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Research paper

Carbohydrate regulation of photosynthesis and respiration from branch girdling in four species of wet tropical rain forest trees

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How trees sense source–sink carbon balance remains unclear. One potential mechanism is a feedback from non-structural carbohydrates regulating photosynthesis and removing excess as waste respiration when the balance of photosynthesis against growth and metabolic activity changes. We tested this carbohydrate regulation of photosynthesis and respiration using branch girdling in four tree species in a wet tropical rainforest in Costa Rica. Because girdling severs phloem to stop carbohydrate export while leaving xylem intact to allow photosynthesis, we expected carbohydrates to accumulate in leaves to simulate a carbon imbalance. We varied girdling intensity by removing phloem in increments of one-quarter of the circumference (zero, one-quarter, half, three-quarters, full) and surrounded a target branch with fully girdled ones to create a gradient in leaf carbohydrate content. Light saturated photosynthesis rate was measured in situ, and foliar respiration rate and leaf carbohydrate content were measured after destructive harvest at the end of the treatment. Girdling intensity created no consistent or strong responses in leaf carbohydrates. Glucose and fructose slightly increased in all species by 3.4% per one-quarter girdle, total carbon content and leaf mass per area increased only in one species by 5.4 and 5.5% per one-quarter girdle, and starch did not change. Only full girdling lowered photosynthesis in three of four species by 59–69%, but the decrease in photosynthesis was unrelated to the increase in glucose and fructose content. Girdling did not affect respiration. The results suggest that leaf carbohydrate content remains relatively constant under carbon imbalance, and any changes are unlikely to regulate photosynthesis or respiration. Because girdling also stops the export of hormones and reactive oxygen species, girdling may induce physiological changes unrelated to carbohydrate accumulation and may not be an effective method to study carbohydrate feedback in leaves. In three species, removal of three-quarters of phloem area did not cause leaf carbohydrates to accumulate nor did it change photosynthesis or respiration, suggesting that phloem transport is flexible and transport rate per unit phloem can rapidly increase under an increase in carbohydrate supply relative to phloem area. Leaf carbohydrate content thus may be decoupled from whole plant carbon balance by phloem transport in some species, and carbohydrate regulation of photosynthesis and respiration may not be as common in trees as previous girdling studies suggest. Further studies in carbohydrate regulation should avoid using girdling as girdling can decrease photosynthesis through unintended means without the tested mechanisms of accumulating leaf carbohydrates.

Keywords: non-structural carbohydrates, NSC, phloem, sink regulation.

Introduction

Plants are thought to balance their carbon budget with feedback from carbohydrate storage (Paul and Foyer 2001, Fatichi et al. 2014), but the controls and mechanisms at whole plant scale remain unclear. The simplest view is that photosynthesis (source) supplies carbohydrates for metabolism and growth

(sink), and the difference between source and sink fluxes determines the size of the storage pool (Chapin et al. 1990, Körner 2003). Clearly the storage pool cannot increase or decrease infinitely, supporting the idea that the pool size must play a role in the feedback regulation of source and sink fluxes.

Feedback regulation in concept states that carbohydrate storage pool size regulates photosynthesis (Boussingault 1868, Ewart 1896, Paul and Foyer 2001), and respiration removes excess carbohydrates when the storage pool becomes too large (Lambers 1982, Amthor 2000, Cannell and Thornley 2000). This carbohydrate regulation has gained attention as a way to increase crop yields (Cui et al. 2003, Reynolds et al. 2005, Ainsworth and Bush 2011), a mechanism that dampens plant response to higher CO_2 in the atmosphere (Körner 2003) and a different perspective for modeling plant growth and ecosystem biogeochemistry (Génard et al. 2008, Yin and Struik 2010, Nikinmaa et al. 2013, Fatichi et al. 2014). However, the importance of carbohydrate regulation in plant growth remains unknown. The storage pool primarily serves as a source of carbohydrates during night and seasonal dormancy, and the pool size could fluctuate widely before triggering any regulation. The changes in storage pool required to trigger feedback regulation of photosynthesis or respiration remain unquantified.

The mechanism of carbohydrate regulation of photosynthesis is well documented at the cellular level, with pathways identified that both increase and decrease photosynthesis through changes in biochemistry and stomatal behavior. When experimental manipulations increase sucrose and starch concentrations in leaves, Rubisco and other Calvin-cycle enzymes decrease, and the rates of RuBP regeneration, carboxylation and electron transport decline (Stitt et al. 1991, Goldschmidt and Huber 1992, Krapp and Stitt 1995). Carbohydrate concentrations also control the expression of photosynthetic and phloem transport genes (Sheen 1990, Krapp et al. 1993, Koch 1996, Chiou and Bush 1998). Stomata may be involved, where accumulating carbohydrates causes stomatal closure perhaps to optimize carbon gain and water use (Mäkelä 1996, Nikinmaa et al. 2013). However, carbohydrate regulation of photosynthesis at the cellular level has been tested mostly in vitro with high levels of carbohydrate concentration (Paul and Foyer 2001). It has been field tested in some crops, but only on a few fruit trees and even less on non-agricultural trees (Sweet and Wareing 1966, Herold 1980, Harrell and Williams 1987, Myers et al. 1999, Urban and Alphonsout 2007, Domec and Pruyn 2008, Nebauer et al. 2011). Results from crops may not apply to trees because trees face increasing barriers to water transport and phloem function as they grow taller (Hellkvist et al. 1974, Bauerle et al. 1999, Woodruff 2014), and thus may be chronically carbon limited or over supplied (Ryan and Yoder 1997, Woodruff et al. 2004, Ryan et al. 2006).

The removal of excess carbohydrate in respiration is thought to occur through respiratory pathways that yield less energy for a consumed substrate (Lambers 1982, Rasmusson et al. 2004, van Dongen et al. 2011). Respiration in plant mitochondria has multiple pathways that vary in the efficiency of ATP production. The most efficient is the cytochrome pathway with a step to produce ATP using electrochemical potential across the inner mitochondrial membrane. Less efficient pathways bypass this step, using alternative oxidases, uncoupling protein or NAD(P)H dehydrogenases (Millar et al. 2011, van Dongen et al. 2011). These bypassing pathways yield less ATP for a substrate and are often suggested as a mechanism to remove excess carbohydrates (Lambers 1982, Millar et al. 1998, Amthor 2000, Cannell and Thornley 2000). It remains unclear whether these bypassing pathways actually remove excess carbohydrates to balance carbon budget at whole plant level (Lambers et al. 2008). In tomato plants, elevated CO_2 concentration increased leaf carbohydrates, respiration and transcripts of enzymes involved in the bypassing pathways as expected from the carbohydrate removal model (Li et al. 2013). In contrast, elevated CO_2 decreased respiration in *Opuntia*, despite enhanced growth and increased activity of a bypassing pathway (Gomez-Casanovas et al. 2007). Transgenic tobacco cells without the bypassing pathways accumulated carbohydrates, increased growth and reduced respiration under nutrient stress (Sieger et al. 2005), but across grass species in the field, the activity of a bypassing pathway increased with relative growth rate (Millenaar et al. 2001).

