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The role of leaf hydraulic function and anatomy in the acclimation of tropical forest trees to drought

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May 2016
Declaration

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or qualification.

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May 2016
Abstract

Seasonality in the Amazon Rainforest is predicted to become more extreme, with dry seasons increasing in length and severity, while severe episodic droughts are expected to occur with greater frequency. Drought stress can reduce the capacity of the rainforest to sequester carbon, and severe drought events can switch the region from being a net sink to a temporary source of carbon to the atmosphere. A key component in the drought-induced carbon flux is tree mortality, and there is evidence of strong feedbacks globally and regionally in the Amazon with climate change. Although the exact cause of drought-induced mortality in trees is difficult to ascertain, recent data suggests that reduced functionality of the water transport pathway (hydraulic failure) is an important factor. Hydraulic vulnerability in trees is often assessed using measurements of the capacity of stems and branches to cope with the strongly negative internal water pressures associated with drought. However, leaves play a vital role in protecting the integrity of the ‘upstream’ hydraulic pathway by influencing the rate of transpiration and thus the tension in the water column. Therefore, the physiology of leaves can be informative of, and influence, tree species’ sensitivity to drought.

This thesis uses a long-term large-scale rainfall exclusion experiment in the Eastern Amazon to examine the possible link between leaf physiology and drought sensitivity (or tolerance) by different taxa, and the capacity of mature, upper canopy Amazonian trees to respond to drought via plastic changes in leaf physiology. The plasticity in response to experimental drought and the differences between taxa classed as drought-sensitive and drought-resistant based on drought induced mortality records were tested by the study of leaf water relations (Chapter 2), leaf anatomy (Chapter 3) and foliar water uptake (Chapter 4).

No consistent differences were found between drought-resistant and drought-sensitive species suggesting that the sensitivity of these species to drought may be due to other aspects of plant physiology. However, a limited response to the imposed drought conditions was detected across all taxa and included reductions of osmotic potential at full turgor and turgor loss point (Chapter 2), and increases in the thickness of the upper epidermis and the leaf internal cavity volume (Chapter 3). Interestingly, drought-
sensitive taxa showed more seasonal osmotic adjustment than drought-resistant taxa, indicating that short-term responses to drought (e.g. season) are not representative of the capacity for adjustment in response to long-term water deficits. No significant changes occurred in leaf size, thickness, stomatal and vein density, the quantity of the inner leaf tissues (i.e. the palisade and spongy mesophyll) and mesophyll cell size, in response to the experimental drought.

The experiments on foliar water uptake in Chapter 4 revealed that this rarely-considered process occurs in all taxa, but the response to the drought treatment differed among taxa. Using a simple model, foliar water uptake was scaled up to canopy level. Under normal conditions (i.e. no drought) canopy foliar uptake was calculated to be $29.9 \pm 2.3 \text{ mm year}^{-1}$ from rainfall alone, but this increased to a maximum of $51.9 \pm 2.3 \text{ mm year}^{-1}$ when including the input of dew in the dry season. However, lower water potential in the drought plot causing increased rates of foliar water uptake, led to estimates of $38.7 \pm 3.0 \text{ mm year}^{-1}$ (rainfall only) and $68.9 \pm 2.9 \text{ mm year}^{-1}$ (including dry season dew).

Taken together, these results demonstrate that Amazonian trees show some limited capacity for acclimation to drought through the changes in leaf physiology measured in this thesis. Low turgor loss point is associated with dry climate-adapted plants, so the finding that this parameter reduced in response to the drought reveals some potential for Amazonian trees to acclimate with the predicted changes in moisture availability. However, the limited response of leaf anatomy to long-term drought might suggest that acclimation may only occur within a narrow range. The finding that six common Amazonian tree genera can take water up through their leaves has considerable implications for understanding the Amazon water budget, in terms of the contribution of dew and light rainfall to canopy water status, but also the implications it has for the hydraulic vulnerability of trees in rainforests right across the Amazon basin.
Lay summary

The rainforests of Amazônia play a substantial role in regulating global hydrological and carbon cycles. However, the capacity for maintaining these vital functions appears to be at risk from changes in climate. In addition to a long-term predicted warming trend, seasonality in the Amazon region is predicted to become more extreme, with dry seasons increasing in length and severity, while severe episodic droughts are expected to occur with greater frequency. Such reductions in rainfall reduce the amount of carbon taken up and can even turn the rainforest into a net source of carbon to the atmosphere contributing to climate change. A key component in the drought-induced carbon flux is tree mortality, so there is strong feedback between global climate change and tree mortality in the Amazon.

Due to a fundamental trade-off between carbon uptake and water loss in plants, trees have evolved to operate very close to the threshold for hydraulic failure; that is, the point at which the water transport system breaks down. Leaves play a crucial role in regulating the flux of water through plants and, therefore, protecting the water transport pathway. This thesis uses a long-term large-scale rainfall exclusion experiment in the Eastern Amazon to examine the link between leaf physiology and drought sensitivity (or tolerance) of different taxa, and the capacity of mature, upper canopy Amazonian trees to respond to drought via plastic changes in leaf physiology. Leaves were examined with respect to their potential for adjusting water pressure and storage in cells (leaf water relations, Chapter 2), anatomical parameters (changes in the amount of each leaf tissue, Chapter 3), and the capacity for absorbing water directly from the environment e.g. rain and dew (foliar water uptake, Chapter 4).

No consistent differences were found between drought-resistant and drought-sensitive species (selected on the basis of mortality response to drought) suggesting that the sensitivity of these species to drought may be due to other aspects of plant physiology. However, a limited response to the imposed drought conditions was detected across species and included increases in solute concentration of cell sap (Chapter 2), and thickness of the upper epidermis (Chapter 3). High solute concentration is an important mechanism for enabling plants to live in drier conditions, and so this finding
suggests that Amazonian tree species can, within limits, acclimate to lower levels of rainfall.

All species in this study were found to be capable of foliar water uptake (chapter 4), and this is the first time that this rarely-considered phenomenon has ever been reported in Amazonian rainforest species. The occurrence of foliar water uptake considerably influences the perceived interaction between the Amazonian hydrological cycle and drought sensitivity of trees. Although predicting the role foliar water uptake will play under different climate conditions is challenging, because little is known about the underlying biology, and how changes in humidity and temperature will affect this process.

Taken together, the results of this thesis demonstrate that Amazonian trees show some limited capacity for acclimation to drought through changes in leaf physiology, but that differences in leaf physiology and anatomy are unlikely to account for differences in drought sensitivity among taxa. The finding that six common Amazonian tree genera can take water up through their leaves has considerable implications for understanding the Amazon water budget, in terms of the contribution of dew and light rainfall to canopy water status, but also the implications it has for the hydraulic vulnerability of trees in rainforests right across the Amazon basin.
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“To every problem, however complicated, there is a single, elegant solution which one will discover if one looks hard enough.

This solution will turn out to be wrong.”

Rothchild’s Rule
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The role of leaf hydraulic function and anatomy in the acclimation of tropical forest trees to drought

Chapter 1

Introduction

1.1.1 Summary of Chapter 1

Some model predictions and empirical reports suggest that Amazonian rainforests are destined to experience greater drought stress in the future (Cai et al., 2014, Fu et al., 2013, Jupp et al., 2010, Boisier et al., 2015) potentially leading to elevated tree mortality (Boisier et al., 2015), a large net flux of carbon dioxide into the atmosphere (Brienen et al., 2015, Gatti et al., 2014, Reichstein et al., 2013), changes in the global hydrological cycle (Lawrence and Vandecar, 2015) and a considerable loss of biodiversity (Da Silva et al., 2005). Not all model predictions agree (Li et al., 2006, Jupp et al., 2010, Joetzjer et al., 2013, Huang et al., 2013). One aspect of Earth system models that requires improvement is the interaction between vegetation and the atmosphere (Bonan, 2008, Beer et al., 2010), particularly in the Amazon region (Davidson et al., 2012), which requires a better understanding of the interaction between drought events and tree mortality (Allen et al., 2015, Meir et al., 2015a, Phillips et al., 2009).

The precise cause of drought-induced mortality is challenging to ascertain in natural communities (Meir et al., 2015a, Phillips et al., 2010, Hartmann et al., 2013), but there is a general consensus emerging that hydraulic limitations precipitate physiological deterioration that ultimately leads to tree death (Gleason et al., 2015, Rowland et al., 2015a, Hartmann et al., 2013, Anderegg et al., 2013, Anderegg et al., 2012, Mitchell et al., 2013, Nardini et al., 2013). While most research on hydraulic vulnerability is focused on the capacity for stems to maintain continuity in the water transport pathway under drought stress, there are good reasons and much evidence to suggest that leaves
play a role in the ability of plants to cope with drought (Johnson et al., 2011, Hao et al., 2008, Chen et al., 2010, Bouche et al., 2016).

This chapter provides a brief explanation of how vegetation-climate interactions are approached in a scientific context, and why it is necessary to gain a robust understanding of this relationship to predict the interdependent future of the Amazon and global climate. The likely mechanisms for tree mortality are discussed in addition to the role that leaf physiology plays in conferring resistance to hydraulic stress. The value of using ecosystem-scale experiments is reviewed in the context of establishing the physiology underlying drought-induced tree mortality and plasticity of the relevant traits. Finally, a concise overview of the thesis aims and justification for each of the research chapters is given.

1.1.2 Coupling of vegetation with global climate

The first models characterising the interaction between vegetation and the atmosphere represented the energy fluxes determined by radiation absorption and emission, the interception of precipitation, surface drag, and evapotranspiration (Charney et al., 1975, Sellers et al., 1986, Dickinson et al., 1993, Pitman et al., 1991), and were formulated to operate within Global Circulation Models (Bonan, 1995). Further development required combining plant-atmosphere models with biogeochemistry models (e.g. Melillo et al., 1993, Parton et al., 1993), providing a primitive representation of mineral nutrients and carbon cycles, and biogeographical models (e.g. Prentice et al., 1992, Neilson and Marks, 1994), representing the interaction between the global distribution of plant functional types and climate. This led to the emergence of dynamic global vegetation models (DGVMs) which were, in principle, able to incorporate feedbacks between climate, vegetation types, and geochemical cycling (Foley et al., 1996, Brovkin et al., 1997). DGVMs use basic differences in plant functional type (Woodward and Cramer, 1996) to describe the community-level storage and flux of carbon in response to the climate (Beer et al., 2010). Thus, non-equilibrium soil-plant-atmosphere exchanges of greenhouse gases, primarily carbon dioxide, modify the climate which in turn acts on the vegetation, influencing its capacity for sequestration and emission of carbon (Cox et al., 2000).
Carbon storage and gross primary productivity (GPP) vary by biome, tend to be higher in the tropics and, in terms of terrestrial biomes, are highest in tropical forests (Beer et al., 2010, Saatchi et al., 2011). There is a strong relationship between precipitation and GPP such that GPP reduces from 23.3 Mg C ha\(^{-1}\) year\(^{-1}\) in tropical rainforest to 11.3 Mg C ha\(^{-1}\) year\(^{-1}\) in adjacent savanna\(^1\) (Beer et al., 2010) following a gradient of absolute levels of rainfall and seasonality in rainfall (Boisier et al., 2015). Thus, reductions in rainfall in the Amazon could lead to a transition from rainforest to savanna (Salazar et al., 2007, Leonel Da Silveira Lobo, 2001, Phillips et al., 2009), which has lower GPP and biomass (Nogueira et al., 2008), resulting in net emission of carbon. Establishing the climate thresholds that cause a transition from one plant community to another, i.e., the response of plant functional types to climate, has been a critical area of research.

1.1.3 How the climate of the Amazon is expected to change

Precipitation in the Amazon is associated with sea surface temperatures (SST) of both the Pacific and Atlantic oceans (Liebmann and Marengo, 2001, Nobre and Srukla, 1996), principally via the influence of atmospheric moisture content and convection (Butt et al., 2008, Fu et al., 1998). The El Nino-Southern Oscillation, which determines SSTs of the Pacific, exerts some influence on wet season precipitation (Fu et al., 2001, Ronchail et al., 2002) and medium-frequency drought events (Nobre and Srukla, 1996), while the location and temperature gradient of the Intertropical Convergence Zone (ITCZ) in the Atlantic, influences the severity of the dry season, determines the periodicity of severe droughts (Marengo et al., 2008), and has the greatest overall influence on Eastern Amazon rainfall (Fu et al., 2001, Hastenrath and Greischar, 1993, Butt et al., 2008). Changes in the sea surface density due to warming at high latitudes and melting of sea ice are predicted to lead to alterations in thermohaline circulation, causing the ITCZ to move southwards (Dong and Sutton, 2002, Boer et al., 2014) influencing precipitation regimes in the Amazon (Ronchail et al., 2002; Fu et al., 1998).

\(^1\) GPP values for tropical forest and savanna are the median values from six models and the Köppen-Geiger Biome value reported in the supplementary information in Beer et al. 2010. The minimum and maximum values for tropical forest are 21.1 to 26.6 Mg C ha\(^{-1}\) year\(^{-1}\), and for savanna are 0.9 to 16.9 Mg C ha\(^{-1}\) year\(^{-1}\).
There is disparity amongst climate models over the prediction of future levels of precipitation in the Amazon (Li et al., 2006, Jupp et al., 2010, Joetzjer et al., 2013, Huang et al., 2013), potentially due to emphasis on different aspects of convection in the Hadley cell (Lu et al., 2007, Yin et al., 2012). However, models using historical data to constrain future estimates of precipitation, such as decadal trends in dry season length (Fu et al., 2013), inter-annual variability in seasonal rainfall (Jupp et al., 2010), and changes in annual precipitation (Boisier et al., 2015), predict increases in dry season length, a general strengthening of the monsoon seasonal cycle (also recorded by Gloor et al. (2013)) and the likelihood of reduced rainfall over some parts of Amazonia. The future transition to drier conditions in the Amazon is supported by predictions from several recent studies (Joetzjer et al., 2013, Cai et al., 2014), and is also consistent with the apparent increase in frequency of extreme drought events observed in the Amazon over the previous half-century (Lewis et al., 2011, Marengo et al., 2011). Such changes in the precipitation regime strongly influences the carbon cycle in the Amazon, largely mediated by tree mortality (Phillips et al., 2009), which then feeds back to the local and global climate.

A recent prediction of evapotranspiration (ET) throughout the Amazon basin gives a range of 2.39 to 3.26 mm day\(^{-1}\) (Getirana et al., 2014), which, assuming a forested area of 5.5*10\(^6\) km\(^2\), scales up to 13.1 to 17.9 petagrams of water per day. This represents a conservative estimate of over 10 % of global terrestrial ET (Jasechko et al., 2013), a highly significant global flux of water and energy (Malhi et al. 2002). Isotopic analysis suggests that 80 to 90 % of terrestrial ET is derived from transpiration (Jasechko et al., 2013) and 16 to 37 % of Amazonian ET is recycled as local precipitation (dry to wet season, respectively) (Satyamurty et al., 2013). Higher temperatures are expected to cause elevated vapour pressure deficit (VPD) in the future (Scheff and Frierson, 2014, Sherwood and Fu, 2014), which will exert a selection pressure on vegetation communities (McDowell and Allen, 2015), and will likely lead to a loss of diversity from complex species assemblages. Evapotranspiration can be influenced by vegetation via the regulation of stomatal conductance (Costa et al., 2010, Hasler and Avissar, 2007), particularly during periods of drought stress (Malhi et al., 2002). Because of the energy required to evaporate water (latent heat of vaporisation), the amount of water vapour in the atmosphere influences temperature through the
conversion of sensible to latent heat (Machado, 2000). Reductions in transpiration could increase the proportional contribution of sensible heat leading to higher temperatures (Harper et al., 2014). Thus, the response of Amazonian vegetation to future changes in climate, both in terms of reduced transpiration and potentially elevated mortality, will strongly influence global fluxes of water and energy and will impact contingent ecosystem services.

1.1.4 Drought, the carbon cycle and the Amazon

The mechanisms that contribute to the net exchange of carbon to or from an ecosystem, that is the sum of photosynthesis (carbon uptake) and respiration (carbon emission), are numerous and their interaction with the environment varied. Photosynthesis, the process underlying gross primary production (GPP), is directly influenced by drought because of a fundamental trade-off between carbon uptake and water loss. Both carbon gain and water loss occur via stomata, small pores in leaves, thus water stress causes stomatal closure, which reduces both water loss and carbon uptake. Such limitations to photosynthesis lead to lower growth rates in trees (Wagner et al., 2014, Aubry-Kientz et al., 2015). The response to drought of respiration, on the other hand, can vary depending upon the source of respired CO$_2$ (Meir et al., 2008). Autotrophic respiration, which accounts for 25-65% of total ecosystem respiration (Janssens et al., 2001, Fu et al., 2001) can increase (Rowland et al., 2015b, da Costa et al., 2014, Metcalfe et al., 2010) while, over the short term at least, heterotrophic soil respiration can decrease due to water limitation on the action of soil microbes (Bonal et al., 2008, Meir et al., 2008). Commonly, both photosynthesis and respiration decline in response to seasonal water deficit (van der Molen et al., 2011, Bonal et al., 2016), in which case, the relative decline in each component determines the direction of the net carbon flux (Bonal et al. 2008, Meir et al., 2008). For example, carbon uptake commonly increases in dry season as a result of the proportionally greater reduction in heterotrophic respiration than photosynthesis (Keller et al., 2004, Yan et al., 2013, Baker et al., 2008).

Prolonged and/or severe drought, in addition to tipping the balance of photosynthesis and respiration towards carbon emission (Gatti et al., 2014, Phillips et al., 2010, Lewis et al., 2011, Reichstein et al., 2013), can also incur more substantial costs including a
reduction in leaf area index (Metcalfe et al., 2010, Meir et al., 2009) and elevated tree mortality (Breshears et al., 2005, Allen et al., 2010, Nakagawa et al., 2000, Meir and Grace, 2005, Phillips et al., 2009, Brienen et al., 2015). These added sources of dead plant material, together with the drying of the litter layer vastly increase the flammability of forests and lead to greater frequency and spatial extent of fires (Aragão et al., 2007), leading to huge losses of carbon from the ecosystem (van der Werf et al., 2009, Gatti et al., 2014). Even without the occurrence of fire, mortality reduces the carbon uptake capacity of the forest, and results in a committed future flux of carbon as a consequence of the decomposition of necromass (Meir et al., 2008). Thus, tree mortality is a principal driver of carbon loss from forest ecosystems in response to severe drought.

Continued assessment of the biomass stocks in long-term forest plots have led to the robust conclusion that the Amazon has been a net carbon sink since at least the 1990s (Pan et al., 2011, Brienen et al., 2015) and possibly earlier (Phillips et al., 2009). This trend is consistent with forests globally (tropical, temperate and boreal), and while the causes for carbon uptake differ geographically, the principal factors pertaining to the Amazon are biomass accumulation in mature forests, and growth in secondary forests (Pan et al., 2011). However, the rate of carbon accumulation appears to have been reducing since 1990 (Brienen et al., 2015), and unusually severe dry seasons contribute to reduced uptake or result in a net emission of carbon (Gatti et al., 2014, Phillips et al., 2010, Lewis et al., 2011, Reichstein et al., 2013). A severe Amazonian drought in 2005, referred to as a 1 in 100 year event (Marengo et al., 2008), caused an estimated net loss of carbon to the atmosphere of 1.2 (0.57, 2.01 95 % confidence intervals) petagrams (Phillips et al., 2009), while a satellite estimate based on a comparison with empirically measured conditions in the 2005 drought, suggests that the drought in 2010 was more spatially extensive and resulted in a higher estimated loss of 2.2 (1.2, 3.4) petagrams from the Amazon (Lewis et al., 2011), an amount equivalent to 13 and 24 % of yearly anthropogenic carbon emissions (Phillips et al., 2009), respectively.

Models describing the carbon storage of the Amazon vary in their sensitivity to different climatic effects e.g. temperature, precipitation, humidity and thus produce divergent predictions for identical climate scenarios (Galbraith et al., 2010, Huntingford et al., 2013). Moreover, simulations of the interaction between climate
and vegetation often fail to predict the responses generated in large-scale drought experiments (Galbraith et al., 2010), or the actual decline of carbon uptake in the Amazon (Brienen et al., 2015). The disparity in model outcomes is largely due to uncertainty regarding the physiology underlying plant-environment interactions (Huntingford et al., 2013, Meir et al., 2015b) and this, therefore, is a focal point of research essential for characterising the risk to the Amazon of future climate change.

1.1.5 Identifying the cause of tree mortality

Not all species are equally vulnerable to drought-induced mortality (da Costa et al., 2010, Meir et al., 2015a, Rowland et al., 2015b) implying the existence of a range of strategies for coping with water stress (Mitchell et al., 2013, Tardieu and Simonneau, 1998, Manzoni et al., 2014, McDowell et al., 2008). The same genera were found to be drought resistant or sensitive in two independent long-term drought experiments in the Amazon (Nepstad et al., 2007, da Costa et al., 2010), suggesting consistent taxonomic differences in drought tolerance (Meir et al., 2015b). This conclusion is supported by the findings of a recent study in which taxa intolerant to dry season drought conditions in the Amazon were confined to the wettest areas of the forest, in contrast to the drought tolerant taxa which were distributed throughout (Muelbert et al., 2016). Furthermore, mounting evidence suggests that large trees (diameter at breast height) (Rowland et al., 2015a, Phillips et al., 2010, da Costa et al., 2010, Meir et al., 2015b) and those with lower wood density (Phillips et al., 2010, Aubry-Kientz et al., 2015) appear to be most vulnerable to drought-induced mortality.

In dry conditions, plant water status can be maintained within favourable limits by increasing the capacity for water uptake from the drying soil, and by reducing the loss of water through transpiration. This generates a trade-off because the extraction of water from dry soil requires greater investment in tissues, and may result in a compromise in maximum rates of water transport (Gleason et al., 2015), while reducing water loss, most effectively through lowering stomatal conductance, reduces carbon uptake and photosynthesis. This is the basis for the strategies known as isohydric (Tardieu, 1993), prioritising water conservation through stomatal regulation, and anisohydric, tolerating water stress to maintain levels of photosynthesis, and is fundamental to the hydraulic framework proposed by McDowell et al. (2008).
hydraulic framework builds upon three, non-exclusive, mechanisms for mortality which are: susceptibility to biotic agents – reduced fitness through herbivory and pathogenic infection; hydraulic failure – low water potentials resulting in xylem embolism and dysfunction (see Box 1 for explanation of ‘water potential’); and carbon starvation – lack of sufficient carbohydrate to carry out minimal metabolic function (McDowell et al., 2008). Thus, theoretically, isohydric species are better able to cope with short intense droughts but die of carbon starvation when the drought is of long duration, while anisohydric species can cope with mild droughts of long duration, but are at risk of hydraulic failure if the drought becomes too intense. In reality, carbon starvation is challenging to reveal (McDowell, 2011), and while factors contributing to carbon starvation have been measured in drought stressed trees, e.g. altered respiration (Rowland et al., 2015b, Adams et al., 2009), curtailed photosynthesis (Sevanto et al., 2013, McDowell et al., 2008), and depleted non-structural carbohydrate reserves (Galiano et al., 2012, Sevanto et al., 2013, Mitchell et al., 2013), no studies have unambiguously demonstrated starvation-induced death (Phillips et al., 2010, Hartmann et al., 2013, McDowell and Sevanto, 2010, Sala et al., 2010).

In contrast, the evidence that hydraulic deterioration commonly underpins the cascade of events that eventually leads to death during severe droughts, is accumulating across biomes (Rowland et al., 2015a, Hartmann et al., 2013, Anderegg et al., 2013, Anderegg et al., 2012, Mitchell et al., 2013, Nardini et al., 2013). At low water potentials experienced during drought stress, the continuity of the hydraulic pathway can break due to the formation of bubbles, emboli, in the xylem. The bubbles expand to fill the vessel in a process called cavitation, rendering the vessel dysfunctional and reducing the proportion of active xylem. Whether or not cavitated vessels can be repaired in trees is a contentious issue (Wheeler et al., 2013, Trifilò et al., 2014, Nardini et al., 2011, Urli et al., 2013); nevertheless, cavitation either results in a reduction of hydraulic conductivity which is permanent and requires the growth of replacement tissue, or is temporary and incurs a repair cost (Brodersen and McElrone, 2013, Hacke et al., 2001). Thus, the percent loss of hydraulic conductivity (PLC) is a measure of the damage caused by water stress, and the water potential at which a threshold PLC occurs (e.g. 50 %, known as the P50) is an indication of drought vulnerability. P50 correlates positively with mean annual rainfall (Choat et al., 2012), demonstrating its
usefulness as a proxy for water stress tolerance. However, arguably, a metric more suitable for measuring the capacity of vegetation to cope with sporadically severe drought is the hydraulic safety margin: the difference between a normal and a dangerous level of drought stress e.g. midday water potential and P50. Thus, plants with a bigger safety margin can cope with greater levels of drought stress as compared to the ‘normal’ level experienced daily. The hydraulic safety margin has been found to be less than 1 MPa in 70 % of forest species in a global analysis, and the mean for tropical rainforest trees is 0.39 MPa (Choat et al., 2012).

**Box 1.1**

**Water potential**

Gibbs free energy is a thermodynamic concept inversely related to enthalpy, describing the capacity for a chemical state to do work, its potential energy. Typically the units for free energy are given as J kg$^{-1}$ but, as water is incompressible, it can be expressed volumetrically as J m$^3$ which is equivalent to N m$^{-2}$ and Pa. The free energy of water in plants is referred to as water potential ($\Psi$) and, because of its magnitude, is usually expressed in MPa. The water potential of a plant, or plant part, is the sum of all potential terms, where the $\Psi$ of pure water is zero. Thus:

$$\Psi = \Psi_p + \Psi_g + \Psi_o + \Psi_m$$

where $\Psi_p$ is pressure potential, $\Psi_g$ is gravitational potential, $\Psi_o$ is osmotic potential, and $\Psi_m$ is matric potential. (Matric potential is the term used to describe the potential generated by the adhesion of water molecules to a surface of a porous medium, and is inversely proportional to the size of pores in the medium e.g. capillary action.) Because the water potential of pure water at standard temperature and pressure is zero, the water potential of plants must be less than zero (i.e. negative) to enable the movement of water down a potential gradient.
1.1.6 The relevance of leaves for determining drought sensitivity

The physiology and structure of the xylem determines the extent to which it is prone to embolise under a given level of water stress (Hargrave et al., 1994, Lens et al., 2013). Nevertheless, as the surfaces from which transpiration occurs, leaves exert a large degree of control over the water stress transmitted to the stem xylem, via stomatal control (Brodribb et al., 2003, Chen et al., 2010) and hydraulic resistance (see Box 2 for an explanation of the mechanism by which water moves through plants: the cohesion-tension theory). Despite being such a small fraction of the total plant hydraulic pathway in trees, leaves typically account for around 25% of the total plant hydraulic resistance ($R$) (Sack et al., 2003), with some reported values of over 80% (Nardini and Salleo, 2000). Other things being equal, a proportionally high resistance ($R$) in the leaf reduces the flow rate ($F$) throughout the plant according to the relation $F = \frac{\Delta \Psi}{R}$, where $\Delta \Psi$ is the water potential difference between two points of interest e.g. the roots and leaves, or stem and leaves. The low flow rate generated by the leaf reduces the potential difference over the preceding part of the pathway i.e. the stem and roots. Thus, the damagingly low water potentials occur in the leaves rather than in the stem. Moreover, like in stems, leaf hydraulic conductivity decreases with water potential, but typically in response to higher water potentials, i.e., leaf $P50$s are often higher (closer to zero) than stem $P50$s (Johnson et al., 2011, Hao et al., 2008, Chen et al., 2010, Salleo et al., 2001, Bouche et al., 2016), meaning that leaves act as hydraulic fuses. In other words, as water stress increases, the degree to which leaves and stems are hydraulically coupled decreases, decoupling the stem xylem from the evaporative demand, and reducing the potential for catastrophic xylem failure in the stem (Tyree et al., 1993). This potentially has two advantages: leaves are more expendable than stems (Johnson et al., 2011) due to naturally higher rates of turnover i.e. they are cheaper to replace, and that loss of conductance in leaves should be more readily reversible as it can arise from the regulation of aquaporins (Martre et al., 2002, Shatil-Cohen et al., 2011, Cochard et al., 2007) and conduit collapse (Blackman et al., 2010) rather than cavitation, i.e., they are cheaper to repair (Boyce et al., 2009). This concept of hydraulic fuses is consistent with the vulnerability segmentation hypothesis postulated by Zimmermann (1983): that the terminal parts of branches are more hydraulically vulnerable than the stem, in order to confine water stress-induced
damage to organs that are cheaper to replace. Accordingly, leaves would be expected to incur damage during severe water stress, and indeed canopy dieback is a very well documented response to drought (Matusick et al., 2013, McDowell et al., 2013, Allen et al., 2010, Hoffmann et al., 2011, Galiano et al., 2012, Tyree et al., 1993).

A further advantage of examining the linkage between leaves and drought sensitivity, is that changes in leaf area and certain leaf characteristics (e.g. nutrient, carbon and water content) can be identified remotely (Asner et al., 2014). Understanding the properties of leaves that indicate vulnerability to drought may enable more reliable, and spatially explicit, estimates of forest response to climate change (Meir et al., 2015b).

**Box 2**

**Cohesion-Tension theory**

Water moves as liquid from the soil, through the plant, and into the atmosphere as vapour from inside the leaf, down a water potential gradient, according to the cohesion-tension theory (Dixon, 1914). Throughout the water pathway, the dominant component of water potential changes (among $\Psi_p$, $\Psi_g$, $\Psi_o$, $\Psi_m$, see Box 1 for definitions) but, to generate movement (either as bulk flow or diffusion), water potential must become progressively lower (more negative). Thus, water, retained in the soil through matric potential, has to move into living cells in the root tissue where the main signal of water potential becomes osmotic, and then into the xylem where the driving force is (sub-atmospheric) pressure, and ultimately into the mesophyll cell walls in the leaf as matric potential, from where it evaporates and diffuses along a gradient of vapour pressure.

