Whole-plant Transpiration in *Populus* sp.: Its Determination, Nocturnal Effects and Influence by Form of Nitrogen

by

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Abstract

Water is a crucial factor in the life of land plants. Transpiration (E) is inevitable and it is viewed as the universal cost of accessing a CO_2-rich atmosphere. Although we now know that plants lose water at night, it had been assumed that stomata remained closed after dark. Renewed interest has encouraged more research on night-time E. An overlooked challenge in this area is that of measuring water loss. Most of the current research relies on leaf-level measurements using gas analysers. This presents many complications and although the instruments are simple to operate, it is not a trivial task to measure E properly, especially at night. I present a system that includes instructions to build the hardware platform and the software package, which utilises balances to measure E simultaneously and continuously from multiple specimens. The system is particularly suited to measuring nocturnal E of whole plants since it requires minimal interaction and it is not intrusive for the plant. With this system, a survey of whole-plant nocturnal E was carried out on four species of *Populus* with a range of habitat preference from riparian to upland. This survey characterises night-time water loss in relation to habitat, showing that *Populus* from drier environments may curtail night-time E more readily than riparian species. The survey also investigates the effect of drought on nocturnal E, arguing that night-time sap flow may be associated with one or more physiological functions. Lastly, the effect of two forms of N (NO_3^- and NH_4^+) on whole-plant stomatal conductance is analysed in two hybrid poplars with different growth potentials. NH_4^+ is shown to depress day-time, but not night-time conductance in

comparison with NO_3^-; likely due to a change in stomatal function. Root anatomy was radically changed in a species-dependent manner when grown under different N forms. Some of these changes reflect different metabolic needs associated with each N form, while others may have a direct bearing on plant hydraulics.

In memoriam Miguel Cirelli, my source of ‘newén’

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Many close friends have been by my side, some literally and some metaphorically due to distance, and some both. I am particularly grateful to Walter Araujo, Darío Ganem and Juan Pablo Peralta for their constant encouragement. I have leaned on many others; space does not permit a full list. I would be remiss not to acknowledge their influence on me and therefore their influence on my work, which may be meager but never for lack of conscience, a good deal of which is

reinforced by these rock-solid friends. They are my patrimony and I can only hope that they may be proud of my small achievements as I am proud of their friendship. Thank you.

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Chapter 1. Introduction

Transpiration

Plant transpiration (E) can be described in simple terms by the generally-accepted soil-plant-atmosphere continuum model based on Darcy's law in which water evaporation from the leaf is directly proportional to conductance and driving force, such that:

$$E = g_L\left(\Psi_{leaf} - \Psi_{air}\right) \text{ (eq. 1-1)}$$

$$J = \mathrm{K}_{plant}\left(\Psi_{soil} - \Psi_{leaf}\right) \text{ (eq. 1-2)}$$

$$E \equiv J \text{ (eq. 1-3)}$$

where g_L: leaf hydraulic conductance which includes the mesophyll-to-substomatal chamber and stomatal conductances (g_m and g_s respectively); Ψ: water potential; J: water flux; and K_{plant}: whole-plant hydraulic conductance. Incidentally, this model works on the assumption that, once a steady state is reached, the sap flow (J) is equivalent to the evaporation rate (E) to satisfy the premise that all water lost must be replaced (Sperry 2000).

A few problems arise when trying to determine g_L; such discussion is beyond the scope of this thesis. However, a more common measurement of the conductance to the vapour phase involves substituting the vapour pressure deficit (VPD) of the atmosphere (i.e. the partial pressure of water vapour needed to attain air saturation), as the force driving evaporation, obtaining:

$$g_s = \frac{E}{VPD} \text{ (eq. 1-4)}$$

where g_s represents the stomatal conductance to water vapour.

Traditionally, transpiration has been seen as an unfortunate by-product of land colonization (Raven 2002). Biology textbooks have deemed it as an "unavoidable evil" which stands between the photosynthetic apparatus and the CO_2 resource (Raven et al. 2005). This trade-off between carbon fixation and water loss through the stomata or “the dilemma of opposing priorities” (Raschke 1976) has driven the development of conceptual models based on stomatal optimization of gas exchange as well as the continuous search for improving the ratio of carbon fixed to water lost, i.e. water use efficiency (WUE), of economically important species. Within this view, the increasing evidence that many species maintain substantial stomatal conductance and transpirational water loss during the night has discredited the long-held assumption that stomata remain closed during the night. At the same time, this challenges us to explain the significance, if any, of a process that according to some, should have been fixed in evolution.

Current accounts of average water loss during the night estimate that nocturnal transpiration rates (E_N) range from 5 to 30% of day-time rates (Caird et al. 2007). An intense area of research has then been established in order to assess the magnitude of nocturnal transpiration in different species, habitats and the possible functional role of it. This increasing body of research suggest that a new paradigm might be developing in which transpiration is held as a "design feature" of plants instead of an impediment to productivity (reviewed in Cramer et al. 2009). Although knowledge about nocturnal transpiration (E_N) of trees is increasing and its impact on regional water and carbon balance has been recognized, the costs and benefits of high night-time stomatal conductance (g_N) and high E_N remain largely

unknown. The apparent "wastefulness" of water has prompted the question of whether nocturnal transpiration confers a functional benefit to the plant. Although it is possible that nighttime water loss may not necessarily be adaptive and may simply result from incomplete stomatal control (Cavender-Bares et al. 2007), several hypotheses have been proposed regarding functional benefits that may be associated with nocturnal water loss such as:

a. Maintaining the stomata in a "pre-opened" state may reduce diffusional limitations to CO_2 uptake, allowing photosynthesis to begin without delay at sunrise when VPD is low (e.g. Bucci et al. 2005; Dawson et al. 2007; Auchincloss et al. 2014).
b. Night-time sap flow (driven by transpiration through stomatal opening) may increase the supply of oxygen to xylem parenchyma cells to maintain respiration (Gansert 2003).
c. Nocturnal transpiration may enhance nutrient uptake or nutrient transport to distal parts of the plant by mass flow (Daley and Phillips 2006; Caird et al. 2007).

The possibility that nocturnal transpiration may assist with nutrient uptake by mass flow has recently received a great deal of attention. However, experimental results in this area have been contradictory, possibly due to variability in plant species and experimental conditions such as nutrient concentration, form of nutrients and the methodology used to assess nocturnal water loss (e.g. Scholz et al. 2007; Snyder et al. 2008; Howard and Donovan 2007, 2010; Christman et al. 2009; Kupper et al. 2012; de Dios et al. 2013). Overall, there is no clear

experimental support for any of these hypotheses and the evolutionary and ecological role of nocturnal transpiration as well as the mechanisms underlying night-time stomatal regulation remain as open questions (see de Dios et al. 2013; Auchincloss et al. 2014 and references therein).

Populus and diurnal/nocturnal transpiration

The genus *Populus* contains approximately thirty species distributed in a wide range throughout the northern hemisphere (Eckenwalder 1996). The species are divided into poplars, cottonwoods and aspens, based on genetic and ecophysiological characteristics (Eckenwalder 1996; Hamzeh and Dayanandan 2004). Cottonwoods and poplars are found in riparian areas and other moist habitats, while aspens are generally upland species and occupy more seasonally-dry areas (Kranjcec et al. 1998). Poplars play a major role in the ecology of a wide range of landscapes in the northern hemisphere and they represent an important economic resource as they exhibit some of the fastest growth rates in temperate forests (Stettler et al. 1996; Monclus et al. 2005). The genus is also becoming increasingly important as a model plant, not unlike *Arabidopsis*, for the study of a wide array of molecular mechanisms that are of interest to both forestry and basic plant biology (Taylor 2002).

The fast growth of hybrid poplars has been the main incentive for their establishment in fibre plantations. The government of Canada is also using incentives to encourage hybrid plantations in order to meet Canada's carbon sequestration targets (Natural Resources Canada 2005). Hybrid poplars are highly demanding in both water and nutrients (Blake et al. 1996; Heilman et al. 1996) and,

therefore, meeting growth rate expectations in relatively marginal areas such as the boreal regions, with relatively short growing seasons, requires a deep knowledge of the interaction between water and nutrient use. During the past 10 years a notable amount of research has been done to identify hybrid poplar clones with high productivity (e.g. Bassman and Zwier 1991; Barigah et al. 1994; Rae et al. 2004; Monclus et al. 2005). There has also been considerable work to understand the relationships between growing potential and water use efficiency and how these factors are affected by water stress and nutrition (e.g. Rhodenbaugh and Pallardy 1993; Liu and Dickman 1996; Harvey and van den Driessche 1999; Marron et al. 2002; Monclus et al. 2006).

The high productivity of poplars has been correlated numerous times with large water requirements (Wullschleger et al. 1998 and references therein), especially compared with other temperate species in which the water use can be 1 or 2 orders of magnitude less (see table in Wullschelger et al. 1998). Numerous studies link (or rather correlate) the hydraulic conductivity of various plant parts with productivity, thus hinting at a relationship between plant-water relations and growth (Sperry et al. 1993; Van der Willigen and Pammenter 1998; Atkinson et al. 2003; Tyree 2003). Thus, several questions arise regarding the extent to which nocturnal transpiration might have a significant role in the overall water-balance of *Populus* species. In a meta-analysis that included multiple species with different growth habits and from different habitats, *Populus* appears in the upper range of the reported values of nocturnal water loss (Caird et al 2007). However, what variation and range exist among species of the same genus that occupy different ecological

niches (e.g. riparian versus upland) remains to be explored. Also, it is still unclear how g_N and E_N are regulated by environmental factors such as leaf-to-air vapor pressure difference and soil water content, since contrasting patterns have been found in some species (Dawson et al. 2007; Phillips et al. 2010; Zeppel et al. 2010).

In exploring the relationship between mineral nutrition and nocturnal transpiration, recent studies have focused on the effects of nutrient availability (particularly NO_3^-) on E_N (Howard and Donovan 2010; Kupper et al. 2012). However, results have been contradictory, with either no changes in E_N when plants were grown at different N concentrations (in *P. balsamifera* and *P. angustifolia*, Howard and Donovan 2010) or increasing E with higher N concentration (in *P. tremuloides*, Kupper et al. 2012), while in other species fertilization seems to reduce E_N (Scholz et al. 2007). Clearly, more work is needed elucidate patterns (or point to a lack thereof) of nutrient availability and nocturnal water use. Finally, although it may seems rather obvious, it is necessary to emphasize the need for precise quantification of nocturnal water loss at the whole plant level, since in the majority of cases analyses have been done using measurements at the leaf level, without consideration of scaling issues.

Methods of quantifying nocturnal transpiration

Some methods are more accurate and/or have less uncertainty than others for the estimation of E_N/g_N and their relative contribution to the daily water budget. Because nocturnal transpiration rates are typically low, accurate quantification by widely used instantaneous gas exchange measurements is questionable (Barbour et al. 2005). Moreover, gas exchange measurements are typically performed on the

"youngest fully-expanded leaf", which is not necessarily representative of the entire leaf population (e.g. Wilson et al. 2000; Pataki and Oren 2003). Sap-flow methods typically have attendant uncertainties as to the proportion of the measured flux resulting in bole refilling rather than transpiration from the canopy (Caird et al. 2007). However, some studies have been able to separate transpiration and refilling components by using modeling approach based on refilling time constants (Bucley et al 2011). Other recent studies done at the stand level or with big trees have combined measurements of sap flow with leaf-level gas exchange measurements using an infra-red gas analyser (IRGA) (Moore et al. 2008; Philips et al. 2010; Sellin et al. 2010; Zeppel et al. 2010).

Gravimetric assessment of plant water loss can provide an accurate measurement of whole-plant/canopy transpiration (E) and conductance (g = E / VPD, see equation 1-4) independently of the movement of water into or out of the tissue compartments (Cavender-Bares et al. 2007). It is then surprising that high-resolution gravimetric measurements are rare. Caird et al. (2007) summarized a large number of studies on nighttime conductance that span from 1952 to 2007. Out of the 57 papers, 53% have used gas exchange and/or sap-flow methods, while gravimetric methods (lysimeters or balances) represented 15% of the studies. I followed up this analysis by looking at the methodology employed by papers on nocturnal transpiration that have been published between 2007 and 2014. Out of 36 papers, the use of gas exchange and/or sap flow methods have increased to 88% while gravimetric methods have been used only in 2 cases (4%) (Figure 1-1).

Lysimeters have been used for a long time, with the first lysimeter built in 1875 (Sturtevant 1919). Most lysimeter applications concentrate on larger scales often for agronomical purposes (large grass patches, crops, etc.) although large trees have also been measured (*e.g.* Edwards 1986). Since weighing lysimeters are gravimetric by nature, they provide an accurate measurement of water loss. However, custom-built lysimeters tend to be expensive and cumbersome with special interfaces, and consequently their use in experimental setups is limited and, once again, with larger scales in mind (plot-level as opposed to plant-level scale, *e.g.* Flury et al. 1999; Liu et al. 2002). Regular laboratory balances on the other hand are relatively inexpensive and have almost a commodity status as they are indispensable in virtually any plant biology-related research. Furthermore, properly equipped balances can be easily interfaced through a serial RS-232 port making them equally suitable for use with either a computer or a simple custom micro-controller (μC) device and/or data-logger. The technique described here refers to the use of laboratory bench balances in a greenhouse setting but these balances can be 'ruggedized' to function in moderately harsh environments.

Balances present themselves as great tools for real-time accurate measurements of water loss, and although their use is limited to potted plants, it should be the obvious choice for whole-plant level physiological studies. In their paper, Cavender-Bares et al. (2007) utilize an approach similar to the proposed methodology here. However, they implemented a point-solution for their study and did not describe their system in any detail. Moreover, they recorded a single weight value every 5 minutes and calculated transpiration over that period.

The system presented in Chapter 2 improves on this concept by directly measuring transpiration as the slope of 10-second values over 5 minutes. This improves resolution and smooths environmentally-induced fluctuations. The complete system is composed of three parts: *i*) balances and computer to record weights, *ii*) humidity, temperature and light sensors (plus computer to log), and *iii*) a non-destructive leaf area measuring system which can be analog (such as a ruler) or digital (such as caliper-based, or imaging-based).

Variation of g_N in *Populus* species from different habitats and interaction with drought

The magnitude of water loss occurring during the night depends on g_s and the vapour pressure difference between leaves and the air, as well as canopy structure and wind speed. While g_N has been recorded at up to 90% of daytime conductance, nighttime transpiration rates (E_N) range from 5 to 30% of daytime rates since nighttime VPD is typically lower than during the day (Caird et al. 2007). It has been suggested that g_N and E_N may be most prominent in fast-growing, shade-intolerant tree species from environments with stable and high water availability (Dawson et al. 2007). Nocturnal conductance and transpiration have been measured in natural stands of *Populus balsamifera* (Snyder et al. 2003) as well as in greenhouse-grown *P. trichocarpa* and *P. angustifolia* (Howard and Donovan 2010), and they are in the upper range of values reported in the literature. Depending on environmental conditions, E_N can represent a significant percentage of the diel water budget in

Populus, although how much variation occurs among *Populus* species that occupy habitats with different water availability has not been assessed.

Substantial E_N has been found to prevent equilibration between water potential of soil and leaves resulting in lower-than-expected leaf water potentials, as well as to reduce the magnitude of hydraulic redistribution during the night period in some species (Donovan et al. 2003; Bucci et al. 2005; Kavanagh et al. 2007; Howard and Donovan 2010). It has been proposed that the effect of E_N on hydraulic redistribution may affect plant performance and reduce nutrient cycling during drought periods (Howard and Donovan 2010). In order to explore the involvement of E_N in preventing pre-dawn water potential equilibrium in *Populus*, I have measured the pre-dawn water potential of transpiring (uncovered) and non-transpiring (covered) plants in different *Populus* species. In all cases, pre-dawn water potential from uncovered plants was significantly lower than in covered plants, showing a significant effect of nocturnal transpiration on whole-plant water status (Cirelli, unpublished). The difference between nominally equilibrated and non-equilibrated plants ranged from 1.9 to 0.9 bars, which shows a large degree of variation among *Populus* species. This created the need to survey g_N and E_N in species of the same genus which occupy different habitats.

The magnitude of g_N (and E_N) is generally significantly higher than the minimum leaf conductance (measured after water stress and/or ABA treatment), which suggest that the reported values of g_N are largely due to stomatal opening and should be under guard cell regulation (Howard and Donovan 2010). In fact, it has been observed that under well-watered conditions, g_N declines with increasing

VPD, providing evidence for stomatal regulation of nocturnal transpiration (Caird et al. 2007; Cavender-Bares et al. 2007). However, this is not always the case, and it can be inferred by the various contrasting data available, that stomatal regulation by VPD is highly species-specific (Phillips et al. 2010; Zeppel et al. 2010; Dawson et al. 2007). In addition, the influence of soil water status on E_N has also been found to be variable across species (Dawson et al. 2007; Phillips et al. 2010; Zeppel et al. 2010).

The response of nocturnal stomatal conductance to various environmental conditions (chiefly water availability and atmospheric demand) may or may not interact with the various systems that respond to light. It is possible that its regulation happens through different mechanisms than those operating during the day (Ogle et al. 2012) and that stomatal response to VPD may be subjected to an underlying circadian clock (Howard and Donovan 2010). Different patterns have been observed in different *Populus* species (Howard and Donovan 2010) but they have not been tied to any particular set of variables. Based on data obtained from different oak species, Cavender-Bares et al. (2007) proposed that differences in mean g_N might be related to differences in stomatal pore index (SPI = stomatal density × stomatal length), although their analysis did not discriminate between SPI and actual stomatal opening, which they also suggest as a partial contributor. Whether differences in SPI can explain differences in absolute E_N among *Populus* species remains unknown. This, together with the influence of drought on whole-plant E_N, is part of the focus of Chapter 3.

Effect of nitrogen form on transpiration and conductance in two hybrid poplars with different growth potential

The possibility that nighttime water loss may be regulated in response to soil nutrient availability has received a great deal of attention (e.g. Scholz et al. 2007; Snyder et al. 2008; Howard and Donovan 2007, 2010; Christman et al. 2009, Kupper et al. 2012; de Dios et al. 2013). If maintaining a flux of water toward roots at night can decrease the formation of nutrient depletion zones, then increased E_N could be beneficial when water is plentiful and mobile nutrients are scarce. This idea is supported by the Barber-Cushman model of root nutrient uptake which predicts that increased water flux to the root rhizoplane will decrease nitrate depletion zones around roots (Barber and Cushman 1981). However, no consensus has been found among studies assessing the effect of E_N on nutrients (mostly NO_3^-) uptake (*e.g.* Ludwig et al. 2006; Snyder et al. 2008; Christman et al. 2009). While g_N has declined in response to long-term N fertilization in some species (Scholz et al. 2007), no regulation of g_N in response to long-term nitrogen limitation has been found in other species, *e.g. Helianthus* (Christman et al. 2009) and *Arabidopsis* (Howard and Donovan 2007).

Among Populus species, NO_3^- can modulate E_N but this observation is not consistent, and more precise experimental conditions may be necessary (Howard and Donovan 2010). Hybrid aspen plants (*P. tremula* × *P. tremuloides*) grown under high N conditions are reported to have higher night-time sap flux and higher night-time water use relative to daytime, in contrast to plants under N-limited conditions (Kupper et al. 2012). These authors suggest that lower night-time water flux under

N deficient conditions could be characteristic of fast growing species because "they are adapted to fertile soils". Most of the studies have focused on NO_3^--fertilization, since, as a mobile form of N, its acquisition is more likely to be affected by changes in transpiration than the less-mobile NH_4^+ would be (Cramer et al. 2009; Howard and Donovan 2010). In consequence, NH_4^+ has not been systematically compared with NO_3^- as a possibly different regulatory form of N in connection to water use (Matimati et al. 2014), particularly at night. Matimati et al. (2014) observed that *Phaseolus* seedlings that were prevented from intercepting urea (a source NH_4^+) had a twofold increase in transpiration rates, which could indicate that transpirationally-driven mass flow may also help to deliver N in NH_4^+ form.

The form of N is known to have a significant effect on plant growth. The NH_4^+:NO_3^- ratio optimum for plant growth shows substantial variation among species and, as such, N-form preference is considered to exert a significant influence on community structure and forest succession (Kronzucker et al. 2003; Boudsocq et al. 2012). In boreal forests, the shift in inorganic N from NO_3^- to NH_4^+ has been proposed to be one of the driving forces behind forest succession (Kronzucker et al. 1997; Kronzucker et al. 2003). In this paradigm, early successional species such as *Populus* are expected to grow better in the presence of NO_3^- while conifers are expected to favour NH_4^+. However, neither soil characteristics of aspen-dominated boreal forests (where NH_4^+ is prevalent), nor experiments of N-form in different *Populus* species, fully support this hypothesis. Surprisingly, the effects of N form on physiological responses that are tightly linked

with plant growth, such as plant transpiration and carbon assimilation, have been scarcely analyzed in poplar species.

