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# NEW INSIGHTS IN THE ECOLOGY AND EVOLUTION OF PLANT NITROGEN LIMITATION

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To the Graduate Council:

I am submitting herewith a dissertation written by Rachel Christine Wooliver entitled "NEW INSIGHTS IN THE ECOLOGY AND EVOLUTION OF PLANT NITROGEN LIMITATION." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Ecology and Evolutionary Biology.

Jennifer A. Schweitzer, Major Professor

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**NEW INSIGHTS IN THE ECOLOGY AND EVOLUTION  
OF PLANT NITROGEN LIMITATION**

A Dissertation Presented for the  
Doctor of Philosophy  
Degree  
The University of Tennessee, Knoxville

Rachel Christine Wooliver  
May 2018

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*“Ignis aurum probat”*

–Seneca

## ABSTRACT

Under increasing additions of reactive nitrogen (N) to the planet via anthropogenic N deposition and excess fertilization, some plant species will thrive while others will not. This may seem counterintuitive, as the growth of most plants is thought to be limited by soil N, but recent evidence shows that excess N can reduce plant community composition, alter plant-microbial interactions, and lead to fundamental alterations in plant growth and fitness. Yet, we lack the ability to predict which plant species will be winners or losers in soil N enrichment scenarios. The primary goal of my dissertation was to examine variation in plant growth responses to N enrichment and whether ecological and evolutionary factors explain such variation. These factors, according to current literature, should include aspects of past evolution such as phylogeny and evolutionary differentiation in resource use traits, nutrient co-limitation, and interactions with root-associated microbes. Because variation in plant responses to soil N enrichment challenges the paradigm in ecology that productivity of all plants is N-limited or N co-limited, a second goal of my dissertation was to determine how this and other recent work changes our understanding of the terrestrial N and carbon (C) cycles and feedbacks between soil N gradients and evolution under global change.

In my first chapter, I used a global dataset of plant biomass responses to N fertilization and evolutionary models to show that species vary in the direction and magnitude with which they respond to N enrichment (with more than one in four species responding negatively or neutrally), and that two aspects of past evolution (phylogenetic relatedness and selection associated with constraints on resource use) govern responses to N enrichment. In my second and third chapters, I implemented two greenhouse fertilization experiments and subsets of the 30 functionally diverse tree species within the genus *Eucalyptus* that are native to Tasmania, Australia. The main result from these experiments was that phylogenetic patterns in biomass responses to N enrichment are associated with phylogenetic variation in root function (specific root length and interactions with ectomycorrhizal fungi), but not co-limitation by phosphorus (despite the fact that Tasmanian eucalypts occur across strong soil phosphorus gradients). In my fourth chapter, I reviewed how this and other current research challenges long-held and fundamental assumptions regarding the source, plant use, and microbial transformations of N and provides insights into eco-evolutionary feedbacks and C cycling under global change. Overall, my dissertation has used major theories in plant ecology and evolution to explain the variation in plant responses to global change, and synthesized research that highlights new understanding of the drivers and consequences of terrestrial N cycling.

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## INTRODUCTION

Fossil fuel combustion and agricultural fertilizer applications have tripled rates of nitrogen (N) addition to soils since the preindustrial era (Vitousek & Howarth 1991; Dentener *et al.* 2006) and are predicted to at least double the global rate of N deposition to soils by the end of this century (Lamarque *et al.* 2005). Decades of observational and experimental work have established that such influxes of N (either natural or simulated in experiments using organic or inorganic N fertilization) to soils in natural ecosystems can have both a benefit and a cost for terrestrial ecosystems. The benefit is that greater N inputs to soils tend to stimulate overall plant growth and community productivity (see meta-analyses by Vitousek & Howarth 1991; LeBauer & Treseder 2008; Xia & Wan 2008), facilitating sequestration of atmospheric CO<sub>2</sub> into plant biomass (Reich *et al.* 2006; Norby *et al.* 2010). The cost is a rearrangement of plant communities through changes in composition and reductions in plant species richness (Bobbink *et al.* 2010; De Schrijver *et al.* 2010), which has cascading effects on above- and belowground biodiversity (Siemann 1998; Zak *et al.* 2003) and ecosystem processes such as nutrient cycling (Hooper & Vitousek 1997). These alterations to plant communities suggest that some plant species will be favored over others in N enrichment scenarios, due to the loss of a dimension of niche space (ability to overcome N limitation) and subsequent loss of species that are evolutionarily differentiated to fill that niche (Harpole & Tilman 2007).

Yet, our ability to accurately predict which plants will persist, and which will be lost, as well as our understanding of the ecological and evolutionary factors generating such outcomes, is only in its infancy. Ecologists have attributed variation in plant species' responses to soil N to variation in resource use traits, where species with more resource-acquisitive traits can better use excess N for growth and competitively exclude other species (Gilliam 2006; Harpole & Tilman 2007; Bobbink *et al.* 2010; Cleland & Harpole 2010; De Schrijver *et al.* 2011; Harpole & Suding 2011; Phoenix *et al.* 2012). Other factors that literature suggests should underlie variation in plant resource use are seldom considered, including the degree of co-limitation by other growth-limiting resources (Harpole *et al.* 2011), interactions with symbiotic root-associated microbes (Kivlin *et al.* 2013), and phylogeny (because resource use traits, nutrient stoichiometry, and plant-microbial symbiosis are generally more similar among more closely related plant species; Brundrett 2002; Kerkhoff *et al.* 2006; Cornwell *et al.* 2014; Cavender-Bares *et al.* 2016). The major goal of my dissertation is to build on the long-standing literature on effects of N enrichment on ecosystems by exploring the extent to which each of these factors underpins variation in plant species' responses N, a crucial step in understanding population- and community-level responses to soil N enrichment.

One macroevolutionary tool that is increasingly being recognized as important for predicting plant species' performances in global change scenarios is phylogeny (Edwards *et al.* 2007; Davis *et al.* 2010; Lavergne *et al.* 2013). For example, Davis *et al.* (2010) showed that more closely related angiosperm species share more similar phenological responses to climate warming in natural systems than expected at random (i.e., phylogenetic signal). This means that the response of a particular plant species to climate change would be predictable based on the responses of its closest relatives. Whether plant responses to N enrichment show phylogenetic signal is unknown, except in a group of 14 species native to a Canadian grassland that showed no evidence of phylogenetic signal in responses to N fertilization (Bennett & Cahill 2013). In this case especially, and even in cases where species *do* respond more similarly than expected at random, knowledge about variation in ecological traits that govern these responses and the

processes that have driven the evolution of these traits may help us to better predict plant responses to global change (Suding *et al.* 2008; Elser *et al.* 2010).

A long-standing theory in plant evolutionary ecology, termed the “plant economics spectrum,” holds that there is an evolutionary trade-off between competitive and conservative resource use strategies in plant growth that can lead to macroevolutionary patterns (Grime 1977; Chapin 1980; Westoby 1998; Reich *et al.* 2003; Westoby & Wright 2006; Craine 2009; Reich 2014). According to this theory, high-resource environments have selected for competitive resource use strategies (i.e., combinations of traits that increase the capacity for resource use), and low-resource environments have selected for conservative resource use strategies (i.e., combinations of traits that constrain the capacity for resource use but increase the ability to persist in resource-limiting conditions). In support of the plant economics spectrum theory, much empirical work has shown that plants occurring on infertile soils have evolved lower-quality foliage, greater root:shoot ratios, slower photosynthetic and growth rates, and lower requirements for soil nutrients (Grime 1977; Hobbie 1992), while those occurring on fertile soils have evolved higher specific leaf area, photosynthetic rate, and growth rate (Reich *et al.* 1997; Poorter & Bongers 2006; Wright *et al.* 2010). We should expect that a continuum of competitive to conservative growth strategies (and underlying suites of resource use traits) in plants directly affect their capacities for N use, thus determining variation in growth responses to N across species. Despite the common attribution of variation in plant species’ responses to N to variation in their resource use traits (Gilliam 2006; Harpole & Tilman 2007; Bobbink *et al.* 2010; Cleland & Harpole 2010; De Schrijver *et al.* 2011; Harpole & Suding 2011; Phoenix *et al.* 2012), no study to my knowledge has explicitly addressed whether interspecific variation in plant growth responses to N reflects the evolution of their resource use traits or the selective gradients across which they have evolved.

Similarly, co-limitation by phosphorus (P) or other micro- or macronutrients may also govern plant biomass responses to N enrichment (Elser *et al.* 2007, Harpole *et al.* 2011). Co-limitation occurs when biomass responses to one nutrient (e.g., N) increase with the addition of another nutrient (e.g., P) (Harpole *et al.* 2011). If some species have evolved different mechanisms to relieve P limitation, such as increased P use efficiency or the ability to scavenge for P through associations with fungal symbionts, plant species’ responses to N should be differentially co-limited by P (Cleland & Harpole 2010). Under N enrichment, species whose responses to N are less limited by P (expected winners) should show increases in productivity and abundance over more strongly co-limited species (expected losers).

Because soil fungi mediate plant acquisition of soil resources, soil fungi should also guide variation in plant biomass responses to N enrichment. Mycorrhizal fungi are one guild of symbionts that provide up to 80% of required N and 75% of required P for terrestrial plant species (van der Heijden *et al.* 2008). But with increasing soil N, both theory and evidence suggest that the positive effects and feedbacks of these mutualists on plant growth will decrease (Antoninka *et al.* 2009; Kivlin *et al.* 2013; van der Putten *et al.* 2016), with mycorrhizal fungi even becoming parasitic in some cases (Johnson 1993; Johnson *et al.* 2008). Less is known about how other root-associated fungi, for example septate endophytic fungi whose effects on plant growth vary from negative to positive, interact with mycorrhizal fungi to mediate plant responses to N enrichment in natural systems (Farrer & Suding 2016). Ultimately, to predict community-level consequences of N enrichment scenarios, it is critical to understand integrated plant-soil-microbe responses to N.

That not all plants gain more biomass in response to N enrichment challenges the textbook paradigm that the productivity of all plants is N-limited. But this is not the only assumption regarding multiple aspects in the N cycle that have recently been demonstrated. Since a major revision of the N cycle last occurred (Schimel & Bennett 2004), multiple lines of evidence have shown that the understanding of the determinants and regulators of N pools, biogeochemical mechanisms of N transformations, and the roles of species and species interactions are still being identified. Specifically, studies have quantified biologically relevant N sources from rock weathering sources in plant-soil systems (Morford *et al.* 2011; Houlton & Morford 2015; Morford *et al.* 2016a; Morford *et al.* 2016b), challenging the traditional view of the N cycle that focuses exclusively on atmospheric N inputs that are solely derived from N fixation (Walker & Syers 1976; Cleveland *et al.* 1999; Galloway *et al.* 2004; Galloway *et al.* 2013; Vitousek *et al.* 2013; Stocker *et al.* 2016). Further, the biogeochemical mechanisms of N transformations from decomposition of organic matter to mineralization are still being revealed (Averill & Waring 2017). Last, contrary to the paradigm that disturbance drives N losses in all ecosystems, declines in soil N pools in response to an increasingly important disturbance, fire, can be suppressed in some plant communities (Pellegrini *et al.* 2017). Together this new knowledge has important implications for our understanding of the terrestrial N cycle, the role of deep time (i.e., geology and macroevolution) in the interactions and feedbacks between ecosystem processes and organisms, and C and N cycling in terrestrial systems under global change.

Overall, this dissertation first builds on the well-established literature on the effects of excess N on plant productivity (Vitousek & Howarth 1991; LeBauer & Treseder 2008; Xia & Wan 2008; Bobbink *et al.* 2010; De Schrijver *et al.* 2010) by exploring the evolutionary and ecological mechanisms underlying variation in plant responses to soil N enrichment. To achieve this, I take two approaches, including a meta-analysis of plant responses to N fertilization and empirical fertilization experiments using a phylogenetically characterized group of 30 tree species in the genus *Eucalyptus* that naturally co-occur on the island state of Tasmania, Australia. The Tasmanian *Eucalyptus* system is ideal because species within this genus have evolved across nutrient, climatic, and topographical gradients (Williams & Potts 1996) and should thus have unique resource use strategies. Eucalypts are also dependent on mycorrhizal fungi for nutrient acquisition, being one of the few tree genera known to form dual associations with both ectomycorrhizal and arbuscular mycorrhizal fungi (Brundrett *et al.* 1996). Second, this dissertation synthesizes research that changes our understanding of processes involved in the terrestrial N cycle. Based, in part, on the meta-analysis and empirical work, I review research that contradicts long-held assumptions about the sources, plant use, microbial transformations, and losses of N in ecosystems. Together these results show the importance of understanding eco-evolutionary dynamics and provide important examples of the hypothesized linkages between ecosystem ecology and evolutionary biology (Schoener 2010; Matthews *et al.* 2011). More accurate assessment of the mechanisms and dynamics of terrestrial N cycling are critical to advance as this knowledge may directly influence predictions about C cycling under global change.

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**CHAPTER I**  
**PLANT FUNCTIONAL TRAITS GUIDE MACROEVOLUTIONARY TRADE-OFFS**  
**BETWEEN COMPETITIVE AND CONSERVATIVE GROWTH RESPONSES TO**  
**NITROGEN**

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### **Abstract**

Decades of research show that plants vary in their growth responses to increasing soil nitrogen (N), supporting theory on evolutionary trade-offs between competitive and conservative growth strategies. However, we lack an explicit examination of the evolutionary processes guiding trade-offs in competitive and conservative growth responses to N addition (GRN), processes which should include selection across environmental gradients and constraint within certain plant functional types. To determine current variation in GRN across plants, we collected previously published data on total biomass responses of 125 terrestrial plant species to N fertilization, relative to control soil N conditions. We calculated phylogenetic signal of GRN to assess the influence of shared evolutionary history on variation in N use capacities. To determine whether this variation is consistent with stabilizing selection towards unique N use capacities across environmental gradients and plant functional types, we compared the fit (second order Akaike Information Criterion) of species' GRN data to models that approximate evolution according to genetic drift with and without stabilizing selection across plant functional types, biomes, latitude, mean annual temperature, and annual precipitation. More than one in four species in our analysis responded negatively or neutrally to increasing soil N and responses ranged from a 60% decrease to an 1800% increase in biomass. We identified a significant phylogenetic signal for GRN, and evolutionary models incorporating stabilizing selection plus genetic drift explained more variation in GRN than models incorporating genetic drift alone. Parameter estimates from selection-based models indicate that plant functional types have experienced selection towards GRN values (i.e., evolutionary optima) that differ more than among biomes or across climatic gradients. Overall, our results suggest that phylogenetic relatedness and stabilizing selection associated with functional constraints are two aspects of past evolution that govern whether species will be winners or losers in global soil N addition scenarios.

### **Introduction**

A primary conclusion drawn from decades of ecological research on nitrogen (N) limitation of plant growth (Vitousek & Howarth 1991; LeBauer & Treseder 2008) is that all plants will produce more biomass as N levels in soils increase. However, a host of recent studies have demonstrated that species' growth responses to increasing soil N (hereafter "GRN", which is defined as the change in plant biomass production in elevated N soils relative to ambient or control N soils) differ in both direction and magnitude. For example, a field N addition experiment by Fynn & O'Connor (2005) demonstrated that five of eight South African grass species produce significantly less biomass in response to N addition. Further, a meta-analysis by Lawrence (2003) showed that the responses of 74 tropical tree species to N addition range from no response to more than 20-fold increases in biomass in some species. Variation in plant responses to N, combined with the expected doubling of the global rate of anthropogenically-driven N inputs to soils within the coming century (Lamarque et al. 2005) will result in decreased species richness in most communities (Bobbink et al. 2010). Thus, explaining

interspecific variation in plant GRN will be critical in efforts to predict which species will decline and which will benefit in higher N soils.

Recent research shows that past evolution is a key determinant of interspecific patterns in plant responses to global change (Edwards, Still & Donoghue 2007; Davis et al. 2010) but the evolutionary forces that have shaped current patterns in plant GRN are unclear. Selection should be one such force, according to a central theory in plant ecology of an evolutionary trade-off between competitive and conservative growth strategies (Grime 1977; Chapin 1980; Reich 2014). Termed the worldwide economics spectrum (WES) (Reich 2014), this theory holds that resource-abundant environments select for competitive growth strategies that increase the capacity of plants to exploit available resources, while resource-limited environments select for conservative growth strategies that increase the ability of plants to tolerate and persist in such environments. The continuum of competitive to conservative growth strategies in plants should directly affect their capacities for N use, thus leading to variation in GRN across species. However, no study to date has explicitly addressed whether interspecific variation in GRN reflects the selective gradients across which plants have evolved.

Phylogenetic comparative methods provide a framework to determine which evolutionary processes, including genetic drift and selection, are consistent with observed variation across species. In describing this variation, we might identify a phylogenetic signal, where GRN is more similar among close relatives than expected at random. A significant phylogenetic signal would be expected given evolution according to genetic drift in the absence of strong selection (Revell, Harmon & Collar 2008). Yet, recent studies (Bennett & Cahill 2013; Senior et al. 2013) report different strengths of phylogenetic signal in such responses, indicating that phylogenies alone are not always helpful tools for predicting species' performances in higher soil N environments. Weak phylogenetic signal could be explained by past selection towards one or more evolutionary optima for GRN across species (Revell, Harmon & Collar 2008). Such a prediction would be validated by better fit of evolutionary models that simulate evolution under genetic drift plus unique selective regimes (e.g. different biomes or plant functional types) to current data on species' GRN values than those that simulate evolution under genetic drift alone. Investigating past evolutionary drivers of current N limitation using a phylogenetic comparative framework could provide key insight into past evolutionary drivers of N use across species, and how this extends to community responses to continuing global change (Edwards, Still & Donoghue 2007).

Despite evidence that past evolution informs patterns in species' responses to global change (Edwards, Still & Donoghue 2007; Davis et al. 2010; Senior et al. 2013), existing meta-analyses on plant GRN (Elser et al. 2007; LeBauer & Treseder 2008; Xia & Wan 2008; De Schrijver et al. 2011) have not been conducted within a phylogenetic comparative framework. Nevertheless, such meta-analyses have built upon the foundation laid out by the WES theory for testing hypotheses about the evolution of GRN. Broadly speaking, species evolving in more abiotically stressful environments, for example heathland biomes, or habitats at higher latitudes where soils have lower concentrations of biologically fixed N (Vitousek et al. 2010) and climates are colder and drier, should evolve comparatively lower capacities for N use due to low return on investment in resource acquisition (Reich 2014). Alternatively, species evolving in less abiotically stressful environments, for example grassland biomes, or habitats at lower latitudes where soils have higher concentrations of biologically fixed N (Vitousek et al. 2010) and climates are warmer and wetter, should evolve greater capacities for N use due to high return on investment in resource acquisition (Reich 2014). Accordingly, three meta-analyses (Elser et al.

2007; Xia & Wan 2008; De Schrijver et al. 2011) have shown that species occurring in grassland biomes respond more positively, and species occurring in shrublands less positively or neutrally, to N fertilization. Further, Xia & Wan (2008) demonstrated that GRN increases towards the equator and with increasing annual precipitation. Plant functional types should also be strongly associated with growth strategy because being competitive in one respect (e.g. having finer roots that increase the capacity for resource capture and an annual life history to exploit intermittent influxes of resources) requires being competitive in others (e.g. having higher N use capacity) (Grime 1977; Chapin 1980; Reich 2014). Correspondingly, Xia & Wan (2008) showed that annual herbs grow more in response to N addition than perennial herbs and, together with Elser et al. (2007) and De Schrijver et al. (2011), that grasses grow more in response to N addition than shrubs. A critical next step in understanding variation in plant performances in elevated soil N scenarios is to determine whether these observed differences in GRN are consistent with past selection along environmental gradients and among plant functional types.

We collected previously published data on the growth responses of 125 terrestrial plant species to experimental N addition to soils, relative to control soil N conditions, to first test the hypothesis that 1) GRN varies across plant species in both direction and magnitude, as suggested by the WES theory (Reich 2014) and demonstrated in previous experiments (Lawrence 2003; Fynn & O'Connor 2005) and meta-analyses (Xia & Wan 2008; De Schrijver et al. 2011). The distribution of species' GRN values across a continuum of negative, neutral, and positive values would support this hypothesis. We then calculated phylogenetic signal for our estimates of GRN across species to determine whether shared evolutionary history explains variation in capacities for N use. To test the hypothesis that 2) current variation in GRN across plant species is consistent with selection towards unique capacities for N use across environments and plant functional types, as is also suggested by the WES theory, we compared the fit of species' GRN estimates to models that approximate evolution according to either Brownian motion or Ornstein-Uhlenbeck processes. Better fit of Brownian motion models would indicate that contemporary variation in GRN is consistent with evolution according to genetic drift, in the absence of strong selection. Alternatively, better fit of Ornstein-Uhlenbeck models that incorporate genetic drift plus constant selective pull towards different evolutionary optima across selective regimes (e.g. biomes) or gradients (e.g. absolute latitude) would support our second hypothesis. Evidence for both hypotheses would indicate that past selection has guided the evolution of plant capacities for N use in terrestrial plant species, establishing an important link between plant functional evolution and global patterns of plant winners and losers in rapidly changing environments.

## **Methods**

### *Data collection and calculation of species' GRN values*

We collected previously published data on plant growth responses to experimentally manipulated soil N from 1) studies used in a recent meta-analysis of plant responses to N addition (Xia & Wan 2008) and 2) a search conducted in Web of Science<sup>TM</sup> for publications since 2008, using combinations of the search terms plant biomass, plant productivity, nitrogen addition, nitrogen deposition, and nitrogen fertilization. We limited our data collection to studies reporting (1) biomass production (per individual, pot or plot via direct harvest or allometric estimates), (2) standard error or standard deviation of biomass, and (3) treatment sample size of individual plant species grown in both ambient (control) and elevated soil N treatments. Values for total biomass and standard error or deviation illustrated in figures were extracted using Plot Digitizer v2.6.3

(Huwaldt 2013). Though Xia & Wan (2008) accepted observations of total, aboveground, and belowground biomass responses to N, we limited our data collection to studies reporting total (i.e., above- plus belowground) biomass responses to account for interspecific variation in above- versus belowground biomass allocation. We also included only observations for species within a phylogeny of 30,000 plant species (Zanne et al. 2013; 2014); we used this pruned phylogeny in the analyses described below. Similar to Xia & Wan (2008), we excluded studies of common agricultural and horticultural species as we sought to capture the outcomes of GRN reflecting past natural, rather than artificial, selection. Overall, 122 of the 381 species for which Xia & Wan (2008) collected biomass responses to N addition fit our total biomass and phylogeny criteria. However, we included observations for only 87 of these species in our dataset because we excluded non-vascular (moss) species, species that we consider agricultural or horticultural despite being accepted by Xia & Wan (2008), and species whose responses had been mistakenly categorized as total biomass by Xia & Wan (2008) but were based on aboveground biomass alone. We collected GRN observations for an additional 38 species from our Web of Science™ search species since 2008.

To estimate the evolved (i.e. genetic) variation in GRN across the 125 species included in our dataset, we accepted multiple observations for species (observations which would ultimately be averaged by species) published in different studies or reported across factorial treatments within studies. We included observations in which an individual species received different forms, rates, or cumulative amounts of N; were grown in field or greenhouse conditions, for different lengths of time, or in differing densities; and received different amounts of CO<sub>2</sub>, phosphorus (P), micronutrients, light, or water. In total, we acquired 445 observations of plant GRN from 71 studies (see Appendix S1 in Chapter I Supplementary Material for full data-source references). For each observation we calculated the GRN as the log-transformed response ratio of total biomass in the elevated soil N condition to total biomass in the ambient or control soil N condition, and standard error around each response ratio (Hedges, Gurevitch & Curtis 1999). We then averaged response ratios and standard errors for each of the 125 species represented in the full dataset to use in the phylogenetic comparative analyses described below. Because species were not grown in the same experimental conditions (as is often an issue in ecological meta-analyses), we argue that averaging responses for each species across different studies and treatments yields the most realistic estimates of GRN given environmental variation that plants will experience as global change continues.

#### *Distribution and phylogenetic signal of GRN*

To address our first hypothesis that GRN varies across plant species in direction and magnitude, we calculated the proportions of species with response ratios that were negative (response ratio + standard error < 0), neutral (response ratio ± standard error overlapping 0), and positive (response ratio - standard error > 0). We also calculated the range, phylogenetically-weighted mean (± standard error), and median of species' log response ratios, each of which we back-transformed and reported as percent change in biomass for ease of interpretation.

We obtained a phylogenetic tree for the 125 species included in our dataset by pruning a phylogeny of over 30,000 terrestrial plant species (Zanne et al. 2013; 2014). This phylogeny was reconstructed using aligned sequence data for 7 gene regions (18S rDNA, 26S rDNA, ITS, matK, rbcL, atpB, and trnL-F) and a general time-reversible model with gamma-distributed rate heterogeneity, and branch lengths time-scaled according to estimated divergence times. We used the pruned tree to estimate Pagel's (1999)  $\lambda$ , a maximum likelihood-based estimate of



phylogenetic signal, for species-averaged response ratios using the R package *geiger* v.2.0.3 (Harmon et al. 2008). Pagel's  $\lambda$  varies from 0 (indicating that species' traits are phylogenetically independent) to 1 (indicating that species' traits covary with phylogenetic relatedness among species, as would be expected given evolution according to a Brownian motion process). When calculating Pagel's  $\lambda$  we accounted for within-species variation that may arise from plasticity across experiments and genetic variation among conspecifics by incorporating species-averaged standard errors around response ratios in our estimate of  $\lambda$  (Ives, Midford & Garland 2007). Plant GRN would be considered phylogenetically non-independent if the likelihood of the estimated  $\lambda$  were more than two units greater than the likelihood that  $\lambda$  is equal to 0. Moreover, likelihood of the estimated  $\lambda$  less than two units lower than the likelihood of  $\lambda$  being equal to 1 would be consistent with evolution according to a Brownian motion process. All supplementary and main analyses were performed in R v3.1.2 (R Development Core Team 2012).

As significant phylogenetic signal could be an outcome of more similar experimental environments among closer relatives rather than true similarities in GRN values, we tested whether observed species' responses and the phylogenetic signal of these responses are influenced by the following sources of experimental variation: the form of N applied (i.e., organic, inorganic, or both), growth environment (i.e., greenhouse, field, or both), average experimental duration, average increase in the cumulative amount of N added ( $\text{g m}^{-2}$ ) relative to the control N treatment, average increase in the rate of N added ( $\text{g m}^{-2} \text{yr}^{-1}$ ) relative to the control N treatment, average plant density, and whether co-limiting resources (i.e., phosphorus, micronutrients,  $\text{CO}_2$ , light, and water) were applied across control and elevated soil N treatments. Using phylogenetic linear regressions (see Appendix S2 and Table S1), we found that three sources of experimental variation (N form, P limitation, and micronutrient limitation) explain significant variation in the observed distribution of species' GRN, whereas the other seven sources of experimental variation do not. Most important, none of the ten sources of experimental variation significantly influences our estimate of phylogenetic signal in GRN. These results indicate that although the experimental environment can shape GRN estimates across species, it does not significantly shift the distribution of observed responses across the phylogenetic tree. Thus, we did not incorporate sources of experimental variation into the main analyses described below.