To test carbohydrate regulation of photosynthesis and respiration, studies often manipulate leaf carbohydrate levels using girdling (Myers et al. 1999, Iglesias et al. 2002, Urban and Alphonsout 2007, Cheng et al. 2008, Domec and Pruyn 2008, De Schepper et al. 2010, Fan et al. 2010, Nebauer et al. 2011). Girdling severs phloem and thus stops the export of carbohydrates out of the leaf while leaving xylem intact for water and nutrient transport, allowing photosynthesis to continue (Noel 1970). Carbohydrates should then accumulate in the leaf and simulate a carbon imbalance where source activity exceeds sink activity. These studies tracked physiological changes through measurements of leaf gas exchange, and quantified carbohydrate accumulation with measurements of the concentration of simple sugars and starch (non-structural carbohydrates, NSC) in the leaves harvested at the end of the experiment. Most find that girdling accumulates leaf carbohydrates and reduces photosynthesis (Myers et al. 1999, Iglesias et al. 2002, Urban and Alphonsout 2007, Cheng et al. 2008, Domec and Pruyn 2008, De Schepper et al. 2010, Fan et al. 2010), with few showing that leaf carbohydrate content negatively correlates with photosynthesis rate (Franck et al. 2006, Urban and Alphonsout 2007) or positively correlates with respiration (Urban 2004, Domec and Pruyn 2008, De Schepper et al. 2010). Further studies will make clear the changes in leaf carbohydrates required to trigger the regulation and the generality of this phenomenon.

We tested carbohydrate regulation of photosynthesis and respiration using branch girdling on four tree species in a wet tropical rainforest in Costa Rica. We use girdling with differing intensity

to create a gradient in leaf carbohydrate content. We girdled branches in the upper canopy fully to stop phloem export, incompletely in quarter fractions to reduce phloem export by degree, and surrounded an intact branch with girdled ones to increase phloem export. We hypothesized that increasing girdling intensity would (1) increase leaf carbohydrate content, (2) decrease photosynthesis rate inversely proportional to carbohydrate content and (3) increase respiration rate proportional to carbohydrate content (Figure 1 describes the hypotheses graphically).

Materials and methods

Study site

We conducted this study at La Selva Biological Station, in the Atlantic lowlands of Costa Rica (10°26′N, 84°00′W), with climate categorized in the Holdridge system as Tropical Wet Forest (McDade 1994), with a mean annual rainfall of 4000 mm, mean annual temperature of 26 °C and precipitation averaging >100 mm each month. Measurements were taken from June to September in 2009 and from January to June in 2010. Annual rainfall was ~4500 mm year^{-1} and annual temperature averaged 25 °C for both 2009 and 2010. The soil is an acidic, highly leached, organic matter rich oxisol classified as Mixed Haplic Haploperox (Kleber et al. 2007). The native vegetation is broad-leaved evergreen tropical rainforest.

The site is part of a larger study examining tree species effects on ecosystem processes (ECOS, http://www.nrem.iastate.edu/ECOS/home; Russell et al. 2010, Russell and Raich 2012). The site was cleared of primary forest and converted to pasture in 1956 then grazed until 1987. In 1988, experimental plantations were established with eleven tree species and unplanted control, replicated over four blocks in a randomized

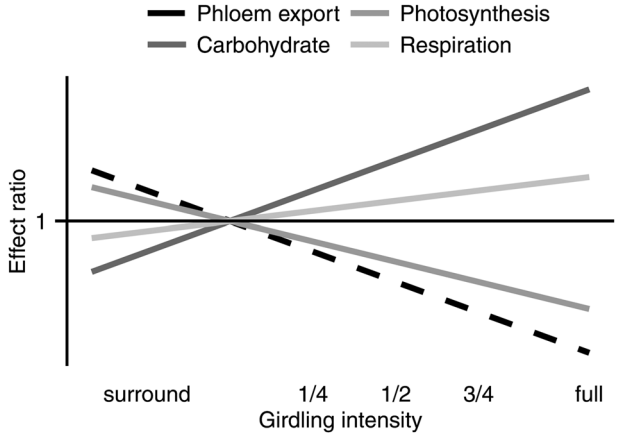

Figure 1. Graphical representation of hypothesis, showing the effect ratios (treatment/control) of the response variables to differences in phloem area created by varying girdling intensity. We predicted that increased girdling intensity would decrease phloem export (dashed line) and photosynthesis (gray line), and increase leaf carbohydrate content (dark gray line) and respiration (light gray line). We measured the latter three (solid lines).

complete block design (Fisher 1995). Understory plants were cleared during plantation establishment and for 3 years afterwards, but then allowed to regenerate naturally. Plots were 50 × 50 m (0.25 ha), with a single-tree species planted in each plot except for the unplanted control. By 2008, only four tree species had survival adequate for whole-plot measurements, and these species were the subjects of this study.

The four species were *Hieronyma alchorneoides* Allemao, *Pentaclethra macroloba* (Willd.) Kunth., *Virola koschnyi* Warb. and *Vochysia guatemalensis* Donn. Sm. All are native to the surrounding primary forest, and *Pentaclethra* is the dominant species of canopy trees at La Selva and the only N-fixing species of the four. By 2008, each species had formed a stand with aboveground biomass similar to the surrounding forest (~8500 g C m^{-2} mean total aboveground biomass, Russell et al. 2010). The stands were growing fast at aboveground net primary productivity of ~1200 g C m^{-2} year^{-1} (Russell et al. 2010). Further details on site history and its carbon and nitrogen cycle characteristics can be found in Fisher (1995), Russell et al. (2010) and Russell and Raich (2012).

The girdling treatments were made on the branches of upper canopy, in one block per species in 2009 and in another block per species in 2010. The branches were 2–4 cm in diameter supporting 5–10 fully expanded sun leaves in the upper canopy, and accessed from a 30 m portable scaffolding tower (Upright Inc., Dublin, Ireland).

Treatments

We varied girdling intensity to generate a gradient of phloem export rate. We girdled in quarter increments (zero, one-quarter, half, three-quarters and full) by removing bark and phloem in a band 1.5 cm wide near the proximal end of the branch. We also surrounded an intact branch with completely girdled ones to increase demand for phloem export for the target branch. Branches not immediately connected to the girdled ones served as controls, to minimize the influence of girdled branches. For 2009 measurements, complete girdle and control treatments were established on four branches per treatment for each species; in 2010, all treatments were made on three to four branches per treatment for each species.