Objectives, hypotheses and overview of the chapters

The main objective of the present thesis was to investigate the importance of nocturnal whole-plant stomatal conductance (G_N) and transpiration (E_N) in the diel plant water budget of *Populus* species, and whether night-time water loss may be functional rather than accidental.

Firstly, Chapter 2 presents an original software package (Amalthea) and a design to create a system for measuring transpiration using laboratory balances based on readily available hardware. The system is modular, capable of multiple-balance synchronisation and is highly scalable from one to one hundred units. Its flexibility accommodates varied applications such as monitoring nighttime transpiration or long term drought treatments. The software runs under GNU/Linux and it requires little computer resources. Reporting of transpiration rates is based on linear regressions of data from pre-set intervals which yields high resolution and provides some level of noise filtering.

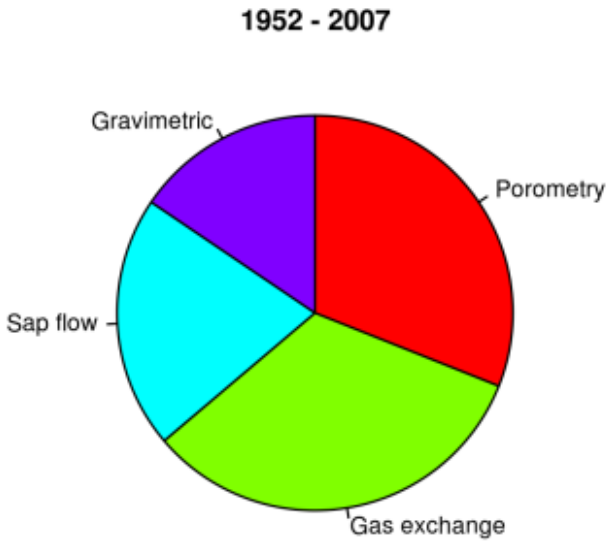
Secondly, Chapter 3 investigates the relationship of day-time transpiration (E_D) and night-time transpiration (E_N) under well watered and drought conditions in four *Populus* species (including a hybrid) from different habitats. In particular, my objectives were: 1—to assess the response of E_D and and E_N to nocturnal VPD under both well watered and drought conditions, 2—to relate G_N maximum values with stomatal (anatomical) characteristics, 3—to compare the contribution of E night to the total diel water budget under well watered and drought conditions in

Populus from contrasting habitats. I hypothesized that riparian species would display higher nocturnal transpiration with little adjustment of nighttime stomatal conductance in response to moderate drought. I performed simultaneous and continuous measurements of transpiration and whole-plant stomatal conductance (G) in whole plants during two weeks using an automated gravimetric technique. I studied four species of *Populus* with a range of habitats from riparian to upland sites with frequent drought. I measured E and G under well-watered conditions, followed by moderate water stress and lastly severe drought. I found that riparian species had higher nighttime G than non-riparian species, and did not significantly reduce their nighttime G in response to moderate stress. On the other hand, non-riparian species showed a decline in nighttime G proportional to the level of drought. However, none of the non-riparian species approached minimal G values until leaf death was imminent and I found evidence of active stomatal involvement under moderate drought. I concluded that active stomatal opening is responsible for nighttime water loss even under drought and that this phenomenon may be adaptive.

Finally, Chapter 4 is concerned with the effects of N form on whole plant physiology in fast- (AP2403) and slow-growing (AP9) poplar hybrids. Specifically, I assessed day- and night-time conductance, net photosynthesis and growth, and foliar nutrient concentrations. I pursued the following hypotheses: i—inorganic nitrogen in the form of NO_3^- increases whole-plant conductance (particularly night-time) relative to NH_4^+; ii—the increased transpiration under NO_3^- nutrition has a secondary effect on the delivery of other nutrients such as K and Ca, for which mass flow is an important form of acquisition and delivery; and iii—plants under NH_4^+

nutrition produce thicker roots with distinct anatomical changes since they appear to be the main site of NH_4^+ metabolism.

Figures


1952 - 2007
Gravimetric
Porometry
Sap flow
Gas exchange

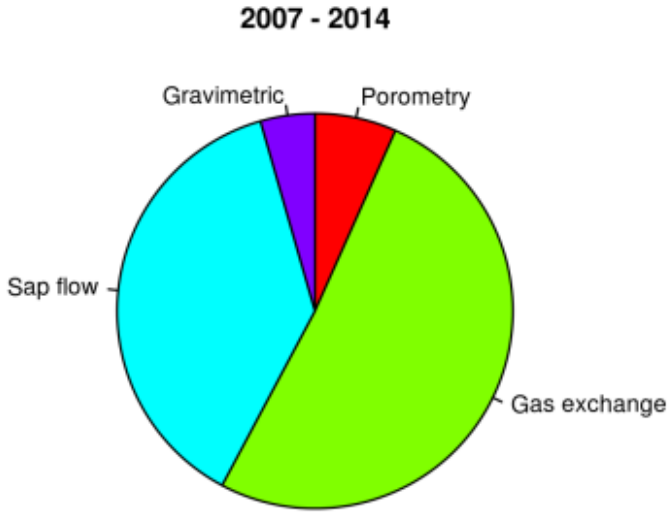

2007 - 2014
Gravimetric
Porometry
Sap flow
Gas exchange

Figure 1-1: Comparison on the percentage of the number of publications using different methods to assess nocturnal water loss. Data from 1952 to 2007 corresponds to 57 papers reviewed by Caird et al. (2007). Data from 2007-2014 is based on the following references: Barbour and Buckley (2007); Caird et al. (2007); Cavender-Bares et al. (2007); Dawson et al. (2007); Fisher et al. (2007); Grulke et al. (2007); Kobayashi et al. (2007); Marks and Lechowicz (2007); Christman et al. (2008, 2009a, 2009b); Moore et al. (2008); Snyder et al (2008); Howard et al. (2009); Novick et al. (2009); Rogiers et al. (2009); Howard and Donovan (2010); Phillips et al. (2010); Prieto et al. (2010); Sellin and Lubenets (2010); Zeppel et al. (2010, 2011, 2012); Buckley et al (2011); Pfautsch et al. (2011); Barbeta et al. (2012); Kupper et al. (2012); Ogle et al. (2012); Rosado et al. (2012); de Dios et al. (2013); Escalona et al. (2013); Rogiers and Clarke (2013); Auchincloss et al. (2014); Rohula et al. (2014).

Chapter 2. Measuring whole-plant transpiration gravimetrically: a scalable automated system built from components[1]

Introduction

Transpiration (E), is one of the most fundamental processes in vascular plants and its inextricable nature governs many aspects of their physiology. Transpiration is linked to carbon gain as both water loss and CO_2 uptake (and consequently assimilation, *A*) occur through stomata and the water-use efficiency (WUE) is often expressed as the ratio E/*A* (Stanhill 1986). High rates of E may foster higher *A* but may also come at a peril for the plant and some authors regard E as a negative side-effect (Kramer and Boyer 1995; Raven et al. 2005; Niklas 1997). Other authors have attributed specific functions to E, from nutrient delivery to leaf cooling (Cramer et al. 2009; Mahan and Upchurch 1988; Dawson et al. 2007).

Plant transpiration is of interest not only in the context of whole-plant physiology but also in a more applied sense since it is tied to production-sensitive factors like drought tolerance and WUE. In addition, the discussion of plant water-use patterns has become of central importance since water availability and distribution are predicted to change around the globe (Naik et al. 2003; Rees and Ali 2007; Ridgwell et al. 2009; Oliveira et al. 2011). Several recent publications highlight the current interest in understanding and improving crop WUE (e.g. Yoo

[1] A version of this chapter has been published. Cirelli et al. 2012. Trees 26: 1669-1676.

et al. 2009) and concerted efforts are underway to uncover its genetic and mechanistic basis (Karaba et al. 2007; Masle et al. 2005; Davies et al. 2002). It is therefore still relevant to develop and improve upon tools to measure whole-plant E accurately and reliably as it will be essential to integrate sub-organismal mechanisms into a whole-organism level.

Many techniques are available to measure or estimate E, from instantaneous leaf-level to landscape-level, each having its own scope and limitations. Arguably, the most popular techniques to measure E are based on porometry or sap-flow meters (Wullschleger et al. 1998; Pearcy et al. 1988). Sap-flow based metrics can be used on very large trees in the field and in many cases they constitute the only option for whole-tree measurements, but they make several assumptions such as constant heat dissipation across the sapwood and uniform cross-sectional flow, although proper calibration can significantly enhance accuracy (Sun et al. 2011). Furthermore, the flow of sap can differ from transpiration due to stem capacitance, particularly at night. These techniques are also unsuitable for most herbaceous plants. Porometric techniques are convenient for instantaneous readings of stomatal conductance (g) in a wide variety of plants. These leaf-level measurements may not be representative of whole-plant responses and water use partly because the porometer strips the boundary layer from the leaf surface with rapid air movement which is more appropriate for measuring water diffusion through stomata and calculating g. When measuring water use at the whole-plant level, the leaf boundary layer (consider e.g. tomentose *vs* glabrous leaves) may well significantly affect WUE. Even when the boundary layer is properly taken into account by porometric

methods, the architecture of the entire plant is inexorably overlooked by leaf-level measurements. While porometry is an invaluable tool, estimations of *E* from leaf-level *g* seldom agree with in-situ whole-plant transpiration measurements (McDermitt 1990).

Gravimetric instruments such as weighing lysimeters can provide reliable, accurate measurements and a resolution-to-scale ratio at the whole-plant level if properly constructed (Yoo et al 2009; Edwards 1986). The nature of lysimeters makes replication cumbersome, due not only to the cost and the work involved in their installation but also because they are field-only stationary instruments by design. Laboratory balances are well suited to function as small-scale weighing lysimeters, are relatively inexpensive, provide remarkable precision and accuracy, and have been used in a number of studies to measure *E*, sometimes with a fine degree of time-resolution (Edwards 1986). Unlike the case of lysimeters, a system with balances can easily incorporate replicates if carefully designed. Despite this, to date no specific software and technical report are available to help researchers build such a system.

Measuring E by means of weighing can be considered a sort of "gold-standard" for whole-plant *E* since a balance is used to account for the loss in (water) mass. Three simple assumptions can be identified: *i*—the water is all lost through the plant (zero soil evaporation), *ii*—the plant gains no mass, and *iii*—the plant loses no mass other than that of water. The first of these assumptions can be controlled, to some extent, by minimising soil exposure and by measuring it (e.g. a pot with no plant). Plant gain or loss in dry weight can be ignored since their

contribution to balance mass can only be detected after long periods of time (much longer than those needed to measure *E*). It is clear that measuring *E* with a high degree of resolution on several plants simultaneously using balances would be a very impractical task unless automated.

Most electronic (digital) balances can be easily interfaced to a computer. Likewise, the task of converting regular balances to weighing lysimeters is conceptually simple. However, the details of wiring multiple balances and coordinating and logging their readings are not trivial. Firstly, installing a multiple-balance system controlled by a computer running a Windows operating system will need customized hardware. Secondly, an off-the-shelf approach using the universal serial bus (USB) as the basis of serial port expansion is problematic since port numbering is not standardised, making the production of the software a technical challenge and highly inefficient. In contrast, this is more feasible under a Unix-like system. Thirdly, there is no available software to coordinate this system and compute E from mass loss.

We have taken advantage of the current availability of "user-friendly" Linux distributions to build a flexible and reliable multiple-balance system capable of over 100 concurrent balances. Here we present an original Free and Open-Source Software (FOSS) called *Amalthea*[2] (Cirelli 2010) and a highly scalable system designed to measure whole-plant transpiration using laboratory balances. The strengths of the system are that it allows multiple balances to be used simultaneously, can be run for long periods with dependable stability and obtains

[2] Named after one of Jupiter's moons, a small satellite subjected to large gravitational forces.

high resolution measurements with good noise filtering, suitable to many types of studies and many scales of plants from small herbs to large potted trees. Moreover, the system can be built entirely with readily available components.

Materials and Methods

System description, materials and installation

The system was designed to be modular so balances could be added or removed and the software quickly reconfigured. We also focused on using easily available and economical parts. Essentially, the system consists of a computer that runs the software, a laboratory scale (balance) and a connection between them. This would comprise a "minimal unit" to which more balances can be added. Below, we provide the general specifications to be met by the hardware and a thorough description of the software.

Hardware requirements

The most minimal installation requires a computer with an available serial port, a balance with serial bi-directional communications capability and a serial communications cable. Multiple balances require an equal number of serial ports. Universal Serial Bus (USB) to RS-232 (RS232) converter cables are used for this purpose and can be connected to a USB hub. The USB approach allows for easy expansion of the system and this was how our final test system was installed. The configuration we used for testing consisted of one computer to which nine balances were connected through two USB hubs. Figure 2-1 shows the connection diagram of a complete system.

Since nine serial ports were required to connect the nine balances, we used two 7-port self-powered "industrial" USB hubs (StarTech ST7200USBM, StarTech.com Ltd., Canada) connected independently (one hub per built-in system port), each with its own DC power supply. The hubs were attached to the computer with 28/24AWG gold-plated type A/B USB cables (Monoprice Inc., USA). Although we chose a star topology, a daisy-chain arrangement of the hubs could have been used and it would be our recommended wiring scheme. The nine serial ports for balance connection were provided by nine USB-to-RS232 converter cables (StarTech ICUSB232, StarTech.com Ltd., Canada).

We used nine top-loading digital balances with a 0.01 g resolution and a maximum capacity of 4500 g (Adam Equipment model PGW 4502e, Adam Equipment, South Africa). The balances were placed on levelled metal greenhouse benches with ad-hoc medium-density fibreboard platforms for stability (1.25 cm thickness). Each balance was numbered and connected to the respective USB-serial port with a null-modem (cross-linked) serial cable (StarTech SCNM9FF, StarTech.com Ltd., Canada). We performed weekly calibrations of the balances to maintain linearity and offset any drift.

All electronic equipment, was connected to an uninterrupted power supply (UPS) (APC Back-UPS CS 350, Schneider Electric SA, France).

Software overview

Amalthea was programmed mainly in the Python language (www.python.org) with a programme wrapper (amalthea-wrapper) written as a bash shell script (www.gnu.org/software/bash/bash.html) which makes use of standard GNU/Linux

utilities. For simplicity, Amalthea runs in traditional linear fashion and relies on the 'cron' dæmon (a time-based scheduling programme) for periodic execution. Upon installation, the configuration script adds an entry to the crontab, a file read periodically by cron to execute scheduled tasks.

Once called by cron, Amalthea will run for the configured period taking mass readings at regular intervals (default is every 10 seconds for 4 minutes). Every reading is sent to the *standard error*[3] *stream* (*stderr*) and at the end of the run, Amalthea compiles all the readings of the period and performs a linear regression thus obtaining a rate of mass loss. Although this rate integrates the mass loss over the entire period, the time values (seconds) comprising the abscissa are averaged and the result is taken as the point value of time for the calculated rate, which allows said rate to be presented as an approximation of the tangent for that time point. The rate obtained is in units of g s^{-1} and it is further converted to mmol s^{-1}. This information is then sent to the *standard output stream* (*stdout*) as a single line of comma-separated values (CSV).

The wrapper script takes both *stderr* and *stdout* and respectively appends the raw values to a CSV file with extension ".raw" and the processed values to a CSV file with extension ".csv". Both files have the same base name which corresponds to the date in the format YYYY-MM-DD (e.g., data collected on July 5, 2011 would be written to the files '2011-07-05.raw' and '2011-07-05.csv').

[3] Not to be confused with the statistical term. For clarification see Rosen et al. (2006).

Environmental monitoring

Measurements of temperature, relative humidity, and light intensity were collected using a custom-built interface to connect the required sensors to the serial port of the same computer running Amalthea. The hardware design and software to read the sensors are publicly available as Creative Commons and open source projects respectively (Cirelli 2011). The designed interface and software allowed the use of the same logging facility as the balances, thus both measurements coincided in time (see discussion).

Plant material and growing conditions

We tested the system with aspen (*Populus tremuloides*) and hybrid poplar (*Populus* sp.) seedlings grown at the University of Alberta. All plants were cultivated in 2.4-liter pots containing Sunshine® Mix #4 / LA4 potting mix (Sun Gro Horticulture Ltd., Vancouver, Canada) and fed with Osmocote® slow-release fertiliser (ScottsMiracle-Gro, Marysville, USA). Air-mixing in the greenhouse was achieved by the use of a low-speed ceiling fan. Supplemental light was provided with Gro-Light fluorescent tubes with a photoperiod of 18 hours, which is representative of mid-summer conditions at this latitude.

During measurement, the tops of the pots were covered with aluminium foil to minimise evaporation. Drain-holes were not covered so as to allow air exchange in the pots. To avoid water percolating through the pots during the experiment, each pot was watered to excess and allowed to fully drain before placing them on the balances. Subsequent additions of water during the experiment were done with the

pots on the balances while administering the water slowly with a large-nozzle[4] wash bottle until the mass was about 100 g less than the initial weigh-in. This was done to avoid percolation.

Quantification of evaporation from pots

The Amalthea system was used to quantify the contribution of water evaporating directly from the pots. Six pots containing live root systems of decapitated plants were watered and allowed to drain completely. There were four 2.5 cm × 2 cm drain-holes on each pot placed on the sides, where the side meets the bottom. The top of each pot was covered with a square of aluminium foil wrapped around the rim and containing a slit from the edge to the middle as would be the case when accommodating the stem of an intact plant. Drain-holes were not covered. The pots were placed on the balances and their mass loss monitored over a period of four days. No exudate from root pressure was present after cutting.

Sensitivity analysis

To assess the adequacy of both the balance resolution and the chosen length of one run, a sensitivity analysis was conducted. A slope of 0.01 g period^{-1} was chosen as the starting point, which, with the default period of 220 s, it represents a rate of 4.54×10^{-5} g s^{-1}. From this slope, a set of data was constructed containing 23 points, one every 10 s for 220 s starting at zero seconds. Each point i was then subjected to the operation $floor(10^n \cdot i) \cdot 10^{-n}$ where n is the number of decimals available and the

[4] This can be achieved by snipping off the tip of the original nozzle to the desired size.

function floor takes only the integer part of the number discarding the decimals. This was done to simulate a conservative scenario in which for a balance to display an increment of one unit of resolution (0.01 in the case of two-decimal resolution), the full mass of this unit has to be added before the balance registers the new mass. For example, under such condition, adding 0.007 g to a balance reading "0.01" will not cause a reading of "0.02"; only adding a full 0.01 g will change the display to "0.02".

A linear regression was fitted to the new "rounded down" data set, and the resulting slope (S_R) compared to the known slope without rounding (S_K). The percentage of variation between the slopes was calculated as $\left(100\frac{S_R}{S_K}\right) - 100$ and this process was repeated for slopes of 0.03 to 0.93 g period^{-1} in 0.09-g increments. A series of slope variations was obtained for *n=1* and *n=2*. In addition, the entire process was also repeated with a period of one half of the original period and another of double length, but maintaining the same reference rates in the series as grams per second, thus varying the rate in grams per period to evaluate the effect of period length.

Results and Discussion

Figure 2-2 shows five consecutive intervals of a transpiring plant during the transition from lights-on to lights-off. Each group of points displays the linear regression fitted to that particular group, the slope of which is the rate of water loss in g s^{-1} unit^{-1}. The differences among the slopes indicate that lengthening the runs would result in a loss of temporal resolution. Some runs may display a degree of

non-linearity and in these cases a quadratic function is a better fit than a linear regression. The reported abscissa corresponding to each slope is the average of the time period, thus the linear regression slope can be taken as a very close approximation to the first derivative of a quadratic function for the same midpoint in time.

Monitoring potted plants for long periods denies the option of sealing the pots as it would create an anoxic environment in the roots. For this reason, we deemed necessary to quantify the water loss from pots with leaky top covers (foil) and open drain-holes. Water evaporation from the pots was linearly correlated with the evaporative demand of the air, expressed as vapour pressure deficit (VPD, figure 2-3a). Figure 2-3b shows a four-day span of evaporation measurements from "empty" pots and a second trace of evaporation modelled from the VPD data. Compared with a transpiring plant, the evaporation rate from the pots is negligible, amounting to no more than 1% of the total measured water-loss (see figure 2-4).

