The role of herbivory in governing tropical nitrogen fixation

William Barker

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The candidate confirms that the work submitted is their own and that appropriate credit has been given where reference has been made to the work of others.

“The very hungry caterpillar then ate through one green leaf. He started to feel better”

– The Very Hungry Caterpillar, Eric Carle

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Abstract

A growing body of evidence suggests that periods of nitrogen limitation on plant growth are common across tropical forests and that nitrogen-fixing trees alleviate this limitation and promote tropical forest carbon sequestration. However, fixers rarely exceed ~5-15% of basal area in tropical forests, limiting the role of fixation in mitigating nitrogen limitation. Existing hypotheses have not sufficiently explained this low abundance of fixers. I examine the previously untested hypothesis that tropical nitrogen fixation is constrained by a high herbivory cost for fixers. I evaluate (1) if nitrogen-fixing trees have higher herbivory than non-fixers; and, whether this cost constrains symbiotic nitrogen fixation in tropical forests by governing (2) the fixation rates of individual trees and (3) fixer demographic traits.

I first conduct a field survey of herbivory on 1,632 leaves from 350 seedlings across 43 tree species in Panama to determine if fixers undergo higher herbivory than non-fixers and use species leaf traits to assess what drives herbivory differences between the two groups. I find that fixers undergo more herbivory and that this constitutes a significant carbon cost. Second, I use greenhouse experiments with 200 seedlings from five fixer species to test if herbivory regulates plant-level fixation rates. Surprisingly, I find up to ten-fold increases in fixation rates following herbivory, possibly to replace lost leaf nitrogen. Third, using a census of >200,000 seedlings over 14 years, with herbivory measured on a subset of seedlings, I determine if herbivory drives differences in the growth, survival, and strength of negative density-dependent effects between fixers and non-fixers. I find that herbivory contributes to stronger negative density-dependence for fixers, which could cap neotropical fixer abundances.

My findings demonstrate that high herbivory for fixer species governs individual fixation rates and may constrain fixer abundances, with combined consequences for the tropical carbon sink.

Table of Contents

List of tables:

Chapter 2

List of figures:

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of appendices

List of equations

I rescaled my proportion of leaf area data so that it was amenable to analysis using a beta regression. As a beta distribution is only suitable for values above 0 and below 1, and my data contained zeroes (leaves that were not attacked), I rescaled my data using the equation:

$$X' = \frac{x(N-1)+0.5}{N}$$

Where x is proportion of leaf damage and N is the total number of individuals (Smithson and Verkuilen, 2006; Murphy *et al.*, 2016).

Abbreviations

- References to the appendices in the thesis text are abbreviated to A after the first usage – so that Appendix 1 becomes A1, Appendix 2, A2 etc.
- Leaf Mass per Area is interchangeably abbreviated to LMA in figure legends and methods.
- Maximum carbon assimilation by photosynthesis (µmol cm$^{2\text{-}1}$ s^{-1}) is abbreviated to Amax in Chapter 2.
- The measure of similarity in leaf chemistry across species, the Chemical Structural and Compositional Similarity, is abbreviated to CSCS in Chapter 2.
- The plant hormone Methyl-Jasmonate is abbreviated to MeJa in Chapter 3.
- The statistical indicator of model fit, the Akaike Information Criterion value, is abbreviated to AIC in Supplemental Tables.

Chapter 1

Introduction

Nitrogen-fixing trees fulfil a critical role in underpinning tropical forest recovery and growth but appear to be constrained across the tropics (Batterman *et al.*, 2013b; Brookshire *et al.*, 2019; Levy-Varon *et al.*, 2019). This cap on fixer abundance could prevent fixers from mitigating nitrogen limitation of the tropical carbon sink, but it remains unclear why tropical nitrogen-fixing trees are not more prevalent in tropical forests (Vitousek and Howarth, 1991; Vitousek *et al.*, 2013). A long standing, but not adequately tested hypothesis has suggested that fixers may be constrained by a high herbivory cost, associated with the trait of fixation (Vitousek and Howarth, 1991; Vitousek and Field, 1999). Therefore, this thesis will examine the role of herbivory in governing tropical nitrogen fixation. I will here explore the relevant literature around first, nitrogen cycling in tropical forests and its importance for the terrestrial tropical carbon sink, and second, herbivory within the tropics and its effects on tropical biodiversity and ecosystem functioning. I will then identify relevant literature gaps in both these areas that motivate my research questions. Finally, I will explain how answering these research questions will have important applications for tropical ecology, biogeochemistry, modelling of the carbon sink and reforestation efforts.

1.1 Nitrogen cycling, nitrogen-fixing trees and the terrestrial tropical carbon sink

Tropical forests make up the largest single component of the terrestrial vegetation carbon sink (Korner, 2009; Pan *et al.*, 2011) including both established mature forest and secondary forest recovering from disturbance (Pan *et al.*, 2011; Brienen *et al.*, 2015; Chazdon *et al.*, 2016; Poorter *et al.*, 2016). Mature tropical forests actively removed excess carbon from the atmosphere during the 1990's and early 2000's and were responsible for 50% of the total terrestrial carbon sink from 1990-2007 (sequestering ~1.19 ± 0.41 Pg C year^{-1}) (Pan *et al.*, 2011; Hubau *et al.*, 2020). Young secondary forests, which now represent >50% of all tropical forests (Chazdon *et al.*, 2009), also play a significant role in taking up carbon from the atmosphere - at rates up to 11 times faster than in mature forests across the Neotropics (at 3.05 Mg C ha^{-1} yr^{-1}) (Chazdon *et al.*, 2016). In 2008, tropical secondary forests covered

~2.4 million km^2 of land in the neotropics, and, over a 40-year period, were estimated to absorb CO_2 equivalent to the emissions of all Latin American and Caribbean countries from 1993 – 2014 (Chazdon *et al.*, 2016). Theoretically, these carbon uptake rates would be expected to increase as atmospheric CO_2 concentrations continue to rise in an effect known as CO_2 fertilization (Lewis *et al.*, 2009; Kirschbaum, 2011; Zhu *et al.*, 2016; Jiang *et al.*, 2020), offering hope that both mature and young forests may be able to mitigate increasing atmospheric CO_2 concentrations and the effects of climate change. However, there is a growing consensus that the continued growth of tropical carbon sink is limited (Brienen *et al.*, 2015; Pugh *et al.*, 2019; Hubau *et al.*, 2020).

A large body of evidence now indicates that carbon uptake in both mature and secondary tropical forests is constrained and, in the case of mature forest, declining (Brienen *et al.*, 2015; Pugh *et al.*, 2019; Hubau *et al.*, 2020). Evidence from hundreds of forest plots demonstrates that the carbon sink potential of Latin American mature forest has been declining since the early 1990's - it is now predicted that the Amazonian sink will cease to function by 2035 - and sinks in Africa began to show a similar effect from 2015 onwards (Hubau *et al.*, 2020). Limits to carbon uptake have also been observed for young forests, with secondary forest in the Brazilian Amazon reaching only 41% of the carbon stored in neighbouring mature forest after two decades (Fleischer *et al.*, 2019). These findings raise the question of what could be limiting tropical forest growth across mature and young forests. To date, several underlying causes have been identified, including increased drought (Phillips *et al.*, 2009; Lewis *et al.*, 2011; Feldpausch *et al.*, 2016; Hubau *et al.*, 2020), above optimum temperatures for photosynthesis and heat related mortality (Phillips and Gentry, 1994; McDowell *et al.*, 2018; Aleixo *et al.*, 2019; Hubau *et al.*, 2020), changes in species composition or demographic rates (Brienen *et al.*, 2015; Esquivel-Muelbert *et al.*, 2019; Pugh *et al.*, 2019) and deforestation (Mitchard, 2018). However, I here focus on a comparatively un-investigated constraint on the growth of tropical forests – limitation by soil nutrients.

Plants are not comprised of carbon alone and key biological functions rely on other nutrients, such as calcium for cell wall development (Demarty *et al.*, 1984), phosphorus to build nucleic acids and membrane phospholipids (Kavanová *et al.*, 2006) and nitrogen for producing nucleic acids and proteins, in particular RUBISCO for photosynthesis (Matson Jr, 1980). The main source for additional macro and micro-nutrients is from the soil and when soil nutrient stores do not meet plant demand plant growth is constrained (Wieder *et al.*, 2015b; Smith-Martin *et al.*, 2017; Zemunik *et al.*, 2018). In the tropics, soils are typically considered to be deprived of rock-derived nutrients, such as phosphorus, and are widely believed to be nitrogen saturated (Walker and Syers, 1976; Hedin *et al.*, 2009; Vitousek *et al.*, 2013; Pajares and Bohannan, 2016). Phosphorus limitation has been shown to constrain tropical plant growth (Turner *et al.*, 2018), but it is possible that other nutrients, such as nitrogen, can also limit plant growth in the tropics (Wieder *et al.*, 2015b; Terrer *et al.*, 2019; Wright, 2019; Du *et al.*, 2020). It is often difficult to identify spatially continuous growth limitation by a given nutrient over tropical ecosystems, as tropical forests may undergo heterogenous patterns of nutrient limitation dependent on the cycling of many nutrients and the interactions between several biological processes (Townsend *et al.*, 2008; Hedin *et al.*, 2009; Batterman *et al.*, 2013b; Pajares and Bohannan, 2016). Specifically, in order to understand the role of nitrogen in limiting tropical forest growth we must therefore first understand the tropical nitrogen cycle.

Although the terrestrial tropics occupy only ~12% of the worlds surface, the tropical nitrogen cycle carries out ~70% of all terrestrial biological nitrogen fixation, the dominant input of new natural nitrogen to ecosystems, and emits ~50% of the worlds nitrous oxide (Townsend *et al.*, 2011). In terms of function, nitrogen cycles in any ecosystem follow a similar pattern. Starting with the nitrogen in plant matter, plant litter is decomposed by microbes into soil organic matter and then into dissolved organic nitrogen and ammonium (Ross, 1993; Cleveland *et al.*, 2006; Hedin *et al.*, 2009; Quesada *et al.*, 2010). Nitrifying bacteria then convert soil ammonium to nitrites, which are in turn converted into nitrates, a readily available source of nitrogen for plant uptake (although, some plants are able to take up ammonium directly from the soil) (Roberston, 1989; Warren, 2013; Pajares and Bohannan, 2016). Those nitrates not taken up by surrounding plants and microbes are converted into nitrous oxide by denitrifying bacteria which is released from the soil into the large atmospheric nitrogen pool (although some nitrifying bacteria can produce nitrous oxide from other chemical forms of nitrogen prior to nitrification) (Roberston, 1989; Pajares and Bohannan, 2016). Importantly, the only major source for atmospheric nitrogen to return to the soil is biological nitrogen fixation, either by free-living bacteria or via symbiotic nitrogen-fixing trees (Cleveland *et al.*, 1999; Batterman *et al.*, 2013b; Vitousek *et al.*, 2013). In tropical forests specifically, productivity largely depends on the rapid break down and release of nutrients from organic matter in a thin layer at the soil surface. As organic matter tends to be nitrogen rich, tropical soils are often high in nitrogen, with high rates of denitrification and nitrogen leaching (Hedin *et al.*, 2009). These losses are balanced by new inputs from biological nitrogen fixation (Hedin *et al.*, 2009). Because of the high nitrogen losses from tropical forests, nitrogen-rich soils were thought to be pervasive across the tropics (Hedin *et al.*, 2009). However, a growing body of evidence suggests that nitrogen limitation of tropical forest growth may be more prevalent than previously thought across tropical forests and across stages of succession (Batterman *et al.*, 2013b; Brookshire *et al.*, 2019; Levy-Varon *et al.*, 2019; Wright, 2019).

Several bodies of evidence suggest that periods of nitrogen limitation are a common occurrence across tropical forests, including high net carbon uptake rates in mature forests (Brookshire *et al.*, 2019), the high rates of nitrogen fixation by trees in tree fall gaps (Barron *et al.*, 2011) and the prevalence and high growth rates of fixers in secondary forest following disturbance (Batterman *et al.*, 2013b), modelling of the global carbon sink and nitrogen inputs to ecosystems (Wieder *et al.*, 2015b), and recent nutrient addition experiments (Wright, 2019). In particular, it is now well established that tropical forests become nitrogen limited after disturbance (Davidson *et al.*, 2004, 2007; Amazonas *et al.*, 2011; Batterman *et al.*, 2013b; Levy-Varon *et al.*, 2019; Sullivan *et al.*, 2019), which promotes nitrogen leaching from the soil and is followed by periods of greater uptake of nitrogen from the soil for plant growth relative to turnover from plant litter (Davidson *et al.*, 2004, 2007; Amazonas *et al.*, 2011). Generally, this early nitrogen limitation is considered to decrease relative to phosphorus limitation throughout succession, as turnover of plant matter returns nitrogen to the local nitrogen cycle (Walker and Syers, 1976; Davidson *et al.*, 2004; Amazonas *et al.*, 2011). However, there is growing evidence that mature forests can also be nitrogen limited, in part due to local scale disturbances such as treefall gaps (Barron *et al.*, 2011; Wright, 2019). A recent meta-analysis of 48 nutrient addition experiments in naturally growing tropical forests found that nitrogen limited plant growth in both mature and secondary forests, with the strongest effect in younger forests (Wright, 2019). A large body of evidence therefore suggests that tropical forests depend on soil nitrogen stores for growth, but nitrogen in the soil is not increasing in pace with atmospheric CO_2, and as has been found for a range of other ecosystems, this may constrain the tropical carbon sink (Fernández-Martínez *et al.*, 2014; Wieder *et al.*, 2015b; Terrer *et al.*, 2019). Therefore, sources of nitrogen external to that stored in the soil may be able to alleviate nitrogen limitation of the tropical carbon sink. In tropical forests, the largest external source of nitrogen comes from nitrogen-fixing trees (Vitousek *et al.*, 2013).

Tropical nitrogen-fixing tree species (from now on referred to interchangeably as fixers) contribute the largest proportion of biologically fixed nitrogen in tropical forests, and so may be crucial in regulating tropical nitrogen cycles and alleviating nitrogen limitation (Vitousek *et al.*, 2013). Fixer species are able to fix nitrogen in symbiosis with bacteria housed in root nodules (*rhizobial* bacteria are most common in the tropics) (Gutschick, 1981; Parker, 2008; Vance, 2008; Vitousek *et al.*, 2013). Rhizobia are abundant in tropical soils and are able to trigger the formation of, and then inhabit, nodules on fixer roots (Kiers *et al.*, 2003; Vance, 2008). Once within the nodule, nitrogen-fixing bacteria can fix atmospheric dinitrogen into forms that are usable by the plant, such as ammonia, in exchange for photosynthetic carbon from the plant host (Vance, 2008). It is difficult to overstate the importance of this symbiotic partnership in the function of global ecosystems, but tropical nitrogen fixation in particular provides the large majority of nitrogen needed for forest regrowth and enhances carbon accumulation by 200% in young forests and total carbon stored in mature forests by ~10% (Brookshire *et al.*, 2019; Levy-Varon *et al.*, 2019). Therefore, fixers fulfil a particularly critical role in tropical forest recovery following disturbance by supplying nitrogen to underpin plant growth.

As well as being essential for forest recovery, the trait of fixation carries a suite of advantages (Batterman *et al.*, 2013b; Adams *et al.*, 2016; Menge and Chazdon, 2016; Gei *et al.*, 2018) and in tropical forests fixation entails a significant growth benefit for fixer species in nitrogen limited conditions – fixers growing in secondary forests have exhibited growth rates nine times greater than their non-fixing neighbours (Batterman *et al.*, 2013b). However, despite this advantage, and high fixer biodiversity across Africa and the neotropics (Lavin, 2016), there is little understanding as to why the fixation trait is not more widespread over ecological and evolutionary timescales, with fixation found largely within one family (the Fabaceae) and fixer abundances capped at 1% of basal area in temperate forest, 1-8.5% in mature forests across Asia, ~9% at a tropical forest site in Central Africa, and at ~5-15% in diverse forest types across the Neotropics (Ter Steege *et al.*, 2006; Sullivan *et al.*, 2014; Gei *et al.*, 2018; Menge *et al.*, 2019).

Several explanations for the low abundance of fixers have been proposed, largely focusing on the high energetic cost of fixation. Fixer bacteria housed in root nodules can break the strong triple bond in the dinitrogen molecule, the chemical form of nitrogen most prevalent in the atmosphere (Gutschick, 1981; Vance, 2008). The biochemical difficulty in breaking this bond is what prevents most organisms from utilising the atmospheric nitrogen pool (Hedin *et al.*, 2009; Vitousek *et al.*, 2013). Fixers, however, are able to utilise this microbial ingenuity to obtain atmospheric nitrogen, albeit at a high energetic cost. The plants exchange photosynthetically derived carbon for fixed nitrogen with their bacterial symbionts, at around 6 g carbon per g nitrogen fixed (Vance, 2008). Alongside this high energetic cost, other constraints on the trait of fixation have also been suggested, from trade-offs with direct uptake and efficiency of the use of nitrogen and other nutrients (Menge *et al.*, 2008, 2010; Vitousek *et al.*, 2013), limitation by other nutrients such as soil phosphorus and molybdenum (molybdenum is an important metal catalyst in the nitrogenase enzyme used by many nitrogen-fixing bacteria) (Vitousek and Field, 1999; Barron *et al.*, 2009; Batterman *et al.*, 2013c; Winbourne *et al.*, 2017; Ament *et al.*, 2018; Dynarski and Houlton, 2018) or a competitive advantage only under low soil nitrogen (Hedin *et al.*, 2009; Batterman *et al.*, 2013b; Sheffer *et al.*, 2015). If the trait of fixation does only benefit plants under nitrogen limitation, then the competitive advantage of fixation, and the abundance of fixing trees, would be expected to vary across tropical forest soil nitrogen gradients.

Young tropical soils typically have low nitrogen concentrations but, due to the high rates of turnover and fixation in the tropics, tend to become more nitrogen rich over time, with this upward trend only levelling off when phosphorus limitation limits turnover of biomass to the soil in old, highly weathered soils (Walker and Syers, 1976; Davidson *et al.*, 2004; Quesada *et al.*, 2010; Amazonas *et al.*, 2011). However, soil fertility is also governed by other factors such as climate, moisture, soil texture and soil fertility. Accordingly, there are a wide range of soil nitrogen concentrations found across tropical forests soils which are not always correlated with soil age (Quesada *et al.*, 2010). For example, whilst little work has been done comparing soil nitrogen concentrations globally across tropical forests, and there are few resources documenting total nitrogen content (Batjes reported that total soil nitrogen content in the tropics was around 20-22 Pg C to a depth of 30cm and 42-44 to a depth of 100cm (Batjes *et al.*, 1996)), across the Latin American tropics, inorganic soil nitrogen concentrations vary from below 4.13 ug mg^{-1} in some mature tropical forests in Costa Rica (Sullivan *et al.*, 2014) to 22.57 ug mg^{-1} in the South Eastern Amazon (mature forests in Panama, where my PhD work was carried out, show an average of 20.53 (Yavitt *et al.*, 2009)). In addition, Quesada et al (2010) found that total soil nitrogen concentrations ranged from 0.07% – 0.9% across the Amazon basin, largely dependent on soil type (see Quesada et al. 2010 Supplemental 1). However, it remains unclear how these gradients of soil nitrogen may drive fixer abundances across tropical forests.

As discussed above, several bodies of research have assessed how the relative abundance of fixers varies across tropical forests – with fixer basal area capped at ~5-15% in tropical forests globally (Ter Steege *et al.*, 2006; Sullivan *et al.*, 2014; Gei *et al.*, 2018; Menge *et al.*, 2019). Yet, to my knowledge, no work has been done comparing these relative abundances across gradients in soil nitrogen concentrations across the tropics. The few studies that do compare total soil nitrogen or inorganic soil nitrogen and fixer abundance do so in one place over a successional gradient of forest recovery from disturbance –nitrogen fixation is highest in young forests where soil nitrogen is low across sites in Panama, Costa Rica, and the South Eastern Amazon, but there is a lot of variation in fixer abundances over successional time across different tropical forests (Batterman *et al.*, 2013a; Gei *et al.*, 2018; Winbourne *et al.*, 2018; Taylor *et al.*, 2019; Wong *et al.*, 2020). Where measures of soil fertility are comparable between studies at different sites, we see no role of soil nitrogen in driving herbivory. Fixer abundance is low in the South Eastern Amazon (0.76% of basal area) (Wong *et al.*, 2020) compared to Costa Rica (29% - high fixer basal area due to stands of the monodominant tree species, *Peteclethra macroloba,* at this site) (Taylor *et al.*, 2019), despite comparable inorganic soil nitrogen availability (22.6 mg Kg^{-1} in control plots in the South Eastern Amazon compared to 25 mg Kg^{-1} – 50 mg Kg^{-1} across forest ages in Costa Rica)

(Taylor *et al.*, 2019; Wong *et al.*, 2020). Furthermore, whilst fixation rates have been found to vary with soil nitrogen across the amazon, fixer abundances have not (Nardoto *et al.*, 2014).

The majority of explanations for the low abundances of fixers therefore either lack support in the literature, as for the role of regional soil nitrogen gradients, or suggest that the fixation trait is only beneficial in certain environments. However, tropical fixers utilize a facultative fixation mechanism, where they are able to downregulate their fixation when nitrogen is readily available in the soil and comparatively cheaper to take up than fixing (Barron *et al.*, 2011; Batterman *et al.*, 2013c, 2013b). Plants need to take up many nutrients for growth and can gain the same resource from different sources, such as nitrogen from atmospheric di-nitrogen or soil nitrates in the case of fixers (Rapport, 1980; Tilman, 1982; Lerdau and Coley, 2002a; McNickle *et al.*, 2009). Therefore the relationship between the uptake rate from different nutrient sources, and their combined effect on plant growth rate, can vary dramatically – giving different optimal foraging strategies under different conditions (Rapport, 1980; Tilman, 1982). Fixers may invest carbon into fine root production to take up soil nitrogen, but as soil nitrogen stores are depleted, this approach becomes comparatively more expensive per unit nitrogen gained (McNickle *et al.*, 2009; Batterman *et al.*, 2013b). At this point, fixers can switch to nitrogen-fixation, instead paying carbon to their symbiotic bacteria in exchange for fixed nitrogen (Batterman *et al.*, 2013b). If soil nitrogen increases again due to biomass turnover, fixers can switch back to taking up soil nitrogen - when the carbon cost of fixation outweighs the cost of producing fine roots in a nitrogen rich soil (Barron *et al.*, 2011; Batterman *et al.*, 2013a, 2013b; Wong *et al.*, 2020).

Variation in foraging strategies between fixers and non-fixers could be used to understand observed low fixer abundances across tropical forests, despite the benefits of the trait of fixation. The R* theory states that when species are competing for one limiting resource, the species which can persist at the lowest resource level (the R* for that species) will outcompete the others, regardless of starting densities (Tilman, 1982). Fixers have a lower R* value for soil nitrogen than non-fixers as they can access the atmospheric nitrogen pool and so should outcompete non-fixers under nitrogen limitation even when starting from very low abundances (Tilman, 1982; Batterman *et al.*, 2013d; Gei *et al.*, 2018). That fixers do not outcompete neighbouring non-fixers in nitrogen limited tropical forests could suggest that fixers and non-fixers are also limited by other factors, like light, phosphorus or molybdenum, and that non-fixers have a lower R* for this second nutrient (Barron *et al.*, 2009; Batterman *et al.*, 2013c; Sheffer *et al.*, 2015; Taylor and Menge, 2018; McCulloch and Porder, 2020). However, fixers can reduce their high demand for nutrients needed to support fixation by instead taking up nitrogen from the soil (adopting the most optimal foraging strategy) (Barron *et al.*, 2011; Batterman *et al.*, 2013d; McCulloch and Porder, 2020). This enables fixers to avoid the costs of fixation when they outweigh the benefits, and thus should allow fixers to persist throughout succession in tropical forests – when tropical forest growth tends to move from nitrogen limited to nitrogen replete (Batterman *et al.*, 2013c). Therefore, there must be an additional cost (or costs) associated with fixation that prevent fixers from becoming more abundant over ecological and evolutionary timescales. Such a cost could be a permanently higher demand for another limiting nutrient, or a possible trade off with nutrient use efficiency, but these hypotheses have already been explored by previous research (Menge *et al.*, 2008; Vitousek *et al.*, 2013). One potential cost that has received little attention, but that may have a major effect on fixers, is herbivory.

That fixers may be exposed to a greater herbivory cost than non-fixers was first suggested over two decades ago, but the relationship between fixation and herbivory remains unclear for tropical nitrogen-fixing trees (Vitousek and Howarth, 1991; Vitousek and Field, 1999). Herbivores have high nitrogen requirements but a poor efficiency of digestion, so it is beneficial for them to target plant tissues with high concentrations of nitrogen (Matson Jr, 1980). Previous studies of tropical plants have found a correlation between increased nitrogen concentration and plant palatability and herbivory (Matson Jr, 1980; Kursar and Coley, 2003; La Pierre and Smith, 2016). Fixers have higher concentrations of tissue nitrogen, relative to non-fixers; therefore, one would expect that fixers undergo higher herbivory than non-fixers (Fyllas *et al.*, 2009). Some empirical evidence supports this, such as fixer relative abundance in grasslands increasing significantly when herbivores are excluded and a preliminary study suggesting some tropical nitrogen-fixing tree species experience higher herbivory than non-fixer species, and that this is due to high foliar nitrogen concentrations (Batterman *et al.*, 2019). However, a recent comparison between a small number of fixer and non-fixer tree species in Costa Rica found no difference in herbivory between the two groups (Taylor and Ostrowsky, 2019), and so it remains unclear if many fixer species undergo high herbivory and whether herbivory would actually constitute a high carbon cost associated with the trait of fixation.

1.2 Herbivory: drivers, deterrents, and possible effects on tropical nitrogen fixation

Herbivory is a widespread and important process in ecosystems globally, with plants and their herbivores making up over half of the macroscopic diversity of life on earth (Coley and Barone, 1996; Kursar and Coley, 2003). To understand the consequences of a high herbivory cost for tropical fixer species, we must therefore explore how herbivory shapes plant evolution, biodiversity, and ecosystem functioning, notably in tropical forests. Since the 1990's, it has been suggested that herbivory pressure is greater at the equator due to a greater diversity and overall number of herbivores and a higher degree of herbivore specialization due to a comparatively warmer and more suitable environment for herbivores – mainly insects – at lower latitudes (Coley and Aide, 1991; Coley and Barone, 1996; Salazar and Marquis, 2012; Lim *et al.*, 2015). Whilst some recent evidence suggests this may only be true for the northern hemisphere (Zhang *et al.*, 2016), or across evergreen species (Lim *et al.*, 2015) and may not affect overall damage rates due to greater investment in defences by plants in the tropics (Salazar and Marquis, 2012), a global pattern persists (Gao *et al.*, 2019), and it remains clear that insect herbivores are more abundant and diverse and herbivory pressure appears higher for many plant species in the tropics (Coley and Barone, 1996; Salazar and Marquis, 2012; Coley and Kursar, 2014). Therefore, understanding the role of this high herbivory, particularly for fixer species, in regulating tropical biogeochemical cycling and atmospheric carbon uptake is especially important in the tropics, where the carbon sink is large.

Before discussing the likelihood and effects of high herbivory for fixers, it is important to lay out some definitions. Herbivory is carried out by a diverse range of animals and it is important to define exactly what I am referring to as a herbivore. Whilst large herbivores fulfil important roles in tropical forests (Villar *et al.*, 2020), the majority of herbivory damage in the tropics is by insect herbivores – an estimated 75% of all leaf area consumed by herbivores on Barro Colorado Island, Panama is consumed by insects (Coley and Barone, 1996; Leigh, 1999). Within insect herbivores it is easier to determine the amount of damage caused to plants by leaf chewing insects, but herbivory by sap sucking insects is also common in the tropics and can reduce growth, reproduction and photosynthesis (Coley and Barone, 1996; Novotny *et al.*, 2006; Zvereva *et al.*, 2010; Coley and Kursar, 2014). However, difficulty in measuring damage from phloem sucking herbivores means that the damage they cause in tropical forests remains largely unquantified. In this thesis, when discussing herbivores, I am therefore referring to leaf chewing insects. It is also important to consider that herbivory and its effects vary widely across plant life stages (Zvereva *et al.*, 2020). In this thesis, I largely discuss herbivory at the seedling scale as herbivory is greater for seedlings than adult trees (~2.5 time greater) and herbivory at this life stage acts as a major bottleneck on species survival (Coley and Barone, 1996; Dyer *et al.*, 2010; Forrister *et al.*, 2019; Zvereva *et al.*, 2020). I will now consider how herbivory by leaf chewing insects could affect fixers, what other traits may drive herbivory patterns across species and what fixers may do to attempt to deter herbivores, and then explore the consequences of a high herbivory cost for fixers for fixer ecology, evolution and tropical nitrogen fixation at ecosystem and biome scales.

Fixer species are expected to undergo higher herbivory than non-fixers due to their high leaf nitrogen concentrations (Matson Jr, 1980; Kursar and Coley, 2003; Batterman *et al.*, 2019). However, this pattern may not manifest between the two groups due to variation in leaf nitrogen and other nutrients across species (Matson Jr, 1980; Coley *et al.*, 1985; Coley and Barone, 1996). Firstly, nitrogen content may not always be higher in fixer species than non-fixer species. For example, plants in the Asteraceae often show tissue nitrogen concentrations comparable with fixer species (Palm *et al.*, 2001). Other leaf nutrients, such as sodium and phosphorus have also been found to drive herbivory and may not covary with leaf nitrogen across fixer and non-fixer species (Perkins *et al.*, 2004; Kaspari, 2020) and non-nutrient leaf traits like leaf area can also drive herbivore preference, with many herbivores targeting larger leaves (Ribeiro *et al.*, 1994; Moles and Westoby, 2000).

Variation in nutrient and photosynthetic leaf traits that may attract herbivores could drive herbivory patterns across fixer and non-fixers, but herbivory may also vary between species groups due to differences in traits that deter herbivores. Anti-herbivore traits consists of adaptations and strategies to deter, impede or avoid herbivores consuming plant tissues, including increasing leaf toughness (Kitajima and Poorter, 2010; Westbrook *et al.*, 2011; Kitajima *et al.*, 2016), decreasing leaf palatability (HoI and Jander, 2008; Kitajima *et al.*, 2016; Schaller *et al.*, 2018), producing trichomes (Coley *et al.*, 2018; Fishbein *et al.*, 2018), harbouring ant mutualisms (Kursar *et al.*, 2009), rapid leaf expansion to avoid herbivory during the vulnerable early stages of leaf development (Kursar and Coley, 2003), and the use of a diverse range of secondary metabolites (Wink, 2003, 2013; Mithöfer and Boland, 2012; Sedio *et al.*, 2017). For tropical seedlings, leaf toughness has been found to be the biggest determinant of herbivory damage, and is best achieved by increasing leaf cellulose density (Kitajima *et al.*, 2016). Chemical defences, whilst of secondary effectiveness in deterring herbivores compared to leaf toughness, can be incredibly diverse and toxic, encompassing phenolic compounds such as tannins, which bind to animal proteins (Coley, 1986; Crozier *et al.*, 2007; Wiggins *et al.*, 2016), or carbon based terpenes that are likely to have a diverse range of functions such as inhibiting ATP-synthases or interfering with insect moulting (Wink, 2003; Fine *et al.*, 2006; Okada *et al.*, 2015). In particular, fixers may invest more heavily in toxic nitrogen-based defences (Wink, 2013). Leaf nitrogen can exist in many forms, and whilst the nitrogen used in photosynthetic machinery is nutritious for insect herbivores (Matson Jr, 1980; Kursar and Coley, 2003), leaf nitrogen can be found in toxic secondary metabolites, such as cyanogenic glucosides, which are broken down to form toxic hydrogen cyanide when leaf tissues is damaged by leaf chewing herbivores (Mithöfer and Boland, 2012; Wink, 2013), alkaloids, such as nicotine, which can inhibit DNA synthesis and repair or affect the nervous system (Khan and Harborne, 1990; Mithöfer and Boland, 2012; Wink, 2013, 2018), and overexpressed essential amino acids, like tyrosine in certain *Inga* species (Bixenmann *et al.*, 2016) and non-protein amino acids (Bromberg *et al.*, 2005; Wink, 2013). These nitrogen-based compounds could increase in step with leaf nitrogen concentration and therefore obscure the role of leaf nitrogen in driving herbivory.