**1.1.7 The value of large-scale ecosystem manipulation experiments**

Much observational research describes the response of forests to natural variation in climate, including rare extreme events (Phillips et al., 2010, Brienen et al., 2015, Gatti et al., 2014). These studies are invaluable for revealing ecosystem trends to natural events, but are limited in their capacity to illuminate the detailed physiological mechanisms pertaining to individual species due to their unpredictable occurrence and the number of variables that change coincidently (Wu et al., 2011, Leuzinger et al., 2015b).
Large-scale ecosystem manipulation experiments, by selectively altering the environment *in situ*, are the only way to identify causal links between the environment and specific traits or ecosystem processes. This process cannot be perfect due to the impossibility of altering any single variable entirely independently of other variables, e.g. it is difficult to influence soil moisture input without also influencing vapour pressure above the soil surface, but does provide opportunities for study that would not be possible without experimental manipulation.

Figure 1.1. The through-fall exclusion plot in Caxiuanã, Brazil, with plastic panels arranged to intercept 50% of the through-fall precipitation.

An open question pertaining to the response of the Amazon to the 2005 and 2010 droughts is whether the elevated mortality was principally a result of the early death of senescent or physiologically compromised individuals (Lewis et al., 2011), or the death of an otherwise healthy demographic (e.g. species, size or age class) which is particularly vulnerable to drought (Phillips et al., 2009, Phillips et al., 2010). While the first point is difficult to rule out in the context of observational research, the findings of two large-scale drought experiments in the eastern Amazon corroborate the tentative conclusion of Phillips et al. (2010), based on a pan-tropical study, that large
trees are more vulnerable than small trees (da Costa et al., 2010, Rowland et al., 2015a, Nepstad et al., 2007), whilst also finding consistent differences in drought vulnerability between some genera (Nepstad et al., 2007, da Costa et al., 2010, Rowland et al., 2015b, Meir et al., 2015b). Identifying differences in the physiology between drought vulnerable and resistant taxa could help pinpoint the physiology of drought vulnerability, and thus reveal functional traits particularly suitable for use in vegetation models.

Another important advantage of large-scale experimental manipulations is the capacity to detect phenotypic plasticity. Plasticity in plants occurs at scales from molecular to whole organism (e.g. nutrient content in leaves to the whole organism growth rate), and over short to long time periods (e.g. drought-induced canopy dieback to coarse root placement) (Nicotra et al., 2010, Schlichting, 1986). The current rate of climate change will outpace the evolutionary (i.e. genotypically-constrained) response of canopy trees, given their typical generation time. However, phenotypic plasticity could, to some extent, act as a buffer (Nicotra et al., 2010, Valladares et al., 2007), depending on the plasticity of the traits underlying mortality. For example, plants subjected to ongoing experimental drought may acclimate to the conditions through e.g. changes in canopy leaf area and rooting depth, and thus be less susceptible to sporadic severe droughts, in a manner analogous to cold hardening in plants - the practice of exposing plants to controlled levels of cold conditions to make them more tolerant to sub-zero temperatures (Weiser, 1970). In the same way, gradual reduction in precipitation due to climate change may result in changes to plant phenotypes such that they are less susceptible to sporadic droughts. Plasticity in leaves is known to occur in many traits including life span, area, stomatal density, leaf mass per area and mesophyll characteristics (Sack et al., 2006, Sultan, 2000, Nobel et al., 1975) demonstrating the role of leaves in the acclimation of plants to their local environment. Leaves that emerge following episodic drought-induced canopy dieback are likely to develop under similar conditions as the leaves they are replacing and so will have similar characteristics and be equally sensitive to drought. However, leaves emerging under drought conditions may express traits better adapted to cope with drought stress. Hence, experimental manipulations enable the identification of plasticity that occurs over longer time scales than those produced by episodic drought.
1.1.8 Thesis aims

Despite the diversity in leaf shape and structure, there is a surprising degree of commonality in the internal tissues, and tissue structure, in leaves: much diversity exists, but leaves generally consist of the mesophyll (usually differentiated into two parts), vasculature with bundle sheath cells, and the epidermis. The similarity in the internal structure and physiology of leaves may be expected given their common purpose, i.e., balancing carbon uptake with water loss, but nonetheless, predictable differences might be expected to occur in response to relevant climatic variables such as water availability. Indeed, environmental water availability has been shown to correlate with some key leaf water relations parameters (Chapter 2) (Bartlett et al., 2012). Less is known about how internal anatomy relates to water availability or with leaf water relations. Although, in the same way that, e.g., the xylem is specialised for water transport, or the palisade mesophyll for photosynthesis (Chabot and Chabot, 1977, Catoni et al., 2015), it is conceivable that other aspects of anatomy contribute substantially to other factors such as hydraulic capacitance or nocturnal rehydration. If this was the case, growth under drought conditions may favour the plastic development of some tissues over others and/or, taxa more resistant to drought may have consistent differences in leaf anatomy that facilitate tolerance to, or recovery from, drought (Chapter 3). Another aspect which could considerably influence the capacity of a plant to cope with water stress is the potential for water acquisition by leaves directly from the environment: foliar water uptake. This phenomenon appears to be common in certain biomes (Goldsmith et al., 2013) and enables the efficient scavenging of small amounts of water on the leaf surface from rainfall and alternative types of precipitation including fog and dew (Chapter 4). Measuring the occurrence of foliar water uptake will be informative about the importance of dew deposition to the dry season water budget in Amazonian trees, and the relevance of hydraulic safety margins as a means of calculating hydraulic vulnerability.

The aspects of leaf physiology mentioned above, water relations, anatomy and foliar water uptake, were measured in trees subjected to a long-term, large-scale through-fall exclusion experiment (TFE) in Caxiuanã National Forest Reserve, in the eastern Amazon Rainforest. Approximately 50% of incident rainfall, in an area of 1 hectare of rainforest, has been diverted away from the site since the project’s inception in 2002.
Thus, the trees have been subjected to reduced water availability for over a decade before the first measurements for this study were taken in 2013, allowing time for long-term responses in physiological plasticity to occur. In Chapter 2 there is a more detailed explanation of experimental setup. Furthermore, using mortality records, genera were identified as drought sensitive or resistant. This experimental set up, therefore, offers exemplary conditions to test for differences in leaf traits that might confer resistance to drought, and to test plastic responses to drought. Thus, written in the style of scientific papers (Chapter 2 is published in *New Phytologist* (Binks et al. 2016), and Chapter 3 has been accepted for publication in *Tree Physiology*), Chapters 2 to 4 look at the aspects of leaf physiology outlined above and address whether they show plasticity in response to an experimentally imposed drought, and if they differ significantly between genera identified as drought sensitive or resistant based on drought-induced mortality.

1.1.9 Research chapter overview

Chapter 2 – Leaf water relations

Plants need to maintain turgor pressure within living cells in order to maintain normal physiological function. Turgor pressure is generated through the accumulation of solutes in the membrane-bound part of the cell (symplast), and the concentration of solutes (the osmotic potential) determines the water potential at which turgor becomes zero, the turgor loss point. The turgor loss point has been found to correlate with stomatal closure (Brodribb et al., 2003), shows a high degree of plasticity in many species over seasonal time scales (Bartlett et al., 2014), and is a strong indicator of drought resistance (Bartlett et al., 2012). In Chapter 2, leaf water relations traits, including turgor loss point, are compared between seasons (wet and dry) and treatment (control and TFE), to address the question of whether short term plasticity (season) indicates the potential for longer term plasticity (treatment). The significance of leaf water relations, and their plasticity, is also examined with respect to drought sensitivity.

Chapter 3 – Leaf anatomy

Leaf anatomy varies spectacularly between species, from the thorn-like structures on cacti to the table-top-size fronds of banana plants; there are consistent differences
between typical leaf types from different biomes (Schimper, 1903), and even variation in leaf traits within species spanning climate gradients (Geeske et al., 1994, Cunningham et al., 1999, Warren et al., 2005, McLean et al., 2014). Plants adapted to drier areas tend to have smaller, thicker leaves with higher stomatal and vein density than those from wetter areas (Maximov, 1929, Cutler et al., 1977), suggesting that these traits are important for optimising performance, or safety, under different hydrological regimes. Plasticity in leaf anatomy, including the proportional abundance of internal tissues, is examined with respect to the experimental drought and drought sensitivity of different taxa in Chapter 3.

Chapter 4 – Foliar water uptake

The phenomenon of foliar water uptake, leaves absorbing water through their epidermis, was recorded as early as 1727 (Hales, 1727). In the last few decades the subject has attracted interest and has been recorded in multiple biomes from deserts (Yan et al., 2015) to montane cloud forests (Goldsmith et al., 2013), but not in tropical rainforests. The capacity for leaves to take up water directly from intercepted precipitation has the potential to challenge existing theory pertaining to drought vulnerability by enhancing the prospect of cavitation repair, enabling leaves to attain water potentials greater than that determined by height and soil water availability, and by allowing access to a water source previously considered unavailable for plants. Research has indicated that Amazonian tree species may have particularly low hydraulic safety margins (Choat et al., 2012) which may be explained by a widespread capacity for foliar water uptake. In Chapter 4, the capacity for foliar uptake in the study taxa is analysed and a simple model scaling leaf water uptake to the level of the canopy is presented. Plasticity in foliar water uptake in response to imposed drought is also examined together with its relevance to drought sensitivity.
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Chapter 2

Plasticity in leaf-level water relations of tropical rainforest trees in response to experimental drought

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Author contributions:

OB designed the study, collected and analysed the data, and wrote the paper. OB, MM and PM designed the research. PM and ACLC conceived and implemented the experiment. LR, AARO and BC assisted data collection, LF enabled data collection, and SSV provided equipment. LR, MM, PM and AN all helped with manuscript preparation, and MM contributed to data analysis.

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2.1 Summary

The tropics are predicted to become warmer and drier, and understanding the sensitivity of tree species to drought is important for characterising the risk to forests of climate change. This study makes use of a long-term drought experiment in the Amazon Rainforest to evaluate the role of leaf-level water relations, leaf anatomy and their plasticity in response to drought in six tree genera. The variables (osmotic potential at full turgor, turgor loss point, capacitance, elastic modulus, relative water content and saturated water content) were compared between seasons and between plots (control and through-fall exclusion) enabling a comparison between short- and long-term plasticity in traits. Leaf anatomical traits were correlated with water relations parameters to test if water relations differed among tissues. The key findings were that (i) osmotic adjustment occurred in response to the long-term drought treatment; (ii) species resistant to drought stress showed less osmotic adjustment than drought-sensitive species; (iii) water relations traits were correlated with tissue properties, especially the thickness of the abaxial epidermis and the spongy mesophyll. These findings demonstrate that cell-level water relations traits can acclimate to long-term water stress, and highlight the limitations of extrapolating the results of short-term studies to temporal scales associated with climate change.
2.2 Introduction

The Amazon accounts for half of the world’s tropical rainforest (Fritz et al., 2003), contains approximately 123±31 Pg of carbon in woody biomass (FAO, 2010, Malhi et al., 2006, Saatchi et al., 2007), contributes over 10% to the world’s biodiversity (Da Silva et al., 2005, Lewinsohn and Prado, 2005) and is suggested to influence rainfall patterns as far away as Asia (Lawrence and Vandecar, 2015). Many of the ecosystem functions and services carried out by the forests of the Amazon basin are dependent on its hydrologic regime (Boisier et al., 2015). Yet Earth system models have been used to suggest that the hydrology of the Amazon may change drastically under future climate change scenarios through increases in dry season length, long-term soil drying, and increased frequency and intensity of drought events (Boisier et al., 2015, Reichstein et al., 2013, Christensen et al., 2013, Fu et al., 2013). Such shifts in climate may result in higher tree mortality (Allen et al., 2010, Phillips et al., 2009), threaten biodiversity and increase the possibility of climate feedbacks, the magnitude and direction of which remain uncertain. Currently, vegetation models used to represent the dynamic response to climate in Earth System Models (Dynamic Global Vegetation Models, DGVMs) lack the capability to predict ecological responses to drought within tropical forests reliably (Meir et al., 2015a, Powell et al. 2013), in part due to poor representation of how soil water stress influences leaf scale processes (Rowland et al., 2015b). To improve such representations, a greater empirical understanding of how soil water stress impacts leaf-level processes is necessary.

According to the cohesion-tension theory (Dixon and Joly, 1895), water moves down a free energy gradient (water potential, Ψ) from soil to the leaves (following a pressure gradient along the xylem). For a plant to maintain its transpiration stream during drought the leaves must be able to generate and sustain lower Ψ than the soil (Bowman and Roberts, 1985). The presence of solutes in the symplast (usually represented as osmotic potential, Ψπ, with more negative values indicating higher solute concentration) enables leaves to reach lower Ψ than the soil while maintaining turgor pressure. Thus, a lower osmotic potential enables a plant to function while drawing water from drier soil (Bowman and Roberts, 1985). Consequently, both osmotic potential at full turgor (ΨπF) and the water potential at turgor loss point (ΨπLP) are good predictors of plant sensitivity to drought stress (Bartlett et al., 2012).
is influenced by both the bulk modulus of elasticity (\(\varepsilon\) – the difference in turgor per relative change in cell volume) and \(\Psi_n\), which appears to be the stronger determinant (Bartlett et al., 2012, Lenz et al., 2006). Additional water relations parameters derived from pressure volume curves e.g. capacitance, relative water content at \(\Psi_{\text{lp}}\), saturated water content, can also affect the drought sensitivity of a plant.

Osmotic adjustment to seasonal water stress is common and has been the focus of much research (see Bartlett et al. 2014 for review). However, few, if any, studies have directly addressed the question of how the capacity for seasonal adjustment equips species for long-term shifts in water availability. Is there a physiological limit to osmotic adjustment determined by typical dry season water availability? Do species showing greater seasonal variability in water relations stand a better chance of coping with long-term climate changes? Understanding the variation and plasticity of leaf tissue-level parameters is essential to answering these questions and determining ecosystem-level response to environmental change.

Recent evidence suggests that tissues within leaves may be functionally ‘sequestered from one another’ (Buckley et al., 2015, Buckley, 2015, Rockwell et al., 2014). Leaf tissues are likely to experience different levels of hydration during transpiration (Rockwell et al., 2014, Buckley et al., 2015), and may be hydraulically compartmentalised (Canny et al., 2012, Nardini et al., 2010, Blackman and Brodribb, 2011). Given the evidence that the palisade mesophyll maintains turgor during transpiration (Canny et al., 2012, Buckley et al., 2015), we hypothesise that it may have a more negative osmotic potential than other cell layers. If that was the case, one might predict a correlation to emerge between palisade relative thickness and tissue-level osmotic potential. Furthermore, Canny et al. (2012) also observed that spongy mesophyll cells ‘easily lose water’ compared to the palisade matrix cells, so we suggest that the spongy mesophyll acts as a hydraulic buffer. A relationship could thus be postulated between spongy mesophyll volume (excluding airspaces) and tissue-level capacitance (Canny et al., 2012). Linking drought stress vulnerability with pressure volume traits and leaf anatomy could both strengthen the current understanding of leaf function and facilitate the identification of traits indicative of drought sensitivity or tolerance.
This study aimed to test whether tropical rainforest species can acclimate to changes in water availability on both a short time scale, represented by seasonal differences, and a long time scale, using a long-term (>12 years) through-fall exclusion experiment (TFE) in the Caxiuanã National Forest Reserve, State of Para, in Brazil. I correlated tissue-level pressure volume parameters with leaf anatomical traits for indications of whether particular cell types contribute disproportionately to some PV traits, thus examining linkages between tissue form and function. The following hypotheses were tested:

1. Acclimation to long-term soil moisture deficit results in greater osmotic adjustment and changes in elastic modulus than does acclimation to seasonal differences in soil moisture availability. Thus, osmotic potential at full turgor and turgor loss point are expected to be more negative, and elastic modulus more positive, in response to the long-term drought treatment than in response to dry season changes.

2. Drought resistant taxa show greater seasonal osmotic adjustment than drought sensitive taxa.

3. Palisade volume per leaf area correlates negatively with osmotic potential at full turgor and turgor loss point, suggesting higher solute concentration in this tissue. Spongy mesophyll volume per area correlates positively with capacitance, indicating a role as a water storage site.

In summary, this study aimed to test how leaf water relations parameters varied in response to changes in water availability in trees from lowland Amazon rainforest that resulted from seasonal differences in rainfall and a long-term field-scale soil moisture reduction experiment. Changes in parameters due to seasonal variation in rainfall were compared with those arising from an experimentally imposed drought (soil moisture deficit) to explore the adaptive capacity of rainforest tree leaves. The PV parameters were modelled against the absolute and relative values of thickness and volume of the leaf tissues to provide an indication of whether hydraulic differences occur among cell layers, and to facilitate the identification of traits indicative of differential drought sensitivity.
2.3 Materials and Methods

2.3.1 Study Site

The study was undertaken in the Caxiuanã National Forest Reserve in the eastern Amazon (1°43’S, 51°27’W). The site is situated in lowland terra firme rainforest 10-15 m above river level. The site has a mean temperature of ca. 25 °C, receives 2000 – 2500 mm of rainfall annually and has a dry season in which rainfall is < 100 mm per month between June and November. The soil is a yellow oxisol of 3-4 m depth, below which is a laterite layer 0.3-0.4 m thick (Fisher et al., 2007).

2.3.2 Large-scale through-fall exclusion experiment (TFE)

The TFE is one hectare of rainforest, in which canopy through-fall has been reduced by approximately 50 % since January, 2002 (Meir et al., 2015b). An artificial ‘roof’ was constructed from clear plastic panels and wooden guttering at a height of 1-2 m above the ground. The intercepted water is channelled downslope to a point more than 50 m away from the TFE. Both the TFE and the near-by control plot are surrounded by trenches 1-2 m deep to prevent lateral subsurface flow of water into the study plots. The plots, both 1 ha, are divided into 10 m x 10 m subplots and the outermost subplots are excluded from the study to mitigate the potential for edge effects on tree growth. For further details on the experimental setup and key results, see Meir et al. (2009) and Rowland et al. (2015a).

2.3.3 Study specimens and drought vulnerability status

This study uses six of the most common genera on the plots, which have been previously determined as drought-sensitive (Manilkara, Eschweilera and Pouteria), and drought-resistant (Protium, Swartzia, and Licania), through analysis of drought-induced mortality rates (Rowland et al., 2015c, da Costa et al., 2010, Meir et al., 2015b). A genus was determined as drought-sensitive if, on the TFE, it experienced 50 % higher mortality and the death of at least two more individuals, than in the control plot (da Costa et al. 2010). This criteria was re-applied by Rowland et al. (2015c) following 13 years of experimental drought and the results were found to have remained consistent with the determination of da Costa et al. (da Costa et al. 2010). Henceforth, these genera are referred to simply as ‘sensitive’ or ‘resistant’ genera. Where possible a single species was used to represent a genus (Pouteria anomala
(Pires) T.D. Penn., _Manilkara bidentata_ (A.DC.) A.Chev., _Swartzia racemosa_ (Benth.), but more than one species was used where there were too few individuals in a species per plot: _Eschweilera_ is represented by the species _E. coriacea_ (DC.) S.A.Mori, _E. grandiflora_ (Aubl.) Sandwith, and _E. pedicellata_ (Rich) S.A.Mori, _Licania_ by _L. membranacea_ (Sagot ex Laness) and _L.octandra_ (Kuntze) and _Protium_ by _P.tenuifolium_ Engl. and _P. paniculatum_ Engl. This approach was necessary to obtain sufficient numbers of trees within each genus and plot to enable a comparison, and has been adopted in other studies (Butt et al., 2008, van Mantgem et al., 2009). It is acknowledged that relevant interspecific differences do occur within a genus (Abrams, 1990), but in this study, variance amongst individuals within a genus was consistently less than variance among genera as demonstrated by the difference among the random effects ID (tree individual) and Gn (genus) in Table 2.1.

**Table 2.1.** Proportions of variance of model components in percentage, total variance of transformed data and the conditional and marginal $r^2$.

<table>
<thead>
<tr>
<th>Variance (%)</th>
<th>$\Psi_{\pi}^{\text{tlp}}$</th>
<th>$\Psi_{\pi}^{\text{o}}$</th>
<th>SWC</th>
<th>RWC$^{\text{tlp}}$</th>
<th>$\epsilon$</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed</td>
<td>30</td>
<td>32</td>
<td>4</td>
<td>13</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>Random</td>
<td>8</td>
<td>3</td>
<td>27</td>
<td>9</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>ID</td>
<td>33</td>
<td>26</td>
<td>44</td>
<td>19</td>
<td>11</td>
<td>24</td>
</tr>
<tr>
<td>Gn</td>
<td>30</td>
<td>39</td>
<td>24</td>
<td>59</td>
<td>71</td>
<td>55</td>
</tr>
<tr>
<td>Residual</td>
<td>30</td>
<td>39</td>
<td>24</td>
<td>59</td>
<td>71</td>
<td>55</td>
</tr>
<tr>
<td>Total Variance</td>
<td>0.1965</td>
<td>0.3060</td>
<td>0.0537</td>
<td>0.0090</td>
<td>0.3568</td>
<td>0.2809</td>
</tr>
<tr>
<td>$r^2_{\text{conditional}}$</td>
<td>0.70</td>
<td>0.60</td>
<td>0.76</td>
<td>0.41</td>
<td>0.29</td>
<td>0.45</td>
</tr>
<tr>
<td>$r^2_{\text{marginal}}$</td>
<td>0.30</td>
<td>0.32</td>
<td>0.04</td>
<td>0.13</td>
<td>0.13</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Note: the total variance used for calculating the percentages was determined using the product of the variance values derived from the models as per Nakagawa & Schielzeth (2013), and is, therefore, not identical to the ‘Total Variance’ value listed in the table. Variables are turgor loss point ($\Psi_{\pi}^{\text{tlp}}$), osmotic potential at full turgor ($\Psi_{\pi}^{\text{o}}$), saturated water content (SWC), relative water content at $\Psi_{\pi}^{\text{tlp}}$ (RWC$^{\text{tlp}}$), elastic modulus ($\epsilon$) and capacitance (C).
2.3.4 Experimental protocol

Pressure Volume curves

To provide information on seasonal variability in PV parameters, measurements were carried out at the end of the dry season in November, 2013 and the end of the wet season in May, 2014, corresponding to periods of minimum and maximum soil water availability, respectively. The same sets of individuals were sampled in both periods, with the exception of the genus *Eschweilera*, for which three additional individuals were measured on each plot in the dry season. Top-canopy, fully sunlit branches were sampled, and after excision they were recut under water and immediately transported back to the laboratory in water where they were again recut under water filtered to 0.2 μm, and then allowed to rehydrate overnight. Previous studies have demonstrated that rehydrating specimens prior to PV analysis can influence the results, particularly $\Psi_\pi^\circ$, which tends to get higher (closer to zero) as a result of very short-term osmotic adjustment (Meinzer et al., 1986, Meinzer et al., 2014, Kubiske and Abrams, 1991, Yan et al., 2013). For two temperate zone species, Meinzer et al. (2014) showed that some PV parameters correlated strongly with the initial water potential ($r^2$ of 0.78 to 0.94 for the elastic modulus and TLP respectively) in the highly anisohydric species *Juniperus monosperma*, but this relationship was not found in the isohydric species *Pinus edulis*. However, because the purpose of this study was to compare differences of these and several other parameters (e.g., Rowland et al., 2015a; 2015c) in response to long term drought and to seasonal differences in water availability, and not just initial water potential, full rehydration was employed to standardise starting conditions for all samples. Moreover, as there are 10 species in this study, presumably exhibiting different levels of isohydry, quantifying the degree of change with respect to initial water potential in each species would have been challenging given the field conditions. Leaves were selected that were fully expanded, mature and were entirely unblemished, or had < 5 % of their surface covered by epiphylls - lichen, fungus and moss that colonise leaf surfaces. PV curves were made on a minimum of three leaves per genus per plot per season (one leaf per tree and nine leaves overall per sensitivity group) according to the ‘bench drying’ protocol described in Tyree & Hammel (1972). Briefly, as the leaf dried out over a period of 3 – 8 hours, repeated measurements of leaf water potential ($\Psi$) and mass were taken using a Scholander pressure bomb (PMS...
Instruments Co., Corvalis, OR, USA) accurate to 0.05 MPa and mass balance accurate to 0.1 mg, respectively. After the final water potential measurement, the leaves were scanned to determine area using ImageJ software (Schneider et al., 2012) and then dried to constant mass in an oven at 70 °C for > 48 hours. The points were then plotted as $1/\Psi$ against leaf mass enabling the calculation of the parameters: osmotic potential at full turgor ($\Psi_0$, MPa), turgor loss point ($\Psi_{lp}$, MPa), saturated water content (the ratio of water mass to leaf dry mass in a fully saturated leaf, SWC, g g$^{-1}$), relative water content at $\Psi_{lp}$ (RWC$_{lp}$, %), modulus of elasticity ($\varepsilon$, MPa) and hydraulic capacitance ($C$, mol MPa$^{-1}$ m$^{-2}$). Calculations of variables from PV curves were carried out according to Sack & Pasquet-Kok (2011). I recognise that PV data analysis may contain a number of sources of error including the decision of which points to include to identify $\Psi_{lp}$. While it is very difficult to account for all possible error sources in a single analysis framework, I employed a maximum likelihood approach based on mixed effects modelling to avoid inflating degrees of freedom in nested samples and check normality assumptions (see below for details on statistical analysis).

**Morphological Traits**

All samples for the tissue analysis were taken in the wet season. Small squares of leaf, approximately 8 mm to a side, were taken from midway along the leaf between the midrib and edge of the lamina and were hand sectioned using a Euromex MT.5500 cylinder microtome. Images of the sections were taken with a Moticam 2 digital camera on a Motic B3 microscope. A magnification of x40 was used where the leaf was thin enough to view a whole section, from upper to lower cuticle, in one image. For thicker leaves it was sometimes necessary to use a magnification of x10 to ensure each tissue measurement was taken on a single ‘transect’, thus providing reliable proportional measurements. Where measuring all tissue layers on one image was not possible, multiple images were used per single leaf section – these values were only employed for absolute tissue measurements but were excluded from the analysis of proportional measurements. The values for each tissue thickness (abaxial epidermis – Ab, palisade – Pal, spongy mesophyll – SM and adaxial epidermis – Ad) for each tree are means taken from a single measurement from two leaves per tree.
Cavity volume of leaves (CV, otherwise referred to as leaf airspaces) was measured by subtracting the mass of fully hydrated leaves from the mass of the same leaves after perfusion with water. Branches were allowed to hydrate overnight and leaves were only used if adjacent leaves had a water potential higher than -0.2 MPa. The leaves were then weighed before being perfused with water at a pressure of 18 KPa for a minimum of 20 hours and then reweighed. The risk of emboli forming in the petiole before perfusion was minimised by taking the initial weight with a small section of branch attached to the leaf. The petiole was then severed at its base with a razor blade under water filtered to 0.02 µm and attached to a silicon tube, the excised branch segment was then weighed and subtracted from the initial weight. Two leaves per individual were measured, all leaves being measured for area and dry mass. Cavity volume was expressed as volume per area (µm$^3$ µm$^{-2}$), which is equivalent to thickness per leaf section (µm) and so directly comparable to the other tissue thickness measurements.

The tissue measurements, cavity volumes and PV analysis were all carried out on different leaves to avoid the effects of one leaf manipulation influencing the others. Therefore, each set of measurements was averaged per individual to enable the correlation analysis. Both the tissue and the cavity volume measurements were only carried out in the wet season, but because genus was found to be the largest source of variance and because the seasonal effects were only found for SWC and RWC$^{lp}$, I pooled the results for the PV parameters across season to maintain the largest possible sample size.

### 2.3.5 Statistical analysis of drought treatment effects on PV parameters

Results for the response of PV parameters to the drought treatment were analysed using linear mixed effects models using the packages lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2014) in R (R Core Team, 2015). As the focus of this study is understanding sensitivity and resistance to drought and not the effect of taxon, genera and individuals (trees) were included as nested random effects. Therefore, large differences between species within a genus would be represented by high variance in the random effect category ‘ID’ (tree individual), because the variance in the ID term groups the inter-individual and inter-species variance (Table 2.1). Models
were initially constructed using all variables and interactions (e.g. Treatment*Season*sensitivity status), and were manually simplified by systematically removing non-significant variables and interactions. The best model (Table 2) was selected on the basis of AIC. The distribution of the data was assessed using the profile function as per Bates et al. (2015) and the data were transformed accordingly. The conditional and marginal $r^2$ were calculated as per Nakagawa et al. (2013).

Table 2.2. Models used to describe variables.

<table>
<thead>
<tr>
<th>Response Variable</th>
<th>Symbol</th>
<th>Units</th>
<th>Transformation</th>
<th>Model structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turgor loss point</td>
<td>$\Psi_{\text{tlp}}$</td>
<td>MPa</td>
<td>log(-1*Y)</td>
<td>T<em>V</em>S</td>
</tr>
<tr>
<td>Osmotic potential at full turgor</td>
<td>$\Psi_0$</td>
<td>MPa</td>
<td>log(-1*Y)</td>
<td>T<em>V</em>S</td>
</tr>
<tr>
<td>Saturated water content</td>
<td>SWC</td>
<td>g$<em>{\text{water}}$ g$</em>{\text{dry, mass}}$</td>
<td>log(Y)</td>
<td>T+S</td>
</tr>
<tr>
<td>Relative water content at TLP</td>
<td>RWC$_{\text{tlp}}$</td>
<td>%</td>
<td>arcsin(Y/100)</td>
<td>S</td>
</tr>
<tr>
<td>Capacitance</td>
<td>$C$</td>
<td>mol MPa$^{-1}$ m$^{-2}$</td>
<td>log(Y)</td>
<td>T<em>V</em>S</td>
</tr>
<tr>
<td>Elastic modulus</td>
<td>$\epsilon$</td>
<td>MPa</td>
<td>$Y^{0.34}$</td>
<td>T<em>V</em>S</td>
</tr>
</tbody>
</table>

Model terms are as follows: T – treatment (TFE or control), V – drought vulnerability status (sensitive or resistant), S – season (dry or wet). In all models tree individual nested inside genus was a random effect used to adjust only the intercept.