#### *Identifying potential selective regimes and gradients*

We chose four groups of discrete selective regimes given that each has acted as a selective force for the evolutionary trade-off between competitive and conservative growth strategies (Grime 1977; Chapin 1980; Reich 2014). The first group was native biome (boreal forest, temperate forest, tropical rainforest, wetland, grassland, tundra, heathland or desert), which we categorized according to the original publications from which we derived growth responses to N addition. If unavailable in these publications, we obtained biome information from the USDA plants database, and if species spanned multiple biomes we categorized them according to where they occur most commonly. The other three groups of selective regimes were growth form (grass, forb, tree, or shrub), life history (perennial or annual), and root type (fibrous root or taproot). We categorized species' growth forms and life histories according to the original publications from which we derived growth responses to N addition. We determined root type by the absence or presence of a taproot at any point in development, as reported in the USDA Forest Service Fire Effects Information System. If growth form, root type, or life history information were not

available in the above sources, we categorized species according to published data in Web of Science™ database.

We also chose three continuous environmental gradients to represent selective forces on GRN: 1) absolute value of latitude, 2) mean annual temperature, and 3) annual precipitation. We obtained longitudes and latitudes for each of the 369 of the original 445 observations for which authors reported geographic location where species occurred naturally. We imported these longitudes and latitudes into ArcGIS v10.1 (Environmental Systems Research Institute, Inc.) to extract mean annual temperature and annual precipitation from the WorldClim database (Hijmans et al. 2005) at a 10 arc-minute resolution. We then averaged latitudes, mean annual temperatures, and annual precipitation by species. In total, we obtained latitude, mean annual temperature, and annual precipitation estimates for 114 of the 125 species in our analysis.

### *Evolutionary models*

To test the hypothesis that current variation in GRN is consistent with stabilizing selection towards unique optimal capacities for N use across selective regimes and gradients, we compared the fit of the following models to species-averaged response ratios: 1) those approximating evolution according to a Brownian motion process and 2) those approximating evolution according to the Ornstein-Uhlenbeck process.

We implemented models of discrete selective regimes (biome, growth form, root type, and life history) in the R packages corHMM v1.13 (Beaulieu, O’Meara & Donoghue 2013) and OUwie v1.40 (Beaulieu et al. 2012). The OUwie package offers a set of seven unique models (see Table 1.1 for a description of each) allowing the trait optimum, rate of stochastic motion, and strength of selection to vary across selective regimes and can incorporate estimates of within-species variation (standard error of response ratios). We report model fit as the second order Akaike information criterion (AICc) scores, delta ( $\Delta$ ) AICc, and weight (the likelihood of each model divided by the sum of likelihoods across models; Johnson & Omland 2004), with the best fitting model(s) yielding  $\Delta$ AICc less than 2. To characterize the direction and magnitude of the GRN towards which species in each regime have evolved while accounting for model uncertainty, we calculated weighted estimates of optima for GRN across regimes (i.e. across different biomes), wherein parameter estimates of better-fitting models contribute more to the weighted estimates. We then back-transformed estimates of GRN to percent changes in biomass for ease of interpretation. Better fit of models based on Ornstein-Uhlenbeck processes, and non-overlapping standard error intervals of responses to N among regimes, would suggest that species in different regimes have experienced selection towards different capacities for N use.

We implemented models of continuous selective gradients (absolute latitude, mean annual temperature, and annual precipitation) using phylogenetic linear regressions in the R package phylolm v2.3 (Ho & Ané 2013). We modeled each predictor separately because our aim was not to build a model explaining maximum variation in GRN values, but rather to determine whether species’ GRN values increase or decrease with stress-associated gradients, as predicted by the worldwide economics spectrum theory. For each model we incorporated a covariance matrix expected from either a Brownian motion or an Ornstein-Uhlenbeck process. Before analysis, annual precipitation was log-transformed to achieve normality. We report model AICc,  $\Delta$ AICc, and weight. To determine the direction and magnitude of the effects of absolute latitude, mean annual temperature, and annual precipitation on GRN, we examined the slopes and p-values of best-fitting models. Better fit of models assuming an Ornstein-Uhlenbeck process, and

**Table 1.1. Current growth responses of 125 plant species to experimental nitrogen addition are better explained by Ornstein-Uhlenbeck models that approximate evolution according to genetic drift plus stabilizing selection than Brownian motion models that approximate evolution according to genetic drift alone.** Here we report second order Akaike Information Criteria (AICc),  $\Delta$ AICc, and weights for the seven evolutionary models implemented in the R package OUwie (Beaulieu et al. 2012). All models estimate a rate of stochastic evolution ( $\sigma$ ), but Brownian motion (BM) models assume traits are normally distributed around the ancestral trait value and increase in variance over time, whereas Ornstein-Uhlenbeck (OU) models introduce some level of attraction ( $\alpha$ ) that ‘pulls’ the evolution of that trait towards some optimal value ( $\theta$ ). For each model, subscripts describe the parameters ( $\sigma$ ,  $\alpha$ , and  $\theta$ ) that are allowed to vary across evolutionary regimes. We assigned species to the following evolutionary regimes: grass, forb, tree or shrub (growth form), boreal forest, temperate forest, tropical rainforest, wetland, grassland, tundra, heathland, or desert (biome), fibrous root or taproot (root type), or perennial or annual (life cycle). Best fitting models ( $\Delta$ AICc < 2) for each group of regimes are bolded. Models for which data were insufficient are blank.

Group		Model						
		BM <sub>a</sub>	BM <sub><math>\sigma</math></sub>	OU	OU <sub><math>\theta</math></sub>	OU <sub><math>\theta,\sigma</math></sub>	OU <sub><math>\theta,\alpha</math></sub>	OU <sub><math>\theta,\sigma,\alpha</math></sub>
Growth form	AICc	318.75	300.76	251.03	253.28	252.35	253.83	258.11
	$\Delta$ AICc	67.72	49.73	0	2.24	1.31	2.79	7.08
	Weight	0	0	0.47	0.15	0.24	0.12	0.01
Biome	AICc	318.75	311.65	251.03	259.86	260.27	-	-
	$\Delta$ AICc	67.72	60.62	0	8.83	9.24	-	-
	Weight	0	0	0.98	0.01	0.01	-	-
Root type	AICc	318.75	314.38	251.03	251.94	248.62	250.05	250.48
	$\Delta$ AICc	70.13	65.76	2.41	3.32	0	1.43	1.86
	Weight	0	0	0.13	0.08	0.42	0.21	0.17
Life cycle	AICc	318.75	320.85	251.03	252.2	251.45	-	-
	$\Delta$ AICc	67.72	69.81	0	1.17	0.42	-	-
	Weight	0	0	0.42	0.24	0.34	-	-

<sup>a</sup> Model notations differ from those of Beaulieu et al. (2012). See Figure 1.2 and Table S2 in Chapter I Supplementary Material for weighted  $\theta$  and standard error for each evolutionary regime.

a significant slope estimated by these models, would suggest that opposite ends of gradients select for different optimal capacities for N use.

For evolutionary models approximating evolution of GRN according to both discrete and continuous selective agents, we quantified the phylogenetic half-life, which is a measure of evolutionary time in millions of years (MY) in which a trait (in our case, GRN) moves half the distance from the ancestral trait value to the trait optimum estimated by Ornstein-Uhlenbeck models (Hansen, Pienaar & Orzack 2008). A long phylogenetic half-life relative to the age of the phylogenetic tree would indicate that the ancestral influence on GRN values linger through evolutionary time and that stabilizing selection is likely weak or absent. A short phylogenetic half-life relative to the age of the phylogenetic tree would indicate that the ancestral influence on GRN values erodes quickly as species evolve and that GRN has evolved according to stabilizing selection (Diniz Filho et al. 2012).

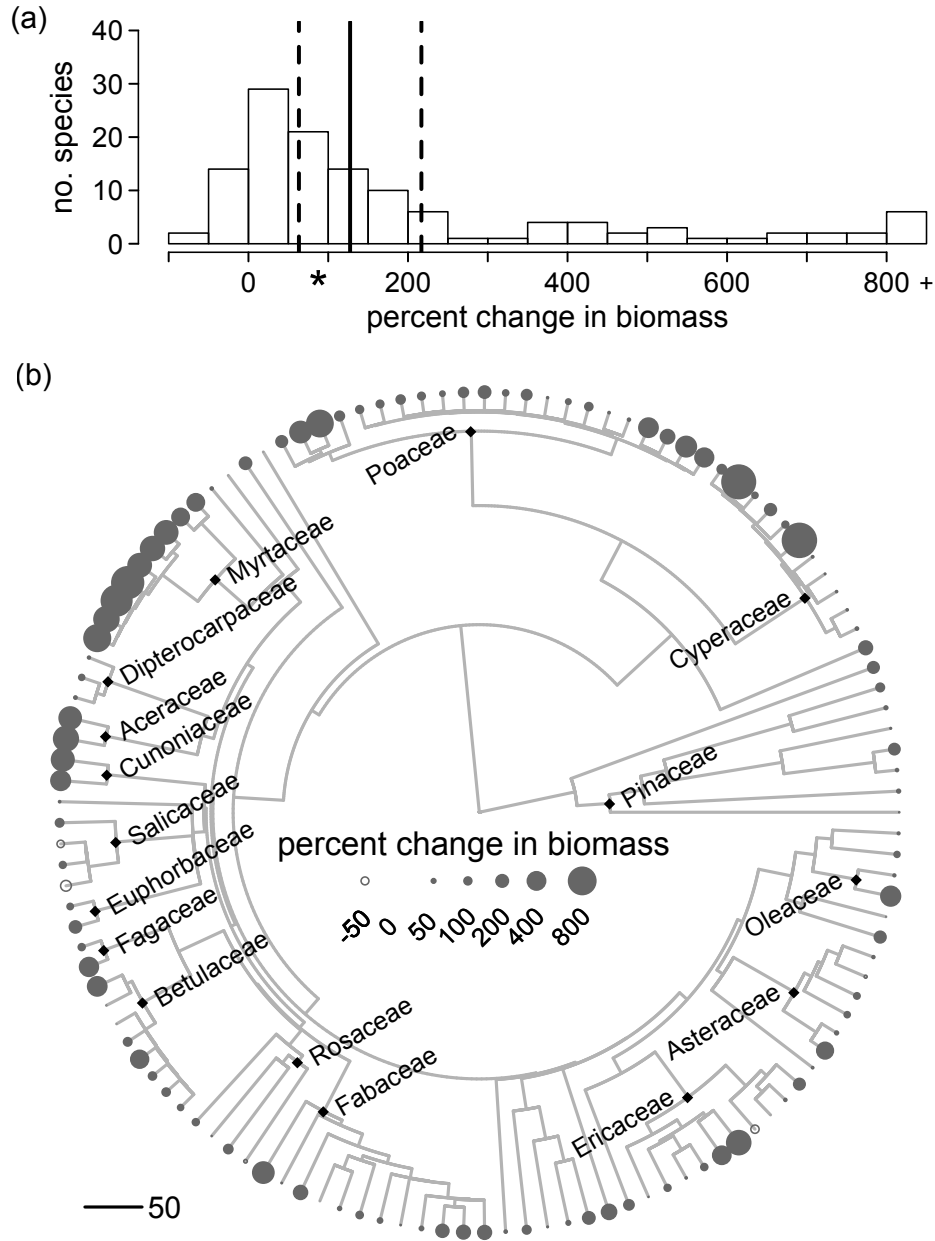
## Results

### *Distribution and phylogenetic signal of GRN*

Consistent with our first hypothesis that species' GRN values vary in direction and magnitude, of 125 total species, 2, 31, and 92 species exhibited negative, neutral, and positive biomass responses to N addition, respectively, with responses ranging from a 60% decrease in biomass (*Salix pulchra*) to a more than 1800% increase in biomass (*Carex vaginata*) (Figure 1.1a). Overall, species gained an average of 128% more total biomass in response to N fertilization with lower and upper standard errors respectively at 63% and 217%, and a median response of an 88% increase in biomass (Fig. 1.1a). The maximum likelihood estimate of phylogenetic signal of GRN was 0.778 (log likelihood = -133.705). GRN was phylogenetically structured (log likelihood of  $\lambda$  equaling 0 = -145.197), but the distribution of observed values of GRN for species across the phylogeny was not representative of evolution according to a Brownian motion process (log likelihood of  $\lambda$  equaling 1 = -157.328) (Fig. 1.1b).

### *Evolutionary models*

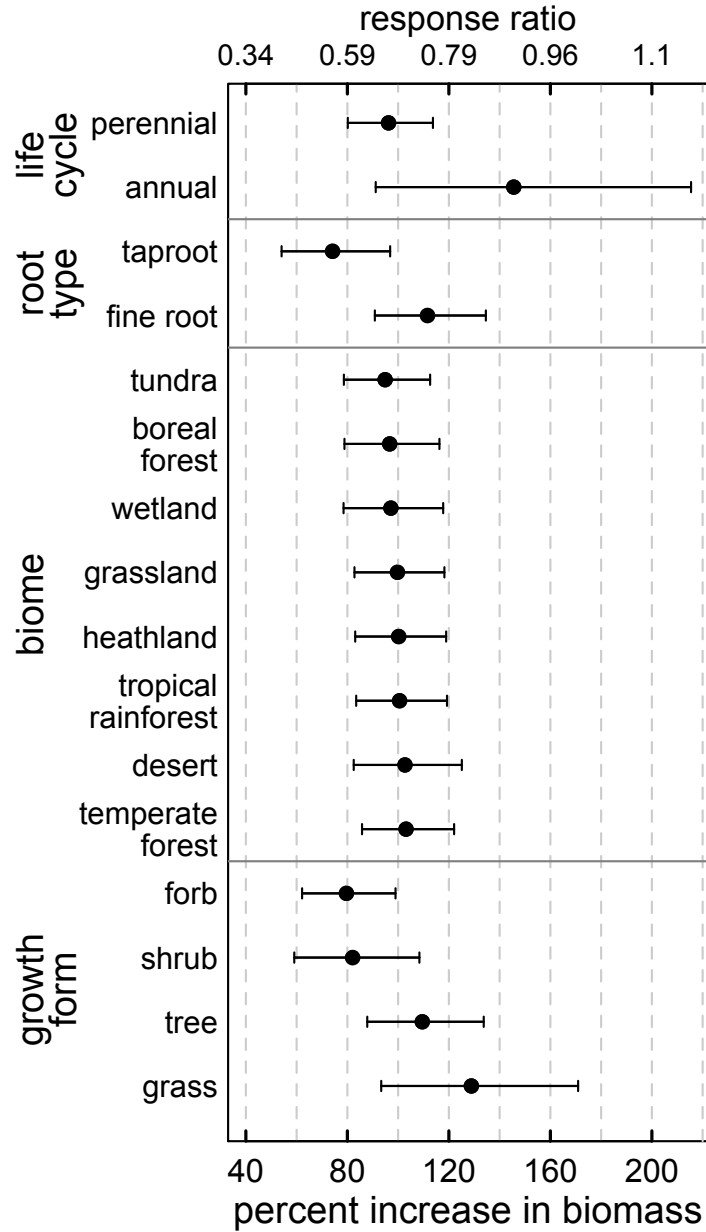
Although Ornstein-Uhlenbeck models explained significantly more variation in GRN than Brownian motion models (Tables 1.1 and 1.2), we found little evidence for our second hypothesis that plants have evolved towards significantly different capacities for N use across selective regimes (Fig. 1.2) or gradients (Fig. 1.3). Overall, weighted parameter estimates are consistent with selection towards positive GRN for each biome, growth form, root type, and life history category (Fig. 1.2) and across absolute latitude, mean annual temperature, and annual precipitation (Fig. 1.3). Although standard errors around these estimates are large, percent increases in biomass were more than one and one-half times greater for: 1) grass species compared to shrub and forb species, 2) species with fine roots compared to species with taproots, and 3) for annual species compared to perennial species (Fig. 1.2). Percent increases in biomass varied little across biomes (Fig. 1.2) due to overwhelming support for the Ornstein-Uhlenbeck model that approximates evolution towards a single evolutionary optimum (OUM model; Table 1.2). Phylogenetic half-lives ranged from 10.25 to 10.81 MY (OUwie models) to 4.88 MY (phylogenetic linear models). See Table S2 for weighted parameter estimates (evolutionary optima, standard errors of evolutionary optima, and phylogenetic half-lives) of OUwie models.



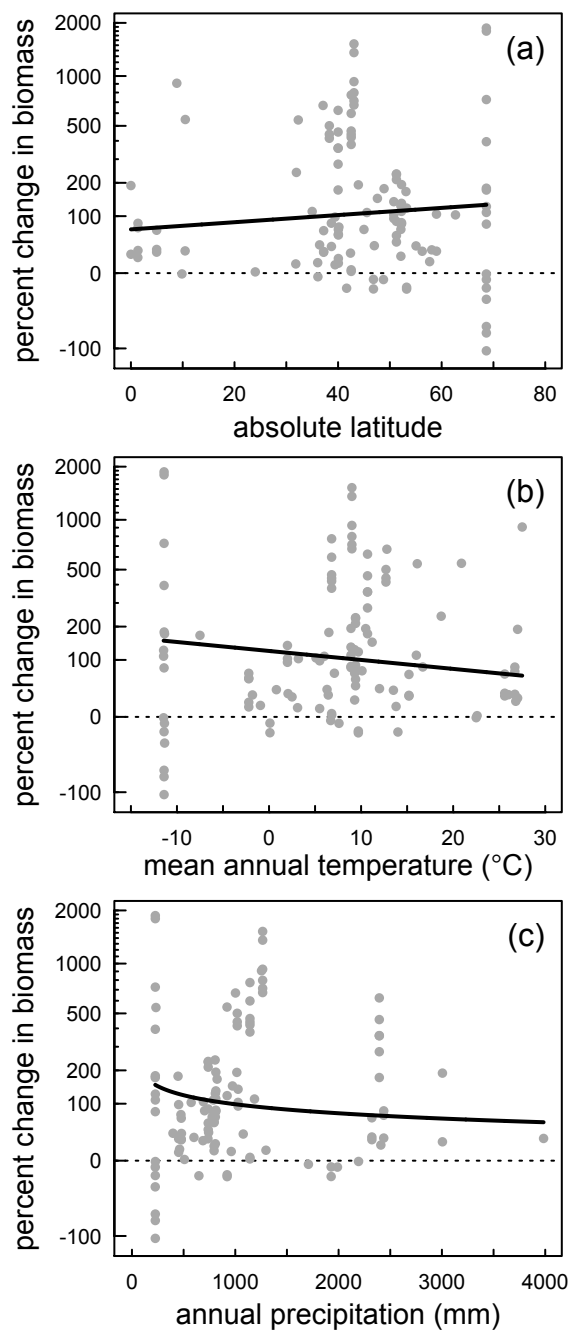
**Figure 1.1. Biomass responses of 125 terrestrial plant species to nitrogen addition vary significantly and are phylogenetically structured.** (a) More than one in four respond negatively or neutrally to experimental nitrogen addition. On average, species gain 128% (solid line) more biomass with added soil nitrogen, with upper and lower standard errors at 63% and 217%, respectively (dashed lines). The asterisk denotes median response to nitrogen (88% increase in biomass). (b) Distribution of responses across the species phylogeny (Pagel's  $\lambda = 0.778$ ). Positive and negative responses are shown as filled and open circles, respectively, across a phylogeny pruned from a recent time-scaled phylogenetic reconstruction of over 30,000 terrestrial plant species (Zanne et al. 2013; 2014). Node labels denote 16 of 36 plant families represented in our dataset. Terminal branch lengths are extended by 15 million years to aid visualization, and the scale bar below the phylogeny represents 50 million years.

**Table 1.2. Ornstein-Uhlenbeck (OU) models that approximate evolution according to genetic drift plus stabilizing selection across absolute latitude, mean annual temperature, and annual precipitation provide significantly better fit for growth responses of 114 plant species to experimental nitrogen addition than Brownian motion (BM) models that approximate evolution according to genetic drift alone.** We report the change in second order Akaike Information Criterion (AICc),  $\Delta$ AICc, slope estimate ( $\beta$ ), and p-value of  $\beta$  (P) for each phylogenetic linear model. Ornstein-Uhlenbeck model weights were 1 in each case.

Model	Gradient											
	Absolute latitude				Mean annual temperature (°C)				Log annual precipitation (mm)			
	AICc	$\Delta$ AICc	$\beta$	P	AICc	$\Delta$ AICc	$\beta$	P	AICc	$\Delta$ AICc	$\beta$	P
BM	416.11	138.15	0.03	<0.001	401.57	125.03	-0.04	<0.001	393.46	116.84	-0.77	<0.001
OU	277.96	0	0.004	0.37	276.54	0	-0.01	0.14	276.62	0	-0.16	0.15



**Figure 1.2. Plant species' nitrogen (N) use capacities are consistent with evolution according to stabilizing selection towards positive values across selective regimes.** Despite large error bars ( $\pm$ SE), estimated evolutionary optima for growth responses to N addition are more than one and one-half times greater for: 1) grass species compared to shrub and forb species, 2) species with fine roots compared to species with taproots, and 3) for annual species compared to perennial species. Numbers next to error bars represent sample size. Species log response ratios of biomass in elevated vs. ambient (control) soil N treatments used in evolutionary modeling (top x-axis) were back-transformed here to percent increases in biomass (bottom x-axis) to aid interpretation.



**Figure 1.3. Plant growth responses to nitrogen addition do not vary significantly across (a) absolute latitude, (b) mean annual temperature, and (c) annual precipitation.** Responses are the percent changes in total biomass from ambient to elevated soil nitrogen conditions for 114 plant species. Regressions incorporate covariance matrices based on an Ornstein-Uhlenbeck process and thus approximate evolution according to drift plus stabilizing selection.



## Discussion

Using published growth data on a total of 125 plant species that represent over 350 million years of evolution, we have shown that species have evolved a continuum of conservative to competitive N use strategies (Fig. 1a). This both complements the worldwide economic spectrum (WES) theory of a universal evolutionary trade-off between the ability of plants to exploit resources and conserve resources (Grime 1977; Chapin 1980; Reich 2014) and challenges the conventional perception that all plants are N-limited (Vitousek & Howarth 1991; LeBauer & Treseder 2008). Our results suggest that a substantial portion of species (more than one in four) produce less biomass or do not respond to N addition. Such species will be vulnerable to population decline and competitive exclusion with N addition (Bobbink et al. 2010; De Schrijver et al. 2011). Our results also corroborate existing evidence that plant responses to global change are generally more similar among more closely related species (Davis et al. 2010; Senior et al. 2013) (Fig. 1b), supporting that phylogenies can be useful tools for predicting species responses to anthropogenic inputs of N to soils. Notably, forest tree species in the Myrtaceae family (seven and two species within *Eucalyptus* and *Amomyrtus* genera, respectively) exhibit greater responses to N on average, which might be an outcome of shared inheritance of adaptations to low concentrations of soil P (Attiwill & Adams 1996). However, this study is the first to demonstrate that trade-offs between conservative and competitive N use strategies are consistent with selection-driven evolution, with more differential selection across plant functional types than across environmental gradients.

Although our results fail to support our hypothesis that different environments are associated with selection for different capacities for N use, we find that plants have experienced stabilizing selection towards positive growth responses to N addition. This outcome is not surprising as N is an important building block in the structures and proteins involved in photosynthesis and is necessary for the production of plant biomass (Evans 1988). Combined with estimates of the phylogenetic half-life of GRN, which indicate that the ancestral influence on trait values erodes within 10 million years of evolution (one-fifth the length of scale bar shown in Fig. 1), our results point to stabilizing selection as a mechanism that has reduced our estimate of phylogenetic signal such that variation in GRN does not fully reflect the amounts of shared evolutionary history among species.

Empirical evidence suggests that temperature, soil moisture, and soil fertility gradients induce a functional trade-off between rapid growth rate and ability to persist where resources are limiting (Cavender-Bares, Kitajima & Bazzaz 2004; Ordoñez et al. 2009; Bobbink et al. 2010; Moles et al. 2014; Zanne et al. 2014). However, we did not identify significantly different selective peaks for N use strategies among biomes or across absolute latitude, temperature, and precipitation gradients (Figs. 2 and 3). Although our power to confirm the absence of selective optima across environments is low, here we have accounted for non-independency due shared evolutionary history. Failing to account for such non-independency may have led to Xia & Wan's (2008) identification of significant decreases in GRN with absolute latitude and increases with annual precipitation, because these patterns might merely be outcomes of closer relatives occurring in more similar environments (Adams 2008). Still, we have found evidence against the prediction that more stressful environments induce species to evolve more conservative growth strategies with respect to N. An explanation for this result is that ecologically similar species are less likely to co-occur due to increased competition (Hardin 1960). Multiple studies have demonstrated that at a global scale, much more variation in plant functional traits (up to one-half) exists within communities than between them (Reich, Walters & Ellsworth 1997; Moles et al.

2014; Freschet et al. 2011). As such, examining selective gradients for N use capacity within separate biomes, where the identity and number of selective agents (including species interactions) that drive trade-offs in growth strategies likely change, could provide a more integrative description of macroevolutionary patterns in GRN. For example, evidence indicates that natural variation in soil N is an important source of selection on functional traits associated with plant growth strategies (Ordoñez et al. 2009). However, the intensity of selection should differ between biomes, for example between tundras, where soil N more strongly limits plant growth, and tropical forests, where soil P and light more strongly limit plant growth (Vitousek et al. 2010).

Our results encourage further research to determine whether plant functional type has imposed different selective constraints on N use strategy. Despite large standard errors around evolutionary optima (Fig. 2), substantial differences in the evolutionary optima themselves (i.e. between grasses and forbs/shrubs, fine- and taproot species, and annuals and perennials) should be taken into consideration. For example, low sample size could have inflated error around the evolutionary optimum for annuals and masked a real difference in the evolutionary optima between annual and perennial life histories. Associations between plant functional types and responses to global change could partially explain the lack of associations between global environmental gradients and responses to global change, given that multiple functional types co-occur within each of these gradients. Our dataset could be expanded by the collection of both a wider range of species' GRN data and other functional traits relevant to the evolutionary trade-off between competitive and conservative growth strategies. For example, identifying correlations between leaf, root, and stem functional traits and plant responses to globally increasing soil N, among other agents of global change, would build upon the connections among past evolution, plant function, and responses to global change established here.

### *Conclusions*

Our synthesis provides a foundation for understanding not only how much variation exists in plant responses to global change, but also how this variation is largely a reflection of a combination of species ecological and evolutionary histories. Specifically, we show that although plants have experienced selection for high N use capacities, closer relatives respond more similarly and selection has operated differently across plant functional types but not across environments (Figs. 2 and 3). As such, conservation-oriented questions should shift towards asking for whom (i.e. specific lineages and functional types), and not where (e.g. specific biomes), the most pressing conservation need exists, given rising inputs of anthropogenic N to soils. A function-based perspective could also aid in efforts to more accurately identify species that exhibit the most positive GRN as the least vulnerable, and the strongest potential drivers of ecosystem function and community interactions, in future global change scenarios. Further research is required to determine how persisting species will realistically interact with one another and with soil microbial communities (van der Putten et al. 2016), yet our study suggests that species within communities will be more phylogenetically related and functionally similar in the future than at present.