Photosynthesis and stomatal conductance measurements were made periodically for 6–20 days after treatment for all replicates on fully expanded leaves. Branches were then cut under water, transported with cut ends in water to the lab, measured for respiration and leaf area and dried for quantification of leaf mass per area (LMA) and NSC. A few branches did not survive through the end of the photosynthesis measurements because of wind damage or herbivory by leaf cutter ants.

Measurements of response variables

Photosynthesis and stomatal conductance were measured as a light saturated rate, and photosynthetic capacity assessed as the

response to CO_2 concentration (A–C_i curve). Measurements were made on five fully expanded leaves per branch with an open-system portable infrared gas analyzer (IRGA) (LI-6400, LI-COR, Inc., Lincoln, NE, USA). Light saturated photosynthesis rate and stomatal conductance were measured under a reference CO_2 of 390 μmol mol^{-1}, at an air flow rate of 500 μmol s^{-1} and with a saturating level of photosynthetic photon flux density of 2000 μmol m^{-2} s^{-1} after an acclimation time when the readings stabilized. Because the site was fairly remote and rain was frequent, we could not control for measurement time of day, temperature and humidity. Measurements were made between 07:00 and 16:00 h, under temperature ranging from 24.5 to 39 °C. Measurements were excluded from the analysis if vapor pressure deficit at the leaf surface exceeded 3 kPa. Response of photosynthesis rate to CO_2 concentration (A–C_i curve) was taken to estimate maximum rates of caboxylation (V_{cmax}) and electron transport (J_{max}), on a subset of foliage by varying the reference CO_2 concentration (400, 300, 200, 100, 50, 400, 600, 800 μmol mol^{-1}). A subset of *Hieronyma* leaves were measured for midday leaf water potential using a pressure bomb (Model 3000, Soilmoisture Equipment Corp., Santa Barbara, CA, USA).

Foliar respiration was measured on detached foliage at night using a chamber made from clear polycarbonate connected to an IRGA. The branches were cut under water in the afternoon, placed in a floral tube with water without exposing the cut surface to air, and the cut surface kept in water until after respiration measurement. The foliage was taken back to the lab and measured at night between 20:00 and 02:00 h in the dark and under ambient temperature. Detached and attached foliage had similar respiration rate in a previous study at La Selva (Cavaleri et al. 2008) and in several other studies (Mitchell et al. 1999, Turnbull et al. 2005). The foliage was placed inside the chamber and sealed with neoprene gaskets, and the seal checked with a flow meter. The chamber was 1580 ml in volume, and the air inside was mixed with a small fan. The chamber was connected to an IRGA: open-system LCA-3 (Analytical Development Company, Hoddeson, UK) for 2009 measurements, or a lab-built closed-system with Li-820 (LI-COR, Inc.) and CR10X data logger (Campbell Scientific, Logan, UT, USA) for 2010 measurements. The open-system IRGA drew ambient air from a 19 l mixing container to maintain stable concentration of reference CO_2 during measurements. The airflow rates through the chamber ranged between 270 and 340 μmol s^{-1}. Both instruments were regularly calibrated with a CO_2 standard. Foliar respiration rates measured at ambient temperature were standardized to 25 °C using estimated Q_{10} specific to each species from a previous study (*Hieronyma* = 1.6, *Pentaclethra* = 2.6, *Virola* = 1.6, *Vochysia* = 1.8; Asao et al. 2015).

Carbohydrate response was quantified by measuring NSC content, LMA and carbon (C) and nitrogen (N) contents of foliage dried immediately after respiration measurements. The foliage was measured for leaf area with a leaf area meter (LI-3100, LI-COR, Inc.), dried for 48 h at 65 °C and measured for leaf dry mass, ground to a fine powder and measured for leaf N and C with a C–N analyzer (TruSpec CN, LECO, Inc., St Joseph, MI, USA). The foliage was measured for NSC content using an enzymatic assay (Wong 1990, Hoch et al. 2002). Briefly, ~2 mg of powdered leaf was extracted with 0.75 ml distilled water in closed centrifuge tubes fitted with silicone O-rings in a block heater set at 120 °C for 3 h for starch, and a separate ~4 mg powder was extracted with 1.5 ml distilled water at 100 °C for 1 h for glucose and fructose. Glucose and fructose (and other hexoses) were enzymatically converted to glucose-6-phosphate with hexokinase on a 96-well plate and measured photometrically. Sucrose was hydrolyzed to glucose and fructose using invertase at 40 °C for 1 h, and starch to glucose using amyloglucosidase at 40 °C overnight. Resulting monosaccharides were measured as described above, and concentration calculated by subtracting free glucose and fructose concentrations from sucrose and starch assay values. The concentrations were then converted to per leaf area basis using LMA. All values of NSC content are expressed as g glucose equivalent per unit leaf area, and C and N content values are expressed as g per unit leaf area.

Units of response variables

We expressed the measured values on leaf area basis instead of the more common dry mass basis because leaf area is constant in fully expanded leaves (barring herbivory), while leaf mass will change if carbohydrates accumulate or deplete. Consider for example that girdling accumulates NSC in leaves without changing photosynthesis. Using mass basis for concentration (mg NSC g^{-1} dry mass) will underestimate the accumulation of NSC because both the numerator and the denominator increase. Worse, using mass basis for photosynthesis (μmol CO_2 g^{-1} dry mass) will falsely give a decreased rate because the denominator increases. Unlike mass, leaf area is constant and thus a superior basis for expressing at least photosynthesis and respiration, if not NSC. Furthermore, a common denominator for x and y simplifies the interpretation of regressions. Area-based NSC content in g m^{-2} (x/a) regressed against photosynthesis in μmol CO_2 m^{-2} s^{-1} (y/a) will yield a slope of photosynthesis per NSC in μmol CO_2 g^{-1} s^{-1} (y/x), a value directly testable in regression analysis for a relationship between NSC and photosynthesis. However, mass-based NSC concentration in mg g^{-1} (x/b) regressed against area-based photosynthesis in μmol CO_2 m^{-2} s^{-1} (y/a) will yield a slope of μmol CO_2 g dry mass g^{-1} NSC s^{-1} (yb/xa). The slope yb/xa is directly proportional to y/x only if b/a remains constant over the range of y and x, and in cases of leaf area (a) and mass (b), b/a is expected to change as carbohydrates accumulate or deplete.

