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1Leaf carbon and water status control stomatal and non-stomatal 2limitations of photosynthesis in trees.

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7

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23Summary

- 24 • Photosynthetic rate is concurrently limited by stomatal and non-stomatal limitations.
25 However, the controls on non-stomatal limitations to photosynthesis (NSL) and their
26 coordination with stomatal control on different timescales remain poorly understood.
27 According to a recent optimization hypothesis, NSL depends on leaf osmotic or water status,
28 and is coordinated with stomatal control so as to maximize leaf photosynthesis.
- 29 • Drought and notching experiments were conducted on *Pinus sylvestris*, *Picea abies*, *Betula*
30 *Pendula*, and *Populus tremula* seedlings in greenhouse conditions to study the dependence
31 of NSL on leaf osmotic and water status, and its coordination with stomatal control, on
32 timescales of minutes and weeks, to test the assumptions and predictions of the optimization
33 hypothesis.
- 34 • Both NSL and stomatal conductance followed power-law functions of leaf osmotic
35 concentration and leaf water potential. Moreover, stomatal conductance was proportional to
36 the square root of soil-to-leaf hydraulic conductance, as predicted by the optimization
37 hypothesis.
- 38 • While the detailed mechanisms underlying the dependence of NSL on leaf osmotic or water
39 status lie outside the scope of this study, our results support the hypothesis that NSL and
40 stomatal control are coordinated to maximize leaf photosynthesis, and allow the effect of
41 NSL to be included in models of tree gas-exchange.

42**Key words:** Drought, leaf osmotic concentration, notching, stomatal conductance, xylem transport,
43leaf water potential, non-stomatal limitation.

44

45Introduction

46

47Leaf photosynthesis is subject to both stomatal and non-stomatal limitations (hereafter NSL)
48(Buckley & Diaz-Espejo, 2015). Stomatal limitations refer to restrictions in the supply of CO₂ by
49diffusion through the stomata to the intercellular spaces within leaves. Non-stomatal limitations
50refer to all other processes limiting the rate of leaf photosynthesis; these include the diffusion of
51CO₂ from the leaf-intercellular spaces to the sites of the dark reactions of photosynthesis in
52chloroplasts (i.e. a finite mesophyll conductance), and biochemical limitations to photosynthetic
53efficiency (Grassi & Magnani, 2005).

54

55 Generally, stomatal and non-stomatal limitations to photosynthesis operate concurrently and are
56 coordinated with each other (Flexas *et al.*, 2008; Gago *et al.*, 2016). For example, both limitations
57 occur during drought (Drake *et al.*, 2017) when soil water availability decreases and/or vapour
58 pressure deficit (VPD) increases. While it has long been recognized that stomata react rapidly to
59 environmental changes (Jarvis *et al.*, 1976) and internal leaf water status (Sperry *et al.*, 1993), more
60 recently it has become evident that NSL also operate on the same time scale as stomatal limitations
61 (Flexas *et al.*, 2008; Flexas *et al.*, 2012; Kaiser *et al.*, 2015). However, what is lacking is a
62 quantitative understanding of the controls on NSL and their co-ordination with stomatal limitation.
63 Such an understanding is central to our ability to predict adaptive responses of plants to climate
64 change, such as increased drought (McDowell *et al.*, 2011; Choat *et al.*, 2012; Klein & Hartmann,
65 2018).

66

67 Recently it has been conjectured that co-ordination of stomatal and non-stomatal limitations to
68 photosynthesis might be understood from the viewpoint of maximizing net leaf photosynthetic rate
69 (A) within a whole-tree level framework (Hölttä *et al.*, 2017) that also involves co-ordination
70 between sugar source, transport and sink processes. Because sugar accumulation in mesophyll cells
71 is tightly coupled to sugar export by the phloem (Nikinmaa *et al.*, 2013), especially in passive
72 loading species (Turgeon, 2010), this conjecture is also closely related to the one of maximizing
73 phloem transport (Nikinmaa *et al.*, 2013; Huang *et al.*, 2018). According to this hypothesis, as
74 stomatal conductance (g_s) increases (under constant environmental conditions) there is a tradeoff
75 between increases in A through increased leaf-intercellular CO_2 concentration (c_i), and decreases in
76 A through NSL associated with decreased leaf water potential (Dewar *et al.*, 2018) and/or an
77 associated increase in leaf sugar concentration (a potential mechanism in presented in box 1) as
78 observed in numerous species (Jeannette *et al.*, 2000; e.g., Turnbull *et al.*, 2002; Franck *et al.*, 2006;
79 Hüve *et al.*, 2006; Quentin *et al.*, 2013; Cano *et al.*, 2014; Kelly *et al.*, 2014; Ramalho *et al.*, 2014;
80 Kitao *et al.*, 2015; Hölttä *et al.*, 2017). As a result, there is a maximum in the A - g_s curve at which
81 stomatal and non-stomatal limitations to photosynthesis are in balance. If the stomata would open
82 further from their optimal opening rate, internal CO_2 concentration would increase, thus increase the
83 supply of CO_2 , but at the same time, the increasing water loss rate by transpiration would decrease
84 leaf water potential and increase osmotic concentration so that in the increase in non-stomatal
85 limitations would lead to a decrease in gross assimilation rate (Fig. 1). Within this framework (Fig.
86 1), factors influencing stomatal limitation to photosynthesis include the maximum rates of
87 carboxylation (V_{cmax}) and electron transport (J_{max}), CO_2 concentration and photosynthetically active

88 radiation (PAR). Factors influencing NSL to photosynthesis include VPD, soil water potential (Ψ_s)
 89 and soil-to-leaf hydraulic conductance (K_{sl}), with K_{sl} itself dependent on Ψ_s and on xylem
 90 vulnerability characteristics (e.g. PLC_{50} , the xylem water potential for 50% loss of conductance). In
 91 addition, NSL also respond to sugar transport as shown by its response to decreasing sink strength
 92 (Azcón-Bieto, 1983; Goldschmidt & Huber, 1992; Nakano *et al.*, 2000; Hölttä *et al.*, 2017).

93 This theoretical conjecture has led to several unique experimentally testable predictions that
 94 distinguish it from other models of stomatal conductance. For example, in the analytically solvable
 95 model of Dewar *et al.* (2018), in which non-stomatal limitation to photosynthesis is assumed to be
 96 linearly related to leaf water potential, and xylem embolism is ignored, the optimal stomatal
 97 conductance and photosynthetic rate are related by

$$98 \quad g_s = \frac{A}{c_a - \Gamma^*} \left(1 + \frac{\xi}{\sqrt{D}} \right), \quad (1)$$

99 where c_a is the atmospheric CO_2 concentration, Γ^* is the photorespiratory CO_2 compensation point,
 100 D is VPD, and ξ is a simple combination of various physiological parameters of the model (Dewar
 101 *et al.*, 2018, their table 4):

$$102 \quad \xi = \sqrt{\frac{K_{sl} |\psi_c|}{1.6} \left(r_{x,0} + \frac{2\Gamma^*}{\alpha_0 Q} \right)}, \quad (2)$$