Our analysis demonstrates that identifying broad patterns in plant responses to global change factors merits the consideration of biologically relevant models of evolution—including those allowing one or more selective regimes (Ornstein-Uhlenbeck models)—to clarify how past evolution can inform winners and losers in global change scenarios. Further research can expand upon these models and results, to test a 'nitrogen economics spectrum' hypothesis, analogous to

the WES theory, that functional traits themselves have converged in concert in any one taxon upon a slow, medium, or fast strategy for N use. These functional traits will likely have strong impacts on the evolution of plant interactions with their soils and vice versa (Evans et al. 2016; terHorst & Zee 2016). Continued research on plant function in rapidly changing environments, coupled with advances in phylogenetic comparative methods and functional evolution, will elucidate how past evolution governs future alterations of plant community composition and function induced by global change.

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**CHAPTER II**  
**PHYLOGENY IS A POWERFUL TOOL FOR PREDICTING PLANT BIOMASS**  
**RESPONSES TO NITROGEN ENRICHMENT**

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### Abstract

Increasing rates of anthropogenic nitrogen (N) enrichment to soils often lead to the dominance of nitrophilic plant species and reduce plant diversity in natural ecosystems. Yet, we lack a framework to predict which species will be winners or losers in soil N enrichment scenarios, a framework that current literature suggests should integrate plant phylogeny, functional trade-offs, and nutrient co-limitation. Using a controlled fertilization experiment, we quantified biomass responses to N enrichment for 23 forest tree species within the genus *Eucalyptus* that are native to Tasmania, Australia. Based on previous work with these species' responses to global change factors and theory on the evolution of plant resource-use strategies, we hypothesized that (1) growth responses to N enrichment are phylogenetically structured, (2) species with more resource-acquisitive functional traits have greater growth responses to N enrichment, and (3) phosphorus (P) limits growth responses to N enrichment differentially across species, wherein P enrichment increases growth responses to N enrichment more in some species than others. We built a hierarchical Bayesian model estimating effects of functional traits (specific leaf area, specific stem density, and specific root length) and P fertilization on species' biomass responses to N, which we then compared between lineages to determine whether phylogeny explains variation in responses to N. In concordance with literature on N limitation, a majority of species responded strongly and positively to N enrichment. Mean responses ranged three-fold, from 6.21 (*E. pulchella*) to 16.87 (*E. delegatensis*) percent increases in biomass per g N m<sup>-2</sup> yr<sup>-1</sup> added. We identified a strong difference in responses to N between two phylogenetic lineages in the *Eucalyptus* subgenus *Symphyomyrtus*, suggesting that shared ancestry explains variation in N limitation. However, our model indicated that after controlling for phylogenetic non-independence, eucalypt responses to N were not associated with functional traits (although post-hoc analyses show a phylogenetic pattern in specific root length similar to that of responses to N), nor were responses differentially limited by P. Overall, our model results suggest that phylogeny is a powerful predictor of winners and losers in anthropogenic N enrichment scenarios in Tasmanian eucalypts, which may have implications for other species.

### Introduction

Nitrogen (N) is the predominantly limiting nutrient to net primary productivity across terrestrial ecosystems (Vitousek and Howarth 1991, LeBauer and Treseder 2008); however, N does not limit biomass production equally across terrestrial plant species (Bobbink et al. 2010, De Schrijver et al. 2011). For example, Wooliver et al. (2016) found that across 125 terrestrial plant species, average total biomass responses to N enrichment range from -6% to 187% for each gram of N m<sup>-2</sup> yr<sup>-1</sup> added. Such variation in N limitation explains why increased rates of N inputs to plant communities often lead to greater community productivity but lower species richness due to competitive exclusion of less nitrophilic species (Bobbink et al. 2010). This downward trajectory in plant diversity should continue because fossil fuel combustion and agricultural



fertilization are predicted to more than double the rates of anthropogenic N enrichment to soils by the end of the century (Lamarque et al. 2005). Because plant diversity has consequences for above- and belowground biodiversity as well as ecosystem functioning (Zak et al. 2003, Whitham et al. 2006), it is important to determine possible factors associated with variation in plant species' performances under N enrichment scenarios.

Phylogeny may be a predictor of plant responses to N enrichment. Specifically, phylogenetic analyses have shown that plant species' responses to global change are more similar among close relatives than would be expected at random (Edwards et al. 2007, Davis et al. 2010, Becklin et al. 2014, Wooliver et al. 2016). These patterns are likely reflections of plant functional traits that are important in resource use and show phylogenetic signal (Cavender-Bares et al. 2009, Cornwell et al. 2014), suggesting that phylogenies can be used to predict plant winners and losers in N enrichment scenarios. However, phylogenetic patterns in plant responses to N enrichment do not always persist at smaller phylogenetic scales (i.e., groups of more closely related, co-occurring species; Bennett and Cahill 2013), because of greater divergence or convergence of functional traits towards the tips of phylogenies (Ackerly 2004, Cavender-Bares et al. 2009, Cadotte et al. 2013). For example, Cavender-Bares et al. (2004) found that resource-use traits such as shoot transpiration are evolutionarily convergent in a group of 17 oak species that co-occur in North Central Florida, USA. Therefore, functional traits that influence resource-use strategies may serve as better predictors of winners and losers in N enrichment scenarios than phylogeny.

Decades of theoretical and experimental work suggest that plant functional traits directly influence capacities for resource acquisition and use: an outcome of an evolutionary trade-off between resource-acquisitive and resource-conservative growth strategies (Grime 1977, Chapin 1980, Wright et al. 2004, Craine 2009, Reich 2014). According to this theory—termed the plant economics spectrum—species' functional traits (specifically, those affecting resource uptake, transport, and processing capacities; Violle et al. 2007) will evolve to be resource-conservative (low capacity for resource use) in low-resource environments and resource-acquisitive (high capacity for resource use) in high-resource environments as a result of the return on investment in resource acquisition. For example, empirical work has shown that plants occurring on infertile soils have lower-quality foliage, slower photosynthetic and growth rates, and lower requirements for soil nutrients (Grime 1977, Hobbie 1992, Cavender-Bares et al. 2004). Alternatively, species found on fertile soils have higher specific leaf area and faster photosynthetic rates, which accommodate higher growth rates (Reich et al. 1997, Poorter and Bongers 2006, Wright et al. 2010, Cavender-Bares et al. 2004). It might intuitively follow that species with more resource-acquisitive functional traits should exhibit greater biomass responses to N enrichment than species with more resource-conservative functional traits. However, we are unaware of any study that has investigated the associations between plant functional traits and N limitation, especially at smaller phylogenetic scales.

Co-limitation by phosphorus (P) may also govern plant biomass responses to increased soil N (Elser et al. 2007, Harpole et al. 2011). Co-limitation occurs when biomass responses to one nutrient (e.g., N) increase with the addition of another nutrient (e.g., P) (Harpole et al. 2011). Although global anthropogenic P inputs to the biosphere are now four times greater than that of natural chemical weathering (Falkowski et al. 2000), they are generally not sufficient to relieve P limitation under anthropogenic N deposition scenarios (Vitousek et al. 2010). Species' responses to N should be differentially co-limited by P if species have evolved different solutions to deal with co-limitation by P such as increased P use efficiency or the ability to scavenge for P through

associations with fungal symbionts (Cleland and Harpole 2010). In this case, species whose responses to N are less limited by P (expected winners in N enrichment scenarios) should show increases in productivity and abundance over more strongly co-limited species (expected losers in N enrichment scenarios).

Using a group of 23 *Eucalyptus* tree species that are native to Tasmania, Australia, whose responses to global change factors have been shown to be phylogenetically structured (Senior et al. 2013, Wooliver et al. 2014), we examined whether phylogenetic variation in growth responses to N enrichment across plant species is associated with variation in plant functional traits and nutrient co-limitation. Specifically, we tested three hypotheses: (1) phylogenetic groups differ in growth responses to N enrichment; (2) following the plant economics spectrum theory, species with more resource-acquisitive functional traits in ambient nutrient conditions exhibit greater growth responses to N enrichment (i.e., are more N-limited); and (3) P differentially limits biomass responses to N enrichment across species (i.e., P enrichment increases growth responses to N enrichment more in some species than others). To test these hypotheses, we applied factorial N and P fertilization treatments to the Tasmanian eucalypts in a controlled greenhouse environment. For each individual we quantified total biomass and three functional traits: specific root length (SRL), specific stem density (SSD), and specific leaf area (SLA). These traits are proxies for nutrient-uptake capacity, the ability to transport resources from roots to leaves, and photosynthetic activity, respectively (Perez-Harguindeguy et al. 2013). We implemented a hierarchical Bayesian model, which allowed us to estimate multiple unknown parameters (nutrient limitation and the effects of functional traits and P on N limitation) while accounting for uncertainty within and across species. A benefit of this model is that different levels of the hierarchy (i.e., within and across species) inform one another, allowing for better estimation for groups with less information (e.g., species with fewer biomass and functional trait observations due to lower survivability). We expected to find strong differences in biomass responses to N between phylogenetic groups, greater biomass responses to N in species with more resource-acquisitive functional traits (greater SLA, lower SSD, and greater SRL) in control nutrient conditions, and different N by P interaction terms across species. Using a single model to examine the associations of phylogeny, functional traits, and P limitation with plant N limitation, we provide a framework for predicting winners and losers in N deposition scenarios, and more important, test for novel linkages between past evolution and plant performance in global change scenarios.

## Methods

### *Study system*

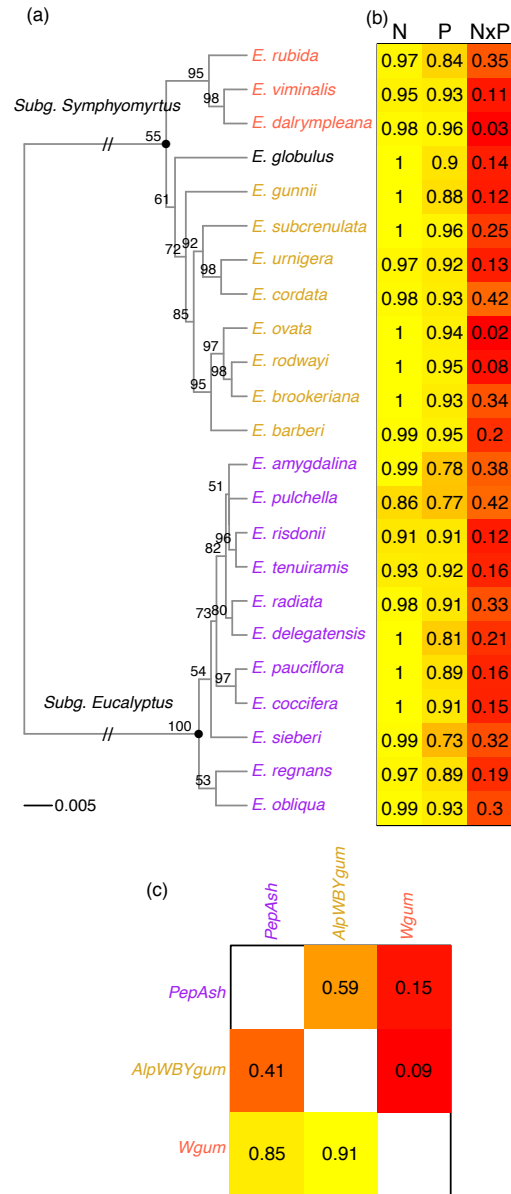
Of the  $\approx 700$  described tree species within the genus *Eucalyptus* (Grattapaglia et al. 2012), 30 are native to the island state of Tasmania, Australia (Williams and Potts 1996, Gray 2008). According to both morphology (Brooker 2000) and genetic data (Steane et al. 2011), the Tasmanian eucalypts belong to two subgenera, *Symphyomyrtus* (470 total species in Australia and 17 in Tasmania) and *Eucalyptus* (108 total species in Australia and 13 in Tasmania; Grattapaglia et al. 2012). We chose to use the Tasmanian eucalypts in our study for three reasons. First, this set of eucalypts contains ecologically diverse and economically important species. The Tasmanian eucalypt species include growth forms from giant rainforest trees to stunted “mallee” shrubs, one of the most widely planted hardwood species across the globe (*E. globulus*), and several rare endemics (Williams and Potts 1996). Because the Tasmanian eucalypts within each subgenus have diverged across multiple soil nutrient, climatic, and

topographical gradients and occupy habitats from coastal to sub-alpine regions and wet to dry sclerophyll woodlands (Williams and Potts 1996), we might expect genetic-based variation in resource-related functional traits within and among species. Second, empirical evidence has shown that species' performances and performance responses to global change are phylogenetically structured. For example, species within the *Symphyomyrtus* subgenus have faster growth rates than those within the *Eucalyptus* subgenus as seedlings and juveniles, which may explain why the former show greater survival and growth rates in a broader range of environments than the latter (Noble 1989, Anekonda et al. 1999). Subgenera also differ in responses to combined treatments of N fertilization and elevated CO<sub>2</sub>, with species in subgenus *Symphyomyrtus* showing the most positive biomass responses to N and CO<sub>2</sub> (Senior et al. 2013, Wooliver 2014). Third, evidence suggests that species differ in nutrient use strategies. *Eucalyptus* species have evolved in the relatively P-limited soils of Australia (Wild 1958), and Noble (1989) speculated that species within subgenus *Eucalyptus* have adapted to P limitation via greater dependence on mycorrhizal fungi than species within the subgenus *Symphyomyrtus*. Further, within Tasmania itself there are gradients in soil N and P, with species differing in mean soil N and P levels in their natural ranges (see Figure S1 in Chapter II Supplementary Material; data from Rossel et al. 2015), which may lead to variation in nutrient requirements for growth. Despite documented phylogenetic patterns in Tasmanian eucalypt growth responses to N enrichment, and indications that functional traits and nutrient use strategies vary across species, no study to date has addressed whether functional traits or differential co-limitation by P explain variation in biomass responses to N enrichment.

#### *Phylogenetic reconstruction*

We reconstructed the phylogeny of the Tasmanian eucalypts using a set of 3,881 parsimony informative Diversity Array Technology (DArT) markers (Jaccoud et al. 2001) and Metropolis-coupled Markov chain Monte Carlo in MrBayes v3.2 (Ronquist and Huelsenbeck 2003). We used DArT markers because they perform well at resolving phylogenetic relationships among *Eucalyptus* species (Jones et al. 2016). Genetic material for DArT markers was collected from individuals grown from seeds purchased from Forestry Tasmania (<http://www.forestrytas.com.au/>; see Table S1 for information on seed collection zones), including the 23 species in the greenhouse experiment (described below), plus four Tasmanian natives not included in the greenhouse experiment (*E. johnstonii*, *E. nitida*, *E. perriniana*, and *E. vernicosa*), and one non-native (*E. nitens*). The native Tasmanian species *E. archeri*, *E. morrisbyi*, and *E. nebulosa* were not included because seed was either not available or mislabeled. Full details of the phylogenetic reconstruction are provided in Appendix S1.

After pruning out the species not used in our experiment, our phylogeny correctly placed species into their respective subgenera (Fig. 2.1a). Further, species within the subgenus *Symphyomyrtus* formed two phylogenetic lineages—1) white gums and 2) alpine white, black, and yellow gums—while species within the subgenus *Eucalyptus* formed a third phylogenetic group composed of peppermints and ashes (Fig. 2.1a). We used these groupings to address whether phylogenetic groups differ in responses to N enrichment. Because the placement of *E. globulus* (the Tasmanian Blue Gum) into either of the gum lineages is uncertain (for this and other phylogenetic reconstructions, e.g. Steane et al. 2011, Senior et al. 2013, Jones et al. 2016), we excluded it from the lineage-level comparisons described below.



**Figure 2.1. Nitrogen (N) and phosphorus (P) limitation and co-limitation across 23 eucalypt species that are native to Tasmania, Australia.** (a) The species phylogeny, with percent posterior probabilities shown at the top left of each node. To aid visualization we have inserted a break in the branches connecting the subgenera (subg.), both of which constitute >98% of the phylogeny's arbitrary height of one (note the scale bar below the phylogeny). Species are colored by phylogenetic lineages, which include the white gums (red), alpine white, black, and yellow gums (gold), and peppermints and ashes (purple). (b) Percent of iterations in our Bayesian model in which N, P, and N × P coefficients for plant biomass were greater than zero for each species. Values are colored by a gradient from yellow (positive effect of nutrients) to red (negative effect of nutrients). (c) Percent of iterations in our Bayesian model in which N coefficients for biomass were greater for each column lineage compared to each row lineage. Lineages correspond to the phylogeny in (a). Values are colored from yellow (more positive responses to N) to red (more negative responses to N).

### *Greenhouse experiment*

We established a controlled greenhouse experiment at the University of Tennessee, Knoxville, in which we applied factorial treatments of N and P fertilization to 23 Tasmanian eucalypt species (12 in subgenus *Symphyomyrtus* and 11 in subgenus *Eucalyptus*), with five replicates per treatment/species combination arranged in a completely randomized design. We conducted the experiment in a greenhouse rather than a field setting to isolate genetic variation in plant functional traits and biomass production. Of the 1,150 plants (5 N fertilization levels x 2 P fertilization levels x 23 species x 5 replicates), 931 survived; we collected SLA, SSD, SRL, and total biomass data from 722. Individuals were grown from seeds purchased from Forestry Tasmania (see Table S1 for information on seed collection zones). For germination, we soaked seeds of each species in a petri dish between two pieces of Whatman number 1 filter papers (110 mm diameter; Whatman, Maidstone, Kent, UK) with 10 mL of 100 ppm gibberellic acid solution. After 24 h we spread the seeds over a 5 cm layer of moist non-fertilized potting soil (General Purpose Pro-Mix® BX, Premier Horticulture Inc.) and covered the seeds with another 0.5 cm layer of the potting soil. After 2 weeks of growth we transplanted 50 seedlings per species into separate 270-cm<sup>3</sup> pots containing the non-fertilized potting soil and initiated monthly N (urea) treatments at each of five rates (0, 1.2, 2.4, 5, and 10 g m<sup>-2</sup> yr<sup>-1</sup>) to 10 individuals of each species. Though exceeding the predicted global average rate of N deposition to forests by the end of this century (~0.9 g N m<sup>-2</sup> yr<sup>-1</sup>, Lamarque et al. 2005), these rates fall within the range of current N deposition rates across the globe, which reach up to 17 g N m<sup>-2</sup> yr<sup>-1</sup> (Berendse et al. 1993). To determine whether species' responses to N are differently co-limited by P, we applied P (Triple Super Phosphate, Bonide Products Inc., Oriskany, New York) at the rate of 1.2 g m<sup>-2</sup> yr<sup>-1</sup> (a rate which relieves P limitation in Australian plantations; May et al. 2009) to five individuals of each species within each N application rate. After five months of growth we transplanted individuals into 2 L pots containing the non-fertilized potting soil to avoid root-binding. For the duration of the experiment (11 months) individuals were watered three times weekly to keep the soil near field capacity (moist but not saturated) and to prevent plant growth limitation by water. Daily temperatures in the greenhouse varied between 21 and 24 °C. We acknowledge that soil microbial communities in this study came only from local greenhouse sources and thus were not representative of soil microbial communities in Tasmania. Although such communities—especially mycorrhizal communities of both arbuscular or ectomycorrhizal fungi that dually colonize eucalypts (Adams et al. 2006)—play major roles in plant responses to nutrient additions (Johnson 1993), testing the effects of soil microbial communities on eucalypt performance was outside the scope of this study.

### *Data collection*

To quantify species' biomass responses to N and P and interspecific variation in functional traits, we measured SLA, SSD, SRL, and total biomass in December 2014 (after 11 months of nutrient treatments) for each individual in the greenhouse experiment following the sampling methods of Perez-Harguindeguy et al. (2013). We measured SLA by averaging the area (cm<sup>2</sup>) of two randomly selected, relatively young but fully expanded, outer canopy leaves on the terminal shoot per individual divided by their respective masses (g) after being oven-dried at 70 °C for 72 hours. We measured SSD by dividing the oven-dried mass (mg) of a 5 to 10 cm section of the main stem by its fresh volume (mm<sup>3</sup>), excluding individuals with a main stem less than 5 cm long (91 of the 931 surviving individuals). We measured SRL by dividing the total length (cm) of the root system (scanned in a 20 x 15 cm tray and quantified using WinRhizo, Regent

Instruments Inc., Quebec, Canada) by its oven-dried mass (g). For individuals with more roots than could be scanned without obscuring underlying roots (253 of the 931 surviving individuals), we randomly sampled and scanned a ~1.5 g (fresh weight) subsection to quantify SRL. A linear model suggested that SRL of individuals with whole root systems scanned was significantly greater than individuals with random root sections scanned, but this is to be expected as plants with lower biomass—those with whole root systems scanned—tend to have finer roots (Kallioikoski et al. 2010). For each individual, we summed the leaf mass used to calculate SLA, stem mass used to calculate SSD, oven-dried root biomass used to calculate SRL, and the remaining above- and belowground oven-dried biomass to obtain total biomass. Because species grew under the same conditions, we considered the functional traits of individuals receiving no additional N or P to represent genetic variation and thus species' placements along the resource-acquisitive to resource-conservative growth strategy spectrum. The dataset of SLA, SSD, SRL, and total biomass of all individuals in the experiment are provided in Data S1.

### Analysis

To estimate species' biomass responses to N enrichment and whether these responses are associated with shared ancestry, specific suites of functional traits, and P limitation, we created a hierarchical Bayesian model. Here we provide a broad overview of the model's structure that is essential to understanding how we tested our hypotheses. Model specifications are provided in Appendix S2, with descriptions of data, index, and parameters descriptions listed in Tables S2 and S3.

We modeled biomass and functional trait data of individuals for which all values were present ( $n_{\text{obs}} = 722$  of the original 1150 individuals), log-transforming biomass and functional traits before analysis to increase conformance to normality. For each individual ( $i$ ) of each species ( $j$ ), log-biomass ( $y_i^{(j)}$ ) and log-functional traits ( $\vec{w}_i^{(j)}$ ) were modeled as:

$$y_i^{(j)} \sim \text{Normal}(\vec{\beta}_j \mathbf{X}_i, \sigma_j^{[y]}) \quad (1)$$

$$\vec{w}_{i,\ell}^{(j)} \sim \text{Normal}(\vec{\phi}_{\ell,j} \mathbf{X}_i, \sigma_{\ell,j}^{[w]}) \quad (2)$$

$\vec{\beta}_j$  is a vector of biomass coefficients (intercept, response to N, response to P,  $N \times P$  interaction) for species  $j$  and  $\mathbf{X}$  is the corresponding matrix of predictors.  $\Phi$  is a 3-dimensional array of coefficients that represent responses of each functional trait  $\ell$  for each species  $j$  to  $\mathbf{X}$ . We hierarchically modeled species-level  $\vec{\phi}_{\ell,j}$  coefficients around a central value ( $\vec{\phi}_{\ell}^*$ ) so that parameter estimates would be partially pooled across species (Gelman and Hill 2007). The standard deviations ( $\sigma$ ) were similarly modeled. To model the effect of functional traits on biomass responses to N, the  $\beta$  coefficients were estimated with a second-level regression.

$$E(\beta_{j,k}) = \gamma_k + \vec{\delta}_k \cdot \vec{\phi}_{j,k=1} \quad (3)$$

The expected value of  $\beta_{j,k}$  is determined by the overall intercept for predictor  $k$  ( $\gamma_k$ ), the species-level intercepts for each functional trait ( $\vec{\phi}_{j,k=1}$ ), and the effect of  $\phi_{\ell,j,1}$  on  $\beta_{j,k}$  ( $\delta_{k,\ell}$ ). The actual values of  $\beta_{j,k}$  were modeled with a matrix normal distribution that allowed for covariance structures: correlation among coefficients within species ( $\Sigma^{[\beta]}$ ) and phylogenetic correlation among species ( $\Sigma^{[\lambda]}$ ). A phylogenetic covariance matrix  $\mathbf{V}$  was derived from our phylogeny and treated as a fixed parameter. Within the model,  $\Sigma^{[\lambda]} = \mathbf{V}$ , except the off-diagonal elements were scaled by  $\lambda$  (Pagel 1999), a parameter that accounts for phylogenetic covariance between biomass and functional traits and ranges from 0 (no phylogenetic covariance between biomass

responses to N and traits) to 1 (covariance between biomass responses to N and traits matching phylogenetic distances among species).

The model was implemented using Stan (Carpenter et al. 2016), a system for Bayesian inference that uses Hamiltonian Monte Carlo to efficiently sample the posterior distribution (Hoffman and Gelman 2014), via the rstan package (v2.11.1; Stan Development Team 2016) in R (v3.3.0; R Foundation for Statistical Computing 2016). We ran 3 chains for 2000 iterations each, with the first 1000 used for adaptive warmup. We checked for convergence using the  $\hat{R}$  statistic (Gelman and Hill 2007) and by examining trace plots. We examined model fit with posterior predictive checks. See Data S1 for the model code and R code used in model implementation and hypothesis testing (described below).

### *Hypothesis testing*

We characterized the strength of parameters by calculating the percentage of posterior iterations in which the parameter is positive (or in specified cases, negative). Post-hoc pairwise differences between species, lineages, or subgenera for a parameter were calculated as the percent of iterations in which each species, lineage, or subgenus had a greater value than each other species, lineage, or subgenus. For all parameters or comparison between parameters, we used the value of 90% to represent a strong effect or difference. Before testing our hypotheses, we examined  $\vec{\beta}_N$ ,  $\vec{\beta}_P$ , and  $\vec{\beta}_{NP}$  (the coefficients for species-level biomass responses to N, P, and N+P, respectively) to quantify nutrient limitation and co-limitation for each species.  $\vec{\beta}_N$ ,  $\vec{\beta}_P$ , and  $\vec{\beta}_{NP}$  coefficients that were strongly positive for a particular species would suggest N-limitation, P-limitation, and nutrient co-limitation, respectively, in that species. Alternatively,  $\vec{\beta}_N$ ,  $\vec{\beta}_P$ , and  $\vec{\beta}_{NP}$  coefficients that were strongly negative would suggest that plants suffer from non-optimal nutrient balance due to factors such as nutrient toxicity (Goyal and Huffaker 1984) or decreased benefit from mycorrhizal fungi (Johnson 1993).  $\vec{\beta}_N$ ,  $\vec{\beta}_P$ , and  $\vec{\beta}_{NP}$  coefficients that were neither strongly positive nor negative would suggest that plant growth is neither limited nor co-limited by N and P.