The transpiration over a two-day continuous run of an ~40-cm aspen seedling can be seen in figure 2-4. This seedling was representative of the six plants measured. This figure also shows a measure of evaporative demand experienced by the plants during the same period. It is evident from these data that, the effect of light on stomatal opening notwithstanding, VPD was the principal driver of transpiration in these plants. It is also noteworthy that transpiration and atmospheric conditions were each measured independently, yet there is great parallelism between both traces with some segments clearly recognisable in both data (arrows in figure 2-4). This emphasises the close relationship between VPD and *E*, not only

as the main driver but also acting on smaller scales, which Amalthea is able to detect (the particular drop in VPD – first arrow – can be explained by the opening of a ventilation window in the greenhouse). Comparatively high-amplitude oscillations are visible from midday onward lasting about two hours. These are partly in response to variations in VPD and natural light intensity, but may also exhibit an intrinsic nature (i.e. an oscillatory component is still present after normalising). Regardless of the cause, these oscillations are not an artifact of the system which is capable of reliable measurements and high accuracy, as seen for example after the first arrow in figure 2-4.

Two poplar clones with contrasting characteristics (primarily in growth rate) were simultaneously monitored for five consecutive days (and nights) to test the system and its handling of multi-species configuration while obtaining data to compare their transpiration responses. For clarity and brevity, figure 2-5 shows two of the five days and the traces have been corrected by leaf area to make direct comparison possible.

Area-correction is a necessary post-processing step to obtain a fine level of resolution, especially when measuring for long periods. However, the system can be made area-aware *while* collecting data, through the proper configuration option. This is advisable only when measuring times are short (1 or 2 days) or when measuring plants that have negligible expansion rates in the desired time-frame. During long periods of monitoring fast-growing plants, leaf area increments can be appreciable as was the case with the clones showed in figure 2-5. The expansion rates in this case were ~38 $cm^2\ day^{-1}$ for ap2403 and ~12 $cm^2\ day^{-1}$ for ap9. Thus,

we configured Amalthea to collect data assuming a leaf area of 1 and transpiration was corrected afterwards with individual leaf-area functions.

It can be seen that, despite oscillations in the data, Amalthea consistently measured higher *E* on a leaf-area basis in the slower-growing ap9 clone. The traces in the right panel of the figure have been subjected to a line-smoothing algorithm (Tukey's running-median smoothing) for visual clarity only, while the left panel shows the original data. Overall, oscillations in *E* appear larger than those in figure 2-4 due to the fact that the absolute whole-plant rates measured were much smaller, but became amplified when standardised to the per-square-meter scale. Naturally, direct "signal" (*E* in this case) amplification will also amplify noise, although since these data are a time series more specialised can be applied such as frequency domain (highly relevant to circadian rhythm studies).

Since all the individual data points collected by Amalthea (both "raw" and "compiled") are synchronised within seconds of each other among balances, and since all the files contain the same number of points, averages of the entire transpiration patterns of two or more plants are simple to produce. Thus, the synchronised replicates of which Amalthea is capable and the response envelopes they allow are an important advantage of this system. Comparing two or more envelopes that are one-standard-deviation wide incorporates a robustness not attainable otherwise, but indispensable when working with natural genetic variability or when accounting for every independent variable is not feasible. The average trace of an envelope is also a more reliable signal to use for analysis when cyclic or circadian fluctuations are of interest.

As explained in the Methods section, the main programme in our software only communicates with the balances and performs the regressions. A helper programme (*amalthea-config*) part of the package configures files and coordinates how the data logging is to be done. However, the actual recursion essential to any logging software is carried out by *cron*. This is a key design choice; by outsourcing the repetitive execution to another, standard programme, we can take advantage of the resulting coordination not only with multiple instances[5] of Amalthea but also with other components. We chose to build a custom environmental hardware package and software (both available as open source) in order to obtain coordinated measurements of transpiration *and* evaporative demand. This is an important feature because, following the procedure outlined in the previous paragraph (e.g. point-by-point division), it allows further standardisation of the data according to environmental conditions. Combining transpiration and environmental data point-for-point can be an invaluable tool which allows the comparison of transpirational behaviours of plants measured sequentially in time (e.g. through phenological stages) since there can always be a correspondence with driving factors.

We used balances with two-decimal precision (0.01 g) which, together with the described intervals (220 s and 10 s sub-interval), provided an ideal combination of resolution and noise tolerance for the plant sizes and transpiration rates measured. Sensitivity analyses were performed to investigate the viability of 0.1-g precision and different measuring intervals. These simulations showed that using

[5] In computer science, *instance* refers to each individual copy of an active programme or process (i.e. executing in memory).

the system with a balance capable of a 0.01-g resolution, it is possible to measure rates greater than 0.03 g period^{-1} with an accuracy of 5% of the true rate or better. At the chosen period of 220 s, 0.03 g represents ~0.0075 mmol s^{-1} which is in the range of the measured pot water loss. Simulations with 0.1-g precision showed that 220 s is not enough to measure such low rates accurately. However, doubling the period improved the accuracy, making it possible to measure 0.0555 mmol s^{-1} within less than 5% of the actual rate. The reader should be aware of the trade-offs between period length and accuracy due to round-off errors and between balance precision and signal-to-noise ratio under different environments and transpiration rates. Ultimately, the system is flexible enough to adapt to different situations given proper assessment of the conditions: e.g. it is unlikely that a small *Arabidopsis* plant can be measured with a 0.01-g balance precision in fast-moving air, while conversely, 0.1-g precision would be sufficient for a potted tree with a leaf area of 0.5 m^2.

The system presented in this paper has the advantage, over other methods, of relying on fewer assumptions and giving a precise whole-plant *E* measurement with a number of true replicates as opposed to an estimation. This is particularly adept to model validation and verification. Its applications are wide-ranging although clear limitations emerge from the current hardware. Some of these limitations can be circumvented with careful planning and consideration of the experimental requirements such as weather-proofing or load-sharing, amongst others. Special attention should be paid to connecting and disconnecting converter cables since a change in port number can create a configuration problem and result

in mixed data, but since port numbering is sequential in the Linux kernel it is easy to predict port assignment (a more in-depth instruction is provided in the 'Readme' file when downloading the software). The number of ports is also limited by the system, to 127 devices per bus. In many cases, the 'different' USB ports on a computer are on the same bus, detracting from the total 127 maximum. For this and other technical reasons, we recognize a limit of 17 daisy-chained hubs to provide a total of 103 ports (i.e. possible number of balances). Larger systems would be possible by adding more buses or by combining computers, the latter afforded by the use of *cron* as the logging control given that all computers are regularly synchronised with a time server. No single system devised to measure E is suited to every experimental condition or research question and Amalthea is likely to complement other techniques and form part of a repertoire of tools to investigate transpiration and its regulation.

Gravimetric techniques have always been reliable but highly impractical on a larger scale in terms of space (multiple samples) and time, especially without automation. Studies that look at transpiration which have used automated balance measurements (e.g. Medrano et al. 2005; Cavender-Bares et al. 2007; Dodd et al. 2008; Dodd et al. 2010) often implement a non-described point-solution[6] software that, although of value to the study, is neither scalable nor universal as evidenced by the presentation of single-plant measurements. Both the open source software and the hardware connection scheme put forth in this paper are designed to be

[6] Point-solution or point product refers to a product that is used only for a particular situation but does not address the technical aspects required for a more general implementation.

scalable, versatile and simple to implement and use. Since the design is modular, it is entirely possible to “mix-and-match” balances, to combine balances, or to use all or some of the balances as potometers. We encourage researchers interested in transpiration to visit the software website for more information and to obtain help on downloading and running the programme.

Figures

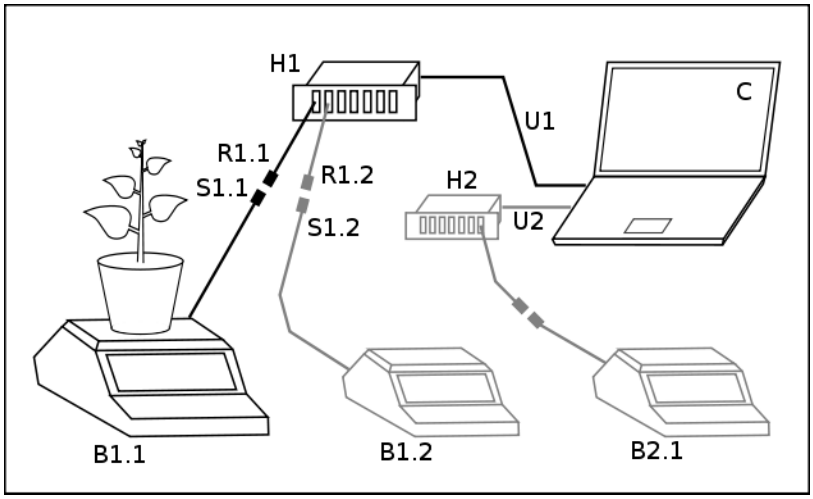


Figure 2-1. (C) Computer running Amalthea; (U[*i*]) standard USB cable; (H[*i*]) USB hub; (R[*i.j*]) USB-to-RS232 cable; (S[*i.j*]) serial communications cable (bi-directional); (B[*i.j*]) weighing balance. The chain formed by (U1)-(H1)-(R1.1)-(S1.1)-(B1.1) is a complete system unit. Repeats of (R)-(S)-(B) can be added to (H1) to create more units until all ports are exhausted; addition of a [(U)-(H)] unit to the computer allows further expansion (depicted here by (U2)-(H2)). A single-unit system is possible by connecting (R1.1)-(S1.1)-(B1.1) directly to (C).

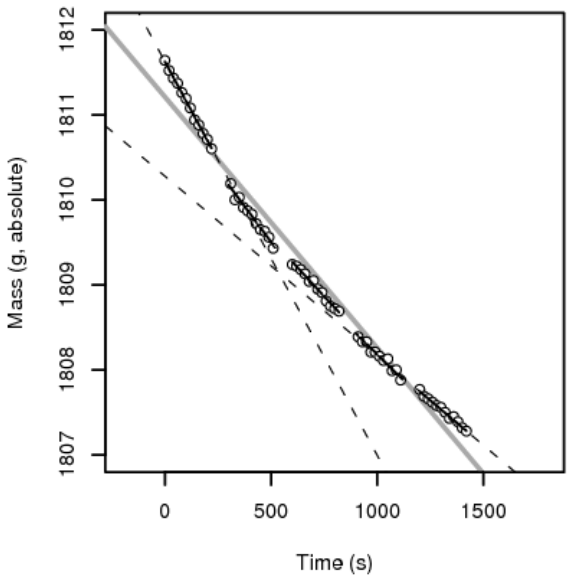


Figure 2-2. Five runs of "raw" mass data (25 min) in the transition between light (first two runs) and dark (last three runs). Individual linear regressions are fitted to each run by Amalthea (thin black lines) and rate information derived from them. An overall linear regression was fitted here for comparison (thick grey line), and to show that periods longer than 5 min are unsuitable if fine granularity is desired. Dashed lines show extended regression lines of the first and last runs.

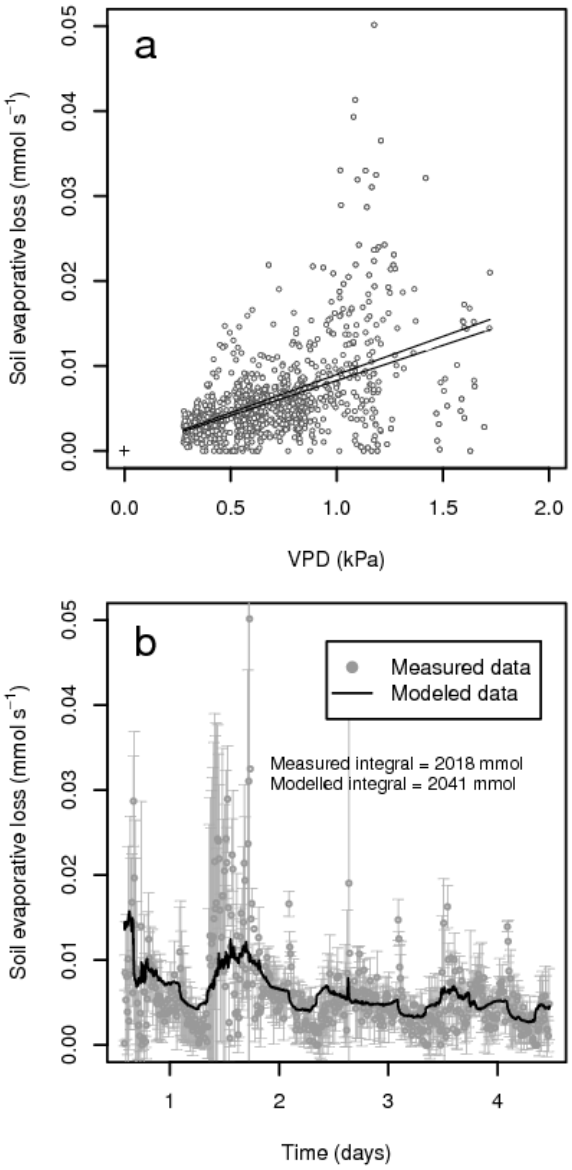


Figure 2-3. (a) Linear regression of soil water evaporation in response to evaporative demand. The fitted regression line is not shown; instead the lines are the 95% confidence interval of the regression. "+" marks the origin (*x=0, y=0*) through which the function was forced since evaporation should not be detectable at 100% relative humidity. Closed circles are influential points which were not discarded. (b) Monitoring evaporation from covered pots for four days. Open circles are the means of 3 pots collected simultaneously every 5 min; bars indicate standard deviations. The black line shows the expected evaporation based on the regression in (a).

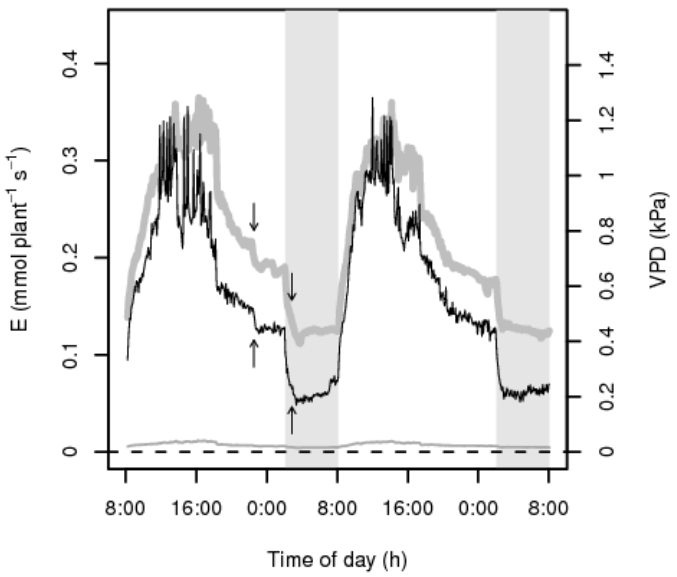


Figure 2-4. Two-day traces of a transpiring 30-cm aspen seedling (thin black line, left axis) and the evaporative demand of the air measured close to the plant canopy (thick grey line, right axis). '*' and '§' mark sections in both traces which parallel each other; each trace was measured simultaneously but independently. The thin grey line above the zero-line (dashed) shows the estimated contribution to *E* of direct water evaporation from the pot. Nighttime is indicated by grey vertical bars.

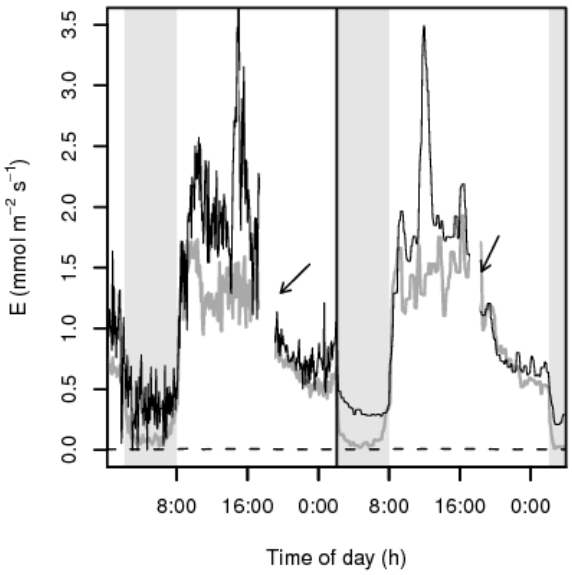


Figure 2-5. Monitoring *E* of clones ap9 (thin black line) and ap2403 (thick grey line) over two days. Transpiration rates have been corrected by leaf area. Arrows point to gaps in the data when the system was paused to water the plants. In the second day shown, the traces have been smoothed for visual clarity (right panel). Nighttime is indicated by grey vertical bars. Traces represent individual plants.

Chapter 3. *Populus* species from diverse habitats maintain high night-time conductance under drought

Introduction

The loss of water during the night challenges some of our preconceptions regarding plant function. Despite long-standing assumptions to the contrary, evidence is mounting for appreciable nocturnal transpiration (E_N) in many tree species (e.g. Zeppel et al. 2011; Phillips et al. 2010; Caird et al. 2007; Wullschleger et al. 1998). However, the extent and magnitude of this phenomenon remain sparsely documented and the notion that stomata remain closed in the absence of light still influences our general models of water loss (Dawson et al. 2007; Phillips et al. 2010; Daley and Phillips 2006). Fisher et al. (2007), Marks and Lechowicz (2007), and Dawson et al. (2007), have reported that stomata might be "leaky", while others suggest that they are responsible only for negligible nocturnal water loss (e.g. Dixon and Grace 1984; Pickard 1989; Cienciala et al. 1992; Saugier et al. 1997). In many cases, the inherent technical problems of accurately measuring night-time transpiration also contribute to the lack of conclusive data (Seginer 1984; Fisher et al. 2007).

Daytime transpiration (E_D) has been extensively studied since it is necessarily linked to photosynthesis. Despite the growing body of literature that documents night-time transpiration and explores its implications (e.g. Caird et al. 2007; Dawson et al. 2007; Zeppel et al. 2010; Cramer et al. 2008), some basic points still require more attention. Specifically, the level of inter- and intra-specific

variation in E_N and its underlying control (Zeppel et al. 2010; Phillips et al. 2010); the ratio of E_N/E_D within and across species (Caird et al. 2007) and how it is affected by water relations; the ability of stomata to fully close (Cramer et al. 2009); and the possible functional role behind E_N.

A wide range of E_N has been observed across tree taxa. While some species such as red maple and red oak do in fact appear to have negligible levels of night-time water loss (Daley and Phillips 2006), other species can have E_N values in excess of 30% of day-time E (Snyder et al. 2003). It has been suggested that night-time stomatal conductance (g_N) and E_N may be most prominent in fast-growing, shade-intolerant tree species from environments that experience high water availability (Caird et al. 2007; Dawson et al. 2007; Marks and Lechowicz 2007). However, this relationship has been drawn mostly from comparisons among unrelated (or distant) species that occupy radically different environments (Caird et al. 2007; Dawson et al. 2007). Comparisons among closely-related species within a genera have seldom been performed (e.g. Cavender-Bares et al. 2007 and Phillips et al. 2010 in oaks and eucalypts respectively).

Populus is a genus of ecological and economic importance with a wide geographic distribution. In North America, there are approx. 21 species of *Populus*, including poplars, hybrid poplars (naturally occurring), cottonwoods and aspens (Eckenwalder 1996; Hamzeh and Dayanandan 2004). Some species such as *P. deltoides* and *P. trichocarpa* are mainly obligate riparian, while others such as *P. tremuloides* can dominate upland sites (Kranjcec et al. 1998; Lieffers et al. 2001). In addition, *Populus* differ greatly in their stomatal control (Lu et al. 2010) as well

as in stomatal anatomy (Caird et al. 2007). Nocturnal conductance and transpiration have been measured in natural stands of *P. balsamifera* (Snyder et al. 2003) as well as in greenhouse-grown *P. trichocarpa* and *P. angustifolia* (Howard and Donovan 2010), and they are in the upper range of values reported in the literature (as part of a meta-analysis by Caird et al. 2007). However, g_N and E_N have not been assessed systematically among different *Populus* and, therefore, the inter-specific variation in E_N and its possible link with habitat distribution remain unclear. Comparison of contrasting *Populus* might provide further insight as to how E_N is related to habitat as well as to stomatal characteristics in closely related species.

Most of the studies that have assessed E_N in tree species rely on variants of the sap-flow technique to estimate both transpiration and refilling. This technique can provide valuable information in the field, although careful attention has to be paid to the methodology, especially to decoupling flow due to bole recharging (stem capacitance) from that due to water loss from leaves (Clearwater et al. 1999; Dawson et al. 2007; Phillips et al. 2010; Sun et al. 2011). Porometer-style measurements are also commonly used to obtain E_N, but the nature of these measurements makes it impossible to continuously monitor whole plants as porometers are typically designed to work at the leaf level and long-term measurements are not viable. In addition, the majority of the available data pertains to mature trees (usually from sap-flow data) but little information is available on seedling and/or saplings (Marks and Lechowicz 2007). Although issues of capacitance (i.e. storage of water in the bole) and hydraulic redistribution are of less importance in young tree seedlings, water use and availability are likely major

determinants of establishment and competition outcomes (Cooper et al. 1999; Sher et al. 2003). The evaluation of night-time transpiration patterns might provide further insight into recruiting and establishment dynamics.