103 where ψ_c is the critical leaf water potential, $r_{x,0}$ is the carboxylation resistance in the absence of
 104 NSL, α_0 is the photosynthetic quantum yield in the absence of NSL and Q is the leaf photosynthetic
 105 photon flux density. Mathematically, eqn (1) is almost identical in form to the relationship derived
 106 by Medlyn *et al.* (2011) from the alternative optimization hypothesis (Cowan & Farquhar, 1977)
 107 that stomata maximize leaf photosynthesis for a given amount of leaf transpiration (rather than
 108 through a tradeoff between stomatal and non-stomatal limitations),

$$109 \quad g_s = g_0 + \frac{A}{c_a} \left(1 + \frac{g_1}{\sqrt{D}} \right), \quad (3)$$

110 in which g_0 and g_1 are empirical parameters (g_1 is related to the marginal water cost of plant carbon
 111 gain); eqns (1) and (3) are identical when $g_0 = 0$ and $g_1 = \xi$ in eqn (3) and $\Gamma^* = 0$ in eqn (1). Both of
 112 these alternative optimization hypotheses successfully predict the observed close correlation

between g_s and A (Wong *et al.*, 1979) that forms the basis of the empirical Ball-Woodrow-Berry stomatal model and its variants (Ball *et al.*, 1987; Leuning, 1995).

However, a key difference is that while g_1 in eqn (3) is a priori unknown and must be determined by fitting eqn (3) to data, ξ in eqn (1) is fully determined as a function of measurable physiological parameters; in particular, ξ is predicted to be proportional to $\sqrt{K_{sl}}$ (Dewar *et al.*, 2018, their Table 194). Thus, while eqn (3) successfully encapsulates the dependence of g_s on all other factors shown in Fig. 1, it does not explicitly describe a dependence on K_{sl} , whereas numerous experiments have shown that stomatal conductance is positively related to hydraulic conductance (Sperry *et al.*, 1993; Nardini & Salleo, 2000). In contrast, eqn (1) predicts a positive relationship between g_s and K_{sl} via the parameter $\xi \propto \sqrt{K_{sl}}$, reflecting the role K_{sl} plays in determining the extent of NSL through its influence on leaf water potential. In eqn (1) the term $\frac{\xi}{\sqrt{D}}$ tends to dominate the factor in brackets; with $\Gamma^* \approx 0$, eqn (1) then becomes

$$g_s \approx \frac{A}{c_a} \frac{\xi}{\sqrt{D}} \propto \frac{A}{c_a} \sqrt{\frac{K_{sl}}{D}}. \quad (4)$$

Thus, all else being equal, g_s is predicted to be proportional to $\sqrt{K_{sl}}$, a result also found numerically from the model of Hölttä *et al.* (2017), which included xylem embolism, and which assumed that NSL was linearly related to leaf sugar concentration rather than leaf water potential. Indeed, an analytically solvable version of this sugar-based model, in which xylem embolism is ignored (Dewar & Hölttä, unpublished), also leads to eqn (4), which therefore appears to be a robust prediction of the hypothesis that NSL and stomatal control are coordinated so as to maximize leaf photosynthesis

However, the dependence of g_s on K_{sl} predicted by eqn (4) is yet untested, as are the underlying assumptions that non-stomatal limitations to photosynthesis are related to either leaf water status or leaf sugar status, and act on the same time scale as stomatal limitation. The overall objective of this study was to provide experimental tests of the conjecture that stomatal and non-stomatal limitations to photosynthesis are co-ordinated so as to maximize leaf photosynthetic rate (Fig. 1). The specific hypotheses were:

- 1) NSL increase with decreasing leaf water status and/or increasing leaf sugar/osmotic status;
- 2) These correlations are similar across timescale and species;

142 3) NSL and stomatal limitation to photosynthesis are coordinated on similar time scales and for
143 different species;

144 4) The key model prediction of eqn (4), particularly with regard to its implication for the
145 relationship between g_s and K_{st} , is supported by experimental data

146 To achieve these aims, we used saplings from four common boreal species (*Pinus sylvestris*, *Picea*
147 *abies*, *Populus tremula* and *Betula pendula*) to conduct two experiments in which leaf water and
148 sugar status were manipulated on different time scales: (i) a drought treatment to induce changes in
149 photosynthetic rates over a time scale of weeks; and (ii) a notching experiment, where xylem
150 hydraulic conductance was artificially reduced to induce changes in photosynthetic rates over a time
151 scale of minutes/hours.

152

153 **Materials and Method**

154

155 All experiments were performed at the greenhouse and laboratory facilities at the Viikki campus of
156 the University of Helsinki (Helsinki, Finland).

157

158 *Drought experiment*

159 Potted saplings of *Pinus sylvestris* (L.), *Picea abies* (L. H. Karst.), *Populus tremula* (L.) and *Betula*
160 *pendula* (Roth.) were used for this experiment. Aspen and birch were acclimated for five weeks,
161 and pine and spruce were acclimated for four weeks to greenhouse conditions (PAR $\sim 800 \mu\text{mol m}^{-2}$
162 s^{-1} , temperature $\sim 20^\circ\text{C}$, relative humidity $\sim 55\%$, ambient $[\text{CO}_2] \sim 400 \text{ ppm}$) prior to the first
163 measurements. Initial heights and diameters of the trees are shown in Table 1.

164

165 During the experiment, all trees were kept in the greenhouse under stable environmental conditions.
166 Two treatments were applied to the seedlings: (1) control, in which seedlings were automatically
167 watered twice a day by flooding the table on which they were kept, and (2) drought, in which
168 seedlings received no irrigation after the beginning of the experiment. The drought treatment started
169 on the third week of April 2017.

170

171 The following measurements were performed on two control saplings and three droughted saplings
172 per week of sampling: leaf gas exchange under ambient conditions, $A-c_i$ and light curves, leaf water
173 potential, and leaf osmotic concentration (see below for details). The weeks (W) of sampling for
174 each species were as follows:

175

176• Pine: W0, W2, W3, W5, W6

177• Spruce: W0, W2, W3, W4, W5

178• Aspen: W0, W1, W2

179• Birch: W0, W1, W2, W3

180

181The duration and frequency of measurements were decided based on a priori estimation of species
182drought sensitivity. On week 0, 3 seedlings per species were sampled; these measurements
183correspond to background values.

184

185*Notching experiment*

186Potted saplings of four *Pinus sylvestris*, seven *Populus tremula* and six *Betula pendula* were used for
187this experiment. The seedlings were approximately 1 meter in height and 2 cm in diameter at the
188base. Measurements were made during three periods: spring 2015 for the pines, later summer and
189early autumn 2016 and late summer 2017 for the broadleaves. Measurements conducted for pine in
190the notching experiment have already been published (Hölttä *et al.*, 2017).

191

192Seedlings were brought inside the laboratory three days prior to the measurements and were well
193watered. The seedlings were kept in constant environmental conditions (PAR ~ 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$,
194temperature ~ 22 °C, VPD ~ 0.01 mol mol⁻¹, ambient [CO₂] ~ 450 ppm) for 3 to 8 hours before the
195experiment. During the measurements, the saplings were notched at approximately 20 cm below the
196point of leaf gas exchange measurement to decrease xylem hydraulic conductance and induce rapid
197changes in leaf water and osmotic potentials, and leaf gas exchange. Notching was done by
198gradually cutting the stem xylem with a scalpel so that the xylem hydraulic conductance would
199decrease gradually (Sperry *et al.*, 1993).