Fixers may invest more in defences that non-fixers to offset the high herbivory cost associated with nitrogen-rich leaves, or alternatively, a relationship between leaf nitrogen and herbivory may not manifest across species due to the highly coevolved nature of plant-herbivore interactions. Insect herbivores in the tropics often have a very narrow host range (Coley and Barone, 1996; Dyer *et al.*, 2007; Endara *et al.*, 2018). In order to escape herbivory damage from these specialised herbivores, plants evolve increasingly novel defences, to which, in turn, herbivores evolve resistance (Kursar *et al.*, 2009; Endara *et al.*, 2015, 2018). One plant species can therefore have a suite of physical and chemical defences effective in deterring only one group of herbivores (Becerra, 2015; Endara *et al.*, 2017). The species-specific effectiveness of various defensive traits means that, whilst fixer defence investment overall may obscure the role of leaf nitrogen in driving herbivory, no one defensive trait will reduce herbivory across many species. A recent comparison of herbivory between four fixer species and three non-fixers in Costa Rica found that species specific differences in leaf chemistry explained herbivory and that there was no difference between the two groups (Taylor and Ostrowsky, 2019). This pattern of herbivory and defence makes it difficult to understand the underlying mechanism behind tropical herbivory patterns, but more importantly, may contribute to the immense biodiversity found in the tropics, and within fixer species themselves (Endara *et al.*, 2015).

Plant and insect diversity may be driven by the coevolution of plant-host and insect herbivores (Becerra *et al.*, 2009; Becerra, 2015). The vast majority of tropical fixers are rhizobial and in the Fabaceae, which one of the most diverse plant families in the world (Sprent, 2009). Research carried out on many species in the fixer genus *Inga* suggests that tropical plants may speciate by evolving novel herbivore defences compared to the defensive traits of their most closely related species (Kursar *et al.*, 2009; Endara *et al.*, 2015, 2017; Sedio *et al.*, 2017). *Inga* species, which are fixers, have been found to differ more in defensive characteristics than in reproductive or resource acquisitive traits, suggesting that such species occupy niches formed by developing novel resistance to herbivore pressure (Endara *et al.*, 2015). If fixers do undergo higher herbivory due to their high leaf nitrogen concentrations, fixer species may be under more pressure to evolve new defensive traits, and this may therefore explain not only why fixer abundances are low across the tropics, but also why fixers are so diverse.

A high herbivory pressure for fixers driving the evolution of complex defences raises the question of how fixers fit into existing theories to explain the variation in herbivore defence across plant species. On average, ~10% of all biomass produced by plants is consumed by herbivores (Coley *et al.*, 1985). Given this substantial cost, plants invest heavily in traits to deter herbivores, but not all plant species invest to the same degree. Several theories have been expounded to explain why plant species vary in their defence investment (Ehrlich and Raven, 1964; Feeny, 1976; Coley *et al.*, 1985; Coley, 1993; Lerdau and Coley, 2002b; Kursar and Coley, 2003; Fine *et al.*, 2006; Becerra *et al.*, 2009). The most widely supported is the resource allocation (RAH) hypothesis, which posits that natural selection will favour slow-growing but well defended plants in low-resource environments and fast-growing, poorly-defended plants in high-resource environments (Coley *et al.*, 1985; Endara and Coley, 2011; Koffel *et al.*, 2018). For species in low-resource environments with slow growth rates, the loss of leaf tissue represents a far greater cost than for species in fertile environments that are able to rapidly replace lost leaf area. This theory suggests that fixers with access to atmospheric nitrogen but growing in nitrogen poor, high light secondary forests should grow quickly and invest little in defences. However, the same fixer species can persist in the shady, nitrogen-rich understorey of mature forests by downregulating their fixation rates (Barron *et al.*, 2011; Batterman *et al.*, 2013b). In this low-resource environment, fixers would be expected to invest heavily in defence and exhibit slow growth rates. As fixer species can make the transition from fast growing to slow growing depending on if they can access the benefits of fixation, they may suffer an opportunity cost in both environments by not being able to develop specialised high growth-tolerance or low growth-defence traits.

A separate theory to characterise defence investment across species has been developed using the fixer genus *Inga,* and defines species as growth or escape specialists depending on their rate of leaf expansion rather than whole plant growth rates (Kursar and Coley, 2003; Coley *et al.*, 2006). For many species, the vast majority of herbivory damage occurs on young leaves as they are expanding (Kursar and Coley, 2003). Young leaves are not able to toughen until they have finished expanding and so make inviting targets for insect herbivores (Coley and Barone, 1996; Moles and Westoby, 2000; Kursar and Coley, 2003). Kursar and Coley (2003) identified that some species minimise the risk of herbivory during early leaf development by expanding rapidly, whilst other species have leaves that expand slowly but are well chemically defended (Kursar and Coley, 2003). It remains unclear whether the strategies categorised under the defence-continuum hypothesis framework may prevent a high herbivory cost for fixer species. For example, despite little genetic variation within the *Inga* genus, two *Inga* species, *Inga umbeliffera* and *Inga goldmannii,* utilise either defence or escape anti-herbivore strategies, but both show comparable levels of herbivory (22% leaf tissue lost over the course of the study) (Bromberg *et al.*, 2005).

Herbivory is therefore likely to drive high biodiversity and the evolution of anti-herbivore defences for fixer species, but high herbivory for fixers may also constrain the biogeochemical role of nitrogen-fixing tree species in tropical forests with important consequences for the tropical carbon sink. The effects of herbivory on the role of tropical nitrogen fixation in the tropical nitrogen and carbon cycles are therefore the focus of my thesis. A higher cost of herbivory, relative to non-fixers, may constrain the fixation rates of individual trees. Tropical fixers utilize a facultative fixation strategy, undergoing nodulation and fixation only when soil nitrogen is low and there is sufficient light and phosphorus for biomass generation (Batterman *et al.*, 2013b, 2013c). As described above, this allows fixers to avoid the energetic cost of fixation when there is sufficient nitrogen in the soil, and to fix only when it offers a competitive advantage (Barron *et al.*, 2011; Batterman *et al.*, 2013b; Sheffer *et al.*, 2015). Nitrogen and carbon lost to herbivory will need to be replaced in order to regain lost net primary production. However, a high carbon cost of herbivory may reduce the carbon available for exchange with symbiotic bacteria for fixed nitrogen, causing the *rhizobia* to cease fixing. Therefore, a trade-off may exist between the benefits and costs of fixation under high herbivory, which may constrain facultative fixation rates at times when fixation would have provided a competitive advantage to the plant, such as in the early stages of succession (Batterman *et al.*, 2013b).

Reduced competitive ability for fixers under herbivory may govern fixer abundances (Maron and Crone, 2006). A higher cost of herbivory may prevent the trait of fixation from being more widespread over ecological and evolutionary time scales, and too high of a cost could remove the fixation trait by negative selection (Vellend, 2010; Castillo *et al.*, 2014). Negative selection occurs when a trait is less competitive, and thus is removed from a population over time by natural selection (Pigliucci, 2007; Vellend, 2010; Castillo *et al.*, 2014). There are two ways that a higher herbivory cost may limit, but not remove, the trait of fixation over time. First, the negative selection against fixation, under higher herbivory, may be offset by positive selection for some benefit of having continual access to nitrogen, such as rapid growth in nitrogen limited conditions (Kursar and Coley, 1991). Second, the trait of fixation may be sustained, but at low fixer abundances, via spatial distributions that limit competition between fixers and non-fixers by negatively affecting seedlings at high densities (Janzen, 1970; Terborgh, 2012; Comita *et al.*, 2014).

Seedling growth rate, survival and abundance can be governed by negative density-dependent selection (Janzen, 1970; Harms *et al.*, 2000; Bagchi *et al.*, 2014). Under herbivory, negative selection tends to increase with the density of individuals of a species, as herbivores are able to feed and reproduce more rapidly on densely-packed plants (Harms *et al.*, 2000; Dyer *et al.*, 2010; Terborgh, 2012; Forrister *et al.*, 2019). This density-dependent selection would cause fixer growth and survival to be more negatively affected by fixer density than that of non-fixers. Removal of individuals by negative density-dependent selection therefore reduces fixer abundance by limiting the amount of fixer seedlings able to grow over a given area - the strength of negative density-dependent effects has been negatively correlated with overall species abundance for seedlings of tropical trees in Panama (Comita *et al.*, 2010). Alternatively, negative selection against fixers may remain constant regardless of density, if herbivores seek out fixer seedlings and attack them at both low and high densities, in which case fixer survival rates would be lower than non-fixer survival regardless of density.

A higher herbivory cost for fixers, relative to non-fixers, could therefore affect two critical components for tropical nitrogen cycling: (1) the fixation rates of individual trees; and, by reducing fixer seedling growth and survival (2) the abundance of fixer trees (see Fig. 1). By governing these two factors, herbivory may reduce the competitive ability of fixers, and explain why they are not more abundant over ecological and evolutionary timescales (Ter Steege *et al.*, 2006; Hedin *et al.*, 2009; Gei *et al.*, 2018; Menge *et al.*, 2019). Additionally, these two factors will govern the total input of nitrogen by fixers into terrestrial tropical systems (see Fig. 1) (Vitousek and Howarth, 1991; Hedin *et al.*, 2009; Vitousek *et al.*, 2013). Understanding the role of herbivory in the tropical nitrogen cycle will allow us to predict whether nitrogen can equilibrate with increased CO_2, and thus whether the tropical carbon sink will increase with rising atmospheric carbon (Brienen *et al.*, 2015; Wieder *et al.*, 2015b; Brookshire *et al.*, 2019; Terrer *et al.*, 2019), or if it will become increasingly constrained as the growth demand for nitrogen stimulated by rising atmospheric CO_2 increases. Therefore, this thesis will determine if herbivory can reduce individual fixation rates and constrain the abundance of fixers in the tropical forest biome.

1.3 Context for the importance of my findings

Answers to the questions raised in this thesis will be vital for many theoretical and applied applications, such as constraining Dynamic Global Vegetation, components of Earth System Model, estimates of biological nitrogen fixation and its effects on (1) our understanding of the future tropical carbon sink and (2) the role of the terrestrial biosphere in moderating future climate change (Cleveland *et al.*, 1999, 2010; Wang *et al.*, 2007; Wieder *et al.*, 2015a). In addition, my findings will aid species selection for reforestation efforts and our understanding of the natural recovery of secondary forests as a climate mitigation strategy (Voigtlaender *et al.*, 2012; Batterman *et al.*, 2013b; Sang *et al.*, 2013; Brookshire *et al.*, 2019; Levy-Varon *et al.*, 2019). Finally, the findings from my thesis are likely to become more important as the effects of herbivory in tropical forests become stronger and more pervasive with climate and land use change (Hahn *et al.*, 2015; Morante-Filho *et al.*, 2016; Zavala *et al.*, 2017; Hall *et al.*, 2020; Johnson *et al.*, 2020).

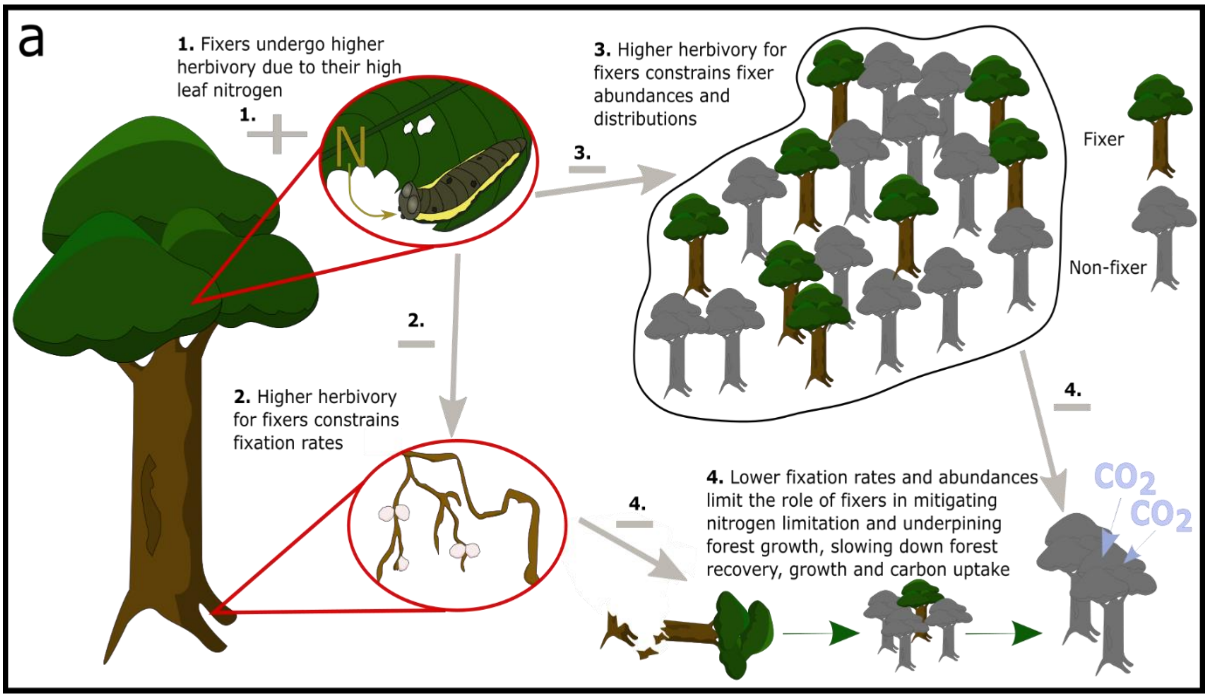


Figure 1. The role of herbivory in governing tropical nitrogen fixation. *A conceptual figure showing how I expect high herbivory for fixers, driven by high leaf nitrogen (1), to reduce the fixation rates of individual nitrogen-fixing trees (2), to constrain the abundances of fixer individuals (3), and the consequences this will have on forest growth and recovery after*

disturbance and the response of tropical forests to rising CO2 and climate change (4). Minus symbols represent a negative effect of each step on the next, whilst a positive symbol represents a positive effect.

First, my findings will have important applications for modelling of interactions between the nitrogen cycle and the tropical carbon sink and the future of the terrestrial biosphere. It is likely that the Dynamic Global Vegetation component of current Earth System Models overestimate the capacity of tropical nutrient cycling to meet the demands for plant growth under rising atmospheric CO_2 concentrations (Wieder *et al.*, 2015a). In particular, current modelling of nitrogen fixation, as the largest biological source of external nitrogen for tropical forests, predominantly estimates fixation based on forest net primary productivity, derived from estimates that now seem unrealistic in the light of recent evidence (Cleveland *et al.*, 1999; Wang *et al.*, 2007; Batterman *et al.*, 2013b; Wieder *et al.*, 2015a; Wurzburger and Hedin, 2016). This measure does not consider the important species-specific differences known to influence fixation rates (Batterman *et al.*, 2013b; Wurzburger and Hedin, 2016), the variation in fixation rates across forest types or age (Davidson *et al.*, 2007; Barron *et al.*, 2011; Batterman *et al.*, 2013b; Sheffer *et al.*, 2015; Levy-Varon *et al.*, 2019), or the role of ecological species interactions such as herbivory (Ritchie and Tilman, 1995; Vitousek and Field, 1999; Menge *et al.*, 2008). More importantly, current estimates of tropical nitrogen fixation do not incorporate the ability of fixers to adjust fixation rates to environmental factors, such as increasing nitrogen demand under CO_2 fertilization, and instead treat fixation as static (Gerber *et al.*, 2010). Therefore, in this thesis, by determining the role of herbivory in governing tropical nitrogen fixation at the tree and species scale, I will highlight some of the limitations of the current methods for estimating nitrogen fixation at the ecosystem scale and how it is incorporated into biogeochemical and Dynamic Global Vegetation models.

Second, my findings will have importance for reforestation and forest recovery as a tactic for mitigating climate change. Due to anthropogenic disturbance, the proportion of tropical forests made up of young, recovering forests is growing and currently accounts for >50% (Chazdon *et al.*, 2009; Cook-Patton *et al.*, 2020). A large body of evidence demonstrates that nitrogen-fixing trees underpin the recovery of secondary forests and increase the carbon sequestered in biomass once these forests reach maturity (Batterman *et al.*, 2013b; Brookshire *et al.*, 2019; Levy-Varon *et al.*, 2019). By including nitrogen-fixing trees in replanting schemes, the rate of forest recovery can be increased, soil fertility improved, and the carbon sink potential of secondary forests enhanced (Voigtlaender *et al.*, 2012; Sang *et al.*, 2013; Brookshire *et al.*, 2019; Levy-Varon *et al.*, 2019). However, fixer seedlings from many species may face high herbivory costs that could constrain fixation rates or reduce seedling survival, particularly at high densities (Ritchie and Tilman, 1995; Batterman *et al.*, 2019; Forrister *et al.*, 2019). These herbivory effects could limit the efficiency of including fixers in seed mixes for replanting, and therefore, my findings may be able to guide species selection for reforestation efforts where nitrogen fixing species are incorporated.

Finally, my findings are likely to become more relevant as climate change and increases in the rate of land use change continue. Herbivory has been found to increase under high concentrations of atmospheric CO_2, likely due to downregulation of plant defence under elevated CO_2 levels (Zavala *et al.*, 2017; Hall *et al.*, 2020; Johnson *et al.*, 2020) (as predicted by the resource acquisition hypothesis, see above (Coley *et al.*, 1985; Endara and Coley, 2011)). Furthermore, edge effects following forest disturbance increase herbivory as the loss of larger predators in disturbed habitats allows populations of insect herbivores to grow (Hahn *et al.*, 2015; Morante-Filho *et al.*, 2016). As herbivory damage becomes greater under increasing land use change and CO_2 concentrations, we may expect to see even greater herbivory costs for fixers. Importantly, higher herbivory for fixers may also constrain the fixation rates and abundances of fixers across the tropics, reducing the amount of nitrogen supplied to tropical forests and limiting growth of the carbon sink under rising atmospheric CO_2 concentrations (Wieder *et al.*, 2015b; Brookshire *et al.*, 2019; Terrer *et al.*, 2019).

1.4 Questions and hypothesis

Based on the current understanding and knowledge gaps outlined above, I will address three key research questions:

1. Do fixers undergo higher herbivory than non-fixers?
2. Does herbivory regulate individual-scale fixation rates in tropical nitrogen-fixing trees?

3. Does herbivory govern the growth, survival and strength of negative density dependent effects for fixer species in a way which could constrain fixer abundances and distributions?

Using these research questions, I will test the central hypothesis that:

Herbivory governs tropical nitrogen fixation

1.5 Thesis outline

Each of the key research questions outlined above, alongside research topics related to each question respectively (see chapter objectives below), will make up a chapter of my thesis, with the aim of testing the central hypothesis. Fixers may undergo higher herbivory than non-fixers, and if this high herbivory constitutes a significant carbon cost for affected plants, could constrain tropical nitrogen fixation by reducing plant-level fixation rates or limiting the abundance of fixer trees. Therefore, in this thesis, the first chapter will determine if fixers undergo higher herbivory than non-fixers, estimate the carbon cost of herbivory, and, using species leaf traits, assess if any leaf trait explains differences in herbivory between the two groups. The second chapter will investigate how high herbivory for fixers affects the fixation rates of individual trees and explore how this herbivory affect is governed by soil nitrogen, level of herbivory and plant species antiherbivore strategy. Finally, the third chapter will explore how herbivory may constrain fixer abundances by reducing fixer seedling growth and survival and/or increasing the strength of negative conspecific density-dependent effects for fixer species. Each chapter is written as a stand-alone paper, as I aim to submit them for publication following my PhD. I will focus on the biogeochemical consequences of a high herbivory cost for fixers and therefore not directly consider the role of herbivory in governing fixer biodiversity or evolution, but I will discuss these topics when relevant.

1.6 Chapter objectives

1 Introduce the literature surrounding the role of nitrogen-fixing trees in tropical forests and tropical herbivory, and identify key literature gaps in both areas

1.1 Discuss how tropical nitrogen fixation may be constrained and explain how herbivory may govern fixation in tropical forests

1.2 Provide context for the potential applications of this research

1.2 Introduce key research questions, outline thesis structure and list objectives for each chapter

2. Determine whether fixers undergo greater herbivory than non-fixers using my survey of herbivory on seedlings in mature tropical forest, Panama

2.1 Generate estimates of the carbon cost of herbivory and compare it to the energetic cost of nitrogen fixation.

2.2 Use species leaf trait data to assess whether leaf nitrogen, or any other leaf characteristic associated with the trait of fixation, could explain herbivory differences between fixers and non-fixers.

3 Identify if herbivory affects fixation rates for fixer seedlings in greenhouse experiments

3.1 Investigate how soil nitrogen and level of herbivory governs the herbivory effect on fixation, to better understand how nitrogen and carbon demand influence the fixation response to herbivore damage

3.2 Determine how plant species antiherbivore strategy governs the effect of herbivory on fixation

4 Summarize the findings from my thesis, conclude whether I have answered my research questions and contextualise my findings with discussion of the relevant literature

4.1 Discuss my findings and the relevant literature in reference to each chapter

4.2 Explore the significance of my findings in understanding the diversity of the fixer functional group and the evolution of defence investment for fixer species

4.3 Outline how my findings have improved my understanding of tropical nitrogen cycling and the role of the terrestrial tropical carbon in mitigating future climate change

4.4 Confirm how my findings are applicable for research into tropical nitrogen fixation, improving Dynamic Global Vegetation modelling and informing tropical reforestation programs.

Chapter 2

High herbivory is a major cost for tropical nitrogen-fixing tree species

2.1 Abstract

Recent observations of nitrogen limitation on the terrestrial tropical carbon sink raise the fundamental question of what limits new nitrogen from entering tropical ecosystems, where the carbon sink in mature and recovering forests is particularly large. Nitrogen-fixing tree species provide the main source of new nitrogen to tropical forests, but it remains unclear what factors govern tropical nitrogen fixation. Here I examine the previously untested hypothesis that herbivory constrains tropical nitrogen fixation. I evaluate whether fixers undergo higher herbivory than non-fixers, if the resulting herbivory cost is substantial and if high herbivory for fixers is due to herbivores targeting their nitrogen rich leaves. I analysed 1,632 leaves from 350 seedlings of 43 tropical tree species in Panama and find that (a) fixers undergo 29% (+/- 7%) higher herbivory than non-fixers despite high variation across both species groups, (b) that this loss of leaf area constitutes a 3.3% (+/- 3%) larger carbon cost for fixer seedlings which was greater than the energetic cost of fixation and (c), unexpectedly, that leaf nitrogen does not govern herbivory. My findings demonstrate for the first time that herbivory constrains tropical nitrogen fixers and may promote nitrogen limitation on the tropical carbon sink.

2.2 Introduction

Increasing evidence suggests that the ability of tropical forests to sustain a long-term carbon sink in mature and secondary forests may be limited by nutrients (Brookshire *et al.*, 2019; Levy-Varon *et al.*, 2019; Wright, 2019). Observations of high symbiotic nitrogen fixation rates in tree fall gaps (Barron *et al.*, 2011), mature tropical forests with high net carbon uptake rates (Brookshire *et al.*, 2019) and tropical secondary forests recovering from disturbance (Batterman *et al.*, 2013b; Levy-Varon *et al.*, 2019) point to nitrogen limitation as being pervasive (Wright, 2019). The trait of nitrogen fixation provides a substantial growth benefit to tropical trees capable of fixation during periods of nitrogen limitation by ensuring a constant supply of atmospheric nitrogen (Batterman *et al.*, 2013b). Given this substantial benefit of nitrogen fixation to tropical trees, it remains unclear why the trait of fixation is not more widespread: nitrogen fixers represent only 1% of the basal area of trees in temperate forests, 1-8.5% in Asian mature tropical forests, ~9% at a tropical forest site in Central Africa and 5-15% in diverse Neotropical forests (Ter Steege *et al.*, 2006; Hedin *et al.*, 2009; Sullivan *et al.*, 2014; Gei *et al.*, 2018; Menge *et al.*, 2019).

Previous hypotheses to explain constraints on tropical symbiotic nitrogen fixation point to high carbon costs associated with the trait (Vitousek and Howarth, 1991; Menge *et al.*, 2008, 2017; Sheffer *et al.*, 2015). Breaking the triple-bond on the dinitrogen atom requires substantial reducing power, leading to a high carbon cost for producing fixed nitrogen relative to acquiring nitrogen from the soil (Vance, 2008). Building and maintaining nodules also presents an additional carbon cost directly associated with fixation (Gutschick, 1981; Vance, 2008). While these constraints may explain the low abundances of temperate fixers, which maintain a constant fixation rate per unit biomass regardless of soil nitrogen supply, they cannot alone account for tropical fixers, whose facultative fixation allows them to downregulate fixation when the benefit of fixation is small, such as when growing in nitrogen-rich soil (Batterman *et al.*, 2013b; Levy-Varon *et al.*, 2019). Tropical fixers therefore can avoid these fixation-associated costs and should remain competitive and abundant even when soil nitrogen is high and the costs of fixation outweigh the benefits (Menge *et al.*, 2008; Barron *et al.*, 2011; Batterman *et al.*, 2013b). These fixation-associated costs that can be avoided when fixation is not needed are therefore insufficient to explain why the trait has not spread across more tropical trees over ecological and evolutionary time. Tropical fixers must face an additional cost associated with fixation that is constant regardless of how much trees are actively fixing (Menge *et al.*, 2008, 2010; Vitousek *et al.*, 2013).

Several constant fixation-associated costs have been previously proposed, with many often lacking empirical support (Menge *et al.*, 2008; Vitousek *et al.*, 2013). One cost that has been understudied and that may affect tropical fixers even when they are not actively fixing is high herbivory (Vitousek and Howarth, 1991; Vitousek and Field, 1999; Menge *et al.*, 2008). In neotropical forests in particular, herbivory pressure is high and is mainly carried out by insects that target high nitrogen plant tissues to meet their nitrogen requirements for growth (Matson Jr, 1980; Coley and Barone, 1996; Coley *et al.*, 2006; Becerra, 2015). Nitrogen-fixing species contain high leaf nitrogen concentrations relative to non-fixing species, regardless of fixation rates (Fyllas *et al.*, 2009; Batterman *et al.*, 2013c; Epihov *et al.*, 2017). This pattern could lead to elevated herbivory irrespective of fixation rates and an elevated carbon cost for fixer species. A high carbon cost due to herbivory relative to the benefits of fixation would reduce fixer growth and competitive ability, ultimately suppressing fixer abundance in tropical forests.

A constraint on fixation by herbivory has been proposed in several theoretical frameworks, but support has been limited (Vitousek and Howarth, 1991; Ritchie and Tilman, 1995; Vitousek and Field, 1999; Ritchie and Raina, 2016; Taylor and Ostrowsky, 2019) and direct relationships between fixation, leaf nitrogen and herbivory remain unexamined. Herbivores in the tropics target a complex suite of leaf traits and species attributes, and often times only attack plant species with specific traits they have co-evolved with (Wiggins *et al.*, 2016; Endara *et al.*, 2017; Coley *et al.*, 2018). Furthermore, whilst certain leaf traits such as leaf nitrogen have been shown to attract herbivores , defensive leaf traits and the rate at which a leaf expands through its vulnerable early stages of growth can reduce herbivory levels and obscure the role of attractive leaf traits (Kursar and Coley, 1991, 2003; Kitajima *et al.*, 2016; Sedio *et al.*, 2017). Fixers have been observed to deploy toxic secondary metabolites, indigestible leaf fibres like cellulose, and tough leaves to deter herbivores (Kursar *et al.*, 2009; Endara *et al.*, 2017; Taylor and Ostrowsky, 2019). Many traits that may govern herbivory are often linked with other attributes, such as the link between the expansion rate of young leaves and leaf area of mature leaves (Moles and Westoby, 2000; Fine *et al.*, 2006; Gianoli and Salgado-Luarte, 2017; Defossez *et al.*, 2018). Therefore, due to this complex nature of tropical herbivory, it may be difficult to determine if leaf nitrogen, or any other trait associated with fixation, drives high herbivory across many species.

I here test three specific hypotheses: first, that fixers undergo higher herbivory than non-fixers; second, that this higher herbivory constitutes a fixation-associated carbon cost substantial enough to constrain fixation; and third, that high herbivory for fixers is driven by high leaf nitrogen concentrations. To examine my hypotheses, I quantified herbivory on 1,632 mature leaves from 350 seedlings and saplings across 23 fixer species and 20 non-fixer species from mature forest in Panama. On a subset of my plants, I tracked active herbivory rates for 226 young leaves over 3 months. I next used my field-based measures to estimate the carbon cost of herbivory for fixers. Finally, I combined my herbivory measures with data on nutrient concentrations, physical traits and chemical profiles of leaves and other species attributes to examine whether leaf nitrogen or another trait governs herbivory. I find that fixers undergo higher herbivory than non-fixers and that this herbivory represents a major carbon cost for fixers species, but that herbivory is not driven by leaf nitrogen. My findings can help explain why fixers are not more abundant in tropical forests and suggest that fixers may have a limited ability to support a tropical carbon sink under rising atmospheric CO_2.

2.3 Methods

Species selection and study site

To compare herbivory across the wide range of fixer and non-fixer species present in biodiverse lowland moist tropical forests, I sampled individuals from 23 fixer species (Sprent, 2009) and 20 non-fixer species in the 50-Ha plot on Barro Colorado Island, Panama (9°9′ N,79°51′ W), during the wet seasons of 2017 and 2018. To determine if high herbivory was general across fixer species, I sampled nearly every fixer species present at the site (23 out of 26 species). We then selected 20 non-fixer species that covered the same range of species abundances across the 50ha plot. The study site receives a mean annual rainfall ~2600mm of rainfall and has a mean annual temperature of 27°C. Monthly means vary by 2°C (Leigh, 1999).

I focused my study on seedlings because herbivory acts as a major bottleneck at this life stage (Comita *et al.*, 2010; Queenborough *et al.*, 2013). All seedlings sampled were included in the on-going seedling census in the 50-ha plot, which was established in 2001. In the census, seedlings are defined as trees <1cm in basal diameter and with a stem height from 200mm-3000mm. Lianas and non-woody plants were not included in my study.

Quantifying herbivory

From the 13/06/2017 to the 17/07/2017, I non-destructively scanned up to six (4.9 on average) randomly selected leaves per individual for 195 fixer seedlings and 169 non-fixer seedlings within the field. To scan leaves, I used a hand-held document scanner (TaoTronics) capable of taking scans with 1050 Dots per Inch (DPI). I quantified the leaf area lost to herbivory in cm^2 from my leaf scans using ImageJ (Schneider *et al.*, 2012). For all leaves where the edge was damaged by herbivory, I cloned and used a complete leaf edge to estimate the undamaged leaf area. In addition, I tagged one young leaf each from 226 of these seedlings and scanned them again in November 2017 to quantify herbivory rates. This allowed us to account for young leaves that may undergo higher herbivory (Kursar and Coley, 2003) than more mature leaves, and allowed us to determine leaf turnover rates (see Appendix 1 (A1)). I calculated herbivory rate as the difference in leaf area between when they were first scanned and again after three months, divided by the estimated total leaf area at time point one (the area including leaf tissue lost to herbivory). This allowed us to easily include entire leaflets lost to herbivory over the study. For leaves which grew more leaf area than they lost to herbivory over the study, I calculated herbivory rate as the difference in percentage of leaf area missing at time point one and two. Importantly, these two methods give the same value for leaves that did not produce more leaf area or lose leaflets and together allowed us to assess my whole dataset.