2.3.6 Regression analysis of leaf anatomy and PV parameters

The PV parameters were compared to the absolute and proportional thicknesses of leaf tissues using multiple linear regression in R (R Core Team). Of the PV parameters, $\Psi_0$ and $\Psi_{\text{tlp}}$ represent conditions in the symplast, while the other parameters, SWC, RWC$_{\text{tlp}}$, $C$ and $\epsilon$ represent the entire water-occupied volume of the leaf. The elastic modulus, $\epsilon = \frac{dP}{dRWC_{\text{leaf}}}$ where $P$ is the turgor pressure, can be calculated to represent only the symplast (and therefore the influence of turgor on cell wall expansion); however, bulk $\epsilon$ is used here to avoid errors arising from the extreme extrapolation of the PV curve to the y intercept needed to return $\epsilon$ for only the symplast (Andersen et al., 1991), and to minimise the impact of the necessary assumption that the apoplastic fraction remains constant throughout the PV analysis (Tyree and Richter, 1981). Therefore, the SWC, RWC$_{\text{tlp}}$, $C$ and $\epsilon$ all represent conditions of both the symplast and the cell walls, the ratio of which will vary between tissue layers depending on cell
geometry and cell wall thickness. Absolute measurements of tissue thickness ($\text{Ab}_{\text{abs}}$, $\text{Pal}_{\text{abs}}$, $\text{SM}_{\text{abs}}$, $\text{Ad}_{\text{abs}}$) represent volume per area ($\mu\text{m}^3 \, \mu\text{m}^{-2} = \mu\text{m}$), and correlations of PV parameters with absolute measurements would indicate a functional link between the tissue type and the PV parameter. For example, a correlation between $\Psi_\pi^0$ and $\text{Pal}_{\text{abs}}$, but not $\text{Pal}_{\text{prop}}$ (palisade thickness as a proportion of leaf thickness), would indicate that a thicker palisade leads to or requires higher $\Psi_\pi^0$. On the other hand, correlations of PV parameters with proportional measurements of tissue thickness ($\text{Ab}_{\text{prop}}$, $\text{Pal}_{\text{prop}}$, $\text{SM}_{\text{prop}}$, $\text{Ad}_{\text{prop}}$) would indicate which tissues are particularly influential on the overall leaf-level value, and possibly are different from the leaf average. A significant relationship between a PV parameter and proportional tissue thickness might suggest that the properties of the tissue in question are important in determining the overall leaf-level values.

Because $\Psi_\pi^0$ and $\Psi_{\pi}^{\text{HP}}$ are fundamental properties of the symplast which can become decoupled from cell volume through changes in cell size and cell wall thickness (Fig. S2.1), the same analysis was carried out by calculating the symplast volume of each tissue (Supporting Information 2.6). The symplast volume was calculated using mean cell size and cell wall thickness, and by assuming that spongy mesophyll cells were spherical, palisade cells were cylindrical and epidermal cells were cuboids. It was not possible to measure cell wall thickness with sufficient accuracy due to the resolution of the images (S2.1), so mean cell wall thickness was derived from values presented by Buckley et al. (2015) for 14 species (n=13 for spongy mesophyll). Given the additional error associated with the assumption of cell shape, the adoption of a mean cell wall thickness taken from other species, the reduction in the degrees of freedom due to the difficulty in measuring cell size accurately (mean df = 25 for tissue thickness and 17 for symplast volume) and the similarity in the results between the tissue thickness and the symplast analysis, the tissue thickness results are presented here while the alternative analysis is given in Supporting Information Table S2.1.

Tissue thicknesses, cavity volume and PV parameters were all averaged for each individual tree before performing the regression analyses. The cavity volume was subtracted from the total spongy mesophyll volume to give a value of water-saturated spongy mesophyll volume, but the interaction between spongy mesophyll and cavity
volume was analysed for significance. This required the assumption that the cavity volume in the palisade layer was negligible compared to that in the spongy mesophyll, which was consistent with the images (Fig. S2.1). Absolute and proportional tissue thicknesses were modelled separately to highlight the different effects and to reduce correlation among independent predictors. Thus, the starting structure of the models was $Y \sim \text{Pal} + \text{Ad} + \text{Ab} + \text{SM} \times \text{CV}$ for both absolute and proportional measurements, where $Y$ stands for the response variable and the sign $\sim$ stands for ‘as a function of’. Models were simplified by sequentially removing the factors that did not contribute significantly to increasing model log-likelihood. At each simplification successive models were compared using AIC values using a chi-square test. Variance inflation factors for all variables in the final models were found to be $<3$, indicating very limited autocorrelation among independent variables.

2.4 Results

2.4.1 $H_1$ Imposed drought versus seasonal effects

Water-relation parameters varied highly by season and treatment, with no common pattern existing across all parameters. Significant treatment effects were detected for $\Psi_{\text{tlp}}$ ($P = 0.041$), $\Psi_0$ ($P = 0.038$) and $\varepsilon$ ($P = 0.030$), while no significant effects were found for SWC, RWC$_{tlp}$ and $C$ (Table 2.3). Both $\Psi_{\text{tlp}}$ and $\Psi_0$ (which were highly correlated, $r^2 = 0.94$), were lower (more negative) in the TFE compared to Control, while $\varepsilon$ was larger in the TFE than the control. In contrast, significant seasonal changes occurred for SWC, RWC$_{tlp}$ and $C$, while no significant seasonal effects were detected in $\Psi_{\text{tlp}}$, $\Psi_0$ and $\varepsilon$ (Table 2.3, Fig. 2.1). Values of SWC and RWC$_{tlp}$ were higher and those of $C$ lower in wet season. Thus, $\Psi_0$, $\Psi_{\text{tlp}}$ and $\varepsilon$ had stronger (long-term drought) treatment than seasonal effects, consistent with hypothesis 1. Trends in both $C$ and $\varepsilon$ were opposite to those expected from differences in water availability between season and treatment; $C$ was highest in the dry season and in the control plot, with opposite trends for $\varepsilon$. Interactions between treatment and season were found only for capacitance, which showed no seasonal difference in the TFE, but an increase in the dry season in the control plot (Fig. 2.2).
Table 2.3. Probability values and coefficients for the fixed effects included in the mixed models listed in Table 1; factors with a dash were not included in the final model, and values where $P < 0.05$ are in bold.

<table>
<thead>
<tr>
<th>Variable</th>
<th>S</th>
<th>T</th>
<th>V</th>
<th>S:T</th>
<th>T:V</th>
<th>T:S</th>
<th>T:S:T</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Psi_{\pi}^{\text{tlp}}$</td>
<td>$0.334$</td>
<td>$0.041$</td>
<td>$0.599$</td>
<td>$&lt;$0.001</td>
<td>$0.131$</td>
<td>$0.084$</td>
<td>$\textbf{0.007}$</td>
</tr>
<tr>
<td>Coef.</td>
<td>$0.09$</td>
<td>$0.25$</td>
<td>$-0.14$</td>
<td>$-0.55$</td>
<td>$-0.21$</td>
<td>$-0.29$</td>
<td>$0.52$</td>
</tr>
<tr>
<td>$\Psi_{\pi}^{o}$</td>
<td>$0.317$</td>
<td>$0.038$</td>
<td>$0.509$</td>
<td>$&lt;$0.001</td>
<td>$0.089$</td>
<td>$0.053$</td>
<td>$\textbf{0.004}$</td>
</tr>
<tr>
<td>Coef.</td>
<td>$0.13$</td>
<td>$0.33$</td>
<td>$-0.2$</td>
<td>$-0.71$</td>
<td>$-0.33$</td>
<td>$-0.41$</td>
<td>$0.79$</td>
</tr>
<tr>
<td>SWC</td>
<td>$0.015$</td>
<td>$0.068$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Coef.</td>
<td>$0.05$</td>
<td>$-0.08$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RWC$_{\text{tlp}}$</td>
<td>$&lt;$0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Coef.</td>
<td>$0.07$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\epsilon$</td>
<td>$0.068$</td>
<td>$0.03$</td>
<td>$0.876$</td>
<td>$0.156$</td>
<td>$0.055$</td>
<td>$\textbf{0.027}$</td>
<td>$0.044$</td>
</tr>
<tr>
<td>Coef.</td>
<td>$0.35$</td>
<td>$0.49$</td>
<td>$-0.04$</td>
<td>$-0.39$</td>
<td>$-0.53$</td>
<td>$-0.67$</td>
<td>$0.77$</td>
</tr>
<tr>
<td>C</td>
<td>$0.007$</td>
<td>$0.05$</td>
<td>$0.953$</td>
<td>$\textbf{0.044}$</td>
<td>$\textbf{0.014}$</td>
<td>$0.059$</td>
<td>$\textbf{0.018}$</td>
</tr>
<tr>
<td>Coef.</td>
<td>$-0.42$</td>
<td>$-0.38$</td>
<td>$-0.02$</td>
<td>$0.45$</td>
<td>$0.55$</td>
<td>$0.49$</td>
<td>$-0.74$</td>
</tr>
</tbody>
</table>

Footnote: Factors are Season (S), Treatment (T) and Sensitivity status (V). Variables are turgor loss point ($\Psi_{\pi}^{\text{tlp}}$), osmotic potential at full turgor ($\Psi_{\pi}^{o}$), saturated water content (SWC), relative water content at $\Psi_{\pi}^{\text{tlp}}$ (RWC$_{\text{tlp}}$), elastic modulus ($\epsilon$) and capacitance (C).

2.4.2 $H_2$ Drought sensitivity status versus seasonal variation

Drought sensitivity status alone had no significant impact on any of the parameters, but there were significant interactions between sensitivity and season for $\Psi_{\pi}^{o}$ ($P < 0.001$), $\Psi_{\pi}^{\text{tlp}}$ ($P < 0.001$) and $C$ ($P = 0.044$, Table 3, Fig. 2.3). In resistant species $\Psi_{\pi}^{o}$ and $\Psi_{\pi}^{\text{tlp}}$ showed little seasonal variation but in sensitive species both parameters were higher in the wet season. This is opposite to hypothesis 2, that resistant genera would show greater seasonal variation. However, the reverse trend was evident for $C$, in which greater seasonal changes occurred in resistant species. Significant three-way interactions occurred between treatment, season and sensitivity status for $\Psi_{\pi}^{\text{tlp}}$ ($P = 0.007$), $\Psi_{\pi}^{o}$ ($P = 0.004$), $\epsilon$ ($P = 0.044$) and $C$ ($P = 0.018$), due to a large treatment effect amongst resistant species in the dry season, which was largely absent in the wet season and for the sensitive species.
Figure 2.1. Comparison between seasonal and plot effects of PV parameters. On the left hand side (panel a) white bars represent the control and grey bars the TFE; on the right hand side (panel b) white bars represent wet season and grey bars dry season. Bars display the mean +/- 1 standard error and significance is denoted by asterisks, whereby * < $P=0.05$; ** < $P=0.01$; *** < $P=0.001$; $P=0.05 < \cdots < P=0.10$.

Annual rain in the drought plot is $\approx 90$ mm month$^{-1}$, in the control plot is $\approx 180$ mm month$^{-1}$, in the wet season (averaged between TFE and control plot) is $\approx 210$ mm month$^{-1}$ and in dry season is $\approx 60$ mm month$^{-1}$. $\Psi^{\text{tlp}}$ is turgor loss point, $\Psi^{\text{wp}}$ is osmotic potential at full turgor, SWC is saturated water content and RWC$^{\text{tlp}}$ is relative water content at $\Psi^{\text{wp}}$. 
2.4.3 Variance in drought treatment analysis

The $r^2_{conditional}$, showing the total amount of variance explained by the models, varied from 0.29 for $\varepsilon$ to 0.76 for SWC (Table 2.1). The greatest proportion of explained variance in the mixed effects models was accounted for by the experimental (fixed) effects in $\Psi_{\pi}$ and $\varepsilon$ but by random differences from genus to genus in the other variables. The variance attributed to individuals within a genus was typically a small proportion (3 – 11 %) of total variance (with the exception of SWC, 27 %) indicating that traits varied little among individuals within these genera. The modelled fixed effects accounted for between 4 and 32 % of total variance, and were highest for $\Psi_{\pi}^{tlp}$ and $\Psi_{\pi}^{o}$ with 30 and 32 % respectively.

2.4.4 H$_3$ PV traits and tissue correlations

Contrary to expectation, there was no correlation between $\Psi_{\pi}^{o}$ and either Pal$_{abs}$ or Pal$_{prop}$, but Pal$_{abs}$ was significantly negatively correlated with $\Psi_{\pi}^{tlp}$ and the relationship between Pal$_{prop}$ and $\Psi_{\pi}^{tlp}$ was marginally significant (Fig. 2.4a, Table 2.4). SM$_{prop}$ correlated with $C$ (Fig. 2.4b) and $\Psi_{\pi}^{o}$, and, interestingly, SM$_{abs}$ had highly significant positive correlations with $\Psi_{\pi}^{tlp}$, $\Psi_{\pi}^{o}$ (Fig. 2.4c), SWC and RWC$_{tlp}$. As SM$_{abs}$ correlated strongly with leaf thickness ($R^2 = 0.76$, $P << 0.001$), they were employed in separate models to determine whether the correlations with SM$_{abs}$ arose simply as a function of leaf thickness. Neither $\Psi_{\pi}^{tlp}$ nor $\Psi_{\pi}^{o}$ correlated with leaf thickness, while both SWC
and RWC$^{\text{tlp}}$ did ($R = 0.48$, $P = 0.003$, and $R^2 = 0.61$, $P < 0.001$ respectively) albeit less strongly than with SM$_{\text{abs}}$. Ab$_{\text{prop}}$ correlated with $\Psi$$_{\pi}^{\text{tlp}}$ (Fig. 2.4d) and $\Psi$$_{\pi}^{0}$, but Ab$_{\text{abs}}$ correlated only with $\Psi$$_{\pi}^{\text{tlp}}$. On the other hand, Ad$_{\text{abs}}$ correlated with $\Psi$$_{\pi}^{\text{tlp}}$, $\Psi$$_{\pi}^{0}$, C and $\epsilon$, but Ad$_{\text{prop}}$ only correlated with RWC$^{\text{tlp}}$. The absolute measurements of cavity volume did not correlate with any of the variables but significantly improved the strength of the models for $\Psi$$_{\pi}^{\text{tlp}}$, SWC and C, while CV$_{\text{prop}}$ only significantly improved the model.

Figure 2.3. Season and drought sensitivity status effects for osmotic potential at full turgor ($\Psi$$_{\pi}^{0}$, $P < 0.001$), osmotic potential at turgor loss point ($\Psi$$_{\pi}^{\text{tlp}}$, $P < 0.001$) and capacitance ($P = 0.044$). Grey bars are resistant species, white bars are vulnerable species. Bars display the mean +/- 1 standard error.
for C. The models were initially performed with response variables transformed as in the mixed models; however, the transformation made little difference to the model results and so transformations were not used to facilitate interpretation of the model coefficients.

Table 2.4. Slope coefficients for regressions of PV parameters against tissue thickness, expressed in either absolute (top section) or proportional units (lower section).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$\Psi_{\pi}^{\text{Hl}}$ (MPa)</th>
<th>$\Psi_{\pi}^0$ (MPa)</th>
<th>SWC</th>
<th>RWC (%)</th>
<th>C (mol MPa$^{-1}$ m$^{-2}$)</th>
<th>$\varepsilon$ (MPa)</th>
</tr>
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<td>Absolute tissue thickness (µm*10$^{-3}$)</td>
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<td>9.31***</td>
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<td>-</td>
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<td>-</td>
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</tbody>
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Note: Tissue parameters with a dash were not included in the final model. Significance is denoted by asterisks, whereby * < P=0.05; ** < P=0.01; ***<P=0.001; P=0.05 < · < P=0.10, and significant values are in bold. The significance, P, percent of explained variance, $R^2$, and the degrees of freedom df, are given for each model. Variables are turgor loss point ($\Psi_{\pi}^{\text{Hl}}$), osmotic potential at full turgor ($\Psi_{\pi}^0$), saturated water content (SWC), relative water content at $\Psi_{\pi}^{\text{Hl}}$ (RWC$_{\text{tlp}}$), elastic modulus ($\varepsilon$) and capacitance (C). Absolute measurements of tissue thickness are given in µm*10$^{-3}$, which gives units for the slope as e.g. ‘slope’ * 10$^{-3}$ MPa µm$^{-1}$.
Figure 2.4. Relationships between PV parameters and tissue thickness. The Pearson product-moment correlation coefficient for: a, $r = -0.44$; for b, $r = 0.32$; for c, $r = 0.47$; for d, $r = -0.55$. $\Psi_{\pi}^{\text{tlp}}$ is turgor loss point and $\Psi_{\pi}^{\text{0}}$ is osmotic potential at full turgor.
Hypothesis 3, i.e. that Pal should correlate with $\Psi_\pi^o$, while SM should correlate with $C$, can be rejected in terms of there being no correlation between palisade thickness and $\Psi_\pi^o$, although the correlation between $SM_{\text{prop}}$ and $C$ may suggest that the spongy mesophyll plays a role in water storage. Interesting correlations that were not predicted include the negative correlation between the palisade thickness and $\Psi_\pi^{\text{tlp}}$, the negative correlations between $Ab_{\text{prop}}$ and both $\Psi_\pi^{\text{tlp}}$ and $\Psi_\pi^o$ and the positive correlations between $SM_{\text{abs}}$ and $\Psi_\pi^{\text{tlp}}$, $\Psi_\pi^o$, SWC and RWC$^{\text{tlp}}$.

2.5 Discussion

This study reveals how leaf water relations in Amazonian rainforest trees respond to long-term experimental drought and whether these responses are related to: (i) seasonal leaf water relations; (ii) differential rates of drought-induced mortality; (iii) leaf tissue morphology. Overall, the studied trees, independent of drought-sensitivity status, showed greater acclimation to the experimental soil moisture deficit than to seasonal variation in water availability, primarily via osmotic adjustments ($H_1$). The designation of drought sensitivity of a species (based on mortality response; da Costa et al. 2010) was only important in these data with respect to differences in seasonal acclimation: drought-sensitive species underwent greater levels of seasonal osmotic adjustment than resistant species ($H_2$, Table 2.3), but a significant difference of the sensitivity status per se was not found. Lastly, palisade thickness did not correlate with osmotic potential at full turgor, but $SM_{\text{prop}}$ did correlate with leaf hydraulic capacitance ($H_3$, Table 2.4). The data imply that caution is needed in ascribing acclimation capability to drought based on short-term (seasonal) data: they demonstrate that tissue-level water relation traits can acclimate to long-term water stress, but that seasonal osmotic adjustment may not be an adaptive advantage in coping with extended drought stress.

2.5.1 $H_1$ Imposed drought versus seasonal response in $\Psi_\pi^o$, $\Psi_\pi^{\text{tlp}}$ and $\epsilon$

Consistent with $H_1$, $\Psi_\pi^o$, $\Psi_\pi^{\text{tlp}}$, and $\epsilon$ all showed a significant response to the drought treatment and no seasonal effect. Stable osmotic gradients, such as those between the symplast and apoplast, require energy to be created and maintained as they involve moving molecules up a gradient of osmotic potential (Nobel, 1999). Moreover,
excessively high solute concentrations, due to dehydration, run the risk of causing membrane damage (Steponkus, 1984, Bryant et al., 2001). The cost and risk associated with increasing solute concentration is, therefore, likely to result in a physiological maximum solute concentration. The finding that $\Psi_\pi^0$ is significantly different between plots, but not season, indicates that the magnitude of seasonal osmotic adjustment does not represent a physiological limit for longer term water deficits and is therefore not a good indicator of a species’ capacity to cope with long-term reduction in water availability. The higher $\varepsilon$ in the TFE is consistent with the general negative correlation between $\varepsilon$ and $\Psi_\pi^0$ (Niinemets, 2001, Bartlett et al., 2012), and the combination of the changes in these two parameters contributes to drought resistance by creating a greater change in $\Psi$ for a given amount of water loss, thus facilitating water uptake from drier soils without turgor loss (Bowman and Roberts, 1985). It is not known what determines the maximum capacity for adjustment in osmotic properties or the elastic modulus and, therefore, the adaptation of the trees in this study could not have been predicted without a long term experiment. The ability of trees to adapt to long term changes in water availability is fundamental to predicting how tropical forests are going to respond to climate change and, if overlooked, could lead to inaccurate projections of future vegetation-climate interactions.

2.5.2 H2 Seasonal plasticity and drought vulnerability

Several studies have indicated that osmotic adjustment is linked to drought resistance (Kubiske and Abrams, 1991, Tschaplinski et al., 1998, Mitchell et al., 2008), suggesting that drought-resistant species should show greater seasonal variation in osmotic traits (H2). In contrast to this expectation, it was the drought sensitive species that showed greater seasonal osmotic adjustment (Fig. 2.3), while the resistant species showed very little. The drought sensitive species had significantly higher (less negative) $\Psi_\pi^0$ and $\Psi_\pi^{\text{DP}}$ in the wet than dry season, which should, presumably, lead to lower maintenance costs than the resistant species. On this basis, drought sensitive species might be expected to have lower respiration than the resistant species. However, there is no correlation between $\Psi_\pi^0$ and leaf dark respiration amongst these species ($P = 0.4, R^2 = 0.02$, data not shown) and previous work has demonstrated that the leaves of the sensitive species on the drought plot had higher leaf dark respiration,
especially in the dry season (Rowland et al., 2015c). Capacitance also showed an interaction between season and vulnerability status, but with a reverse trend to the osmotic parameters, in which the resistant genera showed seasonal variation while the sensitive genera showed little response (Fig. 2.3). The finding that most osmotic adjustment happened in the sensitive species may indicate that, rather than being an active strategy to reduce sensitivity to water stress, it may be an indirect result of another process. It is also worth stressing that no significant effect of the sensitivity status was found on leaf NSC concentrations and that this last parameter even increased slightly in the dry season in all species (Rowland et al., 2015c).

There was an apparent divide between the parameters in this study that responded significantly to the drought treatment, $\Psi_\pi^o$, $\Psi_\pi^{tlp}$, and $\varepsilon$, and those that responded more to seasonality, SWC, RWC$^{tlp}$ and $C$ (Table 2.3). SWC, $C$ and RWC$^{tlp}$ have also been suggested to play a role in drought resistance (Niinemets, 2001, Kubiske and Abrams, 1991, Hao et al., 2008) but, in this case, their response to the experimental drought was not significant ($P > 0.05$), despite their short term response to seasonal water availability. Given the mechanistic nature of the links between PV parameters, the disparity in responses between the two groups of traits may be seen as surprising. It is possible that the difference between the groups is caused by seasonal changes in cell wall properties, hence not affecting $\Psi_\pi^o$ and $\Psi_\pi^{tlp}$, which are only properties of the symplast, while $\varepsilon$ would be influenced only slightly by the changes in water content of the cell walls. Another, potential explanation might be ontogenetic changes, whereby leaves of a similar age change systematically throughout the year. However, immature leaves were intentionally avoided and an analysis of variability in mean leaf area across seasons demonstrated that leaves were fully expanded (unpublished data). Therefore, it is concluded that while seasonal differences alone were not significant in the osmotic parameters or $\varepsilon$, there were non-significant seasonal trends (Fig. 2.1) which enabled significant variation in the other parameters.

2.5.3 H$_3$ Correlations between anatomical and water relations traits

It was hypothesised that the thickness of the palisade layer would correlate negatively with osmotic potential at full turgor (i.e. that leaves with thicker palisade would have more negative $\Psi_\pi^o$) and that the spongy mesophyll would correlate positively with
capacitance. We find no evidence that the palisade thickness (calculated as either total or as symplastic fraction) influences leaf osmotic potential at full turgor and in this respect our data reject H3; however, the correlation of SM\textsubscript{prop} with C suggests that the spongy mesophyll may affect leaf-level capacitance (Table 2.4, Fig. 2.4b). The analysis of symplastic fractions (Table SI) yielded no correlations between capacitance and SM, perhaps arguing for a capacitive role of the apoplast of the SM. While neither of the palisade measurements correlated with Ψ\textsubscript{p}, the correlation of Pal\textsubscript{abs} and weak correlation of Pal\textsubscript{prop} with Ψ\textsubscript{plp} (P = 0.021 and P = 0.052 respectively, Table 2.4, Fig. 2.4a) could imply osmotic adjustment in the palisade layer in response to dehydration. Thus, it is unlikely that the osmotic potential of the palisade layer is significantly below that of the bulk leaf value when the leaf is hydrated (above Ψ\textsubscript{plp}), but it is possible that solutes are generated in, or moved into, the palisade in response to leaf dehydration. These correlations disappeared when using the symplastic fractions of Pal and Pal\textsubscript{prop}, but the available degrees of freedom were drastically reduced for this analysis.

2.5.4 Other correlations between anatomy and PV traits

The strong negative correlation between the proportional thickness of the abaxial epidermis with both Ψ\textsubscript{p} and Ψ\textsubscript{plp} (Fig. 2.4d, also Table S2.1 for the symplastically adjusted values) implies that either leaves with low osmotic potentials benefit from having a thicker abaxial epidermis, or that the abaxial epidermis has a lower Ψ\textsubscript{p} than the rest of the leaf (Mott, 2007). The latter hypothesis is in line with the findings of Buckley et al. (2015) that the upper and lower epidermal layers are hydraulically independent. Stomata close in response to a threshold leaf water potential (Brodribb et al., 2003), and thus by having an osmotic potential lower than the leaf average, turgor in the abaxial epidermis would be higher than the leaf average, enabling stomata to remain open when the epidermis is close to bulk leaf Ψ\textsubscript{plp}. This strategy would be associated with anisohydric behaviours, which is consistent with recent findings from the same trees (unpublished data).

The absolute thickness of the spongy mesophyll appears to play an influential role in determining leaf PV values (Table 2.4, Table S2.1). The strong correlation of SM\textsubscript{prop} with Ψ\textsubscript{p} (Fig. 2.4c) could indicate both that the SM has a higher osmotic potential (closer to 0) than the other tissues, and/or that the structure of the SM compensates for
the effects of low osmotic potential. The first possibility (higher osmotic potential, closer to 0) is consistent with the significant positive correlation between SM_{prop} and C (Table 2.4, Fig. 2.4b), although these results will have been influenced by two samples with particularly low SM_{prop}, and there is also no correlation between the symplastic volume and C (Table S2.1). The second point, that the structure of the spongy mesophyll compensates for low (more negative) bulk leaf \( \Psi_{\pi} \), supports the view that the spongy mesophyll offers a low resistance (high conductance) pathway for lateral hydraulic flow, in contrast to the palisade mesophyll (Wylie, 1946). Because water moves down a water potential gradient, flow can be increased by either increasing the gradient or the conductance according to the relation \( F = \Delta \Psi * K \), where \( F \) is flow rate and \( K \) is conductance. A thick spongy mesophyll, represented here without the cavity volume, can have large lateral connectivity (Fig. S2.2a, Wylie, 1946), potentially increasing hydraulic conductance within the leaf, and so reducing the need for low osmotic potential required for maintaining turgor with low water potentials.

2.5.5 Variance accounted for by individual and genus

The percent variance accounted for by ID (individual tree within a genus, random effect) was low for most parameters, with the exception of SWC. In contrast, the variance accounted for by genus was relatively high (Table 2.1), indicating that the variation within a genus is lower than variation among genera, and hence that there is some conservation of these parameters by taxonomic group. Bulk elastic modulus had the lowest variance among genera, suggesting convergence on a similar strategy regarding cell wall rigidity; conversely SWC had high variance, suggesting divergence among genera in overall water content.

2.5.6 Wider implications and summary

There is mounting evidence that hydraulic processes are fundamental to understanding drought-induced tree mortality (Rowland et al., 2015a, Anderegg et al., 2012, Hartmann et al., 2015), and consequently there is increasing interest in how knowledge of hydraulic responses could inform ecosystem models. This study demonstrates that the six focal tropical tree genera can: (i) perform osmotic adjustment in response to long-term (decadal-scale) reductions in soil water availability over and above those
associated with seasonal variation; (ii) that seasonal osmotic adjustment does not act as an indicator of increased resilience to long-term drought stress; and strengthens the case that (iii) different leaf tissues respond to hydraulic demands in different ways. While these findings only cover six genera, they suggest that, in contrast to those found in drier ecosystems (Kubiske and Abrams, 1991, Tschaplinski et al., 1998, Mitchell et al., 2008), maintaining osmotic homeostasis may be a more successful drought-resistance strategy than relying on osmotic adjustment in tropical rainforest communities.

Results such as these are vital for understanding how we can predict plant responses under future water stress in tropical forests, for which further empirical understanding of both long- and short-term responses to drought conditions is urgently needed.

2.6 Supporting Information

2.6.1 Correlation analysis of symplastic tissue volume versus leaf water relations

Osmotic potential at full turgor and turgor loss point pertain only to the symplast volume of cells. If differences exist in these parameters between tissue types within the leaf one might expect a correlation to exist between the thickness of the particular tissue with the osmotic parameter. However, such relationship, if one exists, could become decoupled from tissue thickness due to changes in cell size and apoplastic fraction. Therefore, a separate analysis was performed to examine the correlations without the volumetric apoplastic fraction.