Our main objective was to assess the day- and night-time transpiration of four species of *Populus* with habitats ranging from riparian to upland, including a hybrid poplar clone with drought-tolerant parents. We measured whole seedlings directly by continuous whole-plant gravimetric readings of water loss from individual plants over a span of at least one week. In particular, we aimed to quantify whole-plant stomatal conductance (G) during the night across different species of *Populus*, to obtain minimum values of nocturnal E and G under drought, to analyse the ratio night-/day-time conductance (G_N/G_D) under well-watered and drought conditions, and to compare the anatomy of stomata among species. We hypothesize that riparian species will display higher nocturnal conductance and will not adjust night-time stomatal conductance in response to moderate drought.

Materials and Methods

Plant material and growth conditions

Four *Populus* species were measured in this study: *P. deltoides*, *P. trichocarpa*, *P.* × *petrowskyana* (hybrid of *P. nigra* × *P. laurifolia*), and *P. tremuloides*. These species represent a gamut of habitat preference and drought tolerance, from riparian habits to drier upland environments (Table 3-1). All plants were grown from seed except plants of *P.* × *petrowskyana* which were cloned from stem cuttings. Plants were grown in 2.4 - l pots filled with potting mix (Sunshine® LA4 mix, Sun Gro

Horticulture Canada Ltd.) with 20 g of slow-release fertilizer (13:13:13 N:P:K). All species shared the same growing conditions in the greenhouse which provided a mix of natural and supplemental light, under a 16-hour photoperiod. The average daily light intensity (photosynthetically-active radiation) during the measurement period was 532 ± 85 μmol m^{-2} s^{-1}, with daily maximums between 800 and 1000 μmol m^{-2} s^{-1}.

Measurement of water loss

Four plants of each species were measured continuously by a gravimetric method (Amalthea system from Cirelli et al. 2012). Briefly, the Amalthea system consists of a balance array where all the balances are coordinated by a computer which logs the data. The computer also synchronises the gravimetric measurements with a mid-canopy sensor array to simultaneously record light, temperature, and relative humidity. Measurements were collected every 5 minutes, and each measurement consisted of the average and slope of a linear regression from 23 samples taken at 10-second intervals (for details, refer to Cirelli et al. 2012).

The plants were, on average, 50 cm in height and held between 25 and 30 fully-expanded leaves. Leaf area was calculated on a leaf-by-leaf basis by non-destructively measuring minimum and maximum caliper (i.e. width and length). Calculation was based on previously constructed calibration regressions by destructively sampling a wide range of leaf sizes from each species under the same growing conditions, and correlating the product of width and length with the actual area of each leaf thus obtaining a calibration factor (slope of the regression). The correction factors ranged between 0.65 and 0.76 with every R^2 higher than 0.98.

Width and length of every leaf was then measured every two days and an area-time function was adjusted for each plant to correct the transpiration values point-for-point a posteriori (see Cirelli et al. 2012).

All transpiration values were normalized by vapour pressure deficit (VPD) thus transforming *E* to whole-plant or "total" stomatal conductance (G = E/VPD). The use of VPD as the evaporative driving force assumes that the average leaf temperature is the same as the air temperature. G applies to the entire soil–plant–atmosphere continuum and should not be confused with either hydraulic or stomatal conductance at the leaf level. In addition, we are making the distinction between *transpiration* (E) which is the instantaneous rate of water loss expressed in mmol m^{-2} s^{-1} (not taking into account the VPD), and *water-use* (U) expressed in g m^{-2} per period (day—U_D or night—U_N), in which E is integrated over some period (e.g. the entire night) in order to show the actual amount of water lost (usually expressed in grams or alternatively as a percentage as in U_N/U_D).

Watering regime and drought treatment

Immediately preceding gravimetric measurements, plants were watered and pots were allowed to percolate fully. After percolation had ceased, the bottom of every pot was repeatedly blotted with paper towels until no more liquid was present. To minimize water loss from the soil surface, the tops of the pots were covered with aluminum foil. Drain holes were left uncovered as it was previously determined that this only contributed up to 1% of the total loss (as described in Cirelli et al. 2012). Each pot was then weighed and this weight represented the percolation point for the pot. During measurements under well-watered conditions, pots were watered

daily, on the scales, to 100 g less than its percolation weight to avoid water spillage onto the balances. After 5 days under these well-watered conditions, water was withdrawn in a controlled manner to observe the decline in night-time transpiration. This treatment involved the daily addition of 50% of the water that each plant had individually transpired since the last watering (one full day, since watering was done at the same time each day). Once the leaves had lost turgor (stage 2 as described by Lu et al. 2010), water was withheld completely to obtain a possible minimum night-time *E* under extreme drought conditions.

Leaf surface anatomy

Stomatal impressions of both abaxial and adaxial surfaces were collected for anatomical analysis. The impressions were taken with clear nail polish and lifted from the surface with clear tape. Six digital images of each surface from independent samples were taken and processed in GIMP (GNU Image Manipulation Program, http://www.gimp.org). Processing consisted of marking the length and width (opening) of each stoma outlined by the peristomatal groove on a transparent layer. The resulting line drawings were extracted as separate images and analysed in Fiji (Schindelin et al. 2012). The image extracted consisted of a collection of crosshair-like drawings of varying widths and lengths which represented the stomata on the original image. We used the particle analysis function of ImageJ to obtain the Feret's diameter (also known as maximum caliper) of each particle-stoma. This measurement included both the length and width of the stomatal opening including the stomatal ledge (ridge).

Leaf samples were also taken for SEM imaging. Small (~1.5 mm^2) pieces of fresh leaves were cut and plunged into 2.8% glutaraldehyde in phosphate buffer pH 7.2. Primary fixation lasted for 24 hours at 4 °C, followed by dehydration in an alcohol series consisting of 25%, 50%, 70%, 85% and 95% ethanol before final dehydration in absolute ethanol. No secondary fixation was performed. Dehydrated samples were dried in a CO_2 critical-point dryer, mounted to show both the abaxial and adaxial surfaces, and sputter-coated with 150 nm of gold.

Calculation of stomatal pore index

Stomatal pore index is commonly defined as a fixed "aperture" value resulting from the product of the stomatal density and the square of the mean stomatal pore length (Sack et al. 2003). However, this value does not take into account the dynamic nature of stomata, which causes them to act as variable resistors in the hydraulic pathway by varying their width. Therefore, and since we obtained measurements of stomatal width, we constructed a "variable" version of SPI, noted here as SPI_v.

Other authors have approximated the stomatal pore area to the area of an ellipse of semi-major axis $l/2$ and semi-minor axis $w/2$ (e.g. Field et al. 2011), where l is the stomatal pore length and w the pore width. We first approximated the pore area to twice the area occupied by the arc of a parabola of length l and vertex $w/2$. However, we also measured individual pore areas from SEM preparations to compare with the estimates. In all cases, calculating the area as an ellipse overestimated the actual area of the opening, while using the parabola calculation underestimated the actual area by about the same amount. We therefore combined

the two equations to obtain a more precise estimate of the stomatal pore area such that:

$$SPI_v = \frac{(3\pi + 8)\sum_{i=1}^{n} l_i w_i}{24a}$$

where *l*: stomatal pore length, *w*: stomatal pore width, and *a*: area of the sample. Measurements of l and w included the stomatal ridges which was necessary for accuracy and speed, since in many cases the only visible boundary was the groove between the ridge and the guard cell proper. However, we obtained the average ridge size (r = length from the groove to the beginning of the stomatal opening) for each species from SEM samples. Subsequently, we incorporated this variable into the ellipse and parabola equations to obtain:

$$SPI_v = \frac{(3\pi + 8)\sum_{i=1}^{n} 4r^2 + (l_i - 2r)w_i - 2l_i r}{24a}$$

Nighttime cuticular conductance

In *P. tremuloides* which has hypostomatic leaves, cuticular conductance was assessed on the adaxial surface. To avoid transpiration from the abaxial side, a thick (~3-5 mm) layer of petroleum jelly was smeared along the margins, forming a continuous band. A piece of Parafilm M® was pasted over the leaf using the petroleum jelly to serve both as glue to hold the film and as a water vapour seal. In the other species with amphistomatic leaves, both sides were exposed to dehydration. For these species conductance was estimated as half the average of the largest group of points on the dehydration curve for which the slope was non-significant (constant G).

In all cases, fishing line was used to form a small loop which was glued to the petiole (close to the lamina) with methacrylate glue and secured with a strip of Parafilm. The petiole was cut close to the lamina and sealed with a dab of petroleum jelly. We used a freestanding lightweight wooden structure from which the leaf could be hung. The wooden base plus hanging-leaf assembly was placed on a 4-decimal digital balance (Mettler AE200, Mettler Toledo, Ohio, USA) with the weighing chamber open to allow for air circulation. Immediately following placement of the leaf on the balance, lights were turned off and since the procedure was started after sundown, the room remained dark for the duration of the measurement.

The same software used for whole-plant measurements was used to interface with the high-precision balance. Temperature and humidity were measured also in synchrony with the balance to obtain a measurement of leaf-to-air conductance as explained for the whole-plant measurements. The mean value obtained from the adaxial-only transpiration was considered one-half that of the entire leaf since only one surface was exposed.

Data analysis and statistics

Data processing was done in the R language for statistical computing (http://www.r-project.org). The transpiration data as well as the environmental data for each plant was separated into "day" and "night" for each 24-h period. Each full day of logging (once every 5 minutes) consisted of 194 daytime and 94 night-time measurements; each point of which was the result of 23 records within the 5-minute period (see Cirelli et al., 2012). We performed repeated measures ANOVAs on the daily

averages of G, E, and U during the well-watered period, where each individual plant was used as a source of error in the 'within' treatment.

Results

Night-time transpiration and water use

All species showed a substantial amount of night-time whole-plant stomatal conductance (G) and transpiration (E) that remained above zero throughout well-watered conditions and drought (Fig. 3-1). Night-time G was significantly different between species ($P < 0.05$, table 3-2). Under well-watered conditions, *P.* × *petrowskyana* and *P. tremuloides* had the lowest absolute water use per night (U_N), transpiring on average 138 g m^{-2}, while *P. deltoides* had the highest U_N at 340 g m^{-2}. In relative terms however, the minimum recorded $U_{N:D}$ (U_N / U_D) was 9% for *P.* × *petrowskyana* and the maximum was 20% for *P. trichocarpa*, while the average across species was 15% (Table 3-2). In all cases, differences in transpiration and conductance were mostly driven by changes in VPD since light integrals were similar across days and species.

P. × *petrowskyana* showed high G_D and relatively low G_N under well-watered conditions (Table 3-2), resulting in the largest difference observed between G_D and G_N (~1.91 mmol m^{-2} s^{-1} kPa^{-1}) in any of the species. *P. trichocarpa* showed the smallest difference between G_D and G_N (~0.86 mmol m^{-2} s^{-1} kPa^{-1}). *P. tremuloides* had moderate values of G_D, which were close to the median of ~1.6 (Table 3-2). *P. deltoides* had the highest average night-time G both in well-watered and drought conditions, whereas the overall mean values excluding *P. deltoides*

were 0.48 ± 0.2 mmol m^{-2} s^{-1} kPa^{-1} in well-watered conditions and 0.38 ± 0.2 mmol m^{-2} s^{-1} kPa under drought. G_D was also highest in *P. deltoides* (Table 3-2), although not statistically different from *P.* × *petrowskyana* ($P = 0.99$).

During the course of drought, all species showed a marked decline in day-time G. *P. deltoides* showed lower daytime G under drought mainly in response to higher VPD since E remained high (Fig. 3-1). *P. trichocarpa* also showed this pattern although to a lesser degree. The other species had a decline in both night time G and E under drought. Nighttime conductance was markedly reduced in *P.* × *petrowskyana* and *P. tremuloides*, but not so in *P. deltoides* or *P. trichocarpa* (Table 3-2). The contrast between these two groups was maintained after applying plant-specific VPD corrections. When drought became severe, all species showed similar G_N (about 0.2 mmol m^{-2} s^{-1}) except *P. deltoides*, which nonetheless presented a decline not seen during moderate drought conditions (Fig. 3-2a). The ratio of E_N/E_D tended to increase for all species as drought progressed since the rate of reduction of E_N was proportionately less than that of E_D (Fig. 3-2b).

In all species, night-time conductance was negatively correlated with VPD. The slope of this relationship was greater in plants subjected to drought (Fig. 3-3). *P.* × *petrowskyana* was the species with the least difference in G_N-VPD slopes between the well-watered and drought conditions, while it was at the same time the species with the largest difference in means between such conditions.

Cuticular conductance

Leaves of all species were mostly glabrous, with an occasional trichome along leaf veins. The mean cuticular conductance (g_c) for all species was one order of

magnitude below G_N (range of g_c/G_N 0.230 to 0.019) under well-watered conditions (Table 3-2). Under drought, the mean ratio was 0.24 with a range of 0.52 to 0.088. None of the slopes of g_c versus time were significant ($P > 0.01$) in the species in which g_c was measured directly, while the opposite was true for measurements of either bare leaf or leaf with a blocked (sealed) adaxial surface (Fig. 3-4).

Stomatal characteristics and stomatal pore index

Some *P. tremuloides* leaves had a few stomata concentrated around major veins on the adaxial side in some of the leaves, but as most did not have adaxial stomata we consider the plants hypostomatic. *P. trichocarpa* showed a low stomatal density on the adaxial side, averaging 11% that of the abaxial surface and being regularly distributed. Scanning electron micrographs of the leaf surface contained both open and closed stomata in every sample (Fig. 3-5). The outer ledges on the pore side of the guard cells were visible in most stomata in all four species, an example of which can be seen in Figure 3-5c.

Stomatal pore index (SPI_v, table 3-3) was lowest in *P. tremuloides*, owing to both lower stomatal counts and comparatively narrower stomata. *P. × petrowskyana* on the other hand, showed the highest SPI_v (Table 3-3), greatly due to more widely open stomata (higher mean values of stomatal width). We found a positive correlation between G and SPI_v (Fig. 3-6).

Discussion

The most striking finding was the occurrence of night-time transpiration in all species under drought, whether in obligate riparian species such as *P. deltoides* or,

surprisingly, in upland species such as *P. tremuloides*. Nocturnal transpiration was observed under well-watered conditions in all four *Populus* species and evidence strongly suggests that stomata are active during the night since VPD was shown to affect G (i.e. non-linear response of E to VPD; Fig. 3-3). Further evidence of active stomatal involvement comes from measurements of cuticular transpiration, which show that the cuticle is only responsible for a small fraction of the leaf transpiration in the dark (Fig. 3-4). When subjected to moderate drought, two groups could be identified in terms of night-time water loss which were consistent with habitat preference: those that maintained high levels of G (and E) after dark comparable to well-watered conditions (*P. deltoides* and *P. trichocarpa*), and those that showed a marked decline in water use (*P. tremuloides* and *P.* × *petrowskyana*). In either group, notably, night-time G remained significantly higher than cuticular conductance during moderate stress, indicating stomatal participation in a water loss process that could be construed as deleterious to the water relations of trees; this is especially intriguing in *P. tremuloides* that frequently experiences water shortages. The different responses of G_N between these 2 groups of species were not associated with the rates of day-time G or E; on the contrary, sharp contrast in absolute G can be observed within groups in Figure 3-2. We expand on each of the above points below.

All species showed a negative correlation between G_N and VPD under well-watered conditions and all the slopes were significant (Fig. 3-3). Howard and Donovan (2010) found that night-time g_s increased during the night in *P. angustifolia* but remained constant in *P. balsamifera* despite both species being

exposed to the same, declining VPD conditions, suggesting the action of a circadiam rhythm in *P. angustifolia*. We did not find a trend independent of VPD or evidence suggesting a circadian rhythm in any of the four species. Outside of tree species, the night-time VPD response we encountered agree with the observations of Barbour and Buckley (2007) who also show an active nocturnal stomatal response in *Ricinus*. Accordingly, we would expect that if appreciable night-time conductance resulted from residual stomatal openness or simply from cuticular permeability, G_N (and thus night-time g_s) should not present a discernible trend under varying atmospheric demand. An example of such non-responsive stomata can be seen in droughted *Quercus* spp. at night (Cavender-Bares et al. 2007).

Values of night-time conductance under drought were between 25 and 55% of the daytime conductance (these values can be derived from table 3-3 as G_N/G_D). Under a 16-hour photoperiod and nights with 50% of the daytime VPD, this can translate to an integrated night-time water use of about 10% of the diurnal budget during drought, as was the case in *P. tremuloides* and *P. trichocarpa*. The continued loss of water at night under water stress is in conflict with the optimization hypothesis (Cowan 1977; Katul et al. 2010 and references therein); this hypothesis has already been challenged by the occurrence of night-time transpiration under well-watered conditions but loss of water at night during drought is a much stronger contradiction. Moreover, the fact that stomata appear to remain open at night even under stress, albeit to a lesser degree in non-riparian species, suggests that this water loss is at a time when C gain is not possible and water conservation would appear paramount.

High nocturnal transpiration is not unexpected in anisohydric riparian species such as *P. deltoides*, although we had anticipated that drought would exert more self-regulation at night when no carbon gain is to be had from opening stomata. Nevertheless, we could see a steeper decline in G_N in response to VPD under drought, compared to well-watered, in both riparian and non-riparian plants (Fig. 3-3). It is noteworthy that the high day- and night-time conductance of droughted *P. deltoides* and *P. trichocarpa* plants happened in seedlings, which are often recruited to temporarily flooded high river banks but die once the water level drops. In this case, their narrow distribution range is reflected in their water-use patterns.

We could only produce one-sided measurements of cuticular conductance in hypostomatic leaves, but the bare dehydration method which exposed both sides of the leaf yielded similar values in amphistomatic species. In all cases, cuticular conductance was significantly lower than G_N, confirming that stomata were involved in nightly water loss. We also obtained anatomical evidence that, even in high-transpiring amphistomatous species, stomata seem perfectly capable of closing fully (see Figure 3-5 for examples of closed stomata of *P. deltoides* and *P. trichocarpa*). A higher magnification image of a closed stoma of *P. deltoides* (Fig. 3-5c) shows that prominent cuticle-covered outer ledges (and presumably the inner ledges as well) can overlap and form a tight seam upon closing, likely sealing the stoma when closed. In addition, as already discussed, the non-linear relationship between whole-plant conductance and night-time VPD both in non-droughted and droughted situations (Fig. 3-3) provides further evidence that stomata remain open,

in excess of their minimum, whether as a whole or, probably, as part of a patchy network (Pospíšilová and Šantrůček 1994). Thus, at least in the species we analysed, we state that night-time transpiration appears to not simply be due to "incomplete stomatal closure" but more likely has other physiological benefits.

It is interesting to note that daytime conductance in well-watered conditions appeared to correlate with stomatal pore index. Sack et al. (2005) also found a similar correlation in tropical species. The SPI_v we report, however, conveys a more realistic value of pore area since we were able to measure the average width and length of every stoma in the sample and we approximate the opening geometry averaging an ellipse and a fusiform shape. When the G_N/G_D ratio was applied to the daytime SPI_v, the resulting values agreed with the correlation (Fig. 3-6).

We modelled G_N based on the effects of a theoretical minimum SPI based on the smallest stomatal opening that we could find for each species, assuming this minimum would be obtained if all stomata showed the same level of closure. Since we found completely closed stomata on most species, the minimum SPI resulted in zero. The values calculated for *P.* × *petrowskyana* and *P. trichocarpa* were higher since the minimum measured stomatal width was greater than zero for the adaxial side of *P.* × *petrowskyana* and the abaxial side of *P. trichocarpa*. The calculated minimum G_N for these species was still well below the measured G_N (Fig. 3-6). This provides further insight into the role of stomata in night-time transpiration, suggesting deliberate opening as the main driver.

There are several hypotheses to explain a functional role of night-time transpiration. There is some evidence that nutrient availability is linked to nocturnal

conductance (Scholz et al. 2007; Barber 1995; Marschner 1995). However, under a rapid drought cycle, it is unlikely that nutritional status would take precedence in any but the most extreme nutritional situations, especially since most growth processes slow down long before carbon fixation becomes limiting due to stomatal limitations (Hsiao and Acevedo 1974). On the other hand, the flow of sap may provide an important source of oxygen to internal tissues, particularly those with a high metabolic requirement, even in situations where conserving water may be critical. It is conceivable that O_2 levels could fall dangerously low without the replenishment from xylem sap flow (Eklund 2000; Gansert 2003; Daley and Phillips 2006) particularly within larger stems; lack of O_2 could hasten heartwood formation and slow down the recovery after drought. It is not clear, however, whether internal tissues are actually susceptible to low oxygen such that reduced sap flow might have an impact (Spicer and Holbrook 2005).