Calculating carbon costs of herbivory

I consider two aspects of an herbivory-associated cost: (1) a structural carbon cost of leaf area lost to herbivory and (2) a photosynthetic opportunity carbon cost since lost leaf area reduces carbon assimilation via photosynthesis over time. I do not consider a potential third additional cost of replacing lost leaf nitrogen due to herbivory. Finally, I calculate the direct carbon cost of fixing nitrogen to evaluate the magnitude of the herbivory-associated costs relative to the fixation-associated costs.

I estimated the structural carbon cost at the leaf scale using species-specific leaf traits (described below). I first generated total leaf mass and leaf mass lost to herbivory by multiplying species-specific leaf mass per area (LMA) by either the total leaf area or leaf area lost to herbivory, respectively. I then multiplied the total leaf mass and leaf mass lost to herbivory by species-specific per-mass carbon concentrations to estimate total leaf carbon and the carbon lost to herbivory.

As well as removing structural leaf carbon, herbivory also reduces the leaf area available to assimilate carbon by photosynthesis. To estimate this photosynthetic opportunity carbon cost, I used the mean photosynthetic rate (maximum carbon assimilation, Amax) observed in field-grown seedlings of a common species in Panama, *Alseis blackiana* (Pasquini and Santiago, 2012), converted Amax values from µmol cm$^{2\ -1}$ s^{-1} to µg cm$^{2\ -1}$ s^{-1} and multiplied this value by the leaf area lost to herbivory per leaf. I assumed 12 hours of photosynthesis per day and 365 days per month and expressed the carbon cost as g C year^{-1}. To generate estimates of nutrient costs and total leaf nutrients per seedling, I multiplied the average structural carbon cost, photosynthetic opportunity cost and total leaf carbon and nitrogen across sampled leaves (up to 6) for each individual by its total number of leaves. I square root transformed costs across seedlings to meet normal distributions and then took the mean for fixer and non-fixer seedlings (the mean value was then squared to derive meaningful estimates). To account for the carbon costs of herbivory on non-fixers, I used the difference between average herbivory costs for each group to quantify the herbivory cost for fixer species. I compared all carbon costs as a percentage of annual net primary production, calculated as the annual photosynthetic capacity for each seedling minus the ~47% of carbon lost to respiration each year, an average found across plant species from a meta-analysis using over 200 studies (Collalti and Prentice, 2019).

Finally, I compared these herbivory-associated costs to the carbon cost attributed specifically to fixing nitrogen. I consider the maximum direct cost of fixing nitrogen for my seedlings by assuming all plant leaf nitrogen was acquired by fixation. This is a conservative estimate of the importance of the herbivory cost relative to direct fixation costs since many seedlings do not fix nitrogen when growing in the understory (Taylor and Menge, 2018) , in which case the herbivory-associated cost would be comparatively larger. I estimated maximum nitrogen fixation costs by multiplying seedling total leaf area by species-specific leaf nitrogen concentration. I then convert this total seedling leaf nitrogen to carbon using the common ratio of six grams of carbon per gram of nitrogen fixed (Vance, 2008). If plants received all nitrogen from the soil, then this fixation-associated cost would be 0.

Species attributes and leaf traits

To determine what might drive differences in herbivory between fixers and non-fixers I combined my data with species level leaf traits and attributes. I tested for differences in leaf nutrient concentrations (nitrogen, carbon, phosphorus, potassium and calcium), physical defence traits (cellulose, hemicellulose, lignin and silicon concentrations), measures of leaf toughness (lamina toughness, vein toughness, lamina density, work to shear and LMA) and chemical similarity metrices between the two groups to determine if any traits could drive patterns of herbivory across my sample species (full methods can be found in A2: Supplemental Methods; see A4: Supplemental Table 1 for all traits).

For nutrient and physical defence traits, three leaves were sampled from the highest point of the crown for the largest six and smallest six individuals of each species in the 50-ha plot. Leaves were sampled across species and light environment (Westbrook *et al.*, 2011). Since my herbivory data was collected for seedlings under the canopy, I used leaf trait data from shade leaves. Nutrient concentrations were analysed using nitric acid digestion (200mg of dried leaf samples with 2ml of 70% nitric acid) and an elemental analyser (dry combustion and gas chromatography, with thermal conductivity detection). An adapted previous method of Van Soest et al (1991) was chosen to calculate the cellulose, hemicellulose, silicon and lignin concentrations per unit leaf dry mass (see Appendix A of Westbrook et al., 2011). Leaf toughness was measured as resistance to fracture, either per unit dry mass (density corrected fracture toughness), unit volume (fracture toughness (J cm^{-2})) or unit cut length (Work to shear (J cm^{-1})). These measures were included alongside lamina density (g cm^{-3}) and Leaf Mass per Area (LMA, g cm^{-2}) (for protocol, see Westbrook et al., 2011, Kitajima et al., 2012, Kitajima et al., 2016).

Chemical similarity metrices were derived from methanol extracted homogenized leaf tissue. Molecules were identified using ultra high-performance liquid chromatography, electrospray ionization and molecular fragmentation, and tandem mass spectrometry of molecular fragments (Sedio *et al.*, 2017) . All pairwise combinations of said compounds were then used to calculate two variables, first, the nearest neighbour Chemical Structural and Compositional Similarity (nnCSCS), which measures the similarity in leaf secondary metabolites between the two most chemically similar pair of species in the 50-ha plot, and second, the mean Chemical Structural and Compositional Similarity (mCSCS), which measures the mean similarity across all pairs of species in the 50-ha plot (for more information on all species attributes and leaf traits, see A2: Supplemental Methods).

Statistical analyses:

The proportion of leaf area lost across leaves was highly right skewed and contained many zeroes (zero inflated) across both the young and old leaves I sampled (as there were many undamaged leaves). To account for this, I used two approaches. First, I analysed individual leaves using a hurdle model approach. The first step of the hurdle considered the incidence of herbivory, which is a binary measure of leaves either with herbivory (leaves with any leaf area missing) or no herbivory (no leaf area missing). For the incidence of herbivory, I ran a binary logistic regression using the lme4 package (Bates *et al.*, 2014) to test if there was a difference in the incidence of herbivory between fixer and non-fixer species. The second step of the hurdle model considered the proportion of leaf area lost only from leaves with area missing, referred to interchangeably as Proportion$^{\text{damaged}}$, which was a continuous measure. For Proportion$^{\text{damaged}}$, I ran a mixed effects model with normal distribution and square root transformed the proportion of leaf area missing to meet assumptions of normality. This model evaluated whether there was a difference in the leaf area lost on leaves attacked by herbivores between fixers and non-fixers. Together, this hurdle approach allowed us to assess whether more fixer leaves were attacked and/or whether more fixer leaf area was eaten per attack than for non-fixers, allowing us to ultimately understand the mechanism by which herbivory affected fixer and non-fixer seedlings.

Second, I analysed individual seedlings with a mixed effects beta regression. This approach allowed us to better assess if fixer seedlings lost more leaf area overall by considering the entire data set together, including leaves that did not have area missing (zero values) and leaves with damage. For this method, I summed the amount of leaf area missing across leaves per individual seedling, including leaves with no herbivory damage, referred to interchangeably as Proportion$^{\mathrm{all}}$. This approach reduced the number of zeroes in the dataset since there were fewer individuals with no herbivory than there were leaves with no herbivory and made a unified statistical approach possible. I ran the mixed effects beta regression model using the glmmTMB package (Brooks *et al.*, 2017). A beta distribution is well suited to model variables with upper and lower limits, such as proportions (Smithson and Verkuilen, 2006). However, as a beta distribution is only suitable for values above 0 and below 1, and my data contained zeroes, I rescaled my data using the equation:

Equation 1

$$X' = \frac{x(N-1)+0.5}{N}$$

Where x is proportion of leaf damage and N is the total number of individuals (Smithson and Verkuilen, 2006; Murphy *et al.*, 2016).

In all my models, my hurdle models and my beta regression model, I included the trait of fixation as a fixed effect to test for differences in herbivory between the two groups (see Model 2.1 below). To control for variation in seedling and leaf size, I also included stem length and leaf area. Leaf area was standardized (centred on zero and divided by one standard deviation) within species to account for species level variation in leaf size. Stem length was scaled across all seedlings (centred on zero and divided by one standard deviation). All models also included species identity as a random effect to account for species specific variation in herbivore damage. The 20m^2 plot in which the seedling was found was likewise included as a random effect to account for spatial autocorrelation in modelling Incidence and Proportion$^{\mathrm{all}}$, but not for Proportion$^{\mathrm{damaged}}$ as this led to overfitting with the smaller dataset. In addition, when modelling incidence of herbivory, individual seedling identity was included as a random effect to account for taking repeated measures from the same individuals (since I sampled up to 6 leaves per seedling). To test whether the trait of fixation or some other trait associated with the Fabaceae drove the Incidence of herbivory or the Proportion^{all} for fixers, I also ran a binary logistic regression and a beta model using only fixation as a fixed effect. However, to determine if fixation drove herbivory, or some other Fabaceae associated trait, in these models the trait of fixation specified whether a seedling was from a fixer species, a non-fixing member of the Fabaceae or any other non-fixer species, rather than just separating species into fixers or non-fixers as in previous models. I used pseudo R^2 values to determine how much of the observed variance in herbivory measures was explained by my models.

To determine if leaf traits governed the difference in herbivory between fixer and non-fixer species, I identified leaf traits that differed between the fixer and non-fixer groups (Wilcoxon rank tests). I then restructured the models described above to include the variables that differed to generate new models, by replacing the trait of fixation as an explanatory variable with each of the identified leaf traits. For example, when testing the role of leaf nitrogen, I ran a model using species leaf nitrogen concentration, standardized leaf area and stem length as fixed effects and species and plot identity as random effects. I then ran these models for all species, or just fixer and non-fixer species alone. For the Proportion^{damaged} I ran simple linear regression, excluding random effects, to avoid overfitting with the reduced dataset.

Model 2.1: Variable specification for my model testing for a difference in Proportion^{all} between fixer and non-fixer species (fitted using a beta regression, see above).

$$\begin{aligned} Proportion^{all} \sim\ & Fixation + Standardized\ leaf\ area + Stem\ length \\ & + (1|species) + (1|plot) \end{aligned}$$

Model 2.2: Variable specification for my model examining the role of a given leaf trait found to differ between fixer and non-fixer species (here leaf nitrogen concentration) in driving Proportion^{all} across species.

$$
\begin{aligned}
Proportion^{all} \sim\ & Leaf\ nitrogen\ concentration + Standardized\ leaf\ area \\
& + Stem\ length \\
& + (1|species) + (1|plot)
\end{aligned}
$$

Model 2.3: Variable specification for the beta regression used to predict species level Proportion^{all}. Species is now specified as a fixed effect and fixation is removed from the model.

$$
\begin{aligned}
Proportion^{all} \sim\ & Species + Standardized\ leaf\ area + Stem\ length \\
& + (1|plot)
\end{aligned}
$$

Finally, I used my models to predict the probability of herbivory and average mean leaf area lost to herbivory across fixer and non-fixer species to account for variation due to seedling size, leaf area, species identity and location in the plot. I restructured all models so that species identity was included as a fixed effect and fixation (or any trait other than standardized leaf area and stem length) was omitted (compare variable specification in Model 2.1 and 2.3 above). Next, I used my restructured model of the incidence of herbivory across seedlings to predict the probability of herbivory per species and my two restructured models of the proportion of leaf area lost to predict the average leaf are lost to herbivory per species (using the predict() function from the bootpredictlme4 function for the binary logistic regression model and the linear mixed effects model and the predict.GLMM() function from the glmmTMB package for the beta regression model (Brooks *et al.*, 2017; Duursma, 2020)). Species for which I had less than 10 leaves were excluded from my probability model to prevent singularity. I then ran Wilcoxon Rank tests to determine if there was a difference in the average predicted value between fixer species and non-fixer species for each measure of herbivory. All statistical analyses were carried out in R version 3.5.1 (R Core Development Team, 2018).

2.4 Results and discussion

I found support for my first hypothesis that fixers undergo higher herbivory than non-fixers across all three metrics of herbivory on mature leaves. My three metrics were (1) a binary measure of the incidence of herbivory across leaves (2) the proportion of leaf area lost to herbivory for leaves that were damaged by herbivores (Proportion^{damaged}) and (3) the proportion of leaf area lost to herbivory for all leaves (Proportion^{all}). For the incidence of herbivory, 21% more fixer leaves had been attacked than non-fixer leaves (see Fig 2.1a, A5: Supplemental Table 1). At the individual seedling scale; fixers also had a significantly higher proportion of leaf area loss for all leaves, with and without herbivory (Proportion^{all}; 9.6% vs 7.6%; see Fig. 2.1e; A5: Supplemental Table 3), and a non-significant trend towards having a higher proportion of leaf area loss only for leaves with herbivory (Proportion^{damaged}; 9.3% vs 6.8%; see Fig. 2.1c; A5: Supplemental Table 2). When considering species, fixers also had a higher predicted incidence of herbivory, Proportion^{all} and Proportion^{damaged}, when controlling for variation in leaf size, seedling height and position of seedling in the 50-ha plot (See Figs. 2.1b-f, 2.2). The proportion of leaf area lost to herbivory ranged between 0 and 39% for species in both functional groups and, while fixers underwent more herbivory on average, being a fixer did not always ensure higher herbivory for some fixer species (see Fig. 2.2), as found previously (Taylor and Ostrowsky, 2019). Unlike for mature leaves, I found no difference in any herbivory measure across young leaves (see A3: Supplemental Fig. 1; A5: Supplemental Tables 4, 5, 6). Even when controlling for other factors (described below), I found that fixation status explained herbivory patterns across species, suggesting a direct link between the fixation trait and herbivory.

My finding of higher herbivory for fixers contrasts with former comparisons between tropical fixer and non-fixer tree species carried out on fewer species, but is line with reported higher herbivory for grassland fixer species (Ritchie and Tilman, 1995; Ritchie and Raina, 2016; Taylor and Ostrowsky, 2019). Taken together, these results suggest that a fixation associated trait attracts herbivores to fixer species. High herbivory for seedlings will affect adult fixer abundances as this life stage is a major bottleneck on species reaching the canopy (Forrister *et al.*, 2019). Previously, herbivory rates of ~1% on the mature leaves of tropical seedlings have been found to drive seedling mortality the following year (Eichhorn *et al.*, 2010). High herbivory associated with the trait of fixation could therefore explain why fixers are constrained across the tropics, but to understand how, I must first quantify the cost of herbivory for fixer seedlings.

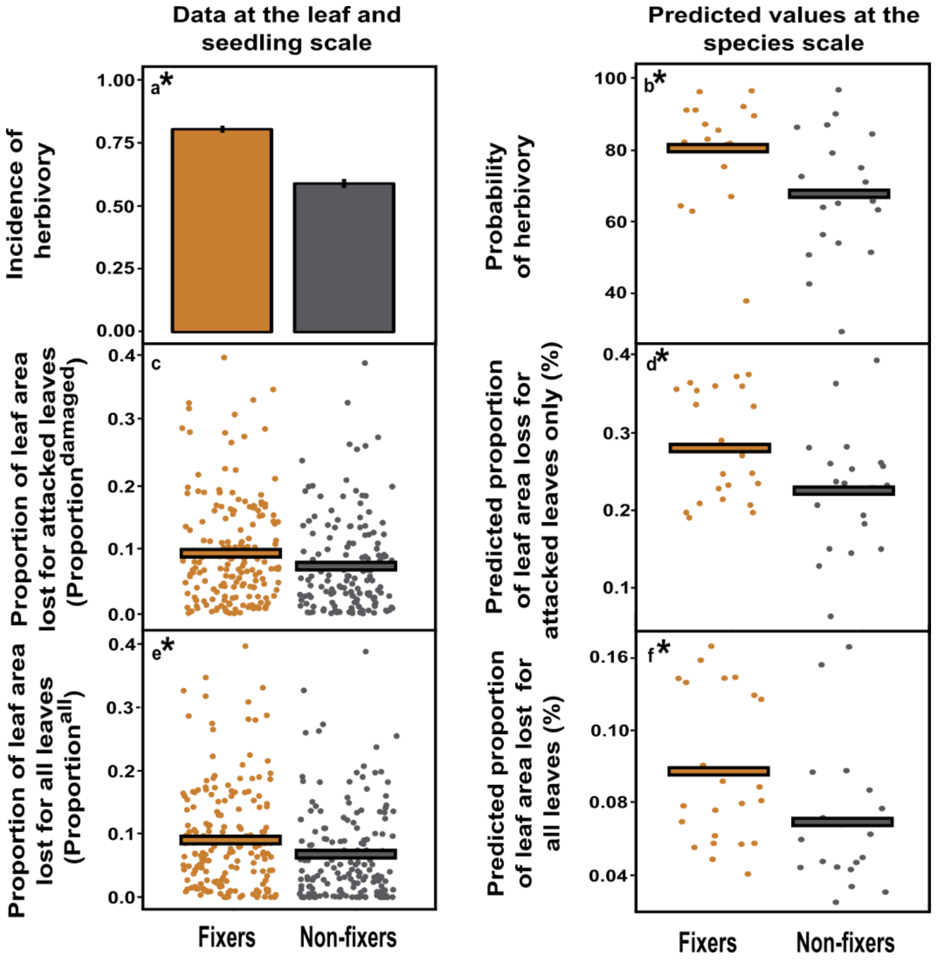


Figure 2. 1. ***The difference in herbivory for fixer and non-fixer species****. At the leaf and seedling scale, the incidence of herbivory on leaves (a), the proportion of leaf area lost to damaged leaves from each seedling (Porportion^{damage}, c) and the proportion of leaf area lost to all leaves from each seedling (Porportion^{all}, e), and at the species scale, the probability of herbivory on leaves (b) the predicted proportion of leaf area lost to herbivory on damaged leaves from each seedling (d) and the predicted proportion of leaf area lost to herbivory including all sampled leaves from each seedling (f) for fixers (orange) and non-fixers (grey). Predicted values were derived from my modelling of the incidence of herbivory (b), Porportion^{damaged} (d) and Proportion^{all} (f). Asterisks denote statistically significant differences between fixers and non-fixers in my mixed effects models (a, c, e) and in Wilcoxon rank tests (b, d, f).*

I next examined my second hypothesis, that high herbivory for fixers constitutes a significant carbon cost that could constrain the trait of fixation, by estimating structural carbon costs and photosynthetic opportunity costs for both groups. I found that the combined structural cost and photosynthetic opportunity cost was ~13.7%% of annual net carbon sequestration for an average fixer seedling (the photosynthetic opportunity cost alone was 13.0%, see Table 2.1). This fixation associated cost was 3.3% more than for non-fixer seedlings on average and greater than the one-time carbon cost of paying bacteria to fix 100% of leaf nitrogen (2.6% of annual carbon sequestration, see Table 2.1) (Vance, 2008) – a cost which is likely to be overestimated as fixer seedlings in the understorey fix at low rates and derive much of their leaf nitrogen from the soil (Barron *et al.*, 2011; Taylor and Menge, 2018).

Importantly, the high photosynthetic opportunity cost for fixers will be compounded every year and so therefore continually reduces carbon available for the growth of new photosynthetic leaf area, thus further limiting resource acquisition. Ultimately, this high cost could increase fixer seedling mortality and reduce the chances of fixer seedlings reaching the canopy and reproducing (Eichhorn *et al.*, 2010; Quiroz-Pacheco *et al.*, 2020). Seedling transplantation experiments have established that the combined effect of herbivory and soil conditions can determine tropical tree species abundance and distribution (Fine *et al.*, 2004). Tropical fixers downregulate fixation rates when nitrogen is readily available in the soil, when they are outside of their optimum soil conditions, to avoid paying the energetic costs of fixation when they outweigh the benefits (Barron *et al.*, 2011; Batterman *et al.*, 2013c, 2013b). However, these cumulative, constant herbivory costs will affect fixers in high nitrogen soil when fixer seedlings gain no benefits from fixation and therefore could explain why fixers are not more abundant in mature tropical forests (Vitousek and Howarth, 1991; Vitousek and Field, 1999).

Table 2. 1*. *The fixation associated cost of herbivory. *Showing the annual net primary production, structural carbon cost, photosynthetic opportunity cost, and fixation cost for fixing all leaf nitrogen in g C and as a percentage of annual net photosynthetic capacity for fixer and non-fixer seedlings.*

Variable	Fixer	Standard Error	Non-fixer	Standard Error
Number of seedlings	185	-	165	-
Annual net primary production (g C plant^{-1})	15.4	0.15	9.1	0.1
Structural carbon cost (g C plant $^{-1}$)	0.1	0.03	0.05	0.02
Structural carbon cost as a percentage of annual net primary production (%)	0.7	0.2	0.6	0.2
Photosynthetic opportunity cost (g C plant^{-1})	2.0	0.1	0.9	0.08
Photosynthetic opportunity cost as a percentage of annual net primary production (%)	13.0	0.7	9.9	0.9
Cost of fixing all leaf nitrogen (g C plant^{-1})	0.4	0.02	-	-
Cost of fixing all leaf nitrogen as a percentage of annual net primary production (%)	2.6	0.13	-	-

As high herbivory is associated with the trait of fixation, I would expect there to be leaf traits unique to fixers that promote herbivory, especially fixer high leaf nitrogen. However, I found little evidence to support my third hypothesis, that high leaf nitrogen concentrations drove high herbivory for fixers, or to pinpoint any other variable that could explain the difference in herbivory between fixers and non-fixers such as leaf chemical or physical defences. If a trait like high leaf nitrogen that attracts herbivores drives high herbivory for fixers, I would expect it to be higher in fixer leaves and to show a positive relationship with measures of herbivory consistent across fixer species, non-fixer species and all species grouped together. For a trait like leaf toughness that deters herbivores to explain high herbivory for fixers, I would expect the trait to be lower in fixer leaves and to have a negative relationship with measures of herbivory consistent across both groups together and separately.

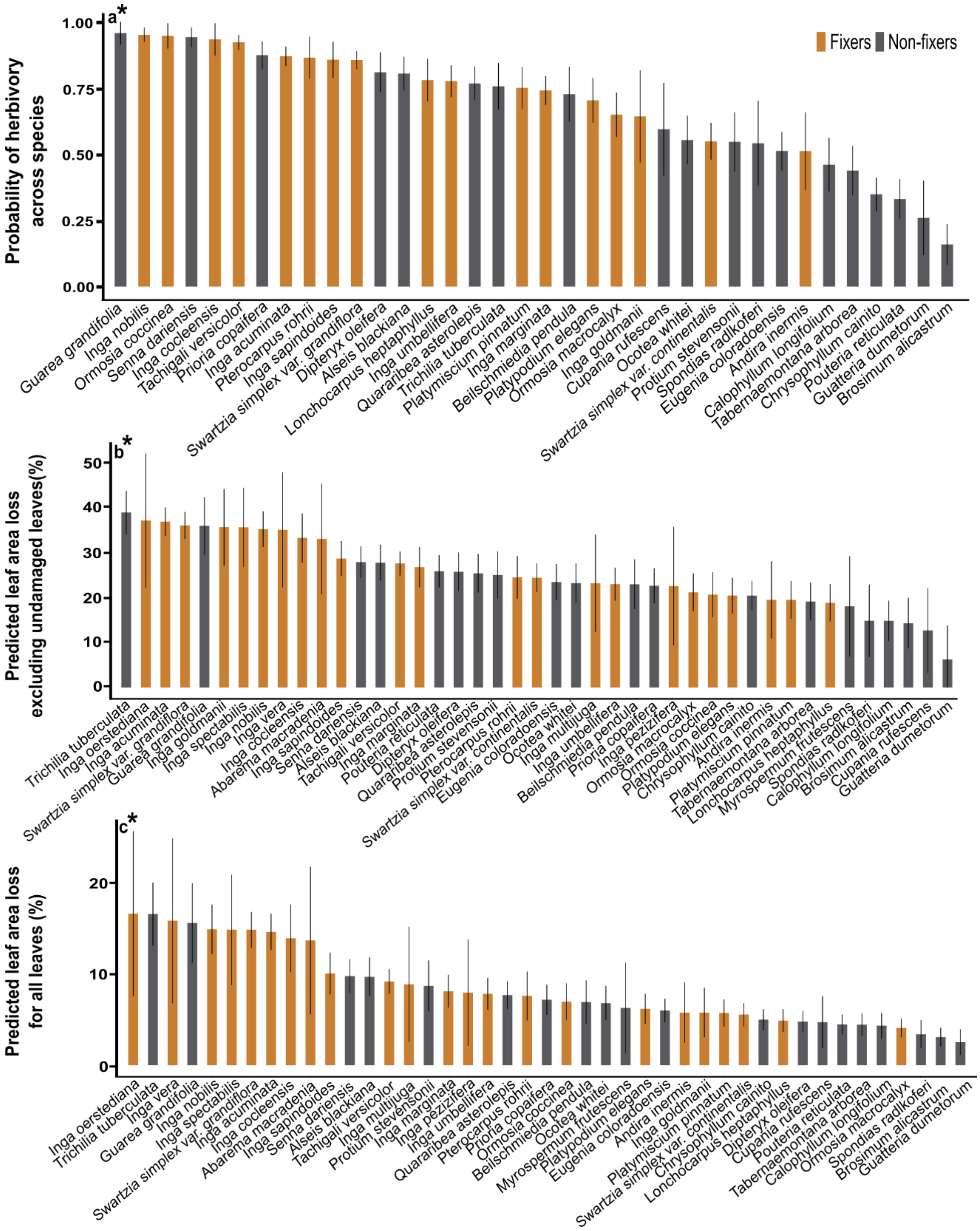


Figure 2. 2 The difference in herbivory for fixer and non-fixer species, as predicted by my mixed effect model when controlling for species and plot identity. *The distribution of the predicted probability of herbivory on leaves (a) the distribution of the predicted proportion of leaf area lost to herbivory on damaged leaves per seedling (b) and the distribution of the predicted proportion of leaf area lost to herbivory including all sampled leaves per seedling*

(c) for fixers (orange) and non-fixers (grey). Predicted values were derived from my modelling of incidence of herbivory (a), Porportion^{damaged} *(b) and Proportion*^{all} *(c). Error bars represent standard error of model estimates for each species. Asterisks denote statistically significant differences between fixers and non-fixers from Wilcoxon rank tests.*

Of the leaf variables I considered in my analysis (see A4: Supplemental Table 1), I identified six leaf traits that differed between fixers and non-fixers and that could theoretically attract or deter herbivores: nitrogen (3.1 g g^{-1} (leaf tissue) versus 2.3 g g^{-1}, respectively), carbon (46.0 g g^{-1} versus 44.4 g g^{-1}, respectively), potassium (7.4 mg g^{-1} versus 11.2 mg g^{-1}, respectively), cellulose (3.1 g g^{-1} versus 2.3 g g^{-1}, respectively), lignin (0.2 g g^{-1} versus 0.2 g g^{-1}, respectively) and leaf area per seedling (117.3 cm^2 versus 59.1 cm^2, respectively) (See A3: Supplemental Fig. 2). Of these traits, only leaf area was positively correlated with any measure of herbivory, the incidence of herbivory, when considered across fixer species alone, non-fixer species alone and all species together (see Table 2.2 for the role of leaf area in driving the incidence of herbivory, Proportion^{damaged} and Proportion^{all} of leaf area lost to herbivory; See A6: Supplemental Tables 1-15 for the effects of the other five leaf traits). However, I found that greater leaf area did not drive the percentage of leaf area lost for fixer species (only for non-fixer species, and across all species when grouping fixer and non-fixers together; see Table 2.2).

Our results therefore suggest that fixers have larger leaves which are more likely to be attacked, but that greater leaf area for fixers does not explain why fixers lose a greater percentage of their leaves. This discrepancy indicates that leaf area alone cannot explain why fixer species undergo higher herbivory than non-fixers. The lack of a correlation between greater leaf area and leaf area loss for fixer species may instead signify that fixers have, on average, both high herbivory and high leaf area, but that this high herbivory for fixers is driven by another unrelated leaf trait or traits. Whilst several hypotheses have been proposed to explain why larger leaves may be more likely to undergo herbivory - they may be better landing pads for insect herbivores, more suitable places for egg laying, or, as larger leaves take longer to expand, undergo more herbivory during the vulnerable early stages of leaf expansion (Feeny, 1976; Ribeiro *et al.*, 1994; Moles and Westoby, 2000) - the question therefore remains why leaf area, nor any other trait, could not explain why fixers lost more leaf area than non-fixers overall.

***Table 2. 2*. The effect of leaf area in driving the incidence of herbivory, the proportion^{all} of leaf area lost to herbivory, and the proportion^{damatged} of leaf area lost to herbivory.** Analyses were conducted across all species, fixer species alone and non-fixer species alone (number of observations: 350 (184 fixers, 166 non-fixers)). Stem length was included to control for variation in seedling size. For each response variable, both fixed effects were included, only changing the data set between models. Seedling, species and plot identity were included as random effects. Significant p values are in bold.

Response variable	**Dataset**	**Variables**	**Estimate**	**Z Value**	**p value**	**R^{2}m**	**R^{2}c**
Incidence of herbivory	All species	Leaf area	1.4	6.2	**4.20E-10**	0.31	0.49
		Stem length	-0.026	-0.29	0.77		
	Fixers species	Leaf area	1.4	4.9	**1.20E-06**	0.4	0.52
		Stem length	0.052	0.37	0.71		
	Non-fixer species	Leaf area	1.2	3.4	**0.00071**	0.15	0.36
		Stem length	-0.046	-0.38	0.7		
Proportion^{all} of leaf area lost to herbivory	All species	Leaf area	0.017	1.9	0.052	0.015	0.22
		Stem length	-0.0012	-0.16	0.88		
	Fixers species	Leaf area	0.0091	0.87	0.38	0.01	0.31
		Stem length	0.0061	0.57	0.57		
	Non-fixer species	Leaf area	0.029	1.9	0.071	0.025	0.25
		Stem length	-0.012	-1.1	0.27		
Proportion^{damaged} of leaf area lost to herbivory	All species	Leaf area	0.14	2.7	**0.0064**	0.029	0.22
		Stem length	0.025	0.49	0.62		
	Fixers species	Leaf area	0.098	1.5	0.13	0.02	0.3
		Stem length	0.0097	0.13	0.89		
	Non-fixer species	Leaf area	0.22	2.3	**0.022**	0.038	NA
		Stem length	0.033	0.47	0.64		

It is possible that another unknown leaf trait underpins herbivory for fixers, although I find this unlikely given that I analysed 17 key leaf variables widely recognized to govern herbivory (see A4: Supplemental Table 1). Instead, I find the lack of one governing factor for herbivory is consistent with the large body of evidence suggesting that plant-herbivore relationships are highly co-evolved and governed by complex species-specific suite of defence traits (Wink, 2013; Endara *et al.*, 2015, 2017; Coley *et al.*, 2018; Taylor and Ostrowsky, 2019). Fixers had a higher incidence of herbivory and proportion of leaf area lost for all leaves, but that there was no difference in the proportion of leaf area lost just for damaged leaves (see A5: Supplemental Tables 1-3). Thus, I found that fixer leaves are attacked more frequently than non-fixer leaves, but that the same amount of each individual leaf is consumed for fixers and non-fixers per attack. This mechanism of herbivore attack indicates that, whilst some traits may attract herbivores to fixer species, fixer defensive traits may prevent herbivores from eating large amounts of leaf area per attack. Investment in defensive secondary metabolites that adversely affect larval growth could lead to this pattern of herbivory, such as the overexpression of the essential amino acid tyrosine in some *Inga* species (Coley *et al.*, 2018). These defence traits may have obscured the role of one fixation associated trait in driving herbivory across many species in a way I were unable to detect due to the highly coevolved relationships between herbivores and their plant hosts (Kursar *et al.*, 2009; Endara *et al.*, 2015, 2017). Closely related fixers can invest in very different defences to deter specialist coevolved herbivores, but as these defences deter only the specialist herbivore, the effect of that defence trait on reducing herbivory is difficult to quantify across many species (Kursar *et al.*, 2009; Endara *et al.*, 2017; Coley *et al.*, 2018). A previous comparison of leaf traits and defence characteristics between fixers and non-fixers also found that species-specific differences in plant-herbivore interactions most explained variation in herbivory across the two groups (Taylor and Ostrowsky, 2019). Leaf nitrogen concentration itself is a good model of how one leaf trait can govern herbivory in many different ways across species.