2.6.2 Methods

Cell sizes were measured by analysing sections of leaf, approximately 10 µm thick, on transmission light microscope images using ImageJ software. Spongy mesophyll cells were assumed to be spherical and represented by a single measurement of diameter, palisade mesophyll cells were assumed to be cylindrical and were represented by measurements of length and diameter, and epidermal cells were assumed to be cuboids, square in paradermal section, represented by measurements of length and thickness. Five measurements of each were made per leaf section, and each tree was represented by two leaves, one section from each. All measurements were averaged per tree for the correlation analysis with the PV parameters.
For the correlation analysis (see Section 2.3.6 for further details) cell volumes were calculated based on the measurements described above. The apoplastic volume of each cell was calculated using average cell wall thicknesses taken from Buckley et al. (2015). The values were means taken from 14 species (13 for spongy mesophyll) and are as follows in µm +/- 1 standard error: Ad_{cell\_wall} = 1.87 +/- 0.16, Pal_{cell\_wall} = 1.15 +/- 0.09, SM_{cell\_wall} = 1.31 +/- 0.14, Ab_{cell\_wall} = 1.71 +/- 0.15. Thus, the symplastic fraction was determined by subtracting the apoplastic volume from the total cell volume and dividing it by total cell volume. The spongy mesophyll, for example, would be:

\[
V_{sm\_T} = \text{Volume of spongy mesophyll cell (µm}^3\text{)}
\]

\[
V_{sm\_s} = \text{symplast volume of SM cell (µm}^3\text{)}
\]

\[
F_{sm\_s} = \text{symplastic fraction of SM cell}
\]

\[
r_{sm} = \text{radius of SM cell (µm)}
\]

\[
T_{sm\_cw} = \text{Thickness of the cell wall (µm)}
\]

\[
V_{sm\_T} = \frac{4}{3}\pi r_{sm}^3
\]

\[
V_{sm\_s} = \frac{4}{3}\pi(r_{sm} - T_{sm\_cw})^3
\]

\[
F_{sm\_s} = V_{sm\_s} / V_{sm\_T}
\]

As all of the tissue volumes in this paper are normalised by leaf area (µm$^3$ µm$^{-2}$ = µm), multiplying the thickness of tissue by the symplastic fraction gives the ‘thickness’ of symplast i.e. the volume of symplast per area:

\[
SM_{abs} \times F_{sm\_s} = SM_{symp} (µm)
\]

Because this was just an analysis of symplast volume, the cavity volume was not analysed in these models. Otherwise, the analysis was conducted in exactly the same way as the analysis of the tissue thickness. Therefore, the starting structure of the models, using SM as an example, was:

\[
Y \sim Ad_{symp} + Pal_{symp} + SM_{symp} + Ab_{symp}
\]
To make this analysis correspond to the analysis of tissue thickness, the symplastic thickness of each tissue was also found as a proportion of total leaf thickness. Proportional measurements were not found by summing the fractional symplastic contribution because this resulted in a high degree of interdependence between values. Thus, $SM_{prop\_symp} = SM_{symp} / \text{leaf thickness}$, and not $SM_{symp} / (Ad_{symp} + Pal_{symp} + SM_{symp} + Ad_{symp})$.

Figure. S2.1 Transmission light microscope images of leaf sections of a) *Pouteria anomala*, b) *Eschweilera coriacea* and c) *Swartzia racemosa*. The pictures show densely packed palisade mesophyll layers with very little air space, and varying amounts of air space in the spongy mesophyll layer.
2.6.3 Results and discussion

Mean cell volume of all tissue layers increased significantly with tissue thickness. This was expected in the case of the epidermal layers, which are one cell thick, and for the palisade, which was often one cell thick (Fig S2.1), but not expected for the SM ($R^2 = 0.16$, $P = 0.002$). Because SM cells were assumed to be spherical, the surface area to volume ratio was expected to decrease non-linearly with volume and, therefore, the relationship between symplastic fraction and SM thickness was also predicted to be non-linear (Fig. S2.2).

![Figure S2.2](image.png)

**Figure S2.2.** Relationships between a) the symplastic fraction of the spongy mesophyll, and b) the spongy mesophyll symplast volume per area with spongy mesophyll thickness. Plot a) $y = 0.250966 \times x^{0.1767}$, $R^2 = 0.19$ and $P = <0.001$. Plot b) $y = 0.250966 \times x^{1.1767}$, $R^2 = 0.92$ and $P << 0.001$

The analysis of symplastic volumes with PV parameters provided results similar to those of the tissue thickness (compare Table S2.1 with Table 2.4). The absolute values of spongy mesophyll correlate with $\Psi_{\pi}^{\text{tlp}}$, $\Psi_{\pi}$, SWC and RWC$^{\text{tlp}}$ in both analyses, although in the symplastic analysis the proportional measurements also correlate with $\Psi_{\pi}^{\text{tlp}}$, SWC and RWC$^{\text{tlp}}$. Perhaps the biggest difference between analyses is that neither the absolute or proportional measurements of the palisade correlate with anything. As the analysis of tissue thickness combines the apoplast and symplast, it is possible that disparity between the two analyses (symplast vs thickness) indicates a
functional role for the apoplast. However, the results of the symplast analysis must be interpreted with caution due to the assumptions (listed below) made to derive symplast volume and to the reduced degrees of freedom of the analysis.

Assumptions required to determine symplast volume:

- Cells accurately represented by designated shape e.g. SM cell is spherical, palisade is cylindrical, epidermal cells are cuboid.
- Cell walls are a comparable thickness to the 14 species measured by Buckley (2015)
- Cuticle accounts for negligible proportion of leaf thickness.
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<th>$\Psi_{\pi}^{o}$ (MPa)</th>
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<th>RWC (%)</th>
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Note: Tissue parameters with a dash were not included in the final model. Significance is denoted by asterisks, whereby * $P<0.05$; ** $P<0.01$; *** $P<0.001$; P $0.05 < \cdot < P=0.10$, and significant values are in bold. The significance, P, proportion of explained variance, $R^2$, and the degrees of freedom, df, are given for each model. Variables are turgor loss point ($\Psi_{\pi}^{\text{tip}}$), osmotic potential at full turgor ($\Psi_{\pi}^{o}$), saturated water content (SWC), relative water content at $\Psi_{\pi}^{\text{tip}}$ (RWC$^{\text{tip}}$), elastic modulus ($\varepsilon$) and capacitance (C). Absolute measurements of tissue thickness are given in $\mu$m$^{*}10^{-3}$, which gives units for the slope as e.g. ‘slope’ $*10^{-3}$ MPa $\mu$m$^{-1}$.
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Chapter 3

Limited acclimation in leaf anatomy to experimental drought in tropical rainforest trees

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Author contributions:
OB designed the study, collected all of the leaf anatomy and leaf water relations data, analysed the data, and wrote the paper. LR collected the data for predawn water potentials and gas exchange and provided permission for its use in this manuscript. OB, MM and PM designed the research. PM and ACLC conceived and implemented the experiment. LR and AARO assisted data collection, LF enabled data collection, and SSV provided equipment. LR, MM and PM all helped with manuscript preparation, and MM contributed to data analysis.

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3.1 Summary

Dry periods are predicted to become more frequent and severe in the future in some parts of the tropics, including Amazonia, potentially causing reduced productivity, higher tree mortality and increased emissions of stored carbon. Using a long-term (12 year) through-fall exclusion (TFE) experiment in the tropics, I test the hypothesis that trees produce leaves adapted to cope with higher levels of water stress, by examining the following leaf characteristics: area, thickness, LMA, vein density, stomatal density, the thickness of palisade mesophyll, spongy mesophyll and both of the epidermal layers, internal cavity volume and the average cell sizes of the palisade and spongy mesophyll. I also test whether differences in leaf anatomy are consistent with observed differential drought-induced mortality responses among taxa, and look for relationships between leaf anatomy, and leaf water relations and gas exchange parameters. My data show that trees do not produce leaves that are more xeromorphic in response to 12 years of soil moisture deficit. However, the drought treatment did result in increases in the thickness of the adaxial epidermis (TFE 20.5 ± 1.5 μm, control 16.7 ± 1.0 μm) and the internal cavity volume (TFE 2.43 ± 0.50 mm$^3$ cm$^{-2}$, control 1.77 ± 0.30 mm$^3$ cm$^{-2}$). No consistent differences were detected between drought-resistant and drought sensitive taxa, although interactions occurred between drought-sensitivity status and drought treatment for the palisade mesophyll thickness ($P = 0.034$) and the cavity volume of the leaves ($P = 0.025$). The limited response to water deficit probably reflects a tight co-ordination between leaf morphology, water relations and photosynthetic properties. This suggests that there is little plasticity in these aspects of plant anatomy in these taxa, and that phenotypic plasticity in leaf traits may not facilitate the acclimation of Amazonian trees to the predicted future reductions in dry season water availability.
3.2 Introduction

A key issue in the prediction of future climate change is understanding how forests, significant stores of carbon (Pan et al., 2011, Grace et al., 2014), will respond to current and future changes in temperature and water availability (Bonan, 2008). Tree mortality has reportedly increased in response to episodic severe drought (Breshears et al.; 2005, Allen et al., 2010), including in the tropics (Nakagawa et al., 2000, Meir and Grace, 2005, Phillips et al., 2009, Brienen et al., 2015), and understanding the physiology underlying drought-induced mortality is essential for estimating forest sensitivity to drought (Christensen et al., 2013, Allen et al., 2015, Meir et al., 2015b). Although spatial variation in predicted rainfall patterns is substantial, current consensus suggests that precipitation extremes in the tropics, and especially in Amazonia, are likely to become more frequent, with extended dry seasons of particular note (Christensen et al., 2013, Fu et al., 2013, Reichstein et al., 2013, Boisier et al., 2015). These changed conditions will exert a selection pressure affecting the next generation of trees, but the persistence of the current generation depends on their capacity for acclimation or resilience in the face of climate change. Investigating the capacity of trees to cope with drought in tropical forests is consequently of paramount importance in estimating the magnitude of biosphere-atmosphere feedbacks.

Species differ in their ability to cope with water stress (da Costa et al., 2010, Meir et al., 2015a, Rowland et al., 2015b, Bartlett et al., 2012, Choat et al., 2012) and establishing exactly what traits account for this differential susceptibility is complex, particularly in species-diverse communities. Globally, there have been reports of drought-induced tree mortality with the implicated cause being either carbon starvation or hydraulic failure, or a mixture of the two (Hartmann et al., 2013, Anderegg et al., 2012, Galiano et al., 2012, McDowell, 2011, Anderegg et al., 2013, McDowell et al., 2013). For the tropics, however, there have been few studies, with initial suggestions hinting at a role for progressive carbon starvation (Metcalf et al., 2010) superseded by more recent evidence pointing towards hydraulic deterioration as a principal trigger for drought-induced mortality (Rowland et al., 2015a). Water stress in plants is commonly represented by the percent loss of hydraulic conductivity (PLC) of the xylem, whereby P50, the leaf or branch tissue water potential at 50 % PLC, is used as a metric of the drought resistance of a plant or species, with higher (less
negative) P50 indicating less resistance to the loss of conductivity through embolism. Attempts have been made to map P50 onto xylem anatomy, where wood density, conduit diameter and conduit wall thickness have been found to be weakly predictive of cavitation resistance (Hacke et al., 2001, Hajek et al., 2014, Gleason et al., 2015). Certain characteristics of leaves have also been shown to be associated with drought resistance in plants, e.g., turgor loss point (Bartlett et al., 2012) and the elastic modulus (Bowman and Roberts, 1985), but the mechanistic relationships between leaf anatomy and drought resistance remain poorly understood.

Distinct morphological characteristics of leaves occur in environments of especially low or high water availability, and are termed xeromorphic and hygromorphic, respectively. Xeromorphic leaves tend to be small in area, with a multi-layered epidermis, thick cuticle, compactly-arranged mesophyll with little air space, high stomatal density and high vein density (Maximov, 1929, Cutler et al., 1977). By contrast, hygromorphic leaves, tend to show the opposite features (Schimper, 1903, Roth, 1985). In certain species spanning gradients of rainfall, traits such as leaf area, thickness, specific leaf area, density, and stomatal morphology have been shown to vary with water availability (Geeske et al., 1994, Warren et al., 2005, Cunningham et al., 1999, McLean et al., 2014). Drought experiments have also shown reduced cell size in the mesophyll and epidermis, and increases in cell wall thickness, stomatal density, vein density and cuticle thickness in droughted vs non-droughted plants (Morton and Watson, 1948, Shields, 1950, Cutler et al., 1977, Maximov, 1929).

Most of the experimental research on the adaptations of leaf anatomy to water stress was conducted over half a century ago on mesophytic crop plants (Morton and Watson, 1948, Shields, 1950, Cutler et al., 1977, Maximov, 1929), but their relevance is current in the context of predicted changes in the hydrological regimes of tropical rainforests. Moreover, recent work has attempted to improve the mechanistic understanding of water movement from veins to stomata in leaves, by modelling the hydraulic pathway through cells, cell walls and as vapour through the internal airspaces (Buckley et al., 2015, Rockwell et al., Rockwell et al., 2014a, Buckley, 2015). Therefore, understanding the plastic response of leaves at the tissue level, may well be informative of the influence of cell structure to the hydraulic pathway, and how this effects water use at the leaf level. Thus, the question arises: can trees respond to long-term
reductions in water availability by producing leaves that exhibit more xeromorphic characteristics? Such a capacity for acclimation could confer a significant advantage for long-lived canopy tree species (Nicotra et al., 2010), and could be important for determining the sensitivity of a forest to drought and the difference in drought sensitivity among species. Existing studies addressing this question in natural communities are observational (Geeske et al., 1994, Cunningham et al., 1999, McLean et al., 2014), thus, here I test the plasticity of leaf morphology experimentally, in tropical rainforest trees.

This study uses a long-term (>12 years) through-fall exclusion (TFE) experiment in the lowland Amazon Rainforest (da Costa et al., 2010, Meir et al., 2015b) to address the following questions: (i) do trees respond to long-term imposed soil moisture deficit through changes in leaf structure or anatomy; and (ii) do differences in anatomy, or anatomical plasticity, explain contrasts in drought sensitivity among taxa? I also examine any further associations between leaf anatomy, water relations and gas exchange traits to drought using multivariate analyses.

The expectation was that xeromorphic traits should be found particularly under conditions where leaves have to cope with drought stress. In particular, higher stomatal and vein density, smaller cell size in the spongy and palisade mesophyll, and lower cavity volume were expected to occur in the experimentally-droughted forest and/or in individuals from drought-resistant genera. Additionally, to highlight potential links between anatomical and physiological properties of leaves, multivariate analyses were carried out combining leaf tissue properties with plant water relations traits and gas exchange parameters.

3.3 Methods

3.3.1 Study Site

The field work was conducted in the Caxiuanã National Forest Reserve in the eastern Amazon Rainforest (1°43’S, 51°27’W). The field site is situated in lowland terra firme rainforest approximately 10-15 m above river level, has a mean temperature of ca. 25 °C, receives 2000 – 2500 mm of rainfall annually and has a dry season in which rainfall is < 100 mm per month between June and November.
3.3.2 Large-scale through-fall exclusion experiment

The TFE experiment consists of a 1 ha plot from which approximately 50% of canopy through-fall has been excluded using plastic panels located 1-2 m above the ground since 2002. A 1 ha control plot, < 50 m from the TFE, which has received normal rainfall, was also studied. The plots are divided into 10 m x 10 m subplots, of which the outer-most subplots were excluded from the study to account for possible edge effects on tree growth. Further details on experimental setup and results can be found in Meir et al. (2009), Fisher et al (2007), Metcalfe et al. (2010), da Costa et al. (2010) and Rowland et al. (2015b).

3.3.3 Study specimens and drought vulnerability status

All measurements were taken from six genera common to both the TFE and the control plot of which Manilkara, Eschweilera and Pouteria have been classified ‘drought sensitive’ and Protium, Swartzia, and Licania as ‘drought resistant’, based on analysis of rates of drought-induced mortality (da Costa et al., 2010, Rowland et al., 2015b). These will be subsequently referred to as sensitive and resistant species. The sensitivity status of a genus is based on mortality in response to the imposed drought conditions. Where possible, a single species was used to represent a genus (Pouteria anomalata (Pires) T.D. Penn., Manilkara bidentata (A.DC.) A.Chev., Swartzia racemosa (Benth.)), but more than one species was used where there were too few individuals in a species per plot. So Eschweilera is represented by the species E. coriacea (DC.) S.A. Mori, E. grandiflora (Aubl.) Sandwith, and E. pedicellata (Rich) S.A. Mori, Licania by L. membranacea (Sagot ex Laness) and L.octandra (Kuntze) and Protium by P.tenuifolium Engl. and P. paniculatum Engl. This approach was necessary to obtain sufficient numbers of each genus per plot to enable a comparison of drought sensitivity groups, i.e., in order that drought-sensitive and -resistant taxa were represented by three genera in both plots and was used in two previous studies undertaken at the same site (Rowland et al., 2015b, Binks et al., 2016). In Binks et al. (2016) variance was consistently greater among genera than amongst individuals within genera. Similarly, in this study variance was also greater among, than within, genera in 13 out of 17 traits: the exceptions being leaf area, leaf mass per area and the proportional tissue thicknesses of the spongy mesophyll and abaxial epidermis (Table 3.1).
Table 3.1. Variance accounted for by separate components in the mixed models and the conditional and marginal $r^2$ of each model.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Fixed</th>
<th>Gn</th>
<th>ID</th>
<th>Residual</th>
<th>$r^2_{\text{conditional}}$</th>
<th>$r^2_{\text{marginal}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf thickness</td>
<td>5.8</td>
<td>58.3</td>
<td>29.9</td>
<td>6.0</td>
<td>0.94</td>
<td>0.06</td>
</tr>
<tr>
<td>Leaf area</td>
<td>2.0</td>
<td>32.8</td>
<td>33.9</td>
<td>31.3</td>
<td>0.69</td>
<td>0.02</td>
</tr>
<tr>
<td>Adaxial epidermis thickness (Ad)</td>
<td>1.8</td>
<td>71.5</td>
<td>&lt;0.1</td>
<td>26.7</td>
<td>0.73</td>
<td>0.02</td>
</tr>
<tr>
<td>Spongy mesophyll thickness (SM)</td>
<td>8.0</td>
<td>45.5</td>
<td>27.8</td>
<td>18.8</td>
<td>0.81</td>
<td>0.08</td>
</tr>
<tr>
<td>Palisade mesophyll thickness (Pal)</td>
<td>7.2</td>
<td>39.8</td>
<td>35.9</td>
<td>17.0</td>
<td>0.83</td>
<td>0.07</td>
</tr>
<tr>
<td>Internal cavity volume (CV)</td>
<td>9.0</td>
<td>55.2</td>
<td>29.9</td>
<td>5.9</td>
<td>0.94</td>
<td>0.09</td>
</tr>
<tr>
<td>Abaxial epidermis thickness</td>
<td>4.2</td>
<td>45.1</td>
<td>24.5</td>
<td>26.1</td>
<td>0.74</td>
<td>0.04</td>
</tr>
<tr>
<td>Proportional Ad thickness</td>
<td>16.7</td>
<td>63.1</td>
<td>7.2</td>
<td>13.0</td>
<td>0.87</td>
<td>0.17</td>
</tr>
<tr>
<td>Proportional SM thickness</td>
<td>19.0</td>
<td>26.2</td>
<td>33.0</td>
<td>21.8</td>
<td>0.78</td>
<td>0.19</td>
</tr>
<tr>
<td>Proportional Pal thickness</td>
<td>11.8</td>
<td>33.1</td>
<td>28.5</td>
<td>26.6</td>
<td>0.73</td>
<td>0.12</td>
</tr>
<tr>
<td>Proportional CV thickness</td>
<td>18.1</td>
<td>47.4</td>
<td>-</td>
<td>34.5</td>
<td>0.76</td>
<td>0.06</td>
</tr>
<tr>
<td>Proportional Ab thickness</td>
<td>14.3</td>
<td>15.7</td>
<td>40.2</td>
<td>29.8</td>
<td>0.70</td>
<td>0.14</td>
</tr>
<tr>
<td>Vein density</td>
<td>1.9</td>
<td>69.3</td>
<td>6.7</td>
<td>22.0</td>
<td>0.78</td>
<td>0.02</td>
</tr>
<tr>
<td>Stomatal density</td>
<td>3.8</td>
<td>39.9</td>
<td>38.6</td>
<td>17.8</td>
<td>0.82</td>
<td>0.04</td>
</tr>
<tr>
<td>Leaf mass per area</td>
<td>4.1</td>
<td>13.1</td>
<td>44.8</td>
<td>38.0</td>
<td>0.62</td>
<td>0.04</td>
</tr>
<tr>
<td>SM cell volume</td>
<td>23.8</td>
<td>20.5</td>
<td>14.7</td>
<td>40.9</td>
<td>0.59</td>
<td>0.24</td>
</tr>
<tr>
<td>Pal cell volume</td>
<td>20.3</td>
<td>49.0</td>
<td>10.1</td>
<td>20.6</td>
<td>0.79</td>
<td>0.20</td>
</tr>
</tbody>
</table>

For each of the variables, an attempt was made to measure at least two leaves per individual tree, and three individual trees per genus per plot which would have resulted in 18 leaves from nine individuals per drought sensitivity status, per plot. Unfortunately, it was not always possible to achieve this number of samples, due to difficulties obtaining suitable leaves and performing the analysis under field conditions. Moreover, peculiarities in some of the specimens made particular analyses difficult or impossible; for example, leaves from the genus *Manilkara* were densely packed with sclereids, which obscured the vasculature and made accurate analysis of vein density impossible under the conditions available. Therefore, the minimum and mean number of leaves per: genus, genus per plot, and drought sensitivity status per plot are detailed in Table S3.1 for each analysis. The minimum number of leaves per
sensitivity status per plot, which is the analytical unit, ranged from 7 (relative internal cavity volume) to 93 (leaf area and leaf mass per area), with a median of 13.

All leaves were taken from branches sampled at the top of dominant trees only and exposed to direct sunlight for a portion of the day; shaded leaves were not used in this analysis. After excision, the branches were transported in buckets of water back to the field station, and were recut under water filtered to 0.2 μm and allowed to rehydrate overnight. The leaves used in all analyses were fully expanded and mature. Experimental procedures were carried out in May and June in 2014 with the exception of pressure volume curves, which were also measured in October and November of 2013.

3.3.4 Lamina anatomy

Small squares of leaf of area > 0.5 cm$^2$ situated midway between the leaf tip and base, and midrib and margin of the leaf were sectioned using a hand-held microtome. The sections were photographed at a magnification of x40 in cases where this enabled the whole depth of the leaf to be observed in one image, and at x10 for thicker leaves. Images were analysed in ImageJ to obtain thickness values in microns for the adaxial (Ad) and abaxial (Ab) epidermis layers, the palisade mesophyll (Pal) and the spongy mesophyll (SM). The thickness of each tissue layer was also presented as a percentage of total leaf thickness (T, μm) and indicated by the subscript ‘prop’ e.g. Ad$_{prop}$.

Mean cell volume for the palisade and spongy mesophyll was calculated by assuming that palisade cells were cylinders and spongy mesophyll cells were spheres. Mean values for the length and width of palisade cells, and the width of spongy mesophyll cells, were determined from five cells per leaf section, and averaged between two leaf sections per individual tree.

3.3.5 Mesophyll cavity volume

Branches collected during the afternoon were covered and left to rehydrate overnight and specimen leaves were used only if adjacent leaves had a water potential higher than -0.2 MPa. Specimen leaves were then perfused with water for > 20 hours at a pressure of 18 kPa. The cavity volume (CV) was determined by subtracting the fresh leaf mass from the perfused leaf mass expressed as mm$^3$$_{air}$ space cm$^{-2}$ leaf surface.
3.3.6 Vein density

Small squares of leaf approximately 1 cm$^2$ in size taken from midway between the tip and base, and midrib and margin of the leaf were cleared using 5 % NaOH and briefly 10 % NaClO when necessary to remove the last of the colour (Scoffoni et al., 2010). The cleared leaf sections were then placed in a 1 % solution of toluidine blue for several seconds before being rinsed in water; this process was repeated until sufficient dye was judged to have infiltrated the sample and the veins were clearly visible. The samples were photographed using a Moticam 2 digital camera on a Motic B3 microscope (Motic, Canada). An objective lens of x10 magnification was used for most images, but x4 magnification was used where Student’s t-test revealed no significant difference ($P > 0.05$) in the vein densities calculated from either magnification. Vein density (VD, mm mm$^{-2}$) was derived by tracing and measuring vein length in a known area using ImageJ software (Schneider et al., 2012).

3.3.7 Stomatal density

Dental impression gel was used to cover a minimum area of 2 cm$^2$, situated midway between the tip and base, of the abaxial surface of four leaves per individual and the adaxial surface of one leaf per individual. Clear nail varnish was applied to the surface of the dental impressions, peeled off and photographed using a Leica DFC420 C camera mounted on a Leica DMLB 100S transmission light microscope (Leica, Germany). Two photos were taken per leaf impression, and stomatal density (SD, mm$^{-2}$) was derived by counting the number of stomata in an area of 0.1 – 0.15 mm$^3$ using ImageJ.

3.3.8 Area and LMA

Fresh leaves were scanned and the images analysed using ImageJ to find leaf area. Leaves were dried to constant mass in an oven at 70 °C for > 48 hours and weighed on a mass balance accurate to 0.1 mg.
3.3.9 Pressure volume analysis

Pressure volume (PV) curves were carried out as per the bench-drying protocol described in Tyree and Hammel (1972). Briefly, leaves were taken from branches that had been rehydrated by being allowed to stand overnight in a bucket of water filtered to 0.2 µm. The leaves were allowed to dehydrate over a period of 3 – 8 hours, during which time water potential and mass were measured repeatedly using a Scholander pressure bomb (PMS Instruments Co., Corvalis, OR, USA) and mass balance accurate to 0.1 mg, respectively. After the final set of measurements, leaves were scanned to enable the determination of area using ImageJ software (Schneider et al., 2012) and then dried at 70 °C in an oven for > 48 hours to find dry mass. The parameters osmotic potential at full turgor ($\Psi_{\pi}$, MPa), turgor loss point ($\Psi_{\pi}^{\text{tlp}}$, MPa), saturated water content (the ratio of water mass to leaf dry mass in a fully saturated leaf, SWC, g g$^{-1}$), relative water content at $\pi_{\text{tlp}}$ (RWC$^{\text{tlp}}$, %), modulus of elasticity ($\epsilon$, MPa) and hydraulic capacitance ($C$, mol MPa$^{-1}$ m$^{-2}$) were calculated as per (Sack and Pasquet-Kok, 2011). Differences in PV parameters across drought sensitivity status and plots are reported in a separate paper (Binks et al., 2016). Here, I present a correlation analysis including PV, leaf anatomy and gas exchange parameters in the Supporting Information.

3.3.10 Photosynthesis

For a detailed description of how the gas exchange parameters were measured, refer to Rowland et al. (2015b), in which these parameters were presented in the context of the experimental drought. Photosynthesis was measured on canopy top branches using LICOR 6400 portable photosynthesis systems (LI-COR, Lincoln, NE, USA). The parameters $V_{c\text{max}}$ (the maximum rate of rubisco carboxylation) and $J_{\text{max}}$ (the maximum rate of electron transport) were derived from A-C$_{i}$ curves performed under saturating photosynthetically active radiation (PAR), and data were temperature corrected to 25 °C following Sharkey et al. (2007). To measure dark respiration ($R_{\text{dark}}$) leaves were covered in tin foil for 30 minutes prior to, and during, gas exchange measurements, and these data were also temperature corrected to 25 °C according to Atkin & Tjoelker (2003).
3.3.11 Predawn water potentials

Water potential was measured in three leaves per individual tree, between 5.30 and 7.00 in the morning at the end of the dry season (October) in 2013. Values were averaged for each individual tree.

3.3.12 Analysis

The data were analysed using linear mixed effects models in the packages lmer and lmerTest in R (R Core Team, 2015). Because the study was focused on finding treatment and drought sensitivity effects, and not the responses of individual genera, genus was designated as a random effect, and tree individual was nested inside genus, where there were >2 individuals per genus per plot. This statistical design removes the variance attributable to individuals within genera, and between genera, in order to selectively find the influence of the fixed effects e.g. treatment and drought sensitivity status. Models were simplified by comparing their respective AIC (Table 2). The distributions of all of the variables were checked for normality using the Shapiro-Wilk test and either log or power transformed depending on the starting distribution. The powers employed for transformation were determined using a box-cox transformation function in the MASS package in R (Venables, 2002). The marginal and conditional $r^2$ were calculated according to Nakagawa & Shielzeth (2013).

All of the anatomy variables were tested for correlations with the parameters derived from the PV analysis, predawn water potential ($\Psi_{pd}$) and gas exchange (correlation matrix Table S3.2). The analysis was carried out using Pearson correlation analyses in the R package ‘psych’ (Revelle, 2015) and all variables were transformed as per the mixed effects models (Table 3.2).

A principal component analysis (PCA) was performed on the thicknesses of leaf tissues, vein and stomatal density and the gas exchange-derived parameters $V_{cmax}$, $J_{max}$ and $R_{dark}$ to highlight possible relationships between anatomy and photosynthesis. Absolute values of tissue thickness were used because of the relevance of distance, i.e. tissue thickness, to the molecular diffusion processes.
Table 3.2. Data transformations and final model structures used in analysis for the effect of treatment (T, control plot vs TFE) and drought-sensitivity status (S, sensitive or resistant) on tissue parameters, and gas exchange parameters used in PCA. The random effects were tree individual nested inside genera for all models with the exception of CV\textsubscript{prop}, for which tree individual was not used because of sample size limitations (see Table S1).