The evidence presented here clearly shows that there is a wide range of G_N among members of the same genus and species. The source of this variation appears in part to be the stomatal pore area, although this alone cannot explain the differences in G_N/G_D that span across species. The more drought tolerant species showed some reduction in G_N under drought earlier in the drought cycle. However, those species that reduced E_N under drought still maintained a level of G_N several fold greater than g_c, thus stomatal involvement seems apparent. The evidence suggests that night-time sap flow through transpiration may be based on physiological needs. We believe this warrants further investigations into the nature and role of night-time conductance in a wide range of species.

Tables and Figures

Table 3-1. Species studied; ranked by estimated drought tolerance, from least to most tolerant.

Species	Characteristics
P. deltoides	Exclusively riparian. Tends to dominate river banks.
P. trichcarpa	Wetland species, cannot compete under drier conditions.
P. tremuloides	Frequently found in upland sites. Known to respond quickly to drought (low stomatal conductance, Lu et al., 2009).
P. × petrowskiana	Hybrid of *P. nigra* × *P. laurifolia*. Both parents are drought tolerant. In previous experiments this clone showed the highest level of isohydry.

Table 3-2. Average whole-plant conductance (G) and water-use under well-watered and drought conditions, and minimum night-time conductance (G_N) under the maximum level of drought achieved before total leaf desiccation (Max. Dr.). Different superscript letters indicate statistically significant differences ($p < 0.05$). Bold numbers show the maximum value in the column. All units are mmol m^{-2} s^{-1} kPa^{-1} except where % is indicated.

Species	Well-watered			Drought				Max. Dr.	
	G_D	G_N	$U_{N:D}$ (%)	G_D	G_N	$U_{N:D}$ (%)	G_N dec.[1,2] (%)	G_N	g_c*
Populus deltoides	**2.45**	**0.93**[a]	17	**2.40**	**0.92**	18	1(0)	**0.73**	0.0646
Populus trichcarpa	1.62	0.76[ab]	20	1.49	0.71	**22**	6(8)	0.17	0.0149
Populus tremuloides	1.64	0.56[bc]	15	0.91	0.37	19	33(33)	0.17	0.0543
Populus × petrowskyana	2.43	0.52[bc]	9	1.07	0.28	14	**46**(46)	0.14	0.0318
Means	1.57	0.57	15	1.16	0.47	18		0.23	0.0422

[1]Represents % decline of G_N under drought with respect to G_N under well-watered conditions.

[2]Numbers in parentheses are VPD-adjusted (see Methods).

*g_c: cuticular conductance.

Table 3-3. Stomatal characteristics. Different superscript letters within the same column indicate statistically significant differences ($p < 0.01$). adax: adaxial side; abax: abaxial side.

Species	Stomatal density *mm*$^{-2}$			Relative[1] stomatal ratio	Stomatal length (adax / abax)	Stomatal width (adax / abax)	Stomatal pore index (adax + abax)
	adaxial	**abaxial**	**total**	*adax:abax*	*µm*	*µm*	*×10*$^{-3}$
P. deltoides	89±10	120±19	209	43:57	24.5/20.2	5.0/4.9	11.37^{b}
P. trichcarpa	20± 8	175± 8	195	10:84	25.8/22.0	5.3/4.3	10.63^{b}
P. tremuloides	-	116±17	116	0:56	- /22.2	- /5.3	4.93^{c}
P. × *petrowskyana*	46± 8	147±23	193	22:70	23.5/21.8	5.0/6.3	17.07^{a}

[1]Relative to the total density of *P. deltoides*.

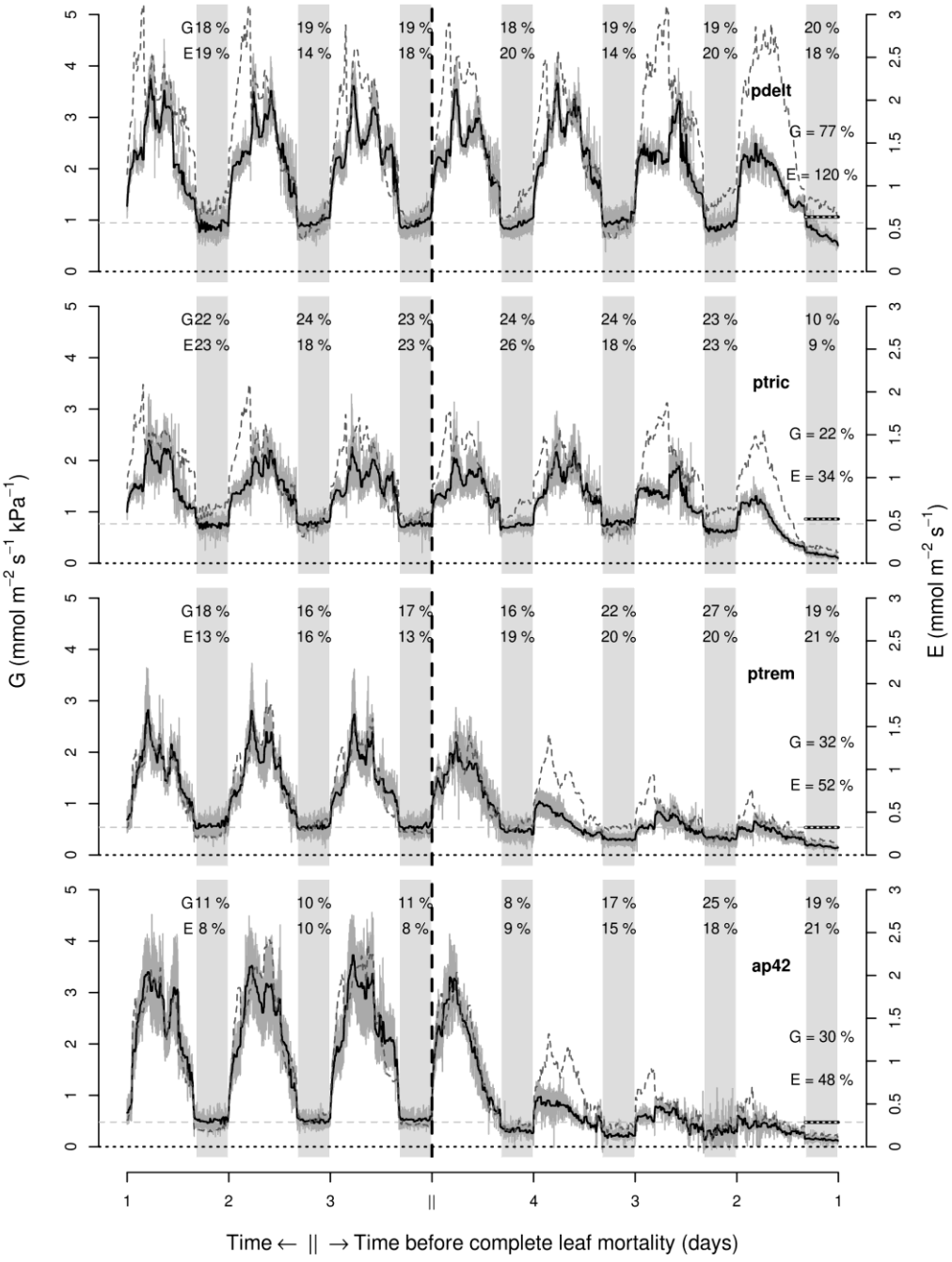

G18 % 19 % 19 % 18 % 19 % 19 % 20 %
E19 % 14 % 18 % 20 % 14 % 20 % 18 %
pdelt
G = 77 %
E = 120 %
G22 % 24 % 23 % 24 % 24 % 23 % 10 %
E23 % 18 % 23 % 26 % 18 % 23 % 9 %
ptric
G = 22 %
E = 34 %
G18 % 16 % 17 % 16 % 22 % 27 % 19 %
E13 % 16 % 13 % 19 % 20 % 20 % 21 %
ptrem
G = 32 %
E = 52 %
G11 % 10 % 11 % 8 % 17 % 25 % 19 %
E 8 % 10 % 8 % 9 % 15 % 18 % 21 %
ap42
G = 30 %
E = 48 %
G (mmol m−2 s−1 kPa−1)
E (mmol m−2 s−1)
1 2 3 || 4 3 2 1
Time ← || → Time before complete leaf mortality (days)

Figure 3-1. Whole-plant conductance (G, solid black trace) and transpiration (E, dashed black trace) of the four *Populus* species before and after drought (point-for-point average of 5 plants per species). pdelt: *P. deltoides*; ptric: *P. trichocarpa*; ptrem: *P. tremuloides*; ap42: *P.* × *petrowskyana*. G is shown with its standard deviation (gray envelope). Light-gray vertical bands indicate the night period. The first 3 days correspond to well-watered conditions, followed by the last 4 days of drought ending in total leaf mortality. A black dotted horizontal line denotes the zero mark; a gray dashed horizontal line marks the average night-time G (five-night average) during the well-watered period. Night-time G and E are indicated also as a % of previous day-time period. On the last night, additional percentages of G and E are shown, which represent the G and E of that night, as a percent of the five-day average rates under well-watered conditions.

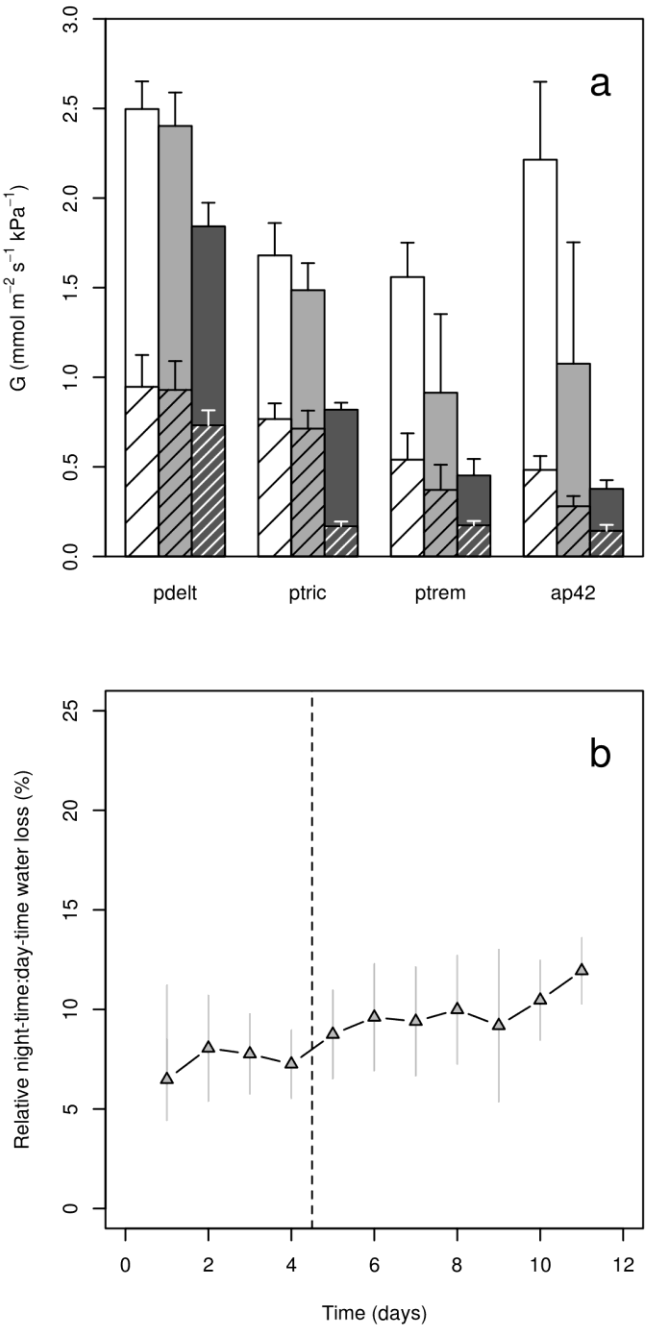


Figure 3-2. Summary of day- and night-time whole-plant stomatal conductance (G) by drought intensity (a) and general relative response to drought (b). Overlapping bars in (a) are non-cumulative and show G_D (solid bars) and G_N (striped bars) under well-watered conditions (light bars), moderate drought (medium gray bars) and severe water shortage (dark bars). Error bars are one-sided standard deviations. pdelt: *P. deltoides*; ptric: *P. trichocarpa*; ptrem: *P. tremuloides*; ap42: *P.* × *petrowskyana*. (b) Ratio of night to daytime conductance G_N/G_D of all species combined under 4 days of well watered conditions and then increasing drought.

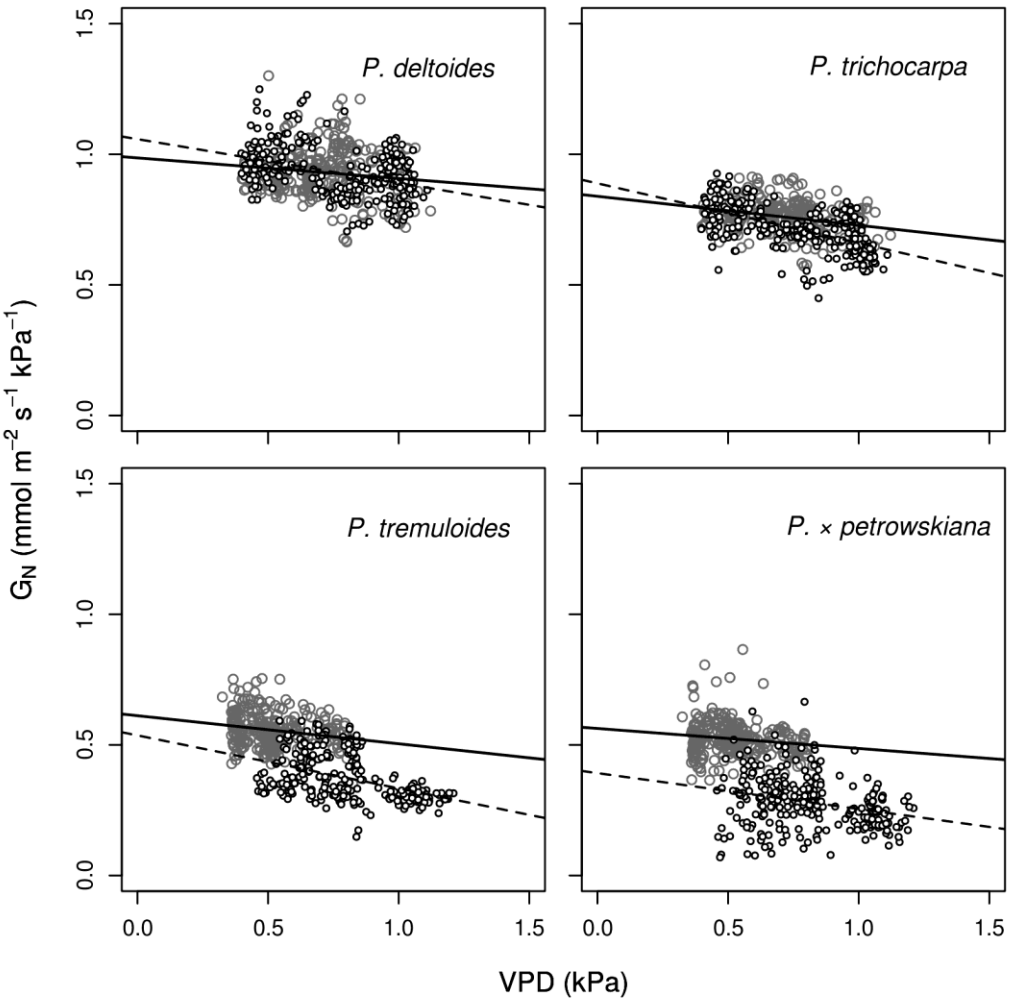


Figure 3-3. Night-time whole-plant stomatal conductance (G_N) in response to night-time VPD under well-watered (larger gray points, solid line) and moderate drought (smaller black points, dashed line) conditions. All linear regression slopes showed a significant decline; *i.e.* slopes significantly different from zero ($P < 0.05$).

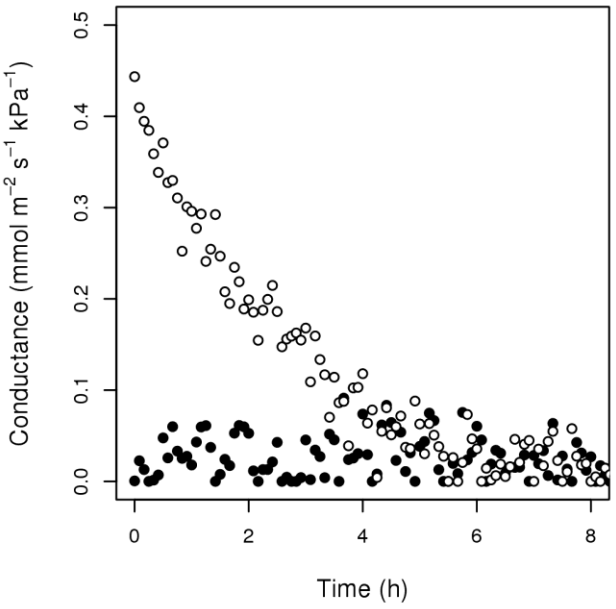


Figure 3-4. Night-time leaf-level conductance of excised leaves measured from weight loss (air drying). Abaxial conductance (stomatal + cuticular) is shown with open symbols and adaxial conductance (cuticular) with closed symbols. The conductance of each side was measured after the opposite side had been sealed (see Materials and Methods).

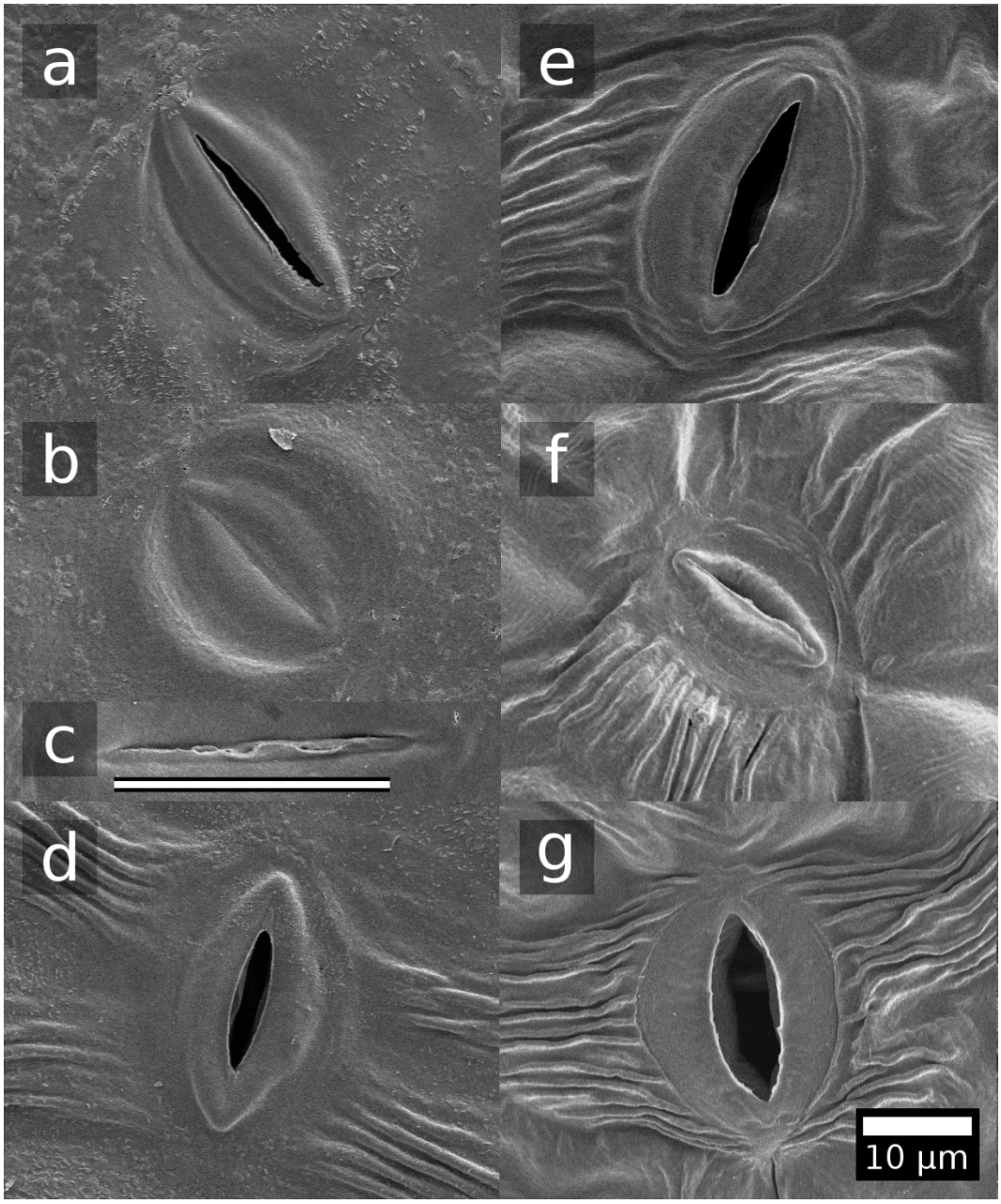


Figure 3-5. Scanning electron microscope images of a representative stoma for each species. (a) *P. deltoides*, open; (b) *P. deltoides*, closed; (c) higher magnification image of a closed *P. deltoides* stoma which was prepared by air-drying the sample to better preserve the cuticle covering the outer ledges; (d) *P. tremuloides*, open; (e) *P. trichocarpa*, open; (f) *P. trichocarpa*, closed; (g) *P.* × *petrowskiana*, open. All images were taken at the same magnification except (c) where the magnification bar represents 10 µm.