200

201*Leaf gas exchange measurements*

202Net CO₂ assimilation rate or net photosynthetic rate (*A*), stomatal conductance (*g_s*) and transpiration
203rate (*E*) were measured with a portable gas-exchange system (GFS-3000, Walz, Effeltrich,
204Germany). Leaf internal CO₂ concentration (*c_i*) was calculated by the portable gas-exchange
205system. For each seedling, after at least 15 min acclimation to darkness, dark respiration rate was
206measured for 5 minutes by measuring the CO₂ efflux rate in the dark. Gross photosynthetic rate (*P*)
207was then calculated as the sum of net CO₂ assimilation rate and the 5-min averaged dark respiration
208rate. Prior to measurements, a leaf from the broad-leaved species and eight needles from the pine

209were positioned inside a closed cuvette. Environmental conditions in the cuvette were set close to
210the ambient environment in the greenhouse (see above).

211

212For the drought experiment, measurements of leaf gas exchange were conducted under ambient
213conditions (see above), CO₂ response curves (hereafter referred to as $A-c_i$ curves) were made with
214the following sequence of CO₂ concentration (in ppm): 400, 200, 100, 50, 400, 600, 800, 1200. Four
215to five measurements were taken at each CO₂ level; $A-c_i$ curves were then fitted using the
216PlantEcophys package in R (Duursma, 2015). We used the default assumption of this package that
217mesophyll conductance (g_m) was infinite during the fitting of the $A-c_i$ curve, because setting a finite
218value of g_m would have interfered with our goal of estimating non-stomatal limitation from actual
219measurements. As a result, the apparent maximum rate of carboxylation V_{cmax} and the apparent
220electron transport rate J_{max} were extracted from the fitted $A-c_i$ curves. Therefore, changes in apparent
221 V_{cmax} and J_{max} reflect changes not only in biochemical limitation but also in g_m . For the notching
222experiment, leaf gas exchange was recorded every minute from prior to notching until the end of the
223experiment.

224

225A digital photograph of the leaves inside the cuvette was taken for estimating leaf area. The area
226covered by the leaf was calculated *a posteriori* with imageJ (Schneider *et al.*, 2012). Original leaf
227gas exchange (obtained from setting the GFS-3000 to a leaf area of 1cm²) data were then scaled
228using the measured leaf area using the GFS software (Walz). For pine and spruce, we assumed that
229the gas-exchange area was twice the projected area because both sides of the needles have stomata
230(As the needle surface are slightly curved rather than perfectly flat, the actual leaf area is likely to be
231slightly higher than twice the projected area. Thus, we are likely slightly overestimating the gas-
232exchange per area.).

233

234Because ambient CO₂ concentration and light level were kept constant in our experiments, the ratio
235of A to c_i was used to evaluate changes in NSL. This procedure assumes a quasi-linear $A-c_i$
236relationship, which is reasonable if changes in c_i are small. To confirm the validity of using A/c_i as a
237measure of NSL, we also evaluated NSL by comparing how much each value of A measured during
238the notching experiment deviated from the $A-c_i$ curve measured before the notching, whenever this
239was possible (see Supplementary Materials Notes S1 and Fig. S1).

240

241Osmotic concentration measurements

242 Leaf osmotic concentration (mol kg^{-1}) is the sum of the concentrations of all dissolved solutes in
243 leaf sap. Leaf osmotic concentration determines leaf osmotic potential, a component of leaf water
244 potential, and is strongly influenced by leaf sugar status.

245

246 In the drought experiment, leaves and needles were collected for leaf water potential and osmotic
247 concentration measurements directly after the leaf gas exchange measurement. In the notching
248 experiment, leaves and needles were collected several times during each experiment, the collection
249 time being recorded and later matched with the gas exchange measurements. The collected leaves
250 and needles were immediately frozen in liquid nitrogen in order to break cell membranes and
251 release symplastic contents to the apoplast (Kikuta & Richter, 1992; Callister *et al.*, 2006). Osmotic
252 concentration samples from the drought and notching experiment were either immediately
253 processed or stored at -80°C . Prior to measurements, samples were allowed to thaw in a sealed
254 plastic tube. Immediately after thawing (to avoid the degradation of di- and polysaccharides) the sap
255 of the leaves or needles was extracted by either pressurizing them in a syringe (drought experiment;
256 extraction was always performed by the same user applying maximum pressure to minimize
257 artifacts) or by centrifuging (notching experiment) them at 14000 g for 10 minutes (Heraeus Fresco
258 17 Centrifuge, Thermo Scientific, Massachusetts, USA). The collected liquid was measured with an
259 osmometer (Osmomat-030 Freezing point osmometer, Gonotec, Berlin, DE) immediately after
260 extraction to avoid water evaporation from the samples and microbial contamination.

261

262 *Leaf water potential and soil-to-leaf hydraulic conductance measurements*

263 Leaf water potential (Ψ_{leaf} , Pa) was measured with a pressure chamber (PMS-1000, PMS
264 instruments, Albany, OR, USA). In the drought experiment, leaf water potential was measured for
265 each seedling at pre-dawn (Ψ_{PD}) prior to any other destructive measurements, and immediately after
266 the leaf gas exchange measurements. Because late spring and early summer nights in Helsinki
267 (latitude 60.17°) are very short and saplings may have insufficient time to equilibrate hydraulically
268 with the soil, the saplings were covered with black plastic bags for the whole night and Ψ_{PD} was
269 measured in the morning just before removing the bags.

270

271 In the drought experiment, soil water potential (Ψ_{s}) was assumed equal to Ψ_{PD} . In the notching
272 experiment, sampling for measurements of leaf osmotic concentration and water potential were
273 done simultaneously, and Ψ_{s} was assumed equal to zero as the soil was well-watered. Soil-to-leaf
274 hydraulic conductance was then estimated as

$$K_{sl} = \frac{E}{\Delta\Psi}, \quad (5)$$

where $\Delta\Psi$ (Pa) is the soil-to-leaf water potential difference ($\Psi_{PD} - \Psi_{leaf}$ for the drought experiment, Ψ_{leaf} for the notching experiment).

Testing the predicted relationship between g_s and K_{sl}
 We used the experimental results to test eqn (4), the theoretical prediction of the optimization hypothesis, as follows. Because ambient CO_2 concentration (c_a) was kept constant, substitution of eqn (5) into eqn (4) gives

$$g_s \propto \frac{A}{\sqrt{D}} \sqrt{\frac{E}{\Delta\Psi}}. \quad (6)$$

However, measurements of the two sides of eqn (6) are not independent, because g_s on the left-hand side was calculated from measurements of E , which also appears on the right-hand side. However, by substituting $g_s = E/1.6D$ and re-arranging the resulting equation so that E appears only on the left-hand side, eqn (6) may be re-expressed in the equivalent form (See note S3 for the details):

$$E \propto A^2 \frac{D}{\Delta\Psi} \equiv r, \quad (7)$$

in which the left- and right-hand sides can be measured independently. We therefore tested the prediction of the stomatal optimization model in the form of eqn (7); because this is a proportionality rather than an equality, for convenience, we compared the normalised value r/r_{max} to the measured value of E , where r is the right-hand side of eqn (7) and r_{max} is the maximum value of r .