The lack of a role for leaf nitrogen specifically in explaining herbivory may be due to the use of nitrogen-based defences by fixer species. The chemical forms of leaf nitrogen present in fixer leaves can vary from nutritious to toxic both within and between species (Matson Jr, 1980; Wink, 2013). Whilst the nitrogen present in photosynthetic proteins is a key dietary source for insect herbivores (Matson Jr, 1980; Kursar and Coley, 2003), nitrogen-based defence compounds like essential and non-protein amino acids, amines and cyanogenic glucosides are often highly toxic and are more common in fixer than non-fixer species (Wink, 2003, 2013). Importantly, the distribution of these compounds across fixer species does not follow a phylogenetic pattern (Wink, 2013), suggesting that the coevolved nature of plant-herbivore interactions governs the chemical form that nitrogen takes in fixer leaves. It is possible that higher herbivory for fixer species has led to the evolution and coexistence of speciose genera, as closely related species differentiate by evolving novel defences, both nitrogen and non-nitrogen based, to escape high herbivore pressure (Kursar *et al.*, 2009; Endara *et al.*, 2015). Inga species, for example, show higher variation in defence traits than they do in functional traits for resource acquisition (Endara *et al.*, 2015). This finding therefore highlights the exciting possibility that high herbivory for fixers may have shaped the evolution of plant-herbivore interactions and contributed to tropical biodiversity.

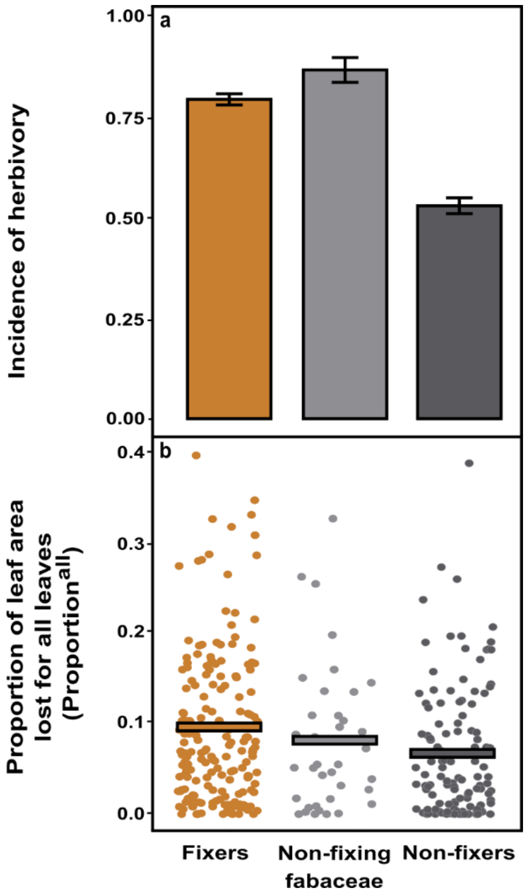


Figure 2. 3 **High herbivory common across the Fabaceae.** At the leaf and seedling scale, the incidence of herbivory on leaves (a), and the proportion of leaf area lost to all leaves from each seedling (Porportion^{all}, b) for fixers (orange), non-fixing Fabaceae (light grey) and non-fixers (dark grey). Error bars in panel a represent standard error. Bars in panel b represent the mean value for each functional group.

All of the nitrogen-fixing species in our sample, along with the vast majority of fixers in the tropics, are phylogenetically clustered within the Fabaceae family (Sprent, 2009). It is therefore important to consider whether higher herbivory for fixers is associated with the functional trait of fixation, or is prevalent amongst species in the Fabaceae, regardless of fixation status. We predicted high probabilities of herbivory for the three non-fixing Fabaceae species in our sample - *Dipteryx oleifera*, *Senna Dariensis* and *Prioria copaifera* (See Fig 2.2a). Furthermore, when comparing average herbivory across fixers, non-fixers and non-fixing Fabaceae we found, first, that fixers underwent higher herbivory than non-fixers when excluding non-fixing species from the Fabaceae, but second, that there was no difference in herbivory between fixing and non-fixing Fabaceae species (see Fig, 2.3).

Despite my small sample size for non-fixing members of the Fabaceae, my findings suggest that some Fabaceae associated trait may drive high herbivory for fixers, rather than the trait of fixation itself. One possible trait that could cause high herbivory for fixers and non-fixing Fabaceae is high leaf nitrogen, which is common across the Fabaceae and independent of nitrogen-fixation status (Fyllas *et al.*, 2009). Alternatively, species may be identified by herbivores as fixers even after they have lost the ability to fix nitrogen. The trait of fixation has evolved and been lost many times throughout the evolution of the Fabaceae (Werner *et al.*, 2014; Griesmann *et al.*, 2018; Velzen *et al.*, 2018), and it is possible that the evolution of herbivore preference cannot reliably distinguish between closely related fixing and non-fixing species phylogenetically clustered within the Fabaceae. Importantly, regardless of whether high herbivory is associated with the trait of fixation, or is common across all species in the Fabaceae, high herbivory for fixing species will have important consequences for tropical nitrogen fixation and will affect tropical nitrogen cycling and carbon sequestration in tropical forests.

This research represents the most extensive survey of herbivory for nitrogen-fixing species to date and reveals a direct link between herbivory and fixation. My findings suggest that herbivory results in a major cost to tropical nitrogen-fixing tree species that constrains fixation over ecological and evolutionary time scales. I found strong support for my first hypothesis that fixer species undergo widespread high herbivory compared to non-fixer species, and found evidence to support my second hypothesis that high herbivory for fixers constitutes a high carbon cost that could constrain the trait of fixation. I find no support for my third hypothesis that high herbivory for fixers is driven by high fixer leaf nitrogen, and instead find a correlation between greater leaf area for fixer species and one out of three of my measures of herbivory. My inability to identify a clear driver of herbivory across species may be due to species-specific investment in defence obscuring the role of fixation associated traits, including nitrogen, in driving high herbivory for fixers.

My findings make a substantial advance on earlier evidence from grassland ecosystems and a handful of tropical tree species in secondary forests (Ritchie and Tilman, 1995; Ritchie and

Raina, 2016; Taylor and Ostrowsky, 2019) and, when combined, suggest that fixers bear high herbivory costs relative to non-fixers across ecosystems and biomes. Furthermore, my identification of widespread high carbon costs across fixer species provides evidence that biogeochemical and dynamic global vegetation models should include herbivory as a constraint on the trait of fixation (Menge *et al.*, 2008, 2010; Vitousek *et al.*, 2013; Wieder *et al.*, 2015a; Peng *et al.*, 2020). Whilst further work is needed to determine the mechanisms that underlie differences in herbivory between fixers and non-fixers, this herbivory cost provides an ecological and evolutionary explanation for why fixer abundances are capped at a maximum of 15% across neotropical forests (Ter Steege *et al.*, 2006; Hedin *et al.*, 2009). Herbivory is likely to be a key factor not only in governing tropical nitrogen fixation but also nitrogen limitation and carbon sequestration by tropical forests.

Chapter 3

A quicker fix: Herbivory may stimulate nitrogen fixation rates in tropical forests

3.1 Abstract

Nitrogen-fixing trees promote tropical forests regrowth after disturbance and are also heavily targeted by herbivores for their nitrogen rich leaves. However, how high herbivory for fixers affects fixation rates remains unclear. Leaf nutrients lost to herbivory could alter patterns of fixation by affecting plant nutrient demand, depending on the level of herbivory, soil nitrogen availability and species antiherbivore strategy. Therefore, I conduct greenhouse experiments on 200 seedlings from five Inga species to test three hypotheses: (1) that herbivory governs fixation rate, (2) that this effect is governed by an interaction between herbivory level and soil nitrogen availability, and (3) that the herbivory-fixation relationship is mediated by species antiherbivore strategy. I found up to tenfold increases in fixation rates (+/- 0.41) following herbivory, but this depended on the three additional factors investigated. This strong relationship between herbivory and fixation may facilitate faster forest regrowth after disturbance. My findings demonstrate a key role for herbivory in governing tropical nitrogen fixation, with consequences for the tropical forest carbon sink.

3.2 Introduction

Tropical nitrogen fixation underlies tropical carbon sequestration, enhancing carbon accumulation by 200% in young forests and total carbon in mature forests by ~10% (Batterman *et al.*, 2013b; Brookshire *et al.*, 2019; Levy-Varon *et al.*, 2019). Yet, despite the critical role that nitrogen-fixing tree species play in tropical forests and the substantial benefits fixation provides for trees when soil nitrogen is limiting, fixer species never exceed 1-8.5% of basal area in Asian mature forests, were found to be at 9% at a tropical forest site in Central Africa and are capped at 5-15% of basal area across the Neotropics (Ter Steege *et al.*, 2006; Hedin *et al.*, 2009; Sullivan *et al.*, 2014; Gei *et al.*, 2018; Menge *et al.*, 2019). This relative scarcity is surprising, given that tropical fixers can avoid fixation associated costs by downregulating fixation when soil nitrogen meets the demands of plant growth (Barron *et al.*, 2011; Batterman *et al.*, 2013c, 2013b). The low abundance of tropical fixers therefore raises the question - what could be constraining the trait of fixation in tropical forests? Work for my first chapter confirmed the previously untested hypothesis that fixers undergo a high herbivory cost due to their high leaf nitrogen concentrations, a limiting nutrient in herbivorous diets (see chapter one). However, how high herbivory affects fixation rates in tropical forests remains unknown.

High herbivory for fixers could result in either upregulated or downregulated fixation rates in individual plants. The direction of this effect may depend on how the loss of two key leaf macronutrients to herbivory – carbon and nitrogen – affect plant nutrient demand. On the one hand, the carbon cost of herbivory could reduce fixation by limiting carbon allocation to the bacteria in exchange for fixed nitrogen (~6-7 g carbon g^{-1} nitrogen fixed), making fixers less competitive in nitrogen-limited conditions (see dashed purple line in Fig 3.1) (Vance, 2008; Ritchie and Raina, 2016). In my first chapter, I found that ~20% more carbon was lost to herbivory than exchanged with bacteria for nitrogen, even at high fixation rates (see Table 2.1). On the other hand, herbivory also constitutes a leaf nitrogen cost and fixers may upregulate fixation in response to herbivory in order to replace lost leaf nitrogen (see solid purple line in Fig. 3.1). Tropical fixers upregulate nitrogen fixation rates when soil nitrogen is lower than plant demand (Batterman *et al.*, 2013c). As nitrogen is a limiting nutrient for plant growth, even small amounts of nitrogen lost to herbivory may increase nitrogen demand and cause fixers to up-regulate fixation as long as carbon from photosynthesis is available (Matson Jr, 1980; Lerdau and Coley, 2002b; Ritchie and Raina, 2016) .

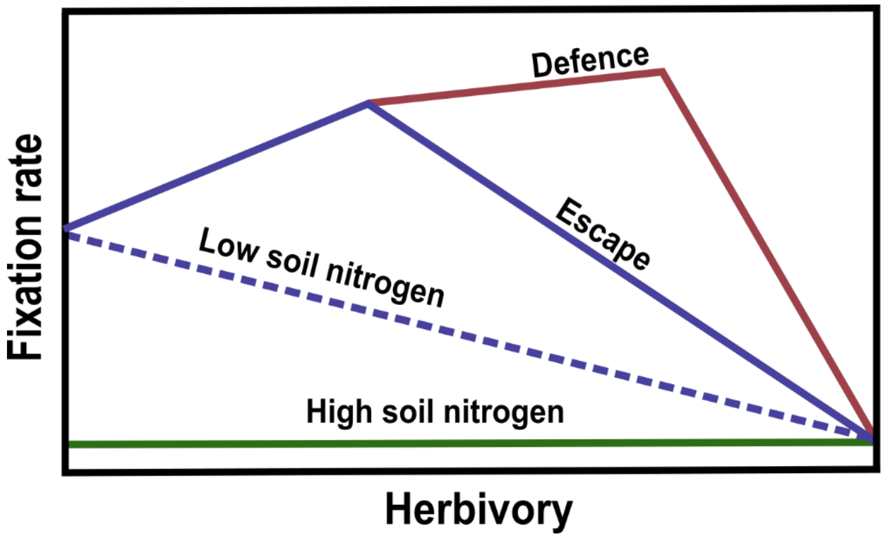


Figure 3. 1. The expected relationship between fixation rate and leaf area lost to herbivory. *The figure shows possible relationships between herbivory and fixation rates depending on soil nitrogen, leaf area lost to herbivory and species antiherbivore strategy. At high soil nitrogen I expect no change in fixation as nitrogen demand is met by uptake from the soil (green line). At low soil nitrogen, I expect herbivory to either lead to lower fixation rates (dashed purple line) or increase fixation rates until a threshold at which herbivory begins to constrain fixation in escape plants (purple line) and further promote fixation in defence plants (red line).* At very high herbivory, I would expect fixation rates to be at zero, regardless of the relationship between herbivory and fixation, as plants will lose too much leaf carbon to be able to pay their mutualistic bacteria (see all lines).

Therefore, any effect of herbivory on fixation will be governed by two factors. First, soil nitrogen availability. In high nitrogen conditions, fixers utilise nitrogen from the soil and fix at low rates (Batterman *et al.*, 2013c), and so herbivory will have little effect on fixation as increased nitrogen demand after herbivory will be met by taking up more nitrogen from the soil (green line in Fig. 3.1) (Barron *et al.*, 2011; Batterman *et al.*, 2013c). In other words, I would only expect herbivory to affect fixation when soil nitrogen is low (purple lines in Fig. 3.1). Secondly, the amount of leaf area lost to herbivory will also govern the effect on fixation. Most herbivory in the tropics is carried out by insects that do not remove the whole leaf, but the quantity of leaf area lost to herbivory can vary widely across individuals of the same species (Kitajima and Poorter, 2010; Kitajima *et al.*, 2012; Becerra, 2015; Endara *et al.*, 2018). For fixers, very high herbivory may reduce plant carbon stocks to the point that they can no longer exchange carbon in return for nitrogen with their symbiotic bacteria, whilst moderate herbivory may not drastically effect carbon stocks but could reduce nitrogen levels and therefore stimulate fixation (Batterman *et al.*, 2013c, 2019). These two factors will likely interact to govern the effect of herbivory on fixation.

Alongside soil nitrogen availability and leaf area lost to herbivory, the relationship between herbivory and fixation will also depend on the strategy used by a given plant species to deter herbivores. Plant species and their associated herbivores comprise more than half of all tropical organisms and multiple, often complementary theoretical frameworks have been expounded to explain general patterns in plant-herbivore interactions (Ehrlich and Raven, 1964; Feeny, 1976; Coley *et al.*, 1985, 2006; Fine *et al.*, 2006; Kursar *et al.*, 2009; Bixenmann *et al.*, 2016; Endara *et al.*, 2017). I here focus on one theory best suited to my study genus, *Inga*; a trade-off between escape and defence in leaf development. Defence plants favour nitrogen-rich leaves that expand at slower rates and utilise a wide suite of chemical defences (Kursar and Coley, 1991; Coley *et al.*, 2006; Kursar *et al.*, 2009). Escape plants tend to produce nitrogen poor leaves that expand rapidly to avoid detection during the vulnerable early stages of leaf development. The strategy utilized by a given species is likely to mediate the response of nitrogen fixation to high herbivory. Defence species may upregulate fixation to produce toxic nitrogen-based defence compounds or to maintain high leaf nitrogen concentrations (red line in Fig. 3.1), whilst fixation may be constrained by herbivory in escape plants, which prioritise carbon for investment into rapid leaf expansion and have nitrogen poor leaves (as shown by the purple line in Fig. 3.1) (Kursar *et al.*, 2009; Wink, 2013). Therefore, defence species may have a greater nitrogen demand, promoting fixation in response to herbivory, whilst escape plants may have a greater carbon demand, suppressing fixation following herbivory damage.

I here test three hypotheses, first, that herbivory governs fixation and leads to either increased or reduced fixation rates, second, that this effect is mediated by the amount of leaf area (and thus leaf carbon and nitrogen) lost to herbivory and soil nitrogen availability, and third, that the antiherbivore strategy used by a species will determine its fixation response to herbivory (see Fig 3.1). To test the first two hypotheses, I used a greenhouse experiment, growing 99 potted seedlings of the neotropical species *Inga bella* across a gradient of soil nitrogen and simulated herbivory damage. To further investigate the first hypothesis and to examine the third hypothesis, I used a separate experiment where I grew

100 seedlings from *Inga* species previously found to specialize in one of two anti-herbivory strategies, escape (*Inga alba* and *Inga laurina*) or defence (*Inga vera* and *Inga spectabilis*) across two simulated herbivory treatments. I find clear evidence that herbivory generally upregulates nitrogen fixation rates, but the degree of which depends on the level of herbivory, soil nitrogen availability and the species antiherbivore strategy. My findings identify a key mechanistic role for herbivory in governing tropical nitrogen fixation with likely consequences for the terrestrial tropical carbon sink.

3.3 Methods

I carried out two experiments to assess the effect of herbivory on the nitrogen fixation rates of individual seedlings. The first examined the role of soil nitrogen availability and herbivory level in governing the herbivory effects on fixation and the second investigated differences in the effect of herbivory on fixation across antiherbivore strategies.

Experimental set up for both experiments

The experiments took place in Gamboa at the Santa Cruz Greenhouse facility in Panama (mean annual temperature: 26.5 °C, mean annual precipitation: 2600mm, Latitude: 9° 06' 60.00" N, Longitude: -79° 41' 59.99" W (Leigh, 1999)) during the rainy season, from September to January (Gallery *et al.*, 2010; Trierweiler *et al.*, 2018) in 2017 to 2018 and then 2018 to 2019. I collected seed from *I. bella* trees located in Fort Sherman, Colon (Latitude: 9° 21' 46.87" N, Longitude: -79° 57' 10.93" W), *I. spectabilis* in Gamboa, Colon (Latitude: 9.1165° N, Longitude: 79.6965° W) , *I. laurina* in El Valle de Anton, Coclé (Latitude: 8.6098° N, Longitude: 80.1317° W) and *I. vera* in Panama City, Panama (Latitude 8.9824° N, Longitude: 79.5199° W) which I then germinated in the shade-house using a well mixed 50:50 sterilized sand and low nutrient soil (Rubrik's Soil) mix in 2.5 L pots. I placed paper towel at the bottom of each pot to prevent sand being washed out during watering. After 10 days, I moved similar sized seedlings to an open sided greenhouse, with consistent light levels across the growing space, for 12 weeks.

To ensure that the appropriate symbionts were present in the soil, I used an inoculum of nodules collected from parent trees of the same species. After eight weeks, I selected one seedling from each treatment to confirm nodulation and then discounted it from the experiment. I also applied a Hoagland's solution weekly, at a molar concentration of 1mM phosphorus, 4mM potassium, 0.06mM magnesium, 0.06mM boric acid, 0.06mM manganese, 0.06mM zinc, 0.06mM copper, 0.06mM calcium, 0.06 mM iron and 0.06mM molybdenum, to ensure that each seedling had all necessary macro and micro nutrients. I watered plants at least once a day using deionized water to avoid water limitation.

Treatments for the soil nitrogen availability and herbivory level experiment

For this experiment, I grew 99 *Inga bella* seedlings across a gradient of soil nitrogen and herbivory damage. I subjected groups of ten or 11 seedlings to six factorial treatments, combining two variables: soil nitrogen content and simulated herbivory level (see A7: Supplemental Table 1). Every week, each pot received either no nitrogen fertilizer (0N), a 3µM nitrogen solution (+N) or a 10µM nitrogen solution (++N), consisting of deionized water and ammonium nitrate. For the simulated herbivory treatments, I either removed no leaf area (0H), 8.5% of leaf area (+H) or 20% of leaf area (++H) from all existing leaves and every new, fully-expanded leaf that was produced over the course of the experiment. These percentages reflect my measurements of the average and extreme herbivory damage for *Inga* seedlings during the wet season in a nearby forest (see chapter one). Leaf area was removed by first measuring the total leaf area using a cm^2 grid and then using scissors to remove the given percentage without cutting the midvein, to prevent amplification of damage to each leaf. I ensured the pots were evenly spaced and organized into three blocks, each containing 33 seedlings, and rotated the pots within each block every 18 days.

Treatments for the antiherbivore strategy experiment

For this experiment, I grew 100 seedlings from *Inga* species previously found to specialize in one of two antiherbivore strategies, escape (*I. alba* and *I. laurina*) or defence (*I. vera* and *I. spectabilis*), under two herbivory treatments (*I. alba*, *I. laurina* and *I. vera* have all been categorized previously as either defence or escape strategists by Kursar et al (2009), *I. spectabilis* is believed to be a defence specialist based on similarity in defensive attributes and leaf morphology to known defence species). I removed either 0% (control) or 20% of leaf area (see A7: Supplemental Table 2). Leaf area was removed as in the soil nitrogen and herbivory level experiment. Additionally, for one species from each antiherbivore strategy group I removed 20% and applied a 100 µM MeJa treatment, dissolved in a 2.5% ethanol solution. MeJa is a plant hormone typically produced in response to herbivory and elicits antiherbivore responses. Previous work has shown that MeJa can better simulate herbivory for many plant species (Toby Kiers *et al.*, 2010; Huang *et al.*, 2015). I removed plants undergoing the MeJa treatment from the greenhouse, sprayed them in a separate location and only returned them to the greenhouse once their leaves had dried to prevent transfer to non-MeJa treatment plants. To ensure a constant application, I sprayed leaves with 8.5ml per treatment. All other seedlings were sprayed with a control of 2.5% ethanol solution. I organized seedlings into 10 blocks by treatment each containing 10 seedlings, to prevent Methyl-Jasmonate application from affecting seedlings from other treatment blocks. I rotated the ten blocks randomly every week and all seedlings grew for 10-14 weeks.

Harvesting and data collection:

For both experiments, I harvested the plants before they became pot bound at 10-14 weeks. I then scanned leaves using a portable document scanner (TT-DS001, Taotronics, Shenzen, China). The open source program ImageJ was then used to calculate leaf area and total leaf area which included area lost to herbivory treatment for seedlings in the first experiment (Schneider *et al.*, 2012). Total leaf area was measured using ImageJ to manually draw in the missing edge of the leaf in seedlings that underwent herbivory treatments before measuring leaf area including the missing portion. This allowed us to account for area removed in experimental treatments in my final measurements of leaf area.

To measure fixation rates for each seedling I conducted Acetylene Reduction Assays. The fixation of acetylene to ethylene by the nitrogenase enzyme can be used as a proxy for the fixation of di-nitrogen to nitrogen in order to quantify fixation rates. For each plant, three nodule bearing root segments were each placed into separate glass jars with a known volume of acetylene. Samples were taken from each jar after 15 and then 30 minutes. I also used a control jar for each seedling, which contained root segments without nodules. Ethylene concentration in the samples was then measured using gas chromatography with a modified Agilent 7890A gas chromatograph (the methanizer was uninstalled for the analysis) with a Carboxen-1006 PLOT 30 m x 0.53 mm I.D column installed and a Flame Ionization Detector. I used a porapack backflush column, helium as a carrier gas, an oven temperature of 165°C, an injection temperature of 230°C and a loop size of 10 µL. To create a standardized curve, I used a sequential dilution of pure ethylene ($R^2 = 0.99$). Integrated results were imported as chromatographs from Chemstation (version E.02.02.1431, Agilent, Santa Clara, USA) and the peak area was integrated manually for each file using Chromeleon V.6.8 (Thermo Fisher Scientific, Waltham, USA). Fixation rate was represented as µmol acetylene fixed per gram nodule dry biomass per hour (µmol g^{-1} $hour^{-1}$).

For biomass determination, I separated plants into leaves, stems, nodules and roots and dried samples to constant weight at 60°C before I weighed them using an analytical balance (sensitive to 0.0001g) for the leaves, stems and roots and a semi-microbalance for the nodules (sensitive to 0.00001g). I analysed dried leaf samples for total nitrogen and carbon content using an elemental analyser (Elementar Varid microQ, Elementar, Langensbold, Germany). In addition to measurements outlined above, I derived ratios of nodule biomass:total biomass and aboveground biomass:belowground biomass, Leaf Mass per Area (LMA) (g $cm^{2\ -1}$) and total plant fixation rates for each plant (plant fixation rate = average nodule fixation rate (µmol g^{-1} $hour^{-1}$)*total nodule biomass (g)).

Statistical analysis:

To determine the effect of treatments and antiherbivore strategy on biomass, fixation rate and foliar nutrient concentrations I ran two-way ANOVA's using R. Linear models or linear mixed effect models were used in lme4, depending on whether the random effect of species improved the model fit (Bates *et al.*, 2014). Models in both experiments were run with and without interactions between the two variables (nitrogen and herbivory treatment in experiment one, herbivory treatment and antiherbivore strategy in experiment two). Residual plots (using the DHARMa package) and Akaike Information Criterion (AIC) values were used to determine which model best fit the data (Hartig, 2019). To account for variation in sample sizes, I ran Type III ANOVA models using the Anova function from the car package on lmer model objects and then the lsmeans package was used to run back adjusted Tukey tests for variables identified as having an effect in the Anova (Lenth, 2016; Fox and Weisberg, 2019). Total plant fixation rate was log transformed to meet assumptions of normality. For response variables that did not meet assumptions of normality even after transformation (nodule biomass in both experiments and fixation rate in the antiherbivore experiment), compound Poisson linear regressions were run using the cplm package to account for zero inflation. In cplm models, the explanatory variables were relevelled as factors to allow for comparisons between each level of each variable.

3.4 Results

Soil nitrogen availability and herbivory level

In my first experiment, nitrogen and herbivory treatments had distinct effects on seedlings. Nitrogen fertilization promoted seedling growth but suppressed nodulation and fixation rates. Herbivory treatments drove compensatory leaf production, shown by increases in leaf area but reduced LMA, and led to an increase in total plant fixation rate at lower herbivory levels.

Effects of nitrogen treatment

Nitrogen promoted biomass production. Plants receiving both moderate nitrogen fertilization (3µM, represented as +N) and high nitrogen fertilization treatments (10µM, ++N) had approximately 25% more leaf biomass (difference between 0N and +N: T = 3.1, p = < 0.05. Difference between 0N and ++N: T = 3.2, p = <0.05, see Fig. 3.2a) and 22% more leaf area under high nitrogen than those receiving no nitrogen treatment (0N) (difference between 0N and ++N: T = 2.9, p = <0.05, see Fig. 3.2b). Total leaf area (accounting for leaf area removed in herbivory treatments) increased only under the 10µM fertilization treatment, by 21% (difference between 0N and ++N: T = 2.9, p = <0.05, see A8: Supplemental Fig. 2b). Stem biomass (ANOVA: p = <0.05) and the ratio of aboveground to belowground biomass (difference between 0N and +N: T = 5.1, p = < 0.0001. difference between 0N and ++N: T = 3.5, p = <0.05) were also higher after both fertilization treatments (see A8: Supplemental Figs. 1b and 1d). Seedlings undergoing fertilization had 2% more foliar carbon at high nitrogen levels (difference between 0N and ++N: T = 3.3, p = <0.05), 18% more foliar nitrogen after moderate nitrogen addition and 21% more after high nitrogen addition (difference between 0N and +N: T = 2.8, p = < 0.05. Difference between 0N and ++N: T = 10, p = <0.0001. Difference between +N and ++N: T = 7.4, p = <0.0001, see

A8: Supplemental Fig. 4a and 4b). Whilst nitrogen promoted biomass growth, it constrained investment in fixation. Fertilized seedlings had 48% less nodule biomass with 3µM nitrogen addition and 98% less with 10µM nitrogen addition (difference between 0N and +N: T = -2.3, p = < 0.05. Difference between 0N and ++N: T = -4.52, p = <0.0001. Difference between +N and ++N: T = -2.34, p = <0.05, see A8: Supplemental Fig. 3a). Moderate and high nitrogen treatments caused seedlings to reduce the ratio of nodule biomass to total biomass by 57% and a 98%, respectively (difference between 0N and +N: T = -3.2, p = < 0.05. Difference between 0N and ++N: T = -5.4, p = <0.0001, see Fig. 3.3a). Finally, nitrogen addition also suppressed total plant fixation rates by 71% at moderate levels of nitrogen fertilization and 97% at high levels of nitrogen fertilization (ANOVA: p = <0.05, see Fig. 3.3b).

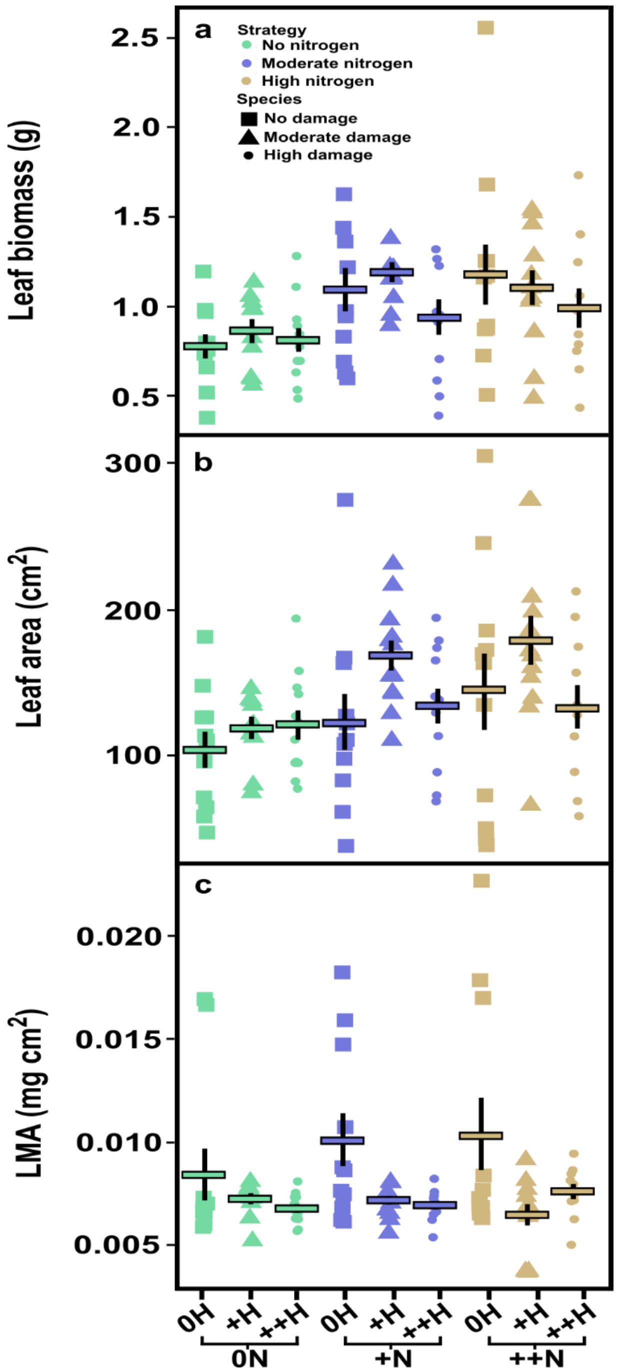


Figure 3. 2. The effect of each herbivory level and nitrogen availability on leaf biomass (a), leaf area (b) and LMA (c). *No nitrogen addition is shown as 0N (in green), the addition of a 3μM nitrogen solution is represented by +N (in blue) and the 10μM nitrogen solution is represented by ++N (in gold). Seedlings that underwent no simulated herbivory treatment are denoted as 0H (squares), those from which I removed 8.5% of leaf area as +H (triangles) and those which lost 20% of leaf area as ++H (circles). Bars represent the mean with standard error for each treatment combination.*

Effects of herbivory treatment

Herbivory drove compensatory leaf production and higher fixation rates, but the effects of herbivory varied with the percentage of leaf area removed. Compared to plants which underwent no herbivory damage (0H), seedlings losing 8.5% of leaf area (+H) ultimately produced 22% more leaf area (ANOVA: p = 0.05, see Fig. 3.2b) and 23% more total leaf area at harvest (difference between 0H and +H: T = 2.9, p = <0.05, see A8: Supplemental Fig. 2b). In addition, plants exhibited a 35% lower LMA when losing 8.5% of leaf area and 28% lower LMA when losing 20% of leaf area (++H) in comparison with undamaged seedlings (difference between 0H and +H: T = -4.2, p = <0.05. Difference between 0H and +H: T = 4.1, p = <0.05, see Fig. 3.2c), although this difference was driven only by eight plants, all under low herbivory treatments, but found across all nitrogen fertilization treatments (when outliers were removed there was no difference between 0H and +H: T = 0.4, p = >0.05. Difference between 0H and +H: T = 0.2, p = >0.05, see Fig. 3.2c). Accordingly, plants under both herbivory treatments had lower ratios of aboveground to belowground biomass (ANOVA: p = < 0.05, see A8: Supplemental Fig. 1c).