Units in area basis become disadvantageous when response variables vary only little among treatments but vary greatly

among treatment replicates. The noise of replicate variation will overwhelm the small response. Leaf area-based NSC likely varies among replicates more than it varies between treatments. We thus used mass-based units in analyzing the response of carbohydrates, and report the results if they depart from the result analyzed using area-based units.

Statistical analysis

We analyzed the effects of girdling treatments on response variables with a linear model ANCOVA with species as a factor and girdling treatments as a scaled independent variable. We assumed that all treatments scale away from control in equal amount (assumed linear contrast coefficients [−1, 0, 1, 2, 3, 4] for treatments surround girdle, control, one-quarter, half, three-quarters and full girdles). These coefficients are justified for partial and full girdles, and only marginally less so for surround girdle. Surround girdle is ordered in relation to treatments and control, and expected response in NSC content for example is surround girdle ≤ control ≤ one-quarter girdle, and so on. Because the treatments are ordered and have the same interval for the most part, treatments should be as a considered scaled variable for a simple linear regression rather than an ordinal variable for monotonic analysis or categorical and unordered for pairwise comparison procedures. Pairwise comparison procedures would needlessly sacrifice power, and monotonic analysis, such as the Abelson–Tukey procedure (Abelson and Tukey 1963), is a type of linear regression that uses fixed contrast coefficients only appropriate when treatments scale non-linearly to a large extent (Abelson and Tukey 1963), an unlikely condition for the treatments in this study. Photosynthesis rate, stomatal conductance and foliar respiration rate were further analyzed with a linear model ANCOVA with three predictor variables, treatment, species and either LMA, C, N or NSC contents as a covariate. Analyzing the 2009 and 2010 data separately and combined yielded similar significance, and we present the result of the combined analysis. All analysis were done in R (R Core Team 2014), with multcomp (Hothorn et al. 2008) and MASS (Venables and Ripley 2002) packages, and plotted with ggplot2 package (Wickham 2009).

Results

Hypothesis 1: increased girdling intensity will increase leaf carbohydrate content

Girdling intensity created no clear, consistent or strong trends in leaf carbohydrate and N content (Table 1), and responses differed among species (Figure 2). Girdling increased area-based glucose and fructose content slightly by 3.4% per one-quarter girdle in all species (Table 1, Figure 2), but only in *Hieronyma* for mass-based glucose and fructose concentration, by 2.2% (1.0 g glucose equivalent m^{-2}, $P = 0.02$, ANCOVA with treatment and species interaction). Girdling intensity slightly increased LMA by 5.5% and C content by 5.4% per one-quarter girdle only in *Virola* (7.1 g dry mass m^{-2}, $P = 0.02$, and 3.8 g C m^{-2}, $P = 0.01$, ANCOVA with treatment and species interaction, Figure 2), did not change starch content (Table 1), and marginally decreased N content in *Hieronyma* by 3.3% and increased in *Virola* by also 3.3% per one-quarter girdle (decreased by 0.07 g N m^{-2}, $P = 0.01$, and increased by 0.11 g N m^{-2}, $P < 0.01$, ANCOVA with treatment and species interaction, Figure 2). The responses were small compared with the variation within treatments (Figure 2). Area-based measures of carbohydrate contents were well related to each other, but less for starch (C with glucose and fructose, $P < 0.01$, $R^2 = 0.84$; C with LMA, $P < 0.01$, $R^2 = 0.98$; C with starch, $P < 0.01$, $R^2 = 0.41$; glucose and fructose with starch, $P < 0.01$, $R^2 = 0.47$). Sucrose content was near zero for all samples and not shown.

Table 1. Predictor variable *P* values for ANCOVA models of girdling intensity and species explaining either C, N, LMA, glucose and fructose, starch or NSC (glucose, fructose and starch). Values <0.05 are in bold.

Response variables	Predictor variables		
	Girdling intensity	Species	Girdling × Species
C content (g C m^{-2})	0.17	**<0.01**	0.10
N content (g N m^{-2})	0.26	**<0.01**	0.08
LMA (g m^{-2})	0.09	**<0.01**	0.15
Glucose + fructose (g m^{-2})	**<0.01**	**<0.01**	0.98
Starch (g m^{-2})	0.49	**<0.01**	0.93

Hypothesis 2: increased girdling intensity will decrease photosynthesis inversely proportional to carbohydrate content

Increased girdling intensity decreased photosynthesis only under full girdling, and *Vochysia* did not respond at all (Figure 3). Girdling intensity was significant in linear ANCOVA for species other than *Vochysia* ($P < 0.01$, $P = 0.82$ for *Vochysia*), but not if full girdling was excluded from analysis ($P = 0.9$ for all species). By the end of treatment duration, full girdling reduced light saturated photosynthesis rate to 31–41% of control in *Hieronyma* (4.1 under full girdle vs 10 μmol CO_2 m^{-2} s^{-1} for control), *Pentaclethra* (1.8 vs 13 μmol CO_2 m^{-2} s^{-1}) and *Virola* (1.8 vs 13 μmol CO_2 m^{-2} s^{-1}), but remained similar in *Vochysia* (10 under full girdle vs 11 μmol CO_2 m^{-2} s^{-1} for control; all at $P < 0.05$ in pair-wise contrast with control, Figure 3). Photosynthesis rate started to decline on the second day after full girdling, continued to decline to the fourth day, and remained low thereafter. Other girdling intensities created no trend in photosynthesis rate (ANCOVA without full girdle, $P = 0.90$, Figure 3). The decline in photosynthesis rate was accompanied by a decline in photosynthetic capacity, with V_{cmax} and J_{max} declining only under full girdling and in three species, similar to photosynthesis. By the end of the treatment duration, V_{cmax} and J_{max} declined to 8.2–24% of control