Statistical analysis

All statistical analyses were performed in R version 3.3.3 (R Core Team, 2017). Due to the different structure of the drought and notching experiments, they were analyzed separately using different methods.

For the drought experiment, the dependences of P , g_s , P/c_i , V_{cmax} and J_{max} on leaf osmotic concentration and water potential were fitted to a power-law, using a linear model after logarithmic transformation of the data (see Table 2). The influence of VPD was tested as a covariate, and the fixed-effect variables of species and treatment (control and stressed) as well as their interaction were also added to the model. Similar methods were used to test the relations between: 1) P/c_i and

306 g_s : the variables were log-transformed, leaf temperature was included as a covariate and species was
307 included as a fixed effect in the model; 2) $r/r_{\max}$ and E : no transformation was needed and species as
308 a fixed effect.

309
310 For the notching experiment, since several measurements were performed on each sapling
311 following the treatment, a unique identifier for each sapling was assigned and used as a random
312 term. Then, the effect of osmotic concentration and water potential on P , g_s and P/c_i were tested
313 with a mixed-effect linear model (package nlme, Pinheiro *et al.*, 2017), with logarithmic
314 transformation of the data as required (see Table 3). The influence of leaf temperature on P/c_i and
315 of VPD on P and g_s , were tested as covariates. Initially, the full model was tested with the effect of
316 osmotic concentration or water potential, species, treatment and their interactions, and then the
317 model was simplified when possible based on the AIC (Akaike information criterion). Model
318 selection and testing were performed according to Zuur *et al.* (2009). The model with the lowest
319 AIC that was not significantly different from the full model was then selected. Similar methods
320 were used to test the relations between: 1) P/c_i and g_s : the variables were log-transformed, leaf
321 temperature was included as a covariate and species was included as a fixed effect in the model; 2)
322 $r/r_{\max}$ and E : no transformation was needed and species as a fixed effect.

323

324 Results

325

326 Leaf gas-exchange

327

328 The drought treatment successfully created a wide range of values of leaf water potential and leaf
329 osmotic concentration for the four species used in the experiment (Fig. 2). P and g_s decreased with
330 increasing leaf osmotic concentration (Fig 2A) and decreasing leaf water potential (Fig. 2B). The
331 model with leaf osmotic concentration explained more of the variance than the model with leaf
332 water potential (see model R^2 values in Table 2. Note that the provided R^2 is for the whole model
333 and thus accounts for the influence of all explanatory variables such as species and treatment kept in
334 the final model as described in Table 2). All the observed relations showed a significant effect of
335 treatment (control versus stressed) and species, either independently or interactively (Tables 2 and
336 S1, Figure 2). Power-law fits with species-average exponents of -3.43 and -3.47 were obtained for
337 P and g_s versus osmotic concentration, respectively, and -1.27 and -1.54 for P and g_s versus leaf
338 water potential respectively (see Table S1 for species specific values).

339

340Notching resulted in a decrease in leaf water potential, P and g_s , and an increase in leaf osmotic
341concentration in all species. P and g_s decreased significantly (Table 3) with increasing leaf osmotic
342concentration (Fig. 3A and C) and decreasing leaf water potential (Fig. 3B and D). These relations
343followed a power-law fit with a species-average exponent of -2.16 for P versus leaf osmotic
344concentration and -0.78 for P versus leaf water potential; the species-average exponents for g_s
345versus osmotic concentration and g_s versus leaf water potential were -2.25 and -0.6 respectively (see
346Table S2 for species specific values). Species only had a significant effect on the relation between
347 g_s and leaf osmotic concentration (Table 3 and S2). Overall, in contrast to the drought experiment,
348changes in leaf water potential explained more of the variability in P and g_s than leaf osmotic
349concentration.

350

351Although we aimed to maintain the same value of VPD in all experiments (see above), there was
352some variation; differences in VPD had a significant effect (Table 2 and 3) on leaf gas exchange,
353and this was accounted for in the statistical analysis.

354

355Non-stomatal limitations to photosynthesis

356

357Over the whole drought dataset, the photosynthetic efficiency P/c_i ranged from almost zero to
358slightly over $0.06 \mu\text{mol m}^{-2} \text{s}^{-1}$, apparent V_{cmax} varied from 4 to $83 \mu\text{mol m}^{-2} \text{s}^{-1}$ and apparent J_{max}
359varied from 8 to $137 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 4), decreasing with increasing leaf osmotic concentration
360(Fig 4 A, C and E) and decreasing leaf water potential (Fig. 4 B, D and F). The P/c_i versus osmotic
361concentration and P/c_i versus leaf water potential relationships were highly significant when fitted
362with a power-law function (Table 2), the species-average exponents being -0.04 and -0.009,
363respectively (Table S1). V_{cmax} decreased significantly with increasing leaf osmotic concentration
364with a species-average power-law exponent of -1.91 (Table 2; Table S1), while the effect of leaf
365water potential on V_{cmax} was not significant (Table 2). The J_{max} versus leaf osmotic concentration
366and J_{max} versus leaf water potential relationships had species-average exponents of -1.51 and -0.23,
367respectively (Table 2; Table S1 for species specific results). For P/c_i , apparent V_{cmax} and apparent
368 J_{max} , the model with leaf osmotic concentration explained more of the variation than the model with
369leaf water potential (see model R2 in Table 2).

370

371In the notching experiment, P/c_i decreased with increasing leaf osmotic concentration and
372decreasing leaf water potential (Fig. 5), with species-average power-law exponents of -0.032 and -

3730.010, respectively (Table 3, Table S2 for species specific results). There was no effect of leaf
374temperature on either of these relations but there was a significant difference between species for
375both of them.

376

377*Non-stomatal vs. stomatal limitations to photosynthesis*

378

379 P/c_i decreased simultaneously with decreasing g_s as the drought progressed (Fig. 6A). The relation
380was highly significant ($p < 0.001$) and has a saturating shape indicating that the decrease in P/c_i was
381much steeper at lower values of g_s (e.g., $< 100 \mu\text{mol m}^{-2} \text{s}^{-1}$). There was no treatment or species
382effect. Similarly, during notching P/c_i decreased significantly with decreasing g_s (Fig. 6B) following
383a power-law relation with an average exponent of 0.015 (Table 3, table S2). The relation was not
384affected by changes in leaf temperature but was affected by the interaction between g_s and the
385species.

386

387In the notching experiment, changes in P/c_i were consistent with changes in measured assimilation
388at a given c_i versus assimilation predicted for that c_i value based on the $A-c_i$ curve (suppl. Mat. Note
389S1 and Fig. S1).

390

391*Relation between stomatal conductance and hydraulic conductance*

392

393In the drought experiment, a strong positive linear relationship ($p < 0.0001$, $R^2 = 0.57$ Fig. 7A) was
394found between measured values of E and modelled E values obtained (after normalization) from
395eqn (7). This finding supports the theoretical prediction of the optimization hypothesis, that
396stomatal conductance is proportional to the square root of the soil-to-leaf hydraulic conductance
397from soil to leaf. There was no species difference in the observed relation.