Herbivory had no effect on leaf carbon or nitrogen concentrations (see A8: Supplemental Figs. 4a and 4b) but seedlings in the moderate herbivory treatment showed total plant fixation rates 89% higher than that of undamaged seedlings (ANOVA: $p = < 0.05$, see Fig. 3.3b). This higher fixation rate was observed in half of the plants subjected to no nitrogen fertilization and 8.5% leaf area removal, whilst the other half showed no upregulation in fixation despite undergoing the same treatment.

Antiherbivore strategy experiment

In my second experiment, I identified reduced growth for individuals undergoing herbivory treatments, with nodulation and fixation rate varying across herbivory treatments and antiherbivore strategies. Defence specialists tended to be larger but fixed less nitrogen when not under herbivory treatments. Interestingly, herbivory reduced nodule biomass in both defence and escape specialists but led to upregulated fixation rates per unit nodule mass in defence seedlings.

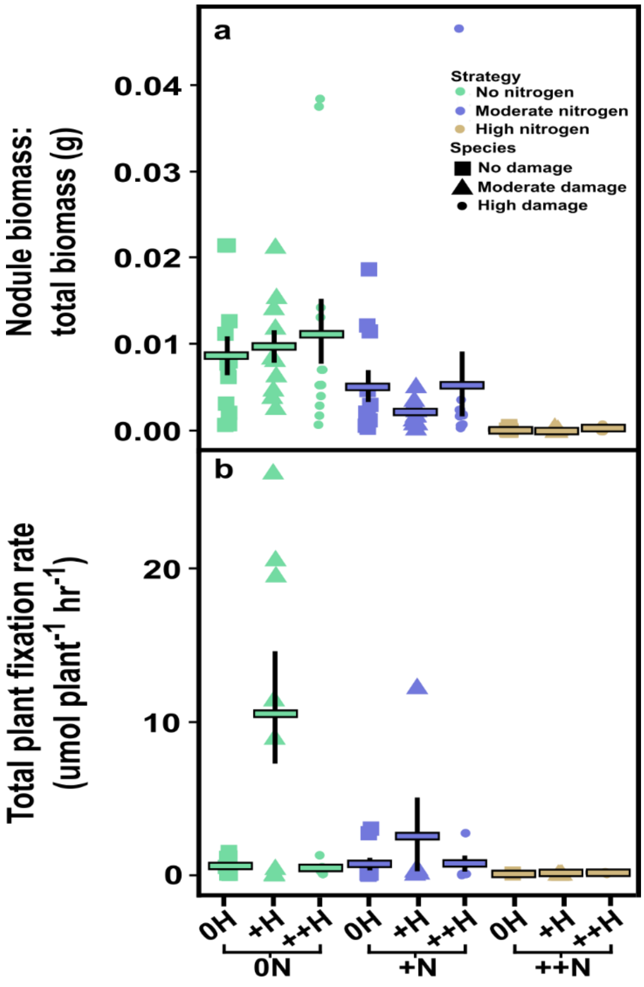


***Figure 3. 3**. **The effect of each herbivory level and nitrogen availability on the ratio of nodule biomass to total biomass (a) and total plant fixation rate (b).** No nitrogen addition is shown as 0N (in green), the addition of a 3µM nitrogen solution is represented by +N (in blue) and the 10µM nitrogen solution is represented by ++N (in gold). Seedlings that underwent no simulated herbivory treatment are denoted as 0H (squares), those from which I removed 8.5% of leaf area as +H (triangles) and those which lost 20% of leaf area as ++H (circles). Bars represent the mean with standard error for each treatment combination.*

Effects of herbivory treatment

Herbivory treatments suppressed growth and biomass accumulation. Plants undergoing either 20% leaf area removal or leaf damage in combination with MeJa application had lower total biomass ($T = -5.1$, $p = < 0.0001$ and $T = -4.4$, $p = < 0.05$, respectively, see Fig. 3.4), leaf biomass ($T = -4$, $p = < 0.0001$ and $T = 4.1$, $p =$

<0.05, respectively, see A8: Supplemental Fig. 6a), leaf area (T = -3.6, p = <0.05 and T = -3.7, p = <0.05, respectively, see A8: Supplemental Fig. 6b), LMA (ANOVA: p = <0.05, see Fig. 6c) and root biomass compared to undamaged plants (T = -3.1, p = <0.05 and T = -2.6, p = <0.05, respectively, See A8: Supplemental Fig. 5b). Two variables were reduced in seedlings following leaf area removal alone: Stem biomass (T = -4.1, p = <0.05, see A8: Supplemental Fig. 5a) and the ratio of above ground to below ground biomass (T = -3.3, p = <0.5, see A8: Supplemental Fig. 5c). These results were largely consistent across my study species.

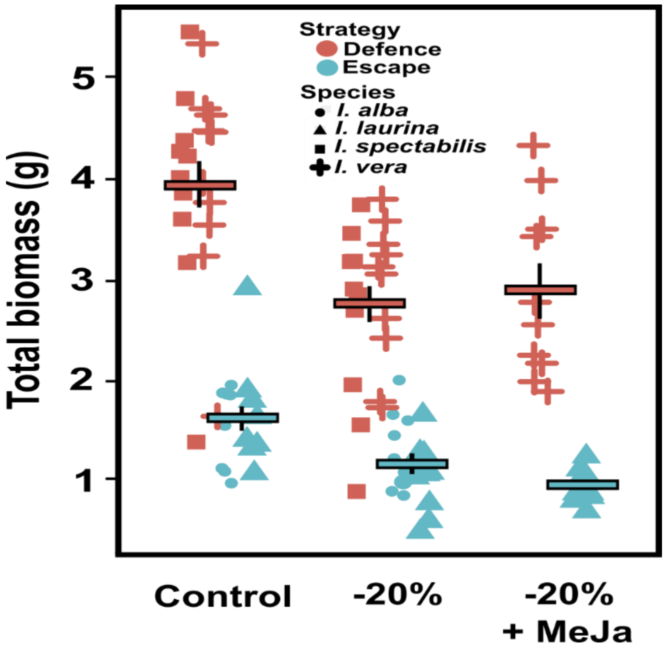


***Figure 3. 4**. The effect of herbivory on total biomass for escape and defence specialists.*
Antiherbivore strategy for each seedling is represented by colour, with defence specialists in red and escape specialists in blue. Individual seedlings from each species are represented by shape with I. alba shown as circles, I. laurina represented by triangles, I. spectabilis represented by squares and I. vera represented by crosses. Bars represent the mean with standard error for each treatment combination.

For *I. laurina* plants undergoing herbivory treatments with MeJa application, total nodule biomass was 87% lower than that of control plants (relevelled compound Poisson regression: T = -4.42, p = <0.001, see Fig 3.5a). However, no change was detected in the ratio of nodule biomass to total plant biomass for any given species (see A8: Supplemental Fig. 7a). Neither fixation rate, foliar carbon or foliar nitrogen varied across herbivory treatments (see A8: Supplemental Figs. 7b, 8a and 8b). However, total plant fixation rate was affected by treatment in interaction with antiherbivore strategy (ANOVA: p = <0.05, see below for pairwise comparisons, see Fig. 3.5b).

Effects of antiherbivore strategy:

Only seedling size and leaf nitrogen concentrations were dependent on antiherbivore strategy. Both defence specialist species were larger across all herbivory treatments (T = 1.9, p = < 0.001, see Fig. 3.4), with more biomass in leaves (T = 0.82, p = < 0.001, see A8: Supplemental Fig. 6a), stems (T = 1.1, p = < 0.001, see A8: Supplemental Fig. 5a) and roots (T = 0.9, p = < 0.001, see A8: Supplemental Fig. 5b), and greater leaf area (T = 1.2, p = < 0.001, see A8: Supplemental Fig. 6b), although one escape species, *I. laurina*, showed greater LMA than the defence specialists (T = -0.002, p = < 0.001, see A8: Supplemental Fig. 6c). Defence and escape species did not differ in terms of their investment in nodulation nor in the ratio of above-ground biomass to below-ground biomass (see Fig 3.5a and A8: Supplemental Fig. 5c). Foliar carbon did not differ between defence and escape specialists, but defence species had 6% more foliar nitrogen (ANOVA: p = < 0.05, see A8: Supplemental Figs. 8a and 8b).

Effects of the interaction between herbivory treatment and antiherbivore strategy

Fixation rates did not differ between antiherbivore strategies, but total plant fixation rate was the only variable showing a significant interaction between herbivory treatment and antiherbivore strategy (p = < 0.05). In particular the defence specialist *I. vera* had much higher total plant fixation rates following leaf area removal and MeJa treatment, with a value 32% greater than escape specialists (T = 2.9, p = < 0.05, see Fig. 3.5b). The defence specialist *I. spectabilis* also appeared to fix nitrogen at faster rates following leaf removal without MeJa application, but this difference was not significant.

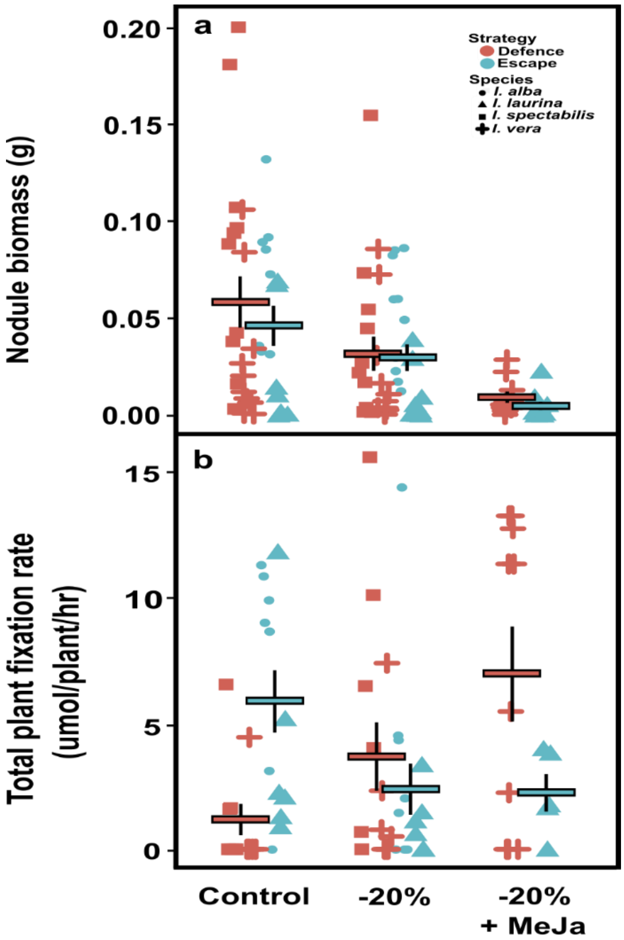


Figure 3. 5. The effect of herbivory on the ratio of nodule biomass to total biomass (a) and total plant fixation rate (b) for defence and escape specialists. *Antiherbivore strategy for each seedling is represented by colour, with defence specialists in red and escape specialists in blue. Individual seedlings from each species are represented by shape with I. alba shown as circles, I. laurina represented by triangles, I. spectabilis represented by squares and I. vera represented by crosses. Bars represent the mean with standard error for each treatment combination*

3.5 Discussion

Overall, my findings support each of my three hypotheses. First, that fixation rates are governed by herbivory, second, that this relationship in mediated by soil nitrogen availability and level of herbivory and third, that fixation response to herbivory depends on antiherbivore strategy. In support of my first hypothesis, I found that herbivory governed fixation, and that fixation rates were generally higher following herbivory. My results suggest that fixers upregulated fixation after herbivory to replace lost leaf nitrogen and indicate that the loss of leaf nitrogen represents a greater cost to fixer seedlings than the loss of carbon in nitrogen limited, high light conditions (as in my greenhouse experiments). Fixers have been found to downregulate fixation in low light conditions (Taylor and Menge, 2018), and therefore herbivory may lead to lower fixation rates when light, and therefore carbon, is limiting. Interestingly, across both experiments, whole plant fixation rates changed in response to herbivory regardless of nodule biomass. This indicates that fixers regulate fixation rates plasticly within each nodule in response to herbivory, rather than by increasing or reducing the number of nodules.

Having established that herbivory governs fixation, I next investigated the role of soil nitrogen availability and herbivory level in mediating the herbivory-fixation relationship. As expected, I found that soil nitrogen most strongly regulates fixation, but that herbivory also governs fixation rates when soil nitrogen is low. In nitrogen-limited conditions, moderate herbivory damage stimulated nitrogen fixation, but higher levels of damage suppressed fixation rates (see solid purple line in Fig. 3.1). Leaf nitrogen lost to moderate herbivory likely increased nitrogen demand and fixation rates, to allow for compensatory growth in the case of *I. bella* (seedlings of this species produced greater leaf area under herbivory) (Garcia and Eubanks, 2018). At higher levels of herbivory, the loss of carbon-rich leaf tissue likely caused carbon demand to exceed nitrogen demand, leading fixers to downregulate the amount of carbon exchanged to nitrogen-fixing bacteria despite increasing requirements for nitrogen (Batterman *et al.*, 2013c, 2019). These findings support my second hypothesis, that the level of herbivory damage and soil nitrogen availability interact to determine the herbivory effect on fixation.

Interestingly, despite higher average fixation rates under moderate herbivory and low soil nitrogen, I saw two distinct fixation responses to leaf area removal. Half of the seedlings upregulated fixation, whilst the other half showed no increase, as in other treatment combinations. This observation raises the question of what else may downregulate or prevent fixation when seedlings are undergoing herbivory. One possibility is that there were low quantities of symbiotic bacteria in our inoculum which was harvested from adult Inga trees, but not adult trees specifically of *Inga bella*. Alternatively, our inoculum may have contained a combination of symbiotic and ‘cheater’ bacteria that form nodules but do not actively fix atmospheric nitrogen (Kiers *et al.*, 2003; Porter and Simms, 2014). Low counts of symbiotic bacteria present, or competition between fixing and cheating bacteria in some pots could have prevented upregulation of fixation under herbivory in specific seedlings. That I found much higher nodulation across all species in our second experiment, in which inoculum was collected directly from the base of the same adult trees from which seeds were harvested, supports this idea. However, as all seedlings received the same inoculum, that I found upregulated fixation only in seedlings growing under low nitrogen and medium herbivory still offers some support for my hypotheses.

I next assessed how escape or defence strategies governed the effect of herbivory on fixation. As predicted, herbivory promoted fixation in defence plants only, suggesting that herbivory increases nitrogen demand in defence species and carbon demand in escape species. Defence strategists upregulated fixation despite showing no increase in leaf biomass or foliar nitrogen concentrations. Instead, fixation may have allowed production of nitrogen-based defence compounds, such as the non-protein amino acid tyrosine, which has been found to be induced by herbivory in *Inga* species (Bixenmann *et al.*, 2016). In escape plants, herbivory may increase demand for carbon more than nitrogen, as escape plants require carbon for leaf expansion, which could come at the detriment of paying carbon to nitrogen-fixing bacteria (Menge *et al.*, 2008; Batterman *et al.*, 2013c). These findings support my third hypothesis, that the antiherbivore strategy used by a species will determine its fixation response to herbivory and may help explain variation in fixation rates across fixer species. Average fixation rates across fixer species vary greatly, with *Inga* and *Tachigali* genera likely providing the majority of fixed nitrogen in secondary tropical forests (Batterman *et al.*, 2013b). Variation in the fixation response to herbivory, depending on species defence strategies, may explain why certain species fix more than others under high herbivory pressure for fixers (see chapter one).

Species are classified along a spectrum of defence-escape depending on their leaf expansion rates and use of chemical and biotic defences (Kursar *et al.*, 2009). *I. bella* was also the only species to show no reduction in biomass following herbivory, instead producing new compensatory leaves. This response cannot be explained by the theoretical framework of the escape-defence trade-off, but does offer support for an additional theory, the Resource Allocation Hypothesis (Coley *et al.*, 1985). The Resource Allocation Hypothesis proposes that slow growing species deter herbivores via investment in defence and fast-growing species tolerate herbivory via compensatory growth, as replacing lost leaf tissue costs less than defence investment for species with high growth rates (Coley *et al.*, 1985). The literature suggests that escape and defence specialists have comparable growth rates *(*1.4 cm dbh yr^{-1} for *I. laurina* and 1.56 cm dbh yr^{-1} for *I.vera* in adult trees (Turner *et al.*, 2018)) suggesting that growth rate does not co-vary with antiherbivore strategies. However, these two theoretical frameworks are not mutually exclusive and fixation responses to herbivory may vary with both growth rate and antiherbivore strategy. Future research should use theory to guide species selection and examine the relationship between herbivory and fixation under different hypothetical frameworks.

Alongside identifying a relationship between herbivory and fixation, my work also demonstrates the importance of methodology when simulating herbivory, especially for nitrogen fixers. MeJa application elicits antiherbivore responses and better simulates herbivory for many species (Toby Kiers *et al.*, 2010; Shahzad *et al.*, 2015). I found lower nodulation but higher fixation rates when combining leaf area removal and MeJa application than compared with leave removal alone, which suggests that fixers under herbivory produce fewer new nodules whilst upregulating fixation rates in existing nodules. I recommend that future work on herbivory in nitrogen-fixing species incorporates MeJa into simulated herbivory treatments.

Finally, I assess how my novel findings impact wider understanding of the role of nitrogen fixation in tropical nutrient cycling and carbon sequestration. Over 50% of tropical forests globally are recovering from either natural or anthropogenic disturbance (Chazdon *et al.*, 2009). Nitrogen fixing trees fulfil a critical function in these early stages of tropical forests growth by supplying an external source of nitrogen. This is particularly so during early succession when nitrogen fixation can double the rate at which tropical forests can sequester atmospheric carbon dioxide (Batterman *et al*., 2013a). Herbivory also fulfils an important biogeochemical role, as herbivores increases the rate of turnover of leaf nutrients to the soil and spread nutrients more evenly across the ecosystem (Metcalfe *et al*., 2014). My research reveals how these two major biogeochemical processes may interact and amplify each other. I identified a 10-fold increase in plant level fixation following herbivory for some species, meaning that herbivory may stimulate nitrogen input into tropical forests as well as accelerate the transport of this nitrogen to the soil. This finding has two key consequences of importance for tropical biogeochemistry. First, Global Vegetation Dynamics Models, the terrestrial component of Earth System Models, do not incorporate the interaction between herbivory and nitrogen fixation (Wieder *et al.*, 2015b), strongly suggesting that the role of both herbivory and of nitrogen fixation in tropical nutrient cycling is being underestimated. Second, as herbivory varies across tropical rainfall or soil nutrient gradients, the effects of herbivory on nitrogen fixation and thus the role of fixation are also likely to vary across tropical forests (Metcalfe *et al.*, 2014; Weissflog *et al.*, 2018; Kaspari, 2020). This may help to explain the large variation in fixation rates measured across the tropics. Further work is required to test these predictions about fixation at the ecosystem scale; first to test if my findings are consistent across all life history stages, second, to determine what proportion of fixer species upregulate fixation in response to herbivory, as a minority of my species downregulated fixation following herbivory damage, and third, to assess the effects of high herbivory on other factors that affect tropical nitrogen fixation, like fixer growth and abundance (i.e. the number of fixers that are likely to be fixing in a given forest).

To my knowledge, this is the first experiment to directly examine the effects of herbivory on nitrogen fixation. Extrapolation of my results suggest that herbivory is likely to play an important role in governing the tropical nitrogen cycle, with consequences for the tropical carbon sink. My findings that herbivory reduced fixer biomass or LMA regardless of upregulated fixation rates also suggest that high herbivory is a cost for fixers. Therefore, herbivory is an appropriate constraint on fixation in biogeochemical modelling in tropical forests that may explain why fixer abundances are capped at ~5-15% across the tropics (Ter Steege *et al.*, 2006; Hedin *et al.*, 2009). While the role of soil nitrogen in governing fixation has been well explored by previous research (Barron *et al.*, 2011; Batterman *et al.*, 2013b, 2013c), my study takes the first step to develop the mechanistic understanding of fixation in tropical forests beyond abiotic factors, demonstrating an important interaction between nitrogen fixation and herbivory. My findings therefore suggest that fixation is likely to be especially important in high herbivory tropical environments, and particularly so in forests recovering from anthropogenic disturbance.

Chapter 4:

Know thy neighbour: Herbivory contributes to greater negative density-dependent effects for fixer seedlings compared to non-fixers in a tropical forest

4.1 Abstract

A growing body of evidence suggests that periods of nitrogen limitation in tropical forests are more common than previously predicted, and that nitrogen-fixing trees can enhance forest growth when soil nitrogen is limiting. The recent observation of a high herbivory cost for fixer species suggests that herbivory may place a demographic constraint on the trait of fixation, but it remains unclear to what degree high herbivory affects the growth, survival and abundance of fixer seedlings. I here compare the growth, survival and strength of negative conspecific density-dependent effects for > 37,000 fixer and non-fixer seedlings, and, for the separate set of 350 seedlings analysed in my first chapter, I examine the role of herbivory in explaining differences between the two groups. I find that fixer seedlings have higher survival than non-fixers (0.4 +/- 1.4e-4 versus 0.26 +/- 1.3 +/- 1.3e-5, respectively), but that fixers suffered greater conspecific density-dependent effects across a range of scales on both growth (-0.63 +/- 0.2 versus -0.04 +/- 0.01 over a 1m^2 area) and survival (-5.4 +/- 0.9 versus -0.47 +/- 0.04 over a 1m^2 area). Importantly, I identify high herbivory for fixers seedlings as a likely cause of strong negative density-dependent effects for fixers – herbivory on fixers was more common at high seedling densities (effect of density on herbivory: 0.29 +/- 0.15 for fixers versus -0.07 +/- 0.1 for non-fixers). My findings suggest that the benefits of fixation lead to higher survival for fixer seedlings, but that herbivory represents a significant cost associated with the trait at the population scale. This herbivory driven density-dependent cost is likely to constrain fixer seedling distributions, explain why fixer abundances appear to be capped in tropical forests and could limit the role of nitrogen fixation in forest recovery.

4.2 Introduction

Tropical forests make up the single largest proportion of the terrestrial carbon sink (Brienen *et al.*, 2015). A large body of research now suggests that, in spite of rising atmospheric CO_2, continued growth of this sink is limited by soil nutrients (Turner *et al.*, 2018; Brookshire *et al.*, 2019; Levy-Varon *et al.*, 2019; Wright, 2019). Other nutrient sources than from the soil can therefore mitigate limitation of the carbon sink and plant growth and photosynthesis is limited in particular by the availability of soil nitrogen. By providing a source of atmospheric nitrogen, nitrogen-fixing trees therefore underpin tropical forest growth and also benefit substantially from the trait of fixation, exhibiting enhanced growth rates compared to their non-fixing neighbours during periods of nitrogen limitation (Barron *et al.*, 2011; Batterman *et al.*, 2013b). However, given the advantages of fixation, it remains unclear why fixers are not more prevalent across tropical forests, with abundances capped at 1-7% across Asian mature forests and at 5-15% across the Neotropics (Ter Steege *et al.*, 2006; Hedin *et al.*, 2009; Gei *et al.*, 2018; Menge *et al.*, 2019). Previously, aims to understand the costs associated with fixation have focussed on the high energetic cost of breaking the triple bond of atmospheric dinitrogen (Menge *et al.*, 2008, 2010; Vance, 2008; Vitousek *et al.*, 2013). This difficulty entails a high carbon cost for plants supplying photosynthate to symbiotic bacteria in exchange for nitrogen (Vance, 2008). However, tropical fixers are able to downregulate fixation when nitrogen is readily available in the soil and so can avoid paying the energetic cost of fixation when it outweighs the benefits (Batterman *et al.*, 2013b, 2013c). Instead, recent evidence demonstrates that fixation could be constrained by a previously un-investigated cost: high levels of herbivory.

Fixer herbivory is *a priori* a credible constraint to fixer abundances as it constitutes a comparatively high carbon cost. Fixer seedlings undergo higher herbivory than non-fixers (see chapter two). This greater quantity of leaf area lost to herbivory for fixers comprises both a loss of structural leaf carbon and a photosynthetic opportunity cost, as the loss of photosynthetic area reduces the potential to assimilate carbon over time. Recent estimates suggest that these costs combined equal ~13.7% of annual net carbon sequestration capacity, versus 3.3% for deriving all leaf nitrogen from fixation (this fixation cost is likely overestimated, as fixers would be expected to derive some leaf nitrogen from the soil, see Table 2.1). Such a large herbivory cost at the seedling stage is likely to affect adult fixer tree abundance, as this period is a major bottleneck in the life history of tropical trees, but the mechanism by which herbivory could constrain fixer abundances remains unclear (Comita *et al.*, 2010; Eichhorn *et al.*, 2010; Forrister *et al.*, 2019).

I would expect the high herbivory cost associated with fixation to affect fixer abundances in two ways. First, high herbivory could limit fixer seedling growth and survival (Blundell *et al.*, 2001; Eichhorn *et al.*, 2010). The greater loss of leaf carbon to herbivory for fixer seedlings could limit the carbon available to each seedling for growth, and over time, cause fixers to be outcompeted in reaching the canopy by non-fixers undergoing less herbivory. Over time this could lead to increased mortality for fixers. High herbivory could also affect survival directly, for example by introducing pathogens to leaf tissue (Kursar and Coley, 2003; Schuldt *et al.*, 2017). By affecting either growth rate or survival for fixer seedlings, herbivory would prevent a proportion of fixer seedlings from reaching maturity and overall fixer abundances would be reduced, regardless of fixer distribution (Eichhorn *et al.*, 2010).

Second, high herbivory for fixers could also constrain fixer abundances in a spatial manner, by creating strong negative density-dependent effects (Comita *et al.*, 2010, 2014; Dyer *et al.*, 2010; Forrister *et al.*, 2019). Negative density-dependent effects arise when local neighbour density has an adverse effect on a given seedling's growth or survival (Janzen, 1970; Harms *et al.*, 2000; Terborgh, 2012). These effects can be driven by overall seedling density, as more seedlings compete for the limited resources in a given area, or by the number of seedlings of the same species – the conspecific seedling density (Comita *et al.*, 2010; Terborgh, 2012). Individuals surrounded by a higher number of conspecific neighbours have been previously found to exhibit lower growth and survival rates. The majority of research suggests that this negative effect is due to facilitating shared natural enemies such as herbivores that are specialised to target certain plant species, rather than due to intraspecific competition (Comita *et al.*, 2010; Dyer *et al.*, 2010; Bagchi *et al.*, 2014; Downey *et al.*, 2018; Forrister *et al.*, 2019). Fixer seedlings face high pressure from herbivores (see first chapter), and natural enemy pressure for any given seedling is expected to increase with conspecific density (Janzen, 1970; Harms *et al.*, 2000; Dyer *et al.*, 2010; Endara *et al.*, 2018). These effects may combine to drive higher attack rates for fixers with high numbers of conspecific neighbours, as densely packed fixers will be easier to locate and facilitate herbivore feeding and reproduction, and to produce high negative density-dependent effects for fixers relative to non-fixers. In tropical seedlings, susceptibility to negative density-dependent effects has been linked to species abundance, with more abundant species facing weaker negative density-dependent effects; however, no work has assessed if the same pattern translates to functional groups, such as fixers and non-fixers (Comita *et al.*, 2010).

I would expect a high herbivory cost to have a negative effect on the growth and survival of fixers, and/or to increase the strength of negative density-dependent effects for fixer seedlings, either of which could constrain fixer abundances and thus limit net nitrogen fixation at the ecosystem scale. Therefore, I here test three hypotheses. First, that tropical forest fixer seedlings have lower growth and survival than non-fixer seedlings; second, that these fixers undergo greater negative density-dependent effects than non-fixers; and, third, that observed differences in growth, survival and the strength of negative density-dependent effects between the two groups can be explained by higher herbivory for fixer species. To examine my hypotheses, I utilize data on the survival, growth and species identity for >37,000 seedlings recorded in the 50-ha plot on Barro Colorado Island, Panama from 2001 – 2014, and I also use the targeted herbivory data that I collected for analysis in my first chapter from 350 seedlings across the 50ha plot in 2017, for which growth and survival was then recorded in 2018. I find that fixer seedlings have higher survival than non-fixers, and yet that they also undergo stronger negative density-dependent effects. I identify herbivory as a likely cause for these stronger negative density-dependent effects for fixers. My research suggests that herbivory may constrain tropical nitrogen-fixing tree abundance and identifies, for the first time, how negative density dependence may affect functional groups differently in tropical forests, with consequences for tropical biogeochemical cycling and the continued growth of the tropical carbon sink.

4.3 Methods

Seedling and adult tree censuses

To compare growth rates, survival and the strength of negative density-dependent effects between fixers and non-fixers, I used data from the seedling census in the long term 50-ha plot on Barro Colorado Island, Panama (9°9′ N,79°51′ W), established in 2001 (Comita *et al.*, 2010) (the 50-ha plot was first established in 1980 (Condit, 1998)). The site has a mean annual rainfall of ~2600mm and exhibits a mean annual temperature of 27º C. Monthly means vary by 2ºC (Leigh, 1999). Lianas and other non-woody plants are also documented but I excluded these growth forms from my study (Comita *et al.*, 2010). For a list of species present in the census, and the number of seedlings recorded in 2001 from each species, see A12: Supplemental Table 1.

In the seedling census, seedlings are characterized as trees <1cm in basal diameter and with a stem height from 200-3000mm. Seedlings that surpassed 3000mm in height over this time period grew out of the census and were no longer monitored. First, I measured relative growth rate as the difference in the natural log of stem length between each census interval, divided by the number of days of the census interval (Hoffmann and Poorter, 2002). Negative growth rates were not included, as they most likely represented stems that had snapped due to browsing by large herbivores or debris falling from the canopy.