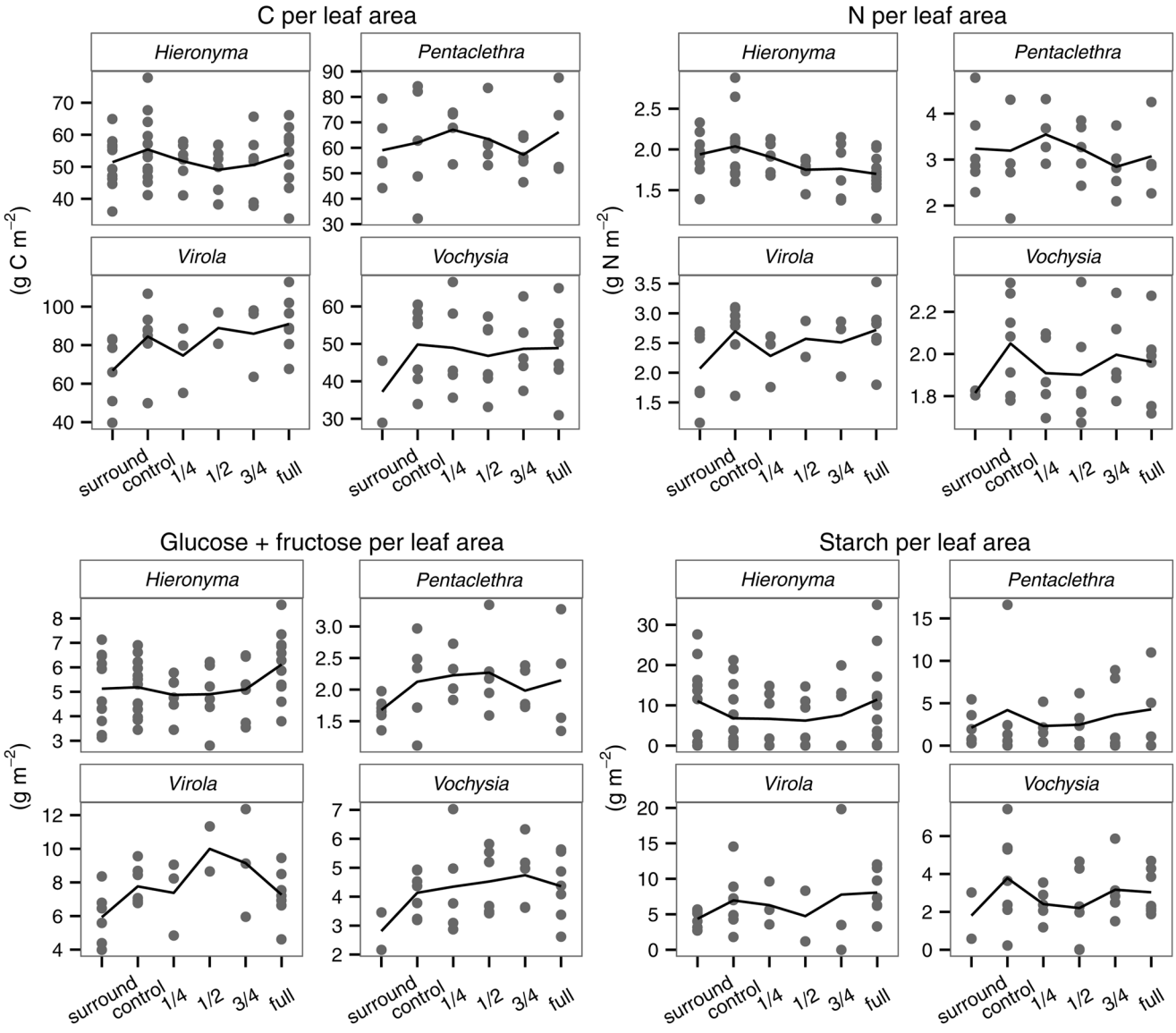

Figure 2. Effects of girdling treatments on C, N, glucose and fructose, and starch content on a leaf area basis (g m^{-2}). The effects show that the increased girdling intensity created no clear trends. Girdling intensity slightly increased glucose + fructose content in all species ($P < 0.01$), C and N contents in *Virola* ($P = 0.01$) and decreased N content in *Hieronyma* ($P = 0.01$). Lines connect treatment means. Response of LMA was virtually identical to C content, with tight relationships between LMA and C content for each species ($P < 0.01$, $R^2 = 0.98$), and not shown here.

in *Hieronyma* (12 vs 50 μmol CO_2 m^{-2} s^{-1} for V_{cmax}; 27 vs 135 μmol CO_2 m^{-2} s^{-1} for J_{max}), *Pentaclethra* (3.1 vs 38 μmol CO_2 m^{-2} s^{-1}; 7.4 vs 129 μmol CO_2 m^{-2} s^{-1}) and *Virola* (6.6 vs 42 μmol CO_2 m^{-2} s^{-1}; 15 vs 114 μmol CO_2 m^{-2} s^{-1}), but remained similar in *Vochysia* (29 vs 38 μmol CO_2 m^{-2} s^{-1}; 58 vs 86 μmol CO_2 m^{-2} s^{-1}; all at $P < 0.05$ in pair-wise contrasts with control). Girdling at less than full had no effect on intercellular concentration of CO_2 ($P = 0.21$).

Stomatal conductance declined in concert with the decline in light saturated photosynthesis rate for full girdle treatments. The decline in stomatal conductance for the full-girdle treatments started within the first 5 days and remained low until the end of the treatment ($P < 0.01$). The decline was 17–26% of control in *Hieronyma* (0.074 under full girdle vs 0.31 mol H_2O m^{-2} s^{-1} for control), ~17% of control for *Pentaclethra* (0.033 vs 0.19 mol H_2O m^{-2} s^{-1}) and 25% of control for *Virola* (0.11 vs 0.42 mol H_2O m^{-2} s^{-1}); *Vochysia* showed no decline (0.21 vs 0.28 mol H_2O m^{-2} s^{-1}; all at $P < 0.05$ in pair-wise contrasts with control). The decline in stomatal conductance was unlikely to have been an artifact of girdling decreasing leaf water potential, because for the one species measured (*Hieronyma*), leaf water potential was unaffected by full girdling at the end of leaf measurements. Stomatal conductance remained unchanged under partial girdling in all species (ANOVA without full girdle, $P = 0.77$).

The decline in photosynthesis under full girdling was not related to accumulation of leaf carbohydrate nor N content

(Figure 4), and may have been an artifact of girdling. Photosynthesis was unrelated to glucose + fructose content ($P = 0.24$, Figure 4) even though photosynthesis decreased under full girdling and glucose + fructose content and increased with girdling intensity. Though only slightly, full girdling diverged from other girdling treatments in the relationship between photosynthesis and leaf C, starch and N content (Figure 4). Under girdling intensities less than full, photosynthesis slightly increased with leaf C and starch ($P < 0.01$), but slightly decreased instead under full girdling ($P < 0.01$). Under girdling intensities less than full, photosynthesis increased with N content per unit leaf area ($P < 0.01$), but decreased slightly under full girdling ($P < 0.01$). These results suggest that full girdling decreased photosynthesis as an unintended artifact, and not because leaf carbohydrates accumulated or leaf N decreased.