<table>
<thead>
<tr>
<th>Leaf properties</th>
<th>Response Variable</th>
<th>Symbol</th>
<th>Transformation</th>
<th>model structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>structural</td>
<td>Leaf area</td>
<td>A</td>
<td>log</td>
<td>T*S</td>
</tr>
<tr>
<td>properties</td>
<td>Leaf thickness</td>
<td>T</td>
<td>log</td>
<td>T*S</td>
</tr>
<tr>
<td></td>
<td>Leaf mass per area</td>
<td>LMA</td>
<td>log</td>
<td>T*S</td>
</tr>
<tr>
<td></td>
<td>Vein density</td>
<td>VD</td>
<td>y^2</td>
<td>T*S</td>
</tr>
<tr>
<td></td>
<td>Stomatal density</td>
<td>SD</td>
<td>-</td>
<td>T*S</td>
</tr>
<tr>
<td>Tissue properties</td>
<td>Spongy mesophyll</td>
<td>SM</td>
<td>-</td>
<td>T*S</td>
</tr>
<tr>
<td></td>
<td>thickness</td>
<td>Ad</td>
<td>log</td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>Adaxial epidermis</td>
<td>Ab</td>
<td>log</td>
<td>T*S</td>
</tr>
<tr>
<td></td>
<td>thickness</td>
<td>CV</td>
<td>-</td>
<td>T*S</td>
</tr>
<tr>
<td></td>
<td>Internal cavity volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Proportional SM</td>
<td>Sm\textsubscript{prop}</td>
<td>y^2</td>
<td>T+S</td>
</tr>
<tr>
<td></td>
<td>thickness</td>
<td>Ab\textsubscript{prop}</td>
<td>log</td>
<td>T*S</td>
</tr>
<tr>
<td></td>
<td>Proportional Ad</td>
<td>Ad\textsubscript{prop}</td>
<td>log</td>
<td>T*S</td>
</tr>
<tr>
<td></td>
<td>thickness</td>
<td>CV\textsubscript{prop}</td>
<td>√y</td>
<td>T*S</td>
</tr>
<tr>
<td></td>
<td>SM cell volume</td>
<td>SM\textsubscript{cell} volume</td>
<td>√y</td>
<td>T*S</td>
</tr>
<tr>
<td></td>
<td>Pal cell volume</td>
<td>Pal\textsubscript{cell} volume</td>
<td>log</td>
<td>T*S</td>
</tr>
<tr>
<td>Gas exchange parameters</td>
<td>Rubisco carboxylation</td>
<td>V\textsubscript{cmax}</td>
<td>-</td>
<td>PCA</td>
</tr>
<tr>
<td></td>
<td>Electron transport</td>
<td>J\textsubscript{max}</td>
<td>-</td>
<td>PCA</td>
</tr>
<tr>
<td></td>
<td>Dark respiration</td>
<td>R\textsubscript{dark}</td>
<td>-</td>
<td>PCA</td>
</tr>
</tbody>
</table>

3.4 Results

Leaf traits by genus are presented in Table 3.3. Leaf thickness varied from 78 to 370 \(\mu m\) with a combined mean and standard error of all genera and treatments of 187.6 ± 7.2 \(\mu m\) and a mean relative thickness with standard error of palisade, spongy
mesophyll, abaxial and adaxial epidermis of 29.5 ± 1.3, 52.9 ± 1.49, 8.1 ± 0.3 and 11.5 ± 0.9 % respectively (Fig 1).

**Table 3.3.** Mean value by genus of each of the leaf tissue parameters +/- one standard error.

<table>
<thead>
<tr>
<th></th>
<th>Drought Sensitive</th>
<th>Drought Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eschweilera</td>
<td>Manilkara</td>
</tr>
<tr>
<td>T (µm)</td>
<td>164.7 ± 4.7</td>
<td>247.2 ± 15.9</td>
</tr>
<tr>
<td>A (cm²)</td>
<td>69.5 ± 4</td>
<td>29.9 ± 1.5</td>
</tr>
<tr>
<td>Pal (µm)</td>
<td>35.9 ± 1.3</td>
<td>66.7 ± 8.1</td>
</tr>
<tr>
<td>SM (µm)</td>
<td>100.6 ± 4.7</td>
<td>150.5 ± 9.1</td>
</tr>
<tr>
<td>Ad (µm)</td>
<td>15.9 ± 0.8</td>
<td>13.1 ± 1.5</td>
</tr>
<tr>
<td>Ab (µm)</td>
<td>12.3 ± 0.6</td>
<td>15.4 ± 1.9</td>
</tr>
<tr>
<td>CV (mm³ cm⁻²)</td>
<td>2.1 ± 0.3</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>VD (mm mm⁻²)</td>
<td>8.4 ± 0.3</td>
<td>-</td>
</tr>
<tr>
<td>SD (mm⁻¹)</td>
<td>409.7 ± 12.2</td>
<td>317.3 ± 20.6</td>
</tr>
<tr>
<td>LMA (g m⁻²)</td>
<td>91.1 ± 1.7</td>
<td>125.8 ± 5.8</td>
</tr>
<tr>
<td>Pal_prop (%)</td>
<td>22.2 ± 1.1</td>
<td>26.3 ± 2.1</td>
</tr>
<tr>
<td>SM_prop (%)</td>
<td>60.6 ± 1.6</td>
<td>63.7 ± 2.2</td>
</tr>
<tr>
<td>Ab_prop (%)</td>
<td>7.6 ± 0.4</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>Ad_prop (%)</td>
<td>9.6 ± 0.4</td>
<td>4.9 ± 0.6</td>
</tr>
<tr>
<td>SM_cell_volume (µm³)</td>
<td>3456 ± 421</td>
<td>2614 ± 224</td>
</tr>
<tr>
<td>Pal_cell_volume (µm³)</td>
<td>1220 ± 106</td>
<td>3695 ± 263</td>
</tr>
</tbody>
</table>

Significant differences in response to treatment ($P < 0.05$) amongst all taxa combined were found for the absolute measure of cavity volume (TFE 2.43 ± 0.50 mm³ cm⁻², control 1.77 ± 0.30 mm³ cm⁻², Table 4) and the thickness of the adaxial epidermis (TFE 20.5 ± 1.5 µm, control 16.7 ± 1.0 µm, Table 3.4, Fig. 3.2). Total leaf thickness ($P = 0.070$), palisade thickness ($P = 0.086$), the proportional cavity volume ($P = 0.069$) and LMA ($P = 0.098$) were found to be marginally significant ($0.05 < P < 0.1$). None of the measured variables showed significant differences between the two drought sensitivity classes (Table 3.4). However, CV and palisade thickness showed significant interactions between treatment and sensitivity status ($P = 0.025$ and 0.034, respectively), whereby the treatment effect was stronger (i.e., values were lower in the control plot) amongst the resistant compared to the sensitive genera (Fig 3.3).
Figure 3.1. Absolute (a) and proportional (b) tissue thicknesses with standard error bars. Dark grey fraction of the spongy mesophyll bar represents the mean ‘thickness’ of the leaf cavity (total cavity volume / leaf area).

The variance accounted for by the fixed effects (See Table 3.2 for model structures) varied from 1.8 % for the adaxial epidermis to 23.8 % for the spongy mesophyll cell volume with a mean of 10.1 % over all of the variables (Table 3.1). In all but 4 out of 17 traits variance was higher among genera than within genera, averaging 42.7 % and 27.1 %, respectively, suggesting that the analysis of the effects was robust to pooling. Of the 4 traits where more variance occurs within a genus, leaf area, SM$_{\text{prop}}$, Ab$_{\text{prop}}$ and LMA, none were found to have significant treatment or drought sensitivity effects.

The correlation matrix of all the anatomical parameters, the PV parameters, the gas exchange parameters and predawn water potentials is given in Table S3.2. The
thickness of the spongy mesophyll and adaxial epidermis correlated with more parameters than the other tissue layers, suggesting that they were tightly associated with other leaf traits, although they appeared to operate antagonistically, i.e., Ad_{prop} and SM_{prop} were negatively correlated with each other, and had opposite associations with the other leaf traits. Both Ad and Ad_{prop} were positively correlated with \( \varepsilon \) and \( \Psi_{PD} \). The photosynthesis parameters correlated positively with both palisade and spongy mesophyll thickness and negatively with vein and stomatal density, but did not correlate with the thickness of the epidermal layers.

**Table 3.4.** Probability values of the fixed effects included in the mixed models listed in Table 1. Factors with a dash were not included in the final model and significant results at \( P < 0.05 \) are in bold.

| Variable  | Treatment | Drought sensitivity | Interaction
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>0.070 ·</td>
<td>0.421</td>
<td>0.181</td>
</tr>
<tr>
<td>A</td>
<td>0.666</td>
<td>0.826</td>
<td>0.834</td>
</tr>
<tr>
<td>Ad</td>
<td><strong>0.038</strong></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SM</td>
<td>0.543</td>
<td>0.488</td>
<td>0.757</td>
</tr>
<tr>
<td>Pal</td>
<td>0.086 ·</td>
<td>0.618</td>
<td><strong>0.034</strong></td>
</tr>
<tr>
<td>CV</td>
<td><strong>0.009</strong></td>
<td>0.565</td>
<td><strong>0.025</strong></td>
</tr>
<tr>
<td>Ab</td>
<td>0.195</td>
<td>0.893</td>
<td>0.611</td>
</tr>
<tr>
<td>Ad_{prop}</td>
<td>0.634</td>
<td>0.266</td>
<td>0.750</td>
</tr>
<tr>
<td>Sm_{prop}</td>
<td>0.786</td>
<td>0.417</td>
<td>0.147</td>
</tr>
<tr>
<td>Pal_{prop}</td>
<td>0.848</td>
<td>0.632</td>
<td>0.212</td>
</tr>
<tr>
<td>CV_{prop}</td>
<td>0.069 ·</td>
<td>0.832</td>
<td>0.054 ·</td>
</tr>
<tr>
<td>Ab_{prop}</td>
<td>0.972</td>
<td>0.173</td>
<td>0.832</td>
</tr>
<tr>
<td>VD</td>
<td>0.756</td>
<td>0.540</td>
<td>0.379</td>
</tr>
<tr>
<td>SD</td>
<td>0.797</td>
<td>0.638</td>
<td>0.470</td>
</tr>
<tr>
<td>LMA</td>
<td>0.098 ·</td>
<td>0.591</td>
<td>0.278</td>
</tr>
<tr>
<td>SM_{cell_volume}</td>
<td>0.914</td>
<td>0.083 ·</td>
<td>0.896</td>
</tr>
<tr>
<td>Pal_{cell_volume}</td>
<td>0.193</td>
<td>0.131</td>
<td>0.098 ·</td>
</tr>
</tbody>
</table>

In the PCA, combining anatomical and gas exchange traits, the first and second axes explained 57.6 % and 16.2 % of the variance, respectively (Fig. 3.4). Palisade thickness was grouped with \( J_{max} \) and \( V_{cmax} \) at high values of both axis 1 and 2, in the
opposite quadrant to R_{dark}. For the other parameters, there was a gradient of high vein and stomatal density, at low values of axis 1 and high values of axis 2, to high thickness of spongy mesophyll and epidermis in the opposite quadrant, which was orthogonal to the photosynthesis traits.

Figure 3.3. Interaction plots between drought sensitivity (grouped by genus) and treatment for (a) internal cavity volume and (b) palisade thickness. Plots show means +/- one standard error. The cavity volume shows significant treatment (P = 0.009) and interaction between treatment and drought sensitivity (P = 0.025) effects, while the palisade shows only significant interaction effects (P = 0.034).

3.5 Discussion

The results of this study reveal that little anatomical change occurred in response to the soil moisture deficit following 12 years of imposed through-fall exclusion. In addition, the drought sensitivity classification in these tropical forest trees, as determined by their increased mortality risk during drought stress, is not linked to specific leaf anatomy traits. I hypothesised that leaves would become more xeromorphic in character in response to the treatment, i.e., thicker, with smaller area, having lower internal cavity volume and higher stomatal and vein density. In fact, the only traits that did vary significantly in response to the treatment was the thickness of the adaxial epidermis (Fig. 3.2) and the cavity volume, the second of which, contrary to expectation, increased in response to the imposed drought (Fig. 3.3a).
Figure 3.4. First two axes of the PCA showing distribution of tree individuals based on absolute tissue thicknesses, vein and stomatal density, and the gas exchange parameters $V_{\text{c,max}}$, $J_{\text{max}}$ and $R_{\text{dark}}$. Each point represents an individual tree. The spongy mesophyll (SM) is a measure of the tissue thickness (volume per area) without the cavity volume.

3.5.1 Drought sensitivity status

There were no significant differences in leaf anatomy based on sensitivity status, implying that other aspects of plant physiology determine sensitivity to water stress. However, the interaction between sensitivity status and treatment for cavity volume and palisade thickness indicates a possible link between drought sensitivity and plasticity in these traits. Values of cavity volume were similar among sensitive and resistant genera in the TFE, whereas the values of the resistant genera in the control plot were lower than the overall mean (Fig 3.3a). In other words, the acclimation response to the drought stress brought the value for CV amongst the resistant taxa in the TFE in-line with the values of the sensitive taxa in the TFE, bringing into question any drought-related benefit of plasticity in this trait. Therefore, there is no strong evidence in our dataset to suggest that the leaves of drought-resistant species are consistently different from those of drought-sensitive species.
Despite the strong general relationship between palisade thickness and photosynthesis (Chabot and Chabot, 1977, Catoni et al., 2015, Smith et al., 1998, Hanba et al., 2002), and also observed in this data (Table S3.2), earlier work by Rowland et al. (2015b) on the same experiment demonstrated no effect of plot or drought sensitivity on photosynthetic capacity or leaf nitrogen content. Therefore, the palisade is significantly thicker for the resistant species on the TFE (with a marginally significant difference between plots, Fig 3.3b, Table 3.4), but this has not resulted in higher photosynthetic capacity as determined by maximum rates of Rubisco carboxylation ($V_{\text{cmax}}$) or electron transport ($J_{\text{max}}$) (Rowland et al., 2015b). Moreover, the lack of significant change in N content suggests that Rubisco content (or Rubisco activation) has not changed considerably between treatments. These findings demonstrate that, at least in this case, the change measured in palisade thickness is not related to the maximum photosynthetic capacity. However, at least one other experiment has shown that the palisade mesophyll thickness increased in response to water deficit (Boughalleb and Hajlaoui, 2011), suggesting that this trait may also influence water use within the leaf, or be influenced by water status during ontogeny.

3.5.2 The drought effect

The cause of the higher CV in the drought plot (Fig. 3.3a) is unknown as this is a feature of leaf morphology that has been explored little in the context of water stress, and the response of CV to long-term conditions of low VPD is not consistent among studies (Leuschner, 2002, Aliniaieifard et al., 2014). I speculate that a higher internal cavity volume may reduce internal vapour pressure, both because of the effect of the larger cavity (greater distance between adjacent cell walls) and/or because of longer apoplastic path lengths resulting in lower local water potentials, with the consequence of potentially increasing photosynthetic water use efficiency (Mediavilla et al., 2001). See Appendix I for a theoretical exploration of how the ratio of H$_2$O to CO$_2$ could be influenced by the structure of the mesophyll.

Several previous studies have linked thicker adaxial epidermis with drought resistance (Boughalleb and Hajlaoui, 2011, Bacelar et al., 2004), although the causes for this remain uncertain. The results of this study show that the adaxial epidermis is thicker in the TFE and correlates with predawn water potential (Table S3.2), while the results
of a previous study, on the same taxa and individuals, showed that the thickness of the adaxial epidermis correlates negatively with osmotic potential at full turgor ($\Psi^0$) and turgor loss point ($\Psi^\text{tlp}$), and positively with the elastic modulus ($\varepsilon$) (Binks et al. 2016, and also Table S3.2). If it is assumed that these correlations arose because turgor and/or osmotic properties in the Ad differ from those in other parts of the leaf, then the Ad would appear to be a particularly drought resistant tissue e.g. low $\Psi^\text{tlp}$ and $\Psi^0$, and high $\varepsilon$. Thus, a thicker Ad would be linked to higher drought resistance which may explain why it is significantly thicker in the drought plot (Table 3.4, Fig. 3.2).

3.5.3 Water deficit and leaf expansion during ontogeny

The Lockhart equation explains the mechanical relationship between turgor pressure and the rate of cell expansion ($E$) in which $E = m(\Psi_p - \Psi_p^{\text{min}})$ where $\Psi_p$ is turgor pressure, $\Psi_p^{\text{min}}$ is the threshold turgor pressure below which growth does not occur, and $m$ is the cell wall extensibility (Lockhart, 1965). Leaves will, therefore, be smaller if their expansion phase occurs during periods of water stress (Shields, 1950). Because vascular tissue and stomata are differentiated prior to expansion, reductions in leaf size effected during growth can be associated with increases in vein and stomatal density (Hsiao, 1973, Schoch et al., 1980, Carins Murphy et al., 2014). However, no differences were detected in leaf area, cell size, vein or stomatal density in response to the drought treatment, indicating that turgor pressure must not have dropped below the threshold minimum for long enough during the leaf expansion phase to influence these parameters in the mature leaves. All of the study species were evergreen but a partial leaf flush occurs at the beginning of the wet season (unpublished data) during which most leaves complete their growth. Thus, the wet season water supply on the TFE appears to be non-limiting to growth, which may be facilitated by reduced stomatal conductance to maintain adequately high cell turgor, and the reduction in leaf area index on the TFE (Metcalf et al., 2010).

One factor that is interesting to consider in relation to the effects of experimental drought is the potentially challenging aspect of separating mechanical effects of reduced turgor on growth, from the active expression of plastic traits that facilitate drought resistance i.e. passive versus active plasticity (Valladares et al., 2007). In the context of leaves, this leads to the question of whether the traits that emerge as a
consequence of expansion under sub-optimal turgor pressure e.g. smaller area, higher vein and stomatal density and smaller mesophyll cells, actually provide an adaptive advantage for drier conditions, or are simply the product of drought stress during ontogeny. The similarity between leaf traits that emerge in response to experimental drought (Cutler et al., 1977, Maximov, 1929), and those that differ along precipitation gradients (Geeske et al., 1994, Cunningham et al., 1999, Warren et al., 2005, McLean et al., 2014) presumably suggests the former: that these traits offer an adaptive advantage in coping with water stress.

3.5.4 Correlations between leaf anatomy, gas exchange, predawn water potential and pressure volume traits

The correlation matrix revealed that several leaf tissues appear to be associated with many water relations and gas exchange traits (Table S3.2), suggesting that these tissues are particularly representative of overall leaf physiology despite the limited treatment effects. These results must be interpreted with caution as the traits in this analysis are not independent; for example, leaf thickness is the sum of the thicknesses of the all tissues layers, and similarly $\Psi_{\pi}^{t_{lp}}$ is a function of $\Psi_{\pi}^{o}$ and $\varepsilon$, so these traits inevitably correlate. Having said that, the proportional thickness of the adaxial epidermis and the absolute thickness of the spongy mesophyll correlated with a larger number of traits than the other tissues, and always in opposite directions (Table S3.2). For example, SM correlates positively with $\Psi_{\pi}^{t_{lp}}$, SWC and RWC and negatively with SD, VD and $\Psi_{PD}$, while Ad$_{prop}$ shows the opposite relationships. Thus, leaves with thicker adaxial epidermis and thinner spongy mesophyll and, therefore, low $\Psi_{\pi}^{t_{lp}}$ and high $\Psi_{PD}$, should be associated with greater drought resistance.

A high value of the elastic modulus is generally associated with drought resilience because it results in a greater change in $\Psi$ for a given amount of water loss (Bowman and Roberts, 1985), thus increasing the potential gradient and the capacity for rehydration. The correlation matrix reveals a positive relationship between $\varepsilon$ and $\Psi_{PD}$ supporting this theory, suggesting that high elastic modulus could be advantageous for nocturnal drought stress recovery in these taxa. In a previous study (Binks et al., 2016), $\varepsilon$ was not found to vary significantly with drought sensitivity status but was significantly higher across all groups in the TFE than the control plot.
3.5.5 Principal component analysis

Given the association of the palisade mesophyll with photosynthesis, it is not surprising that it is clustered with \( J_{\text{max}} \) and \( V_{\text{cmax}} \) in the PCA (Fig. 3.4). However, it is surprising that stomatal and vein density are on a vector orthogonal to the photosynthesis traits, as in other studies they have been shown to correlate positively with photosynthesis (Walls, 2011, Brodribb et al., 2010, Muller et al., 2014, Zhang et al., 2014), while, in this dataset, they correlate negatively (Table S3.2). Acting in the opposite direction to VD and SD are the other leaf traits, SM, Ab, and Ad, which may suggest that increases in the thickness of these tissues can compensate for the functions of otherwise higher vein and stomatal density. Past research analysing the movement of dye from leaf veins into the surrounding tissue indicates that the epidermal layers play a role in lateral water transport (Wylie, 1943), which might explain the negative correlation between vein density and the epidermal layers (Fig. 3.3, Table S3.2).

3.5.6 Acclimation to drought

The level of acclimation detected in this study was lower than expected, suggesting a limitation to levels of plasticity in the measured traits in response to the experimental drought. The conditions that are thought to favour the selection for phenotypic plasticity are predictable variations in the environment within certain limits. If, in a given environment, a particular abiotic factor fluctuates very little, unpredictably, or to too extreme an extent for plastic responses to incur a significant increase in fitness, then phenotypic plasticity is not selected for in a population (Valladares et al., 2007). Thus, it is possible that the taxa in this study have limited capacity to acclimate to drought because of the historical stability or unpredictability in water availability. Other factors that may contribute to the limited response include the concept of ‘integrated phenotype’ where traits are so tightly interdependent that changes in one aspect of physiology impact, perhaps negatively, on other aspects (Valladares et al., 2007, Gianoli, 2001), or the effects of resource limitation inhibiting a plastic response (Van Kleunen and Fischer, 2005). Moreover, plants are rarely specialised to cope with more than one kind of abiotic stress (Niinemets and Valladares, 2006), so perhaps the same is true of plasticity in certain traits, and that the studied taxa show higher levels of plasticity in response to e.g. different levels of irradiance, which may be more advantageous to rainforest species.
Although the changes that were expected did not occur, e.g. higher stomatal and vein density, smaller cell size in the spongy and palisade mesophyll and lower cavity volume, other traits that have been found to arise in droughted plants, such as, cell wall and cuticle thickness (Shields, 1950, Schimper, 1903, Maximov, 1929) were not measured in this study. So it is possible that such changes did occur but were not detected. However, leaf thickness and LMA (or its inverse measure, specific leaf area) have been found to vary with water availability, and had marginally significant treatment effects (Table 3.4). Therefore, larger sample sizes might have revealed significant plot effects for these parameters.

3.5.7 Summary

Changing climate is likely to exert selection pressure on the next generation of forest trees, but as the climate change is so rapid, the persistence and vigour of the current generation will be dependent to some extent on their ability to resist or acclimate to the new conditions (Nicotra et al., 2010). The taxa in this study responded to the imposed drought via changes in aspects of leaf anatomy that were not known to influence drought resistance, whilst exhibiting none of the expected changes. This might indicate that the experimental drought is not severe enough to influence leaf anatomy in the way expected, or that the traits typically associated with drought resistance are tightly constrained by other aspects of plant physiology. In the latter case, the restricted capacity for acclimation is suggestive of high sensitivity of this forest to climate change. The extent to which leaf anatomy determines the capacity of plants to cope with changes in water availability could have wide-reaching implications in understanding the drought sensitivity of plants; yet it is the subject of little research (Morton and Watson, 1948, Shields, 1950, Cutler et al., 1977, Maximov, 1929) and has not been explored in an ecological context. Given that rainfall regimes are predicted to continue changing (Stocker, 2014), and that the tropics in particular are likely to undergo more frequent and severe dry seasons (Christensen et al., 2013, Reichstein et al., 2013, Boisier et al., 2015, Fu et al., 2013), an improved understanding of these subjects could be invaluable to future estimates of forest vulnerability.
3.6 Supporting Information

Table S3.1. The number of leaves used in the analysis for each variable. The minimum and mean number of leaves is shown ‘per genus’, ‘per genus per plot’, and ‘per drought sensitivity status per plot’. The genus represented by the minimum number of leaves is shown where Esch is *Eschweilera*, Prot is *Protium*, Pout is *Pouteria*, and Man is *Manilkara*. In the drought sensitivity per plot category, the minimally represented group is indicated where the control plot is A, the drought plot is B, drought resistant species are R and drought sensitive species are S. Thus, AS represents the drought sensitive species in the control plot.

<table>
<thead>
<tr>
<th>Variable</th>
<th>total</th>
<th>genus</th>
<th>mean</th>
<th>genus per plot</th>
<th>mean</th>
<th>drought sensitivity per plot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf area</td>
<td>400</td>
<td>57, Esch</td>
<td>67</td>
<td>23, Esch</td>
<td>33</td>
<td>93, AS</td>
</tr>
<tr>
<td>Leaf thickness</td>
<td>68</td>
<td>6, Prot</td>
<td>11</td>
<td>2, Prot</td>
<td>6</td>
<td>13, AR</td>
</tr>
<tr>
<td>Leaf mass per area</td>
<td>392</td>
<td>56, Esch</td>
<td>65</td>
<td>23, Esch</td>
<td>33</td>
<td>93, AS</td>
</tr>
<tr>
<td>Vein density</td>
<td>60</td>
<td>7, Pout (0, Man)</td>
<td>12</td>
<td>2, Lic</td>
<td>6</td>
<td>10, AS</td>
</tr>
<tr>
<td>Stomatal density</td>
<td>112</td>
<td>4, Lic</td>
<td>19</td>
<td>0, Lic</td>
<td>10</td>
<td>18, BR</td>
</tr>
<tr>
<td>Spongy mesophyll thickness</td>
<td>72</td>
<td>6, Prot</td>
<td>12</td>
<td>2, Prot</td>
<td>6</td>
<td>14, AR</td>
</tr>
<tr>
<td>Palisade mesophyll thickness</td>
<td>76</td>
<td>6, Prot</td>
<td>13</td>
<td>2, Prot</td>
<td>6</td>
<td>15, AR</td>
</tr>
<tr>
<td>Adaxial epidermis thickness</td>
<td>72</td>
<td>6, Prot</td>
<td>12</td>
<td>2, Prot</td>
<td>6</td>
<td>14, AR</td>
</tr>
<tr>
<td>Abaxial epidermis thickness</td>
<td>66</td>
<td>6, Prot, Man</td>
<td>11</td>
<td>2, Prot, Man</td>
<td>6</td>
<td>13, AR</td>
</tr>
<tr>
<td>Internal cavity volume</td>
<td>56</td>
<td>5, Pout</td>
<td>9</td>
<td>2, Pout</td>
<td>5</td>
<td>13, BR</td>
</tr>
<tr>
<td>Proportional SM thickness</td>
<td>61</td>
<td>4, Man</td>
<td>10</td>
<td>1, Man</td>
<td>5</td>
<td>12, AR</td>
</tr>
<tr>
<td>Proportional Pal thickness</td>
<td>61</td>
<td>4, Man</td>
<td>10</td>
<td>1, Man</td>
<td>5</td>
<td>12, AR</td>
</tr>
<tr>
<td>Proportional Ab thickness</td>
<td>61</td>
<td>4, Man</td>
<td>10</td>
<td>1, Man</td>
<td>5</td>
<td>12, AR</td>
</tr>
<tr>
<td>Proportional Ad thickness</td>
<td>61</td>
<td>4, Man</td>
<td>10</td>
<td>1, Man</td>
<td>5</td>
<td>12, AR</td>
</tr>
<tr>
<td>Proportional CV thickness</td>
<td>29</td>
<td>3, Prot</td>
<td>5</td>
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Table S3.2. Pearson correlation coefficients (r) above the diagonal, and probability values below the diagonal, derived from Pearson correlation analyses. No correction for multiple tests applied. P values < 0.05 and their associated r statistic are highlighted in bold and variables are arranged from the highest number of correlations in the top left, to the lowest in the bottom right. Symbols as defined in Table 1 with additional variables: absolute and proportional palisade mesophyll thickness (Pal and Pal_prop, respectively), osmotic potential at full turgor (Ψᵦ), turgor loss point (Ψ_tlp), saturated water content (SWC), elastic modulus (ε), hydraulic capacitance (C), relative water content at Ψₛₚ (RWCₚ), predawn water potential Ψₚ, electron transport driving regeneration of RuBP (Jₘₐₓ), the maximum rate of rubisco carboxylation (Vₖₐₚₙₐₓ), and dark respiration (Rₚₚₐ₅). The table starts on the following page.
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\end{array}\] | C    | -0.34 | 0.23 | -0.44 | 0.11 | 0.11 | 0.13 | -0.4 | 0.19 | -0.04 | 0.05 | -0.38 |
| Ab     | 0.01 | 0.05 | | -0.16 | 0.07 | -0.22 | -0.28 | 0.05 | 0.26 | 0.07 | 0.04 | 0.19 | 0.04 |
| \(\text{J}_{\text{max}}\) | 0.96 | 0.14 | 0.38 | -0.35 | 0.86 | 0.36 | 0.29 | -0.23 | 0.38 | -0.51 | 0.12 | -0.31 |
| SD     | 0.9 | 0.02 | 0.75 | 0.06 | -0.28 | -0.05 | -0.43 | 0.2 | -0.34 | -0.07 | -0.19 | -0.02 |
| \(V_{\text{cmax}}\) | 0.85 | 0.49 | 0.21 | 0 | 0.14 | | 0.28 | 0.27 | -0.12 | 0.28 | -0.5 | -0.02 | -0.17 |
| \(\text{Pal}_{\text{cell\_volume}}\) | 0.27 | 0.55 | 0.12 | 0.04 | 0.82 | 0.11 | 0 | -0.71 | -0.18 | -0.38 | -0.34 | -0.06 |
| LMA    | 0.1 | 0.46 | 0.8 | 0.09 | 0.04 | 0.12 | 1 | -0.09 | 0.47 | -0.3 | 0.28 | -0.36 |
| PD     | 0.02 | 0.01 | 0.14 | 0.14 | 0.3 | 0.46 | 0 | 0.61 | 0.25 | 0.23 | 0.39 | 0.22 |
| CV     | 0.12 | 0.31 | 0.73 | 0.03 | 0.12 | 0.13 | 0.39 | 0.01 | 0.17 | -0.13 | 0.89 | -0.22 |
| \(R_{\text{dark}}\) | 0.73 | 0.78 | 0.83 | 0 | 0.7 | 0 | 0.03 | 0.08 | 0.15 | 0.47 | 0.17 | 0.29 |
| \(C_{\text{prop}}\) | 0.11 | 0.78 | 0.35 | 0.52 | 0.43 | 0.9 | 0.1 | 0.14 | 0.04 | 0 | 0.39 | -0.11 |
| Area   | 0.94 | 0.02 | 0.86 | 0.07 | 0.92 | 0.32 | 0.76 | 0.03 | 0.21 | 0.24 | 0.09 | 0.58 | 0.16 |
3.7 References


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Chapter 4

Foliar water uptake in the Amazon

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OB designed the study, collected and analysed the data, and wrote the paper. OB, MM and PM designed the research. PM and ACLC conceived and implemented the experiment. LR and AARO and assisted data collection, LF enabled data collection, and SSV provided equipment. MM and PM helped with manuscript preparation.
4.1 Abstract

Foliar water uptake, the absorption of water directly through the leaf surface, has been found to occur in multiple biomes but has not been recorded in tropical rainforests. It is not known how common foliar uptake is in the tropics, but predictions of increased tree mortality in the Amazon, due to changes in rainfall frequency and longer dry seasons, have focused interest on the ability of trees to cope with altered precipitation regimes. This phenomenon allows trees to make use of occult precipitation such as fog and dew forming on leaf surfaces and also light precipitation, which would otherwise not contribute significantly to the forest water budget. Therefore, direct uptake of water through leaves increases the available water and may play a role in determining the sensitivity of forests to drought. This study addresses the following questions: 1) can six common Amazonian tree genera imbibe water directly through their leaf surfaces 2) does the capacity for foliar water uptake differ between taxa that are drought sensitive or resistant and 3) to what extent does foliar water uptake contribute to the forest water budget? The results demonstrate that all the study genera can take up water through their leaf surfaces, indicating that foliar water uptake may be a common strategy throughout the Amazon. There was conflicting evidence regarding the difference between drought-sensitive and drought-resistant taxa depending on whether the surface water was deposited as condensation or rain, although in the latter case foliar uptake was significantly higher in resistant taxa. Modelled estimates indicate that the amount of water taken up through this pathway could realistically account for between 2.2 and 4.4 % of water transpired annually. A rough preliminary calculation suggests that the carbon gain from the increase in hydration status due to foliar uptake could be approximately 0.34 petagrams. The evidence presented in this study suggests that foliar water uptake may be a common strategy throughout the Amazon and could be strongly influential of how trees respond to altered precipitation regimes in the future.
4.2 Introduction

Foliar water uptake from occult precipitation, events that wet the canopy but not the soil (Burgess and Dawson, 2004), could alleviate dry season drought stress in the rainforests of Amazônia, if it is a widely occurring strategy. The forests of the Amazon basin comprise a globally significant store of carbon (Saatchi et al., 2011) and, through the sequestration and emission of carbon dioxide, act as a powerful regulator of the global carbon cycle (Le Quere et al., 2013). During strong droughts, a decline or reversal of the biomass carbon sink has been observed, indicating a net regional emission of carbon dioxide, and underlining the strong sensitivity of these ecosystems to reductions in water availability (Gatti et al., 2014, Phillips et al., 2009, Lewis et al., 2011). Elevated tree mortality was considered the principal cause of the large reduction in carbon gain following the 2005 drought (Phillips et al., 2009) and the impact of drought-related mortality events globally has been noted (Allen et al., 2010, Allen et al., 2015, IPCC, 2013). Although the hydrology of Amazônia is spatially variable (Gloor et al., 2013), future drought stress is likely in several regions of the basin, principally through an increase in frequency and severity of the dry season, but also from a possible increase in future extreme events such as El Nino, and climatic warming via its impacts on vapour pressure deficit of the atmosphere (Boisier et al., 2015, Fu et al., 2013). Understanding the interaction between tree growth and mortality in response to precipitation inputs, particularly in the dry season, is consequently crucial for determining the vulnerability of the Amazon and predicting future climate trends.