Second, I recorded seedling survival from 2001 to 2014. I considered any seedling present at the start of the census to have survived if it was still alive and measured at the end of the census interval in 2014 or if it had grown out of the size-class. Recording survival in this way is ecologically relevant as I were assessing if each seedling made it through the seedling to sapling bottleneck, or if it still had a chance to do so (Comita *et al.*, 2010; Eichhorn *et al.*, 2010).

Finally, I measured the conspecific, confunctional and overall seedling density for each seedling across the plot. Conspecific density was recorded as the number of seedlings of the same species as a given focal seedling, confunctional density as the number from the same functional group – fixer or non-fixer – and overall density as the total number of seedlings, each over a given area. The 50-ha plot is divided into 1,250 20m^2 plots, which each contain 16 evenly spaced 1m^2 plots. Therefore, I recorded conspecific, confunctional and overall density as the number of seedlings of the same species at two scales, in the 1m^2 plot and the 20m^2 plot in 2001. I also used the census of adult trees (>1 cm dbh) in the 50-ha plot, recorded every five years from 1985 - 2015, to determine adult tree density in each 20m^2 plot (Condit, 1998; Harms *et al.*, 2001; Condit *et al.*, 2019). I used the year 2000 adult tree census as this was the closest to the start of my seedling census in 2001. By incorporating adult tree density, I could also test for the effect of proximity to conspecific or heterospecific adult trees on seedling survival (as well as confunctional or heterofunctional trees). To ensure that my measurements of seedling density were able to capture any negative density-dependent effects on growth and survival, I also used an additional measurement of seedling and adult density in my analysis alongside the number of seedlings in a given $1m^2$ or $20m^2$ plot – the number of conspecific and heterospecific seedlings and the number of adult trees that were located within a 5m, 10m and 20m radius from each seedling – and compared between the two measurements. Importantly, this measure incorporated seedlings and adults that were neighbouring a given focal seedling but that were within a different $20m^2$ plot.

Herbivory measurements on seedlings

To determine if herbivory could be driving differences in growth, survival or the effect of conspecific neighbour density between fixers and non-fixers, I used the herbivory data collected from a subset of 184 fixer seedlings and 166 non-fixer seedlings in the 50-ha plot in 2017 for my analysis in chapter one. Sampled individuals represented 23 fixer species (identified from Sprent, 2009) and 20 non-fixer species. I then used the 2017 to 2018 seedling census interval to determine how much these seedlings had grown and how many had survived one year after the herbivory measurements were taken (since 2017, when herbivory on these seedlings was surveyed). Alongside growth and survival, I also utilized measurements of conspecific or total seedling density in the $1m^2$ and $20m^2$ plots – or, in other words, the number of overall seedlings and seedlings of the same species found in each plot in 2017. Incorporating measures of density allowed us to test for density-dependent effects on growth and survival and to see if herbivory had stronger negative effects on seedlings at high density sampled in 2017-2018.

To measure herbivory rate, I scanned five randomly selected leaves per seedling using a hand-held document scanner (TaoTronics) capable of taking scans with 1050 Dots per Inch (DPI). To quantify leaf area lost to herbivory I used the open source program ImageJ (Schneider *et al.*, 2012). I measured total leaf area (the original full leaf area) and actual leaf area (the leaf area with herbivory) and then used the difference between these values to calculate the leaf area lost. Where the edge of the leaf was damaged by herbivory, I used a complete leaf edge from the same leaf to estimate the undamaged leaf area. To allow analysis of the highly right-skewed and zero-inflated data (many leaves were not attacked) I used two measures of herbivory (Murphy *et al.*, 2016). First, I recorded a binary measure of the incidence of herbivory at the leaf scale, as either with herbivory damage (1) or without (0). When examining relationships between seedling growth rate and the incidence of herbivory, incidence was averaged across leaves per seedling to allow for seedling level analyses. Therefore, incidence of herbivory is here analysed at the leaf and at the seedling level. At the seedling level, incidence is not a binary measure but instead represents the proportion of attacked leaves per seedling. Second, I used a continuous measure of the proportion of leaf area lost to herbivory. I summed the proportion of leaf area lost to herbivory across sampled leaves for each seedling, as there were fewer seedlings with no damage across its sampled leaves than there were leaves with no damage (i.e. less zero values). Proportion of leaf area lost is therefore always analysed at the seedling level. See chapter one for a full description of herbivory methods.

Statistical analysis

To test for differences in growth, survival, and the strength of negative density-dependent effects between fixer and non-fixer seedlings, I used linear modelling approaches run with the R package lme4 (Bates *et al.*, 2014). I modelled growth rate by using a repeated measures linear mixed effects model with a gaussian distribution. In this model, growth rate for every seedling each year was included, with seedling identity as a random effect to control for repeated measurements from the same seedling, and was log transformed to meet assumptions of normality. For survival, I used binary logistic regression. In both models, I specified either fixation alone, to test for absolute differences in growth or survival between the two groups, or fixation, conspecific density, heterospecific density and an interaction between fixation and conspecific density as fixed effects, to test for differences in the effect of conspecific density between the two groups. I also included species identity as a random effect to control for variation in growth or survival between species and the 20m^2 plot identity to control for spatial autocorrelation in the response variables in all models (see Model 4.1 as an example). All variables were scaled by subtracting the mean and dividing by the standard deviation.

I ran the models three times, first using conspecific seedling density in the 1m^2 plot, then conspecific seedling density in the 20m^2 plot and finally conspecific adult density in the 20m^2 plot as fixed effect. I also ran models using the conspecific density of seedlings and adults within a 5m, 10m and 20m radius of each seedling and compared the results to the previous models which used the density within the 1m^2 and 20m^2 plots, to ensure that the correct measure of seedling density was used. To test for density-dependent effects driven by high densities of seedlings in the same functional group, all analyses were also repeated using confunctional density rather than conspecific.

Any observed negative effects of conspecific seedling density could actually be due to a total increase in overall seedling density, regardless of species identity, as greater overall seedling density is likely to increase competition. Therefore, I next re-ran my models using overall seedling density and compared then to my original conspecific density models using an anova, to ensure that conspecific density did explain more variance than seedling density overall. Finally, a large discrepancy in the range of conspecific densities at which fixer and non-fixer seedlings were found could bias differences in the estimates of negative density-dependent effects between the two groups (Detto *et al.*, 2019). To account for this, I re-ran my models once more using a dataset constrained to the maximum and minimum conspecific densities observed for fixer seedlings.

Model 4.1:

$$Growth\ rate\ or\ survival \sim fixation + conspecific\ density + heterospecific\ density \\ + fixation{:}conspecific\ density.$$

I next analysed my smaller dataset from 2017 for which I had herbivory measurements. Over the one-year census interval very few seedlings died, which prohibited comparisons of survival between the two groups. Instead, a non-parametric Wilcoxon rank test was used to determine if fixer seedlings survived more or less than non-fixers. For growth rate, I specified the proportion of leaf area lost to herbivory and stem length as fixed effects to test if herbivory affected growth rate while controlling for seedling size. I then ran models with proportion of leaf area lost to herbivory and stem length as fixed effects alongside either conspecific density, heterospecific density and an interaction between conspecific density and herbivory, or total seedling density alone and in interaction with herbivory, to test whether herbivory affected growth rate only at high conspecific or overall seedling densities (see Model 4.2 as an example). I next ran models using measures of herbivory as the response variable, either the incidence of herbivory or the proportion of leaf area lost to herbivory, and either conspecific density or total density as fixed effects, alone or in interaction with fixation, to determine if herbivory increased with measures of seedling density (see Model 4.3 as an example). Models were then repeated using confunctional density in place on conspecific density to test for the role of functional density in driving herbivory and effecting seedling growth and survival in interaction with herbivory. Again, across all models I included the 20m^2 plot and species identity as random effects and all variables were scaled by subtracting the mean and dividing by the standard deviation. All analysis was carried out in R version 4.02 (R Core Development Team, 2018).

Model 4.2:

$$\begin{aligned} Growth\, rate \sim\ & proporton\ of\ leaf\ area\ lost\ to\ herbivory + stem\ length \\ & + conspecific\ density + heterospecific\ density \\ & + proportion\ of\ leaf\ area\ lost\ to\ herbivory{:}\, conspecific\ density. \end{aligned}$$

Model 4.3:

$$\begin{aligned} Incidence\ or\ proportion\ of\ leaf\ area\ lost\ to\ herbivory \sim & fixation \\ & + conspecific\ density + heterospecific\ density \\ & + fixation{:}\, conspecific\ density. \end{aligned}$$

4.4 Results

I find that fixer seedlings survive more than non-fixers (see Fig. 4.1b; A10: Supplemental Table 1), but that fixers face much greater negative density dependent effect (see Figs. 4.2, 4.3; A10: Supplemental Tables 2, 3). These effects are in part driven by high herbivory for fixers species; the incidence of herbivory per seedling was greater for fixers at higher overall seedling density in the 20m^2 plot (see Fig. 4.4a, 4.6; A9: Supplemental Fig. 3; A11: Supplemental Table 11), and had stronger negative effects on growth rates for all seedlings growing at high conspecific seedling density in the 1m^2 plot (Fig. 4.5a; A11: Supplemental Table 2).

Differences in seedling growth rate and survival between fixers and non-fixers

There was no difference in seedling growth rate across the census between the two groups (see Fig. 4.1a; A10: Supplemental Table 1). Fixers exhibited higher survival than non-fixers (0.41 versus 0.26, respectively; see Fig. 4.1b; A10: Supplemental Table 1). However, when controlling for conspecific seedling density at the 1m^2 and 20m^2 plot scale, models showed that the trait of fixation had a negative effect on seedling survival (see Figs. 4.2b, 4.3b; A10: Supplemental Tables 2 and 3).

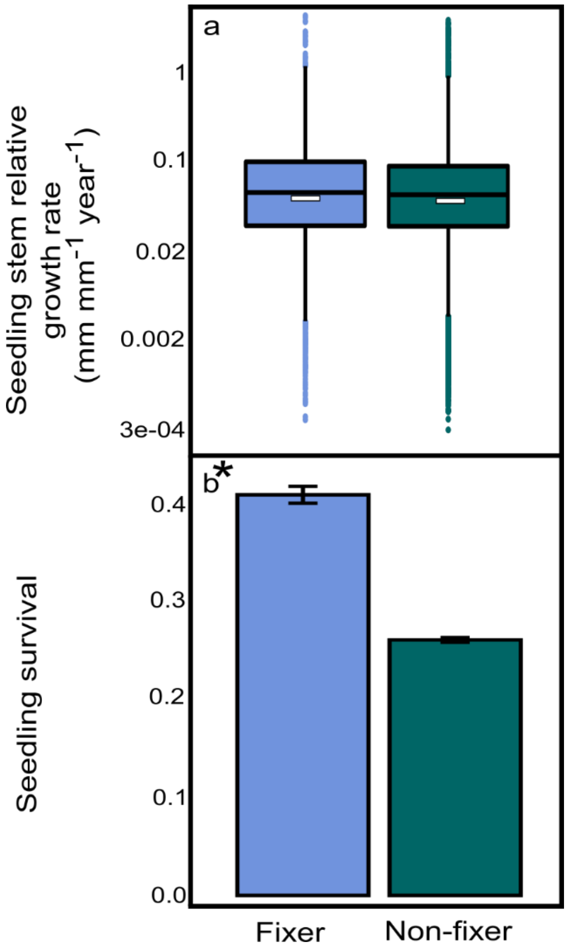


Figure 4. 1. The difference in stem growth rate (a) and seedling survival (b) between fixers and non-fixers from 2001-2014. *White bars in panel a represent mean values and error bars in panel b represent standard error. Seedling stem growth rate is shown on a log scale. Asterisks indicate a significant difference between the two groups in my linear modelling.*

The differences in density-dependent effects between fixers and non-fixers.

I consistently found stronger conspecific negative density-dependent effects on growth rate and survival for fixer seedlings when compared to non-fixers. When modelling growth rate, the negative effect sizes of conspecific seedling density for fixers were -0.63 and -0.39 in the 1m^2 and 20m^2 plot respectively, versus -0.04 and -0.03 for non-fixers. I found similar results for survival, where the negative effect size across the two plot sizes was -5.4 and -5.5, versus -0.47 and -0.01 for non-fixers (see Figs. 4.2, 4.3, 4.2 for effects on growth and survival at the 1m^2 scale; A9: Supplemental Fig. 2 for at the 20m^2 scale). Conspecific adult density reduced growth rates for all seedlings and had a very slight positive effect on seedling survival overall, with a stronger negative effect for fixer seedlings (See A10: Supplemental Tables 2, 3). Conspecific density also had greater negative effect on fixer survival when using the conspecific seedling density within a 5m, 10m or 20m radius of a focal seedling as explanatory variables, and on growth rate within a 5m radius. (see A10: Supplemental Tables 9 and 10). Conspecific adult density within a 5m radius negatively affected growth rate for all seedlings (see A10: Supplemental Tables 11 and 12). The proportion of variance explained by when using these radius models or when using density in the 1m^2 and 20m^2 plot was comparable (see A10: Supplemental Tables 9-12). Across all models, my models using conspecific and heterospecific density explained more of the variance in growth and survival than those using overall seedling density (i.e., they had lower AIC values than models with overall seedling density, see A10: Supplemental Table 6).

Alongside a greater negative effect of conspecific density, I found a stronger negative effect of confunctional density for fixer species. Fixer seedlings growing surrounded by a high density of other fixers, regardless of neighbour species identity, showed lower growth rates in the 1m^2 plot compared to non-fixers surrounded by other non-fixers (-0.51 versus -0.04, see A10: Supplemental Table 4). For survival, a high density of seedlings from the same functional group had a greater negative effect for fixers than non-fixers in both the 1m^2 and 20m^2 plot (-2.9 versus -0.32 in the 1m^2 plot, respectively, and -3.6 versus -0.14 in the 20m^2 plot, see A10: Supplemental Table 5). Again, models using confunctional density as explanatory variables performed better than models using overall seedling density (see A10: Supplemental Table 6).

Differences in negative density-dependent effects between fixers and non-fixers could be caused by discrepancies in the observed range of seedling densities between the two groups. The conspecific densities of fixer species ranged from 0 – 19 in the 1m^2 plot and 0 – 51 in the 20m^2 plot and were lower than the conspecific densities of non-fixer species, which ranged from 0 - 130 and 0 - 319 across both scales, respectively. However, when re-running my models using a dataset constrained to only include seedlings within the range observed for fixer species, I detected fixers had reduced but still stronger negative conspecific density-dependent effects compared to non-fixers across most scales. (see A9: Supplemental Fig. 3; A10: Supplemental Tables 7, 8). When using this constrained dataset, negative conspecific density-dependent effects on growth were still greater for fixer than non-fixer seedlings in the 1m^2 plot (-0.56 versus -0.11, respectively; see A10: Supplemental Table 7) and on survival in both the 1m^2 and 20m^2 plot (-3.5 versus -0.61, respectively, in the 1m^2 plot and -3.1 versus -0.61, respectively, in the 20m^2 plot; A10: Supplemental Table 8).

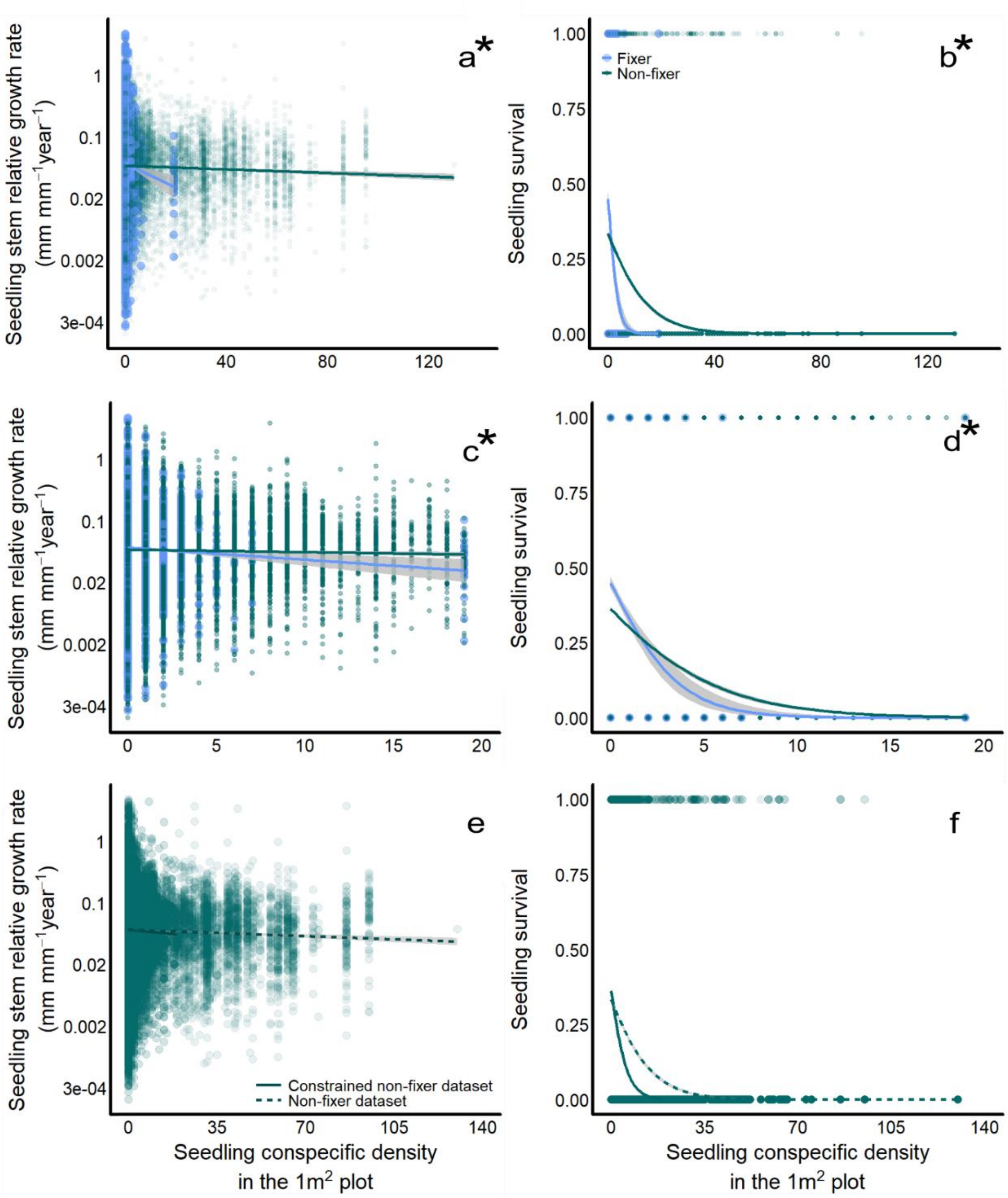


***Figure 4. 2*. The difference in the strength of negative density-dependent effects on growth and survival for fixer and non-fixer seedlings.** *Showing the relationship between conspecific density in the 1m^2 plot and seedling stem relative growth rate (panels a, c, e) and seedling survival (b, d, f) from 2001-2014 for fixers (blue) and non-fixers (green) across the whole dataset (a and b), the dataset when constrained to conspecific densities at which fixers were observed (c and d), and for non-fixers across both the whole dataset and the constrained dataset. Seedling stem relative growth rate is shown on the log scale. Lines are fitted using a linear model with a Gaussian distribution (a, c, e) and a general linear model with a binomial distribution (b, d, f) between the two variables in each panel. Grey shading on lines represents standard error and asterisks represent significant differences between the relationships for fixers and non-fixers from my mixed effects models including species and plot identity as random effects (no statistical comparison was made between lines in panels e and f).*

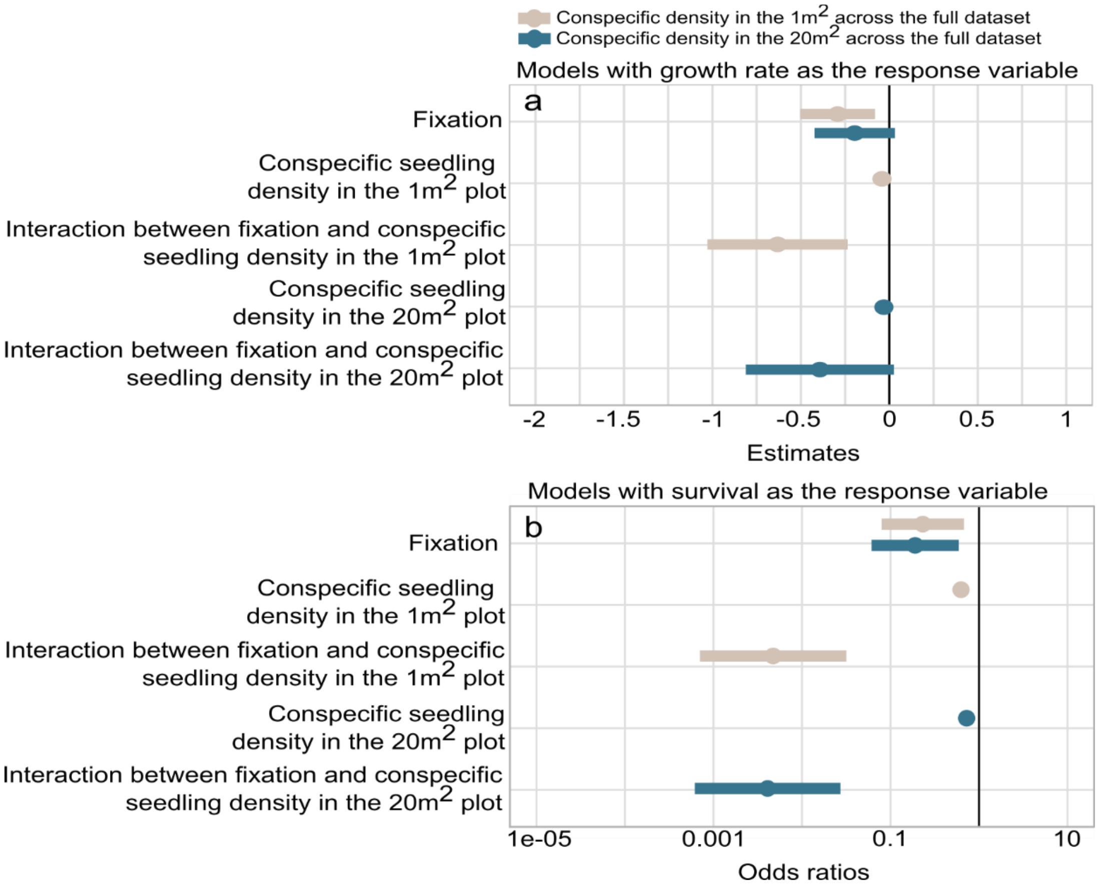


Figure 4. 3*. *The strength of negative density-dependent effects on survival and growth rate. *Coefficient estimates from models regressing seedling growth rate (a) and seedling survival (b) from 2001-2014 against either conspecific density in the 1m^2 plot or the 20m^2 plot. In panel a, the X axis represents changes in stem relative growth rate (in mm). The X axis is panel b is the log odds ratio of survival from my binomial regression (in other words, the odds of survival), where >1 represents an increased chance of survival and <1 represents a reduced chance of survival. The effect of conspecific density in interaction with fixation represents the negative conspecific density-dependent effect for fixers, whilst conspecific density alone represents the size of the effects for non-fixers. Models included the random effects of species identity and 20m^2 plot to control for variation across species and autocorrelation. The black line marks zero on the X axis.*

The role of herbivory in driving demographic traits

Some measures of herbivory drove differences in growth and the strength of negative density-dependent effects between fixers and non-fixers for seedlings from 2017-2018 (those seedlings sampled for my herbivory analysis in chapter one in 2017 and then censused in 2018). However, as 93% of seedlings survived, I was unable to reliably model differences in survival between the two groups. I found that herbivory was greater for fixer seedlings at higher density. The incidence of herbivory, a binary measure of whether a leaf had herbivory or not, was positively correlated with overall seedling density in the 20m^2 plot for fixer species. (see Fig. 4.4; Fig. 4.6a and 4.6b, A11: Supplemental Table 11). There was no effect of conspecific seedling density on the incidence of herbivory (see A11: Supplemental Table 7), and no effect of any measure of density on herbivory in the 1m^2 plot (see A11: Supplemental Tables 7-12). I then found that the incidence of herbivory at the seedling scale (the proportion of leaves with herbivory per seedling) reduced growth rates in a conspecific density-dependent manner in the 1m^2 plot for all seedlings (there was no density-dependent effect in the 20m^2 plot, see Fig. 4.5a; A11: Supplemental Table 2).

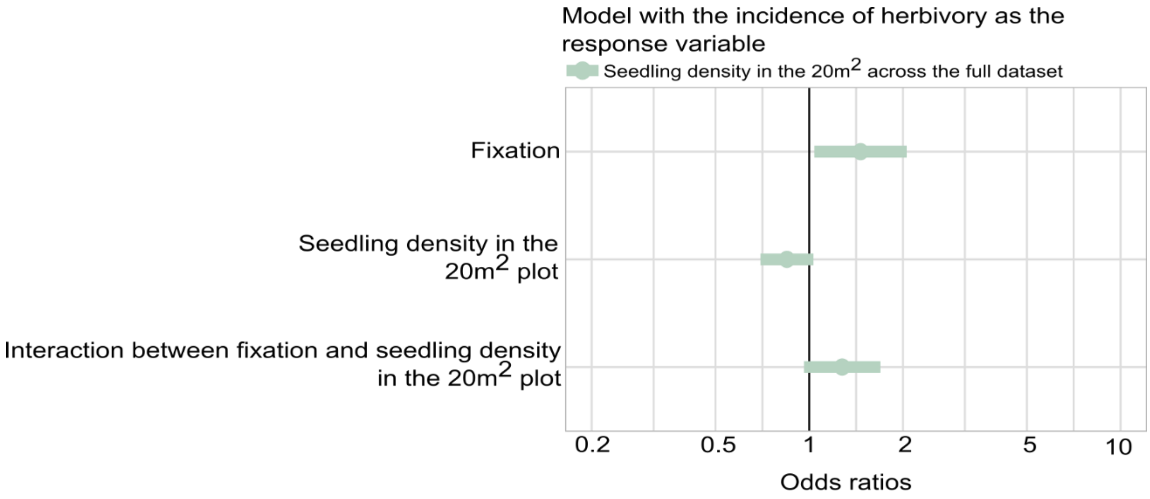


Figure 4. 4 **The coefficient estimates from regressing the incidence of herbivory on my sampled seedlings against either total seedling density in the 20m^2 plot.** The X axis is panel b is the log odds ratio of survival from my binomial regression (in other words, the odds of survival), where >1 represents an increased chance of survival and <1 represents a reduced chance of survival. The model included the random effects of species identity and 20m^2 plot to control for variation across species and autocorrelation. The black line marks zero on the X axis.

My second measure of herbivory, the proportion of leaf area lost to herbivory, did not increase with any measures of seedling density (see A11; Supplemental Table 7-12) and showed no density-dependent effect on seedling growth, but did reduce growth rates for all seedlings, regardless of seedling density (see Fig. 4.6c and 4.6d; A11: Supplemental Tables 4, 5). Finally, herbivory did not increase with any measure of functional density (see A11: Supplemental Tables 8 and 9) and there was no interactive effect between the functional density of seedlings and either measure of herbivory on growth at any scale (see A11: Supplemental Tables 3 and 6).

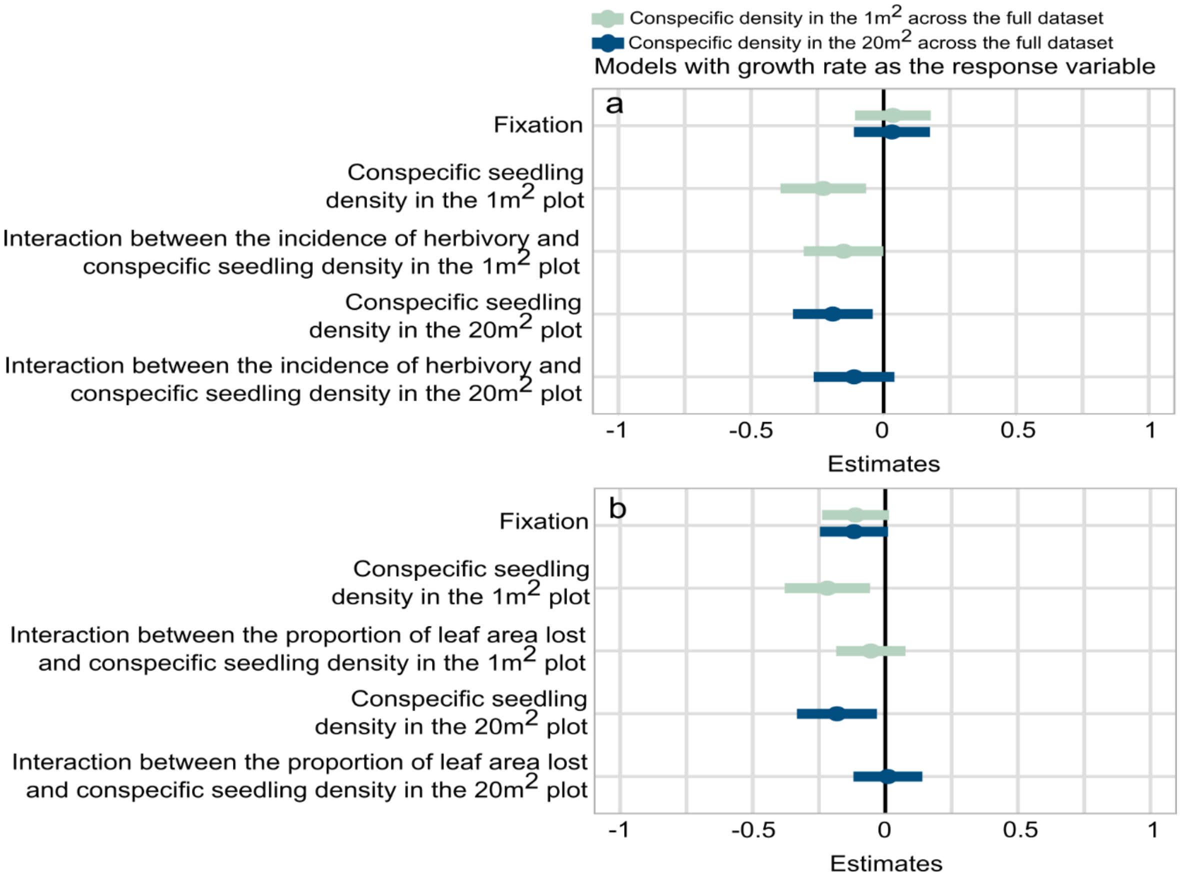


Figure 4. 5 **The strength of measures of herbivory and negative density-dependent effects seedling growth rate.** Coefficient estimates from models regressing seedling growth rate from 2017-2018 against either conspecific density in the 1m^2 plot or the 20m^2 plot. In both panels, the X axis represents changes in stem relative growth rate (in mm). Models included the random effects of species identity and 20m^2 plot to control for variation across species and autocorrelation. The black line marks zero on the X axis.