Hypothesis 3: increased girdling intensity will increase respiration proportional to carbohydrate content

Respiration rate decreased with girdling intensity for *Pentaclethra*, but was unaffected by any treatment for the rest of the species ($P = 0.31$, Figure 5). Respiration rate differed among species and averaged 0.77, 0.66, 0.76 and 0.88 μmol CO_2 m^{-2} s^{-1} for *Hieronyma*, *Pentaclethra*, *Virola* and *Vochysia*

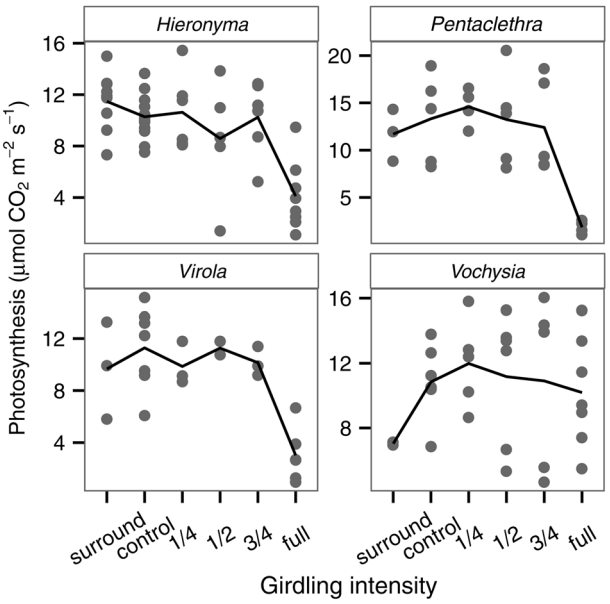

Figure 3. Response of light saturated photosynthesis rate to girdling intensity at the end of treatment duration. Girdling intensity decreased photosynthesis rate only under full girdling, in *Hieronyma*, *Pentaclethra* and *Virola*, but not in *Vochysia*. Lines connect treatment means.

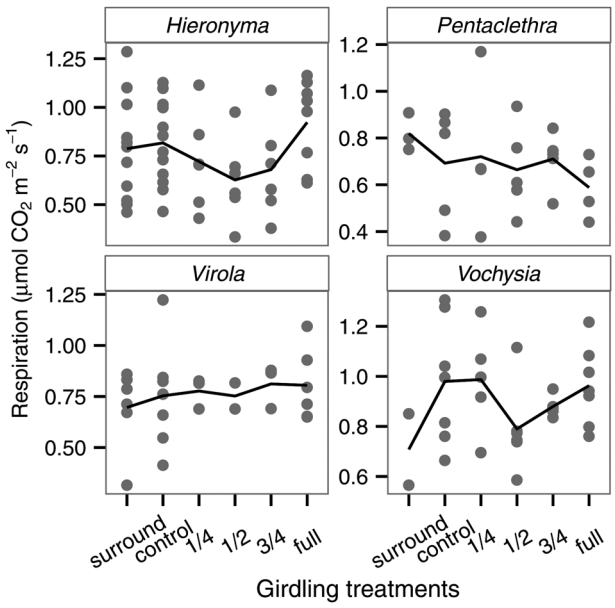

Figure 5. Foliar respiration shows no response to girdling treatments. Lines connect treatment means.

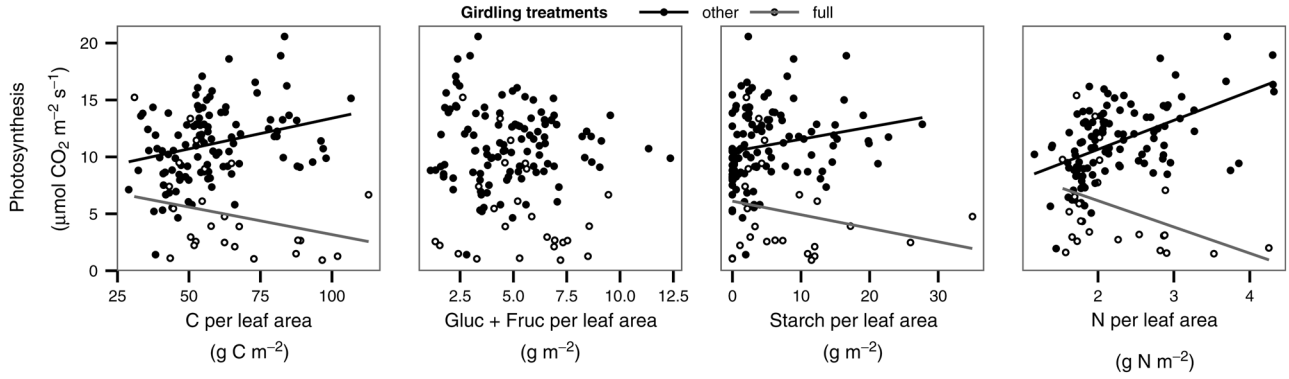

Figure 4. Correlations between light saturated photosynthesis rate and per leaf area contents of C, glucose + fructose, starch or N, showing that full girdling reduced photosynthesis by mechanisms unrelated to leaf carbohydrate or N contents. Open circles and gray lines represent values and trend lines for full girdling intensity, and filled circles and black lines represent values and trend lines for other girdling intensities and control. Photosynthesis was unrelated to glucose and fructose content ($P = 0.24$). Photosynthesis decreased under full girdling but increased under other treatments with C (top right, $P < 0.01$, $R^2 = 0.39$; lines drawn with $y = -0.048x + 8.0$ for full girdling, and $y = 0.054x + 8.0$ for other treatments), starch ($P < 0.01$, $R^2 = 0.40$; $y = -0.12x + 6.1$ for full girdling, and $y = 0.11x + 10.5$ for other treatments) and N ($P < 0.01$, $R^2 = 0.47$; $y = -2.4x + 10.5$ for full girdling, and $y = 2.6x + 5.1$ for other treatments).

($P < 0.01$). Respiration rate increased with N in three species ($P < 0.01$) and marginally in *Vochysia* ($P = 0.06$). Respiration rate increased with glucose + fructose and C content in *Hieronyma* and *Virola*, but decreased with C content in *Pentaclethra* and *Vochysia* ($P < 0.01$). Foliar respiration increased as starch content increased in *Hieronyma* ($P < 0.01$), but was unrelated to respiration in the three other species ($P > 0.18$).

Discussion

The results did not support any of our hypotheses (Figure 6). Most of the measured variables (carbohydrate concentrations, photosynthesis, and photosynthetic capacity and respiration) did not vary with girdling intensity less than full in any species. Only glucose and fructose content increased with girdling intensity, and while full girdling lowered photosynthesis in three of four species, the decrease in photosynthesis was unrelated to glucose and fructose content. Respiration did not respond to the treatments. These results suggest that leaf carbohydrates may remain unchanged under whole tree carbon imbalance, and even when they do change, leaf carbohydrates may not directly regulate photosynthesis or respiration.