*Hypothesis 1:* To test whether phylogenetic groups differ in growth responses to N, we averaged all iterations of  $\vec{\beta}_N$  across species of each subgenus and across species of each lineage; we then performed pairwise comparisons for  $\vec{\beta}_N$  between subgenera and lineages. Strong differences in responses to N would support our hypothesis that phylogenetic groups differ in growth responses to N.

*Hypothesis 2:* To test whether more N-limited species are those with more resource-acquisitive functional traits, we examined the effects of  $\vec{\phi}_{k=1}$  on  $\beta_N$  ( $\vec{\delta}_{k=2}$ , hereafter referred to as  $\vec{\delta}_N$ ).  $\delta_{N,\ell}$  can be interpreted as the change in  $\vec{\beta}_N$  (i.e., the response of log-biomass to N) with each unit increase in the species-level average of log functional trait  $\ell$ . Strongly positive (>90% iterations greater than zero)  $\delta_{N,\ell}$  for SLA and SRL and negative (>90% iterations less than zero)  $\delta_{N,\ell}$  for SSD would support this hypothesis. Alternatively,  $\delta_{N,\ell}$  coefficients which are neither strongly positive nor negative would suggest that capacity for nutrient uptake from soils (indicated by SRL), capacity for nutrient transport from roots to leaves (indicated by SSD), and photosynthetic activity (indicated by SLA) do not influence N use capacity. If functional traits are phylogenetically conserved (e.g. greater on average in one lineage compared to another), our model would identify functional trait effects on biomass responses to N enrichment as effects of phylogeny.

*Hypothesis 3:* To test whether P differentially limits species' responses to N, we performed pairwise comparisons of  $\vec{\beta}_{NP}$  among species. Strong differences would support the hypothesis that P differentially co-limits species' responses to N enrichment.

## Results

### *Nutrient limitation and co-limitation across species*

Overall, most eucalypt species responded positively to increasing N addition (Figs. 2.1b and 2.2). The estimated responses to N enrichment were strongly positive for 22 out of 23 species (Fig. 2.1b). When back-transformed, mean responses ranged three-fold: from 6.21 (*E. pulchella*) to 16.87 (*E. delegatensis*) percent increases in biomass with each  $\text{g N m}^{-2} \text{yr}^{-1}$  added. Mean percent increases in response to P enrichment were larger than those in response to N enrichment: back-transformed mean responses ranged two-fold from 20.80 (*E. sieberi*) to 40.07 (*E. coccifera*) percent increases in biomass with  $1.2 \text{ g P m}^{-2} \text{yr}^{-1}$  added (compare biomass at low vs. high P at  $0 \text{ g N m}^{-2} \text{yr}^{-1}$  in Fig. 2.2). However, a fewer number (only 15) of the 23 species showed strong positive responses to P enrichment than to N enrichment (Fig. 2.1b). In contrast to expectations, P enrichment did not strongly affect species' responses to N enrichment; the percent of iterations in which the NxP interaction term was greater than zero did not reach 90% for any species (Fig. 2.1b; compare slopes of biomass across N between low and high P in Fig. 2.2). Rather, for three species, *E. dalrympleana*, *E. ovata*, and *E. rodwayi*, P addition strongly *reduced* biomass responses to N enrichment (Fig. 2.1b). For example, the mean responses of *E. dalrympleana* to N enrichment without and with P addition were 7.48 and 1.30 percent increases with each unit N, respectively.

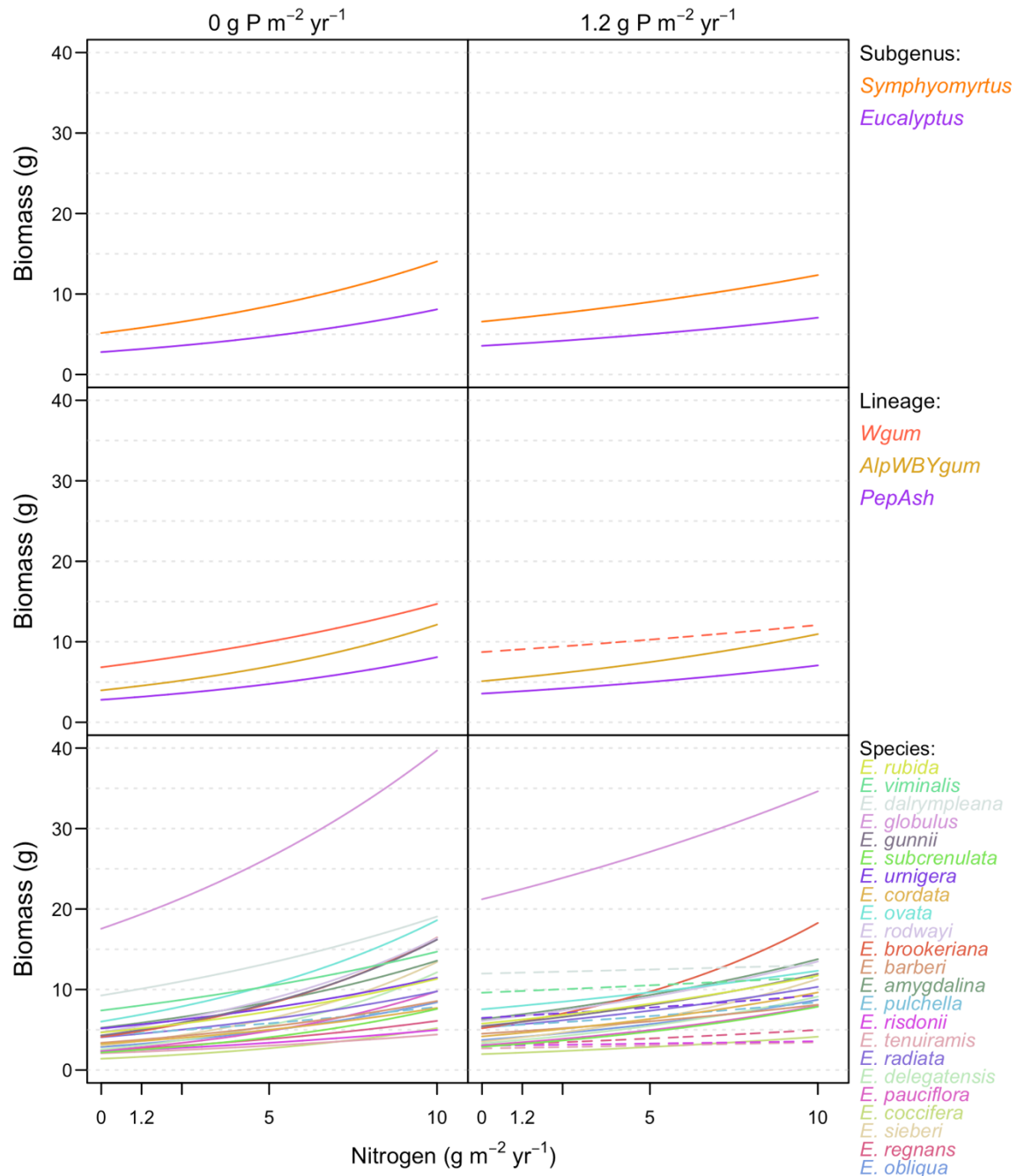
### *N limitation across phylogenetic groups*

In support of our first hypothesis, pairwise comparisons of lineage responses to N revealed that for 91% of iterations, the alpine white, black, and yellow gum lineage responded more to N than the white gum lineage (Fig. 2.1c). When back-transformed, mean percent increases in biomass with each  $\text{g N m}^{-2} \text{yr}^{-1}$  added were 11.80 (alpine white, black, and yellow gums), 11.21 (peppermints and ashes), and 8.01 (white gums). The pairwise comparison between subgenera revealed no strong difference in subgenus responses to N; for 61% of iterations, species within subgenus *Eucalyptus* responded more strongly to N than species within subgenus *Symphomyrtus*.

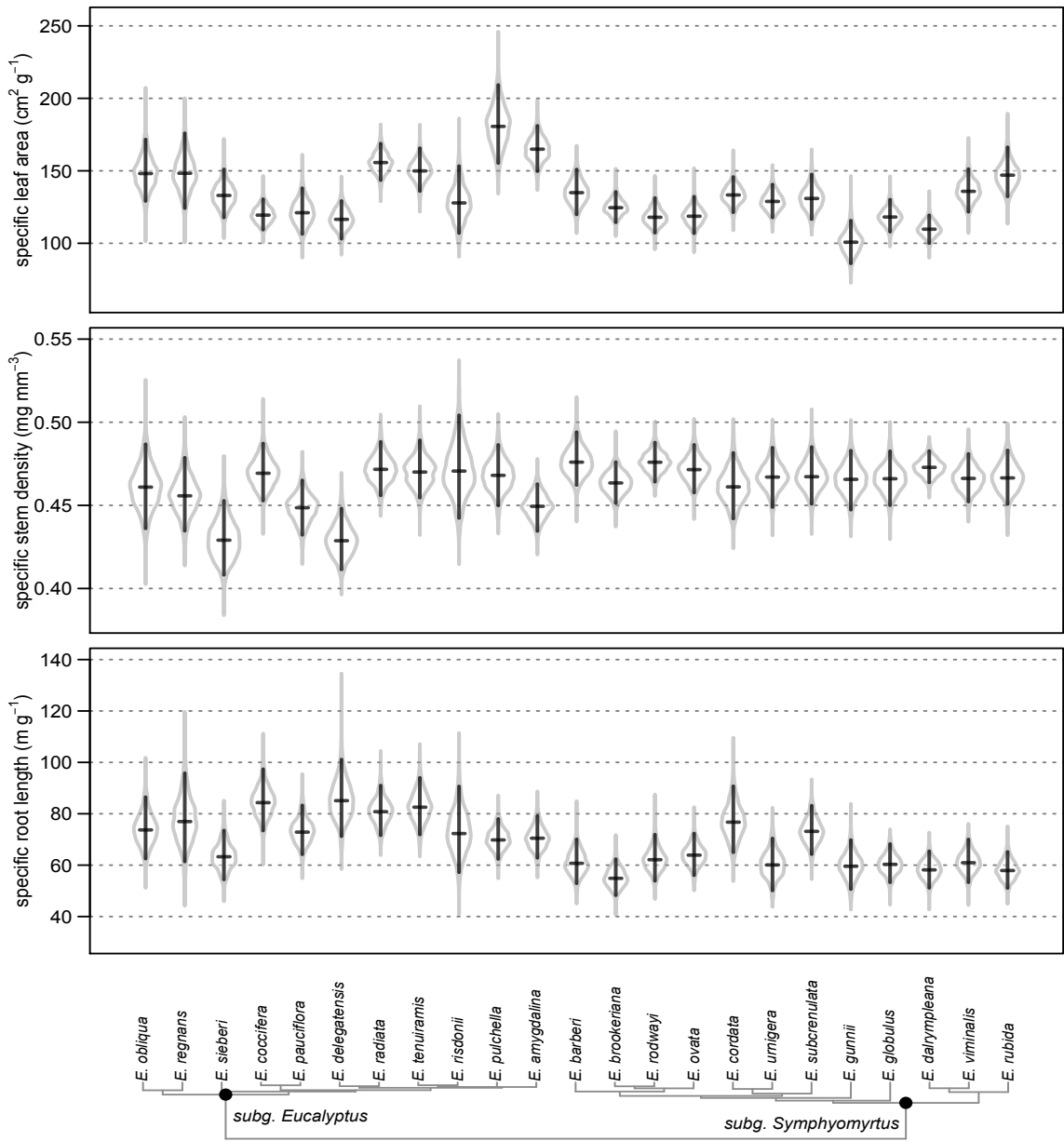
### *Associations between functional traits and N limitation*

Species' mean SLA intercepts estimated from the Bayesian model varied from  $101 \text{ cm}^2 \text{g}^{-1}$  (*E. gunnii*) to  $181 \text{ cm}^2 \text{g}^{-1}$  (*E. pulchella*); mean SSD intercepts varied from  $0.428 \text{ mg mm}^{-3}$  (*E. sieberi*) to  $0.477 \text{ mg mm}^{-3}$  (*E. barberi*); and mean SRL intercepts varied from  $5,487 \text{ cm g}^{-1}$  (*E. brookeriana*) to  $8,548 \text{ cm g}^{-1}$  (*E. delegatensis*) (Fig. 2.3). However, in contrast to our second hypothesis, we found no strong associations between species' functional traits and biomass responses to N enrichment (Fig. S2). This was after we corrected for covariation between species' functional traits and species' biomass coefficients due to phylogenetic non-independence (mean  $\lambda = 0.203$  with 95% credibility interval between 0.017 and 0.5570). The mean effect of SLA was  $-0.12$ , with 20% of iterations estimating effects *greater* than 0; the mean effect of SSD was  $-0.45$ , with 77% of iterations estimating effects *less* than 0; and the mean effect of SRL was  $-0.01$ , with 47% of iterations estimating effects *greater* than 0 (Fig. S2).





**Figure 2.2. Biomass response curves showing nitrogen (N) limitation and co-limitation by phosphorus (P) across 23 eucalypt tree species that are native to Tasmania, Australia.** Response curves represent changes in biomass across a gradient of N ( $0\text{-}10 \text{ g m}^{-2} \text{ yr}^{-1}$ ) with and without P enrichment (right and left, respectively). Responses are shown at the subgenus (top), lineage (middle), and species (bottom) levels. Lineages include peppermints and ashes (PepAsh), alpine white, black, and yellow gums (AlpWBYgum), and white gums (Wgum). Solid and dashed lines represent plant responses to N that were strong (>90% of posterior iterations from our Bayesian model greater than 0) and weak (<90% of posterior iterations from our Bayesian model greater than 0).



**Figure 2.3. Species-level variation in functional traits across Tasmanian eucalypts.** Traits include specific leaf area (SLA; top panel), specific stem density (SSD; middle panel), and specific root length (SRL; bottom panel) in standard potting mix with no additional nutrients (i.e., the trait intercepts in our Bayesian model). Species are arranged according to their phylogeny (shown below). Vertical black lines represent 95% credibility intervals.

### *Nutrient co-limitation across species*

In contrast to our third hypothesis, pairwise comparisons of NxP coefficients did not reveal strong differences between species. That is, for no pair of species did NxP coefficients of one species exceed those of another species for more than 90% of iterations (Fig. S3).

## **Discussion**

We found that phylogeny is an important tool for predicting plant responses to N enrichment. Specifically, we found overall that Tasmanian eucalypt species are N-limited (Figs. 2.1b and 2.2) but vary three-fold in their responses to N enrichment, corroborating evidence that continued anthropogenic increases in soil N will lead to greater community productivity (Kulmatiski et al. 2007, LeBauer and Treseder 2008) and lower species richness through competitive exclusion of less nitrophilic species (Bobbink et al. 2010, De Schrijver et al. 2011). Post-hoc pairwise comparisons among phylogenetic lineages showed that on average some groups have stronger responses to N addition than others (Fig. 2.1c), suggesting that phylogeny can be used to predict winners and losers in future N deposition scenarios. We found that although species vary in functional traits that are commonly used to represent resource-use strategies (Fig. 2.3), these traits do not underlie variation in responses to N enrichment (Fig. S2). This finding suggests that the plant economics spectrum (Grime 1977, Chapin 1980, Wright et al. 2004, Craine 2009, Reich 2014) will not be a useful tool to predict winners (more nitrophilic species) and losers (less nitrophilic species) for Tasmanian eucalypt communities, and perhaps other communities, under increased rates of N deposition. Finally, we found that P does not differentially limit species' responses to N enrichment (Fig. S3). This indicates that, although Tasmanian eucalypt species have evolved in P-limited soils (Wild 1958), their solutions for dealing with limitation by P (Cleland and Harpole 2010) do not generate strong variation in how P affects seedling performances in future N deposition scenarios.

### *Effects of phosphorus on N limitation*

In accordance with evidence that productivity can be limited by N or P (Elser et al. 2007, Harpole et al. 2011), we found overall that Tasmanian eucalypt species respond positively to N or P enrichment. Thus, N and P limitation may play a role in governing eucalypt seedling productivity and increasing rates of anthropogenic N enrichment of soils should increase productivity in eucalypt forests. However, in contrast to evidence that plant growth can be co-limited by soil N and P (Elser et al. 2007, Harpole et al. 2011), we found that P addition does not strongly increase responses of Tasmanian eucalypts to N enrichment overall (Figs. 2.1b and 2.2). In fact, responses of three species to N may be reduced by P enrichment (Fig. 2.1b), a sub-additive nutrient response (i.e., a change in biomass in response to both N and P enrichment that is less than the sum of the responses to each nutrient added independently) that previous work suggests is not uncommon in community-level productivity (Harpole et al. 2011).

Sub-additive responses to N and P fertilization in eucalypts could stem from several factors which may not be mutually exclusive. One such factor might be the interaction between plants and mycorrhizal fungi, on which eucalypts have been shown to depend strongly for nutrient acquisition (e.g., Bougher et al. 1990). Eucalypts are known plant genera that form dual associations with both arbuscular mycorrhizal (AM) and ectomycorrhizal (EM) fungi, generally forming associations predominantly with AM fungi as seedlings and switching to EM fungal associations as they mature (Adams et al. 2006). The timing of this switch varies across species (dos Santos et al. 2001) and may be an important factor driving variation in species' responses to

nutrient enrichment. Though we have pursued subsequent fertilization experiments that include live soil inocula collected from trees in Tasmania to understand how native soil microbiomes influence eucalypt responses to N (which we plan to publish in separate manuscript), inoculating the plants with mycorrhizal fungi from their natural ranges in Tasmania was not possible for this study. Rather, spores of ‘weedy’ mycorrhizal fungi that are not typically representative of natural communities are common in greenhouse settings. Species whose responses to N appear to be reduced by P enrichment may have shown such responses due to a shift in mycorrhizal fungal communities from mutualistic at low soil P levels to parasitic at high soil P levels (Johnson 1993). Alternatively, the overall growth of plants in this study may have not been compatible with (and potentially not as much affected by) foreign mycorrhizal fungal communities, as shown in previous inoculation experiments with *Eucalyptus* (Malajczuk et al. 1982, Burgess et al. 1994), due to co-evolution with unique mycorrhizal fungal communities during long periods of geographic isolation in Australia (Bougher 1995). The impact of mycorrhizal fungi (especially those that commonly associate with Tasmanian eucalypts) on resource uptake was beyond the scope of this experiment, but future work to inoculate eucalypts with native soil fungi, score mycorrhizal colonization, and sequence soils for fungal DNA in fertilization experiments could provide insight into whether soil fungi mediate the responses of Tasmanian eucalypt seedling responses to soil nutrient enrichment. Alternatively, high levels of N and P may have led to nutrient toxicity (Goyal and Huffaker 1984), where uptake of excess nutrients becomes metabolically and physiologically costly, or to increased photosynthesis, transpiration losses, and soil moisture loss, thereby exacerbating limitation by soil moisture (W. S. Harpole, personal communication).

Sub-additive eucalypt responses to N and P fertilization have important implications for our understanding of plant responses to nutrients in both agricultural and natural settings. For example, *E. globulus*, because it is fast-growing and has large flowers that facilitate breeding studies, is one of the most prominent species for pulpwood production across the globe (Williams and Potts 1996). We identified a moderately reduced response to N enrichment with the addition of P fertilizer (Figs. 2.1b and 2.2), indicating that the N + P fertilizers used in agricultural trials (reviewed by May et al. 2009) may lead to less production than would fertilizers including N or P alone. Although our results suggest that P limitation may be an important factor governing responses to anthropogenic increases in soil N for some eucalypt species, co-limitation by P may not drive differential responses to N in these scenarios.

#### *Associations between functional traits and N limitation*

The plant economics spectrum theory argues that a plant’s evolved capacity for resource use reflects an evolutionary trade-off between resource-acquisitive and resource-conservative growth strategies (Grime 1977, Chapin 1980, Wright et al. 2004, Craine 2009, Reich 2014). Recent work has demonstrated that functional traits that predict where species fall along the resource-acquisitive to resource-conservative spectrum contribute to plant responses to global change. For example, plant species with more resource-acquisitive functional traits (including high photosynthetic capacity and short leaf lifespan) at ambient temperatures gain more biomass in response to warming (Gornish et al. 2014). The eucalypt species in this study show strong genetic variation in their functional traits (Fig. 2.3), suggesting that they occupy different places along the resource-acquisitive to resource-conservative spectrum. However, we find that Tasmanian eucalypts not only represent a small portion of phylogenetic variation but also span only a small portion of the range of functional traits observed across terrestrial plant species

(from 9 to 1137 cm<sup>2</sup> g<sup>-1</sup> for SLA, from nearly 0 to 1.331 mg mm<sup>-3</sup> for SSD, and from 608 to 75,681 cm g<sup>-1</sup> for SRL; Kattge et al. 2011). Such small variation in functional traits may explain why our model revealed no strong associations between resource-use traits and growth responses to N enrichment (Fig. S2), nor between resource-use traits and biomass production in control nutrient conditions, as might also be predicted by the plant economics spectrum theory. Given the global variation in terrestrial plant functional traits (Kattge et al. 2011) and responses to N enrichment (Wooliver et al. 2016), the hierarchical Bayesian model implemented in this study should recover any significant associations that truly exist between functional traits and growth responses to N enrichment should it be broadened to an analysis of plant species across the globe.

#### *Phylogenetic variation in N limitation*

Our results corroborate previous evidence showing that phylogenies are important for explaining variation in plant species' growth responses to N enrichment (Wooliver et al. 2016). Specifically, we show that species within the white gum lineage respond less positively to N than do species within the other two lineages (Figs. 2.1b and 2.2). Such a pattern indicates that species within the white gum lineage may share functional traits that limit the ability to use excess N for growth compared to species within the other two lineages. Although our analysis revealed that responses to N are not associated with leaf, stem, or root functional traits, additional post-hoc pairwise comparisons showed that species within the white gum lineage have consistently lower SRL (indicative of a more resource-conservative growth strategy; Reich 2014) in control nutrient conditions than species within the other two lineages (Fig. S4) which we note is likely not an artifact of a lower proportion of individuals whose whole vs. subsampled root system was scanned to calculate SRL (Fig. S5). This pattern may not have been detected in the Bayesian model because the model controls for phylogenetic covariance between functional traits and biomass responses to N enrichment. That is, our model is constructed so that effects of functional traits on biomass responses to N enrichment are interpreted as effects of phylogeny should those traits be phylogenetically conserved. We also note that lower SRL may contribute to greater growth of white and blue gums overall, notably the Tasmanian blue gum *E. globulus* (Fig. 2.2), and may underlie the greater overall growth rates of species within the subgenus *Symphyomyrtus* than those within the *Eucalyptus* subgenus seen here and in previous studies (Noble 1989, Anekonda et al. 1999). Together these results suggest that traits determining species' positions along a root economics spectrum are phylogenetically-based and that this phylogenetic basis to trait variation could underlie species responses to anthropogenic N enrichment of soils.

Additional post-hoc pairwise comparisons revealed that there are also strong species-level differences in responses to N enrichment (Fig. S6). For example, the response of *E. brookeriana* (black gum) to N enrichment was greater than responses of *E. viminalis* (white gum), *E. dalrympleana* (white gum), *E. globulus* (blue gum), and *E. urnigera* (alpine white gum) for 90% or more of iterations in the model. However, long-term N fertilization trials in natural eucalypt communities are required to determine whether nitrophilic eucalypt species will competitively exclude other, less nitrophilic species under future N deposition scenarios (Bobbink et al. 2010). These data overall suggest that who will win and who will lose in these scenarios, as highlighted above, is strongly governed by shared ancestry. Thus, phylogenies represent powerful tools for predicting shifts in plant community composition as rates of N deposition continue to rise. However, future work is needed to pinpoint the proximate

determinants, such as interactions with soil microbes or plasticity in functional traits such as SRL, that underlie phylogenetic patterns in plant performance responses to N deposition.

### *Conclusions*

We have explored variation in species' responses to N enrichment for seedlings of 23 eucalypt species that are native to Tasmania, Australia, and whether this variation is explained by phylogeny, specific functional traits, or co-limitation by P using hierarchical Bayesian modeling. While these outcomes are not mutually exclusive, we found that the growth of a majority of the eucalypt species is strongly limited by N and shared evolutionary history explains variation in the degree of N limitation. However, results show that seedling responses to N are not associated with co-limitation by P, nor has the evolution of functional traits guided variation in responses to N in this experiment (although we find a phylogenetic pattern in root traits similar to that of responses to N). Because Tasmanian eucalypt species represent only a small portion of functional variation across terrestrial plant species, we suggest that the analysis we implemented may identify associations of growth responses to N enrichment with P limitation and functional variation across species that exhibit greater variation in N use strategies. However, our results indicate that even within groups of closely related, co-occurring species, phylogeny will likely be a powerful tool for predicting winners and losers in N deposition scenarios. Continued work to identify ecological mechanisms that underlie phylogenetic patterns in plant responses should provide insight into why these patterns exist and how they will play out across landscapes under global change.

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**CHAPTER III**  
**SOIL FUNGI UNDERLIE A PHYLOGENETIC PATTERN IN PLANT GROWTH**  
**RESPONSES TO NITROGEN ENRICHMENT**

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### Abstract

Under increasing anthropogenic nitrogen (N) deposition, some plant species will thrive while others will not. Previous work has shown that plant phylogeny can predict these responses, and that interactions with mycorrhizal fungi are a mechanism that drives variation in plant responses to N enrichment. Yet, much of this work has ignored the roles of other root-associated fungi and whole soil fungal communities in driving these responses. We tested whether soil fungi mediate responses of plant growth and plant-soil feedbacks (between close and distant plant relatives) to N enrichment by implementing a greenhouse experiment in which we applied factorial treatments of N fertilization, host-specific soil inocula, and fungicide to 15 eucalypt tree species that co-occur on the island state of Tasmania, Australia and form two phylogenetic lineages within the subgenus *Symphyomyrtus*. Conspecific-conditioned soil fungi enhanced growth responses to N enrichment for plants within one lineage (lineage 1) but depressed growth responses to N enrichment for plants within another lineage (lineage 2). Lineage-specific shifts in ectomycorrhizal (ECM) colonization were consistent with previous evidence that more vs. less successful strategies under N enrichment are those where carbon allocation to mycorrhizal fungi is reduced vs. maintained, respectively. The latter was also accompanied by a stronger reduction in root colonization of non-filamentous fungi (of unknown function) under N enrichment. Plant-soil feedbacks were neutral for lineage 1 but negative for lineage 2 (i.e., greater growth in soils conditioned by opposite vs. same lineage individuals), but were not altered by N enrichment or fungicide. Lineage-level differences in root colonization suggest that these feedbacks could be driven by differential plant responsiveness to dark septate endophytes and non-filamentous fungi, the colonization of which seemed to benefit plant growth. Our results confirm that interactions with soil fungi (ECM fungi in particular) underlie phylogenetic patterns in tree species' growth responses to N enrichment and may thus influence which plants win or lose under future N deposition scenarios. We provide some of the first evidence (albeit from controlled rather than natural conditions) that N deposition may not play a strong role in shifting plant-soil feedbacks.