The association of plants with the hydrological cycle is typically portrayed as the soil-plant-atmosphere-continuum, where water moves from the soil, through the plant, evaporates from the leaf surfaces, and precipitation from atmospheric moisture then replenishes soil water (Tyree et al., 2002). However, where vegetation cover is dense, the water from some precipitation events, such as dew and fog (so called ‘occult precipitation’), gets intercepted by foliage and most does not reach the soil. The more complex the vegetation structure, the higher is the surface capacitance of the vegetation, and consequently light and/or brief rainfall events may not reach the ground in tropical forests. In the classical view, occult precipitation events do not contribute directly to plant water status, but there is mounting evidence that water
uptake by leaves, or foliar uptake, plays a significant role in diverse ecosystems. Foliar uptake has been found to occur in desert ecosystems (Yan et al., 2015), savanna (Oliveira et al., 2005), the Mediterranean (Fernandez et al., 2014, Gouvra and Grammatikopoulos, 2003), temperate forests (Anderegg et al., 2013, Boucher et al., 1995, Stone, 1957, McDowell et al., 2008, Simonin et al., 2009), tropical montane (Goldsmith et al., 2013) and subtropical (Eller et al., 2013) cloud forest, and has been reported in conifers (Limm et al., 2009), broadleaf trees (Fernandez et al., 2014) and herbaceous vegetation (Gouvra and Grammatikopoulos, 2003). However, I found no studies reporting foliar uptake in tropical rainforest trees.

Drought events severe enough to cause elevated tree mortality and a net loss of carbon from the Amazon occurred in 2005 (Phillips et al., 2010) and 2010 (Lewis et al., 2011). Although reduced photosynthesis during drought leading to insufficient supply of assimilates to metabolism (‘carbon starvation’) is thought to contribute to drought-induced mortality (Galiano et al., 2012, McDowell et al., 2013, McDowell et al., 2011), recent experimental evidence suggests that hydraulic deterioration is the principal trigger for elevated tree mortality during drought in the Amazon rainforest (Rowland et al., 2015a). Hydraulic vulnerability can be represented by the hydraulic safety margin (HSM), i.e. the difference between a ‘typical’ level of drought stress, e.g., the midday leaf water potential, and the water potential that precipitates hydraulic failure or, more practically, 50 % loss of hydraulic conductance (P50). In a global analysis, 70 % of 226 species had HSMs < 1 MPa (Choat et al., 2012), while the mean and standard error for tropical rainforest species was 0.39 ± 0.15 MPa (data taken from Choat et al. 2012 supplementary information), suggesting a high sensitivity of tropical rainforests to water stress. However, the occurrence of foliar uptake could lead to canopy water potentials that exceed the theoretical maximum values for a given height and soil water potential, and also enhance the capacity for hydraulic repair, i.e., the refilling of embolised conduits. Consequently, plants capable of foliar water uptake could be less drought sensitive than their HSM suggests, and using a standard calculation of HSM may lead to an overestimate of the drought-sensitivity of communities in which foliar uptake is common. Establishing the extent to which vulnerable plant communities can take up water through their foliage is, therefore, of paramount importance.
In this study, I measure the capacity of six common genera (ter Steege et al., 2013) from lowland Amazon rainforest to absorb water through their leaves and, using a long-term (>12 years) through-fall exclusion experiment, examine the extent to which foliar uptake shows plasticity to water availability, and estimate the possible contribution of foliar water uptake to the forest water budget. I address the following questions:

1. Can leaves from Amazonian upper canopy trees take up surface water through their epidermis?

2. Does foliar uptake occur under natural (field) conditions?

3. Does the capacity for foliar uptake change in response to artificially imposed soil moisture deficit, and does it differ between taxa that are drought sensitive or resistant?

4. How much can foliar water uptake contribute to the forest water budget?

In summary, foliar water uptake has been demonstrated in many different biomes but has apparently not been examined in tropical rainforests. It is important to understand the sensitivity of the Amazon to predicted changes in precipitation regime because of the role it plays in regulating the global carbon cycle. However, without knowing if foliar uptake occurs in the Amazon, or how common it is, it is not possible to determine the sensitivity of the Amazon to climate change because the contribution of occult precipitation to dry season water status, and the significance of the HSM to represent hydraulic vulnerability, is unknown. I examine the phenomenon of foliar uptake in six common Amazonian genera, assess the extent to which foliar uptake is influenced by water availability, and generate an estimate of how much dry season occult precipitation could contribute to the Amazon water budget based on a simple model and meteorological data.

4.3 Methods

The experiments were conducted in the Caxiuanã National Forest, in the state of Para, Brazil, at the site of a long-term drought experiment (Meir et al., 2015). At this site, plastic panels were installed at a height of 1-2 m above ground level, diverting 50% of through-fall precipitation from one hectare of rainforest, to a gentle down-hill slope.
more than 50 m away. The site comprises a through-fall exclusion plot (TFE) and a
control plot which is close to the TFE and has similar species composition. See
methods section in Chapters 1 and 2 for a more detailed description of the site and
drought experiment.

4.3.1 Study specimens and drought vulnerability status

All measurements were taken from six genera common to both the TFE and the control
plot of which *Manilkara*, *Eschweilera* and *Pouteria* have been classified ‘drought
sensitive’ and *Protium*, *Swartzia*, and *Licania* as ‘drought resistant’, based on analysis
of rates of drought-induced mortality (Meir et al., 2015, da Costa et al., 2010, Rowland
et al., 2015b). These will be subsequently referred to as sensitive and resistant species.
The sensitivity status of a genus is based on mortality in response to the imposed
drought conditions. Where possible, a single species was used to represent a genus
*Swartzia racemosa* (Benth.)), but more than one species was used where there were
too few individuals in a species per plot. So *Eschweilera* is represented by the species
*E. coriacea* (DC.) S.A.Mori, *E. grandiflora* (Aubl.) Sandwith, and *E. pedicellata*
(Rich) S.A.Mori, *Licania* by *L. membranacea* (Sagot ex Laness) and *L.octandra*
(Kuntze) and *Protium* by *P.tenuifolium* Engl. and *P. paniculatum* Engl. This approach
was necessary to obtain sufficient numbers of each genus per plot to enable a
comparison, and has been used elsewhere (Butt et al., 2008, van Mantgem et al., 2009).
Binks et al. (2016) demonstrated for traits derived from the pressure volume analysis
of leaves that the largest trait variance is primarily found within individual trees and
across genera, rather than within genera (Table S2.1), and this trend is also consistent
with 13 out of 17 leaf anatomy traits measured in Chapter 3 (Table 3.1), reinforcing
the approach adopted here.

4.3.2 Artificial rainfall experiment

Leaves were collected between 11.30 and 12.30 in the afternoon and transported from
the field into the laboratory in a sealed plastic bag that had been blown into to reduce
further water loss. Leaf water potential was taken (Ψ) using a Scholander pressure
chamber (PMS Instruments Co., Corvallis, OR, USA), after which the open end of the
petiole was sealed using cyanoacrylate adhesive (superglue) to prevent non-lamina
water uptake, and the leaf was weighed \((W_i)\). Leaves were supported in a horizontal position by inserting the petiole into a small section of silicon tubing (approximately 20 mm long) which, in turn, was fastened to a freestanding wooden post. Leaves were spirally arranged with an angle of approximately 25° between adjacent leaves to prevent overlap and shading from the experimental ‘rain’ treatment. An even and constant flow of rain was produced by drilling holes 0.8 mm in diameter at 20 mm intervals in the bottom of a bucket whose basal diameter exceeded the diameter of the circle described by the tips of the leaves. The bucket was continually supplied with water throughout the experiment to maintain a minimum hydrostatic head of 400 mm, in order to maintain a constant flow rate. Leaves were subjected to 1 hour of rain in shaded conditions at ambient temperature (26 – 28 °C). Following the rain event the leaves were immediately patted dry with paper towels and placed in sealed plastic bags. The glued tip of the petiole was removed before measuring the final water potential \((\Psi_f)\) and reweighing \((W_f)\) the leaf together with the removed part of the petiole. All leaves were measured for area and dry mass.

### 4.3.3 Humidity and condensation experiment

Leaves were collected as in the artificial rainfall experiment, and their water potential and mass were measured before being put into a sealed chamber with over 98 % relative humidity. Water potential and mass were taken again after 6 and 19 hours in the chamber. The humidity chamber consisted of a sealed plastic box with a depth of approximately 50 mm of water in the bottom, with a mat supported 20 mm above the surface of the water and a damp towel supported 100 mm over the mat. The lid of the box was tightly fitting but was further sealed using cling wrap to avoid gas exchange between the internal and external environments. To calculate actual vapour pressure, thermocouples (copper-constantan type T) were fixed to the mat, the water surface, the underside of the damp towel, the adaxial side of 4 leaves (1 from each species) and the outside of the box, and the average temperature over ten minute intervals was recorded. If the interior of the box reached 100 % RH the temperature of the water surface, mat, leaves and towel would be identical as net evaporation equalled net condensation. However, due to a diurnal temperature cycle of approximately 2.5 °C (mean range ± standard error 25.00 ± 0.10 to 27.46 ± 0.08) outside the chamber, and the difference in specific heat capacity between the open water, the damp towel and the air, there was
usually a temperature difference between the water source (i.e. the open water or the damp towel) and the leaves, indicating an RH < 100 %. The humidity was calculated using wet bulb psychrometric equations whereby the coolest water source (either the water surface or the damp towel) was the wet bulb temperature and the mat on which the leaves were laying was taken as dry bulb temperature. Leaf temperature was always between the temperature of the water surface and the damp towel, therefore creating the possibility of condensation on the leaf surface.

4.3.4 Sap flux

Sap flow sensors (ICT SFM1, ICT International, Australia) were installed in three positions in the canopy of a single tree (*Manilkara bidentata*) adjacent to the canopy tower, in the El Niño dry season of 2015. Two sensors were installed in the upper canopy at approximately 36 m height, one in a branch of diameter 17.5 mm and the other, upstream from the same branch, in a branch of diameter 50.8 mm. The final sensor was installed in the middle canopy, at approximately 32 m in height, in a branch of diameter 16 mm. The data were collected after one week, when the small branches in the upper and middle canopy were cut off and bagged, with the sap flow sensor still attached for a further 18 hours, to enable a zero reference. The bigger branch in the upper canopy was left on the tree, but holes were drilled above and below the sensors to stop sap flux and enable a zero measurement.

4.3.5 Dew collection

Dew was collected using filter papers, 47 mm in diameter, which were weighed on a balance accurate to 0.0001 grams. In order to expose both upper and lower surfaces, the filter papers were inserted onto wires which were fastened vertically onto a board. These experiments were conducted over three nights, each night using a different experimental protocol for the purpose of establishing the best experimental design for future experiments. In the first experiment, on 27/10/15, filter papers were placed at the top of a canopy tower (42 m) and weighed every 2 hours from 20.00 to 06.00 (*n* = 8). In the second experiment (6/11/15), filter papers were placed at two positions on the canopy tower, top (42 m) and in the mid canopy (38 m). At 01.00, and at 03.00, 04.30 and 06.00 five papers were taken from each level, sealed in a plastic bag and taken back to the lab for weighing. In the third experiment (10/11/15), papers were
placed at the same two positons in the canopy tower as they were in experiment 2, but were weighed \textit{in situ} at 00.00, 02.30, 04.00 and 05.30 (\( n = 10 \) per level). In all experiments the filter papers were allowed to equilibrate with atmospheric moisture before the first measurement for at least 1 hour. The use of different protocols in the experiments means that the results cannot be pooled and interpreted as a single dataset.

4.3.6 \textit{Dew formation}

To detect dew, leaf temperature and canopy air temperature and humidity were measured. Thermocouples (copper-constantan type T) were attached to the adaxial surface of four leaves in the upper canopy (36 m) and four leaves in the middle canopy (32 m) using clear silicon sealant. Temperature and relative humidity sensors (HMP45C, Campbell Scientific) were installed on an adjacent tower in radiation shields, one in the upper canopy and one in the mid-canopy, less than 1 m above the leaves that were being measured. The data were logged every 15 minutes (model CR1000, Campbell Scientific) for a duration of eight days.

Dew forms when the temperature of a surface drops below the dew point temperature of the ambient air. Dew point temperature was calculated according to the relationship:

\[ T_{dp} = \frac{237.5}{17.27} \left( \ln \left( \frac{P_{\text{vap}}}{0.6107} \right)^{-1} - 17.27^{-1} \right) \]  

where \( T_{dp} \) is the dew point temperature and \( P_{\text{vap}} \) is the actual water vapour pressure (Monteith and Unsworth, 2013).

4.3.7 \textit{Calculating canopy foliar water uptake (}\( U_c \)\textit{)}

The total water uptake of the canopy \( U_c \) (g H\(_2\)O m\(^{-2}\) ground area) is calculated by the relationship

\[ U_c = K_{\text{cuticle}} (\Psi_{\text{surface}} - \Psi_{\text{canopy}}) P_L L t \]  

where \( K_{\text{cuticle}} \) is the conductance of the leaf cuticle to water (4.47x10\(^{-3}\) ± 5.51x10\(^{-4}\) g m\(^{-2}\) MPa\(^{-1}\) s\(^{-1}\), mean and standard error of all taxa) as determined by lamina rehydration experiments (see Supporting Information, Section 4.7), \( \Psi_{\text{canopy}} \) and \( \Psi_{\text{surface}} \) are the mean
water potential of the canopy and of the surface water (assumed to be 0, i.e. have negligible solute concentration), respectively (MPa), \( P_L \) and \( L \) are the proportion leaf area index taking up water and the leaf area index \( \left( \text{m}^2_{\text{leaf area}} \text{m}^{-2}_{\text{ground area}} \right) \), and \( t \) is time \((\text{s})\) during which uptake occurs. In this study, \( K_{\text{cuticle}} \) represents water taken up by the whole leaf surface area (e.g. both sides) normalised by one-sided leaf area. This was to avoid confusion when comparing values with other leaf area normalised traits e.g. leaf hydraulic conductance.

It is more meaningful to express water uptake in the same units as precipitation (i.e., mm), therefore the conversion factor 0.001 is included as \( 1 \text{ g m}^{-2} = 1 \text{ cm}^3 \text{ m}^{-2} = 0.001 \text{ mm}^3 \text{ mm}^{-2} \). To convert it to an annual value I substituted \( t \) for \( P_p t_y \), where \( P_p \) is the proportion of the year in which precipitation is occurring (unitless) and \( t_y \) \((\text{s yr}^{-1})\) is the number of seconds in a year. Meteorological data has been recorded every 30 minutes since 2001, from a through-canopy walk-up tower in the control plot, providing rainfall data for the canopy uptake model. Therefore, \( P_p \) is obtained by dividing the total number of time intervals in a year in which rain was recorded, over the total number of time intervals annually. Thus the equation for foliar uptake on the basis of the time fraction of precipitation \( U_{c,a} \) \((\text{mmH}_2\text{O m}^{-2}_{\text{ground area}} \text{yr}^{-1})\) becomes:

\[
U_{c,a} = -0.001 K_{\text{cuticle}} \Psi_{\text{canopy}} P_L L P_p t_y \tag{3}
\]

Water potential alters cyclically diurnally and annually so for a more temporally explicit estimate of \( U_{c,a} \), \( \Psi \) cycles were represented thus:

\[
\Psi_{\text{canopy}} = \left( A_D \sin \frac{2\pi t}{\lambda_D} + A_S \sin \frac{2\pi t}{\lambda_S} \right) - 0.9 \tag{4}
\]

Where \( A \) is the amplitude \((1/2 \ (\Psi_{\max} - \Psi_{\min})\) and \( \lambda \) the wavelength (cycle length), -0.9 is the annual mean, and the subscripts denote diurnal (D) and seasonal (S) variation. The model used values based on data from the control plot (unpublished) including water potential with a diurnal range of 1.1 and an annual range (seasonal shift) of 0.5, where at the end of the wet season \( \Psi_{\max} \) is -0.1 and \( \Psi_{\min} \) is -1.2, and at the end of the dry season \( \Psi_{\max} \) is -0.6 and \( \Psi_{\min} \) is -1.7, and diurnal minimum and maximum occur at midnight and midday, respectively. For estimating canopy uptake in the TFE, the water potential parameters were given a diurnal and seasonal range of 1.2 and 1
respectively, which resulted in $\Psi_{\text{max}}$ of -0.1 and $\Psi_{\text{min}}$ of -1.3 in wet season, and $\Psi_{\text{max}}$ of -1.1 and $\Psi_{\text{min}}$ of -2.3 in the dry season.

The proportion of the canopy that gets wet will depend on the intensity and duration of the precipitation event. Thus, $P_L$ (the proportion of the leaf area index that is wet) will be 0 immediately prior to the precipitation event and increase throughout, only becoming 1 when all leaf surfaces of all leaves are covered with a film of water. The leaf area index in the study forest is approximately 5.5 $m^2 m^{-2}$ (Metcalfe et al., 2010b) and it is likely, with such a structurally complex canopy, that $P_L$ rarely becomes 1 and, moreover, the average $P_L$ during a rain event can never be 1 because it always starts from 0. Therefore, in each of the modelled scenarios, $P_L$ is given a constant value of 0.5 for rainfall events resulting in a wet leaf area index of 2.75 $m^2 m^{-2}$.

4.3.8 Canopy foliar water uptake scenarios

Five different scenarios were modelled which are shown in Table 4.1. The factors that differed between models were the duration of dew input per night in the dry season (0, 5, and 10 hours), the amount of leaf area index that was covered by dew (0, 0.5, and 1 $m^2 m^{-2}$), and the cuticular conductance, which was calculated using hydraulic capacitance derived from either the pressure volume analysis (Chapter 2), or from the humidity experiment (Fig. S4.1). Dew was only modelled to occur at night in the six month dry season from June to November.

Scenario 1, with no dew input, was used as the baseline to compare the effect of dew in the other scenarios. Scenarios 2 and 3 demonstrate the effect of the nightly duration of dewfall varying between 5 and 10 hours, with the scenario 3 representing the maximum capacity for canopy foliar water uptake. Conservative conditions were presented in scenarios 4 and 5 in which the effect of cuticular conductance was compared, whilst scenario 5 is suggested to be the most realistic of the scenarios, albeit with a conservative value for dew duration. Each of the scenarios is also presented using the water potential data from the TFE.

4.3.9 Scaling up the findings of the canopy foliar uptake model

The estimate of canopy foliar uptake in scenario 5 (Table 4.1) was used to calculate the total mass of water taken up by the whole Amazon, based on an area of 5.5*10^6
km$^2$ and assuming that the estimate from this study represents the Amazon mean, given that the taxa used here are common in the Amazon (ter Steege et al., 2013). Using the Amazon water use efficiency figure from Zeri et al. (2014), the carbon gain, i.e. the extra carbon assimilated due the increased water status arising from foliar water uptake, was also calculated using the same assumptions stated above. The value for water use efficiency (WUE) was calculated as the ratio of the gross primary productivity (GPP) to the total evapotranspiration (ET) i.e. WUE = GPP/ET from eddy covariance data from a site in the southwestern Amazon, Brazil, state of Rondonia.

Table 4.1. Annual modelled foliar water uptake and standard errors for 5 different scenarios based on 13 years of meteorological data from 2001 to 2014. $K$ represents the experiments from which hydraulic capacitance was derived to calculate cuticular conductance.

<table>
<thead>
<tr>
<th>Scenario</th>
<th>13 yr mean (2001-2014)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>$K$</td>
</tr>
<tr>
<td></td>
<td>m$^{-2}$</td>
</tr>
<tr>
<td>1</td>
<td>PV</td>
</tr>
<tr>
<td>2</td>
<td>PV</td>
</tr>
<tr>
<td>3</td>
<td>PV</td>
</tr>
<tr>
<td>4</td>
<td>HE</td>
</tr>
<tr>
<td>5</td>
<td>PV</td>
</tr>
</tbody>
</table>

$K$ PV = 0.004471 g m$^{-2}$ MPa$^{-1}$ s$^{-1}$ derived using capacitance from pressure volume analysis. $K$ HE = 0.002640 g m$^{-2}$ MPa$^{-1}$ s$^{-1}$ derived using capacitance from humidity experiment (Fig. S4.1). Dew was only applied for six months during the dry season.

4.3.10 Data analysis

In both the artificial rainfall experiment and the high humidity experiment, paired Wilcoxon tests were used to test for a significant increase in water potential following leaf wetting. In the humidity experiment, the comparison was only performed on the data for pre-treatment water potentials and the water potentials after 16 hrs exposure to the treatment i.e. the values for 5 hrs exposure were not included in this analysis in order to avoid the proportionally higher measurement error (due to the smaller effect).

Linear models were used to analyse the effect of genera on the capacity for foliar uptake (change in water potential) in response to the rain and humidity experiments.
Linear mixed models were used to analyse the effects of drought treatment (control plot and TFE) and drought-sensitivity status on foliar uptake, using tree individual nested inside genus as the random effect. Mass change of the leaves, which is required to calculate conductance ($K$), was an unreliable indicator of foliar water uptake in both the artificial rainfall and the high humidity experiment (see explanation in Section S4.7). Instead of a comparison of conductance, therefore, $\Delta\Psi/\Psi$ was used, which is a constant for a given time period ($dt$), capacitance ($C$) and $K$ (see Section S4.8 for detailed explanation), as per the following equation:

$$\Delta\Psi/\Psi = -\frac{dtK}{C}$$

(5)

Capacitance changes little between full hydration and turgor loss point (Tyree and Hammel, 1972), the time component for each experiment was constant, and $K_{cuticle}$ is expected to remain constant, independent of leaf water potential (See Section S4.8, Fig. S4.3), and therefore, $\Delta\Psi/\Psi$ is linearly proportional to conductance. No significant differences were found between treatment (TFE or control plot) or drought sensitivity status (resistant or sensitive) for capacitance derived from the pressure volume analysis, and nor was there an interaction between treatment and sensitivity (Chapter 2). This means that significant differences occurring in $\Delta\Psi/\Psi$ between treatment and drought-sensitivity represent differences in conductance, $K$.

4.4 Results

4.4.1 Foliar water uptake

Water taken up through leaves in a 1 hr artificial rainfall experiment significantly increased water potential from $-1.31 \pm 0.06$ to $-0.68 \pm 0.04$ MPa, mean plus or minus standard error, $P << 0.001$ (Fig. 4.1). Leaves placed in an environment of $> 98$ % relative humidity for 16 hrs significantly increased water potential from $-1.25 \pm 0.06$ to $-0.65 \pm 0.03$ MPa, mean plus or minus standard error, $P << 0.001$ (Fig. 4.2a). Fresh mass per area also increased significantly in the humidity experiment, $P << 0.001$ (Fig. 4.2b). Using a linear model to analyse the interaction between treatment effect (water potentials before and after wetting) and genus, the humidity experiment resulted in significant increases in water potentials for all genera (Fig. 4.3), while the rainfall
experiment resulted in significant increases in water potentials for all genera except *Licania*. Thus, both experiments demonstrate the occurrence of foliar uptake.

**Figure 4.1.** Water potentials of detached leaves collected at midday before and after being in ‘rain’ for one hour. Water potential is significantly higher in Post-rain leaves ($P < 0.001$, one-tailed, paired Wilcoxon test).

**Figure 4.2.** Water potentials a), and change in fresh mass per area b), of detached leaves collected at midday and put into an atmosphere of > 98 % relative humidity. Dashed line in plot b) shows 0 change in mass. One outlying point with a dMass/Area of < -50 in the 16 hrs category not displayed in plot b). Water potential was significantly higher after both 5 hours and 16 hours of the treatment ($P < 0.001$), fresh mass per area was also significantly higher after 5 hours ($P = 0.041$) and 16 hours treatment ($P << 0.001$) according to one-tailed, paired Wilcoxon tests.
4.4.2 Sap flux measurements

The sap flux measurements showed the occurrence of reverse sap flow in a terminal branch of *Manilkara bidentata* in the upper canopy during 6 nights out of the 7 nights recorded (Fig. 4.4a), indicating that water was being taken up by the leaves in situ. Reverse sap flow was not detected in the large branch in the upper canopy (with the possible exception of the fifth night after installation where one point only gave a negative reading, Fig. 4.4b), or in the small branch in the mid-canopy. The sap flux meter in the third branch stopped logging data during the calibration preventing the use of that data. From an average of five nights, the reverse flow tended to occur between the hours of 21.00 and 07.00, with the peak flow happening around 06.00, just before sunrise (Fig. 4.5). During this time there was no rain, but on two nights 0.11 and 0.02 mm of dew were collected in the dew collection experiments. 0.05 mm of dew was also collected over one night prior to the installation of the sap flux equipment. Therefore, dew formation occurred on all occasions that it was collected.
and, despite the sparsity of data, it is likely that dew formation is a common nightly occurrence, at least during the dry season.

4.4.3  *Dew formation*

Despite the positive results of the dew collection experiments, the meteorological data indicate that conditions suitable for dew formation did not occur over the duration of the experiment i.e. leaf temperature never dropped below dew point temperature during the period of these measurements (Fig. 4.6). Unfortunately, this means that there must have been an equipment fault, the most plausible explanation being calibration error in the humidity sensor. Therefore, it was not possible to analyse the relationship between expected dew formation and reverse sap flow.

4.4.4  *Drought sensitivity*

In the rainfall experiment, significant differences in the relative change in water potential, $\Delta \Psi/\Psi$, were found as a function of the genus drought-sensitivity status ($P = 0.005$) and the interaction between genus drought sensitivity and drought treatment ($P = 0.035$, Fig. 4.7). The humidity experiment also showed an interaction between drought-sensitivity status and drought treatment ($P = 0.008$, Fig. 4.8). The response to foliar wetting was opposite between drought resistant and sensitive taxa whereby the resistant taxa absorbed more water in the control plot while the sensitive taxa absorbed more in the TFE (Fig. 4.8).
Figure 4.4. Sap flux of two branches over a period of seven days. Plot a) shows a small branch, diameter 17.2 mm, and plot b) shows a larger branch, diameter 50.8 mm, upstream (closer to the soil) of the branch in plot a). The final 18 hrs of both plots show the calibration, sap flux = 0, when the branch was removed from the tree and bagged. Negative sap flux occurring at night indicates foliar water uptake from dew.
Figure 4.5. The five nights of reverse sap flux that occurred in the small branch (Fig. 4.4a), over-laid on a single time plot, to indicate the typical duration of, and time of maximum, foliar water uptake from dew. The solid black line represents the mean of five nights, and the dashed lines indicate 95 % confidence intervals. The thin grey lines represent each of the five nights.
Figure 4.6. Canopy air temperature (black), mean leaf temperature (blue, n = 4) and dew point temperature (red). Neither air nor leaf temperature drop below dew point which precludes the possibility of fog or condensation formation according to this data. However, on the nights marked by arrows ‘a’ and ‘b’, 0.11 and 0.02 mm of dew was collected, respectively, indicating measurement error of temperature and/or humidity. The horizontal dashed arrow on the plot indicates the time period over which the sap flow data was collected from the branches supporting the leaves measured for temperature (Fig. 4.4).