398

399A similar relationship was found in the notching experiment ($p < 0.0001$, marginal $R^2 = 0.41$,
400conditional $R^2 = 0.66$, Fig. 7B). There was a significant species effect on the slope of the relation
401($p = 0.0006$), with pine having a steeper slope than the broadleaves species

402

403**Discussion**

404

405*NSL increase with decreasing leaf water status and increasing leaf sugar/osmotic status*
406*(hypothesis 1)*

407
408 We found significant quantitative relations between various measures of NSL (i.e. changes in P/c_i ,
409 apparent V_{cmax} and apparent J_{max}) and changes in both leaf osmotic concentration and leaf water
410 potential. The relations were significant despite the high level of noise in the measured variables of
411 interest, especially in the drought experiment which involved sampling different individuals for
412 each measurement.

413
414 Previous studies have linked soil water status to changes in NSL (Drake *et al.*, 2017) or some of its
415 components, such as decreased mesophyll conductance (Noormets *et al.*, 2001; Diaz-Espejo *et al.*,
416 2007) or biochemical limitation through temporary or permanent inactivation of Rubisco (Noormets
417 *et al.*, 2001; Salvucci & Crafts-Brandner, 2004; Feller, 2016). The relative importance of mesophyll
418 and biochemical limitation in NSL is subject to debate and may depend on species, type of stress
419 and its duration (e.g., Cano *et al.*, 2014; Gimeno *et al.*, 2019), making modelling of NSL potentially
420 challenging. While our results do not address these underlying mechanisms explicitly, they extend
421 previous studies by establishing relationships between NSL and leaf (rather than soil) water status
422 and leaf osmotic status (Box 1), thereby addressing in a simple way the need to represent NSL
423 within plant models (e.g., Gimeno *et al.*, 2019) to better predict plant responses to environmental
424 change. In particular, these relationships support the basic assumptions of the optimization theory of
425 Hölttä *et al.* (2017) and Dewar *et al.* (2018), in which NSL and stomatal control are coordinated to
426 maximize leaf photosynthesis.

427
428 We observed a strong relationship between NSL and leaf osmolyte concentration, particularly in the
429 drought experiment in which leaf osmolyte concentration explained more of the variation in NSL
430 than overall changes in leaf water potential. This result has important implications for the response
431 of tree photosynthesis to drought stress. Osmotic potential has been repeatedly demonstrated to
432 decrease during drought stress, in order to maintain the turgor of the mesophyll under decreased
433 water potential (e.g., Sevanto *et al.*, 2014; Salmon *et al.*, 2015; Lintunen *et al.*, 2016). Trees also
434 regulate their leaf osmotic potential to cope with osmotic stress, for example in the case of in
435 mangroves (Rada *et al.*, 1989) or when growing on saline soil, or to avoid frost damage (Levitt &
436 Scarth, 1936; Charrier *et al.*, 2018). Osmotically active solutes in leaves include soluble sugars, as
437 well as ions and amino acids (e.g. proline) that can accumulate under stress (e.g., Talbott & Zeiger,
438 1996; Huang & Jander, 2017). Soluble sugars likely represent a large share of the osmotically active
439 compounds in the leaves of the tree species in our study (Morgan, 1984; Paljakka *et al.*, 2017), but
440 ions such as Na^+ and K^+ are also likely to constitute part of the measured osmolytes (in particular

441 due to the freezing treatment of the samples). Leaf sugar concentration has previously been
442 associated with increasing NSL in some trees and herbaceous plants, both in C3 and C4 metabolism
443 (e.g., Jeannette *et al.*, 2000; Turnbull *et al.*, 2002; Franck *et al.*, 2006; Hüve *et al.*, 2006; Quentin *et*
444 *al.*, 2013; Cano *et al.*, 2014; Ramalho *et al.*, 2014; Kitao *et al.*, 2015). This sugar-mediated
445 repression of photosynthesis is also consistent with earlier studies which showed that decreasing
446 sink strength limits photosynthetic efficiency (Azcón-Bieto, 1983; Goldschmidt & Huber, 1992;
447 Nakano *et al.*, 2000; Hölttä *et al.*, 2017). Specifically, increasing sink strength has been associated
448 with increasing mesophyll conductance in rice (Detmann *et al.*, 2012). However, none of the
449 previous studies have quantified this relationship across multiple species and conditions, thereby
450 hampering its inclusion in models of plant photosynthetic response to stress.

451

452 *These correlations are similar across timescale and species (hypothesis 2)*

453

454 Overall, our results support the growing body of evidence that NSL can take place over periods
455 ranging from days to weeks (e.g., Drake *et al.*, 2017), in particular in the case of plant physiological
456 response to drought. However, the notching results clearly demonstrate that NSL also occurs within
457 minutes, to changes in tree carbon and water status, on timescales comparable to stomatal
458 responses. Such short-term responses of NSL are less-well documented than the long-term response
459 (e.g., Gimeno *et al.*, 2019), even though mesophyll conductance has been shown to be able to
460 respond within minutes to changes in environmental conditions (see for example Flexas *et al.*, 2012
461 and references therein). The species-average power-law exponents describing the P/c_i vs. leaf
462 osmotic concentration and P/c_i vs. leaf water potential relationships were broadly comparable on
463 different timescales (-0.04 and -0.009 in the drought experiment, -0.032 and -0.01 in the notching
464 experiment, respectively).

465

466 Our results also show that despite a common pattern among all species for NSL responses to
467 changes in leaf water and osmotic status, there was some species-specific differences. These results
468 are in agreement with earlier studies showing that the amount of drought-induced NSL may vary
469 according to the species resistance to drought (Ni & Pallardy, 1992), their acclimation to drought
470 conditions (Kubiske *et al.*, 1996), or tree development stage. Furthermore, different species
471 acclimated to different environments may respond differently to drought. For example, Galmés *et*
472 *al.* (2007) showed that in drought-adapted Mediterranean species, drought mostly affected
473 mesophyll conductance and had little effect on biochemical limitation. The observed decreases in

the apparent V_{cmax} and J_{max} during our drought experiment are consistent with such biochemical limitations, which can be expected for boreal species not adapted to drought.

NSL and stomatal limitation to photosynthesis are coordinated on similar time scales and for different species (hypothesis 3)

Although our results show that stomatal and non-stomatal limitations to photosynthesis occurred simultaneously, the former dominated during mild water stress while the latter dominated during severe water stress (Fig. 6 and Supplementary Materials Fig. S1). This result is in agreement with previous studies of NSL responses to drought (Kubiske *et al.*, 1996; Maroco *et al.*, 2002; Sagardoy *et al.*, 2010). It has been found that NSL was present throughout an experimental drought imposed on four Australian species, but its importance increased with increasing drought stress (Drake *et al.*, 2017). Similar results were found for young ashes and oaks (Grassi & Magnani, 2005). While the mechanisms of NSL might differ across species and time scales (e.g. the relative importance of changes in mesophyll conductance versus biochemical limitation), our results clearly show a very similar quantitative response of NSL to osmotic concentration and water potential at short- and long-term scales. The close coordination between NSL and stomatal limitations to photosynthesis supports the theoretical framework (Fig. 1) in which the photosynthesis rate is maximized by balancing between these two limitations to photosynthesis.