### Introduction

Nitrogen (N) enrichment is occurring in soils worldwide due to increasing inputs of reactive N to the atmosphere through fossil fuel combustion and agricultural fertilizer practices (Dentener *et al.* 2006). Growing evidence suggests that plant growth responses to this excess soil N are mediated by root-associated fungi (Jumpponen, Mattson & Trappe 1998; Antoninka *et al.* 2009; Hoeksema *et al.* 2010; Kivlin, Emery & Rudgers 2013; Avolio *et al.* 2014; Mohan *et al.* 2014). For example, mycorrhizal fungi can reduce plant growth responses to N enrichment if mycorrhizal fungi become parasitic under N enrichment (Johnson 1993; Johnson *et al.* 2008). Alternatively, mycorrhizal fungi can increase growth responses to N enrichment if they alleviate co-limitation of plant growth by other resources (e.g., phosphorus or water) under N enrichment (Corkidi *et al.* 2002). Such interactions with soil fungi could in part explain observed variation in N limitation across plant phylogenies (Wooliver *et al.* 2016; Wooliver *et al.* 2017) and ultimately

why some species persist and others do not under natural or simulated anthropogenic N enrichment (Suding *et al.* 2005; Bai *et al.* 2010; Bobbink *et al.* 2010; Cleland & Harpole 2010; Stevens *et al.* 2004; Isbell *et al.* 2013; Avolio *et al.* 2014). For example, in a global meta-analysis of the responses of 125 terrestrial plant species to N enrichment, Wooliver *et al.* (2016) showed that while most species gain significantly more total biomass under N fertilization compared to ambient soil N conditions, more than one in four species respond neutrally or negatively (as low as a 60% decrease in total biomass) to N fertilization. These responses were phylogenetically structured; for example, tree species in the family Aceraceae responded much more positively than those in the family Pinaceae (680% vs. 50% increase in biomass with N fertilization). Although mycorrhizal fungi could play a key role in mediating these responses, much less is known about how other important root-colonizing fungi or whole soil fungal communities respond to N enrichment to mediate plant responses to N in natural systems.

Soil fungal communities as a whole, including both root-associated and free-living fungi, can significantly change in biomass, composition, and function in response to variation in soil N, often more so than soil bacterial communities (Allison, Hanson & Treseder 2007; Lauber *et al.* 2008; Treseder 2008; van Deipen *et al.* 2017), thus they should have strong effects on plant growth responses to N enrichment. Mycorrhizal fungi, which include ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi, are often identified as key mediators of such responses. Recent meta-analyses on the effects of mycorrhizal fungi on plant growth responses to global change have indicated that plant responses to N enrichment are less positive in the presence of mycorrhizal fungi than in their absence (Hoeksema *et al.* 2010; Kivlin, Emery & Rudgers 2013). This may be due to scenarios where mycorrhizal fungi alleviate plant N limitation in ambient N soils (Read 1991; Johnson, Graham & Smith 1997) and become less useful and even parasitic to plants (providing lower returns of resources compared to the amount of plant carbon received) when N enrichment alleviates plant N limitation (Johnson 1993; Corkidi *et al.* 2002; Treseder 2004; Johnson *et al.* 2008).

Mycorrhizal fungi are not the only soil fungi known to mediate plant responses to N enrichment. Dark septate endophytes (DSE) are known to associate with more than 600 plant species and show positive (mutualistic) to negative (parasitic) effects on plants (Jumpponen & Trappe 1998). When mutualistic, DSE can aid in plant uptake of soil resources and deter antagonists including pathogens and herbivores (Mandyam & Jumpponen 2005; Newsham 2011). Inoculation experiments have shown that plant responses to N enrichment are more positive in the presence of DSE than in their absence (reviewed by Jumpponen, Mattson & Trappe 1998), suggesting that these fungi can become more beneficial to plants under N enrichment. But complex interactions between fungal guilds have also been observed, for example mycorrhizal and DSE colonization can reduce colonization by pathogens, and colonization by pathogens can enhance mycorrhizal colonization (Newsham, Fitter & Watkinson 1995; Mandyam & Jumpponen 2005; Maherli & Klironomos 2007; Sikes, Cottenie & Klironomos 2009; Laliberté *et al.* 2015; Zampieri *et al.* 2017). As a result, plant growth responses to N enrichment could be the outcomes of both direct effects of fungal guilds and indirect effects derived from interactions between fungal guilds. Thus, predicting plant winners and losers under anthropogenic N enrichment scenarios could require careful consideration of interactions among multiple types of root-associated fungi within whole soil communities.

Soil fungi may also underlie N-driven changes in the feedbacks between plant species and the distinct soil communities that they condition. ‘Plant-soil feedbacks’ describe these interactions between plants and soil organisms (Bever 1994). Negative plant-soil feedbacks

occur when plants perform worse when growing in soil conditioned by conspecifics ('home' soil) compared to soil conditioned by heterospecifics; these are widespread and can arise from adverse interactions between plants and enemies in their home soils (van der Putten *et al.* 2013). Such feedbacks also promote higher diversity within plant communities (Bever, Westover & Antonovics 1997) through mechanisms like Janzen-Connell effects, wherein offspring perform worse in soils conditioned by adult conspecifics relative to soils conditioned by adult heterospecifics, due to the build up of antagonists to which closely related species are susceptible (Janzen 1970; Connell 1971). Further, through their effects on antagonists and nutrient acquisition, mycorrhizal fungi can induce plant-soil feedbacks that range from negative to positive. For example, recent meta-analysis of 55 North American tree species showed that ECM-associated trees tend to experience positive plant-soil feedbacks while AM-associated trees tend to experience negative plant-soil feedbacks because ECM fungi are thought to provide more nutrients to plants and protection against antagonists that accumulate in conspecific-conditioned soil (Bennett *et al.* 2017). Such effects can lead to monodominance of ECM-associated plants in natural communities (Laliberté *et al.* 2015). However, evidence for the direction and magnitudes with which anthropogenic N enrichment will alter plant-soil feedbacks is mixed. Though in some cases N enrichment can produce more positive plant-soil feedbacks (i.e., greater growth in soil conditioned by heterospecifics vs. home soil) if conspecific mycorrhizae are locally adapted to acquire phosphorus from low-phosphorus soils (Johnson *et al.* 2010), plant-soil feedbacks overall are expected to become more negative under N enrichment due to magnified negative effects of pathogens and reduced mutualistic effects of mycorrhizae (Revillini, Gehring & Johnson 2016).

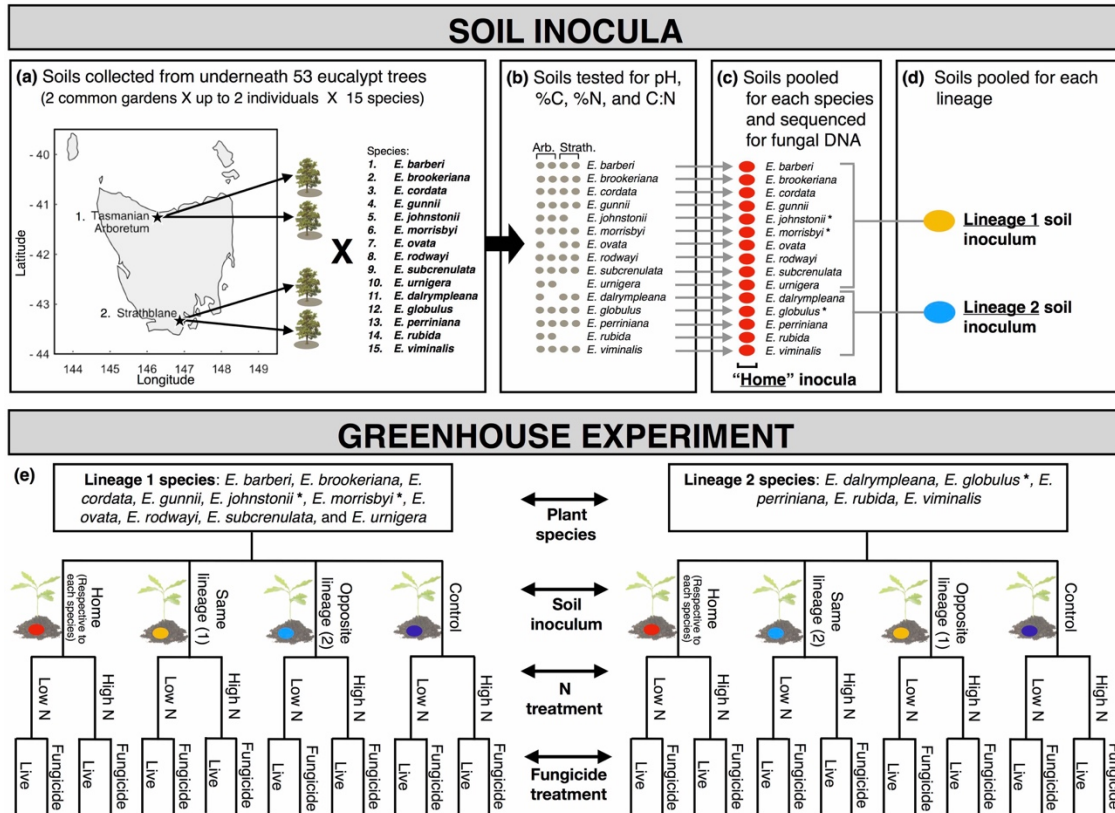
To explore whether conditioning of soil fungal communities by individual species or groups of species interacts with N enrichment to affect plant performance, we undertook a greenhouse experiment in which we applied factorial treatments of soil inocula (that were conditioned by different eucalypt species), N fertilization, and fungicide to eucalypt tree species belonging to two phylogenetic lineages. The predominantly Australian genus *Eucalyptus* is one of the few tree genera that form dual associations with ECM and AM fungi (Brundrett *et al.* 1996). Ectomycorrhizal fungi are known to be particularly important for eucalypt acquisition of P (Malajczuk, McComb & Loneragan 1975; Heinrich & Patrick 1986; Bougher, Grove & Malajczuk 1990; Burgess, Malajczuk & Grove 1993; Jones, Durall & Tinker 1998; Chen, Brundrett & Dell 2000), which is limited in highly weathered Australian soils (Wild 1958). Further, greenhouse experiments have shown that ECM tend to replace AM as the dominant mycorrhizal type within as little as 100 days of eucalypt seedling growth (Lapeyrie & Chilvers 1985; Chilvers, Lapeyrie & Horan 1987; Oliveira, Zambolim & Neves 1995; Chen, Brundrett & Dell 2000; dos Santos *et al.* 2001). A previous study has also shown that ECM is dominant mycorrhizal type for *Eucalyptus grandis* adults in their native ranges (Adams *et al.* 2006). Thus, ECM fungi may be a more ecologically significant symbiont compared to AM fungi for many *Eucalyptus* species and is often viewed as the dominant mycorrhizal type in this genus (Chilvers & Pryor 1965; Wang & Qiu 2006; Tedersoo & Brundrett 2017). Previous work on eucalypts in greenhouse settings has shown variation in plant-soil feedbacks from neutral to negative, wherein performance does not change or increases (respectively) in soils conditioned by increasingly distant relatives (J. K. Senior, unpublished data). Though we should expect *Eucalyptus* (as predominantly ECM) to show positive feedbacks according to Bennett *et al.* (2017), this result could be driven by varying plant responses to plant-conditioned soil pathogens that overwhelm any positive effects of ECM fungi. Here we hypothesized that soil fungi mediate

plant responses to N enrichment. We predicted that when plants are inoculated with conspecific-conditioned soil, N enrichment affects plant growth and colonization by root-associated fungi differently between live and fungicide-treated soils. We also hypothesized that soil fungi mediate how plant-soil feedbacks (between close and distant relatives) change with N enrichment. We predicted that N enrichment alters feedbacks of soils (conditioned by same vs. opposite lineage plants) on plant growth and colonization by root-associated fungi differently between live and fungicide-treated soils.

## Methods

We used 15 of the 17 eucalypt species in the subgenus *Symphyomyrtus* that are native to the island state of Tasmania, Australia (Fig. 3.1a). According to some phylogenetic reconstructions (Woodhams *et al.* 2013; Senior *et al.* 2016), these species form two lineages (hereafter referred to as lineages 1 and 2, respectively): 1) the alpine white, black, and yellow gums (species 1-10 in Fig. 3.1a) and 2) the white and blue gums (species 11-15 in Fig. 3.1a). Because other reconstructions indicate that the Tasmanian blue gum, *E. globulus*, is more closely related to species within lineage 1 than those within lineage 2 (McKinnon *et al.* 2008; Wooliver *et al.* 2017) or may form its own genetically distinct lineage (Jones *et al.* 2016), we took steps in our experimental treatments and statistical analyses (described below) to determine whether placement of *E. globulus* into either (or neither) lineage affected our results. We did this rather than removing *E. globulus* from the study, due to its value as a globally important plantation species (Sands 2013) and focus on this species in literature on mycorrhizal symbiosis (e.g., Burgess, Malajczuk & Grove 1993; Brundrett *et al.* 1996; Chen, Brundrett & Dell 2000).

Building on past greenhouse fertilization experiments showing that these lineages exhibit different growth responses to N fertilization (Wooliver *et al.* 2017), we implemented a greenhouse experiment to test whether conditioning of soil fungi by individual plant species or groups of closely related species (lineages) interacts with N enrichment to affect plant performance. We grew seedlings in potting mix inoculated with 1) soil conditioned by conspecifics (hereafter referred to as ‘home’ soil), 2) soil conditioned by close (same lineage) relatives, 3) soil conditioned by distant (opposite lineage) relatives, or 4) soil conditioned by no plants (control potting mix). Within each soil inoculum treatment, we treated soils of half of the seedlings with a fungicide to determine effects of whole fungal communities on plant growth responses to N enrichment. The control soil inoculum was not used to control for soil fungi (rather, we expected to see high levels of fungal colonization in this inoculum treatment due to ubiquitous ‘weedy’ mycorrhizal fungi that rapidly colonize plant roots in greenhouse settings; e.g., Sikes, Hawkes & Fukami 2016), but to discern whether potential effects of fungicide on plant growth responses to N are driven by soil fungi in the potting mix and greenhouse vs. Tasmanian soil fungi. Individuals within each soil inoculum treatment received factorial treatments of N fertilization applied at a rate of 10 g N m<sup>-2</sup> yr<sup>-1</sup> (within the global range of N deposition rates currently observed) vs. no added N, and non-systemic fungicide (to reduce fungal colonization of plant roots, leaving fungi within aboveground plant tissues unaffected) vs. no fungicide application (to represent live, intact soil fungal communities) (Fig. 3.1e). After 5 months of growth, we measured plant growth and root colonization by fungal guilds identified on eucalypt roots at the end of the experiment to determine whether plant growth responses to N are associated with changes in abundances of root-colonizing fungi. These included ECM, AM, DSE, and non-filamentous fungi (which included yeasts and dimorphic fungi, but whose function and effects on plant growth were unknown).



**Figure 3.1. Schematic of soil inocula preparation and greenhouse experimental design.** (a) We collected soils conditioned by individuals of 15 Tasmanian eucalypt species growing in two Tasmanian common gardens. (b) We quantified abiotic characteristics of the soils collected under each individual, then (c) pooled soils for each species to create ‘home’ soil inocula and characterize soil fungal communities using next-generation sequencing. (d) We then pooled the home soils for each plant lineage to create lineage 1 and lineage 2 soil inocula. (e) To test whether conditioning of soil fungi by individual plant species or lineages interacts with nitrogen (N) enrichment to affect plant performance, we implemented a greenhouse experiment that included factorial treatments of four soil inocula, high vs. low N fertilization, and live vs. fungicide soil treatments to four individuals of each plant species for which we collected soils in the Tasmanian common gardens. Asterisks in (c) and (e) denote the three species (*E. johnstonii*, *E. morrisbyi*, and *E. globulus*) that were not included in lineage-level soil inocula or lineage-level soil treatments.



### *Sampling and pooling soils for inoculum treatments*

To create soil inoculum treatments for our greenhouse experiment, we collected soils conditioned by individuals of each of the 15 species growing in each of two common gardens: the Tasmanian Arboretum, located in northern Tasmania (41.2265 S, 146.3022 E), and a forestry trial at Strathblane, located in southeastern Tasmania (43.3350 S, 146.9451 E) (Fig. 3.1a). We did not collect soils conditioned by the two remaining Tasmanian symphyomyrtes, *E. archeri* and *E. vernicosa*, because they were absent in both common gardens. We collected soils from common gardens rather than naturally occurring trees to reduce the variation in soil microbial communities across tree species attributable to environmental variation, soil type, or variation in surrounding plant communities. We eventually pooled soils from the Arboretum and Strathblane sites, which vary in bioclimatic variables (Hijmans *et al.* 2005; Table S1 in Chapter III Supplementary Material), enabling us to capture microbial communities conditioned by species across differing climates. Because of previously documented ontogenetic changes in AM vs. ECM colonization in eucalypts (Lapeyrie & Chilvers 1985; Chilvers, Lapeyrie & Horan 1987; Oliveira, Zambolim & Neves 1995; Chen, Brundrett & Dell 2000; dos Santos *et al.* 2001; Adams *et al.* 2006), we collected soils conditioned by individuals of different ages (ranging from 2 to 30 years and 6 to 8 years since planting at the Arboretum and Strathblane, respectively) to capture variation in mycorrhizal fungal communities with which *Eucalyptus* individuals interact across developmental stages.

We collected, pooled, and homogenized three evenly spaced 4 x 7 cm soil cores from underneath the canopy of up to two individuals of each species growing in each common garden (N=53 individual soils; Fig. 3.1a,b). We disinfected the soil corer with a 1:10 dilution of the disinfectant Dettol<sup>®</sup> (4.8% chloroxyleneol) between each tree to prevent cross-contamination. We stored soils in individual Ziploc<sup>®</sup> bags and transported them to the University of Tasmania before shipping on ice (such that they were cool but not completely frozen) to the University of Tennessee where they were stored at 4 °C. Chemical analysis of the 53 soil samples showed no differences in soil pH, %C, %N, or C:N associated with plants between plant lineages (see Table S2 for details). This and the low percentage (<1%) of soil inocula in the total soil volume in each pot in our experiment (described below) precludes the possibility that differences in plant growth between individuals inoculated with same and opposite lineage soils in our experiment could be driven by differences in conditioning of soil abiotic (as opposed to biotic) characteristics between plant lineages. To produce home soil inoculum treatments (15 total, each respective to each species), we pooled soils of each species using equal amounts of soil conditioned by each individual at each common garden (Fig. 3.1c). Next-generation sequencing analyses of these soils post-shipping (See Appendix S1) showed that they contained 1,123 fungal OTUs within 111 families and 208 genera. Seventy-three of these families and 87 of these genera have been previously identified in Tasmanian soils (J.K. Senior, unpublished data), thus we were confident that the soils contained fungi naturally encountered by Tasmanian eucalypts. However, PERMANOVA analyses of soil fungal OTU abundances showed no differences in fungal communities (including guild-based subsets of fungal communities) between plant lineages (see Appendix S2), suggesting that lineages do not condition their soils to contain different fungal communities. Yet, plant-soil feedbacks could still arise from differential receptiveness of eucalypts to conditioned soil fungi. To produce lineage-level (same vs. opposite lineage) soil inoculum treatments, we pooled soils of each lineage using equal amounts of soil conditioned by each species within respective lineages (Fig. 3.1d). We excluded *E. globulus*, *E. johnstonii*, and *E. morrisbyi* (denoted with asterisks in Fig. 3.1c,e) from the lineage-level soil inocula and the

lineage-level soil inoculum treatments in the greenhouse experiment due to uncertainty in the phylogenetic placement of *E. globulus* (described above) and low rates of germination of *E. johnstonii* and *E. morrisbyi* seed in our greenhouse experiment (i.e., insufficient replicate seedlings for all treatment combinations).

### *Greenhouse experimental design*

To explore whether conditioning of soil fungi by individual plant species or phylogenetic groups interacts with N enrichment to affect plant performance, we established a 5-month long controlled inoculation and fertilization experiment (Fig. 3.1e) at the University of Tennessee, Knoxville, using seedlings of the 15 subgenus *Symphyomyrtus* species for which we collected soils in the Tasmanian common gardens. We germinated seed from naturally occurring or greenhouse-cultivated populations of each species using the methods of Wooliver *et al.* (2017). After one month, we transplanted 64 seedlings of each species into separate 2 L pots containing 1.5 L fertilizer-free, non-sterilized potting soil (Pro-Mix<sup>®</sup> BX, Premier Tech). While field soils would have most closely simulated natural (abiotic and biotic) conditions, logistical considerations precluded transport of bulk soil from Tasmania to Tennessee. Alternatively, we chose to use potting soil, as it has previously been used to reveal valuable patterns in plant-soil feedbacks in multiple published studies (Pfennigwerth *et al.* 2017; Van Nuland, Bailey & Schweitzer 2017). Immediately prior to transplanting, we mixed 10 mL of each soil inoculum treatment into the potting soil for 16 individuals of each species. Because *E. globulus*, *E. johnstonii*, and *E. morrisbyi* were not inoculated with lineage-level soil inocula, total sample size was 864 individuals (12 species X 16 seedlings X 4 soil inocula treatments + 3 species X 16 seedlings X 2 soil inocula treatments). Of the 16 individuals of each species receiving each soil inoculum, eight were given N (urea in solution with 2 mL water) in monthly applications at a rate of 10 g N m<sup>-2</sup> yr<sup>-1</sup> and the other half were given the same amount of water with no N. Though the higher rate of N fertilization used here is greater than N deposition rates in most terrestrial ecosystems (Lamarque *et al.* 2005), it is within the range of current global N deposition rates (which reach 17 g N m<sup>-2</sup> yr<sup>-1</sup>; Berendse, Aerts & Bobbink 1993) and the range of N fertilization rates used in studies on plant and mycorrhizal fungal responses to N deposition (Johnson *et al.* 2008).

Before mixing into the potting soil, inocula for half of the eight individuals of each species receiving each soil inoculum X N fertilization combination were treated with a non-systemic fungicide (Rovral<sup>®</sup> brand 4 Flowable Fungicide, 41.6% iprodione; Fig. 3.1e) to remove fungal communities while leaving non-fungal soil biotic communities intact. As such, we had four replicates for each species X N fertilization X fungicide treatment combination. The fungicide was mixed with soil inocula in solution at a rate (0.32 g iprodione m<sup>-2</sup>) that has been shown to significantly reduce mycorrhizal fungal colonization (Gange, Brown & Farmer 1990), and thereafter applied to the soil surfaces of ‘fungicide’ individuals in the greenhouse every six weeks (at the same rate) to preclude fungal growth arising from spores. We used a non-systemic rather than a systemic fungicide because the former does not affect foliar infection by pathogenic fungi (Summerbell 1988) or soil invertebrates (Gange, Brown & Sinclair 1993). Though iprodione is 13% N by weight and thus added about 0.35 g N m<sup>-2</sup> yr<sup>-1</sup> to soils in our experiment, ultimately fungicide did not significantly influence plant growth (i.e., did not alleviate N limitation; Tables 3.1 & 3.2). For the duration of the experiment individuals were arranged in a randomized 4-block design and watered three times weekly to prevent growth limitation by water. Daily temperatures in the greenhouse were controlled to vary between 21 and 24 °C.

### *Response variables measured in the greenhouse experiment*

At the end of the experiment we measured total dry biomass and height of seedlings (which are both associated with competitive vigour; Pérez-Harguindeguy *et al.* 2013), and proportion of roots colonized by ECM, AM, DSE, and non-filamentous fungi for all individuals. We measured height (cm) as the distance from the base of the main stem to the tip of the terminal leaf. Shoots were severed from roots and dried at 70 °C for 72 hours to obtain total aboveground biomass. Each root system was then shaken and washed over a 2-mm sieve to remove soil, patted dry with paper towels, and weighed to obtain total wet belowground biomass (g). We took two random samples (~1 g wet weight each) from each root system, one of which we used to quantify proportion of roots colonized by ECM fungi out of 50 roots using a dissecting microscope and the gridline intersect method of Giovannetti & Mosse (1980). Criteria for scoring ECM colonization consisted of the presence/absence of thickened, discolored, and highly branched root tips (Brundrett *et al.* 1996). The other sample was cleared with 10% KOH and stained with trypan blue using the method of Koske & Gemma (1989), mounted and glued on a glass slide with polyvinyl alcohol, and scored for the number of roots colonized by AM, DSE, and non-filamentous fungi out of 50 fields of view using a compound light microscope at 400x and the intersection method of McGonigle *et al.* (1990). Criteria for scoring consisted of the presence/absence of the following structures within roots: 1) AM fungi: non-septate hyphae (>5 µm in diameter) that could be associated with vesicles, arbuscules, or spores (>20 µm in diameter) (Newsham, Fitter & Watkinson 1995; Brundrett *et al.* 1996), 2) DSE fungi: melanized septate hyphae (>5 µm in diameter) that could be associated with microsclerotia (Jumpponen & Trappe 1998), and 3) non-filamentous fungi: spores (<20 µm in diameter) that were not associated with hyphae. See Fig. S1 for images of *Eucalyptus* roots that were scored as colonized by AM, DSE, or non-filamentous fungi, or not colonized by fungi. Colonization by ECM, AM, and DSE fungi (averaged for each species in home, low N, live soils) was not correlated with relative abundances of the respective fungal group in home soil inocula (quantified through sequencing, described in Appendix S1), suggesting that eucalypts are differentially receptive to their home soil fungi. We estimated total dry belowground biomass (g) for each individual by subtracting water weight (the mean percent water in wet biomass for a subsample of 10 individuals' root systems) from the total wet belowground biomass measured initially. We added total dry above- and belowground biomass to obtain total biomass (g).

### *Statistical analyses*

We performed all statistical analyses in R (R Core Team 2016). All main analyses included a main effect of focal plant lineage (rather than species) because previous work has shown lineage-based growth responses of the Tasmanian eucalypts to N fertilization (Senior *et al.* 2013; Wooliver *et al.* 2017). Analyses included *E. globulus* in lineage 2, but (where possible) we conducted supplementary analyses that included *E. globulus* in lineage 1 or excluded *E. globulus* altogether). Using the lme4 package (Bates *et al.* 2015) we implemented linear mixed effects models to analyze plant growth (total biomass and height, transforming response variables to increase conformance to normality), and generalized linear mixed effects models to analyze binomially distributed root colonization data (ECM, DSE, and non-filamentous fungi). We analyzed model results using analysis of variance, obtained least-squares means and standard errors of means using the lsmeans package (Lenth 2016), and analyzed additional models on data subsets to explore interactions among or between main effects. We identified AM structures in only 16 plants in the experiment and the number of roots colonized by AM fungi within these

plants averaged less than one out of 50. With so few individuals having non-zero AMF colonization, models of AM colonization would lack the statistical power to detect true differences between/among treatments. As such, we believe these results would be misleading and do not report them.