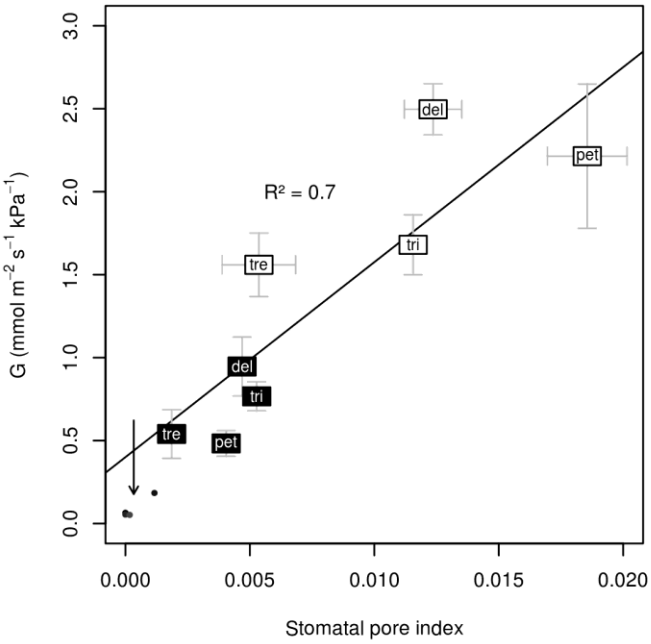


Figure 3-6. Whole-plant stomatal conductance (G) under well watered-conditions in relation to stomatal pore index (SPI). White boxes represent G_D while black boxes represent G_N. X and Y error bars are standard deviations. Black dots correspond to the modelled conductance assuming an SPI where all stomata are at the minimum measured opening.

Chapter 4. N form affects whole-plant conductance and root anatomy in two contrasting *Populus* hybrid clones

Introduction

Plants exert tight control over water movement by varying the resistance to water flow at different checkpoints along its path. Although the role of transpiration (E) in various functional processes continues to be a controversial topic, some clues point to a link between mineral nutrition and the regulation of water flow, nitrogen being of particular interest in this respect (Gorska et al. 2008; Cramer et al. 2009; Matimati et al. 2014).

Mineral N available to plants occurs primarily in two forms: nitrate (NO_3^-) and ammonium (NH_4^+). NO_3^- is most commonly found in early successional environments after a disturbance and it is characteristically labile. In many species, NO_3^- seems to have a stimulatory effect on plant water transport, affecting different physiological functions of both roots and leaves. A localized increase in soil [NO_3^-] can lead to a rapid increase in root hydraulic conductance in herbaceous species (Clarkson et al. 2000; Gloser et al. 2007; Gorska et al. 2008; Cramer et al. 2009). This coupling of NO_3^- and water conductance appears to be driven by changes in the hydraulic properties of the cell membrane, mediated by intracellular [NO_3^-] (Gorska et al. 2008). Therefore, NO_3^- might act as a hydraulic signal to rapidly coordinate responses at the whole plant level (Gorska et al. 2008). Unlike NO_3^-, NH_4^+ has not been shown to increase root hydraulic conductance (Guo et al. 2007).

Some studies suggest that increased nutrient acquisition and delivery could be achieved through transpiration-mediated mass flow (Ludwig et al. 2006; Cramer et al. 2009; Matimati et al. 2014; Cirelli, unpublished data). In this context, NO_3^--induced changes in root hydraulic conductivity might contribute to the acquisition of N by mass flow, while also contributing to the flow of other soil-mobile nutrients toward the rhizosphere (Cramer et al. 2009). It has been argued nocturnal transpiration (E_N) is linked to nutrient uptake under situations of nutrient limitations (Ludwig et al 2006; Scholz et al 2007), but this has not been supported by other studies (Howard and Donovan 2007, 2010; Kupper et al 2012). Furthermore, the impact of form of N on E_N remains unaccounted for. Maintaining E_N could be beneficial when water is plentiful and mobile nutrients are scarce. As NO_3^- is more mobile than NH_4^+, it may be possible that plants under NO_3^- nutrition display a higher E_N than those under NH_4^+.

The form of N is also known to induce changes in root morpho-anatomy. In several species, plants tend to develop thinner roots under NO_3^- nutrition than under NH_4^+ nutrition (Anderson et al. 1991; Cruz et al. 1997). In two hybrid poplar clones (*Populus maximowiczii* × *P. balsamifera*), NO_3^--fertilized trees had higher fine:coarse root ratios and higher specific root length (length-to-mass ratio) than NH_4^+-fertilized trees (Domenicano et al. 2011). It has been suggested that the thinner roots produced under NO_3^- would reduce the resistance to lateral water flow (Cruz et al. 1997), although the cytological basis underlying N-form-mediated changes in root diameter and specific root length are poorly documented (Walch-Liu et al 2006). A study performed in Arabidopsis showed that a localized supply

of NO_3^-, but not NH_4^+, stimulates the development of lateral roots by increasing rates of cell production in the root tips (Zhang et al. 2007). The authors concluded that the developmental change was triggered as a result of the signaling properties of the NO_3^- ion itself. However, and despite the importance of NO_3^- signaling, the effects of N form on anatomical features have not been described for a wide range of species, including *Populus*.

The physiological and morpho-anatomical changes induced by the form of N are also associated with differences in growth. Issues of NH_4^+ toxicity aside, the activation of the regulatory mechanisms by which plants adjust their metabolism of specific N forms may, secondarily, impact carbon assimilation and water relations. Nitrate needs to be reduced to NH_4^+ in order to be used by the plant and this is energetically expensive steps, because the reduction of NO_3^- to NO_2 by nitrate reductase (NR), and the subsequent reduction of NO_2^- to NH_4^+ by nitrite reductase (Gerendás et al. 1997) requires energy. However, the costs for the reduction of NO_3^- to NH_4^+ is lower if the conversion takes place in the leaves instead of roots because it can be coupled directly to the electron transport chain. On the other hand, NH_4^+ seems to have an inhibitory effect on photosynthesis which, at least in vitro, has been proposed to be related to the uncoupling of electron transport from photophosphorylation in chloroplasts due to NH_4^+ accumulation in leaves (Gerendás et al 1997). Agricultural crop species supplied with NH_4^+ generally have lower water use efficiency (WUE) than those supplied with NO_3^- (Cramer et al. 2009). Information regarding N-form-mediated effects on transpiration, carbon assimilation and water use efficiency has not been assessed in *Populus* species.

In this study we assess the effects of N form on whole plant physiology in a fast (AP2403) and slow (AP9) growing poplar hybrids. Specifically, we assess day- and night-time conductance, net photosynthesis and growth, and foliar nutrient concentrations. We also investigate developmental changes at the leaf and root level. We pursue the following hypotheses: (i) inorganic nitrogen in the form of NO_3^- increases whole-plant conductance (particularly night-time) relative to NH_4^+, (ii) the increased transpiration under NO_3^- nutrition will have a secondary effect on the delivery of other nutrients such as K and Ca, for which mass flow is an important form of acquisition and delivery, and (iii) plants under NH_4^+ nutrition will produce thicker roots since they appear to be the main site of NH_4^+ metabolism. Given that higher growth rates have been associated with higher conductance (Tyree 2003) and fast-growing poplar clones have been shown to be more sensitive to N fertilization than slow-growing clones (Li et al. 2012), we expect that N form would have an overall greater effect in AP2403.

Materials and Methods

Plant material and general growth conditions

Clones of two *Populus* hybrids were grown from cuttings provided by Alberta-Pacific (Al-Pac, Canada); AP2403, a fast-growing (*P. deltoides* × *P.* × *petrowskyana*) × *P.* × *petrowskyana* hybrid (*P.* × *petrowskiana* being the hybrid *P. nigra* × *P. laurifolia*), and AP9, a slow-growing *P. balsamifera* × *P. deltoides* hybrid. The characterization as 'fast'- and 'slow'- growing is based on growth data from Al-Pac's evaluation of different hybrids in field plantations in central Alberta, Canada

(Dr. Barbara Thomas, personal communication). The same characteristics were observed in our own trials in potted plants growing outside on a roof-top patio.

Frozen cuttings of each clone, ~ 10 cm in length, were soaked in tap water for 24 hours at room temperature, then rinsed and planted in Rootrainers® propagation trays (Beaver Plastics, Acheson, Alberta, Canada) containing LA4 potting mix (Sun Gro Horticulture, Canada). Once planted, the cuttings were watered once with a high-P liquid fertilizer containing indole-3-butyric acid to aid initiation of adventitious roots. The planted cuttings were put in a roof-top greenhouse where they remained for the duration of the experiment. When plants had an average of 10 leaves, they were removed from the Rootrainers® and their roots carefully washed to remove as much of the potting mix as possible, then transplanted to 5-l pots. A mix consisting of equal parts washed silica sand and perlite was used as substrate to minimize the cation exchange capacity around the roots. After transplantation, plants were allowed to grow for 4 weeks before applying the treatments, to an average height of 70 cm with 26 leaves for AP2403 and 77 cm with 30 leaves for AP9. During this time, plants were given 14-14-14 NPK in liquid form once immediately following transplantation and once after 2 weeks. Plants were watered twice daily to offset the fast-draining quality of the substrate and they never showed any signs of water stress. Supplemental light was provided by grow light fluorescent tubes and the daily photosynthetically-active radiation average was 400 μmol m^{-2} s^{-1}. Average temperature throughout the experiments was 21 ± 3 °C.

Application of N in different forms

Sixteen plants of each hybrid were randomly selected and watered with distilled water 8 times in the course of 2 days, each time allowing enough water (approximately the volume of the pot) to percolate so that remaining nutrients adsorbed to the substrate could be leached out. Plants were then watered with a complete nutrient solution with a [N] of 2 mM either as $NaNO_3$ or NH_4Cl. This concentration was selected to reflect average N levels in boreal forest soils (Larcher 2003). The pots were placed in a holding tub and twice watered to excess with the nutrient solutions to fully flush existing nutrients from the soil. The same nutrient solutions were used to water the plants twice daily for the remainder of the experiment. An exception to this was that once, midway through the experiment, pots were well-flushed with distilled water to avoid accumulation of salts in the substrate. The nutrient solution was prepared in 6.5 pH 0.4 mM phosphate buffer every 2 days from separate stocks. The composition of the nutrient solution is presented on table 4-1.

Transpiration and whole-plant conductance

The Amalthea system (see Cirelli et al. 2012) was used to continuously monitor transpiration (E) by gravimetric water loss. Briefly, 8 top-loading balances were used (Adam PGW 4502e, Adam Equipment, USA) to simultaneously monitor water loss in 4 plants of each treatment for 7 days, one hybrid at a time. Because of the constraint imposed by the amount of balances available, the hybrids were measured sequentially, first AP2403 and then AP9. Thus, the beginning of treatment for AP9 was delayed 7 days with respect to AP2403 to offset the time chosen to monitor

transpiration. The balances were arranged in two rows along a greenhouse bench, such that each row of balances was below a row of light fixtures. The balances were connected via USB-to-serial cables to a USB hub and finally to a laptop running the Amalthea software under Ubuntu 10.04. Power for the balances and laptop was provided by a voltage-stabilising uninterruptible power supply. The balances were calibrated with reference weights before each set of measurements.

Four plants of each treatment were randomly selected. After being watered and fully drained, each pot was blotted dry and its top covered with aluminium foil. Drain holes were left uncovered. Each plant was randomly assigned to a balance, and extra plants from both treatments were placed around the balances to provide a minimal canopy and minimize edge effects. Every day the system was paused, one balance at a time, to water that plant with the appropriate nutrient solution by the amount lost since last being watered; no water drained from the pot. This was done once in the morning and once in the evening. The measurement of transpiration continued for 7 days to provide a short-term measurement after initial treatment. The same procedure was repeated a second time for each clone, 5 weeks after the beginning of treatment on the same plants, to provide a long-term measurement. This second measurement also lasted 7 days.

Throughout the transpiration measurements, light, temperature and humidity were monitored by sensors placed in mid canopy and above the canopy as well. Sensor assemblies were constructed specifically for this task following the open-sourced specifications of the microviron project (http://microviron.sourceforge.net). This allowed the transpiration measurements

and the environmental measurements to be synchronized point-for-point. Whole-plant stomatal conductance (G) was calculated as E divided by the atmospheric vapour pressure deficit (VPD) once the evaporation rates had been converted to actual E by leaf area (see below). The calculation of $G = E/VPD$ assumes that the average leaf temperature is equal to the surrounding air temperature. Although under higher light intensities the effect of radiation on leaf temperature becomes significant, appropriate modelling was performed to account for leaf heating. For brevity, these calculations are not shown. However we report that allowing for a temperature difference of 2 ºC between treatments did not alter the differences observed nor the interpretation of the results. In absolute terms, not accounting for leaf heating results in the overestimation of conductance during the day by about 15 to 20% for maximum allowed temperature increases of 2 to 5 ºC. Night-time results are unaffected.

Leaf area and growth measurements

One day prior to starting the transpiration measurements, the leaf area of each plant was recorded non-destructively. The incremental leaf area of every plant was recorded 4 more times, once every 2 days for a total of 5 data points. To measure the area, the width (W) and length (L) of each leaf was taken using a Vernier calliper. The area of each leaf was calculated as $a = WLf$, where f is a species-specific factor (0.6237 for AP2403 and 0.6924 for AP9) previously determined by a calibration constructed from destructive measurements (data not shown). A quadratic function was fitted for each plant using the 5 leaf area points obtained, versus time expressed in UNIX epoch seconds. The output from Amalthea contains

a time value associated with every point, which is also in UNIX epoch seconds (also known as "timestamp"). The timestamps from the balances were used as input for the area function of every plant. This provided a value of leaf area for every plant and for each data point in the water loss output from Amalthea, with which to calculate transpiration on a leaf-area basis.

The same procedure was repeated a second time for the measurements of long-term effects. Every time a leaf area measurement was taken, height was also recorded. Once the experiments had ended plants were harvested, separated into leaves, stems, and roots, and dried in an oven at 70 °C. The weight of the separate dry parts was determined. The last 3 fully-expanded leaves of every plant were dried and weighed separately, then ground for chemical analysis.

CO_2 response curves and stomatal limitations

To analyse limitations to photosynthesis (A), CO_2 response curves (A/C_i curves) were constructed using a LI-COR 6400 (LI-COR, Lincoln, Nebraska, USA) infra-red gas analyser (IRGA). During the second set of transpiration measurements (long-term effects), plants were removed from the balances one at a time to be measured with the IRGA. The measurements were taken between the hours of 10:00 and 13:00. Because of the time required to create the A/C_i curves, only 3 to 4 plants were measured in one day for a total of 6 plants per treatment. Priority was given to constructing the A/C_i curves during approximately the time of day when photosynthesis is naturally at its maximum. CO_2 was provided by cartridges and progressively higher concentrations were used in the IRGA chamber in the order: 400 (for initial reference in atmospheric conditions), 50, 100, 200, 300, 400, 400,

600, and 800 ppm. Light intensity was set at 1500 µmol m^{-2} s^{-1} PAR so as to not be limiting. In every case, the conditions of temperature and humidity inside the IRGA chamber were matched to those measured in the open air around the vicinity of the plants prior to starting the response curve. Since our primary interest was to assess stomatal limitations to photosynthesis, we chose to fit a single, exponential-rise-to-maximum, model for the entire curve of the form $y = o + a(1 - e^{-bx})$. The function was fitted using the pooled values from each plant in a treatment. Stomatal limitations were calculated as:

$$L_S = \frac{A_0 - A_L}{A_0}$$

where A_0 is the assimilation rate at 400 ppm internal CO_2, (C_i = 400 ppm, i.e. without limitations) and A_L is the assimilation rate at 400 ppm external CO_2 (C_a = 400 ppm).

Leaf nutrient analysis

Upon the beginning of treatments, the youngest visible leaf was marked. Samples were collected from 6 plants from each treatment, starting with leaf number 5 after the marked leaf. The samples were oven-dried and ground in a Wiley mill with a number 40 mesh. Total N and P concentrations were determined with a SmartChem 200 (Unity Scientific, USA) discrete wet chemistry analyzer after sulfuric acid – hydrogen peroxide digestion. The concentrations of K and Ca were determined using a model AA880 atomic absorption spectrophotometer (Varian, Australia).

Anatomy

Impressions of both leaf surfaces were taken to analyse stomatal anatomy. Clear nail polish was applied as a thin coat on either leaf surface, and lifted from the leaf with the aid of clear sticky tape. The impressions were mounted onto glass slides for viewing in the light microscope. Digital images were captured for analysis with ImageJ (Abramoff et al. 2014). Stomata were counted and measured in length.

Leaf discs of the most recent fully-expanded leaf were taken with a hole-punch, from areas occupied by tissue in between veins of order n-1. Samples were immediately placed in vials with FAA and stored on ice for a maximum of 30 min until they were placed under vacuum in the lab, where they were left overnight. The tissue was included in paraffin wax, cross-sectioned and stained with Safranin – Fast-Green. Digital bright-field images were captured for processing and analysis with GIMP (GNU Image Manipulation Program, http://www.gimp.org/) and ImageJ (http://www.imagej.net/). The thicknesses of each epidermis and each mesophyll layer were measured.

Root tips 3 cm in length were collected from each plant. From these excised pieces, a portion less than 1 cm long was taken from the proximal end. Both resulting pieces were placed in FAA and processed in the same way as the leaf tissue. The proximal sample was then prepared for cross-sectioning. We used longitudinal sections of the distal samples to confirm that the features in the cross sections obtained from the proximal samples corresponded to fully-mature tissue. Images of the cross sections were captured and analysed. Tissue boundaries were delineated at the interface between vascular cylinder and cortex, and cortex and

exodermis, in low-magnification images (2.5X objective) where the entire root was visible. The areas of each of these groups were measured. Higher magnification (10X objective) was used to capture images for analysis of vessels in the vascular cylinder. These images included a representative wedge of approximately one-quarter of the root. The lumen area of each vessel in the sample was measured.

Allometric calculations and integrated intrinsic water-use efficiency

Defining t_0 as the beginning of treatment and t_1 as the time when plants were harvested, total plant mass at t_0 was calculated as:

$$m_0 = m_{s0} + m_{s0}q$$

where m_0: total plant mass at t_0, m_{s0}: mass of the shoot at t_0 and q: root:shoot ratio. The individual plant components at t_0 were obtained from:

$$m_{s0} = m_{a0} + m_{b0}$$

where m_{a0}: leaf mass at t_0 and m_{b0}: stem (bole) mass at t_0, given:

$$m_{a0} = \frac{a_0}{\gamma_L}$$

and

$$m_{b0} = \frac{m_b}{\left(\frac{dh}{d_0 h_0}\right)} = \frac{m_b d_0 h_0}{dh}$$

where a_0: leaf area at t_0, γ_L: leaf specific weight, m_b: mass of the stem at t_1, d_0: stem base diameter at t_0, h_0: stem height at t_0, d: stem base diameter at t_1 and h: stem height at t_1.

Leaf specific weight and R:S corresponding to plants from the NO_3^- treatment was used to back-calculate m_0 for plants in the NH_4^+ treatment. This was done because

both groups of plants were fed a low level of NO_3^- during early development, and since the NO_3^- plants grew larger it is more conservative to use them as the basis for m_0 without overestimating Δm.

The integrated intrinsic water-use efficiency was calculated as:

$$\eta_w^i = \frac{\Delta m}{\bar{G}_w}$$

where Δm is the difference in total mass between t_1 and t_0, and Gw is the average of the weighted means of G at t1 and t0 such that:

$$G_w = \frac{\bar{G}_N}{3} + \frac{2\bar{G}_D}{3}$$

where $\bar{G}_N$ and $\bar{G}_D$ are the average night-time and day-time G. Since G was lower in the second measurement (before harvest) than at t_0, $\bar{G}_w$ assumes that the decay in G between t_0 and t_1 was linear.

Statistics

All analysis were performed using the R language for statistical computing (http://www.r-project.org/). Given the natural modal variation during the day, G values were separated in two groups for analysis, one corresponding to the first half of the day-time period and the other to the second half. The sums of the G values for each day-time half and for the night-time period were compared between treatments using a repeated-measures two-way ANOVA where the different days were included in the error term and the hybrids were included in the interaction term.

