 25°C ($V_{\text{cmax, 25}}$); (f) leaf dark respiration per unit leaf area and normalised to 25°C ($R_{\text{dark, 25}}$) and (a) a
784 combined index for each Site_Species taken as the mean of the five trait PIs. Boxplot construction is explained in Fig. 1. Boxes
785 which share the same letter correspond to site mean indices that were not significantly different (Tukey's HSD); the six sites
786 showed comparable levels of plasticity for M_a and N_{area} .

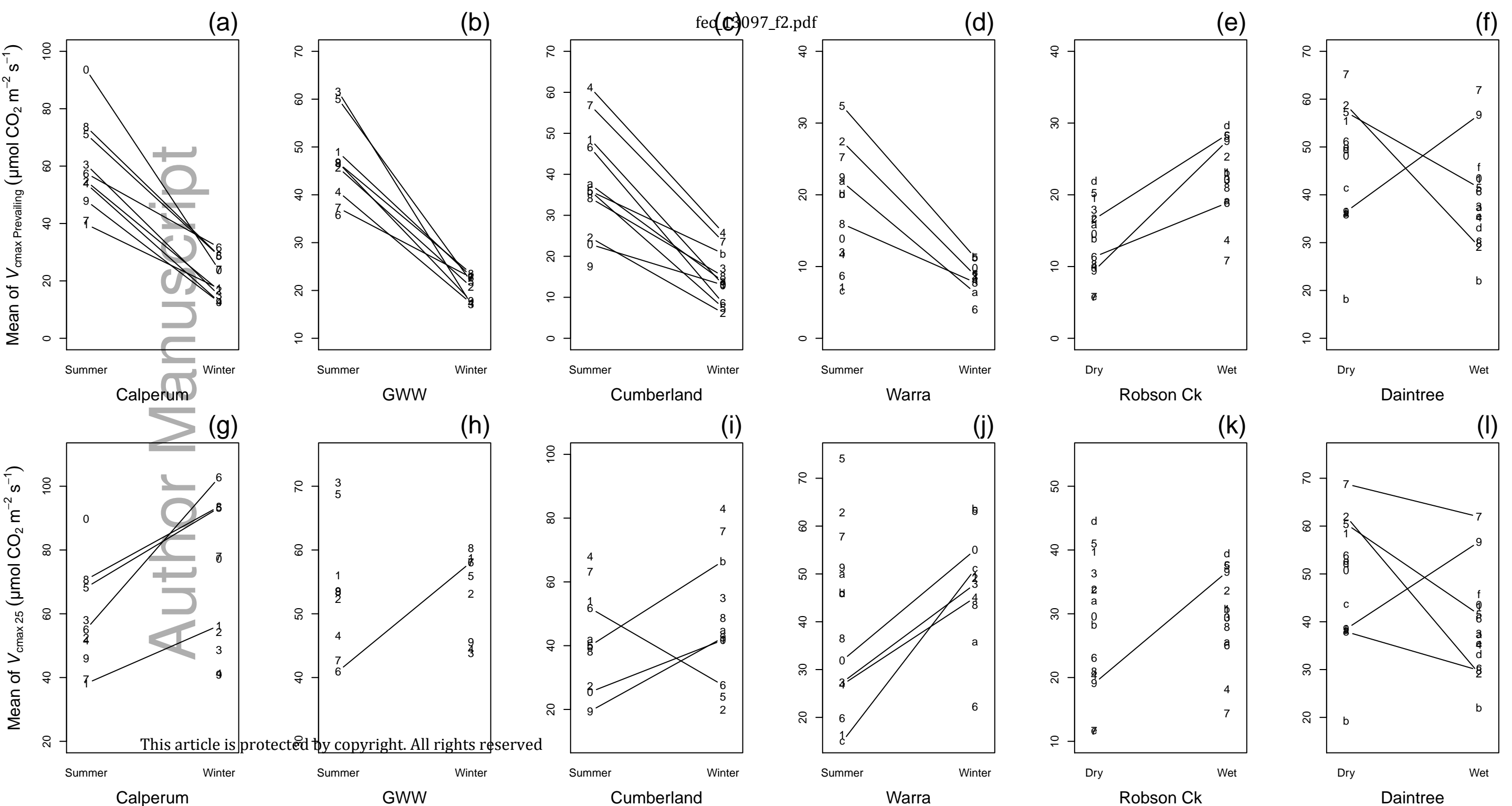
787 Fig. 4 Flexibility in leaf trait-trait relationships: scatterplots of maximal carboxylation capacity ($V_{\text{cmax, 25}}$, left hand panels) and
788 dark respiration ($R_{\text{cmax, 25}}$, right hand panels), both rates expressed per unit leaf area and normalised to a common reference
789 temperature of 25°C and each plotted against leaf Nitrogen per unit area. Paired plots are shown for one site from each of the
790 three spatial clusters: Daintree (Tropical, plots a and b); Great Western Woodlands (Arid, plots c and d) and Warra (Temperate,
791 plots e and f). Each point represents a species average ($n \sim 5$) for distinct seasons: red for favourable and blue for unfavourable.
792 The blue trace lines connect the two seasonal means for a given species; the dashed black line shows the overall trend combining
793 all species and both seasons. Site_Season_Species mean trait values are shown in Table S7.

794 Fig. 5 Spatial variation in leaf trait-trait relationships. Scatterplot of the maximal rate of carboxylation per unit leaf area
795 normalised to a common reference temperature of 25°C in relation to total leaf nitrogen per unit leaf area ([N]_a). Separate panels
796 are shown for each of four species measured at more than one site. Each point represents a single leaf; sites are differentiated by
797 colour and seasons by shape. Points for Alice Mulga relate to the single winter visit to that site. Pearson correlation coefficients
798 are shown where the association was significant ($\alpha = 0.05$).