To address our first hypothesis that soil fungi mediate plant responses to N enrichment, we analyzed data on plant growth and root colonization for the subset of individuals that received the home soil inoculum in the greenhouse experiment. We implemented linear mixed effects models that included three main effects (fungicide treatment, N fertilization, plant lineage) and all interactions, with species (nested within lineage) and block included as random effects. Strong three-way interactions among fungicide treatment, N fertilization, plant lineage for plant growth would support previous evidence of lineage-based responses to N in Tasmanian eucalypts (Senior *et al.* 2013; Wooliver *et al.* 2017) and our first hypothesis that soil fungi mediate plant responses to N enrichment. The same interactions identified for fungal colonization would indicate that plant responses to N enrichment are associated with responses of specific guilds of root-associated soil fungi to N enrichment. Using the model described above, we implemented a supplementary analysis of plant growth and colonization of individuals inoculated with the control inoculum (potting soil) to test whether any effects of soil fungi identified for plant responses to N fertilization for individuals inoculated with home soil were driven by native Tasmanian soil fungi, rather than fungi that occur naturally in potting soil or the greenhouse environment.

To address our second hypothesis that soil fungi mediate responses of plant-soil feedbacks to N enrichment, we analyzed data on plant growth and root colonization for the subset of individuals that received the same or opposite lineage soil inocula in the greenhouse experiment. We implemented linear mixed effects models that included four main effects (fungicide treatment, N fertilization, focal plant lineage, and soil inoculum) and interactions, with species (nested within lineage) and block included as random effects. Strong interactions among fungicide treatment, N fertilization, and soil inoculum for plant growth would support that soil fungi mediate how plant-soil feedbacks between close and distant relatives change with N enrichment, and the same interaction identified for fungal colonization would indicate that plant-soil feedback responses to N enrichment could be driven by responses of specific guilds of root-associated soil fungi to N enrichment.

## Results

### *Home soil fungi mediate plant lineage dependent responses to N enrichment*

For Tasmanian *Symphyomyrtus* individuals inoculated with home soils, we identified three-way interactions ( $p < 0.1$ ) among fungicide treatment, N fertilization, and plant lineage for total biomass, height, ECM colonization, and non-filamentous fungal colonization (Table 3.1). This suggests that soil fungi mediate plant growth responses to N enrichment differentially for plant lineages at relatively small phylogenetic scales (within a subgenus) and that colonization by ECM and non-filamentous fungi underlie these phylogenetically-based responses. To explore these interactions, we ran four separate additional models (one for each plant lineage in live vs. fungicide soil treatments) for each response variable to examine how fungicide affects each lineage's growth and colonization responses to N fertilization (Fig. 3.2). For lineage 1 plants in live soils, biomass and height increased by 47% and 17% respectively with N fertilization (biomass:  $\chi^2 = 5.6_{DF=1, Residual DF=74}$ ,  $P = 0.018$ ; height:  $\chi^2 = 4.6_{1,74}$ ,  $P = 0.032$ ), while colonization by ECM and non-filamentous fungi decreased by 20% and 6% respectively with N fertilization

(ECM:  $\chi^2=20.7_{1,73}$ ,  $P<0.001$ ; non-filamentous fungi:  $\chi^2=4.6_{1,74}$ ,  $P=0.032$ ). However, for lineage 1 plants in fungicide treated soils, neither biomass nor height responded to N fertilization (biomass:  $\chi^2=0.2_{1,75}$ ,  $P=0.666$ ; height:  $\chi^2=0.6_{1,75}$ ,  $P=0.437$ ), while colonization by ECM increased by 23% and colonization by non-filamentous fungi decreased by 21% with N fertilization (ECM:  $\chi^2=6.6_{1,76}$ ,  $P=0.010$ ; non-filamentous fungi:  $\chi^2=20.1_{1,70}$ ,  $P<0.001$ ). For lineage 2 plants in live soils, biomass and height did not respond to N fertilization (biomass:  $\chi^2=2.7_{1,34}$ ,  $P=0.102$ ; height:  $\chi^2=0.5_{1,34}$ ,  $P=0.488$ ), while colonization by ECM did not change and colonization by non-filamentous fungi decreased by 12% with N fertilization (ECM:  $\chi^2=2.2_{1,34}$ ,  $P=0.135$ ; non-filamentous fungi:  $\chi^2=11.9_{1,33}$ ,  $P<0.001$ ). In fungicide treated soils, biomass and height of lineage 2 plants increased by 46% and 20% respectively with N fertilization (biomass:  $\chi^2=9.1_{1,35}$ ,  $P=0.003$ ; height:  $\chi^2=8.2_{1,35}$ ,  $P=0.004$ ), while colonization by ECM decreased by 39% and colonization by non-filamentous fungi did not change with N fertilization (ECM:  $\chi^2=18.6_{1,36}$ ,  $P<0.001$ ; non-filamentous fungi:  $\chi^2=0.03_{1,33}$ ,  $P=0.868$ ). Models of plant growth traits for individuals inoculated with the control inoculum (potting soil) did not identify strong three-way interactions for plant growth (Table S3). Further, models of plant growth traits including *E. globulus* in lineage 1 and excluding *E. globulus* altogether identified the same three-way interactions and trends in responses to N (Table S4).

Overall, fungicide reduced colonization by ECM and non-filamentous fungi by 55% and 45% respectively but increased colonization by DSE fungi by 50% (Table 3.1). However, we identified two-way interactions of fungicide with both N fertilization and plant lineage for DSE colonization. Nitrogen fertilization increased DSE colonization in live soils ( $\chi^2=9.4_{1,106}$ ,  $P=0.002$ ) but did not affect DSE colonization in fungicide treated soils ( $\chi^2=1.0_{1,107}$ ,  $P=0.319$ ); and the fungicide treatment increased DSE colonization significantly more in lineage 2 ( $\chi^2=118.8_{1,70}$ ,  $P<0.001$ ) than in lineage 1 ( $\chi^2=14.3_{1,143}$ ,  $P<0.001$ ).

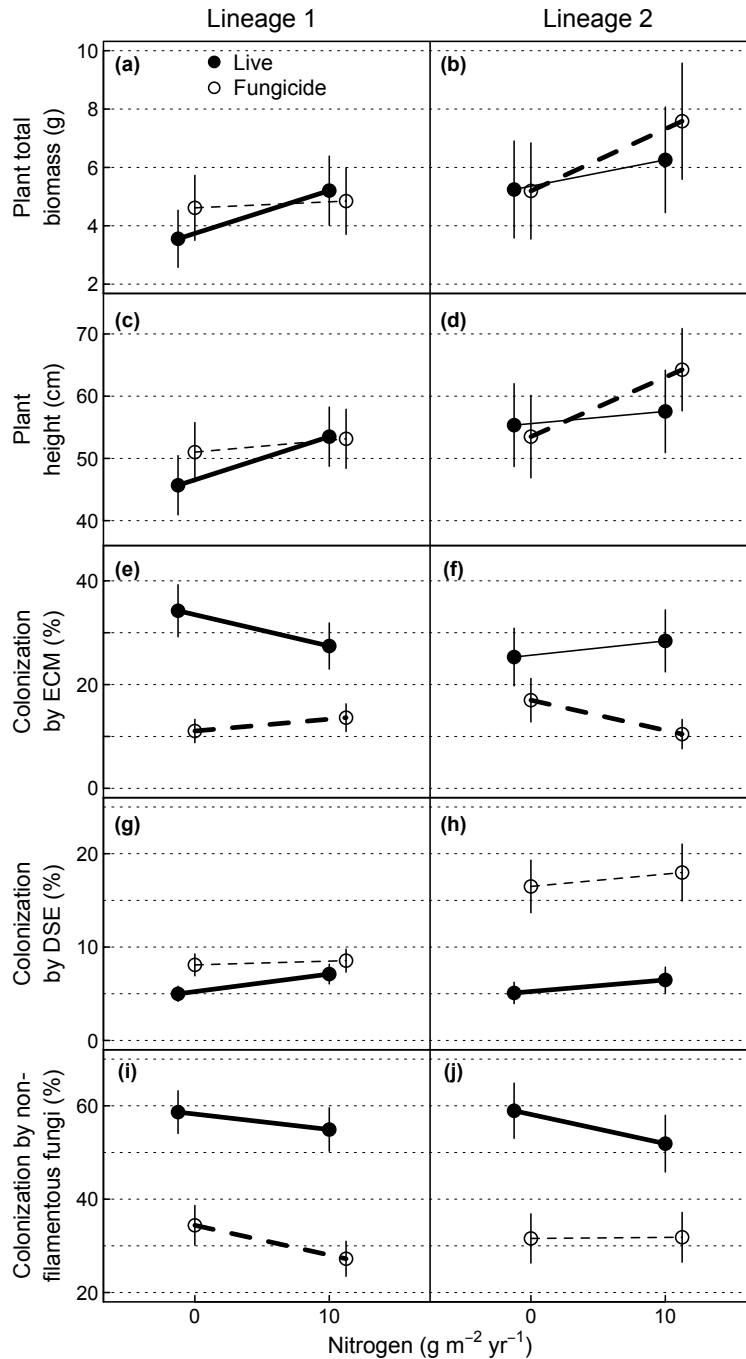
#### *Soil fungi associated with plant lineages underlie plant-soil feedbacks*

For Tasmanian *Symphyomyrtus* individuals inoculated with same or opposite lineage soils, we identified two-way interactions ( $p<0.1$ ) between plant lineage and soil inoculum for total biomass, height, DSE colonization, and non-filamentous fungal colonization (Table 3.2). This indicates that plant lineages differ in plant-soil feedbacks and that colonization by DSE and non-filamentous fungi could underlie these phylogenetically-based plant-soil feedbacks. To explore these interactions, we ran two separate additional models (one for each plant lineage) for each response variable to examine how soil inocula affect each lineage's growth and colonization (Fig. 3.3). For lineage 1 plants, biomass and height did not change between soil inocula (biomass:  $\chi^2=2.6_{1,247}$ ,  $P=0.106$ ; height:  $\chi^2=1.6_{1,247}$ ,  $P=0.214$ ), while DSE colonization decreased by 12% and non-filamentous fungal colonization increased by 7% from same to opposite lineage inocula (DSE:  $\chi^2=14.7_{1,234}$ ,  $P<0.001$ ; non-filamentous fungi:  $\chi^2=9.9_{1,234}$ ,  $P=0.002$ ). For lineage 2 plants, biomass and height increased by 22% and 23% from same to opposite inocula (biomass:  $\chi^2=12.7_{1,121}$ ,  $P<0.001$ ; height:  $\chi^2=9.3_{1,122}$ ,  $P=0.002$ ), indicating negative plant-soil feedbacks, while DSE colonization increased by 9% and non-filamentous fungal colonization increased by 22% from same to opposite lineage inocula (DSE:  $\chi^2=19.2_{1,116}$ ,  $P<0.001$ ; non-filamentous fungi:  $\chi^2=35.6_{1,116}$ ,  $P<0.001$ ). Though root colonization by ECM, DSE, and non-filamentous fungi showed other strong two- and three-way interactions (Table 3.2), we do not explore them here as these interactions did not appear to be important for plant growth.

**Table 3.1. Main and interactive effects ( $\chi^2$ ) of fungicide, nitrogen fertilization, and plant lineage on plant growth traits and colonization of root-associated fungi across 15 Tasmanian eucalypt tree species within the subgenus *Symphyomyrtus* whose soils were inoculated with soil conditioned by conspecific trees (i.e., ‘home’ soils). Effects are derived from linear mixed effects models that include block and species (nested within lineage) as random effects. Bold and italic lettering denote significance at  $\alpha = 0.05$  and  $0.1$ , respectively.**

Effect	Plant growth		Colonization		
	Total biomass	Height	ECM	DSE	Non-filamentous
Fungicide	1.7	2.0	<b>463.1</b>	<b>92.2</b>	<b>659.9</b>
Nitrogen	<b>11.8</b>	<b>9.7</b>	<b>6.8</b>	<b>6.6</b>	<b>26.0</b>
Lineage	0.6	.9	<0.1	<b>5.1</b>	<0.1
Fungicide:Nitrogen	0.9	0.1	1.9	3.2	<0.1
Fungicide:Lineage	<0.1	<0.1	<b>9.6</b>	<b>36.5</b>	1.2
Nitrogen:Lineage	0.6	0.2	<0.1	<0.1	1.2
Fungicide:Nitrogen:Lineage	3.4	3.6	<b>38.9</b>	0.3	<b>7.8</b>
DF (numerator, residual)	1, 227	1, 226	1, 225	1, 211	1, 211

Fungicide, 0 vs. 0.32 g iprodione m<sup>-2</sup> 6 weeks<sup>-1</sup>; Nitrogen, 0 vs. 10 g nitrogen fertilization m<sup>2</sup> yr<sup>-1</sup>; Lineage, plant lineage 1 (alpine white, black, and yellow gums) vs. lineage 2 (white and blue gums); ECM, proportion of roots colonized by ectomycorrhizal fungi; DSE, proportion of roots colonized by dark septate endophytic fungi; Non-filamentous, proportion of roots colonized by non-filamentous fungi.



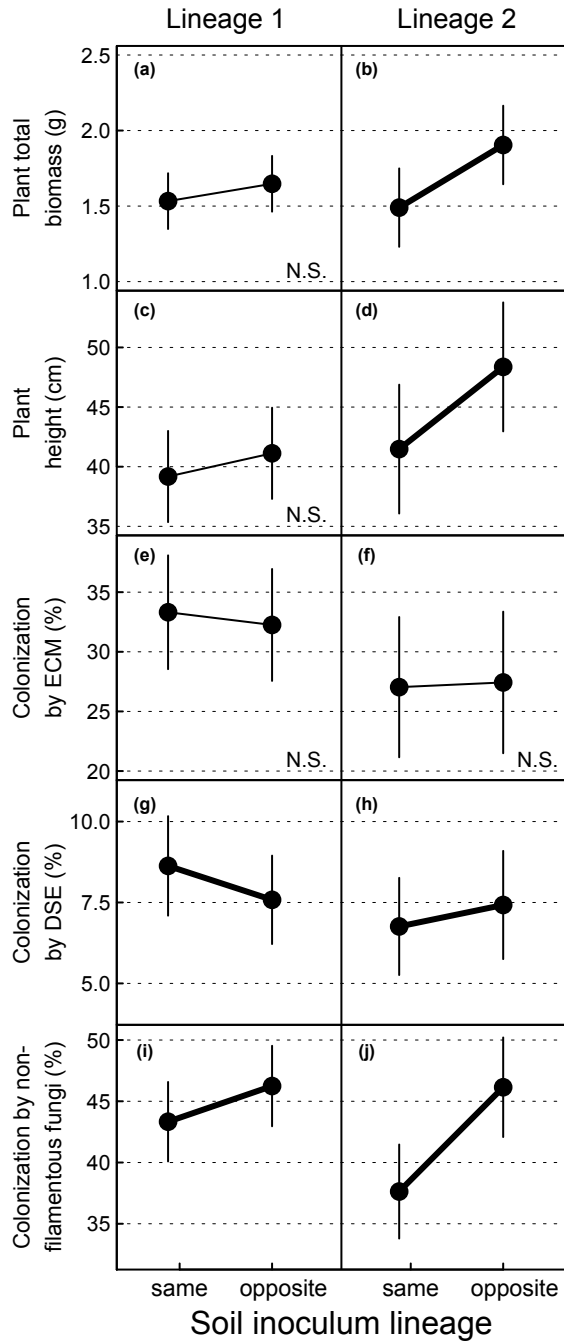
**Figure 3.2. Soil fungi mediate responses of two Tasmanian eucalypt plant lineages to nitrogen enrichment.** Shown are least squares means ( $\pm$ SE) of plant growth and colonization by ectomycorrhizal (ECM), dark septate endophytic (DSE), and non-filamentous fungi each lineage growing in potting mix inoculated with soil conditioned by conspecifics (i.e., ‘home’ soil), with and without nitrogen enrichment (x axis, the unit of which is often used in estimating global N deposition rates), and with fungal communities left intact (live) or removed (fungicide). Bolded lines represent responses to nitrogen enrichment that are statistically significant at  $\alpha = 0.1$ .

**Table 3.2. Main and interactive effects ( $\chi^2$ ) of fungicide, nitrogen fertilization, and plant lineage on plant growth traits and colonization of root-associated fungi across 12 Tasmanian eucalypt tree species within the subgenus *Symphyomyrtus* whose soils were inoculated with soil conditioned by trees in the same or opposite lineage.** Effects are derived from linear mixed effects models that include block and species (nested within lineage) as random effects. Bold and italic lettering denote significance at  $\alpha = 0.05$  and  $0.1$ , respectively.

Effect	Plant growth		Colonization		
	Total biomass	Height	ECM	DSE	Non-filamentous
Fungicide	0	1.1	<b>811.8</b>	<b>444.2</b>	<b>1262.4</b>
Nitrogen	<b>29.2</b>	<b>38.3</b>	<b>55.4</b>	<b>29.6</b>	<b>129.8</b>
Lineage	0.1	0.6	0.6	<0.1	0.7
Soil	<b>11.6</b>	<b>7.3</b>	0.8	0.2	<b>38.9</b>
Fungicide:Nitrogen	<0.1	0.3	<b>17.2</b>	<b>18.5</b>	<b>150.7</b>
Fungicide:Lineage	<0.1	1.1	<b>18.6</b>	1.0	<0.1
Nitrogen:Lineage	1.2	2.1	1.2	<b>19.3</b>	<b>4.1</b>
Fungicide:Soil	0.2	0.8	1.1	0.4	<b>36.9</b>
Nitrogen:Soil	0.7	0.1	<b>5.7</b>	<b>8.2</b>	<b>44.5</b>
Lineage:Soil	<b>5.0</b>	3.0	1.5	<b>34.4</b>	<b>10.7</b>
Fungicide:Nitrogen:Lineage	<0.1	0.1	<0.1	1.2	7.6
Fungicide:Nitrogen:Soil	0.5	<0.1	0.3	<b>34.0</b>	<b>54.3</b>
Fungicide:Lineage:Soil	<0.1	<0.1	0.6	<b>19.4</b>	1.7
Nitrogen:Lineage:Soil	0.2	<0.1	3.6	<b>51.8</b>	1.6
Fungicide:Nitrogen:Lineage:Soil	0.3	1.8	2.5	0.3	0.1
DF (numerator, residual)	1, 359	1, 360	1, 358	1, 340	1, 340

Fungicide, 0 vs. 0.32 g iprodione m<sup>-2</sup> 6 weeks<sup>-1</sup>; Nitrogen, 0 vs. 10 g nitrogen fertilization m<sup>2</sup> yr<sup>-1</sup>; Lineage, plant lineage 1 (alpine white, black, and yellow gums) vs. lineage 2 (white and blue gums); ECM, proportion of roots colonized by ectomycorrhizal fungi; DSE, proportion of roots colonized by dark septate endophytic fungi; Non-filamentous, proportion of roots colonized by non-filamentous fungi.





**Figure 3.3. Plant-soil feedbacks differ between two Tasmanian eucalypt plant lineages.** Shown are least squares means ( $\pm$ SE) of plant growth and colonization by ectomycorrhizal (ECM), dark septate endophytic (DSE), and non-filamentous fungi for each lineage growing in potting mix inoculated with soil conditioned by same vs. opposite lineage plants. Bolded lines represent responses to soil inocula that are statistically significant at  $\alpha = 0.1$ .

## Discussion

Results from a controlled, fully-factorial fungicide, N fertilization, and soil inoculation experiment using forest tree species (Tasmanian eucalypts within the subgenus *Symphyomyrtus*) provide two key insights on plant responses to anthropogenic N deposition. First, soil fungi may underlie phylogenetic patterns in plant growth responses to N enrichment. Specifically, we found that soil fungi (as a whole) enhance the growth responses to N enrichment for one lineage (lineage 1) but decrease growth responses to N enrichment for the other (lineage 2). As such, previously observed phylogenetic patterns in responses to N enrichment across plant species (Wooliver *et al.* 2016; Wooliver *et al.* 2017) could in part be driven by similar interactions with soil fungi for plants with more shared evolutionary history. We identified changes in colonization by ECM fungi that could potentially explain lineage-based responses to N enrichment, with trends consistent with previous evidence that plants that show more positive growth responses to N enrichment are those that allocate less carbon to mycorrhizal fungi under N enrichment (Johnson 1993; Corkidi *et al.* 2002; Johnson *et al.* 2008). We also found that plants that show less positive growth responses to N enrichment maintain their ECM symbioses (suggesting lower nutrient returns of carbon investment in ECM fungi) and show stronger decreases in colonization by non-filamentous fungi, though future work is needed to discern how these fungi affect plant growth under low vs. high soil N. We recognize that other soil biota (e.g., bacteria) could also mediate plant responses to N enrichment, though this was outside the scope of this study and requires further investigation. Second, though plant-soil feedbacks between close and distant relatives within the Tasmanian symphyomyrts are phylogenetically structured, these feedbacks do not change under N enrichment as previous studies have suggested (Revillini, Gehring & Johnson 2016). We found that plant-soil feedbacks on plant growth were on average neutral for one lineage and negative for the other, but whole soil fungal communities did not underlie these feedbacks. We show that this could be an outcome of differential receptiveness to (or showed different levels of root colonization by) DSE and non-filamentous fungi (which seemed to be beneficial for plant growth), but not mycorrhizal fungi.

### *Soil fungi explain a phylogenetic pattern in plant growth responses to N*

Soil fungi are important mediators of plant productivity, and an increasingly relevant challenge in ecology has been to understand how global change impacts plant productivity via alterations to fungal communities (van der Heijden, Bardgett & Straalen 2008). Mycorrhizal fungi in particular associate with at least 80% of all terrestrial plant species (Aerts 2002) and they can provide a majority of required N to plants (Hobbie & Hobbie 2006); thus overall their diversity tends to stimulate plant productivity (van der Heijden *et al.* 1998; Maherali & Klironomos 2007). Consistent with past fertilization and soil inoculation studies, our results show that soil fungi can increase or decrease plant responses to N enrichment (Johnson 1993; Johnson *et al.* 2008; Antoninka *et al.* 2009; Hoeksema *et al.* 2010; Kivlin, Emery & Rudgers 2013; Avolio *et al.* 2014). However, we found that conditioned soil fungi change plant responses to N enrichment which is contingent upon plant evolutionary history of closely related trees. This implies that plant phylogeny can be used as a proxy for trajectories of plant-fungal symbioses under anthropogenic N deposition.

We scored the three types of root-associated fungi (ECM, DSE, and non-filamentous fungi) that are known to influence plant productivity and may thus mediate plant responses to N enrichment, but we found that only ECM fungi responded to N enrichment in a way that may explain plant responses to N enrichment. Specifically, plants within lineage 1 that were

inoculated with home soils (whose growth responses to N enrichment were greater in live soils than in fungicide-treated soils) showed a 21% decrease in colonization by ECM fungi with N enrichment in live soils and a 7% decrease in colonization by non-filamentous fungi with N enrichment in live soils. In these plants, we might expect that a high carbon cost of ECM fungi in ambient soil N explains the decrease in growth with intact soil fungal communities (relative to fungicide conditions). But because plants that can reduce carbon investment in mycorrhizal fungi under N enrichment have been documented as winners in N deposition (Johnson 1993; Corkidi *et al.* 2002; Johnson *et al.* 2008) and ECM fungi are particularly important for eucalypt acquisition of P under conditions of increasingly limited soil P (Malajczuk, McComb & Loneragan 1975; Heinrich & Patrick 1986; Bougher, Grove & Malajczuk 1990; Burgess, Malajczuk & Grove 1993; Jones, Durall & Tinker 1998; Chen, Brundrett & Dell 2000), we predict that greater benefit of ECM fungi for phosphorus acquisition (despite lower carbon allocation to ECM) under N enrichment could have allowed these plants to contribute more carbon to their own biomass. Future studies could use careful isotope tracer experiments to tease apart who (ECM fungi or eucalypt plants) controls the provisioning of N or phosphorus to the plant and carbon to the fungi and how those patterns change depending on soil nutrient status. Alternatively, plants within lineage 2 that were inoculated with home soils (whose growth responses to N enrichment were lower in fungicide-treated soils than in live soils), tended to show no strong change in colonization by ECM fungi with N enrichment in live soils. The decrease in plant performance in live (vs. fungicide) soils in the high N soil suggests that N enrichment could cause ECM fungi to provide less benefit in return for carbon from the plant, corroborating evidence that mycorrhizal associations can become less beneficial to plants under N enrichment (Johnson 1993; Corkidi *et al.* 2002; Johnson *et al.* 2008; Hoeksema *et al.* 2010; Kivlin, Emery & Rudgers 2013; van der Putten *et al.* 2016). At the same time, the decrease in lineage 2 responses to N fertilization could also be an outcome of the stronger (12%) decrease in colonization by non-filamentous fungi under N enrichment compared to lineage 1 plants, but we are unable to tease apart effects of these fungi vs. other interacting soil fungi on plant growth.

The lineage-level differences in plant growth and root colonization responses to N enrichment could be mediated by several physiological differences between the eucalypt lineages. We should expect plant winners (lineage 1) to allocate less biomass to roots and respond to N enrichment with greater plasticity in biomass allocation, such that they allocate more to shoots that harvest light when relieved of N limitation (Johnson *et al.* 2008). But lineages did not differ in shoot biomass, root biomass, root-to-shoot ratio or specific leaf area (a proxy for photosynthetic capacity), nor did one lineage show more plasticity in these traits than the other (though overall, shoot biomass significantly increased, and root-to-shoot ratio significantly decreased, in response to N fertilization; Table S5). However, previous work has shown that lineage 1 plants have significantly greater specific root length than lineage 2 plants in nutrient limited conditions, and lineage 1 plants can significantly decrease their specific root length under N enrichment while lineage 2 plants cannot (Wooliver *et al.* 2017). Altogether, this suggests that plasticity in allocation to fine root production could also allow plants to allocate less to ECM fungi and thus more to biomass under N enrichment.

For individuals inoculated with home soils, we identified AM structures in very few individuals and none of the same three-way interactions for DSE, suggesting that ECM fungi could be contributing more to phylogenetic patterns in eucalypt responses to N enrichment. These ECM fungi were likely native to Tasmania rather than to the potting soil or greenhouse environment, as plant growth traits for individuals inoculated with control soil showed no

interactions among fungicide, N fertilization, and plant lineage; however, sequencing of fungal DNA in post-experiment soil would be needed to confirm this and to identify which fungi drove patterns in plant growth. Overall, ECM colonization was slightly greater for individuals inoculated with control soil (22%) compared to home soil (20%), suggesting the presence of weedy, rapidly colonizing mycorrhizal fungi that are native to greenhouses or potting soil. And for plants inoculated with control soil inocula, biomass significantly increased in response to N enrichment in live soils but did not respond to N enrichment in fungicide soils (with trends suggesting that fungi transition from parasitic to mutualistic with N enrichment). This suggests that plant responses to N are mediated differently between weedy greenhouse fungi and fungi with which plants interact in natural systems. Lastly, we recognize that fungicide increased DSE colonization in only one lineage with no effect on the other, although the contribution of this effect on growth responses to N enrichment requires further investigation.