Measurements that were not cyclically dependent on time were compared between treatments using two-way ANOVAs allowing for an interaction term

between the hybrids and the treatments. These included quantifications of anatomical characteristics such as stomatal density tissue areas as well as growth parameters and foliar nutrients. Where appropriate, one-way ANOVAS were applied after rejecting a non-significant interaction term.

Results

The form of N nutrition affected whole-plant stomatal conductance (G), photosynthesis and growth in both poplar hybrids. The fast-growing clone AP2403 showed 10% lower daytime conductance for NH_4^+-fed plants shortly after beginning treatment (fig. 4-1, 1 week). Nighttime conductance for AP2403 was also lower in the NH_4^+ treatment but only marginally. Conversely, the NH_4^+-fed plants showed slightly increased G in AP9 after one week of treatment but differences were not significant ($p > 0.05$). After longer exposure to different N forms, all NH_4^+-fed plants showed lower G (fig. 4-1, 5 weeks). In both clones, the difference between NH_4^+- and NO_3^--fed plants was only significant during the day ($p < 0.05$). However, nighttime conductance also showed a small decreasing trend in the NH_4^+ treatment in both hybrids. In general, plants showed a decreasing trend over time in conductance, assimilation, and leaf expansion rates regardless of treatment. Differences in G_N were significant between hybrids. No significant interaction was found between hybrids and treatments ($p > 0.05$); indicated differences henceforth are based on one-way ANOVAS.

Overall, projected patterns of water use at constant VPD were different between the clones but did not change with the form of N (table 4-2). During 5 weeks of treatment, AP9 had a projected water use of 94 l when fed NO_3^- and 78 l

when fed NH_4^+. This represents an excess of 50% the water use of AP2403 under NO_3^- and 70% under NH_4^+ (table 4-2); a good part of this difference being driven by nocturnal transpiration (at constant VPD). In both hybrids, the difference in whole-plant day-time conductance between treatments was more accentuated near midday, becoming less pronounced toward the night. Despite the general difference in conductance, the variations within the diel pattern were almost identical between treatments. Both hybrids presented lower conductance in relation to incident light in the NH_4^+ treatment. This was seen as a reduction in the slope of conductance as a function of light, and not simply to lower overall conductance (fig. 4-2).

Plants under the NH_4^+ treatment showed reduced assimilation rates in both clones. The maximum photosynthetic rate at atmospheric [CO_2] was reduced by approximately 30% in AP2403 and close to 20% in AP9. In both clones, the recycling of triose phosphates (plateau portion of the curves) was affected when the N form was NH_4^+ (fig. 4-3). Enzymatic activity also appeared to be lower in the NH_4^+ treatment, as hinted by the decline in the slope of the lower [CO_2]-portion of the A/C_i curves (fig. 4-3). Based on the A/Ci curves, stomatal limitations were slightly lower in the NH_4^+ treatment in AP9. In AP2403 on the other hand, stomatal limitations increased almost twofold in the NH_4^+ treatment compared with the NO_3^- treatment. Water-use efficiency was negatively impacted under NH_4^+ nutrition showing a 14% decline in AP2403 and 31% in AP9 (table 4-3).

Growth was markedly reduced in NH_4^+-fed plants with respect to NO_3^--fed plants. Total dry weight was 20% lower in AP2403 and 29% lower in AP9. Both hybrids showed a reduction of 35% in stem dry weight and 32% in leaf dry weight

(table 4-3). In contrast with the aerial part, root dry weight increased by about 30% in AP2403 in the NH_4^+ treatment, and only by 5% in AP9 (table 4-3) Consequently, root-to-shoot ratios of NH_4^+-treated plants increased twofold in AP2403 and 64% in AP9. Leaf size (and leaf relative expansion rate) was also diminished in both clones by about 20% (LA_5E, table 4-3). NH_4^+ nutrition slowed the rate of leaf appearance (LAR) in both hybrids, from approximately 1 leaf every 2 days, to around 1 leaf every 3 days (table 4-3). Plant height was affected considerably in AP2403 with ~20% reduction in NH_4^+, but not so much in AP9 (table 4-3).

Differences in nutrient concentration between treatments were found in both clones. Nitrogen concentration was lower in the NH_4^+ treatment in both clones, with a significant difference of 17% in AP2403 and 12% in AP9 (table 4-4). Similarly to [N], [K] was consistently lower in the NH_4^+ treatment, but the difference was only significant in AP9 showing a 20% decline (table 4-4). In contrast, [P] was lower in the NO_3^- treatment by about 25% in AP2403 and 28% in AP9. These differences in [P] were highly significant in both clones (table 4-4). [Ca] was also lower in the NO_3^- treatment in both clones. This decline was less pronounced in AP2403 (10%) and was only significant in AP9 which amounted to 24% (table 4-4).

Aside from the change in leaf size, leaf anatomy did not seem to be radically affected by the form of N except for appreciable changes in cell size of the mesophyll. However, leaf thickness did not follow a consistent pattern in both clones in response to N form. Leaves of AP2403 that developed under NO_3^- as the N source were thicker than their NH_4^+ counterparts by 8%. The greater thickness in the NO_3^- treatment was primarily due to a pallisade layer which contained larger

cells. The opposite response was observed in AP9 in which leaves from the NH_4^+ treatment were 7% thicker and mostly because of a thicker spongy mesophyll with a small contribution from both epidermis (fig. 4-4). In the external anatomy, stomatal density of the abaxial side in both clones, showed slightly fewer stomata in NO_3^--fed plants (not significant). This small difference was annulled in AP9 clone in which the trend was reversed on the adaxial surface, while the adaxial side of AP2403 did not show differences (table 4-5).

Large differences were found in root diameter. In both clones, roots developed in NH_4^+ were 29% larger in diameter compared with the NO_3^- treatment (fig. 4-5). When considering the exodermis, the cortex, and the vascular cylinder of the root, tissue proportions on a cross-sectional area basis remained fairly constant in AP2403. In AP9 however, roots from the NH_4^+ treatment had a thinner exodermis and a proportionately larger vascular cylinder in comparison with the NO_3^- treatment (fig. 4-5). The vascular cylinder of NH_4^+-fed plants contained larger vessels. Remarkably, the result was the same in both clones, where the mean vessel diameter was 39 μm in the NH_4^+ treatment and 34 μm under the NO_3^- condition (fig. 4-5). In AP2403, the vessel frequency distribution was evenly shifted toward zero in the NO_3^- treatment compared with the NH_4^+ treatment. In AP9 on the other hand, the distribution was slightly compressed on the right hand side in the NO_3^- treatment, that is, minimum vessel size remained constant. There was a tendency in both clones, to form more vessels in groups of two or more in the NH_4^+ treatment, rather than the mostly solitary arrangement displayed by roots of the NO_3^- treatment (fig. 4-5).

Although vessel size changed equally in both hybrids, in AP9 the larger vessel size of the NH_4^+ treatment was counteracted by a lower vessel density, resulting in comparable vessel-to-xylem area ratios in both treatments. In AP2403, no significant difference in vessel density was found between treatments, which, because of the larger vessel diameter, resulted in significantly larger vessel:xylem area in the NH_4^+ treatment (table 4-6). Nevertheless, taking into account the relationship between total area of vessels to total area of root in cross-section, the different changes shown by the hybrids resulted in an increased vessel:root area in the NH_4^+ treatments. This was more marked in AP2403 (46%) than in AP9 (27%, table 4-6).

Discussion

The form of N fed to the plants had a significant impact on whole-plant conductance. Specifically, G_D was higher in both clones when N was supplied as NO_3^-. The fast growing hybrid AP2403 showed higher transpiration within a week of NO_3^- treatment while the slow growing AP9 did not, and in fact it showed slightly higher E with NH_4^+ fertilization. After five weeks of treatment however, both hybrids showed significantly higher day-time conductance and a small increase in night-time conductance under the NO_3^- treatment compared with NH_4^+. This is a seemingly simple response, but anatomical and physiological evidence suggests a slew of coordinated plant changes which may be both primary and secondary to one or many signals from N form. In addition to the differences in transpiration, we observed differences in photosynthetic rates, growth, leaf anatomy, and profound changes in root anatomy. Most of the changes were paralleled in both hybrids, and

in similar proportions between treatments. We discuss each change and the possible connections between them.

Despite differences in conductance, we found no differences in stomatal density or in potential maximum pore index of either leaf surface between treatments. This strongly suggests that differences in whole-plant stomatal conductance must have been driven by changes in stomatal aperture, either in response to the form of N or to some physiological difference attributable to N form. In both hybrids, the effects of light on G was accentuated by NO_3^- treatment compared to NH_4^+ treatment. In other words, it took higher light intensities for stomata in the NH_4^+ treatment to open to the same level as those in the NO_3^- treatment. It is interesting that both clones responded similarly since, from a hydraulic perspective, their stomatal behaviour differs radically (known from our own trials under drought); AP2403 being typically isohydric in contrast to AP9.

We expected G to be greater in the NO_3^- treatment, particularly during the night, since in most temperate deciduous species NO_3^- reduction occurs more efficiently in the leaves; specifically in *Populus*, nitrate reductase has been measured with much higher activity in leaves than in roots (Rosenstiel et al. 2004). Many plants are also known to accumulate NO_3^- overnight (see Andrews 1986; Larcher 2003) although this has not been mentioned specifically for *Populus*. While day-time whole-plant conductance was in fact greater under NO_3^- in both hybrids, G_N was only slightly higher. We cannot discount that the form of N *per se* may have had a direct effect on nocturnal stomatal opening when in fact we did see a minor difference, albeit not significant. It is evident from the data that there is a strong

interaction involving the stomatal light response and the form of N, but given the link between N form and N content and its possible involvement in stomatal function, it is more probable than the higher stomatal conductance observed with NO_3^- fertilization was secondary to other physiological changes. Thus, the question arises of whether the mechanisms behind the lower nocturnal transpiration that has been observed with increasing N fertilization (Scholz et al. 2007) act separate from, irrespective of, or in conjunction with those that "sense" the form of N.

Leaf [N] was higher in the NO_3^- treatment in both hybrids. NH_4^+ is notorious for entering the root at high rates even in NO_3^- specialists (Britto and Kronzucker 2002) but the net uptake of N as NH_4^+ can be highly inefficient and costly, due to futile import-export cycles and the C investment associated with its incorporation into the plant's glutamate pool. The argument exists that NH_4^+ should not, in principle, hinder photosynthetic performance on the basis of C cost (Miller and Cramer 2004). In our case, the lower leaf [N] of smaller plants suggests a lower net N uptake when supplied as NH_4^+, and this would be consistent with the lower photosynthetic performance of the NH_4^+ treatment which may be more directly impacted by reduced leaf N than by C economics. It must be noted, that although the shoots of NH_4^+-fed plants were generally 50% smaller in both clones, none of the plants showed any of the symptoms of NH_4^+ toxicity (Gerendás et al. 1997; Britto and Kronzucker 2002). The differences in performance with N form continues to vary across studies and species (Rosenstiel et al. 2004, Siemens et al. 2011).

The CO_2 response curves showed that, in both hybrids, NH_4^+ may be affecting photosynthesis in several ways. In AP2403, there were clear stomatal limitations to photosynthesis in the NH_4^+ treatment, which can be linked to the reduced stomatal response to light already mentioned. In AP9 the smaller stomatal opening of NH_4^+ plants did not appear to directly impact assimilation likely as other constraints to photosynthesis became more pressing. The A/Ci curves of both hybrids showed a notably lower plateau in the NH_4^+ treatment indicating an earlier onset of limitations imposed by the rate of triose phosphate utilisation (TPU) (Sharkey et al. 2007). The difference in maxima between treatments was larger in AP9, with TPU limitations acting just above ambient [CO_2], although it is unclear whether or not this might have contributed to lower assimilation at some point since PAR was generally bellow 1000 μmol m^{-2} s^{-1}. In addition, both AP2403 and AP9 had lower slopes in the initial part of the response curve when fed NH_4^+, which would suggest an enzymatic limitation. This last point can be tied back to the leaf [N] issue. It is also conceivable that the decreased stomatal function with respect to light intensity might be related with this very same issue, since guard cells depend on chloroplasts for their light-sensing functions and the phytochrome may be connected to NO_3^- metabolism (Kamiya 1989; Becker et al. 1992).

N form was also associated with differences in the concentrations of other nutrients. In both clones, leaves had lower [P] when fed NO_3^-, although this is likely due to dilution as plants were bigger, and the total foliar content of P did not change with treatment. Leaf [K] was lower in NH_4^+-fed plants of both hybrids, which is in agreement with other reports (e.g. Rothstein and Cregg 2005). In both hybrids,

leaves of NH_4^+-treated plants had higher [Ca] which might appear to contradict previous observations (Riley and Barber 1971), especially given the positive correlation between transpiration and Ca^{+2} accumulation (de Freitas et al. 2011). However, total foliar Ca content was lower in the NH_4^+ treatment despite higher concentrations, suggesting lower acquisition in NH_4^+ treatment Considering an average of 25% increase in total E in NO_3^- representing about 50% more total water loss, differences in leaf N content seem to be largely explained by an almost equally proportional increase in mass flow. Acquisition of soil-mobile nutrients may be aided by the increased water flux as other works suggest (Matimati et al. 2014), however the difference in magnitude of K and Ca contents in the hybrids, suggests the existence of considerable species-specific responses and interactions which should be further evaluated.

At the whole-plant level, the most striking morphological change was a much lower root:shoot ratio in NO_3^--supplied plants. From our perspective, this reinforces, *in vivo*, that the increased root hydraulic conductance observed in the presence of NO_3^- (Gloser et al. 2007) can, and likely does, have radical consequences for whole-plant hydraulics. In our experiments, NO_3^--fed plants not only had higher G (and thus E, under the same conditions), but also upward of 15% the leaf area. This means that the same or smaller root mass had to sustain up to 50% more water demand from the aerial part; and unless NH_4^+-given plants were grossly overbuilt in terms of root hydraulic conductance, this capacity is probably the result of increased transport activity at the root cell membrane level.

The form of N also influenced leaf and particularly of the root anatomy. In AP2403, the thicker leaves were found in the NO_3^- treatment and driven by larger cells in the palisade layer. These deeper and longer palisade cells can increase the vertical stacking and number of chloroplasts, which has been linked to higher photosynthetic efficiency (Oguchi et al. 2003). The changes in leaf anatomy however, were not consistent between hybrids. Leaves of AP9 had a slightly thicker spongy mesophyll and a thicker upper epidermis in the NH_4^+ treatment, but the functional impact of this change in unclear.

The changes that occurred in the roots offered a clearer picture of the effects that N form can have on root development in these species, albeit through different avenues. Firstly, the root diameter of NH_4^+-fed plants was larger in both hybrids. Proportionately, however, the root vascular cylinder was larger only in AP9. Secondly, root under NH_4^+ nutrition had larger diameter vessels; remarkably of the same average diameter in both clones. Lastly, a crucial factor with influence on hydraulics was that vessel density within the root vascular cylinder did not change in AP2403 but decreased significantly in AP9 under the NH_4^+ treatment. In AP9, this change in vessel density compensated for the larger vessels resulting in a vessel-lumen-to-xylem ratio similar to that of the NO_3^- treatment. However, because the vascular cylinder of NH_4^+ plants was larger, the end result was a higher vessel-lumen-to-root area (in cross-section). AP9 also showed a thinner exodermis in the NH_4^+ treatment. In contrast, none of the tissue proportions (vascular cylinder/cortex/exodermis) changed significantly in AP2403, but plants supplied

with NH_4^+ did have a greater vessel—lumen-to-root ratio, driven solely by larger vessels.

Ultimately, we might generalise the different changes in root anatomy of the two clones in response to N form as approaching the same functional response. Roots of plants fed with NH_4^+ had more cortex and more conducting area than those fed with NO_3^-. The modifications to the root induced by NH_4^+ may be costly to the plant on several accounts. Futile export cycles are carbon sinks; of the NH_4^+ that stays in the plant, most of it is likely metabolized in the roots, and it seems logical that a bigger cortex provides more tissue to process the NH_4^+. Much of the resulting glutamine (after glutamate metabolism) will be transported to new leaves via xylem-phloem redistribution (Dickson et al. 1985) which may also impose a cost, whereas the bulk of the NO_3^- may be more efficiently transported to destination directly via the transpiration stream. Another consideration is the extra xylem vasculature in the NH_4^+ treatment, which is an added C cost compared to that of plants in the NO_3^- treatment. We may speculate that the increased conducting area of NH_4^+-grown roots was a compensation for the other limitations on hydraulic conductivity, first at the root cell membrane and then as a result of the long path through the cortex to the vascular cylinder.

It is noteworthy that not only were vessels larger in NH_4^+-treated plants, but the roots had more vessels arranged in groups of two or more, whereas NO_3^--treated plants had mostly solitary vessels. This type of change is not simply modulated by changes in growth rate and cell expansion. This result is indicative of a more profound developmental change in the pattern of fibre and vessel cell

differentiation. We are not aware of other works that describe this specific change; further investigation on the effects of N form on tracheogenesis is required.

To summarize, both hybrids showed reduced whole-plant conductance particularly during the day, reduced photosynthetic performance, and lesser growth when fed NH_4^+ as the N source. The diminished conductance appears to be a function of reduced stomatal opening likely driven by a reduced light response on the part of the stoma. The reduction in stomatal opening imposed stomatal limitations to photosynthesis in high-performing hybrid, although both hybrids showed evidence of enzymatic limitations to photosynthesis. The reduction in total dry weight was driven only by the aerial parts, while the roots were slightly larger. The larger root mass was consistent with roots of larger diameter and this was associated with a greater conducting area. Roots in the NH_4^+ treatment seem overbuilt for water transport while having less demand from the shoot, and this may be a compensatory effect in response to higher resistance to radial flow as a consequence of a thicker cortex.

Tables and figures

Table 4-1. Composition of the nutrient solution used during the treatments.

Element	Concentration (μM)	Source
N	2000.000	$NaNO_3$ or NH_4Cl
K	580.000	KCl, KH_2PO_4
P	520.000	KH_2PO_4, Phosphate Buffer
Mg	90.000	$MgSO_4$
S	90.000	$MgSO_4$
Ca	60.000	$CaCl_2$
B	5.300	H_3BO_3
Fe	3.560	Fe-EDTA
Mn	2.080	$MnCl_2$
Zn	0.260	ZnCl
Cu	0.130	$CuCl_2$
Mo	0.042	Na_2MoO_4
Co	0.028	$CoCl_2$

Table 4-2. Water use during the day (U_D), during the night (U_N) and their sum total (U_T) for both clones under the different N forms, as well as the ratio U_N/U_D. All values are litres except where percent is indicated. The projected consumption period is 5 weeks and a constant VPD of 1 kPa is assumed for comparison purposes.

	AP2403		AP9	
	NO_3^-	NH_4^+	NO_3^-	NH_4^+
U_D	60.0	43.0	76.0	63.0
U_N	3.3	2.2	18.0	15.0
U_T	63.3	45.2	94.0	78.0
$U_N:U_D$	5%	5%	20%	20%

Table 4-3. Growth parameters in relation to form of N fertilization in two poplar clones. DW: dry weight of indicated plant parts. (g); R:S: root-to-shoot ratio; η_w^i: dimensionless ratio of the increment in dry matter to a weighted value of G (i.e. integrated intrinsic WUE); LA: total leaf area; LA_5E: mean leaf area of the five most recently fully-expanded leaves; LAR: leaf-appearance rate. Different superscript letters indicate statistical differences between treatments within the same clone ($p < 0.05$). n = 6.

	AP2403		AP9	
	NO_3^-	NH_4^+	NO_3^-	NH_4^+
Height (cm)	175.0 ±10.3 ^{A}	143.9 ±12.5 ^{B}	143.8 ± 8.2	138.7 ± 9.9
DW Leaf	80.2 ± 9.5 ^{A}	54.3 ±13.5 ^{B}	130.9 ±11.1 ^{a}	88.4 ±14.7 ^{b}
DW Stem	35.1 ± 7.7 ^{A}	22.9 ± 7.0 ^{B}	39.2 ± 4.2 ^{a}	25.4 ± 3.9 ^{b}
DW Root	28.0 ± 3.9	36.9 ± 8.2	19.4 ± 2.0	20.4 ± 4.6
DW Total	143.3 ±22.8 ^{A}	114.1 ±29.5 ^{B}	189.5 ±18.6 ^{a}	134.2 ±24.4 ^{b}
R:S	0.24± 0.04^B	0.48± 0.14^A	0.11± 0.01^b	0.18± 0.02^a
η_w^i	90.9 ±14.5 ^{A}	77.9 ± 9.9 ^{A}	127.0 ±16.2 ^{a}	88.2 ±18.7 ^{b}
LA_5E (cm^2)	121 ±19 ^{A}	94 ± 9 ^{B}	167 ± 5 ^{a}	132 ±22 ^{b}
LAR (n day^{-1})	0.49± 0.11	0.33± 0.05	0.44± 0.05	0.35± 0.07

Table 4-4. Concentration (% dry weight) of N, P, K and Ca in leaves fully developed under different N forms. Significance letters apply to comparisons between treatments but not across clones ($p < 0.05$). Numbers in parentheses indicate total content in the foliar mass (g). n = 6.