Leaf carbohydrate pool has one incoming flux, photosynthesis, and two outgoing fluxes, respiration and phloem export. Even with three-quarters of phloem removed, the pool size, the incoming flux and one outgoing flux did not change. Phloem function must then be flexible.

Flexible phloem transport helps maintain constant leaf carbohydrate content

We hypothesized that partial girdling would trigger carbohydrate accumulation in leaves because partial girdling reduces the number of functioning phloem sieve tubes and thus slow phloem export, if phloem export rate per unit phloem is fixed (Figure 1). The results indicate that phloem export rate per unit phloem had indeed increased under partial girdling. That full girdling severs phloem and dramatically decreases phloem export is confirmed by studies that show the connectivity of photosynthesis and soil CO_2 efflux using full girdling (Högberg et al. 2001, Andersen et al. 2005, Olsson et al. 2005, Binkley et al. 2006, Frey et al. 2006, Levy-Varon et al. 2012). However, with as little as one-quarter of phloem area intact, leaf carbohydrates remained unchanged without any adjustment in C input (photosynthesis) or loss (respiration). Other mechanisms such as increased growth of terminal buds may have played a role, but consider for example a leaf that respires 0.77 μmol CO_2 m^{-2} s^{-1} (mean for this study) during the night, with net photosynthesis averaged for the day in the range of 4.1–11.1 μmol CO_2 m^{-2} s^{-1} (mean under full girdle and control). This leaf would export 1.7–5.4 g C in 24 h with all of the phloem area intact. If partial removal of phloem area reduces the export rate to a quarter, the leaf would accumulate 13–40 g C m^{-1} in 10 days without any changes in photosynthesis or respiration. We did not see a significant growth to account for this accumulation. It is thus difficult to deny that phloem export rate per unit phloem had increased under partial girdling.

For carbohydrate transport to occur through reduced phloem area, the transport path must change across phloem sieve tubes, analogous to changing lanes on a highway. In terminal branches, each functioning phloem sieve tube likely connects to minor veins of a leaf. The phloem sieve tubes directly connected to a leaf, not those connected to another leaf, may conduct most of the phloem export out of the leaf, where the transport path is restricted to the vertical section extended down from the petiole. This sectorial phloem transport has been confirmed in herbaceous plants (Watson and Casper 1984, Marshall 1996, Vuorisalo and Hutchings 1996, Fetene et al. 1997, Preston 1998) and in an oak (De Schepper et al. 2013*a*). Phloem export from a leaf thus occurs through a specific subset of sieve tubes surrounding a branch, and the leaf loses the primary transport conduit when those specific sieve tubes no longer

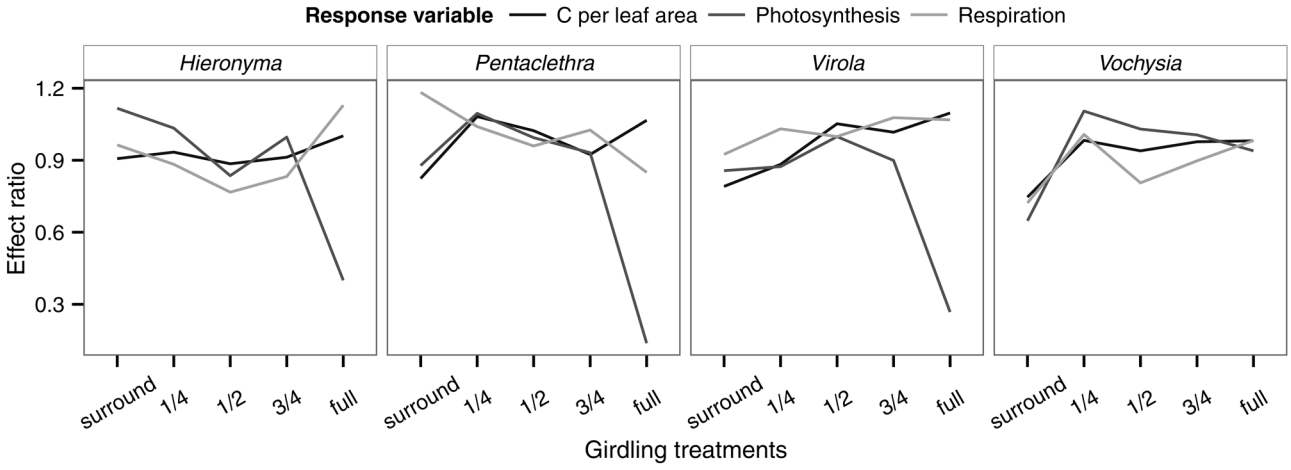

Figure 6. Summary of the effect ratios treatment to control for each response variable under girdling treatments shows that photosynthesis declined under full girdling disproportionately compared with other response variables under the rest of the treatments, and that the responses depended on species.

function. This is what we assumed in our hypothesis that carbohydrate transport does not change lanes and simply comes to a halt at the girdle. Carbohydrates then accumulate in the leaf. However, the results suggest that at least in three of the four species, carbohydrate transport changed lanes from original sieve tubes blocked at the girdle to another set of sieve tubes still functioning that serve a different leaf. De Schepper et al. (2013*a*) also observed that partial girdling rerouted the sectorial phloem flow in oak, and speculated that the mechanism for this lane change may be the leakage and retrieval system along a phloem path. Along a phloem transport path to a terminal sink, carbohydrates continually flow out from phloem to feed lateral sinks, and a portion is reloaded back on to phloem (Thorpe and Lang 1983, van Bel 2003, De Schepper et al 2013*b*). This leakage and retrieval system may allow carbohydrates to flow laterally among phloem tubes, allowing for carbohydrate transport to change lanes in the case of obstruction. The retrieval occurs mainly through active loading pathways (Patrick et al. 2001), and should lead to a higher respiration rate of branches above the partial girdle.

The lane changing of phloem transport would still result in leaf carbohydrates accumulating, unless the remaining phloem sieve tubes have high conductivity capable of supporting increased transport rate per unit phloem. As phloem transport seemed to have rerouted through one-quarter of the original quantity of sieve tubes, each sieve tube may be highly conductive, capable of supporting transport rates much greater than the leaf directly connected can generate. De Schepper et al. (2013*a*) observed reduced phloem flow rate under partial girdling, but we detected no such evidence in three of four species. Phloem conductivity likely differs among species as leaf carbohydrate content slightly increased with girdling intensity in *Virola*, while it remained unchanged even after 20 days under three-quarters girdling in *Hieronyma*. Species difference in phloem conductivity deserves further investigation. It may be a key factor that determines whether carbohydrate regulation of photosynthesis and respiration occur, thus reconciling our results with previous findings.