Numerous studies have shown that the roots of *Eucalyptus* seedlings can be colonized by AM fungi, suggesting that AM fungi can be an ecologically significant symbiont in this tree genus (Malajczuk *et al.* 1981; Reddell & Malajczuk 1984; Chilvers, Lapeyrie & Horan 1987; Oliveira, Zambolim & Neves 1995; Jones, Durall & Tinker 1998; Chen, Brundrett & Dell 2000; dos Santos *et al.* 2001; Adams *et al.* 2006), but only 16 of the 864 seedlings in our study showed any sign of AM (ranging from 2% to 12% colonization). A majority (15) of these individuals had been inoculated with soil conditioned by Tasmanian eucalypts ('home', same lineage, or opposite lineage soil inocula), suggesting that seedlings are more responsive to naturally occurring AM fungi (potentially any of the 15 AM OTUs identified by sequencing analysis; Appendix S1) than to AM fungi in greenhouse settings. Though equally distributed between N levels and between plant lineages, AM presence was greater in live (13 individuals) vs. fungicide soils (3 individuals), and the presence of AM fungi was often associated with lower rates of ECM and DSE colonization (suggestive of competitive exclusion of AM fungi) and higher rates of colonization by non-filamentous fungi (Figs. S2-5). But overall, AM colonization was not associated with plant growth, while colonization by ECM and non-filamentous fungi were associated with lower vs. greater plant growth (Fig. S6). This and several other lines of evidence suggest that ECM fungi could be an even more important symbiont than AM for some eucalypt species. First, absent to low AM colonization in our study has been documented in other greenhouse studies of *Eucalyptus* seedlings inoculated with field soils (Reddell & Malajczuk 1984; Heinrich & Patrick 1986). Second, eucalypts are more dependent on ECM fungi than AM fungi for phosphorus acquisition (Heinrich & Patrick 1986; Bougher, Grove & Malajczuk 1990; Burgess, Malajczuk & Grove 1993; Jones, Durall & Tinker 1998; Chen, Brundrett & Dell 2000). Third, some of the same studies showing AM colonization in eucalypt seedlings have also shown that eucalypt seedling roots transition from AM- to ECM-dominated as they mature, in some cases within as few as 100 days after inoculation (Lapeyrie & Chilvers 1985; Chilvers, Lapeyrie & Horan 1987; Oliveira, Zambolim & Neves 1995; Chen, Brundrett & Dell 2000; dos Santos *et al.* 2001), a shift which has been shown to persist through adulthood in naturally occurring eucalypts (Adams *et al.* 2006). Last, the *Eucalyptus* genus and many species within it are considered to be ECM-dominated (Chilvers & Pryor 1965; Wang & Qiu 2006; Tedersoo & Brundrett 2017). As such, ECM fungi may play stronger roles in mediating eucalypt responses to N enrichment in natural systems than AM fungi.

Though previous work has suggested that Tasmanian eucalypts are susceptible to naturally occurring belowground fungal pathogens (J.K. Senior, unpublished data), and 54 pathogenic OTUs were present in the Tasmanian soils with which plants were inoculated

(Appendix S1), we saw no indication of fungal pathogens on plant roots. Thus, future work is needed to determine how responses of pathogenic fungi to N enrichment, or interactions with mycorrhizal or DSE fungi (e.g., Newsham, Fitter & Watkinson 1995; Mandyam & Jumpponen 2005; Maherali & Klironomos 2007; Sikes, Cottenie & Klironomos 2009; Laliberté *et al.* 2015; Zampieri *et al.* 2017), could alter eucalypt responses to N enrichment in their natural environments.

#### *Sensitivity to soil fungi could explain a phylogenetic pattern in plant-soil feedbacks*

We provide evidence against the prediction that plant-soil feedbacks will become more negative under anthropogenic N enrichment (Revillini, Gehring & Johnson 2016). Specifically, we found that although lineage 1 and 2 plants respectively show neutral and negative feedbacks on growth, these feedbacks do not change under N enrichment. Thus, at least in Tasmanian symphyomyrtes, alteration of plant-soil feedbacks may not be a mechanism through which species become winners or losers under anthropogenic N deposition in the future. Yet, we note that this study was conducted under controlled rather than natural conditions, where other stressors that eucalypts commonly encounter (drought, frost, foliar pathogens, etc.) may alter N-driven plant-soil feedbacks. Further, plants did not show different feedbacks between live and fungicide-treated soils, suggesting that whole soil fungal communities do not alter plant-soil feedbacks. However, we identified different changes in colonization by DSE and non-filamentous fungi between plant lineages that were associated with lineage-based plant-soil feedbacks. Specifically, plants within lineage 2 showed an increase in DSE colonization non-filamentous colonization from same to opposite lineage soils. Given the overall negative feedback for this plant lineage, these patterns suggest that greater colonization by DSE and/or non-filamentous fungi could benefit plant growth. Alternatively, plants within lineage 1 showed a decrease in DSE colonization and an increase in non-filamentous fungal colonization from same to opposite lineage soils. Given the neutral feedback for this plant lineage, these patterns suggest that greater colonization by DSE and non-filamentous fungi in soil of more distant relatives could have had equally beneficial effects on plant growth. The lack of differentiation in fungal communities between plant lineage soils (Appendix S2) and differential colonization between plant lineage soils suggest that plant lineages are differentially receptive to DSE and non-filamentous fungi, which induces different plant-soil feedbacks between lineages. The absence of an effect of N fertilization on phylogenetic-based plant-soil feedbacks suggests that these influences (or lack thereof) on plant growth can be sustained under N enrichment.

#### *Conclusions*

Plant species differentially respond to N enrichment and previous work has shown these responses are contingent upon phylogeny (Wooliver *et al.* 2016; Wooliver *et al.* 2017). Our results establish that plant interactions with soil fungi can underlie such phylogenetic patterns in plant responses to N enrichment. Further, we provide some of the first evidence that N enrichment will not change the magnitude or direction of phylogenetic-based plant-soil feedbacks, though we recognize that this study was conducted under controlled rather than natural conditions and future studies are critical for understanding how other natural stressors may alter N-driven plant-soil feedbacks. Thus, knowledge about both plant evolutionary history and plant interactions with conspecific soil fungi will be critical for understanding variation in performance across plant species as global change continues.

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**CHAPTER IV**  
**NEW INSIGHTS ON TERRESTRIAL NITROGEN CYCLING AND THEIR**  
**RELEVANCE TO PAST, PRESENT, AND FUTURE ECO-EVOLUTIONARY**  
**FEEDBACKS**

This chapter is in preparation by Rachel C. Wooliver, Colin Averill, Lars O. Hedin, Benjamin Z. Houlton, Adam F.A. Pellegrini, Joshua Schimel, Jennifer A. Schweitzer & Bonnie Waring for publication as an invited review at *Functional Ecology*.

### **Abstract**

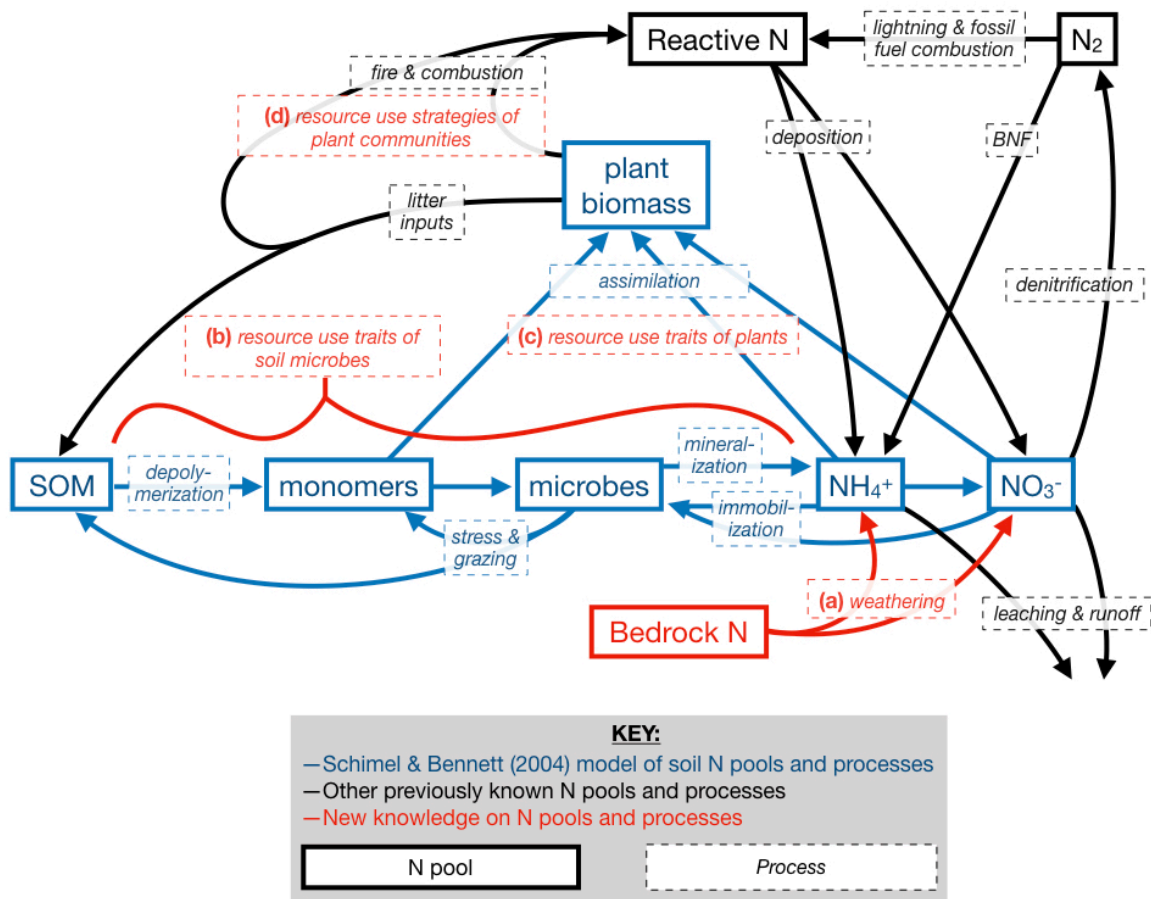
Soil nitrogen (N), in its many forms, plays critical roles in terrestrial ecosystems, but assumptions regarding the source, microbial transformations, plant use, and loss of ecosystem N continue to shift, altering perspectives and management of soil N in terrestrial ecosystems. We integrate recent advances into an updated N cycling framework and discuss consequences for the understanding of eco-evolutionary dynamics and nutrient cycling with global change. One new insight is the significance of N sources from rock weathering in plant-soil systems, which challenges the traditional view of the N cycle that focuses exclusively on atmospheric N inputs. Next, though soil N is often assumed to limit microbial decomposition, N limitation of this process is not always observed. Similarly, though soil N is often seen to limit plant community productivity, less attention has been given to empirical evidence that not all plant *species* respond positively to N enrichment. Last, contrary to the paradigm that disturbance drives N losses in all ecosystems, responses of soil N pools to fire as one form of disturbance range from positive to negative. We describe how this revised knowledge has made it increasingly clear that N cycling is tied to past evolution of microbial and plant resource use traits across soil N gradients (partially formed by global variation in rock N weathering) and their contemporary responses to and controls on N availability. For example, newly revealed nuanced plant and microbial linkages and feedbacks affect ecosystem processes such as decomposition, production, and fire-driven N losses that may reinforce or even weaken historical patterns of N fertility. Through such effects, globally increasing anthropogenic N deposition may alter patterns of eco-evolutionary feedbacks between soil N and organisms, where (in general) higher soil N selects for organisms with 'competitive' resource use traits that lead to rapid N turnover, and lower soil N selects for organisms with 'conservative' resource use traits that lead to slow N turnover. Continually shifting insights about N cycling suggests that how we interpret and teach the N cycle must accommodate this changing knowledge, in particular recognizing the role of evolutionary processes in ecosystem ecology. Moreover, this new understanding can inform our approach to global change as these results have large implications for our understanding of coupled carbon and N cycling and could fundamentally alter how we understand and manage ecosystems in a changing world.

### **Introduction**

Nitrogen (N) has long been viewed as a critical limiting nutrient across ecosystems (Liebig 1842; Vitousek & Howarth 1991; LeBauer & Treseder 2008), but our understanding of the N cycle, co-limitation, and interacting nutrient cycles continues to change in ways that are fundamental and surprising. More than a decade ago, Schimel & Bennett (2004) described a revised soil N cycle, focusing on drivers and gate-keepers of N mineralization based on evidence that plants can acquire organic N, which directly violated older assumptions that plants use only inorganic N and compete poorly against soil microbes for this nutrient. Other major assumptions about N that underlie many theories in ecosystem ecology include: 1) sources of N to terrestrial ecosystems are ultimately derived from the atmosphere through N fixation (where specific soil microbes that convert N<sub>2</sub> to bioavailable N) or from anthropogenic industrial deposition (Walker & Syers, 1976; Cleveland et al. 1999; Galloway et al. 2004; Galloway et al. 2013; Vitousek et al. 2013;

Stocker et al. 2016), even though other sources have long been speculated to be an overlooked source of terrestrial N (Cornwell & Stone 1968; Hendry, McCready & Gould 1984; Holloway et al. 1998; Morford et al. 2011; Dixon, Campbell & Durham 2012; Montross et al. 2013), 2) N is often the predominant nutrient that limits primary productivity and decomposition, followed or co-limited by other nutrients (Vitousek & Howarth 1991; LeBauer & Treseder 2008; Zhang et al. 2008), and 3) disturbances such as clear cutting, anthropogenic N deposition, and fire generally increase N losses from ecosystems through leaching and gaseous pathways (Vitousek & Reiners 1975; Bormann et al. 1968; Vitousek & Reiners 1975; Bayley et al. 1992; Aber et al. 1998; Certini 2005). But these assumptions are now being challenged by recent evidence that ecologically significant N inputs occur from rock weathering, that not all plant biomass and soil microbial activity is N-limited (although both may be limited or co-limited by other site-specific mineral nutrients, especially in tropical ecosystems) due to evolutionary differentiation in resource use strategies, and that some plant communities can suppress N losses from ecosystems after disturbance. Figure 4.1 illustrates how these new insights—the expanded role of N sources through rock N weathering and the mediation of decomposition, production, and fire-driven N losses by variation in plant and microbial resource use—revise the traditional model of N cycling, including amendments by Schimel & Bennett (2004). It is crucial to integrate these changing views of N to better understand major theories and applications in ecosystem ecology and evolution.

An improved understanding of the terrestrial N cycle could clarify current knowledge on linkages and feedbacks between ecosystem N and the evolution of organisms, and thus how internal N cycling operates across ecological space and evolutionary time. Observational and experimental work has suggested that plants and microbes have evolved and coevolved in response to historical soil N gradients and as a result display genetic variation in resource use traits (Thompson 2005). For example, plants that naturally occur in soils with lower inorganic N show (genetically-based) slower growth rate, lower foliar N concentration, and greater dependence on root microbial mutualists than those that naturally occur in soils with higher inorganic N (Cunningham, Summerhayes & Westoby 1999; Cavender-Bares et al. 2004; Johnson et al. 2010; Zhu et al. 2017). This has resulted in a ‘plant economics spectrum’ of competitive to conservative suites of resource use traits from high to low resource environments, respectively (Wright et al. 2004; Reich 2014). Accordingly, N fertilization has been shown to select for plants with faster growth rates and lower dependence on mycorrhizal fungi (Johnson et al. 2008). Similarly, N fertilization has been shown to select for copiotrophic soil microbial taxa that exhibit rapid growth rates and metabolize less recalcitrant substrates over oligotrophic soil microbial taxa that exhibit slower growth rates and metabolize more recalcitrant substrates (Frey et al. 2004; Fierer et al. 2011). Such resource use traits can in turn influence soil N through their effects on N cycling processes (Diaz & Cabido 2001; van der Heijden, Bardgett & Straalen 2007). For example, plants can reinforce or weaken soil fertility patterns through effects of litter quality (e.g., foliar N concentration) on decomposition and plant-microbial interactions (e.g. associations with N-fixing microbes or mycorrhizal fungi) that mediate nutrient inputs and mineralization (Singh, Rawat & Chaturvedi 1984; Hobbie 1992; Hobbie 2015). However, literature on feedbacks between soil N and organisms have failed to recognize rock N weathering as an important source of variation in historical soil N gradients and the role of evolutionary variation in resource use traits in guiding ecological responses to anthropogenic N deposition.



**Figure 4.1. Changing paradigms of the global nitrogen cycle (red) overlaid on the soil nitrogen cycle developed by Schimel & Bennett (2004; blue) and other previously established nitrogen (N) pools and processes (black).** (a) A biologically significant portion of nitrogen inputs to ecosystems originate from parent material; (b) microbial transformations of nitrogen depend evolved variation in microbial resource use traits and abiotic soil characteristics; (c) N limitation of productivity depends on evolved variation in plant resource use traits, including associations with root-associated microbes; and (d) community-level variation in plant resource use strategies can mediate whether fire as a disturbance induces nitrogen losses from ecosystems.

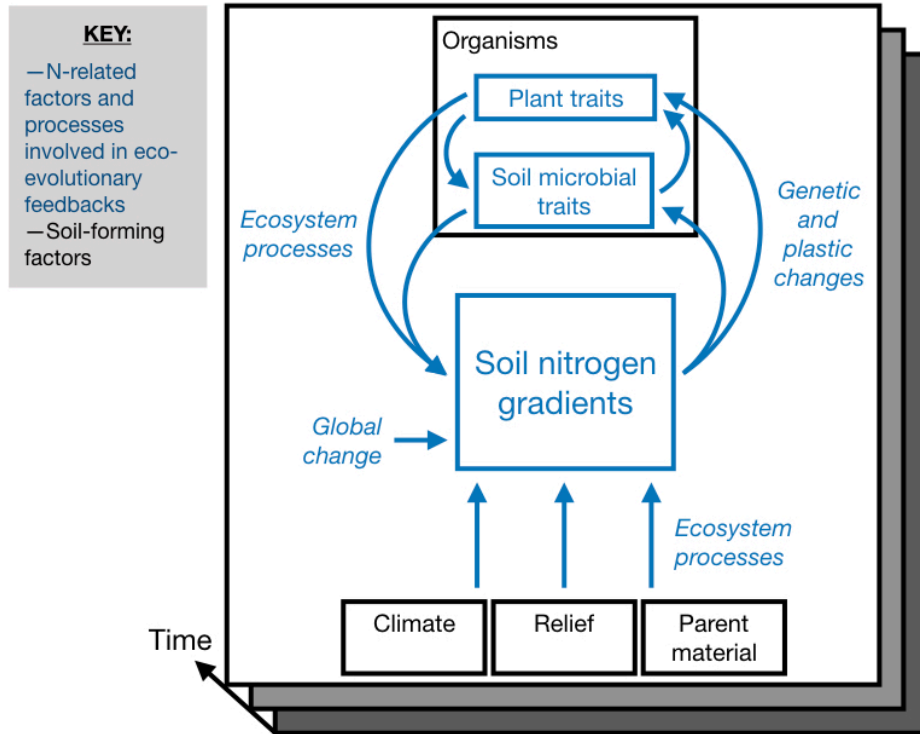
Because the N cycle is coupled with the carbon (C) cycle, our understanding of N limitation of ecosystem processes at local scales will dictate our ability to predict the effects of anthropogenic soil N enrichment and climate change on the global C cycle (Wang & Houlton 2009; Gerber et al. 2010; Finzi et al. 2011). Resource use traits that underlie variation in plant responses to N could explain why plant community productivity is more often co-limited by N and phosphorus (P) than N singly (Elser et al. 2007; Harpole et al. 2011) and why suppression of either plant species or community biomass by natural or simulated N deposition occurs surprisingly often (Aber et al. 1989; Thomas et al. 2010; Harpole et al. 2011; Wooliver et al. 2016). This means that N addition (regardless of source) will not always induce greater rates of C sequestration in plant communities. Furthermore, while N fertilization experiments broadly show suppression of soil microbial activity and decomposition (Janssens et al. 2010; Ramirez, Craine & Fierer 2010; Ramirez, Craine & Fierer 2012), new synthesis suggests this may be more linked to changes in soil chemistry rather than nutritional inhibition of microbial activity (Averill & Waring 2017). This means that microbial responses to natural variation in N availability (through changes in organic matter stoichiometry) may be distinct from microbial responses to N fertilization. Thus, anthropogenic acceleration of the N cycle (i.e., increasing N inputs to soils globally) may not always accelerate productivity and decomposition, and therefore may not always enhance C cycling or storage.

This review uses ecological and evolutionary insights on terrestrial N cycling to show how ecological interactions between soil N and organisms change over evolutionary timescales, and how these feedbacks will operate under global change, with subsequent effects on ecosystem C storage. Specifically, we emphasize the importance of parent material, time, and biotic interactions, as first described in the historical model of Jenny (1941), in eco-evolutionary feedbacks between soil N and organisms. Eco-evolutionary feedbacks occur when evolution via natural selection and subsequent trait variation influences ecosystem processes that drive how ecosystems function (Schoener 2010; Matthews et al. 2011; Van Nuland et al. 2016). Using an eco-evolutionary framework and revised knowledge of N cycling, we describe how (i) rock N weathering could have contributed more strongly to historical gradients in soil N than previously recognized and (ii) past evolution and coevolution of plant and soil microbial resource use traits underlie whether production and decomposition are N-limited, and the magnitude and direction of changes in soil N after fire (Fig. 4.2). This allows us to better link local processes that guide internal N cycling and occur over shorter timescales with global-scale and long-term variation in ecosystem N and coupled ecosystem C. Thus, we conclude by synthesizing implications of this work for our ability to accurately predict future global C pools under global change and suggest directions for making accurate assessments for global models in a changing world.

### **Ecological and evolutionary significance of rock N weathering**

An assumption made by a vast majority of published N cycles is that biological N fixation (BNF) and deposition are the sole sources of N in terrestrial ecosystems (Walker & Syers 1976; Cleveland et al. 1999; Galloway et al. 2004; Galloway et al. 2013; Vitousek et al. 2013; Stocker et al. 2016), even though other sources of N have long been suggested (Cornwell & Stone 1968; Hendry, McCready & Gould 1984; Holloway et al. 1998; Morford et al. 2011; Dixon, Campbell & Durham 2012; Montross et al. 2013). Recent work has revealed that rock-derived N is an ecologically significant source in certain ecosystems, due to advances in understanding the chemistry, denudation, and chemical weathering of rock N sources across terrestrial landscapes (Morford, Houlton & Dahlgren 2011; Houlton & Morford 2015; Morford, Houlton & Dahlgren





**Figure 4.2. Nitrogen guides eco-evolutionary feedbacks (adapted from Van Nuland et al. 2016; blue) between plants to soils in terrestrial systems.** Jenny’s (1941) five soil-forming factors (black boxes) influence ecosystem processes that affect soil available nitrogen (rock nitrogen weathering and biological nitrogen fixation). This creates a historical gradient along which organisms display plastic (individual-level) and genetic (population- and community-level) changes in resource use traits. Organisms can in turn influence ecosystem processes that affect soil nitrogen (production, decomposition, biological nitrogen fixation, fire) that can reinforce or weaken historical gradients of soil nitrogen (eco-evolutionary feedback). Nitrogen deposition is a contemporary agent of global change that directly alters soil nitrogen pools, with consequences for variation in plant traits and subsequent feedbacks.

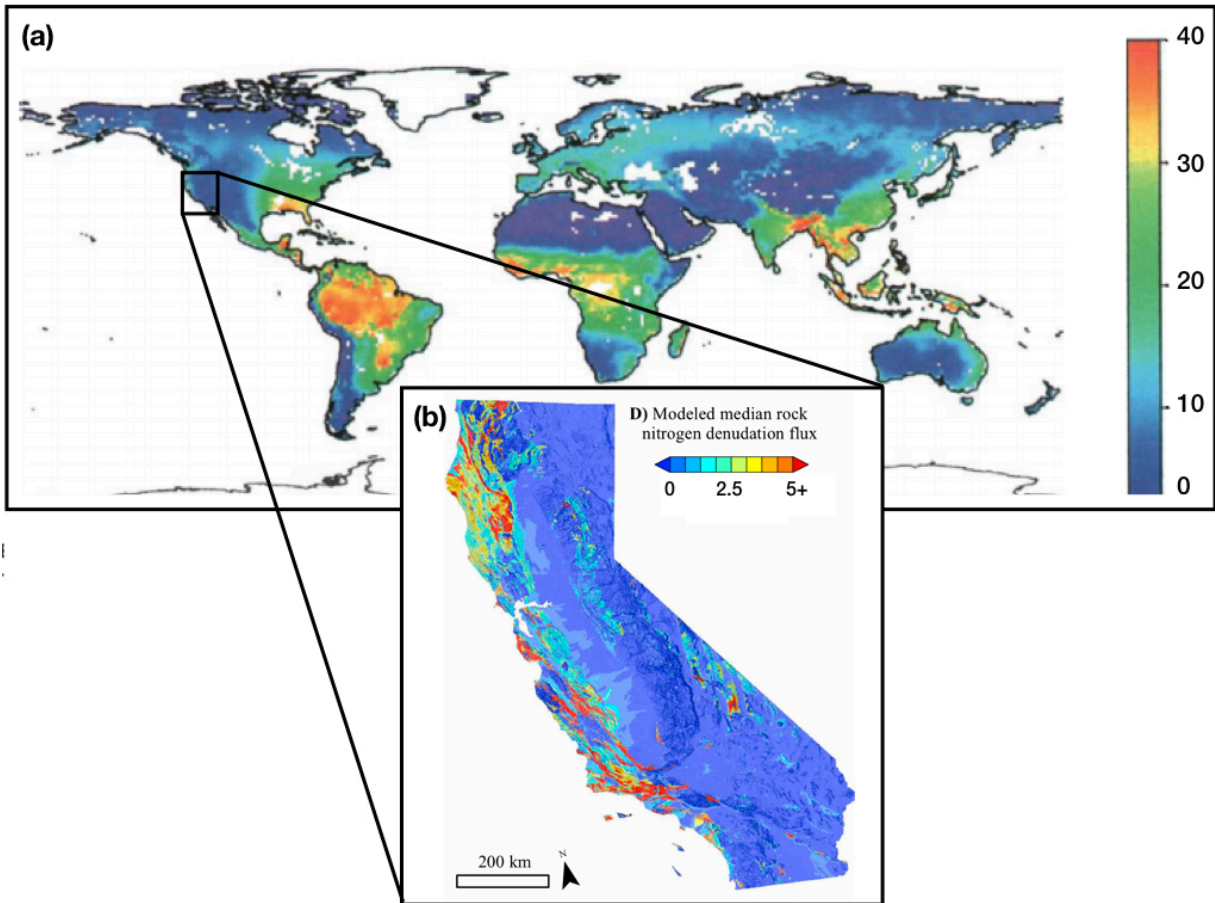
2016a; Morford, Houlton & Dahlgren 2016b; Fig. 1a). This work helps to resolve evidence for ‘missing N’ inputs that have been identified in past syntheses (Binkley, Son & Valentine 2000), in which the accumulation of N in vegetation and soil has been shown to greatly exceed inputs via atmospheric N input pathways (Houlton & Morford 2015). Further, this research argues for synthesis and integration of tectonic uplift, geochemistry, rock weathering, geobiology, and ecological science, with widespread implications for patterns and regulation of soil N gradients, evolutionary responses of terrestrial organisms to such gradients, and consequences for biogeochemical feedbacks with the C cycle and climate system.