Figure 4.7. Mean relative change in water potential in leaves subjected to one hour of artificial rainfall with error bars showing ± one standard error. The effects of drought sensitivity status ($P = 0.005$) and of the interaction between drought sensitivity and plot ($P = 0.035$) are both significant.
4.4.5 Canopy water uptake model

The findings for the five scenarios in the canopy uptake model are presented in Table 4.1. Compared to the control plot, canopy water uptake is approximately 30 % higher in the TFE, reflecting the mean difference in water potentials between plots. The duration of dew input had a large effect on the total amount of foliar uptake, which increased from 37.6 ± 2.3 to 51.9 ± 2.3 mm in the control plot in scenarios 2 and 3, with 5 and 10 hours of dew input per night, respectively. The difference in the canopy water uptake in scenarios 4 (19.9 ± 1.4 mm) and 5 (33.7 ± 2.3 mm) reflect the difference in $K_{\text{cuticle}}$. Transpiration for the same plots was calculated by Fisher et al. (2007) for the years 2001 to 2003 (Table 4.2), based on sap flux measurements, enabling a comparison of water lost via transpiration versus water gained by foliar water uptake. Comparisons are only presented for scenarios 1, 3 and 5 as they represent the minimum, maximum and ‘realistic’ amount of foliar uptake expected. The proportion of total annual transpiration accounted for by water taken up via foliar uptake in the control plot ranged from 2.2 % (2003) to 2.8 % (2001) in scenario 1, while in scenario 3 it was 4.4 % in 2001 and 4.0 % in 2003 (Table 4.3). The TFE was set up in 2001, explaining why estimated transpiration decreased year on year: 1258 mm in 2001, 953 in 2002, and 805 in 2003 (Table 4.2, Fisher et al. 2007). Hence, in the TFE, the foliar uptake as a proportion of transpiration increased from 2001 to 2003.
Figure 4.9. Figure shows suggested allocation of water from continuous rainfall starting at time 0. The solid line, and non-shaded area below, indicates the proportion of the rain that is retained in the canopy, and the shaded area above the solid line represents canopy through-fall. Water taken up via the roots is represented by the dotted line, via the leaves by the dashed line, and water taken by both is represented by the dot dash line. Note that soil water uptake begins a short while after rain starts, while foliar uptake begins as soon as the canopy starts getting wet. The reduced rate of canopy saturation with time occurs due to the rate at which increasingly inaccessible parts of the plant get wet e.g. the underside of leaves. The rates of water uptake, presented here, do not take into account the water potential of the plant.

4.4.6 Scaling model results to the Amazon

Scaling the results of the model to the whole Amazon basin suggested that 185.4 Pg yr\(^{-1}\) of water is taken up via foliar water uptake, and that this equates to a carbon gain of 0.34 Pg yr\(^{-1}\).
Table 4.2. Values for plot-level transpiration taken from Fisher et al. (2007) and leaf area index taken from Metcalfe et al. (2010)

<table>
<thead>
<tr>
<th>Year</th>
<th>Control</th>
<th>TFE</th>
<th>Control</th>
<th>TFE</th>
</tr>
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<tbody>
<tr>
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<td>1258</td>
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<td>5.5</td>
</tr>
<tr>
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<tr>
<td>2003</td>
<td>1223</td>
<td>805</td>
<td>4.2</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Table 4.3. Estimated foliar uptake in years 2001 to 2003, for scenarios 1, 3 and 5 from Table 4.1, with the percentage of the total estimated transpiration. Values for transpiration and leaf area index shown in Table 4.2 and taken from Fisher et al. (2007) and Metcalfe et al. (2010), respectively.

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Plot</th>
<th>2001 mm yr⁻¹</th>
<th>% of E</th>
<th>2002 mm yr⁻¹</th>
<th>% of E</th>
<th>2003 mm yr⁻¹</th>
<th>% of E</th>
</tr>
</thead>
<tbody>
<tr>
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<td>2.8</td>
<td>31.8</td>
<td>2.5</td>
<td>26.9</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>TFE</td>
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<td>3.8</td>
<td>41.6</td>
<td>4.4</td>
<td>34.3</td>
<td>4.3</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
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<td>4.4</td>
<td>53.7</td>
<td>4.3</td>
<td>48.7</td>
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</tr>
<tr>
<td></td>
<td>TFE</td>
<td>76.4</td>
<td>6.1</td>
<td>71.6</td>
<td>7.5</td>
<td>64.1</td>
<td>8.0</td>
</tr>
<tr>
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<td>Control</td>
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<td>35.7</td>
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<td>30.8</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>TFE</td>
<td>53.3</td>
<td>4.2</td>
<td>47.5</td>
<td>5.0</td>
<td>40.2</td>
<td>5.0</td>
</tr>
</tbody>
</table>
4.5 Discussion

4.5.1 Can leaves from Amazonian canopy trees take up surface water through their epidermis?

The results demonstrate that foliar water uptake occurred in all six genera that were analysed (Figs 4.1, 4.2 and 4.3). Of the six genera, *Eschweilera*, *Protium*, *Pouteria* and *Licania* are ranked as the top Amazonian genera in terms of their abundance, *Swartzia* is ranked 17th and *Manilkara* is ranked 73rd (ter Steege et al., 2013). Thus, this study provides strong evidence that foliar water uptake is a common strategy across the rainforests of Amazônia.

4.5.2 Does foliar uptake occur under natural (field) conditions?

The occurrence of reverse sap flow in response to foliar uptake from dew in the small terminal branch (Fig. 4.4a.), but not in the bigger branch upstream (Fig. 4.4b.), indicates that the small amount of water imbibed contributed to replenishing hydraulic capacitance (Fig. 4.5). Water storage increases the amount of time stomata can remain open without incurring dangerously low water potentials (Jansen et al., 2011, Gleason et al., 2014, Scholz et al., 2011), and high capacitance, by buffering transitory changes in evaporative demand, can compensate for vulnerability to hydraulic dysfunction (Tyree and Ewers, 1991, Meinzer et al., 2009).

An attempt was made to measure the conditions that led to dew formation with a view to using past meteorological data to estimate how regularly dew formation occurred and what its contribution might be to the Amazon water budget. Dew formation occurs when the surface temperature drops below the dew point temperature (Beysens, 1995) determined by the ambient water vapour pressure. Unfortunately, these conditions were not recorded during the sampling period (Fig. 4.6) despite actually collecting dew on several nights and observing it on leaf surfaces. Therefore, it is assumed that there was a calibration error with the humidity sensor. Although dew was collected on three nights, differences in methodologies among the three attempts makes a value for the mean nightly dew unreliable and potentially unrepresentative. Moreover, there is some evidence that small dew collection surfaces, such as those used in our preliminary dew collection experiments, can lead to an underestimation of dew formation (Kidron, 1998). As there is no information regarding the frequency of dew
formation, it is not possible to estimate annual dew inputs. However, dew was observed on all nights that measurements were taken in the canopy in the dry season of 2015.

4.5.3 Does the capacity for foliar uptake change in response to artificially imposed soil moisture deficit, and does it differ among taxa that are drought sensitive or resistant?

Our results show that there is no significant difference between plots in $\Delta \Psi / \Psi_i$ in either the rainfall (Fig. 4.7) or the humidity experiment (Fig. 4.8), indicating that, with all taxa combined, there is no plot-level plasticity in this trait (and by inference, cuticular conductance, SI 4.8). However, both experiments showed a significant interaction between drought treatment and drought sensitivity status of the different taxa under study. In the humidity experiment, an effect of the drought treatment was observed in both drought-sensitive and -resistant taxa, but in opposite directions, whereby the resistant taxa have higher $\Delta \Psi / \Psi_i$ in the control plot than the TFE, while in the sensitive taxa, $\Delta \Psi / \Psi_i$ is highest in the TFE (Fig. 4.8). The relationship is slightly different in the rainfall experiment in which there is a drought treatment (plot) effect in the sensitive but not in the resistant taxa (Fig. 4.7). It is not known what caused the difference in results between the experiments, but a possible explanation could be that the resistant taxa have responded to the drought treatment through modifications to the leaf cuticle, making it less permeable to water (to reduce cuticular water loss). Hence, $\Delta \Psi / \Psi_i$ is lower amongst resistant taxa in the TFE in the humidity experiment, but, the mechanical impact of the ‘rain’, in the rain experiment, disturbs the cuticle sufficiently to enable $U_f$ comparable to that in the control plot. That said, the rain generated in this study could be considered gentle by Amazonian standards.

The contrasting response to the drought treatment by the resistant and sensitive taxa in the humidity experiment (Fig. 4.8) might suggest a trade-off between $U_f$ and water loss, i.e., cuticle transpiration (Eller et al., 2016). Some plants reduce cuticle permeability to water in response to drought stress (Kosma et al., 2009, Shepherd and Griffiths, 2006), and if the amount of water potentially lost via cuticular transpiration
exceeded the water taken up through $U_l$, then reducing cuticular permeability would be a more effective way of managing drought stress, i.e., by prioritising water conservation rather than acquisition. The results of the humidity experiment imply that the resistant taxa prioritise water conservation, while the sensitive taxa prioritise water acquisition in response to the drought treatment in the TFE. Assuming that such a trade-off exists, then the value of foliar uptake would presumably be determined by the regularity of occult precipitation events, in other words, whether the amount of water that can only be imbibed through $U_l$ (Fig. 4.9) exceeds water loss via cuticular transpiration. Thus, it seems very likely that occult precipitation plays a strong role in determining species sensitivity to drought in the Amazon.

Predicted changes in climate are likely to result in lower water potentials in periods of drought stress. There was a strong linear relationship between the change in water potential ($\Delta\Psi$) and initial water potential ($\Psi$) for a given time of exposure to moisture (Fig. S4.3). Therefore, the amount of water taken up in a precipitation event is proportional to the level of water stress, such that lower (more negative) water potentials arising as a consequence of longer more severe dry seasons would lead to a larger amount of water imbibed through the leaves. However, it is possible that lower leaf area index cause by drought (McDowell and Allen, 2015, Metcalf et al., 2010a, Carnicer et al., 2011, Matusick et al., 2013) could reduce the maximum rate of foliar uptake during heavy prolonged rain although, in this study, leaf area index expressed in the model was 2.75 while in the TFE it is around 3.6 m$^2$ m$^{-2}$ (Table 2), so there was no effect of reduced leaf area index in the model outcome. Another aspect is that increased temperatures in the future will cause elevated vapour pressure deficit (Scheff and Frierson, 2014, Sherwood and Fu, 2014), reducing the potential for dew formation (Monteith, 1957). These factors acting together could potentially reduce the contribution of foliar water uptake to the Amazon water budget under future climate scenarios.

4.5.4 How much could foliar water uptake contribute to the forest water budget?

Using data from Fisher et al. (2007), from the same plots, I was able to estimate the proportion of transpiration that was accounted for by foliar water uptake in years 2001 - 2003 (Table 2). As a mean from these three years, the amount of foliar uptake was
2.8% of transpiration in the most plausible scenario (scenario 5), and 4.2% in the least conservative scenario (scenario 3, Table 4.3), which included dew input for ten hours every night of the six month dry season. The different outcomes between scenarios demonstrate the importance of establishing realistic values for the parameters which were estimated e.g. the proportion of the canopy that gets wet during a precipitation event (rain and dew) and the duration of dew. Thus, the model for foliar water uptake can be improved upon in the following ways:

- Leaf cuticular conductance needs to be measured more reliably. It is challenging to measure the amount of water taken up through the leaf surface because surface wetting inevitably results in noisy mass data. Therefore, a good way for measuring $K_{\text{cuticle}}$ should be established, or the validity of using $\Delta \Psi / \Psi_i$ (which is easier to measure) as a proxy for $K_{\text{cuticle}}$ should be investigated thoroughly. A convention needs to be established on whether $K_{\text{cuticle}}$ should be expressed on a one- or two-sided basis. This should depend on what proportion of the total leaf surface typically gets wet during a precipitation event e.g. if the underside of leaves seldom gets wet, expressing $K$ by total surface area (both sides) would make sense because $K_{\text{cuticle}}*L$ would then represent a single side of leaves.

- Water potential in this model was simply based on sine functions representing diurnal and seasonal oscillations and did not include feedback between foliar water uptake and water potential. Ideally, canopy conductance should be coupled with some measure of forest capacitance e.g. the change in water potential for a given amount of water uptake, and there should be feedback between water uptake (foliar and root) and canopy water potential.

- The meteorological data must include measurements of dew and fog (Anber et al., 2015). The addition of dew in this model was simply based on the assumption that it occurred for 5 or 10 hrs a night throughout the dry season. Given our observations, and previous research (Alvares et al., 2015) it seems likely that 10 hrs is more representative, but ongoing measurements of leaf wetness would enable a more accurate parameterisation of this important variable.

- The proportion of the canopy that is wetted by a precipitation event is determined by the duration and amount of precipitation and the water storage of the canopy
(Rutter et al., 1971, Herwitz, 1985). In this model I used half the leaf area index, 2.75 m m$^{-2}$, as an approximation of the mean amount of leaf area that was wetted by rainfall, and two values, 0.5 and 1 m m$^{-2}$, to represent the leaf area wetted by dew. However, this is a very large area of uncertainty e.g. Herwitz (1985) found that *Argyroderon peralatum*, a tropical forest tree, could retain up to 8 mm of rainfall over its projected crown area. Thus, any precipitation event resulting in less than 8 mm would wet only fraction of the canopy and, moreover, the rate at which canopy storage approaches saturation is likely to be strongly dependant on the intensity of precipitation. Estimating the proportion of leaf area index contributing to canopy foliar water uptake would, therefore, require measurements of canopy water storage and the rate of saturation to be coupled with detailed meteorological data.

### 4.5.5 Carbon benefit from foliar uptake in the Amazon

According to the simple calculations based on the model results for canopy foliar water uptake, the Amazon could gain approximately 0.34 Pg C yr$^{-1}$ due to the extra hydration afforded by foliar water uptake. To put this in perspective, between 1990 and 2007 the Amazon was predicted to be a net sink of carbon taking up 0.42 to 0.65 Pg C yr$^{-1}$ (Pan et al., 2011), suggesting that the carbon gain due to foliar uptake could be globally significant. The estimate of carbon gain was based on the assumptions that the water uptake measured in the study plot, and the value for water use efficiency taken from research conducted in the Southwest Amazon, were both representative of the Amazon mean. Moreover, the calculation was based on the outcome of modelled scenario 5, which was considered to be the most realistic scenario, but without conducting the measurements outlined above at different locations throughout the Amazon, it is not possible to present confidence limits.

### 4.5.6 What are the implications of widespread foliar water uptake to tropical rainforest function?

Research on foliar uptake has concentrated on two main groups of plants, those in high water stress environments, e.g., drought adapted plants (Yan et al., 2015, Oliveira et al., 2005, Gouvra and Grammatikopoulos, 2003), giant sequoias (Simonin et al., 2009, Limm et al., 2009), and plants in regions where occult precipitation is a significant
water input, e.g., cloud forests (Goldsmith et al., 2013, Eller et al., 2013). Ostensibly, lowland Amazonian trees, inhabiting a region characterised by high rainfall and not notable for fog (but see Anber et al., 2015), would fit into neither of those categories. Nevertheless, during the dry season it is not uncommon for periods of ten consecutive days or more to occur without rain, or longer periods with very little rain (unpublished data). During this time, continuous drying of the soil can lead to acute water stress in trees, stomatal closure, and a reduction in photosynthesis. These regular periods of severe drought stress occurring in the dry season exert the greatest selection pressure on drought-sensitive taxa (da Costa et al., 2010), and should ultimately determine the size of the hydraulic safety margin across the forest. Foliar water uptake, by increasing the potential for embolism repair and making available small but regular inputs of water via dew, potentially alters the level of a species drought-sensitivity. For example, our data suggest that dew contributes to refilling the hydraulic capacitance of the stems (Fig. 4.4) and so it is possible that the value of capacitance for conferring hydraulic resilience is higher in the Amazon than it is in forests without significant $U_f$ or occult precipitation. Thus, the common occurrence of foliar uptake in the Amazon could explain the low hydraulic safety margins measured at the Caxiuanã field site (unpublished data) and amongst Amazonian trees and tropical trees generally (Choat et al., 2012)

Whether or not the capacity for $U_f$ results in greater cuticular transpiration is a question of pressing importance in evaluating the sensitivity of Amazonian species to predicted future climates. If there is no such trade-off, then selection pressure in response to the increasing length and severity of dry seasons will certainly favour those species with highest $K_{cuticle}$, and will also raise the biologically interesting possibility of the cuticle acting as a one-way hydraulic valve. If a trade-off exists, as seems more plausible and is consistent with recent findings (Eller et al., 2016), then the role of cuticle permeability in affecting the hydraulic vulnerability in the Amazon, remains to be addressed. In terms of the consequence of drought duration versus severity, the existence of $U_f$ potentially moves the emphasis onto severity because we know that small amounts of precipitation more efficiently contribute to the ecosystem water budget. A diagram illustrating the relationships between rainfall amount and $U_f$ is presented in Fig. 4.9. Therefore, without $U_f$, the most severe drought could be
classified as the cumulative number of days with precipitation events amounting to less rain than the canopy surface storage. Conversely, considering $U_r$, the most severe drought could be estimated by the cumulative number of days with zero precipitation. Thus, ‘severe’ drought occurs for shorter time periods when small inputs of water can be directly absorbed. Moreover, during heavy rain events when the soil and the canopy become saturated, the maximum rate of water uptake by a tree is higher when water enters leaves in addition to the roots, resulting in more rapid plant-level rehydration and, therefore, hydraulic recovery following drought (Fig. 4.9).

4.5.7 Summary and conclusions

The results of this study demonstrate that foliar water uptake is likely to be a common strategy across the Amazon. Uptake of water from dew occurred on six nights out of seven on which sap flux was measured in the terminal branch of a single *Manilkara bidentata* tree, indicating that dew constitutes a regular supply of water in the dry season. The experimental drought appeared to produce different responses from drought-sensitive and –resistant taxa possibly suggesting a trade-off between foliar water uptake and cuticular transpiration. However, our model showed that water taken up directly through leaves is likely to account for at least 2.2 % of the annual transpiration, and that this value is strongly dependent on water potential and leaf area index, both of which are likely to be effected by reductions in rainfall.

Foliar uptake changes the assumptions regarding what constitutes hydraulic vulnerability, both in terms of the underlying physiology, but also the conditions which lead to significant drought. The precipitation regime in the Amazon is predicted to change, which will inevitably exert a selection pressure on species favouring those with traits better suited to the ‘new’ climate. The challenge is to understand which conditions favour which traits and ultimately learn about the trait distribution across taxa, enabling a projection for the sensitivity of the whole Amazon region under forecast conditions. I identify four topics regarding foliar water uptake in the Amazon that require further research: 1) establishing a rigorous, standard way of measuring $K_{cuticle}$; 2) determining if there is a trade-off between foliar water uptake and cuticular transpiration; 3) measuring canopy surface water storage; and 4) accurately recording occult precipitation including dew and fog input. An understanding of these areas will
enable a more robust conclusion of the significance of foliar water uptake in the Amazon rainforest, present and future.

4.6 Supporting Information

4.7 Determining leaf hydraulic conductance to foliar water uptake

The rate at which surface water is taken up by a leaf is called cuticular conductance, $K_{\text{cuticle}}$ (g m$^{-2}$ s$^{-1}$ MPa$^{-1}$)

$$K_{\text{cuticle}} = \frac{dM}{(A l t (\Psi_{\text{surface}} - \Psi_{l_i}))} \quad (S1)$$

Where $dM$ is the mass difference before and after foliar water uptake, $t$ is the time in seconds during which the leaf was taking up water, $A l$ is the area of the leaf, $\Psi_{\text{surface}}$ is the water potential of the free water on the surface of the leaf (assumed to be 0, so can be removed), and $\Psi_{l_i}$ is the initial water potential of the leaf. Here, $K_{\text{cuticle}}$ is calculated using hydraulic capacitance, $(C, \text{ g m}^{-2} \text{ MPa}^{-1})$, which is the amount of water loss or gain required to change the leaf water potential ($\Psi$) by 1 MPa, normalised by leaf area $A l$ (m$^2$). Because the leaf acts like a capacitor, whereby the rate of charge or discharge (conductance, $K$) is proportional to the water potential of the leaf, the equation for capacitance can be used to determine $K$ (Brodribb and Holbrook, 2003). Thus, the capacitance equation, $V_f = V_0 e^{-t/RC}$, in which $V_f$ and $V_0$ are the respective final and initial voltages, $t$ is the time elapsed, $R$ is the resistance and $C$ is the capacitance, can be rearranged to give conductance, $K = C ln[V_f/V_0] / t$, where $K = 1/R$. Substituting voltage (electrical potential) for water potential, this becomes:

$$K_{\text{cuticle}} = C \frac{ln[\Psi_0/\Psi_f]}{t} \quad (S2)$$

The mean value of $C$ for multiple samples is the slope of the regression line between the change in fresh mass per area ($\Delta M/A$, g m$^{-2}$) and the change in water potential, $\Delta \Psi$. Unfortunately, the $\Delta M$ data from the artificial rain experiment was not reliable because the process of sealing up the petioles with superglue led to a lot of noise in the final mass measurements. Therefore, $dM$ and capacitance could only be used from the humidity experiment. Capacitance measurements derived from the humidity experiment were compared with the mean value derived from the pressure volume analysis (Chapter 2). A comparison is presented in Fig. S4.1.
Figure S4.1. Change in fresh mass versus change in water potential from 16 hours in a high humidity environment, \( P < 0.001 \) and \( R^2_{adj} = 0.12 \). The thin solid line and dashed lines represent the slope with 95 % confidence intervals. The gradient represents capacitance which is \( 2.75 \, \text{g m}^{-2} \, \text{MPa}^{-1} \pm 0.76 \) standard error. The thick solid line represents the mean capacitance derived from pressure volume analysis (Chapter 2) of the same species and individuals at \( 4.65 \, \text{g m}^{-2} \, \text{MPa}^{-1} \).

The capacitance derived from the humidity experiment (\( 2.75 \pm 0.76 \, \text{g m}^{-2} \, \text{MPa}^{-1} \)) is lower than that derived from the PV analysis (\( 4.65 \pm 0.33 \, \text{g m}^{-2} \, \text{MPa}^{-1} \)). This could be because of the mass loss due to respiration in the humidity experiment. The mean respiration rate from all individuals and species is \( 0.77 \, \mu\text{mol m}^{-2} \, \text{s}^{-1} \) (data published in Rowland et al. 2015), which, over the duration of the humidity experiment, 16 hrs, amounts to a loss of \( 1.77 \, \text{g m}^{-2} \) of CO\(_2\). This may also account for the high level of scatter in the relationship between \( \Delta M/A \) and \( \Delta \Psi \) in Figure 1. Therefore, the capacitance value derived from the PV analysis was considered a more reliable figure for determining \( K_{\text{cuticle}} \).
4.7.1 *Time component of equation S2 used to derive* $K_{cuticle}$

Equation S2 shows that time enters the calculation of $K_{cuticle}$ in combination with the change in plant water potential during the same period. I refer to this as the ‘time component’ of water potential changes. A thorough theoretical explanation is given in Section 4.8. The time component from the humidity experiment was thought to be unreliable, both because condensation (of water on the leaves) may have been rate limiting to foliar uptake, and because the long duration of the experiment (16 hrs) could result in an underestimation of $K_{cuticle}$.

A separate experiment was performed to derive the time component. Leaves, collected at midday, were measured for water potential and mass before and after being submerged in water (with petioles remaining dry) for 3 minute time periods. This was repeated 4 times on each leaf, on 72 leaves from the six study genera (Fig. S4.2).

Consistent with the response of the recharging capacitor, the rate of rehydration was initially high and then became less as the initial water potential became closer to 0. Using the capacitance value derived from pressure volume analysis in equation 2, the mean $K_{cuticle}$ value from all the leaves and time periods in the rehydration experiment was calculated as $4.47 \times 10^{-3} \pm 5.51 \times 10^{-4} \text{ g m}^{-2} \text{ s}^{-1} \text{ MPa}^{-1}$. 


Figure S4.2. The water potential of leaves collected at midday and submerged in water for 3 minute intervals, with the petiole remaining out of the water (n = 72). Regression line is a three parameter exponential fit of the form $y = y_0 + a(1-e^{-b*x})$ where $y_0 = -2.17$, $a = 1.03$, $b = 0.01$ and the probability ($P$) < 0.001.

4.7.2 Comparing derived conductance value with published values

No published values for cuticle conductance with reference to foliar water uptake were found. However, there are values for leaf conductance ($K_{\text{leaf}}$), the movement of water through the leaf, and also for the flux of water vapour from the surface of leaves ($K_{\text{cuticle_flux}}$). While neither $K_{\text{leaf}}$ nor $K_{\text{cuticle_flux}}$ accurately represent $K_{\text{cuticle}}$, they can be used to rule out the possibility that the value used for $K_{\text{cuticle}}$ is unrealistically high. This proves to be the case as $K_{\text{cuticle}}$, as calculated here, is more than an order of magnitude lower than the smallest $K_{\text{leaf}}$ value, and less than half of the lowest $K_{\text{cuticle_flux}}$ value in Table S4.1. Thus, while it is impossible to assess how representative the value presented for $K_{\text{cuticle}}$ is without a directly comparable reference, there is no evidence to suggest that it is substantially wrong.
Table S4.1. $K_{\text{leaf}}$ represents water moving through the leaf in the normal direction of flow e.g. from the petiole into the lamina, and $K_{\text{cuticle_flux}}$ represents the flux of water vapour from the leaf surface. The units for both are the same as those used for $K_{\text{cuticle}}$. (The mean value for $K_{\text{cuticle}}$ calculated in this study is $4.47 \times 10^{-3} \pm 5.51 \times 10^{-4}$ g m$^{-2}$ MPa$^{-1}$ s$^{-1}$)

<table>
<thead>
<tr>
<th>$K_{\text{leaf}}$</th>
<th>Study</th>
<th>$K_{\text{cuticle_flux}}$</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.074 to 0.289</td>
<td>Sack et al. 2003</td>
<td>0.036 to 0.072</td>
<td>Hoad et al. 1996</td>
</tr>
<tr>
<td>0.126 to 0.234</td>
<td>Nardini et al. 2012</td>
<td>0.011 to 0.11</td>
<td>Kerstiens 1996</td>
</tr>
<tr>
<td>0.072 to 0.36</td>
<td>Scoffoni et al. 2008</td>
<td></td>
<td>(meta-analysis of 200 species)</td>
</tr>
<tr>
<td>0.054 to 0.576</td>
<td>Brodribb et al. 2004</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.8 $\Delta \Psi / \Psi$ as a means for comparing foliar uptake

Starting from the original equation describing the discharge of a capacitor:

$$\Psi = \Psi_0 e^{-(tK/C)}$$  \hspace{1cm} (S3)

where $\Psi$ is the water potential at time $t$. Because water potentials are negative, this equation actually describes the ‘charging’ rather than discharging of the capacitor or leaf. Also, note that rearranging to give conductance, $K$, results in the following relationship:

$$K = - C \ln \frac{\Psi}{\Psi_f} / t$$  \hspace{1cm} (S4)

which is generally presented as the equivalent $K = C \ln \frac{\Psi_f}{\Psi} / t$ (equation S2) (Brodribb and Holbrook, 2003, Nardini et al., 2012), i.e., without negative sign and with the numerator and denominator reversed. However, I will use equation S4 for the following derivation. Rearrange to isolate the water potential term:

$$\ln \frac{\Psi}{\Psi_f} = - tK / C$$  \hspace{1cm} (S5)
and differentiate:

\[ \frac{d\Psi}{\Psi} = - \frac{dtK}{C} \]  \hspace{1cm} (S6)

Thus, in the context of the experiments, \( dt \) is constant for all samples, the value for \( C \) is an average taken between full hydration and turgor loss point (i.e. also a constant) and, therefore, \( d\Psi/\Psi \) has a linear relationship with \( K \).

Figure S4.3. The change in water potential (\( \Psi \)) per initial water potential of leaves which were subjected to a) one hour of artificial rainfall, and b) 16 hours in a high humidity atmosphere (> 98% RH) resulting in condensation on the leaves. Both relationships were highly significant, \( P << 0.001 \).

4.8.1 Rate dependence of \( d\Psi \) on \( \Psi_i \)

From equation S6 we can rearrange to find that\( d\Psi \) is proportional to \( \Psi_i \).

\[ d\Psi = - \frac{dt\Psi K}{C} \]  \hspace{1cm} (S7)

The internal hydraulic conductance of leaves, \( K_{\text{leaf}} \), varies with leaf hydration (Brodribb and Holbrook, 2003) due to embolism or collapse of conduits (Blackman et al., 2010). Because the xylem is the final part of the hydraulic pathway of water moving into the leaf through the cuticle (Eller et al., 2016), \( K_{\text{cuticle}} \) should be unaffected
by xylem conductance, and therefore, $K_{\text{leaf}}$ and $K_{\text{cuticle}}$ should function largely, if not entirely, independently from each other. If $K_{\text{cuticle}}$ varied with leaf hydration ($\Psi_i$), the relationship between $d\Psi$ and $\Psi_i$ would be non-linear (equation 5, Section 4.3.10). However, there is a linear relationship between $d\Psi$ and $\Psi_i$ suggesting that $K_{\text{cuticle}}$ remains constant throughout the water potentials used in this study (Fig. S4.3).
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Chapter 5

Discussion and conclusions

5.1.1 Thesis Overview

The overall aim of this thesis was to explore the relevance of leaf traits with respect to drought sensitivity by using a long-term through-fall exclusion experiment to generate soil moisture deficit. Specifically, the research set out to determine if tropical rainforest trees from selected taxa responded to experimental drought via plastic changes in leaf physiology and anatomy, and whether there were consistent differences between the leaves of drought sensitive and drought resistant taxa. These questions were addressed in the context of leaf water relations, leaf anatomy and the capacity for leaves to absorb water directly from the environment, in chapters 2 to 4, respectively. In this synthesis chapter, the findings are briefly summarised and presented in an ecological context, and future research priorities are identified.