Though individual sieve tubes may have high conductivity, at branch level partial girdling does decrease the number of functioning sieve tubes, reduce total branch phloem conductivity and increase the resistance to phloem transport. The partial girdling results thus suggest that the species tested increased phloem loading for the branch to counter the increased resistance, and maintained leaf carbohydrate content unchanged. Though debated for trees (Thompson 2006, Knoblauch and Peters 2010, Mencuccini and Hölttä 2010, Jensen et al. 2011), phloem transport is generally accepted to be driven by hydrostatic pressure: carbohydrates are loaded on to phloem sieve tubes for water to follow by osmosis, generating turgor pressure that drives transport downstream (Münch 1930, van Bel 2003, Jensen et al. 2011). Thus phloem loading generates a carbohydrate concentration gradient that regulates the transport rate. Leaf carbohydrates did not accumulate even with three-quarters of phloem severed in most species, indicating that the carbohydrate level was maintained—exported through much fewer phloem sieve tubes—by generating much greater turgor pressure. Thus phloem loading likely increased with girdling intensity to maintain phloem transport rate and leaf carbohydrate content. As woody species tend to have passive loading at the leaf minor veins (Gamalei 1991, Rennie and Turgeon 2009, Fu et al. 2011), the site of regulation may lay downstream. Regulation of phloem loading deserves further attention in order to improve understanding of carbohydrate regulation of photosynthesis and respiration.

Full girdling may affect photosynthesis through changes other than carbohydrate accumulation

Accumulating glucose + fructose content was unrelated to the decline in photosynthesis under full girdling, raising the possibility that an unintended change caused the decline in photosynthesis. Because girdling severs phloem, it stops the export of not just carbohydrates but of all materials including auxin, abscisic acid and reactive oxygen species (Mahouachi et al. 2009, Turgeon and Wolf 2009, Turnbull and Lopez-Cobollo 2013). These materials may have accumulated simultaneously yet independently of carbohydrates, directly causing stomatal closure, which in turn reduced the measured photosynthesis rate (Setter et al. 1980, Harrell and Williams 1987, Roper and Williams 1989). In some girdling studies, photosynthesis declined before carbohydrates accumulated in citrus (Nebauer et al. 2011), only under high temperature and independently of carbohydrate accumulation in young apple (Fan et al. 2010), and not at all in oil palm (Legros et al. 2009).

The unintended changes likely reduced stomatal conductance as well, and the reduction is unlikely to have come from decreased xylem hydraulic conductivity. The reduction was not immediate, occurred only under full girdling, and not at all in *Vochysia*. Though only measured on *Hieronyma*, midday leaf water potential increased under full girdling (–0.74 MPa under full girdling and –1.1 MPa under control; $P = 0.02$), reflecting the stomatal closure observed. Previous girdling studies observed similar reduction in stomatal, leaf conductance or transpiration rate (Harrell and Williams 1987, Proietti 2003, Franck et al. 2006, Urban and Alphonsout 2007, Domec and Pruyn 2008, Wu et al. 2008, Fan et al. 2010, Urban et al. 2010, Nebauer et al. 2011). They attributed the cause to assimilate accumulation because girdling had no effect on predawn and midday water potential or leaf water content (Proietti 2003, Franck et al. 2006, Domec and Pruyn 2008), or because girdling had no effect on internal concentration of CO_2 (Harrell and Williams 1987, Proietti 2003, Urban 2004, Urban and Alphonsout 2007, Wu et al. 2008, Fan et al. 2010). These studies suggest that stomata respond to decreased photosynthesis from assimilate concentration, to

optimize carbon gain and water use (Mäkelä 1996, Nikinmaa et al. 2013). However, it remains unclear which factors, hormones, reactive oxygen species or carbohydrates, caused the decline in stomatal conductance and photosynthesis, and girdling alone cannot provide clear evidence.

Does leaf carbohydrate content indicate plant C balance?

Leaf carbohydrate content mostly remained unchanged and well above zero at all girdling intensities, suggesting that leaf carbohydrate content may be decoupled from whole plant carbon balance under some conditions. Studies have used as evidence the presence or lack of change in non-structural carbohydrate pool to infer carbon balance, for both negative (Lacointe et al. 2004, Palacio et al. 2012) and positive (Hoch et al. 2003, Chew and Bonser 2009, Sanz-Perez et al. 2009). Our results suggest that neither interpretation warrants confidence unless mechanisms regulating leaf carbohydrates become clearer. Leaf carbohydrates do not indicate whole plant carbon balance if carbohydrate content can remain unchanged in fully girdled leaves with decreased photosynthesis, and when carbohydrate export can proceed seemingly unaltered with only one-quarter of phloem intact. However, it is logically impossible for carbohydrate reserves to not reflect plant carbon balance at the whole plant level to some extent. A plant in dark must reduce carbohydrate reserves to keep respiring, for example. Rather, our results show that this logic should not be extended to leaves. Some additional mechanisms regulate leaf carbohydrates regardless of whole plant carbon balance, and mechanisms are likely mediated through phloem transport.

Sink regulation of photosynthesis assumes a passive buildup of carbohydrates when sink activity declines, but this assumption weakens as more precise understanding emerges on the regulation of leaf carbohydrate content and phloem transport. *Arabidopsis* actively regulates leaf starch content diurnally to match day-time gain and night time use (Gibon et al. 2004, Scialdone et al. 2013), and accumulates carbohydrates when starved of C (Smith and Stitt 2007). Our results suggest that some tree species also tightly regulate carbohydrate content through phloem transport and sink activity. Sink regulation of photosynthesis may still occur, perhaps through signaling pathways other than directly by carbohydrate accumulation, and understanding of such pathways would improve our ability to model and predict plant carbon balance.

Conclusion

Girdling intensity did not change leaf carbohydrates in most species tested. Though glucose and fructose slightly increased in all species, total C content and LMA increased only in one species, and starch did not change. Only full girdling lowered photosynthesis in three of four species, but the decrease in photosynthesis was unrelated to the increase in glucose and fructose content. Girdling did not affect respiration. The results suggest that leaf carbohydrate content does not directly regulate photosynthesis or respiration and may be decoupled from whole plant carbon balance by phloem transport. The capacity for phloem transport, both phloem loading and conductivity, may be high, and phloem transport rate and path may change rapidly. Regulation of phloem transport is thus a key factor in understanding how trees regulate carbon balance at the whole tree level. However, girdling may not be an effective method of study, at least in some species, because girdling may induce physiological changes unrelated to carbohydrate accumulation. Carbohydrate regulation of photosynthesis and respiration may not be as common in trees as previous girdling studies have suggested.

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Conflict of interest

None declared.

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