The insight that rocks are a significant source of N in ecosystems undermines a widely held hypothesis about soil formation and nutrient inputs, which proposes that N limitation increases with latitude (Walker & Syers 1976). This hypothesis is founded upon the assumption that the sole source of ecosystem N is through BNF, rates of which tend to be greatest (and thus tend to alleviate N limitation) in lower latitudes that comprise early-succession systems and warmer climates (Cleveland et al. 1999; Hedin et al. 2009; Menge & Hedin 2009; Fig. 4.3a). Because soils are older and more weathered in these systems, P (which, unlike N, is largely derived from rock weathering) becomes the most limiting nutrient (Walker & Syers 1976). However, it is now clear that rock N inputs can relieve N limitation in higher-latitude, younger soils where BNF may not be substantial (Houlton & Morford 2015; Morford, Houlton & Dahlgren 2016a; Morford, Houlton & Dahlgren 2016b). Globally, these rock N inputs do not follow latitudinal gradients as strongly as do BNF inputs (Cleveland et al. 1999; Houlton 2018; Fig. 4.3b). Rather, rock N inputs tend to be highest in early-succession systems with exposed N-rich sedimentary/meta-sedimentary parent materials, wetter (and to a lesser extent warmer) climates, moderate relief, and greater productivity (Houlton & Morford 2015). These rock N inputs could contribute to global gradients of soil N that do not necessarily vary strongly with latitude and explain why global meta-analyses of plant responses to N fertilization have found no support for Walker & Syer’s (1976) hypothesis that N limitation increases with latitude (Harpole et al. 2011; LeBauer & Treseder 2008; Wooliver et al. 2016).

Further, the knowledge that rock N weathering can be an ecologically significant source of N to ecosystems can be incorporated into a framework for understanding eco-evolutionary feedbacks between soil N and organisms (Fig. 4.2). Specifically, global gradients of soil N have historically been shaped by Jenny’s (1941) five factors of soil formation (time, parent material, climate, relief, and organisms) through their effects on the ecosystem processes of rock N weathering and BNF. This provides the foundation for historical soil N gradients along which organisms could have evolved and co-evolved (described above, Cunningham, Summerhayes & Westoby 1999; Cavender-Bares et al. 2004; Frey et al. 2004; Johnson et al. 2010; Fierer et al. 2011; Zhu et al. 2017), demonstrating newly recognized importance of parent material (rocks) in contributing to an abiotic gradient (soil N) along which organisms interact and evolve (Thompson 2005).

### **Evolution and abiotic drivers of microbial N transformations**

Soil microbes mediate how N moves among soil pools, which include soil organic matter (polymeric organic N), soil inorganic N, and plant biomass (Schimel & Bennett 2004; Fig. 4.1). Many theories in ecosystem ecology assume that N limits microbial activity and decomposition, because N is immobilized in leaf litter decay during the early stages of decay, and N-poor organic matter decomposes more slowly (Melillo, Aber & Muratore 1982; Zhang et al. 2008). Counter to these expectations, experiments consistently demonstrate that N fertilization can



**Figure 4.3. (a) Biological nitrogen fixation has been identified as the sole source of nitrogen in ecosystems and tends to follow a latitudinal gradient (where inputs increase from the poles to the equator; adapted from Cleveland et al. 1999), but recent work has shown that (b) rock nitrogen weathering is another biologically significant source of ecosystem nitrogen (adapted from Morford, Houlton & Dahlgren 2016). Rock nitrogen weathering does not follow a latitudinal gradient, undermining the widely held hypothesis that higher-latitude ecosystems are more nitrogen -limited than lower-latitude ecosystems (Walker & Syers 1976). Units are in  $\text{kg ha}^{-1} \text{yr}^{-1}$ .**

actually suppress microbial activity and thus decomposition rates (Janssens et al. 2010; Ramirez, Craine & Fierer 2010; Ramirez, Craine & Fierer 2012), seemingly challenging the assumption that decomposition is limited by N availability. Empirical and theoretical work offer that this apparent contradiction could be an outcome of past evolution of resource use strategies and/or abiotic factors that limit microbial activity. Specifically, long-term fertilization studies in natural systems have shown that higher soil N induces shifts in microbial communities, favoring copiotrophic microbial taxa that can less easily metabolize recalcitrant substrates that could make up the majority of soil C and N pools (Frey et al. 2004; Fierer et al. 2011). At the same time, N fertilization can induce soil acidification, creating toxic soil environments that limit microbial activity (Tian & Niu 2015). Hence, microbial decomposition may be frequently limited by N; however fertilization-induced acidification may counteract the positive effects of N on microbial growth (Averill & Waring 2017, Fig. 4.1b). This suggests that anthropogenic N deposition could become a strong selective force for soil microbial communities (van Diepen et al. 2017), ultimately weakening feedbacks of microbes on soil N through suppression of decomposition, which will be important for understanding future patterns in C cycling.

Within natural systems, soil N and microbes can interact with one another through their respective effects on evolution and ecosystem processes (Fig. 4.2). Across historical soil N gradients, soil microbes have evolved traits that increase fitness in low or high N soils and feed back at the ecosystem level to reinforce soil N. One example is ectomycorrhizal fungi, which can selectively mine organic N from soils and thus exacerbate N limitation of decomposition, furthering their competitive advantage over other microbes that are less able to access organic N (Fig. 4.2, Fernandez & Kennedy 2016, Averill & Hawkes 2016). The sensitivity of soil microbes to acidification, shifts towards more copiotrophic-dominated microbial communities, and subsequent effects on decomposition with N enrichment provide an important linkage among the past evolution of soil microbial resource use traits, ecosystem process, and outcomes for soil N that could emerge under scenarios of anthropogenic N deposition. Specifically, evidence for decreases in microbial activity and biomass in response to the acidity of these new environments (Janssens et al. 2010; Ramirez, Craine & Fierer 2010; Ramirez, Craine & Fierer 2012) suggests that feedbacks, which may have previously reinforced historical patterns in soil N, will weaken.

### **Evolution of plant N use**

Soil N is the nutrient that is often thought to primarily limit terrestrial productivity (Liebig 1842; Vitousek & Howarth 1991), although nutrient co-limitation and limitation by P is also common in many tropical terrestrial systems (Elser et al. 2007; Hedin et al. 2009; Vitousek et al. 2010; Harpole et al. 2011). This assumption is consistent with evidence that fertilization with organic and inorganic forms of N increases community productivity across the globe (Treseder & LeBauer 2008). However, this assumption is not accurate at the species level because N fertilization studies have shown that species' responses to N range from negative to positive (Pennings et al. 2005; Xia & Wan 2008; Thomas et al. 2010; Wooliver et al. 2016). Such variation in N limitation among plant species is one reason why some species persist and others are lost under anthropogenic N enrichment (Stevens et al. 2004; Suding et al. 2005; Bobbink et al. 2010). Thus, understanding variation in N limitation across plant species and the mechanisms that underlie these responses will be important for predicting plant winners and losers under future N deposition scenarios. Recent work has shown that past evolution of resource use strategies (including associations with microbial symbionts) may explain variation in N limitation across plant species (Fig. 4.1c), which highlights the importance of evolutionary time

and biotic interactions in driving eco-evolutionary feedbacks and C sequestration under global change.

Under anthropogenic N enrichment, we should expect conservative resource-use plants to show lower N limitation (and thus be outcompeted), competitive resource-use plants to show greater N limitation (and thus outcompete other plants), and traits of the latter to further stimulate soil N cycling. Empirical work has indicated that the extent to which system-specific genetic and plastic variation in plant resource use traits mediates production responses to increasing N deposition (Fig. 4.2) is context dependent, varying by scale, ecosystem, the breadth of traits examined, and which traits are examined (Suding et al. 2008). For example, meta-analyses of plant responses to N fertilization have shown that plant functional types (e.g. root type and life history) explain more variation in N limitation across species than global climatic or latitude gradients (Wooliver et al. 2016), but N-induced local extinction in North American ecosystems is more often associated with initial species abundances than plant functional types (Suding et al. 2005). Within a group of functionally diverse *Eucalyptus* species that are native to Tasmania, Australia, N limitation is associated with phylogenetic variation in one resource use trait (specific root length) but not others (specific leaf area and specific stem density) (Wooliver et al. 2017). Plastic responses of plant resource use traits also show mixed results. For example, N fertilization studies have found overall increases in specific leaf area and foliar percent N in response to N fertilization (Wright & Sutton-Grier 2012; Wooliver et al. 2017); because these traits are associated with greater rates of litter decomposition across terrestrial plants (Cornwell et al. 2008), this could lead to positive feedbacks on soil N under N deposition scenarios. However, species' productivity responses to N fertilization are not always correlated with litter decomposability (Manning et al. 2008), nor is within-species variability in litter quality always related to decomposability (Jackson et al. 2013), suggesting that N deposition could cause feedbacks between plant traits and soil N to weaken in some systems.

Shared evolutionary history is emerging as a key predictor of species' responses to N (Wooliver et al. 2016), in part due to patterns where more closely related plants tend to be more similar in their resource use strategies, including not only physiological traits described above but also symbioses with root microbial symbionts (Doyle 1998; Brundrett 2002). Plant symbiosis with N-fixing rhizobial bacteria and mycorrhizal fungi are the result of local adaptation to nutrient-limited soils (Lambers et al. 2008; Johnson et al. 2010) and are thus extremely responsive to N availability, often with negative outcomes for plant performance. Specifically, N-fixing plants are among the most vulnerable to local extinction under N fertilization (B. Waring, unpublished data; Suding et al. 2005) and the presence of mycorrhizal fungi tends to suppress overall plant biomass responses to N fertilization (Kivlin et al. 2013). Yet, plants may have some control over such patterns, for example some mycorrhizal plants can allocate less C to mycorrhizal fungi (and thus more C to biomass production) under N fertilization (Johnson 1993; Corkidi et al. 2002; Johnson et al. 2008; Wooliver et al. in review). These plant-microbial interactions have been shown to explain phylogenetic variation in plant responses to N fertilization (Wooliver et al. in review), and suggest that N deposition will generally weaken the positive feedbacks and coevolution between plants and their nutrient-scavenging microbial symbionts (Revillini, Gehring & Johnson 2016).

### **Plant resource use strategies mediate fire-driven N losses**

Both bioavailable and unavailable forms of N are naturally lost from ecosystems through leaching and such losses are known to be mediated by biotic uptake and litter inputs (Vitousek &

Reiners 1975; Hedin, Armesto & Johnson 1995; Lovett, Weathers & Arthur 2002). Disturbances, such as clear cutting, anthropogenic N deposition, and fire, have been shown to accelerate losses by promoting N leaching (Bormann et al. 1968; Vitousek & Reiners 1975; Bayley et al. 1992; Aber et al. 1998; Certini 2005). Fire in particular is one large N loss pathway because it also combusts N stored in aboveground biomass (Kauffman, Cummings & Ward 1994), restricting its turnover into soil N pools. For example, a recent meta-analysis showed that over a 65-year period, recurrent fires in plots across grassland and forest ecosystems reduced soil N by ~38% on average relative to fire protected plots (Pellegrini et al. 2017). Similar losses were observed for C, but not for other limiting nutrients such as calcium and potassium (which were both enriched) and P (which was initially enriched but later declined with sustained burning), suggesting that communities that experience recurrent fires become more N-limited over time.

But what has only recently been recognized is that changes in soil N after disturbance can be mediated by plant community composition, where frequent burning enriches total soil N and C in certain communities (needleleaf forests), but depletes total soil N and C in others (broadleaf forests and savanna-grasslands) (Pellegrini et al. 2017; Fig. 4.1d). Increases in soil N and C in needleleaf forests could be due to greater abundances of N-fixing plants and/or mobilization of resources from smoldering organic material on the forest floor (Pellegrini et al. 2017). This suggests an eco-evolutionary interaction between soil N and plant resource use in communities that experience frequent fire (Fig. 4.2), where soil N pools drive genetic and plastic changes in plant resource use that can either reinforce or weaken soil N pools.

In the case of certain disturbances, ecosystem losses of soil N can elicit plastic or genetic compensatory responses in the resource use strategies of plants. For example, it is thought that recurrent fire selects for plants that can increase tissue C:N (thereby reducing N limitation through reductions in N requirements), and/or plants that associate with N-fixing microbes (thereby reducing N limitation through acquisition of N from otherwise unavailable sources) (Reich et al. 2001; Cavender-Bares & Reich 2012). However, in tropical savannas, frequent burning does not generally select for N fixers and while the C:N ratios of individual species are low, they remained unchanged in spite of large changes in soil N (Pellegrini et al. 2015), suggesting fire can act as a habitat filter favoring plants that have already evolved conservative resource use strategies in ecosystems with historically low soil N. However, it raises new questions as to whether the evolution of plants in fire frequent environments has constrained their ability to respond plastically to fire-driven changes in soil N (Pellegrini et al. 2015). But as previously described, different plant communities that experience frequent fire can either decrease or increase soil N after fire (Pellegrini et al. 2017). This suggests that even though low-nutrient systems may select for plant species with conservative resource use traits, these traits will not always reinforce slow N cycling as suggested by Hobbie (1992).

### **Synthesis and future directions**

Because research that has altered understanding of N cycling has remained isolated within the fields of biogeochemistry, microbial ecology, and plant ecology, the first goal of this review was to integrate recent advances in our understanding of the role of N in ecosystems into a revised N cycle (Fig. 4.1). This N cycle builds on a previous revision of the soil N cycle (Schimel & Bennett 2004) and other known N pools and processes that drive fluxes among pools. The second goal of the review was to highlight how advances in our understanding of the N cycle clarify how soil N can be a major component of eco-evolutionary dynamics (Fig. 4.2), drawing on both ecological and evolutionary ideas and theory. This allows us to better link local

processes that guide internal N cycling and occur over shorter timescales with global-scale and long-term variation in soil N. We have discussed four major advances in our understanding of the terrestrial N cycle. Though these advances challenge long-held assumptions in ecosystem ecology and evolutionary biology, they provide insight into how ecological interactions change over evolutionary timescales (eco-evo feedbacks) and how these feedbacks will operate under global change. First, rocks are a previously unaccounted for and biologically significant source of N in ecosystems. This helps to explain the “missing N” source observed for many terrestrial ecosystem sites (Fig. 4.1a) and global gradients of soil N along which organisms have evolved. Second, according to N fertilization experiments, productivity and decomposition are not always N limited as previously assumed (Fig. 4.1b,c). This can be explained by evolutionary and ecological dynamics that govern plant and microbial responses to nutrient availability, and suggests that anthropogenic soil N enrichment will not always result in the same reactions of C release from and sequestration into biomass. Third, the composition of resource use strategies within plant communities can mediate N losses subsequent to long-term repeated disturbance (Fig. 4.1d), negating the paradigm that disturbance always drives N losses and demonstrating that plants can mediate key ecosystem processes to create feedbacks that reinforce or buffer N losses.

With a better understanding of how N operates within and across ecosystems and across evolutionary timescales come newly identified gaps in our knowledge about how N operates within and across spatial scales. At the local and regional scales, we know that rock weathering can vary due to soil-forming factors, but further work is required to determine what happens to rock N once it is weathered and how much rock N weathering affects ecosystems downslope. Further, though we know that plants can uptake both inorganic and organic forms of N, we have a limited understanding of how much of this N uptake is organic vs. inorganic and how this varies across plant species and environments. A new tool to evaluate this question is microdialysis, which uses root-sized flow-through dialysis membranes to capture small molecules that are free to diffuse down a concentration gradient in the soil, and so to sample the soil in much the way a root does. Related to this issue, we are only beginning to understand how the physical and chemical structure of soils affects N cycling in situ. While extracellular enzymes are clearly central to litter decay, issues remain with interpreting the current potential activity measurements which use small freely mobile artificial substrates and with enzymes in mineral soil, where mineral activities (e.g. as oxidizers) and the nature of organic matter cloud the role of exoenzymes.

At the global scale, a newly recognized source of N in ecosystems (rock N weathering) opens questions about how this unaccounted-for N could be contributing to global C cycling. Further, considering future predictions of increasing anthropogenic N limitation that may release communities from progressive N limitation under increasing atmospheric C, gaps remain in our knowledge of the implications of variation in N limitation for C cycling (sequestration in above- or belowground biomass) under global change. Last, given that some areas are projected to experience more common/intense fires in the future, an important but unanswered question is whether plant resource use strategies and associated control over fire-driven N losses affect ecosystem stability.

#### *Consequences for understanding C cycling in a changing world*

This review suggests that future models of N cycling must integrate evolution of plant and microbial resource use strategies, plant-microbial interactions, variation in inputs and outputs

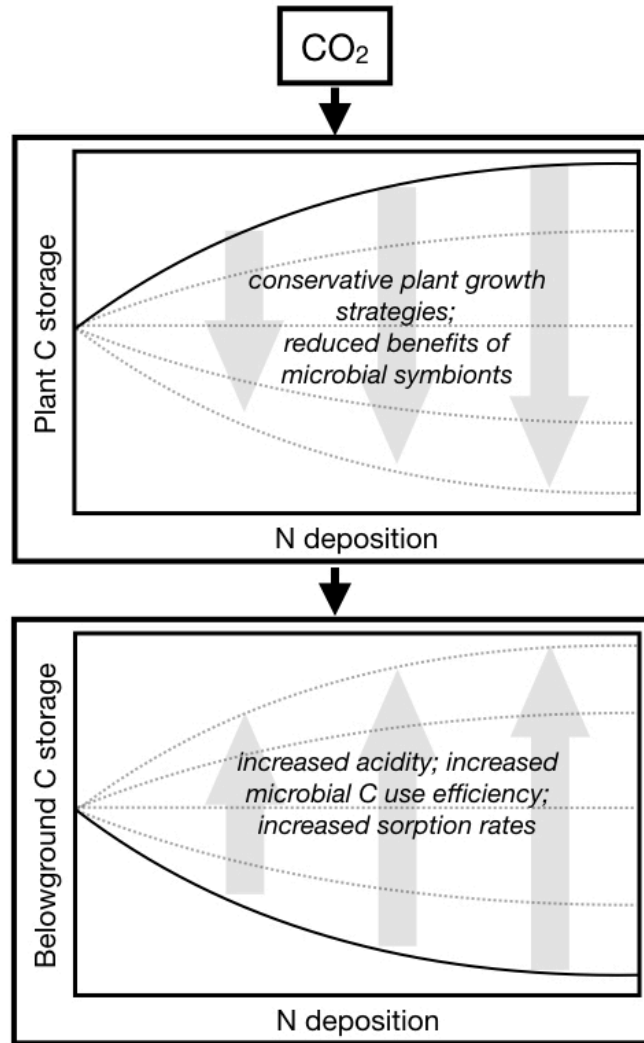
across systems, and soil chemistry. And perhaps the most pressing question is how these factors will also guide coupled C cycling under future global change. Thus, we conclude by synthesizing implications of this work for our ability to accurately predict future global C pools under global change and suggested directions for making accurate assessments for global models in a changing world

First, the net effect of N addition on soil C storage may depend not only on microbial sensitivities to N-induced decreases in pH, but also decomposer nutrient limitation status and also microbial resource use efficiencies. Importantly, all of these effects could cause increases in belowground C storage that are greater than expected (Fig. 4.4). Specifically, by increasing soil acidity, adding N to soils can reduce decomposition of the particulate (unprotected) organic matter pool by microbes that are sensitive to acidic environments. Adding N to soils can also relieve N limitation of soil microbes, which can increase C use efficiency and thus the size of the microbial biomass pool. As a result of the previous two processes, adding N to soils can increase sorption rates of microbially-derived C (biomass, exudates, and necromass) onto mineral surfaces, thus increasing the mineral-associated organic matter pool (Averill & Waring 2017), thereby enhancing belowground C in the form of stabilized, mineral-associated organic matter (Cotrufo et al. 2013). Thus, predicting responses of soil C storage to N enrichment will require careful consideration of microbial nutrient limitation status in the context of other abiotic drivers of C storage, like soil acidity and mineralogy.

Second, the effect of N addition on productivity (the sequestration of atmospheric C in plant biomass) will depend on plant nutrient limitation and microbial symbionts. Both could drive patterns in C sequestration that are lower than expected. Specifically, adding N to soils can reduce productivity of communities containing plants with conservative growth strategies, which are physiologically unable to use the excess N for growth. Adding N to soils can also reduce nutritional benefits of microbial symbionts, thereby reducing nutrient returns for the C that plants invest in the symbionts. Thus, predicting responses of C sequestration to N enrichment will require careful consideration of plant evolutionary history in terms of resource use strategies and interactions with root-associated microbes.

Overall, new insights on terrestrial N cycling, from inputs to within-system turnover to outputs, require that we view coupled ecosystem N and C in a more nuanced way. We have described how these new insights integrate into previously established N pools and processes that mediate movement among these pools, and how they fit into theory on linkages and feedbacks between ecosystems and the evolution of plants and soil microbes. Our attempt to understand such eco-evolutionary dynamics has underscored the importance of biotic interactions and parent material as primary contributors to ecosystem N through deep (evolutionary and geological) time, providing a foundation for inferring ecosystem dynamics under global change.





**Figure 4.4. Nitrogen (N) fertilization does not always result in stimulate sequestration of atmospheric carbon (CO<sub>2</sub>) into plant biomass (which increases plant C storage; solid line in top plot) and decomposition of soil organic C (which decreases belowground C storage; solid line in bottom plot).** As such, in plants, anthropogenic N deposition could decrease C storage by 1) reducing growth of plants with conservative growth strategies that are physiologically unable to use the excess N for growth or 2) reducing nutritional benefits of microbial symbionts. Belowground, N deposition could increase C storage by 1) increasing soil acidity, thereby reducing activity (decomposition) of microbes that are sensitive to acidic environments and increasing the size of the particulate (unprotected) organic matter pool, 2) relieving nitrogen limitation of soil microbes, thereby increasing C use efficiency and the size of the microbial biomass pool, or 3) increasing sorption rates of microbially-derived C (biomass, exudates, and necromass) onto mineral surfaces as a result of the former two processes, thereby increasing the mineral-associated organic matter pool.

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## CONCLUSION

Variation in soil resources has acted as a selective gradient along which plants have evolved, leading to variation in plant traits that can reinforce these gradients through their effects on ecosystem processes. Current global anthropogenic activities are simultaneously increasing a major soil resource for plant growth—nitrogen (N)—consequently shifting plant communities by selecting for some (more N-limited) plant species over (less N-limited) others. Yet, until very recently, we have lacked a basic understanding of the mechanisms that govern this variation in responses to N enrichment among plants. Further, such variation in plant responses to N enrichment contradicts the paradigm that all plants are N-limited, with consequences for our understanding of plant production as well as the linkages and feedbacks between evolution and ecosystem processes under global change. To address these issues, my dissertation has explored evolutionary and ecological drivers of variation in plant responses to soil N enrichment, and how emerging knowledge about N cycle processes clarifies our view of feedbacks between soil N and terrestrial organisms.

My dissertation has yielded four key results. First, responses to N enrichment vary widely across terrestrial plant species (Chapter I) and even among species within the same genus (Chapter II). Second, phylogeny is a strong predictor of species' responses to N enrichment at both of these phylogenetic scales (i.e., within and across genera; Chapters I & II), iterating current evidence that phylogenies are central to our ability to predict winners and losers in global change scenarios. Third, phylogenetic patterns in plant responses to N enrichment can be underpinned by phylogenetic variation in plant traits that are proxies for resource use capacities (Chapters I & II) and phylogenetic variation in interactions with mycorrhizal fungi (Chapter III), indicating that there is a link between past evolution and responses to global change, and pointing to the increasing importance of biotic interactions in assessing plant responses to global change. Last, emerging knowledge about eco-evolutionary processes that drive the terrestrial N cycle—including rock N weathering, biogeochemical mechanisms of N release and transformation, evolution of plant and microbial resources use strategies—highlights how N cycling must be viewed through the lens of deep history (i.e., geological and evolutionary), contemporary responses to and controls on N availability and eco-evolutionary feedbacks, which will determine the balance between sequestration and release of ecosystem carbon (C) in a world that now averages >400 ppm atmospheric CO<sub>2</sub> and is increasing annually.

My dissertation shows that plant N limitation is phylogenetically structured, thus we can use phylogeny as a tool for predicting plant responses to N enrichment. More importantly, phylogenetic patterns in plant N limitation are an outcome of past evolution across resource gradients, as evidenced by evolutionary differentiation in plant resource use strategies and interactions with mycorrhizal fungi. As such, conservation-oriented questions should shift towards asking for whom (defined by your closest relatives that share similar life history, root traits, and ability to control C allocation mycorrhizal fungi, rather than your biome or ability to deal with co-limitation by phosphorus) the most pressing conservation need exists, to preserve plant phylogenetic and functional diversity given rising inputs of anthropogenic N to soils.

Further, I have synthesized shifting paradigms of ecosystem N processes and used this synthesis to clarify the linkage between evolution of organisms across soil N gradients and effects of organisms on soil N, which together give rise to ecosystem-level feedbacks through deep (evolutionary and geological) time. This requires that responses of these feedbacks to anthropogenic N deposition be incorporated into C cycling models as efforts to predict global C cycling will depend on determining the balance between sequestration and release of ecosystem

C that is intimately coupled to the N cycle. Thus, the future understanding of both C and N cycles in a changing world may be greatly informed by merging theory and understanding of feedbacks between evolutionary biology and ecosystem ecology, a synthesis that is only now becoming realized.



## VITA

Rachel C. Wooliver was born in Knoxville, TN. She attended the University of Tennessee where she graduated in 2013 with a Bachelor of Science degree in Ecology and Evolutionary biology. She began her PhD training at the University of Tennessee in 2013 with Dr. Jennifer Schweitzer. She has had multiple grants that were successfully funded during this time, including an NSF East Asia and Pacific Summer Institute Fellowship, Soil Science Society of America Francis and Evelyn Clark Biology Scholarship, and Alexander Hollaender Graduate Fellowship. To date, Rachel has authored and co-authored 5 peer-reviewed articles in journals including *Ecology* and *Functional Ecology*. She also has received awards from multiple professional societies and the University of Tennessee, including a University of Tennessee Excellence in Research Award and two Ecology and Evolutionary Biology Department awards for “Outstanding Publication by a Graduate Student.” Rachel also organized a symposium at the Ecological Society of America conference and served on the Ecology and Evolutionary Biology Seminar Committee for more than one year. She has guest lectured for undergraduates in ecology for several semesters and mentored multiple undergraduate students in scientific research, writing, and presenting, and has peer-reviewed manuscripts for many ecology journals.