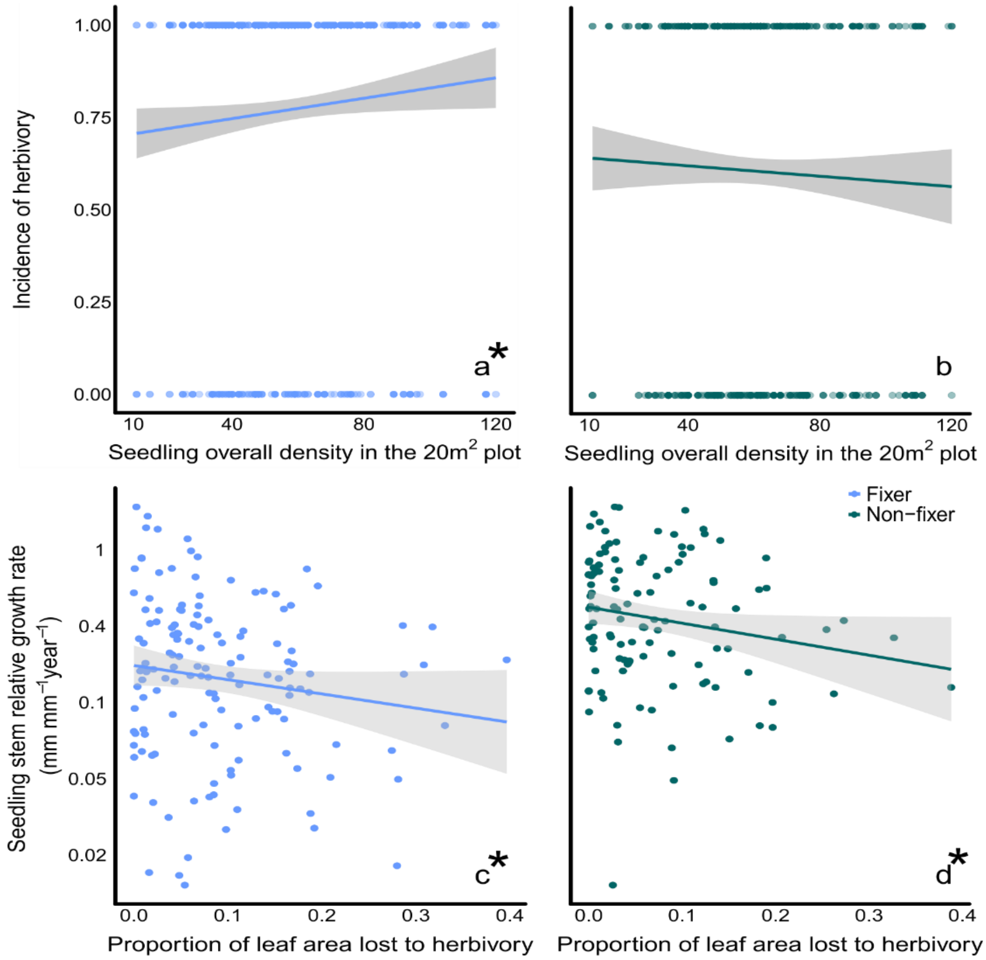


Figure 4. 6 The relationship between herbivory, density and growth rate 2017-2018. *Showing the relationship between the incidence of herbivory and the overall seedling density in the 20m^2 plot for fixers (a) and for non-fixers (b) and between the proportion of leaf area lost to herbivory when surveyed in 2017 and the seedling stem relative growth rate from 2017-2018 for fixers (c) and non-fixers (d). Seedling stem relative growth rate is shown on the log scale. Lines are fitted using a linear model with a Gaussian distribution and grey shading on lines represents standard error. Asterisks represent a significant relationship between the two variables plotted in a given panel.*

4.5 Discussion

More fixer than non-fixer seedlings survived from 2001-2014 in the 50-ha plot on BCI, but fixer individuals experienced far greater negative density-dependent effects on both growth and survival. I found that fixers at higher overall seedling density were more likely to undergo herbivory than those at low densities, and that this incidence of herbivory reduced growth rates for all seedlings at high conspecific seedling density. These findings suggest herbivory could drive high negative density-dependent effects for fixer seedlings and therefore explain why fixers are constrained across tropical forests.

I found no support for my first hypothesis, that fixers have lower growth rates and survival than non-fixers - in fact, fixers had a higher survival rate than non-fixers. High survival for adult fixers has been suggested to explain fixer success in Costa Rica (Menge and Chazdon, 2016). My finding suggests that the benefits of fixation outweigh the high carbon costs of herbivory for seedlings, and potentially that tropical fixers may be able to tolerate herbivory by tailoring fixation rates to their nutrient demands (Barron *et al.*, 2011; Batterman *et al.*, 2013b, 2013c). Loss of leaf area to herbivory removes leaf carbon and nitrogen (Kursar and Coley, 2003), and fixers may be able to compensate for leaf nitrogen lost to herbivores by upregulating fixation, and may downregulate fixation to avoid paying the high energetic carbon cost when plant carbon stocks are reduced following herbivory (see chapter three). However, natural enemies, such as herbivores, can negatively affect growth and survival over density-dependent spatial scales that are not visible in comparisons of growth and survival (Harms *et al.*, 2000; Comita *et al.*, 2010; Dyer *et al.*, 2010; Forrister *et al.*, 2019).

In spite of greater survival for fixer seedlings, I found consistent evidence to support my second hypothesis, that fixers undergo greater negative density-dependent effects than non-fixers. Fixation had a negative effect on survival in my models when including conspecific seedling densities. This effect indicates that fixers only survive more than non-fixers at low conspecific densities. Alongside the effects on survival, the greater negative effects of conspecific density on fixer growth could reduce fixer competitiveness without affecting seedling survival. Fixers with greater growth penalties will take longer to reach the canopy and reproduce, potentially increasing the gap between fixer generations. Tropical fixers downregulate fixation in low light conditions (Taylor and Menge, 2018) and so canopy trees are also more likely to be fixing nitrogen than those growing in the understorey. Thus, by reducing the number of fixers reaching the canopy, negative density-dependent effects on growth could also reduce ecosystem level fixation rates, even if no fixer trees die. High negative density-dependent effects on growth and survival will constrain fixer seedling survival, competitiveness and aggregation during an important life history bottleneck and could translate to lower fixer abundances at the ecosystem scale (Comita *et al.*, 2010; Zhu *et al.*, 2015). By preventing fixers from aggregating at high density, high negative density-dependent effects may also limit the number of fixers able to grow in nitrogen limited sites after disturbance, such as tree fall gaps, and could therefore constrain the role of nitrogen fixation in forest recovery. These results add a new dimension to our understanding of density-dependence – that it may govern the prevalence of functional traits in ecosystems.

My findings link greater negative density-dependent effects with the functional trait of fixation. Previous work has demonstrated that differences in the strength of negative conspecific density-dependent effects between species explain variation in abundance for tropical trees (Comita *et al.*, 2010). Here I highlight how these differences also apply to functional groups, with potential repercussions for ecosystem services, including but not limited to tropical biogeochemical cycling. My finding that confunctional density has a greater negative effect on fixer growth and survival also suggests that density-dependent effects can govern functional group abundance. I account for potential confounding results of comparing groups with different maximum seeding densities by comparing the strength of negative density-dependent effects between species using direct measurements of seedling density and by constraining my dataset to only include seedlings at conspecific densities for which fixers were observed (Detto *et al.*, 2019).

High negative density-dependent effects associated with the trait of fixation may also explain the evolution of reproductive traits for fixer species. Seedling conspecific density depends in part on the species' seed dispersal mechanism (Janzen, 1970; Marchand *et al.*, 2020). Globally, *rhizobial* fixer species have larger seeds than non-fixers that tend to be dispersed by animals (Wilcots *et al.*, 2018). Fixers may have evolved animal dispersed seeds as a mechanism to help spread seeds further from parent trees, where conspecific seedling density, and thus natural enemy presence, is likely to be high (Marchand *et al.*, 2020). Further work should examine how seed size, dispersal mechanism and the strength of conspecific density effects for fixers interact. Strong negative density-dependent effects for fixers could explain why fixer abundances are constrained across tropical forests and, importantly, could be driven by high herbivory for fixer species.

I found strong evidence to support my third hypothesis, that higher herbivory for fixers drove observed difference in growth and the strength of negative density-dependent effects between fixers and non-fixers (I could not test relationships between herbivory and survival due to the low mortality rates for seedlings with herbivory data after only one year). I found that leaf area lost to herbivory reduced growth rates equally for fixer and non-fixer seedlings. In my second chapter, I found that fixers undergo higher herbivory than non-fixers and that this constitutes a high photosynthetic opportunity cost for fixer species (equal to 13% of annual net photosynthetic capacity). This greater reduction in photosynthetic potential for fixer species means that the growth rates of fixers are likely to suffer more than for non-fixers, which undergo less severe herbivory costs. That I observed no difference in growth rates between fixers and non-fixers, despite this herbivory cost, again suggests that there are growth benefits to the trait of fixation, even in the nitrogen rich soils of mature forests that are offset by herbivory. However, by reducing the growth benefits of fixation, so that fixer growth rates are not comparatively greater than for non-fixers, herbivory may reduce the number of fixer seedlings reaching the canopy and reproducing and could therefore limit fixer abundances.

My analysis of the relationship between herbivory and demographic traits for tropical seedlings yielded two novel findings. First, the observation that herbivory reduces growth rates is largely unprecedented for tropical tree seedlings, despite the theoretical expectation that herbivore damage should reduce growth (Eichhorn *et al.*, 2010). Previous research has found that leaf area lost to herbivory has no observable effect on the growth rates of tropical tree seedlings (Coley, 1983; Eichhorn *et al.*, 2010), unless herbivory damage was very high (Blundell *et al.*, 2001). As an exception, herbivore exclusion has been found to facilitate plant growth, but this practice excludes many herbivores guilds beyond just leaf chewing insects (Fine *et al.*, 2004). Where my study differs compared to previous research, is that I analysed leaf herbivory over many species (43), and this large-scale phylogenetic analysis may have prevented species-specific tolerance to herbivory for some species from obscuring general patterns over many species. Second, it was surprising that I could not detect a relationship between herbivory and survival, due to so few seedlings in my sample dying from 2017-2018, as I would expect herbivory to also affect survival given my observation of a negative herbivory effect on growth. Herbivory losses of as little as 1% of mature leaf area have previously been found to affect seedling survival one year on, across 1500 tropical seedlings (Eichhorn *et al.*, 2010). I was unable to examine the relationship between herbivory and survival as only ~7% (24) of my 350 seedlings died after one year, but I may have been able to test for an effect on survival over a larger sample of seedlings with a similar mortality rate.

Herbivory can affect seedling demographic traits consistently across tropical forests, as discussed above, or in a density-dependent manner. I also found a role for herbivory in driving the strength of negative density-dependent effects on growth. The incidence of herbivory for fixer seedlings increased at high overall seedling densities, and, in turn, reduced the growth rates of all seedlings at high conspecific densities. These findings suggest, first, that herbivores preferentially targeting fixer species are able to locate fixer seedlings more easily at high conspecific densities, and second, that herbivory drives negative density-dependent effects across both groups. I therefore identify a clear link between high herbivory for fixers and negative density-dependent effects, indicating that herbivory could constrain tropical nitrogen-fixing tree abundances.

Higher herbivory for fixer seedlings at high overall seedling density suggests that herbivores may not target fixers at the species level. Instead, herbivore preference, and therefore density-dependent effects between species, may be determined by plant defence strategies. Similarity in anti-herbivore defences between neighbouring *Inga* congeners in Panama drives decreased growth and survival, suggesting that herbivory can drive negative density-dependent effects for genera (Forrister *et al.*, 2019). Phylogenetically distant species can have similar anti-herbivore defences (Kursar *et al.*, 2009; Coley *et al.*, 2018). Fixer seedlings may therefore have undergone higher herbivory at high seedling density due to shared defensive strategies with neighbouring heterospecific seedlings.

It is possible that herbivory drives negative density-dependent effects for fixers in conjunction with other factors. Other natural enemies than herbivores can also drive density-dependent patterns of survival (Bagchi *et al.*, 2014; Comita *et al.*, 2014). Experimentally excluding fungal pathogens at the seed to seedling transition reduces seedling diversity, as expected under negative conspecific density dependence (Bagchi *et al.*, 2014). Alternatively, whilst most evidence suggests that natural enemies rather than intraspecific competition drive negative density-dependent effects (Comita *et al.*, 2014), this may not be the case for fixers.

It has previously been suggested that fixers only benefit from nitrogen fixation when soil nitrogen is limiting for plant growth (Taylor *et al.*, 2017; Lai *et al.*, 2018). Fixers tend to colonize low nitrogen soil where the trait of fixation is highly competitive (Batterman *et al.*, 2013b). However, by fixing nitrogen, and through turnover of fixer biomass, local soil nitrogen is increased, and the trait of fixation loses its competitive advantage as taking up nitrogen from the soil is cheaper than fixing (Batterman *et al.*, 2013b). This intraspecific competition could drive negative density-dependent effects between fixers. My finding that fixers growing surrounded by a high density of other fixers show reduced growth and survival, regardless of the species identity of these neighbouring fixers, supports this theory. However, it is also possible negative effects of high fixer seedling density are instead driven by herbivores targeting closely related fixer species (Sprent, 2009). The majority of fixer species, and all fixers in my sample, are phylogenetically clustered in one family, the Fabaceae. Therefore, reduced fixer growth and survival at high confunctional density could instead stem from herbivores specialising on related species within the same family, rather than due to competition between seedlings with the trait of fixation (although, some evidence suggests that closely related fixers may have very different antiherbivore defences) (Kursar *et al.*, 2009; Endara *et al.*, 2017; Coley *et al.*, 2018). Further research should therefore examine the role of herbivores, fungal pathogens, and nitrogen availability and competition between fixer seedlings in driving high negative density-dependent effects for fixers. Regardless of the contribution of other factors, I identify a role of herbivory in driving negative conspecific density-dependent effects that will likely govern fixer abundance and could therefore affect tropical nitrogen fixation.

A growing body of evidence suggests that tropical forest regularly undergoes periods of nitrogen limitation, particularly after disturbance, and that tropical nitrogen fixing trees can mitigate this limitation to underpin plant growth and facilitate forest recovery. However, two factors will contribute to the ability of tropical fixers to alleviate nitrogen limitation on growth – the fixation rates of individual trees and the number and distribution of trees across the forest. High herbivory for fixers is therefore likely to play a role in governing tropical fixation because it affects both of these factors. First, recent evidence suggests that individual tree fixation rates may actually increase under herbivory (see chapter three), and herbivory may also increases the turnover rate of fixed leaf nitrogen from the fixer canopy to the soil, as herbivores remove fixer leaf area and distribute fixed nitrogen through the forest in their deposits (faecal matter, moults) (Metcalfe *et al.*, 2014). This finding suggests that herbivory seems likely to promote tropical nitrogen fixation. However, I here find that herbivory drives strong negative density-dependent effects for fixers which could limit the number and distribution of fixers across tropical forests, constraining the total abundance of fixers and reducing the number of fixer seedlings able to aggregate in areas with low soil nitrogen. Therefore, high herbivory for fixers may increase the fixation rates of individual trees but reduce the total number of nitrogen-fixing trees across tropical forests. Understanding the balance between herbivory effects on individual fixation rates and on fixer abundances will therefore be essential to predict how herbivory will affect tropical nitrogen fixation at the ecosystem scale.

Previous work has examined the effects of density for adult fixers trees with mixed effects (Liao and Menge, 2016; Menge and Chazdon, 2016; Gei *et al.*, 2018), but this research is to my knowledge the first comparison of negative density-dependent effects between these functional groups at the seedling level. I find that fixers seedlings have high survival compared to non-fixers but that they face a far stronger negative conspecific density-dependent effect. A herbivory driven, strong negative density dependence for fixers may explain why their abundances are usually capped at 1-7% in mature tropical forests and at ~5-15% in Neotropical forests (Ter Steege *et al.*, 2006; Hedin *et al.*, 2009; Gei *et al.*, 2018; Menge *et al.*, 2019). This ecological cap could prevent fixers from alleviating nitrogen limitation of the tropical carbon sink and thus may limit the ability of tropical forests to mitigate rising atmospheric CO_2 concentrations (Batterman *et al.*, 2013b; Brookshire *et al.*, 2019; Wright, 2019).

Chapter 5

Conclusion

Tropical nitrogen fixation fulfils a critical role in the growth and recovery of tropical forests. It is therefore important to understand why the abundance of nitrogen-fixing trees never exceeds 8.5% of basal area across mature Asian forests and is capped at ~15% across the Neotropics, despite the benefits of having the capacity to fix nitrogen (Ter Steege *et al.*, 2006; Gei *et al.*, 2018). High herbivory for fixers has been discussed as a constraint on nitrogen fixation for over two decades, but empirical tests of this hypothesis are sparse and particularly so for tropical nitrogen-fixing trees. This thesis aimed to examine the role of herbivory in governing tropical nitrogen fixation and to determine if herbivory could explain why fixer abundances are capped across tropical forests. To do so, I first conducted a herbivory survey on 1,632 leaves, for 350 seedlings from 43 tropical tree species in mature tropical forest to see if fixer species undergo higher herbivory than non-fixers, and combined my herbivory measurements with species leaf trait data to investigate if high leaf nitrogen drove high herbivory for fixers. Second, I used greenhouse experiments with 200 seedlings from five fixer species to explore how high herbivory for fixers affected the fixation rates of individual plants, and how this herbivory effect was governed by soil nitrogen, level of herbivory and anti-herbivore defence strategy. Third, I used a seedling census of growth rate and survival for >200,000 seedlings, with my herbivory measurements on a subset of these seedlings, to determine if fixers have lower growth and survival rates and/or undergo stronger negative density-dependent effects than non-fixers, and whether herbivory drives differences in these demographic traits between the two groups. I will here summarise the findings from each of these analyses and discuss how they inform my understanding of tropical nitrogen fixation.

5.1 Chapter 2: High herbivory is a major cost for tropical nitrogen-fixing tree species

High herbivory for fixers, driven by their high leaf nitrogen concentrations, may explain why fixation is constrained across tropical forests, but the relationships between herbivory, leaf nitrogen and fixation remain uncharacterised. Therefore, I surveyed herbivory across seedlings from 23 fixer and 20 non-fixer seedlings in mature tropical forest, Panama, estimated the carbon cost of the herbivory for fixers, and used species leaf traits to determine what drove differences in herbivory between the two groups. I found that fixer species undergo far greater herbivory than non-fixers and that this constitutes a significant carbon cost associated with the trait of fixation. This novel finding may succeed where other hypotheses have failed in explaining why nitrogen fixation is constrained across the tropics.

Previous hypotheses for why fixers are not more abundant have focused on the high energetic cost of fixation, of ~6-7 g carbon per g nitrogen (Gutschick, 1981; Vance, 2008). However, tropical fixers can downregulate fixation when soil nitrogen is plentiful and can be taken up cheaply from the soil. Therefore fixers can avoid paying the energetic cost of fixation when it outweighs the benefits (Barron *et al.*, 2011; Batterman *et al.*, 2013c, 2013b). Importantly, my estimated carbon cost for herbivory was greater than the carbon cost of fixing even 100% of the leaf nitrogen for an average fixer seedling in my study. Furthermore, this fixation cost is likely to be overestimated for seedlings in the shady, nitrogen rich understory, where fixers are likely to be fixing at low levels and to instead derive their leaf nitrogen from the soil (the energetic cost of fixation would be zero if all leaf nitrogen was derived from the soil) (Hedin *et al.*, 2009; Barron *et al.*, 2011; Batterman *et al.*, 2013b; Sheffer *et al.*, 2015; Taylor and Menge, 2018). This high carbon cost could constrain tropical nitrogen fixation as, first, the cost of herbivory will affect seedlings even when they downregulate fixation rates to avoid the energetic costs of fixation, and second, the seedling stage is a major bottleneck on tropical tree species abundance. Therefore, this high herbivory cost for seedlings poses a constant fixation-associated cost that could constrain the number of tropical nitrogen-fixing trees in tropical forests. I next determined whether leaf nitrogen and other traits known to govern herbivory could explain high herbivory for fixer species.

Surprisingly, given the theoretical expectation for leaf nitrogen to drive herbivory (Matson Jr, 1980; Vitousek and Field, 1999; Kursar and Coley, 2003), I found no evidence for this across fixer and non-fixer species. In fact, when considering a suite of leaf and species traits that I would expect to affect herbivory, including traits relating to chemical and physical defences, I found no variable that could explain why fixers had higher herbivory than non-fixers. Nitrogen has long been expected to drive herbivory for fixers in the biogeochemical literature (Vitousek and Howarth, 1991; Vitousek and Field, 1999; Hedin *et al.*, 2009;

Vitousek *et al.*, 2013), but that no variable could explain herbivory patterns across many species is actually consistent with the large body of research indicating that herbivory is governed instead by a complex suite of defence characteristics, rather than any one variable alone (Kursar *et al.*, 2009; Endara *et al.*, 2017; Coley *et al.*, 2018).

Fixer species may invest in defences to deter herbivores and reduce the cost of herbivory, which could obscure the role of leaf nitrogen in governing herbivory patterns. However, as defence investment is highly species-specific and investment by a given plant species is likely to only deter a particular group of herbivores, measuring one defence variable that reduces herbivory across a large group of species is difficult. This complexity in measuring one effect of a leaf variable across species may be particularly relevant for leaf nitrogen as leaf nitrogen can be used either for nutritious photosynthetic proteins, such as RUBISCO, or for chemical defence compounds, which are commonly utilised by fixer species (Matson Jr, 1980; Kursar and Coley, 2003; Wink, 2013). For example, increasing leaf nitrogen concentrations may be offset by some of that leaf nitrogen existing in compounds damaging to herbivores, such as alkaloids or cyanogenic glucosides (Mithöfer and Boland, 2012; Wink, 2013). Thus, herbivore attraction may not increase linearly with leaf nitrogen concentration as nitrogen could make up toxic compounds in the leaves of fixer species.

A large body of work into plant-herbivore coevolution has highlighted a complex, species-specific pattern of defence investment (Wink, 2003; Kursar *et al.*, 2009; Endara *et al.*, 2017, 2018), but an alternative theory suggests that leaf toughness can predict herbivory damage on seedlings across tropical tree species (Westbrook *et al.*, 2011; Kitajima *et al.*, 2012, 2016). Surveys of leaf toughness and herbivory in mature tropical forests in Panama identified that leaf fracture toughness, due to high cellulose and lamina density, reduces herbivory, but this was not supported by my findings (Kitajima *et al.*, 2016). This discrepancy may be because (a) for some species, the majority of herbivory damage occurs during leaf expansion on new leaves (Kursar and Coley, 2003), and (b) leaf toughness (and chemical defence investment) varies over the lifespan of a leaf (Kursar and Coley, 2003; Kitajima *et al.*, 2016). Kitajima et al (2016) correlated measures of leaf toughness with herbivory on leaves sampled across a range of leaf ages representative for sampled seedlings (Kitajima *et al.*, 2016). I surveyed herbivory across both young and mature leaves for my seedlings, accounting for higher herbivory on expanding leaves, however, my leaf toughness and nutrient data was collected for only mature leaves. By using leaf traits only from older leaves, I may have missed important relationships between herbivory and leaf toughness or other defence measures in young leaves. Future work should examine the role of young leaf traits in driving differences in herbivory between fixer and non-fixer seedlings.

The results from my first chapter show that fixer species undergo higher herbivory than non-fixers and, whilst further work is needed to understand what drives herbivore preference for fixer species, that high herbivory constitutes a significant fixation associated carbon cost that could constrain tropical nitrogen fixation. In order to understand how this cost could constrain fixation, I next investigated what effect herbivory had on the fixation rates of individual plants.

5.2 Chapter 3: A quicker fix: Herbivory may stimulate nitrogen fixation in tropical forests

The high carbon cost that I have identified for fixers due to herbivory in chapter one could govern tropical nitrogen fixation by affecting the fixation rates of individual trees. However, the effects of herbivory on plant level fixation rates have not been established. High herbivory for fixers could either increase or decrease fixation rates, depending on how the loss of two key leaf nutrients – carbon and nitrogen – affect the nutrient demand of the plant. On the one hand, if plant growth is limited by carbon, plants may downregulate fixation after losing leaf carbon to herbivory to conserve carbon reserves for growth, rather than continuing to pay their bacteria carbon for fixed nitrogen (Batterman *et al.*, 2013c). On the other hand, if nitrogen is limiting, plants may upregulate fixation to replace nitrogen lost due to herbivory (Batterman *et al.*, 2013c). How herbivory affects the carbon and nitrogen demand, and the fixation response of the plant, will therefore depend on the nitrogen available in the soil (Batterman *et al.*, 2013c) and on the amount of leaf area lost to herbivory.

How plant nutrient demand is affected by herbivory may also vary depending on the antiherbivore strategy of the plant (Kursar *et al.*, 2009). Inga species have previously been found to differ along a continuum of antiherbivore strategy in young, vulnerable, expanding leaves, along a continuum from defence specialists to escape specialists (Kursar and Coley, 2003; Bromberg *et al.*, 2005; Kursar *et al.*, 2009). Escape specialists have leaves that rapidly expand through the vulnerable early stage of leaf development, but they are poorly defended and so instead may downregulate fixation in response to herbivory to prioritise carbon for growth (Kursar *et al.*, 2009). Defence specialists have slow expanding, well defended leaves and so may upregulate fixation following herbivory, to maintain high nitrogen leaves or to produce nitrogen-based defence compounds (Kursar *et al.*, 2009).

In my greenhouse experiments, seedlings from five species with different antiherbivore strategies were grown in a range of soil nitrogen conditions, across several levels of herbivory and all under high light conditions. I found that, in some species, fixation rates increased by up to ten times in response to herbivory, and that this effect was governed by soil nitrogen, leaf area lost to herbivory and antiherbivore defence strategy. For example, seedlings upregulated fixation rates in response to herbivory only when nitrogen was limiting in the soil. This increase in fixation was likely driven by an increase in leaf area in response to herbivory in some species, which increased demand for nitrogen. This finding suggests that nitrogen and not carbon was the limiting factor for growth and recovery after herbivore attack. My results will likely be consistent with the fixation response to herbivory for trees growing in high light conditions and experiencing nitrogen limitation, as in young secondary forests or treefall gaps, but may not represent the herbivory response of seedlings growing in mature forests. Hence, I would expect herbivory to upregulate fixation for seedlings in secondary forests but herbivory actually may constrain seedling fixation rates in mature forest (this would likely have little effect on overall seedling fixation as rates are likely to be low in mature forests to begin with).

My findings do however raise the question of how adult fixer trees in mature forest will respond to herbivory – I may expect adult trees in mature forests, which are likely to be growing in high nitrogen soil but have access to high light by growing in the canopy, to also upregulate fixation in response to herbivory as photosynthetically derived carbon will not be limiting for growth (Hedin *et al.*, 2009; Taylor and Menge, 2018). A sudden surge in herbivory damage may increase nitrogen demand for adult canopy trees even when growing in high nitrogen soils, shifting their balance of nutrient demand versus soil nitrogen supply toward nitrogen limitation and stimulating bursts of fixation. This effect of herbivory at the canopy level on mature fixers could explain observations of fixation by adult trees in mature forests (Barron *et al.*, 2011; Wurzburger and Hedin, 2016), and why fixation rates have been found to differ between adult trees of the same species (Batterman 2020 – personal communication).

Interestingly, I found that per gram nodule biomass fixation rates increased in response to herbivory and often these increases occurred despite reductions in nodule biomass. In other words, fixation rates were increased metabolically, rather than by producing more nodules that were all fixing at the same rate. This suggests that fixer seedlings can alter fixation rates plastically and rapidly in response to herbivory. The ability to rapidly adjust fixation rates in response to herbivory has not been found for other variables that govern herbivory, such as soil nitrogen and light, where nodulation and fixation rates adjust in unison to environmental conditions (Batterman *et al.*, 2013c; Taylor and Menge, 2018). The evolution of this ability could suggest that herbivory represents a more immediate threat to fixer seedlings than other abiotic factors that limit fixation. Fixers may rapidly upregulate fixation in order to increase growth and replenish lost leaf area, as *Inga bella* did in my first experiment, or to build inducible and toxic nitrogen-based defences (Coley *et al.*, 1985; Fine *et al.*, 2006; Endara and Coley, 2011; Wink, 2013). Whilst there was no change in overall leaf nitrogen content across herbivory treatments for any of my species, the chemical form of this leaf nitrogen could have been altered in response to herbivory without changing leaf nitrogen concentrations in my seedlings. The various response of my fixer seedlings to herbivory fell under two, perhaps complementary theoretical frameworks to explain defence investment and growth responses to herbivory across species, the defence-escape continuum and the Resource Allocation Hypothesis (Coley *et al.*, 1985; Kursar and Coley, 2003; Kursar *et al.*, 2009).

Not all species upregulated fixation in response to herbivory. I next investigated whether fixer response to herbivory varied with antiherbivore defence strategies, characterizing *Inga* species as either defence or escape depending on the chemical defence investment for and expansion rate of young leaves (Kursar *et al.*, 2009). I predicted that escape plants would downregulate fixation in response to herbivory to prioritise carbon for growth, whilst defence plants would upregulate fixation to maintain high leaf nitrogen concentrations and produce nitrogen-based defence compounds. I found that escape plants downregulated fixation under herbivory, and that defence plants upregulated fixation by up to ten times, as expected. This result suggests that loss of leaf area drives demand for carbon in escape plants but increases nitrogen demand in defence plants. These varying responses to herbivory could either increase the input of fixed nitrogen to an ecosystem or reduce it, depending on the relative proportion of antiherbivore defence strategies amongst fixer species. If the majority of fixer species are defence specialists, for example, then herbivory can be largely expected to enhance fixation rates and promote forest growth and recovery - ~60% of the 35 Inga species investigated by Kursar et al (2009) were defence specialists. However, if most fixers are escape specialists, then herbivory may limit the role of nitrogen-fixing trees in mitigating nitrogen limitation of tropical forest growth.

Some species may not clearly fit into either a defence or escape category. I found that *I. bella* showed a different response to herbivory than either my defence or escape species – instead, increasing fixation rates, flushing new leaves, and increasing total leaf area per seedling. This response falls outside the defence-escape continuum, and is instead in keeping with the Resource Allocation Hypothesis (Coley *et al.*, 1985; Endara and Coley, 2011). The Resource Allocation Hypothesis suggests that in high resource environments, natural selection under herbivory will select for fast-growing species with low investment in defence and, in low resource environments, that slow-growing species with high defence investment will be favoured (Coley *et al.*, 1985; Endara and Coley, 2011). Crucially, the Resource Allocation Hypothesis characterizes species based on total plant growth rates, whilst the defence-escape continuum hypothesis categorizes species based on leaf expansion rates (Coley *et al.*, 1985; Kursar *et al.*, 2009; Endara and Coley, 2011). My defence specialists responded to herbivory as expected under both the Resource Allocation Hypothesis and the defence-escape continuum hypothesis, showing no growth response to herbivory despite upregulating fixation rates (Coley *et al.*, 1985; Kursar *et al.*, 2009). These two theoretical frameworks for understanding patterns of defence investment across plant species are not mutually exclusive, and further work should be applied to examine the fixation response to herbivory across a wider range of species selected to test the Resource Allocation Hypothesis.

In the introduction to this thesis, I discussed how, due to fixers at times growing in resource-rich environments (in high light secondary forests where they usually fix) and at times growing in resource-poor environments (in low light mature understorey where they usually do not fix) (Batterman *et al.*, 2013b; Sheffer *et al.*, 2015), fixers may pay an evolutionary cost of not being able to fully specialise to either be fast growing-poorly-defended or slow growing-well-defended. The finding that my well-defended species did not exhibit growth responses to herbivory suggest that, as well as having varying antiherbivore strategies based on leaf expansions rates, fixer species can also either specialise to be growth species or defence species under the Resource Allocation Hypothesis. However, again based on my observation of no growth response to herbivory for defence specialists in high light environments, fixer species do not seem to be able to switch between these two strategies. Accordingly, fixer species may face an evolutionary cost to undergoing high herbivory in both high and low resource environments by not being able to fully specialise to either set of conditions.