The theoretical relationship between g_s and K_{sl} is supported by experimental data (hypothesis 4)

The significant linear relation between modelled and measured E found in both the drought and the notching experiments supports the theoretical prediction that g_s scales with the square root of K_{sl} , based on the hypothesis that NSL and stomatal control are coordinated so as to maximize leaf photosynthetic rate (Hölttä *et al.*, 2017; Dewar *et al.*, 2018). Soil-to-leaf hydraulic conductance plays an important role in this hypothesis, because it determines how much water potential decreases (and osmotic concentration increases) for a given change in stomatal conductance. This prediction is consistent with a large number of experimental studies showing a tight correlation between plant hydraulic conductance and stomatal conductance (Sperry *et al.*, 1993; Cochard *et al.*, 2002; Hernandez-Santana *et al.*, 2016), as well as some earlier models coupling g_s to changes in K_{sl} (Williams *et al.*, 1996; Sperry *et al.*, 2017). In our theoretical framework, the coupling of K_{sl} to g_s occurs through NSL; for a small K_{sl} (in comparison to large K_{sl}), a given transpiration rate induces a

507larger decrease in NSL (Fig. 1), and the stomatal conductance that maximized photosynthesis is
508therefore lower.

509

510*Conclusion*

511

512We established quantitative relationships describing the dependence of non-stomatal limitations to
513photosynthesis on leaf osmotic concentration and leaf water potential on both short (minutes) and
514long (weeks) timescales. Stomatal conductance was found to vary with soil-to-leaf hydraulic
515conductance in a manner consistent with the predictions of the hypothesis that stomatal and non-
516stomatal limitations to photosynthesis are coordinated so as to maximize the instantaneous rate of
517leaf photosynthesis. These results provide a basis for including non-stomatal limitations to
518photosynthesis in models of tree responses to drought in a simple way that does not require a
519detailed representation of the underlying mechanisms.

520

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522

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527

528*Author contribution*

529YS, TV and TH planned and designed the experiment. YS, AL, AD and TC performed the
530experiments. YS analyzed the data with the help of AL and AD. YS wrote the manuscript with the
531help of all co-author and particularly TH and RD.

532

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Box 1: While our experimental design does not allow testing for the physiological mechanisms underlying the relation between stomatal and non-stomatal limitation to photosynthesis with changes in osmotic concentration and water status, they show a coordination of stomatal and non-stomatal response to changes in leaf water and osmotic status that is in agreement with a proposition that a joint molecular pathway aiming at limiting unnecessary water loss by leaves would respond to both water stress and sugar accumulation in leaves (Kelly *et al.*, 2017). Hexokinase has been observed in *Arabidopsis* and tomato (e.g., Granot *et al.*, 2013; Kelly *et al.*, 2013; Kelly *et al.*, 2014; Kelly *et al.*, 2017) to play such a central role in regulating leaf physiological processes in response to changes in sugar metabolism. Under the well-supported assumption that changes in leaf osmolality reflects changes in sugar concentration (Warren *et al.*, 2007; Paljakka *et al.*, 2017, but see above for more details) and that hexokinase would play a similar role in trees than in the previously studied plants, hexokinase would be a good candidate for the coordination mechanism underlying the simultaneous changes in stomatal and non-stomatal limitation in our study (see Fig. 8). Briefly: increasing sugar concentration (for example as a result of decreased water content, impaired phloem transport or osmotic regulation) would stimulate the hexokinase activity. Increased hexokinase activity would decrease mesophyll conductance (Kelly *et al.*, 2014) and could be the sensing mechanisms triggering the downregulation of the photosynthetic machinery with increasing sugar concentration reported before (Stitt & Krapp, 1999; Long *et al.*, 2004). Furthermore, ABA production has been shown to increase with hexokinase signaling caused by increasing sugar concentration (Granot *et al.*, 2013; Kelly *et al.*, 2013) thus causing stomatal closure (e.g., Mitchell *et al.*, 2017).

737Table 1: Average (± 1 S.E.) initial height and basal diameter of the four species of saplings used in
 738the drought experiment.

Species	Initial height (cm)	Initial basal diameter (cm)
Pine	75 ± 8	1.38 ± 0.17
Spruce	57 ± 4	0.83 ± 0.15
Aspen	98 ± 11	0.73 ± 0.09
Birch	58 ± 12	0.55 ± 0.06

Table 2: Result of the statistical test for the relations between P/c_i , V_{cmax} , J_{max} , P and g_s against leaf osmotic concentration and leaf water potential for saplings of four boreal species in the drought experiment. The response variables y (P/c_i , V_{cmax} and J_{max}) were transformed to Y to meet the linear regression requirements. Similarly, the independent variables were transformed into X if needed. An environmental covariate (z) was added to account for possible differences in the measurement conditions. The model tested is $Y \sim z + X * \text{Species} * \text{Treatment}$ (see text for more details). The R^2 for the full model is given. Note that $\log(y+1)$ was used when y was lower than 1 to avoid creating negative values.

Response variable (y)	P/c_i		V_{cmax}		J_{max}		P		g_s	
Transformation (Y)	$\log(y+1)$	$\log(y+1)$	$\log(y)$	$\log(y)$	$\log(y)$	$\log(y)$	$\log(y)$	$\log(y)$	y	$\log(y)$
Independent variable (x)	osmotic concentration		osmotic concentration		osmotic concentration		osmotic concentration		osmotic concentration	
	water potential		water potential		water potential		water potential		water potential	
Transformation (X)	$\log(x)$	$\log(-x)$	$\log(x)$	$\log(-x)$	$\log(x)$	$\log(-x)$	$\log(x)$	$\log(-x)$	$\log(x)$	$\log(-x)$
p-value for X	$2 \cdot 10^{-9}$	0.0011	$4 \cdot 10^{-6}$	ns	$3 \cdot 10^{-7}$	0.039	$2 \cdot 10^{-16}$	$8 \cdot 10^{-13}$	$7 \cdot 10^{-8}$	$8 \cdot 10^{-8}$
Other variables p-values										
VPD	0.0003	0.001	$3 \cdot 10^{-9}$	ns	ns	ns	ns	$1 \cdot 10^{-5}$	0.017	0.003
species	$3 \cdot 10^{-7}$	$2 \cdot 10^{-5}$	$3 \cdot 10^{-8}$	$2 \cdot 10^{-5}$	$6 \cdot 10^{-10}$	$1 \cdot 10^{-17}$	$2 \cdot 10^{-9}$	$9 \cdot 10^{-11}$	$5 \cdot 10^{-8}$	$4 \cdot 10^{-10}$
Treatment	ns	ns	ns	0.082	0.038	0.013	$5 \cdot 10^{-5}$	0.001	$8 \cdot 10^{-7}$	$5 \cdot 10^{-6}$
X:Species	0.0003	ns.	ns	0.072	ns	0.072	0.0048	ns	0.075	ns
X:Treatment	ns	0.0013	0.036	ns	0.061	0.056	ns	$3 \cdot 10^{-7}$	ns	0.001
Species:Treatment	ns	ns	ns	ns	ns	ns	0.0212	0.009	0.032	0.018
X:Species:Treatment	0.04	ns.	0.006	0.0033	0.0045	0.013	0.0237	ns	ns	ns
Model R^2	0.66	0.42	0.74	0.58	0.78	0.65	0.76	0.71	0.68	0.67

ns.: non significant. Excl. excluded from the final model.