5.1.2 Brief summary and implications of results

On balance, the studies in this thesis revealed little acclimation to drought, and no consistent differences between drought-sensitive or -resistant taxa. The most significant response to the drought treatment was the reduction in osmotic potential at full turgor and turgor loss point (Chapter 2), which are the most widely recognised predictors of drought resistance from all of the variables presented in this thesis (Maréchaux et al., 2015, Bartlett et al., 2012). Importantly, the data presented in Chapter 2 revealed that the capacity for seasonal osmotic regulation is not associated with an ability to adjust to long-term changes in water availability, in contrast to this widely held assumption (Bartlett et al., 2014). Moreover, it was the drought-sensitive taxa in this study that showed more seasonal osmotic adjustment than the resistant taxa, indicating that the absolute value in osmotic traits could be more important than plasticity, in broad agreement with the meta-analysis of 283 species conducted by Bartlett et al. (2014). Based on the results from Chapter 2, therefore, it is suggested that the wet season values of osmotic potential at full turgor may be a suitable indicator of drought sensitivity (Chapter 2, Fig. 2.3).
The plastic response of leaf anatomy to the drought treatment was less than expected, although it did result in leaves with thicker adaxial epidermis and higher internal cavity volume (Chapter 3). The results presented in Chapter 2 (Table 2.4), show that the thickness of the adaxial epidermis (Ad) correlates with turgor loss point and the osmotic potential at full turgor, where both of these parameters are lower (more negative) when the Ad is thicker. Whether or not this relationship is causal (it is not possible to determine from this data), this might suggest that Ad thickness, or plasticity in this trait, plays a role in drought resistance. However, there was no difference between drought-sensitive and -resistant taxa, or interaction between drought sensitivity and treatment effect, in this parameter, thereby limiting support for this theory.

The internal cavity volume of the leaves was predicted to decrease in response to the drought treatment but, in fact, did the opposite. This trait is a significant aspect of leaf physiology, which shows plasticity in response to environmental conditions (Leuschner, 2002), changes over gradients of precipitation (Schimper, 1903, Maximov, 1929), and influences hydraulic conductance and gas mixing (Buckley et al., Buckley, 2015, Rockwell et al., 2014). Yet, the influence of cavity volume on gas exchange and transpiration is still poorly understood. It was speculated that higher internal cavity volume could cause a minor reduction in water loss via increases in apoplastic path length and reduced water vapour concentration within the cavities. The lability of this trait could either suggest that changes in size are relatively inconsequential, or that it is a ‘cheap’ optimisation mechanism. Further research is required to elucidate the role of cavity function on leaf performance.

Phenotypic plasticity has the potential to enable resilience to climate change in long-lived species, and therefore has been recognised as an important mechanism to characterise (See Chapter 1, Section 1.1.7 for discussion) (Nicotra et al., 2010). Much research exists on the plasticity of leaf anatomy in response to irradiance and temperature (Meir et al., 2002, Bjorkman and Holmgren, 1963, Carins Murphy et al., 2014), but there is considerably less published work on the effects of water availability, despite the recognised association of certain leaf types with wet or dry environments (Maximov, 1929, Cutler et al., 1977, Schimper, 1903, Roth, 1984). Trait plasticity can be constrained due to knock-on effects to other traits (i.e. enhancing one aspect of
fitness may reduce another), or may not exist as a strategy due to historically stable conditions or unpredictably changing conditions (Valladares et al., 2007). Thus, it is possible that the traits influencing leaf hydraulics, in the studied taxa, are too tightly constrained by associative demands on other traits to respond to the soil drought. Alternatively, it is possible that, with regard to drought effects, leaf traits do not respond plastically to soil drought but to the atmospheric component of drought, high vapour pressure deficit (VPD) only (Leuschner, 2002, Aliniaeifard et al., 2014), in which case such changes would not have arisen in this study. Although, this explanation seems unlikely as the leaves in the droughted trees still routinely incurred lower water potentials than those in the undroughted trees (unpublished data). The limited response in the leaf anatomy of Amazonian trees to the experimental drought, however, suggests that acclimation to drier conditions may be constrained to osmotic adjustment and/or confined to the adjustments occurring in other parts of the plant.

The finding that all of the species in this study absorb water directly through their leaves has profound implications for understanding the globally significant hydrological and carbon cycles of the Amazon, in addition to the ecology and biogeochemistry of Amazônia itself. Calculations of the total canopy water uptake, and the associated carbon exchange, were based on a number of assumptions and therefore are inevitably a first approximation of what appears to be a key flux that has previously been ignored. However, even the smallest estimated values (2.2 – 2.8 % of annual transpiration, as per Scenario 1, Chapter 4, Table 4.3) suggest that foliar water uptake contributes a very significant portion of the total forest water uptake, and also accounts for a considerable flux of carbon from the atmosphere to the vegetation. Moreover, the assumptions necessary in this first study provide a series of focal points for future research. This aspect of Amazonian ecophysiology is a large unknown with respect to how it will influence the response of the forest of the region to future drought. Either the existence of relatively water-permeable leaf cuticles will lead to greater vulnerability to droughts and the higher associated VPD expected in the future because of increased water loss via cuticular transpiration, or foliar water uptake will enable the efficient scavenging of small water inputs enabling a degree of hydraulic resilience to drought. Elucidating and better-quantifying the role of foliar water uptake in the fate of the Amazon is now a matter of pressing importance.
Leaves have been the subject of research for centuries, yet the role of many of their tissues is not precisely defined or understood. The use of free energy gradients to transport water and solutes inevitably results in functional overlap because all tissues must conduct water and contribute to hydraulic capacitance. In leaves, capacitance can follow a biphasic response (Zwieniecki et al., 2007, Blackman and Brodribb, 2011) indicating a differential role amongst tissues and cell types (Canny et al., 2012). Bundle sheath extensions have been demonstrated to reduce hydraulic resistance in leaves (Zsögön et al., 2015), which indirectly supports existing evidence that epidermal layers facilitate the lateral distribution of water in areoles (Wylie, 1943). An increasing body of research also suggests that tissues may have different degrees of hydraulic coupling (Buckley et al., Buckley, 2015, Rockwell et al., 2014, Nardini et al., 2010, Blackman and Brodribb, 2011, Canny et al., 2012) leading to the potential that they may be characterised by different values of their water relations parameters. This study provides tentative support for this hypothesis in terms of the significant correlations between tissue thicknesses and water relations traits in Chapter 2 (Table 2.4), while the correlation matrix in Chapter 3 revealed some interesting relationships, some more expected than others e.g. the positive correlation of cavity volume (CV) with $J_{max}$, but lack of correlation of CV with $V_{cmax}$; the negative correlation of stomatal density with abaxial epidermis thickness; the negative correlation between vein density and spongy mesophyll cell volume; and the positive correlation between the elastic modulus and predawn water potential.

The overall number of correlations with each parameter is informative of the extent to which that tissue represents (and potentially influences) the character of the whole leaf. Thus, the adaxial epidermis and spongy mesophyll appear to be strongly representative of leaf characteristics in antagonistic directions (correlating with 18 and 17, respectively, of 27 traits, Chapter 3, Table S3.2), while leaf mass per area (LMA), which is commonly used as a functional trait to inform models, correlated with only 8 out of 27 traits. In this dataset, LMA did not correlate with any of the gas exchange or water relations traits, suggesting poor suitability as a functional trait of Amazon rainforest trees. A large number of correlations for a given trait may indicate a lack of specialisation and a high degree of functional overlap in tissues, such that a large
volume of one tissue may compensate for a low volume in another. This may account for the poor functional characterisation of the spongy mesophyll: perhaps its function is to support the other tissues in e.g. photosynthesis, water transport and water storage?

5.1.4 Interaction between leaf water relations, anatomy and foliar water uptake

There was an expectation that differences in leaf water relations parameters amongst leaf tissues would lead to contrasting thicknesses of specific tissues between treatments/plots. This mechanism is tentatively hinted at by the results of Chapters 2 and 3, in which the adaxial epidermal thickness correlates negatively with the osmotic potential at full turgor ($\Psi_{\pi}^0$) and both respond significantly to the drought treatment, where Ad is thicker and $\Psi_{\pi}^0$ is lower in the TFE.

When foliar water uptake ($\Delta\Psi/\Psi_i$, i.e., change in water potential over the initial water potential, from the artificial rain experiment) was added to the correlation matrix in Chapter 3 (Table S3.2), it was only found to correlate with osmotic potential at full turgor ($P = 0.04$, Fig. 6.1) and turgor loss point ($P = 0.03$). No other aspects of leaf water relations or anatomy were correlated with even marginal significance to this measure of foliar uptake, suggesting that there were no specialised tissue structures associated with this process (at a scale identifiable from the level of magnification used for measuring the anatomy). No measurements were taken of the leaf cuticle in this study; however, the properties of the cuticle associated with permeability to water are not well understood (Domínguez et al., 2011) and water permeability does not correlate with cuticle thickness (Riederer and Schreiber, 2001). Therefore, detailed histochemical analysis would be required to determine whether the changes in foliar water uptake in response to the drought conditions were a consequence of variation in cuticle composition. The abundance or influence of trichomes was also not quantified, although, of the six genera in these studies, Licania was the only one to have trichomes which occurred only on the abaxial surface.
Future work

The finding, in Chapter 2, that seasonal variation in osmotic parameters occurs more in drought-sensitive than drought-resistant taxa raises some interesting questions. It was speculated that this somewhat counterintuitive relationship indicates that seasonal osmotic change may be a passive response to water availability, perhaps mediated by cell turgor. Adjusting osmotic potential was expected to be more energetically efficient, as the osmotic gradient between the symplast and apoplast is reduced in the wet season when it is required least. However, it may instead indicate the inability to maintain osmotic homeostasis, which would imply that seasonal variation in osmotic potential at full turgor may in fact indicate vulnerability to drought rather than resistance. Given the widely held view that osmotic ‘adjustment’ is a key drought tolerance trait (Bartlett et al., 2014), this hypothesis requires further investigation.

Understanding how each tissue maintains hydration, and how turgor can differ amongst tissues in the course of dehydration is fundamental in elucidating the details of the leaf hydraulic pathway. The correlations in Chapter 2 between leaf anatomy and leaf water relations corroborate the evidence that tissues may be hydraulically
sequestered from one another (Buckley et al., 2015). Although the evidence here is only correlative, the data can be used to generate the hypotheses that the spongy mesophyll has a higher osmotic potential, and that the epidermal layers have a lower osmotic potential than the bulk leaf value. Testing these hypotheses would further clarify the role each tissue plays in the response of leaves to water loss.

An aspect of leaf hydraulic physiology that was not addressed in this thesis was leaf hydraulic conductivity, $K_{leaf}$. In Chapter 1, the relationship between leaf and stem conductance was explained where lower $K_{leaf}$, and higher vulnerability to stress-induced loss of conductivity in leaves, contribute to reducing the risk of damagingly low water potentials occurring the in stem xylem. Thus, $K_{leaf}$ and $P_{50leaf}$ (the water potential at which 50 % loss of hydraulic conductivity occurs) are often coordinated with $K_{stem}$ and $P_{50stem}$ (Johnson et al., 2011, Hao et al., 2008, Chen et al., 2010, Salleo et al., 2001, Bouche et al., 2016), and offer a means to assess whole-plant vulnerability to water stress. Therefore, while no differences were found between drought sensitive and resistant taxa in leaf water relations, anatomy or foliar uptake, it is not possible to rule out the existence of such differences without comparing leaf hydraulic conductance and $P_{50}$. An attempt was made to construct leaf vulnerability curves (i.e. the decline of hydraulic conductance with leaf water potential) in this study using the capacitance method (Brodribb and Holbrook, 2003, Brodribb and Holbrook, 2006), but unfortunately the measurements of conductance were unsuccessful. Leaf conductance appeared to stop, or was immediately reduced to a very low value, in response to the petiole being cut, suggesting that the conduits were rapidly blocked (Appendix II). Therefore, measuring vulnerability curves in these taxa would require the modification of existing methods, or the use of indirect methods such as ultrasonic acoustic emissions (Salleo et al., 2001) or the recently proposed visual technique (Brodribb et al., 2016). Nevertheless, given that hydraulics appear to be a key component of drought-induced mortality in tropical forests (Rowland et al., 2015) and the common association between leaf and stem hydraulic traits, this is an aspect of leaf physiology that deserves further investigation in Amazonian tree species.

Arguably the most pressing questions that arise from this thesis concern foliar water uptake. To accurately determine canopy-level foliar water uptake would require detailed measurements of cuticular conductance, including how it varies by species
and canopy position, what contribution dew makes to the water budget, and how much of the canopy is active in the absorption of water. This last point, specifically, will have a particularly large impact on estimates of canopy uptake, because a canopy with a leaf area index of $5.5 \text{ m}^2 \text{ m}^{-2}$ (as in the field site for this study, Caxiuana National Forest Reserve, Para, Brazil (Metcalfe et al., 2010)) has a maximum absorption area of $11 \text{ m}^2 \text{ m}^{-2}$ including both sides of the leaves. Thus, the amount of the canopy absorbing water can vary from 0 to the theoretical maximum of $11 \text{ m}^2 \text{ m}^{-2}$ as a function of the intensity and duration of precipitation. Establishing realistic parameter boundaries for this relationship is consequently critical for calculating canopy-level foliar uptake.

5.1.6 Final conclusions

Given the potential for future changes in the Amazon hydrological regime, i.e. longer and more intense dry seasons with more frequent periodic droughts (Fu et al., 2013, Jupp et al., 2010, Boisier et al., 2015), understanding the sensitivity of the Amazon to drought has become a research priority. The evidence to date indicates that hydraulic deterioration of the branch xylem is a trigger for the series of processes that lead to drought-induced mortality (Rowland et al., 2015), so the question arises as to whether there are leaf traits associated with drought sensitivity that could facilitate the assessment of species’ drought tolerance. Certain characteristics of leaves do correlate with stem xylem traits (Johnson et al., 2011, Hao et al., 2008, Chen et al., 2010, Salleo et al., 2001, Bouche et al., 2016) and also with habitat water availability (Bartlett et al., 2012, Bartlett et al., 2014, Maréchaux et al., 2015, Lenz et al., 2006) suggesting that leaf physiology plays an important role in determining a species’ capacity to cope with water deficit.

The research presented in this thesis suggests that there are no consistent differences between the leaves of drought-resistant and -sensitive taxa in water relations traits, anatomy, and the capacity for foliar uptake. That being said, without measuring leaf hydraulic conductance and $P50$, it is not possible to rule out that such differences may exist. However, the results of this study might indicate that drought sensitivity in the focal taxa is determined by other aspects of physiology such as the hydraulic safety margins or $P50$ of the stem and/or roots. The osmotic traits and elastic modulus
demonstrated some level of plasticity in response to drought, showing the capacity of plants to acclimate such that leaves could maintain turgor under conditions of lower soil moisture availability than those of non-droughted trees. Leaf anatomy responded to the drought via increases in the adaxial epidermal thickness and the internal cavity volume, although, contrary to expectation it showed no acclimation in other traits.

The occurrence of foliar uptake in Amazonian trees adds another dimension to understanding drought sensitivity of the Amazon. The concept of hydraulic vulnerability may not be adequately described by traditional methods, such as xylem vulnerability curves, and the amount of water available to trees, particularly in the dry season, is likely to have been underestimated in the past. This aspect of Amazonian tree physiology requires investigation in order to properly assess the sensitivity of the Amazon to future climate change.

To conclude, the research conducted for this thesis suggests that the plastic response of leaf physiology in Amazonian trees is restricted to osmotic adjustments with little acclimation in anatomy, that the capacity for leaves for indicating drought sensitivity in the taxa used in this study is limited and that, to get a robust estimate of the drought-sensitivity of Amazonian rainforest, the role of foliar water uptake in the hydrological cycle of the Amazon and hydraulic vulnerability of trees must be recognised and quantified more fully.
5.2 References


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Spongy mesophyll properties influence H$_2$O:CO$_2$ diffusion conductivity

Table A6.1. Model variables

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Variable</th>
<th>Units</th>
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<tbody>
<tr>
<td>g</td>
<td>conductivity</td>
<td>mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>R</td>
<td>resistance</td>
<td>s m$^2$ mol$^{-1}$</td>
</tr>
<tr>
<td>D</td>
<td>Diffusion coefficient</td>
<td>m$^2$ s$^{-1}$</td>
</tr>
<tr>
<td>R</td>
<td>Gas constant</td>
<td>J mol$^{-1}$ K$^{-1}$</td>
</tr>
<tr>
<td>T</td>
<td>temperature</td>
<td>K</td>
</tr>
<tr>
<td>P</td>
<td>Air pressure</td>
<td>Pa</td>
</tr>
<tr>
<td>n</td>
<td>Stomatal density</td>
<td>m$^{-2}$</td>
</tr>
<tr>
<td>a$_s$</td>
<td>Area of stomatal pore</td>
<td>m$^2$</td>
</tr>
<tr>
<td>a$_p$</td>
<td>Cross-sectional area of internal pore space</td>
<td>m$^2$ m$^{-2}$</td>
</tr>
<tr>
<td>l$_s$</td>
<td>Length of stomatal pore</td>
<td>m</td>
</tr>
<tr>
<td>l$_{sm}$ CO$_2$</td>
<td>Path length of CO$_2$ through the spongy mesophyll</td>
<td>m</td>
</tr>
<tr>
<td>L$_{sm}$ H$_2$O</td>
<td>Path length of H$_2$O through the spongy mesophyll</td>
<td>m</td>
</tr>
<tr>
<td>r$_c$</td>
<td>Radius of spongy mesophyll cell</td>
<td>m</td>
</tr>
</tbody>
</table>

The mesophyll is usually treated as a functionally homogeneous leaf tissue layer in terms of modelling the conductivity of water vapour and CO$_2$ (Nobel, 1999). However, several lines of research have demonstrated that most photosynthesis occurs in the palisade mesophyll (Evans, 1999, Nishio et al., 1993) and that water vapour evaporates from sites close to the stomata (Boyer, 1985). Therefore, while most CO$_2$ must diffuse though the whole thickness of the spongy mesophyll, the pathway for water vapour is likely to extend only to the nearest non-cutinised water-saturated cell wall. The structure of the spongy mesophyll (SM) is consequently likely to influence
the conductivity ratio of H\textsubscript{2}O to CO\textsubscript{2}, such that thicker SM (longer path length for CO\textsubscript{2}) will reduce conductivity and bigger intercellular airspaces/pores in the SM will increase the conductivity. Conductance calculations are based on Fick’s 1\textsuperscript{st} law, in which diffusion is proportional to the concentration gradient over the resistance:

\[ E = D \frac{\partial c}{\partial l} \]

where E is diffusion (mol m\textsuperscript{-2} s\textsuperscript{-1}), D is the diffusion coefficient (m\textsuperscript{2} s\textsuperscript{-1}), c is concentration (mol m\textsuperscript{-3}) and l is the path length (m). This can be combined with the ideal gas law to give:

\[ g = \frac{DP}{RT\Delta l} \]

where g is conductivity, P is atmospheric pressure (Pa), R is the gas constant (m\textsuperscript{3} Pa mol\textsuperscript{-1} k\textsuperscript{-1}) and T is temperature (K).

The pathway for the CO\textsubscript{2} can be broken down into the fraction which diffuses through the stomata (g\textsubscript{s}, maximum theoretical stomatal conductivity) and the spongy mesophyll (g\textsubscript{m}). Diffusive resistance (R) is additive such that \( R_{\text{total}} = R_s + R_m \), and because resistance is the inverse of conductivity \( g_{\text{total}} = 1/R_{\text{total}} \).

\[
R_{\text{total CO2}} = \frac{R T l_{\text{sm CO2}}}{D_{\text{CO2}} P a_p} + \frac{R T l_s}{D_{\text{CO2}} P n a_s} \tag{1}
\]

Equation 1 gives the total resistance of the CO\textsubscript{2} pathway where \( l_{\text{sm}} \) is the distance through the spongy mesophyll (m, i.e. the thickness of the spongy mesophyll), \( a_p \) is the cross-sectional area of the spongy mesophyll pore space (m\textsuperscript{2} m\textsuperscript{-2}), \( l_s \) is the length of the stomatal pore (m), \( n \) is the stomatal density (m\textsuperscript{-2}) and \( a_s \) is the area of a single stomatal pore (m\textsuperscript{2}). Thus, \( g_{\text{total CO2}} = 1/R_{\text{total CO2}} \).

The conductivity for water vapour can be figured out in the same way as CO\textsubscript{2} but using a different mesophyll path length. It is not known precisely where in the spongy mesophyll water evaporates from, but the cutinisation of the internal surface of the
guard cells and adjacent cell surfaces in some species (presumably to prevent excessive evaporation) suggests that it occurs close to the stomata. For the purpose of this model it is assumed to occur primarily from the nearest saturated cell wall, which is taken to be a length equal to the average diameter of the spongy mesophyll pore away from the inside surface of the stomata. The diameter of the spongy mesophyll pores is calculated using a 2 dimensional version of an ‘average nearest neighbour’ equation which determines the mean closest distance between randomly spaced particles in a known volume (Bansal and Ardell 1972).

\[ l_{sm H_2O} = \left( \frac{1 - a_p}{\pi r_c^2} \right)^{-1/2} / 2 \]

So \( l_{sm H_2O} \) is the mean closest distance between adjacent cells, \( a_{cv} \) is the cross-sectional area of pore space as a fraction of leaf area and \( r_c \) is the mean radius of a spongy mesophyll cell. This relationship gives an approximation of the distance from the internal side of the stomata to the closest saturated cell wall, although the distance may be longer depending on internal cutinisation. Thus, the model for finding the total resistance of the water vapour pathway is the same as equation 1 but with \( l_{sm CO_2} \) and \( D_{CO_2} \) substituted for \( l_{sm H_2O} \) and \( D_{H_2O} \) respectively. The relative conductivity of the \( H_2O \) to \( CO_2 \) pathways becomes:

\[ \frac{g_{total H_2O}}{g_{total CO_2}} = R_{total CO_2} \frac{RT l_{sm CO_2}}{D_{CO_2} P a_p} + R_{total H_2O} \frac{RT l_{s}}{D_{H_2O} P n a_s} \]

Which reduces to:

\[ \frac{g_{total H_2O}}{g_{total CO_2}} = D_{H_2O} \left( \frac{l_{sm CO_2} n a_s + l_s a_p}{l_{sm H_2O} n a_s + l_s a_p} \right) \]
In equation 3 the ratio of H$_2$O to CO$_2$ conductivity is worked out directly from the resistances by flipping the equation rather than finding 1/R$_T$. This model demonstrates how key parameters influence the ratio of H$_2$O to CO$_2$ conductivity.

**Figure A6.1.** The effect of changes in spongy mesophyll thickness and cavity volume on H$_2$O : CO$_2$ conductivity. The ranges on the x axis span the 1$^{st}$ to 3$^{rd}$ quartile of the data taken from Caxiuana for the variable shown. For all other variables single values were used based on the means from the Caxiuana data i.e. SM thickness is the only changing variable in left panel and CV volume the only changing variable in the right panel.

**Figure A6.2.** Data from Caxiuana illustrating theoretical relationships shown in figure 1 (or not, in the case of SM thickness).

No correlation was found for the maximum theoretical conductivity (g$_{\text{total H}_2\text{O}}$) with the maximum measured conductance of individuals from Caxiuana. The biggest source
of error in the theoretical calculations is likely to be the measurement of stomatal pore size.

### 6.2 Ratio of mesophyll conductivity to stomatal conductivity of CO₂

A system is most efficient when it is co-limited. Consequently it could be expected that, in a leaf maximised for CO₂ uptake, \( g_{mCO₂} \) would be similar to \( g_{sCO₂} \). Where \( g_m > g_s \), maximum photosynthesis would be limited by stomatal conductivity. On the other hand, where \( g_s > g_m \) the upper range of stomatal conductivity would be redundant for CO₂ uptake, resulting in higher water loss per unit carbon assimilated.

![Figure A6.3. Theoretical CO₂ conductivity of the stomata and mesophyll of individuals in Caxiuana. A value above the 1:1 line implies that maximum CO₂ diffusion is determined by conductivity of the spongy mesophyll, not stomatal conductivity.](image)

The mean \( g_s:g_m \) ratio of the individuals in Caxiuana is 0.85, which suggests that the upper 15% of theoretical stomatal conductivity will have no influence on CO₂ uptake. This may suggest that the additional capacity for transpiration could provide an advantage, for example for temperature regulation.

### 6.3 Hypotheses arising from model

The conductivity of the CO₂ pathway is negatively related to the thickness of the spongy mesophyll and positively related to the mesophyll cavity volume. Therefore, to compensate for the increased resistance of thicker SM it would be expected that SM
thickness would correlate with the cross-sectional area of pore space. Indeed, these parameters do correlate significantly in the Caxiuana data (figure 4, $P < 0.05$).

![Cross-sectional area of pore space in the spongy mesophyll as a fraction of leaf area in individuals from the Caxiuana data set.](figure A6.4)

The most direct route for a molecule of CO$_2$ would be in a line perpendicular to the plane of the leaf i.e. the shortest route between the stomata and the palisade. Of course, most CO$_2$ will not travel this route but will move tangentially from the stomata to the palisade. The angle of the tangent from the stomata will be inversely proportional to both leaf thickness and stomatal density: the thicker the leaf the lower the angle, similarly, the more stomata the lower the angle. The bigger the angle the longer the average path length in proportion to the shortest possible path length (figure 5). Therefore, I hypothesise that leaves with thinner spongy mesophyll have higher stomatal density to compensate for the bigger angle and path length. Moreover, because the conductivity of a pore increases exponentially with pore area, as the thickness of the SM increases the advantage of high stomatal density decreases, thus creating a larger benefit to having fewer pores with larger radii. Thus, it is further hypothesised that stomatal size increases with SM thickness. Both of these hypotheses were consistent with the Caxiuana data where stomatal density is negatively correlated to SM thickness ($P < 0.01$, $R^2 = 0.25$) and guard cell length is positively correlated with SM thickness ($P < 0.01$, $R^2 = 0.24$, figure 6).
6.4 Improvements to Model
The model was presented in its simplest form but could be improved upon in various ways.

- Theoretical conductivity was used instead of conductance to omit the need to calculate concentration gradients. The concentration gradient in water vapour
could be calculated with reference to a given leaf water potential. The concentration gradient of CO₂ would have to be determined by a measure of photosynthesis independent of gas exchange, for example fluorescence or RuBisCO concentration [possibly, not thought about this much].

- The mesophyll path length for CO₂ was represented simply by SM thickness, although a more realistic proxy could be calculated using the mean θ (figure 5) from the mean distance between stomata and SM thickness. However, perhaps using the shorter path length value of SM thickness would compensate for the fact that some CO₂ is fixed in the SM, which would have the effect of reducing the mean path length.

- The mesophyll path length for water was represented by the mean narrowest distance between spongy mesophyll pores from the inside of the stomata. In reality, there are often cavities, bigger than the average pore space, immediately behind the stomata. Also, it is also not uncommon for cell walls near the stomata to be cutinised. Therefore, the measure used to represent mesophyll path length for water was probably an underestimate.

### 6.5 References


Chapter 7 - Appendix II
Declining hydraulic conductance in leaf midribs

7.1 Motivation
Attempts to construct leaf vulnerability curves on the genera *Protium, Pouteria, Swartzia* and *Eschweilera* failed because leaf hydraulic conductance appeared to be close to 0 even when measured at high water potentials. It was speculated that this was due to conduit blockage in response to the petiole being severed. However, it was not known where the blockage occurred, or if it was a direct result of mechanical damage incurred in the process of cutting. The following experiment was carried out on several leaves from the genera *Eschweilera, Pouteria* and *Manilkara* to ascertain if the blockage occurred in the midrib, and if it occurred instantaneously (as a result of mechanical damage) or if it was from induced blockages of xylem conduits.

7.2 Methods
Branches around a metre long were collected from canopy top positions in the field and allowed to rehydrate in a bucket of water filtered to 0.02 µm overnight. Under water, the leaves were removed from the branch by severing the petiole with a fresh razor blade and the lamina was cut from the midrib. The petiole and midrib were essentially contained within a tube with a hydrostatic head of 1.4 to 1.7 m at one end (generating a pressure of 14 to 17 KPa), and with the other end draining into a beaker of water on a balance. The tube was sealed around the petiole meaning that water flowed through the petiole and midrib in the natural direction of water movement through the leaf (Fig. 7.1). Flow was recorded for a minimum of 30 minutes per sample. The flow rate was determined by measuring the mass increment on the balance over time, and hydraulic conductance was derived by dividing the flow rate by the pressure difference (MPa) and was normalised per leaf area. Leaf area was measured by analysing scanned images of the lamina using ImageJ.

7.3 Results and Conclusion
In several samples there was no flow rate at all, but where flow did occur it appeared to decrease rapidly for the first several minutes and then continue at a low value (Fig. 7.2). This indicates that the xylem was not blocked as a mechanical consequence of
cutting the petiole, but that it could be physiological wound response. Such a response, especially if it occurs throughout the leaf lamina in addition to in the midrib would prevent measurement of hydraulic conductance on a per leaf basis.

**Figure A7.1.** Apparatus for measuring flow rate through petiole and midrib.
Figure. A7.2 Examples of decline in hydraulic conductance of petiole and midribs of three species.