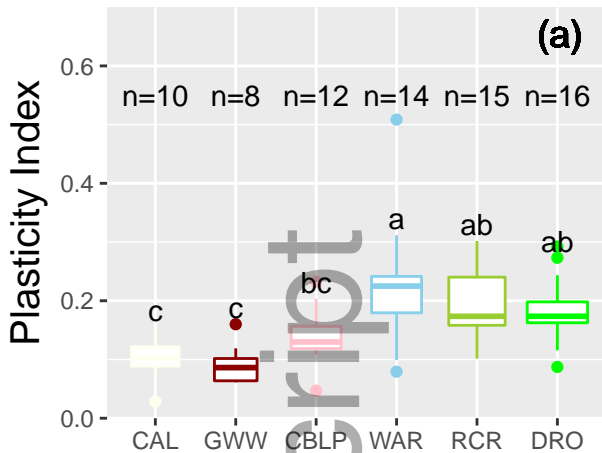
799 Fig. 6 Bar chart showing partitioning of leaf trait variation around the overall mean as derived from a multilevel model with
800 separate random effects for the following factors: Season; Site; Site_ *within* Season; Species_ *within* Site_ *within* Season and
801 Tree.number_ *within* Species_ *within* Site_ *within* Season. Any residual variation in the model was assigned to individual branches
802 *within* a given plant. The leaf traits considered are leaf dry mass per unit area (M_a , g m⁻²); total leaf nitrogen (gN m⁻²); total leaf
803 phosphorus (mgP m⁻²); maximum rate of carboxylation of Rubisco per unit leaf area, normalised to the prevailing air temperature
804 at the time of the campaign **and** to a common reference temperature of 25°C ($V_{\text{cmax Prevailing}}$ **and** V_{cmax25} both $\mu\text{mol m}^{-2} \text{s}^{-1}$);
805 dark respiration per unit leaf area ($R_{\text{dark Prevailing}}$ **and** $R_{\text{dark 25}}$ both $\mu\text{mol m}^{-2} \text{s}^{-1}$).

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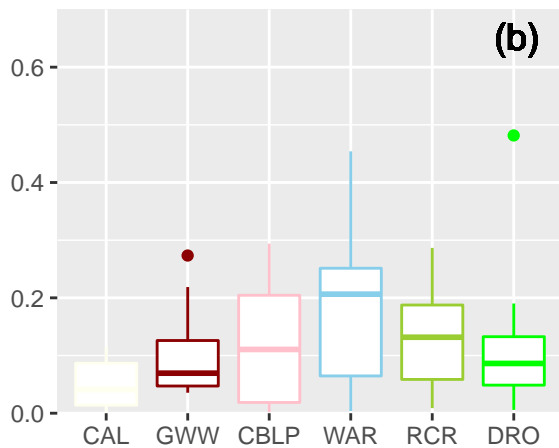




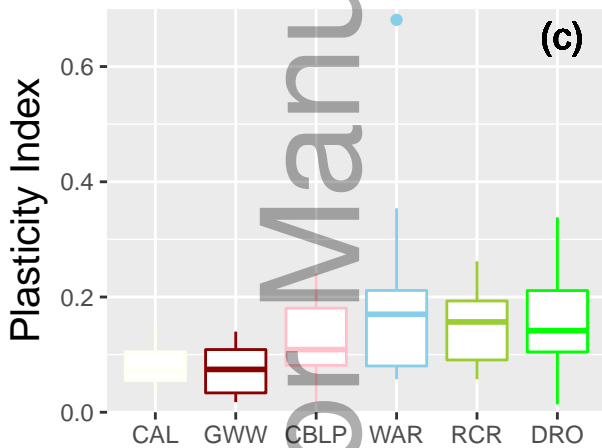
Multiple traits combined



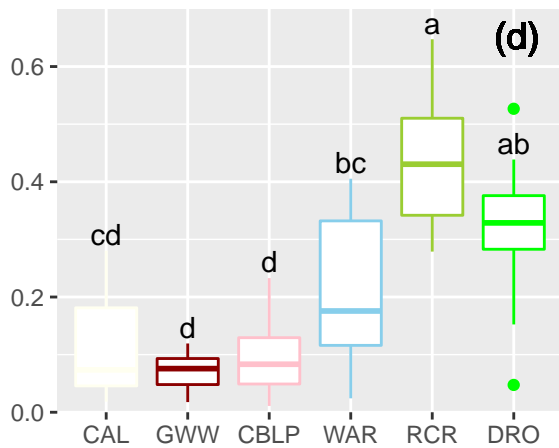
M_a



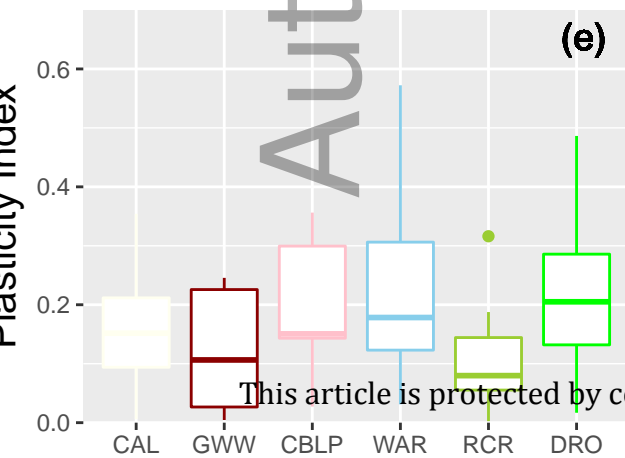
Leaf N_{area}



Leaf P_{area}



$V_{cmax 25}$



$R_{dark 25}$