Table 3: Result of the statistical test for the relations between P/c_i , P and g_s against leaf osmotic concentration and leaf water potential for saplings of four boreal species in the notching experiment. The response variables y (P/c_i) were transformed to Y to meet the linear regression requirements. Similarly, the independent variables were transformed into X if needed. An environmental covariate (z) was added to account for possible differences in the measurement conditions. The original model tested is $Y \sim z + X * \text{Species}$ and simplified when possible (see text for more details), the experimental run was added as a random term. The marginal R^2 and the conditional R^2 for the selected model are given. Note that $\log(y+1)$ was used when y was lower than 1 to avoid creating negative values.

Response variable (y)	P/c_i			P		g_s	
Transformation (Y)	$\log(y+1)$	$\log(y+1)$	$\log(y+1)$	$\log(y)$	$\log(y)$	$\log(y)$	$\log(y)$
Independent variable (x)	osmotic	water		osmotic	water	osmotic	water
	concentration	potential	g_s	concentration	potential	concentration	potential
Transformation (X)	$\log(x)$	$\log(-x)$	$\log(x)$	$\log(x)$	$\log(-x)$	$\log(x)$	$\log(-x)$
p-value fpr X	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
environment (z)	Tleaf	Tleaf	Tleaf	VPD	VPD	VPD	VPD
p-values for z	ns	ns	ns	0.019	0.013	0.0095	0.0015
p values for species	0.009	0.015	0.065	ns	ns	0.032	ns
p values for X:Specie	excl.	excl.	0.004	excl.	excl.	excl.	ns
Marginal R^2	0.31	0.38	0.75	0.30	0.43	0.26	0.49
Conditional R^2	0.38	0.67	0.92	0.43	0.81	0.54	0.79

n.s.: non significant. Excl. excluded from the final model.

Figure 1: A schematic figure of net photosynthetic rate (A) as a function of leaf internal CO_2 concentration (c_i) and leaf water and osmotic status. Stomatal opening causes CO_2 supply to and c_i to increase, but at the same time water potential is decreased and osmotic concentration is increased causing A for a given c_i to be lowered. The maximum photosynthesis rate is achieved at an intermediate, “optimal” stomatal conductance (see insert) to balance between the stomatal and non-stomatal limitations to photosynthesis. The “optimal” stomatal conductance is dependent on e.g. photosynthetic parameters, PAR, ambient CO_2 (c_a), VPD, hydraulic conductance (K_{sl}), and vulnerability to cavitation (PLC_{50}).

Figure 2: Gross photosynthetic rate (P) and stomatal conductance (g_s) as a function of leaf osmotic concentration and water potential for four species exposed to increasing drought stress. Well-water (circle) and drought-stressed (triangle) saplings are used in the same regression per species to cover a wide range of leaf osmolality and water potential, but more details about the statistical model can be found in Tables 2 and S1.

Figure 3: Gross photosynthetic rate (P) and transpiration (E) as a function of leaf osmotic concentration and water potential for three species exposed to notching. Curves are fitted with power regression over the several replicates for each species (more details can be found in tables 3 and S2).

Figure 4: P/c_i (A and B), V_{cmax} (C and D) and J_{max} (E and F) response to changes in leaf osmotic concentration (A, C and E) and water potential (B, D and F) as drought increases for saplings of four common boreal species. Well-water (circle) and drought-stressed (triangle) saplings are used in the same regression per species to cover a wide range of leaf osmotic concentration and water potential, but more details about the statistical model can be found in Table 2 and Table S1. Note the different scales of the x-axis in panels A and B compared to the others, this is the results of unreliable $A-c_i$ curve measurements on very stress plants.

Figure 5: P/c_i response to leaf osmotic concentration (A) and water potential (B) of three species to notching. Curves are fitted with a power regression, more details can be found in tables 3 and S2.

Figure 6: relation between non-stomatal limitation to photosynthesis estimated by changes in P/c_i in response to changes in stomatal conductance, for saplings of four common boreal species exposed to drought (A) and for saplings of three common boreal species exposed to notching (B). For the drought, well-water (circle) and drought-stressed (triangle) saplings are used in the same regression per species to cover a wide range of leaf osmolality and water potential. For the

787notching, curves are fitted with power relation between g_s and P/c_i and over the several replicates
788for each species. (See table 3 for the full statistical test).

789**Figure 7:** relation between $r/r_{\max}$ (see material and methods) and measured E in the two
790experiments presented in this study: (A) saplings of four common boreal species exposed to
791drought. The relation tested between modelled $r/r_{\max}$ and E was highly significant ($y=0.27x$,
792 $p<0.0001$, $R^2=0.54$). There was no difference between species. (B) saplings of three common boreal
793species exposed to notching. The relation tested between relative $r/r_{\max}$ and E was highly significant
794($p<0.0001$, marginal $R^2=0.47$, conditional $R^2=0.79$). Aspen and birch were not statistically different
795($y\sim 0.113x$ for aspen and $y\sim 0.086x$ for birch). Pine was significantly different from the other species
796($y\sim 0.280x$). ($p<0.001$).

797**Figure 8:** A conceptual model demonstrating the mechanisms by which sugar concentration can be
798linked to changes in stomatal and non-stomatal limitation to photosynthesis. Full arrows represent
799processes for which the causal link is established beyond doubt, while dashed arrows represents
800established processes but not as widely demonstrated as the previous one. Dark grey color
801represents environmental drivers and light grey physiological processes and variables.

New Phytologist Supporting Information

Article title: Leaf carbon and water status control stomatal and non-stomatal limitations of photosynthesis in trees.

Authors: Yann Salmon, Anna Lintunen, Alexia Dayet, Tommy Chan, Roderick Dewar, Timo Vesala, Teemu Hölttä

Article acceptance date: [Click here to enter a date.](#)

The following Supporting Information is available for this article:

Notes S1 Assessing non-stomatal limitation to photosynthesis.

Fig. S1 The dependence of the ratio of measured to predicted A on leaf osmolality and water potential.

Table S1 Species and treatment (C: control and D: drought) specific exponent of power function fitted to the data from the drought experiment (details of the transformation can be found in table 2).

Table S2 Species specific exponent of power function fitted to the data from the notching experiment (details of the transformation can be found in table 3). Different letters mean statistically different exponents.

Notes S2 Supporting information for testing the predicted relationship between g_s and K_{sl}

Fig. S2 median, first and third quantiles (upper and lower hinges, upper and lower whisker are the highest and lowest, respectively, values no further from the hinges than 1.5 time to interquantile distance) of K_{sl} during the drought experiment.

Table S3 Best-fit parameters a and b in eqn (S2.3) for the Medlyn et al. (2011) and Dewar et al. (2018) models for the drought and notching experiments.

Notes S2 Showing the mathematical equivalence between testing the relation between r/r_m and E and the relation between g_s and K_{sl}

Notes S1 Assessing non-stomatal limitation to photosynthesis.