	AP2403		AP9	
	NO_3^-	NH_4^+	NO_3^-	NH_4^+
[N]	1.74±0.07^A(1.0)	1.45±0.09^B(0.6)	1.72±0.05^a(2.2)	1.50±0.08^b(1.4)
[P]	0.48±0.10^B(0.3)	0.64±0.11^A(0.3)	0.33±0.01^b(0.4)	0.45±0.07^a(0.4)
[K]	1.45±0.05 (0.9)	1.35±0.08 (0.6)	1.60±0.02^a(2.0)	1.28±0.09^b(1.2)
[Ca]	0.63±0.03 (0.4)	0.71±0.05 (0.3)	0.48±0.05^b(0.6)	0.63±0.11^a(0.6)

Table 4-5. Stomatal density of leaves fully developed under different N form regimes. The density in number of stomata mm^{-2} is shown individually for the abaxial and adaxial surfaces, as well as a combined total. The ratio of abaxial:adaxial is also shown. n = 6.

	AP2403		AP9	
	NO_3^-	NH_4^+	NO_3^-	NH_4^+
Abaxial	281 ±11	287 ±23	265 ±35	275 ±29
Adaxial	20 ± 6	21 ± 3	145 ± 3	137 ±28
Ratio	130 :10	130 :10	18 :10	20 :10
Total	287 ±23	302 ±13	410 ±33	412 ±56

Table 4-6. Vessel density and total vessel cross-sectional area in proportion to total xylem area of roots developed under different N forms. Vessel lumen refers to the total area of vessel lumen in cross-section. '*' indicates a significant difference when comparing treatments of the same clone ($p < 0.05$). n = 6.

	AP2403		AP9	
	NO_3^-	NH_4^+	NO_3^-	NH_4^+
Vessel density (vessels mm^{-2})	148 ±33	152 ±42	162 ± 9	140 ±10*
Vessel lumen / xylem area	0.15	0.21*	0.16	0.18
Vessel lumen/ root area (%)	5.71	8.34	5.47	6.98

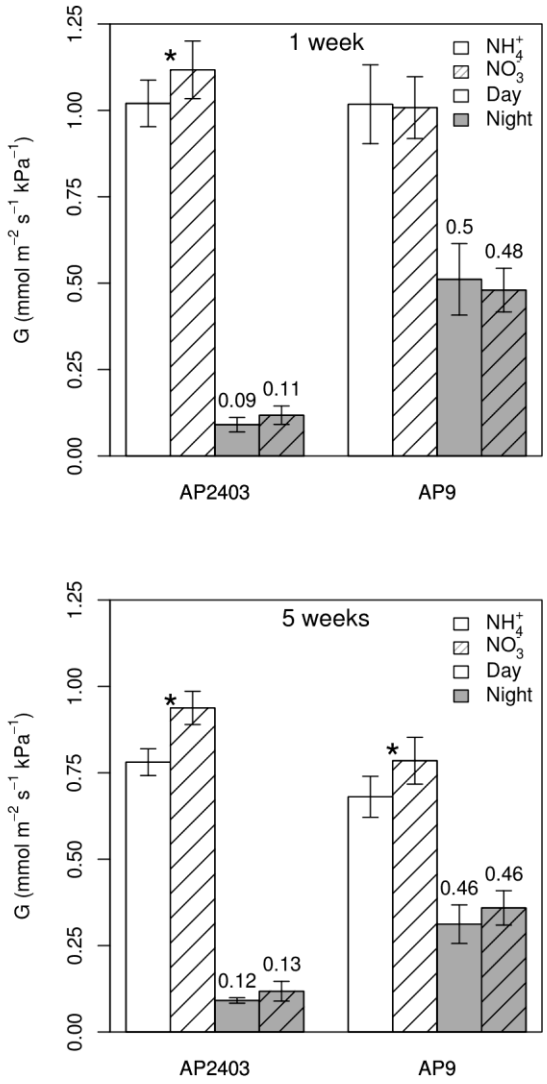


Figure 4-1. Short-term (1 week) and long-term (5 weeks) effects of N form on day- and night-time whole-plant stomatal conductance (G). '*' shows significant differences between indicated pairs ($p < 0.05$). Numbers above nighttime bars show the ratio G_N:G_D for each treatment.

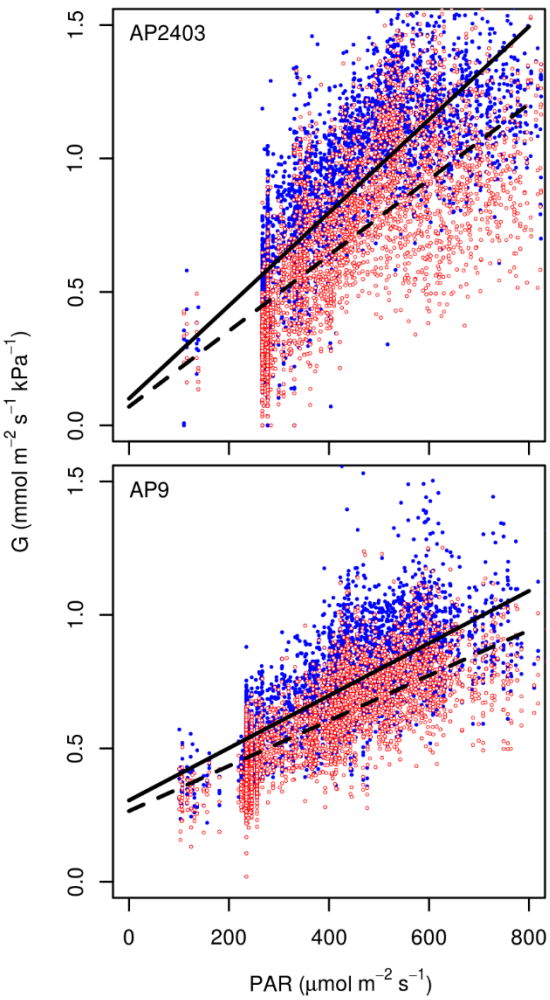


Figure 4-2. Linear regressions of whole-plant stomatal conductance (G) versus photosynthetically-active light radiation (PAR). Blue point, solid line: NO_3^- treatment; red points, dashed line: NH_4^+ treatment.

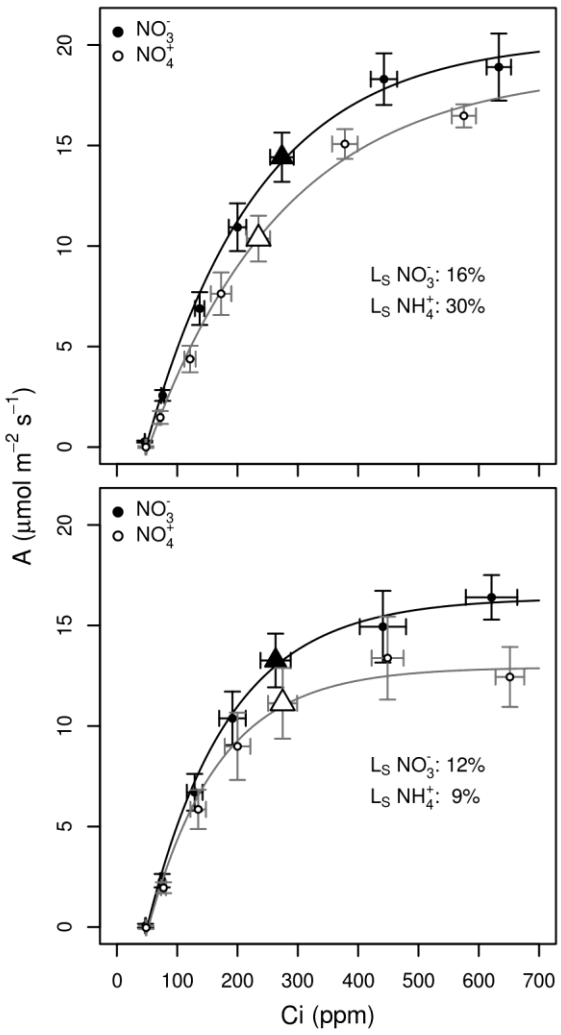


Figure 4-3. A/C_i response curves of plants fed different N forms. Error bars are plus or minus one standard deviation for each point in each axis. The triangles represent the pooled measurements at ambient [CO_2] (typically 3 measurements at 400 ppm, one taken before the A/Ci measurements and two as part of the A/Ci curve).

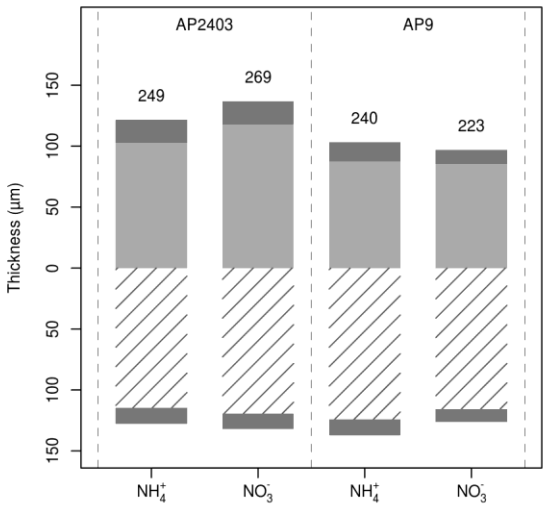


Figure 4-4. Thickness of leaves fully developed under different N sources. One entire bar represents the leaf cross-section profile: upper and lower epidermis (darker gray), pallisade layer (lighter gray) and spongy mesophyll (hatched). Leaf representations are aligned with y=0 at the interface between the pallisade layer and the spongy mesophyll for easy comparison of same layer types across clones and treatments. Numbers above diagrams indicate the total leaf thickness in µm.

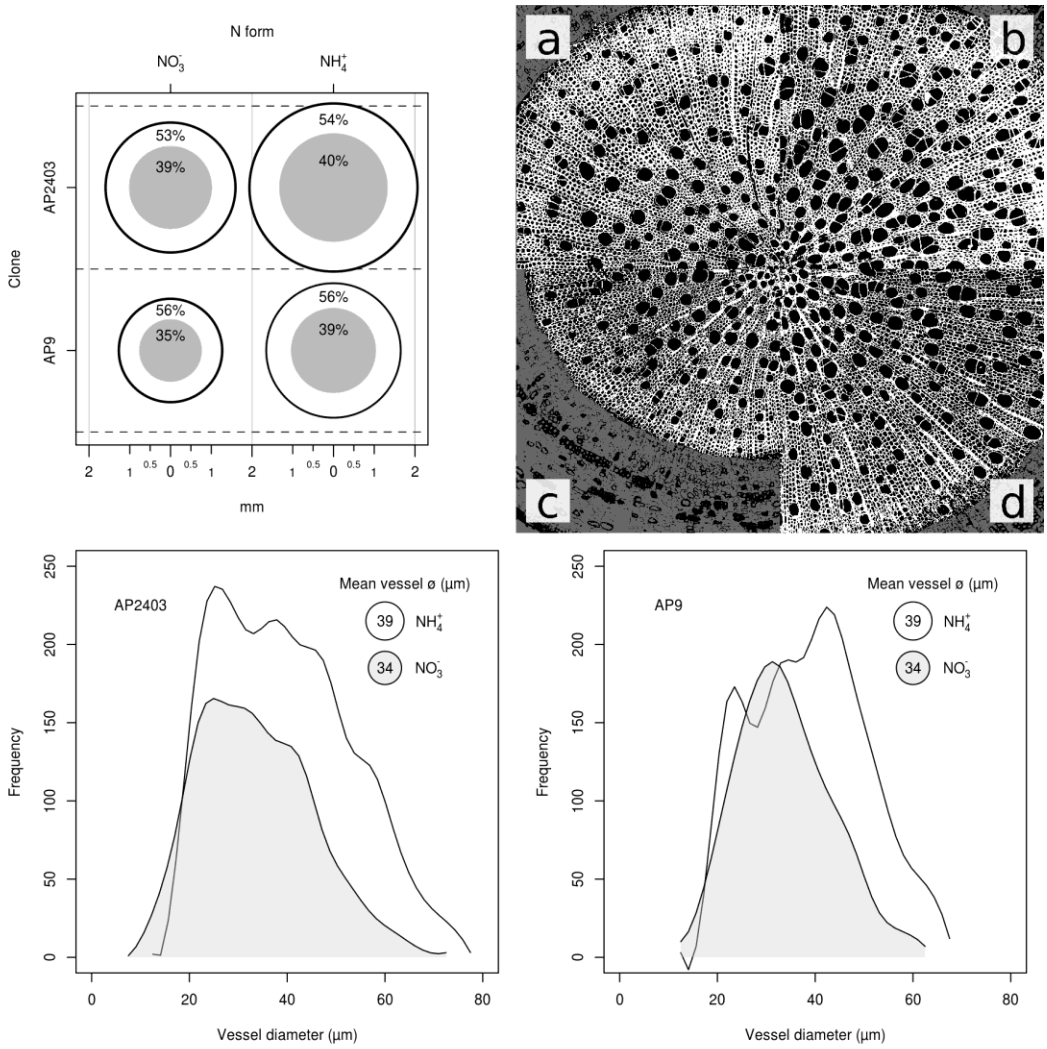


Figure 4-5. Schematic representation of cross-sections of roots developed under different N forms. The exodermis (black), cortex (white), and vascular cylinder (gray). Percentage numbers indicate the proportion of total root cross-sectional area occupied by each tissue with the remainder corresponding to the exodermis. The micrograph plate shows cross-sections of the vascular cylinder (approx. one-quarter) of a: AP2403— NO_3^-; b: AP2403— NH_4^+; c: AP9— NO_3^-; d: AP9— NH_4^+. The lower graphs are the frequency distribution of vessel lumen diameter in the root vascular cylinder in relation to N form. The figure key also shows the mean vessel diameter in microns (the same key applies to the curves).

Chapter 5. Conclusions

The physiology of plant transpiration continues to present challenges both from a theoretical perspective and from the more practical aspect of its empirical determination. The scale of measurement can span an enormous range from the cellular scale to the ecosystem. Within this range, the whole-plant scale is notorious for being overlooked and difficult to measure directly. This is especially true in the case of nocturnal water loss. I have presented "Amalthea", a system which is based on techniques that have been conceptually well-established in the past, in the form of various lysimetry implementations, but it is novel in that it targets specifically the whole-plant scale and it is built from readily available parts afforded by a legacy of proven technologies. Equally important, the system allows for simultaneous replication within experiments in a way not easily achieved (if at all) by other techniques.

This system has several advantages over more widespread approaches. Firstly, it naturally incorporates the whole plant by design and, in doing so, it provides an actual measurement instead of an estimation based on assumptions and/or extrapolations from different scales. Secondly, it combines high sensitivity and accuracy in relation to the scale; but more importantly, this can maintained over long periods since the system does not exert an influence over the plant. It is also forgiving of measuring technique. This is no trivial matter when considering the careful attention that needs to be paid to correctly set up a porometric-based instrument; a task that is prone to be cast aside in regular practice both in field and laboratory environments. McDermitt (1990), of LI-COR inc., has commented that

one should not so much as try to estimate in-situ transpiration from leaf-level data taken from a porometer, perhaps the most widely-used instrument for such measurement.

Leaf-level measurements can provide important information about leaf status and leaf-level processes. In this context, Amalthea can be used simultaneously with a properly set-up porometer in order to provide a full picture of whole-plant plus age- and/or position-dependent leaf measurements. We know that things like leaf age and plant architecture have a big impact on leaf and stomatal function whether directly or indirectly (Reich 1984; personal observation). It is therefore not advisable to extrapolate leaf-level measurements to the whole-plant scale. Rather, these two types of measurement should be complementary to each other.

The use of Amalthea has provided a detailed picture of whole-plant transpiration patterns, particularly during the night, which is challenging to obtain using porometric measurements. This has been useful in investigating differences in day- and night-time water use among *Populus* species from different environments. Although the survey undertaken in this study concerned only four species, it provides a starting point for testing several hypotheses regarding water use at the whole-plant level and comparing it with leaf-level processes. The water use survey that was carried out tested two main hypotheses: i – that species from riparian environments have more night-time water use than upland species, and ii – that night-time transpiration may have functional value as opposed to a mere accident not deleterious enough to have been maladaptive [during evolution].

Night-time whole-plant conductance was positively correlated with an *a priori* ranking of the species according to wetness of habitat, which was not the case with day-time conductance. This would suggest that some environmental pressure exists to limit night-time water loss but not necessarily to always restrict it to the absolute minimum. Because the number of species in the study is small and limited to a single genus, it does not permit generalisations; it does, however, invite for a larger survey across taxa and habitats.

Another feature highlighted by the study was that all species, including those associated with drier landscapes, maintained a level of water loss that was an order of magnitude above cuticular conductance upon experiencing moderate drought. Stomata also appeared to continue to respond to vapour pressure deficit when under drought. These findings support the idea that stomata are not fully closing at night even when stressed, suggesting that there may be a functional value to transpiration in these conditions that has yet to be determined. This has not been previously observed by other studies. One of the proposed advantages of nocturnal transpiration is to maintain water flow to aid with the delivery of oxygen to internal tissues. This is a controversial topic and it is clear that more research is needed in this area.

During well watered conditions, nocturnal transpiration (and thus root and stem water flow) may fulfill other functional roles. One of the main proposed roles is that of nutrient acquisition (and delivery) by mass flow, and while this is also a contentious subject, other studies have shown a connection between nutrient level (particularly N) and night-time transpiration. There is a clear effect of NO_3^- on

hydraulic properties of roots and possibly leaves as it acts as a signal molecule. However, N is most commonly found in the form of NH_4^+ in *Populus*-dominated landscapes and seldom have these two forms of N been compared. The N-form study presented here is the first to compare whole-plant conductance patterns in *Populus* hybrids as affected by NO_3^- or NH_4^+.

In disagreement with other studies (Rosenstiel et al. 2004) and contrary to some predictions (Britto and Kronzucker 2002), the *Populus* clones evaluated in this study displayed what can be considered a strong "preference" for NO_3^-, showing substantially more growth of above-ground parts. However, N form appeared to have little impact over night-time whole-plant conductance. Nevertheless, N form had a marked effect on day-time conductance whereby NO_3^--fed plants showed higher overall conductance than NH_4^+-fed plants. Combined with a lack of change in stomatal index and length, and a difference in the response of transpiration to light intensity, it is concluded that the difference in conductance originates from a change in stomatal physiology.

In addition, root dry mass was slightly lower in NO_3^--fed plants, which underscores the importance of NO_3^- in increasing root hydraulic conductance since the same root mass amply supplied water to a much larger leaf area. Interestingly, the greatest effect of N form was not on root morphology but on root anatomy. The study highlights how two clones altered their root anatomy in different ways in response to NH_4^+, to arrive at a comparable proposed functional modification: to compensate for restrictions to radial water flow imposed by other root

modifications such as a thicker cortex tissue, which in turn would have been induced by the need to metabolise NH_4^+.

As a corollary, it is clear that night-time transpiration is a dynamic process. It is dependent on species and environmental conditions both pertaining to the soil and to the atmosphere. It is also clear that more research would enrich this still underexplored area of whole-plant physiology. Specifically, the following questions need to be addressed in future investigations:

- There is a large variation in night-time transpiration across species. To what extent would this trait be deleterious and susceptible to evolutionary pressure?
- Within a single plant, various degrees of heterogeneity are evident in terms of stomatal function. What is the contribution of leaf age to night-time transpiration?
- Since some plants appear to be nocturnal water-spenders compared with others that maximise day-time conductance while minimising night-time conductance, what is the relationship between night-time and day-time stomatal function?
- A circadian clock appears to affect stomatal function. What is the effect of photoperiod on nocturnal conductance?
- Even upland species can maintain some level of nocturnal transpiration when under water stress. Why is water-flow not abruptly shut down in this situation?

- Nutrients seem to influence transpiration to some extent although it is not clear if this is an indirect effect. What types of species are more likely to change nocturnal transpiration patterns in response to nutrient levels?
- The form of nutrient can have various effects on whole-plant physiology, some of which may directly or indirectly affect transpiration. What interaction exists between different forms of various nutrients, their relative concentrations, and their effect on plant hydraulics and transpiration physiology?

These are questions that can be investigated at different scales, one of which, the whole-plant level, should not be overlooked but rather integrated.

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