To confirm the validity of the use of photosynthetic efficiency (P/c_i) as a proxy for the effect of non-stomatal limitation to photosynthesis, we used $A-c_i$ curves measured prior to notching to predict the expected A for any given c_i if non-stomatal limitations to photosynthesis (i.e. photosynthetic efficiency) remained constant. We then compared the measured A to the predicted A for that given c_i . Hence, changes in stomatal conductance, i.e. changes in the supply of CO_2 to the substomatal cavity, cannot be responsible for the changes in A . A similar approach was used by Drake et al. (2017). Fig S1 demonstrates the ratio of A measured to A predicted as a function of leaf osmotic concentration and leaf water potential.

The relation between $\log(A/A_{\text{predicted}})$ against $\log(\text{osmotic concentration})$ was highly significant ($p < 0.0001$) with no species effect and a small interaction term between species and $\log(\text{osmolality})$ ($p = 0.046$). Overall the model marginal R^2 was 0.37 and the conditional R^2 accounting for the random term (i.e. the experiment identity) was 0.51.

The relation between $\log(A/A_{\text{predicted}})$ against $\log(\text{-water potential})$ was highly significant ($p < 0.0001$) with a species effect ($p = 0.032$) and no interaction between species and $\log(\text{-water potential})$. Overall the model marginal R^2 was 0.53 and the conditional R^2 accounting for the random term (i.e. the experiment identity) was 0.75.

These results are consistent with those obtained from P/c_i and thus support the validity of using the changes in photosynthetic efficiency to assess changes in non-stomatal limitations.

Fig. S1 The dependence of the ratio of measured to predicted A on leaf osmotic concentration and water potential.

Table S1 Species and treatment (C: control and D: drought) specific exponent of power function fitted to the data from the drought experiment (details of the transformation can be found in table 2). Different letters mean statistically different exponents.

Species and treatment	P/c_i		V_{cmax}		J_{max}		P		g_s	
	Osmotic concentration	Water potential	Osmotic concentration	Water potential	Osmotic concentration	Water potential	Osmotic concentration	Water potential	Osmotic concentration	Water potential
	n	l	n	l	n	l	n	l	n	l
Aspen	-0.054 ^a	-0.010 ^a	-1.98 ^a	-0.046 ^a	-1.72 ^a	-0.062 ^a	-4.62 ^a	-1.11 ^a	-4.38 ^{a,b}	-0.98 ^a
Birch	-0.080 ^a	-0.008 ^a	-2.29 ^a	0.203 ^a	-1.76 ^a	0.086 ^a	-4.65 ^a	-1.27 ^a	-6.74 ^b	-2.00 ^a
Pine	-0.023 ^b	-0.012 ^a	-1.39 ^a	-0.297 ^{a,b}	-1.19 ^a	-0.219 ^{a,b}	-2.25 ^{a,b}	-1.05 ^a	-2.66 ^a	-1.37 ^a
Spruce	-0.010 ^b	-0.007 ^a	-1.99 ^a	-0.734 ^b	-1.37 ^a	-0.729 ^b	-2.32 ^b	-1.64 ^a	-0.13 ^c	-1.79 ^a

Table S2 Species specific exponent of power function fitted to the data from the notching experiment (details of the transformation can be found in table 3). Different letters mean statistically different exponents.

	P/c_i Osmotic concentration	P/c_i water potential	P/c_i g_s	P Osmotic concentration	P water potential	g_s Osmotic concentration	g_s water potential
Aspen	-0.027 ^a	-0.013 ^a	0.018 ^a	-1.84 ^a	-1.00 ^a	-1.72 ^a	-0.87 ^{a,b}
Birch	-0.027 ^a	0.007 ^b	0.016 ^a	-2.36 ^b	-0.28 ^a	-2.59 ^b	0.39 ^a
Pine	-0.042 ^b	-0.024 ^a	0.010 ^b	-2.28 ^{a,b}	-1.05 ^a	-2.43 ^{a,b}	-1.32 ^b

Note S2: Supporting information for testing the predicted relationship between g_s and K_{sl}

Figure S2: median, first and third quantiles (upper and lower hinges, upper and lower whisker are the highest and lowest, respectively, values no further from the hinges than 1.5 time to interquantile distance) of K_{sl} during the drought experiment.

To compare the prediction of our model (Dewar et al. 2018) with the prediction of the Medlyn et al. (2011) model, for each model we tested the linearity between the predicted and measured g_s .

The model of Medlyn et al. (2011) predicts that

$$g_s = g_1 \frac{P}{\sqrt{D}} \quad (\text{S2.1})$$

The corresponding prediction of the model of Dewar et al. (2018) is

$$g_s = g_2 \frac{P}{c_a \sqrt{D}} \sqrt{K_{sl}} \quad (\text{S2.2})$$

where K_{sl} is soil-to-leaf hydraulic conductance, and g_2 is a known function of physiological parameters (i.e. not an empirical parameter like g_1), and treated here as a constant. This is eqn (4) of the main text.

To evaluate the linearity between predicted and measured g_s , for each model we selected the values of parameters a and b in the relation

$$g_{s, \text{predicted}} = a (g_{s, \text{modelled}})^b \quad (\text{S2.3})$$

that minimized the distance between the measured points and the model prediction (using the nls function in R). The closer b is to 1, the more linear is the relation.

Table S3 shows that in both the notching and drought experiments, linearity was greater for the Dewar et al. (2018) than for the Medlyn et al. (2011) model, particularly so in the drought experiment.

References

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Table S3: Best-fit parameters a and b in eqn (S2.3) for the Medlyn et al. (2011) and Dewar et al. (2018) models for the drought and notching experiments.

		a	b
Drought	Medlyn	$2.1 \cdot 10^{-4}$	0.68
	Dewar	$3.0 \cdot 10^{-5}$	0.94
Notching	Medlyn	$4.5 \cdot 10^{-3}$	0.79

Note S3: Showing the mathematical equivalence between testing the relation between r/r_m and E and the relation between g_s and K_{sl} .

Equation 4 shows the relation between g_s and K_{sl}

$$g_s \approx \frac{A}{c_a} \frac{\xi}{\sqrt{D}} \propto \frac{A}{c_a} \sqrt{\frac{K_{sl}}{D}} . \quad (4)$$

K_{sl} can be expressed as:

$$K_{sl} = \frac{E}{\Delta\Psi} , \quad (5)$$

Thus, replacing K_{sl} in equation 4 by equation 5 gives:

$$g_s \propto \frac{A}{c_a} \sqrt{\frac{E}{D * \Delta\Psi}} . \quad (4a)$$

Because c_a is constant, the proportionality can be expressed as equation 6:

$$g_s \propto A \sqrt{\frac{E}{D * \Delta\Psi}} . \quad (6)$$

Furthermore: $g_s = E/1.6D$, thus equation 6 can be written:

$$\frac{E}{1.6D} \propto A \sqrt{\frac{E}{D * \Delta\Psi}} . \quad (6a)$$

$$\frac{E}{D} \propto A \sqrt{\frac{E}{D * \Delta\Psi}} . \quad (6b)$$

By rearranging, we obtain:

Which gives:

$$E \propto A^2 \frac{D}{\Delta \Psi} \equiv r \quad (7)$$

Because we are looking at the proportionality between both side of the equation, it is also equivalent to test:

$$E \propto r/r_m \quad (7a)$$