The results from my second chapter show that the high herbivory cost for fixers can govern plant-level fixation rates, generally increasing fixation rates by up to ten times. However, whether herbivory upregulates or downregulates fixation depends on nitrogen availability, herbivory level and species antiherbivore strategy, and herbivory effects on fixation in tropical forests are likely to vary across species, life history stages and forest types. The role of herbivory in governing tropical nitrogen fixation will depend on the fixation rates of individual trees as well as the number and distribution of trees across tropical forests. Therefore, I next investigated whether high herbivory affects fixer abundances by governing fixer survival, growth and/or the strength of negative density-dependent effects for fixer species.

5.3 Chapter 3: Know thy neighbour: Herbivory contributes to greater negative density-dependent effects for fixer seedlings compared to non-fixers in a tropical forest

A high herbivory cost for fixers could constrain fixation by affecting the demographic traits of fixers. Herbivory can reduce growth and survival by reducing the net photosynthetic capacity of seedlings, or by introducing fungal pathogens to damaged leaf tissue (Eichhorn *et al.*, 2010; Schuldt *et al.*, 2017). Herbivory can also cause negative density dependent effects on growth and survival, as clusters of seedlings are easier for herbivores to locate, or because of high intraspecific competition between seedlings of the same species (Janzen, 1970; Comita *et al.*, 2014; Forrister *et al.*, 2019). However, comparisons between the growth, survival and the strength of negative density-dependent effects for fixer and non-fixer seedlings have never been carried out.

First, I compared growth and survival between the two functional groups. I found that fixer seedlings had no growth advantage over non-fixer seedlings in mature tropical forest and had a higher rate of survival. These findings indicate that fixers do not undergo any growth benefits from the trait of fixation by actively fixing in high soil nitrogen, low light conditions, yet nor do they face any growth or survival penalties by downregulating fixation when its costs outweigh the benefits. I observed that herbivory reduced growth rates for both groups evenly. Therefore, alternatively, the high carbon cost of herbivory established in my second chapter may reduce the growth benefits of fixation so that fixers show no overall competitive growth advantage over neighbouring non-fixers.

I next compared the strength of negative density-dependent effects between fixer and non-fixer seedlings. Despite the higher overall survival rate for fixer seedlings, I identified much stronger conspecific negative density-dependent effects on seedling growth and survival for fixers compared to non-fixers. In other words, fixers exhibited high survival but only at low conspecific seedling densities. The negative effects of neighbouring seedlings of the same species could explain why fixers are not more abundant across tropical forests, as strong negative density-dependent effects may remove fixers from the population and prevent fixer seedlings from aggregating at sites with low soil nitrogen where the fixation trait will be particularly competitive (Comita *et al.*, 2010; Barron *et al.*, 2011; Batterman *et al.*, 2013b). The susceptibility to density-dependent effects when surrounded by neighbours of the same species has been previously linked to overall species abundance in the 50-ha plot (Comita *et al.*, 2010), but this is the first time that a link has been found between density-dependent effects and functional groups. I next tried to establish whether herbivory could be driving these density-dependent effects for fixer seedlings.

I identified several indicators that high herbivory for fixers drives negative density-dependent effects for fixer seedlings. First, the incidence of herbivory was greater for fixer seedlings at high overall seedling densities, indicating that herbivores are attracted to clusters of mixed species seedlings where they then preferentially target fixer leaves. Second, I found that this incidence of herbivory reduced seedling growth rates at high conspecific densities for all species, suggesting that a high local density of seedlings from the same species facilitates herbivory for all seedlings. These two findings outline a possible mechanism by which herbivory may contribute towards negative density-dependent effects for fixer species: fixer seedlings undergo more herbivory at high overall seedling density than their non-fixing neighbours which, in turn, leads to high negative conspecific density-dependent effects for fixers if they have neighbouring seedlings of the same species (as feeding and reproduction of herbivores best suited to target fixer species is facilitated by fixer seedlings of the same species growing at high density). Fixers may undergo higher herbivory when surrounded by a mixed group of seedlings due to shared herbivore defences. Herbivores target plant species based on their ability to tolerate plant defences, rather than targeting phylogenetically related species (Endara *et al.*, 2018). Fixers may therefore undergo higher herbivory if fixer seedlings and non-fixer seedlings in close proximity share herbivore defences, but fixers are still preferentially targeted due to some leaf trait associated with fixation (Vitousek and Field, 1999; Endara *et al.*, 2017; Forrister *et al.*, 2019). My findings show that high herbivory on fixer leaves drives density-dependent effects on fixer growth and survival that could explain why fixers abundances are constrained across tropical forests, although it is possible that other factors may also contribute to high negative density-dependent effects for fixers in conjunction with herbivory.

Herbivores have been found to contribute to density-dependent patterns in the survival of seedlings (Dyer *et al.*, 2010; Norghauer *et al.*, 2010; Downey *et al.*, 2018; Forrister *et al.*, 2019; Szefer *et al.*, 2020) alongside fungal pathogens (Bagchi *et al.*, 2014; Comita *et al.*, 2014). Fungal pathogens, for which nitrogen is also a limiting nutrient for growth (Snoeijers *et al.*, 2000), may preferentially target fixer species. Herbivory and fungal pathogens may also drive density-dependent effects at other life stages (Comita *et al.*, 2014; Zhu *et al.*, 2015). It is possible that herbivory or pathogenic attacks at the seed, alongside the seedling level, drive patterns of density dependence for fixer species (Bagchi *et al.*, 2014; Marchand *et al.*, 2020). Fixer seeds tend to be nitrogen rich, well defended and are biotically dispersed to spread germinating seedlings far apart and far from adult trees (Beckman and Muller-Landau, 2011; Beckman, 2013; Vargas *et al.*, 2015; Wilcots *et al.*, 2018), all suggesting that fixers undergo high pressure from natural enemies at the seed stage. Alternatively, strong negative density-dependent effects for fixer species may be due to intense intraspecific competition between fixer seedlings.

Competition between seedlings of the same species, or even closely related species, is likely to be high as they will have very similar demand for light and nutrients and utilise similar strategies to acquire them. The majority of the literature suggests that natural enemies drive density dependence in tropical forests (Bell *et al.*, 2006; Bagchi *et al.*, 2010; Terborgh, 2012; Forrister *et al.*, 2019), rather than competition (Comita *et al.*, 2014), but this may not be the case for fixer species – largely due to the fact that they can fix. Previous work to explain the low abundance of fixers across tropical forests has hypothesized that fixers only benefit from fixation when soil nitrogen is low (Vitousek *et al.*, 2013; Sheffer *et al.*, 2015; Taylor *et al.*, 2017; Lai *et al.*, 2018). Fixers colonize low nitrogen soil where the trait of fixation has a high competitive advantage and begin fixing nitrogen (Batterman *et al.*, 2013b), but by doing so they increase soil nitrogen levels until the trait of fixation is no longer competitive (because taking up nitrogen from the soil is cheaper) (Sheffer *et al.*, 2015), and previous hypotheses have predicted that fixers will then be outcompeted. Phosphorus and molybdenum availability also govern fixation, with phosphorus needed for plant growth and molybdenum to catalyse the fixation reaction, and fixers may downregulate fixation rates when they have used up local soil stores of these nutrients (Barron *et al.*, 2009; Batterman *et al.*, 2013c). However, when soil nitrogen meets plant demands, or when other nutrients limit fixation, tropical fixers can downregulate fixation to avoid the energetic cost of fixation. Therefore, fixers in high soil nitrogen should not face any competitive disadvantage – unless there is another fixation associated cost that constantly affects fixers, like herbivory. If so, herbivory may be the missing piece in explaining why fixers outcompete each other, despite being able to tailor fixation rates to soil nitrogen availability.

Previous attempts to test if the benefits of fixation in low soil nitrogen are reduced by fixing have been coarse, carried out on adult trees over large scales (at the hectare or greater) (Taylor *et al.*, 2017; Lai *et al.*, 2018). My study is the first able to partially test this hypothesis on seedling recruitment and survival at finer scales, and my findings are consistent with the hypothesis that fixers may reduce the competitive advantage of fixation by replenishing soil nitrogen and face a constant cost associated with fixation. Nitrogen limitation may exist for seedlings at very local scales across mature tropical forest, creating a heterogenous map of nitrogen limitation and sites at which fixation will provide competitive benefits (Townsend *et al.*, 2008; Menge and Levin, 2017). By fixing nitrogen at these local scales, fixers could replenish soil nitrogen stores, become less competitive and then under the cost of herbivory, survive less and only at lower densities. Herbivory, attack by fungal pathogens, and the interaction between soil nitrogen availability and intraspecific competition between fixer seedlings may all contribute to negative density-dependence for fixer species. Further work should examine if negative density dependent effects apply to fixers as a functional group, rather than as a function of overall or conspecific seedling density, to test whether competition with seedlings able to fix drives density-dependent effects.

Together, the findings from each of my chapters enrich my understanding of the role that herbivory plays in governing tropical nitrogen fixation. Fixers undergo much higher herbivory, which may stimulate nitrogen fixation rates in secondary forest or canopy trees and therefore could promote increased fixation in secondary forests. However, herbivory constrained plant-level fixation rates in some species and I also found evidence that herbivory contributes to stronger negative density-dependent effects for fixer seedlings. These constraints may limit the numbers and distributions of fixers across tropical forests and reduce the capacity of nitrogen fixation to mitigate nitrogen limitation on tropical forest growth, recovery and carbon uptake. I will now discuss how the findings from each of my chapters combined may affect first, the biodiversity and evolution of tropical fixers, and second, the tropical nitrogen cycle and carbon sink.

5.4 The effects of herbivory on fixer biodiversity and evolution

Both biodiversity and herbivory pressure are high in the tropics. The Fabaceae in particular are an incredibly diverse plant family, and fossil evidence indicates that this has been the case in the tropics for ~ 58 million years (Ter Steege *et al.*, 2006; Wing *et al.*, 2009). Fixers, the majority of which are found in the Fabaceae, also face a high herbivory cost which may drive speciation in the family. However, it remains unclear if herbivory contributes towards the high diversity of fixer species. Therefore, I here discuss how high levels of species coexistence in tropical forests may be facilitated by herbivory under two non-exclusive mechanisms.

First, herbivory and the subsequent evolution of defences by plant species can drive plant speciation (Ehrlich and Raven, 1964; Becerra, 2007; Becerra *et al.*, 2009). The plant host range of tropical herbivores is often very small, likely due to species-specific plant defences that require high specialization by herbivores to overcome or endure herbivore pressure (Coley and Barone, 1996; Coley *et al.*, 2006; Dyer *et al.*, 2007; Novotny *et al.*, 2010; Becerra, 2015). Coevolutionary theory has long postulated that by evolving novel defences, plants can evade their current specialised herbivores. This coevolutionary arms race may create ever increasing cycles of specialization that drives biodiversity for both groups (Ehrlich and Raven, 1964; Becerra, 2007; Becerra *et al.*, 2009). However, evidence for this theory over macroevolutionary scales has been sparse (Futuyma and Agrawal, 2009; Suchan and Alvarez, 2015) and new research instead suggests that, by evading herbivores through the evolution of novel defence traits, plants may attract new distinct groups of herbivores already better adapted to feed on them (Endara *et al.*, 2015, 2017, 2018). Under this model, insect diversity may be decoupled from plant diversity, as insect herbivores instead target plants due to their defence traits rather than their phylogenetic relationship to other palatable plants.

Importantly for understanding the high number of fixer species, both mechanisms of plant-herbivore interaction predict that plant species are able to coexist in similar environments due to occupying different niches formed under herbivory pressure (Endara *et al.*, 2015). In other words, closely related tropical tree species can differentiate along an axis of defence investment in response to herbivory. This pattern of differentiation is consistent across *Inga* species, which exhibit greater variation in defence traits than in traits for acquiring resources or reproduction (in support of this, I found that *Inga* species face very high herbivory pressure, even compared to other fixers – see Fig 2.2) (Becerra *et al.*, 2009; Endara *et al.*, 2017; Coley *et al.*, 2018). Furthermore, patterns of variation in defence are not often phylogenetic, suggesting that the evolution of novel herbivore defences drive speciation rather than the other way round, and this is true for chemical defence traits within the Fabaceae (Kursar *et al.*, 2009; Wink, 2013). Therefore, high herbivory pressure for fixer species could explain why fixer abundances are lower than expected across tropical forests, but also why fixers are so diverse.

The second mechanism by which herbivory could promote higher fixer diversity is through driving strong negative conspecific density-dependent effects for fixers (Dyer *et al.*, 2010; Downey *et al.*, 2018; Forrister *et al.*, 2019). These effects can facilitate high biodiversity by preventing highly competitive species from becoming too common over a given area, such as fixers in low soil nitrogen conditions (Harms *et al.*, 2000; Comita *et al.*, 2010). Accordingly, high density-dependent effects at the species level may explain why fixers, and the Fabaceae, are so speciose – as negative density-dependence could prevent any one fixer species from becoming dominant and promote the evolution of rare fixer species (Comita *et al.*, 2010). Species rarity in mature forests in Panama has previously been linked with strong negative density-dependent effects (Comita *et al.*, 2010). I found several indicators that high herbivory for fixers contributes to the strong negative density-dependent effects that I observed for nitrogen-fixing seedlings.

The phylogenetic clustering of plants able to fix atmospheric nitrogen within the Fabaceae raises the question of whether high herbivory for fixers is driven by a functional trait associated with fixing nitrogen or with a phylogenetic trait associated with the Fabaceae. I found high herbivory for fixers, but also predicted high herbivory at the species level for non-fixing members of the Fabaceae in my sample – *Dipteryx oleifera*, *Senna Dariensis* and *Prioria copaifera*. These species had three of the highest four probabilities of herbivory for non-fixing species (see Fig. 2.2). Although my sample size is small, identifying high herbivory for non-fixing Fabaceae suggests that traits common within the family drive high herbivory for fixer species. One such trait that I would expect to drive herbivory and that is common across the Fabaceae, regardless of fixation status, is leaf nitrogen concentration (Fyllas *et al.*, 2009). Alternatively, the trait of fixation may have been gained and has been lost many times throughout the evolution of the Fabaceae (Werner *et al.*, 2014; Griesmann *et al.*, 2018; Velzen *et al.*, 2018), meaning, first that the trait of fixation has an associated cost for natural selection to act on, such as herbivory, and second, that herbivores specialised to target particular fixer species may continue to target that species even after it has lost the trait of fixation. Regardless, whether fixers undergo high herbivory as a functional group or within their taxonomic group, high herbivory will have important repercussions for tropical nitrogen fixation, with consequences for tropical nitrogen cycling and the tropical terrestrial carbon sink.

5.5 Consequences of my findings for tropical nitrogen cycling and the tropical carbon sink

A growing body of evidence suggests that tropical forests undergo frequent periods of nitrogen limitation, particularly following disturbance (Batterman *et al.*, 2013b; Brookshire *et al.*, 2019; Levy-Varon *et al.*, 2019; Wright, 2019). Nitrogen-fixing trees have been shown to mitigate this nitrogen limitation of plant growth and promote carbon sequestration, enhancing carbon uptake rates by 200% in secondary forest and increasing carbon in mature forest biomass by 10% (Brookshire *et al.*, 2019). The high cost of herbivory that I have identified is likely to govern the role of tropical nitrogen fixation in tropical forests, most directly by altering the fixation rates of individual trees. As discussed above, I find that herbivory can increase fixations rates in individuals of some, but not all species, and may limit the abundances and distributions of fixers across tropical forests.

The role that herbivory plays in governing tropical nitrogen fixation may also vary across life history stages and forest types. In young secondary forests, I would expect herbivory to increase fixation rates and promote faster forest recovery, growth and carbon uptake. It is important to remember that as herbivory occurs across forests naturally, and as previous research into the role of nitrogen-fixing trees in secondary forests has not excluded herbivores, that this herbivory affect is already included in current measures of nitrogen fixation (Batterman *et al.*, 2013b; Brookshire *et al.*, 2019; Levy-Varon *et al.*, 2019). Furthermore, the effects of herbivory may be even stronger than recorded in my survey of herbivory in mature forest, given the higher herbivory rates in forests recovering from disturbance (Coley, 1993; Morante-Filho *et al.*, 2016; van Schrojenstein Lantman *et al.*, 2018). In mature forests, seedling fixation is expected be low due to low light levels and high soil nitrogen (this may not be the case for adult trees with leaves in the canopy) (Hedin *et al.*, 2009; Taylor and Menge, 2018). If herbivory does constrain fixation in low light, high soil nitrogen conditions it may further constrain fixation by seedlings in mature tropical forests.

If my observations of upregulated fixation for seedlings in high light environments are consistent for adult trees with leaves in the canopy, it could answer two key questions in tropical biogeochemistry. First, upregulated fixation on canopy trees under herbivory may explain why fixation has been observed on adult trees in mature forest, despite typically high soil nitrogen levels in mature forests (Barron *et al.*, 2011; Wurzburger and Hedin, 2016). Second, this effect in adult trees may offer a mechanism to explain a paradox in tropical nitrogen cycling. Tropical ecosystems are nitrogen rich, yet export large quantities of bioavailable nitrogen every year (via leaching for example), suggesting that some external source continues to bring nitrogen into tropical ecosystems despite high nitrogen availability in tropical soils (Hedin *et al.*, 2009). Previously, it has been thought that nitrogen-fixing trees could not explain this high input of nitrogen into tropical forests, as fixation by plants is coupled with soil nitrogen availability so that they only fix when soil nitrogen is limiting. Hedin et al (2009) instead suggested a leaky nitrostat model to explain this phenomenon. In the leaky nitrostat model, free living nitrogen-fixing bacteria in the nitrogen poor layer of leaf litter on top of the soil and epiphytic fixers in the canopy cannot access the high quantities of bioavailable nitrogen in tropical ecosystems. These fixers are thus decoupled from the substantial nitrogen stores in tropical ecosystems and fix at high rates, explaining the high levels of nitrogen stored in and exported from tropical forests (Hedin *et al.*, 2009).

My observation of high fixation rates under herbivory, if true for adult trees, may suggest that adult fixer trees can also be decoupled from tropical soil nitrogen stores. Herbivory may stimulate bursts of fixation in canopy trees in mature forests if nitrogen losses to herbivory are significant enough to briefly raise plant nitrogen demand above the soil nitrogen available at its roots. Alongside prompting these spikes in individual fixation for adult trees, herbivores would then facilitate rapid turnover of fixed leaf nitrogen to the soil as nutrient rich insect deposits (faecal matter, moults), contributing to high soil nitrogen stores and exports of bioavailable nitrogen from tropical forests, supporting the leaky nitrostat model (Metcalfe *et al.*, 2014) (see Fig. 5.1). This role of herbivores in facilitating turnover of leaf nutrients to the soil has been previously highlighted and may explain how fixers can underpin plant growth at the forest scale, but the relationship between herbivory, nitrogen and growth in tropical forest remains unexplored.

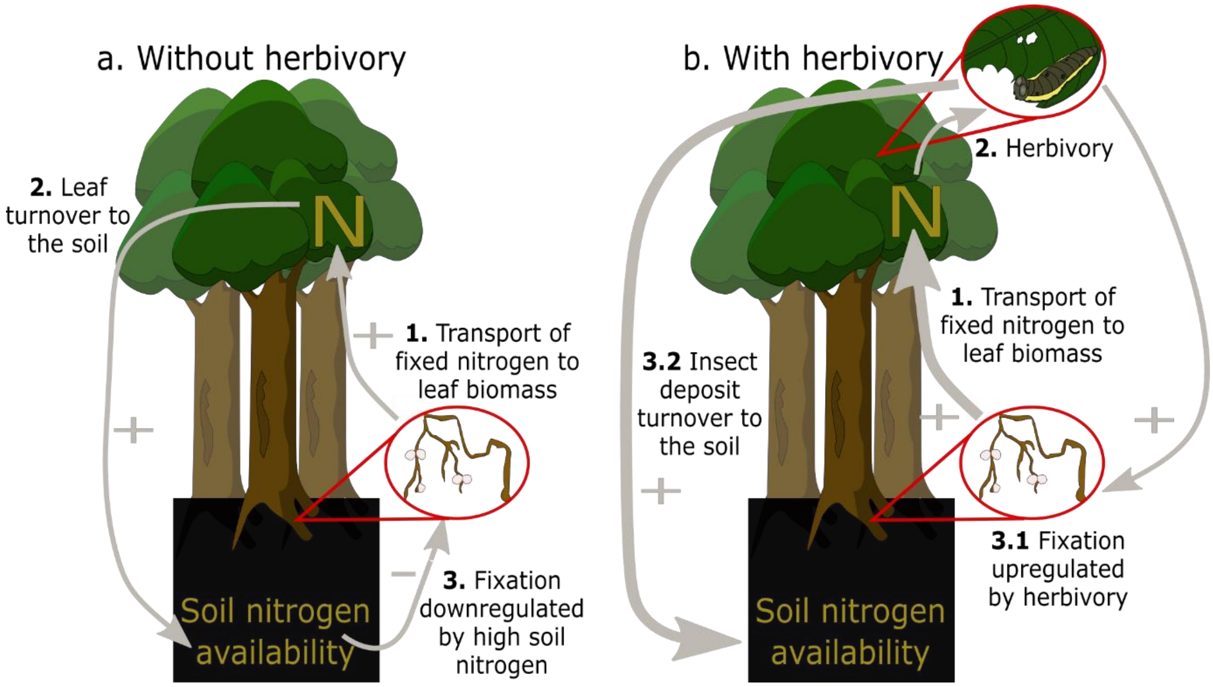


Figure 5. 1**. How fixation by canopy trees under high herbivory may contribute to substantial nitrogen accumulation, recycling and export in tropical forests.** A conceptual figure showing tropical nitrogen fixation by canopy trees without herbivory (a) and with herbivory (b). Without herbivory (a), fixed nitrogen is transported to the canopy (1), where leaf turnover slowly returns this nitrogen to the soil (2), increasing soil nitrogen and reducing plant nitrogen demand and fixation rates (3). With herbivory (b), fixed nitrogen is transported to the canopy (1) where it is eaten by insect herbivores (2), herbivory then causes a loss of leaf nitrogen that briefly increases plant nitrogen demand, stimulating fixation rates to meet plant demand (3.1) and increasing the turnover of nutrient rich insect deposits to the soil, increasing soil nitrogen (3.2). With herbivory in the model, fixation is therefore decoupled from soil nitrogen (no negative effects in conceptual model), leading to a build-up of nitrogen in tropical forests despite high nitrogen availability. Minus symbols represent a negative effect of each step on the next, whilst a positive symbol represents a positive effect. Arrow width represents the strength of the effect compared between the two situations – with and without herbivory.

Herbivory fulfils a critical biogeochemical role in tropical forests. Firstly, herbivory is likely to decrease total photosynthetic capacity across tropical forests and has been shown to reduce litterfall by 12-19%, reducing the amount of carbon sequestered in forests and then turned over as leaf litter and stored as soil carbon (Metcalfe *et al.*, 2014). However, the turnover of insect deposits will be more nutrient rich and so these deposits are likely to increase turnover of nutrients other than carbon to the soil (Bardgett and Wardle, 2003; Jones, 2019). Plant litter is largely nutrient poor and surprisingly recalcitrant to deposition, despite favourable environmental conditions for microbes and other detritivores in tropical forests (Hattenschwiler *et al.*, 2011). Insect deposits will likely be far more amenable to breakdown and therefore will release nutrients more easily into the soil (Bardgett and Wardle, 2003). They will also transport nutrients more evenly throughout the forest. Therefore, my research highlights how herbivory and nitrogen fixation may interact to control biogeochemical cycling in tropical forests. Nitrogen fixation is the main source of external nitrogen for tropical ecosystems (Vitousek *et al.*, 2013), and, as I have identified, the fixation rates of individual trees may increase under high herbivory for fixers. Herbivores will then readily distribute this nitrogen throughout the ecosystem, where it will more readily be released into the soil and will be spread further from the "hotspots" where nitrogen fixer abundance is high – such as nitrogen limited soils following disturbance. Therefore, herbivory may explain how fixation by individual trees can facilitate growth and recovery at the forest scale.

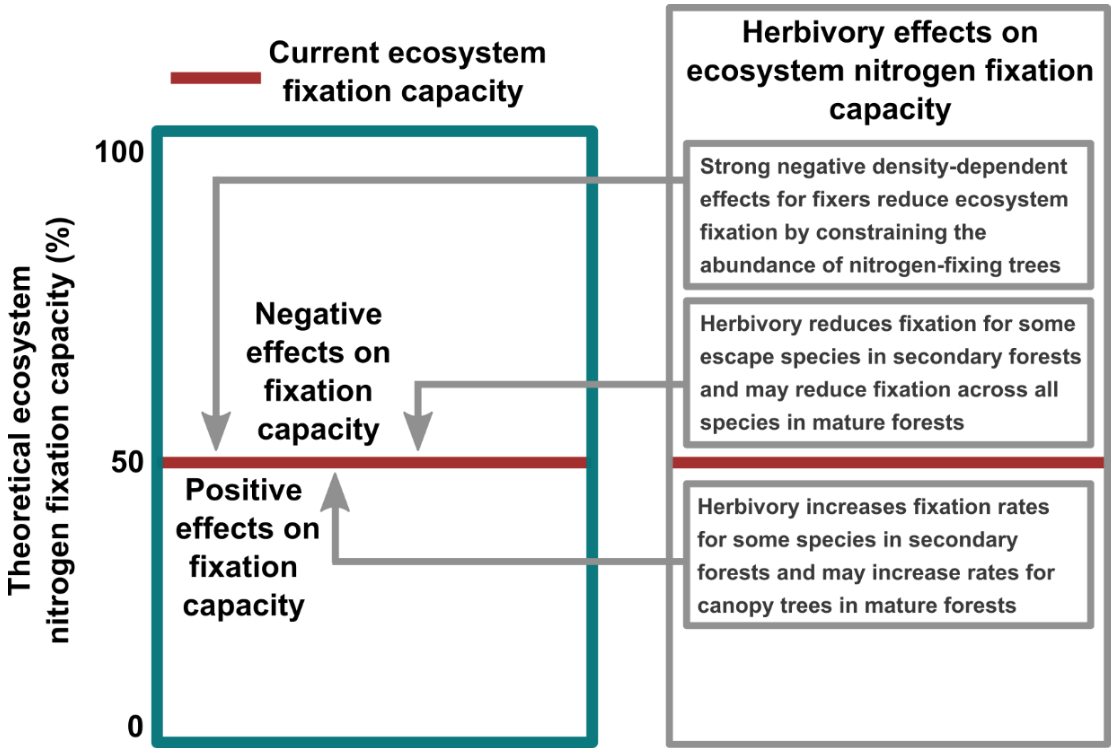


Figure 5. 2. The effects of herbivory on ecosystem nitrogen fixation capacity. *A conceptual figure showing the possible negative and positive effects of a higher herbivory cost for fixer species (in the grey box) on the capacity of tropical forest ecosystems to fix nitrogen (the blue box). Arrows show the expected direction of the herbivory effect (positive or negative) on the current theoretical fixation capacity (the red line).*

I find that ecological interactions may both promote and constrain tropical nitrogen fixation (see Figs. 5.2, 5.3, see Fig. 5.3 for a comparison of how my findings compare to my original hypotheses). The effects of herbivory on fixation rates for seedlings in secondary forests and canopy trees of some fixer species may promote the role of nitrogen-fixing trees in tropical forests. However, herbivory may reduce fixation rates for other species and for seedlings in mature forests. Furthermore, my finding of a herbivory driven strong negative density-dependent effects for fixer seedlings is likely to constrain ecosystem fixation rates (see Fig. 5.2). As discussed above, high negative density-dependent effects for fixers are likely to constrain abundances (Comita *et al.*, 2010), which would limit the total number of points (fixer trees) at which nitrogen can enter the ecosystem. Importantly, this would also reduce the number of fixers that could aggregate in any one place (up to at least 20m^2 as found in my research). This constraint on the density of fixer seedlings may limit the number of surviving fixers able to colonize recently disturbed areas of forest, where soil nitrogen would be expected to be low, and so therefore may limit the rate of forest recovery following disturbance (Batterman *et al.*, 2013b; Brookshire *et al.*, 2019). Further work is needed to establish the cost-benefit ratio of herbivory for tropical nitrogen fixation across life history stages and at the ecosystem scale.

5.6 Applications of my findings

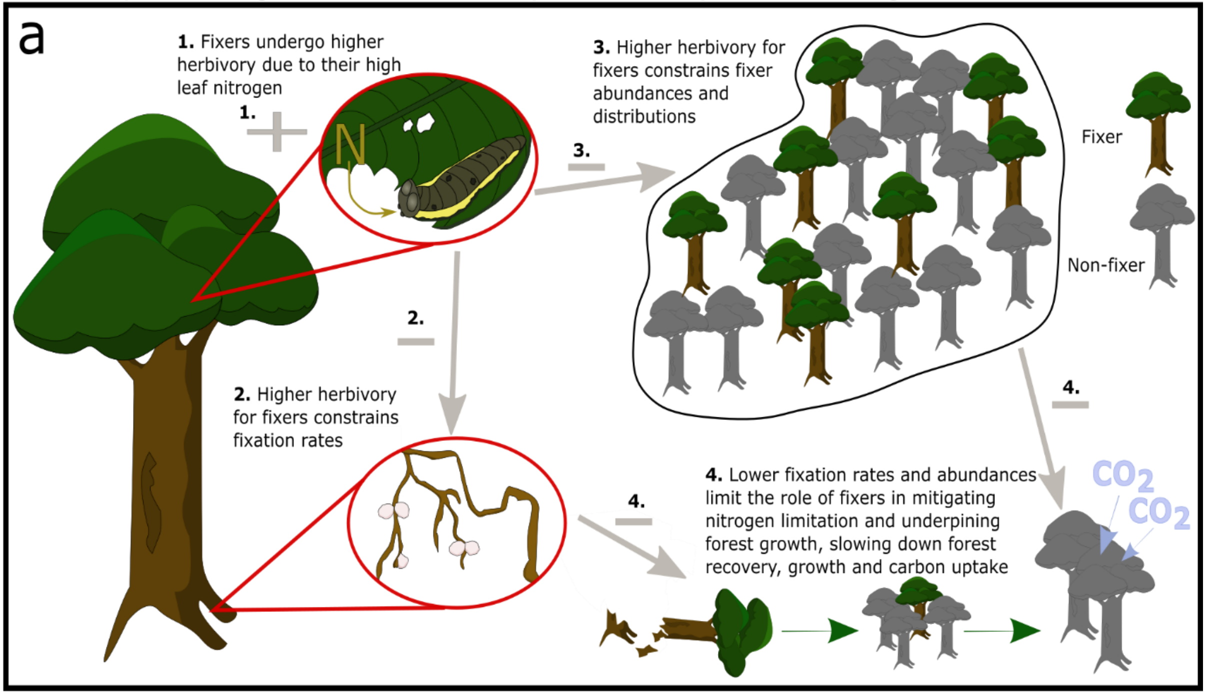
Together my findings suggest that there is a complex relationship between herbivory and tropical nitrogen fixation, but that herbivory is of critical importance to nitrogen cycling and therefore the carbon sink. Accordingly, my findings have important applications for future research concerned with nitrogen fixation, modelling of biogeochemical cycles and the terrestrial tropical carbon sink, and in reforestation efforts as a negative emissions technology to mitigate climate change.

First, my research has important implications for future research into tropical nitrogen fixation. This thesis has highlighted the importance of herbivory in governing tropical nitrogen fixation and has outlined several key knowledge gaps. It remains unclear (1) which fixation associated traits drive herbivory, whether these traits are unique to fixers or within the Fabaceae, and the role that plant defence takes in shaping herbivory for fixers, (2) how herbivory affects fixation rates for seedlings in mature forests, adult canopy trees and across a wider range of fixer species representative of the diverse range of plant antiherbivore strategies, and (3) what are the relative contributions of herbivory, attack by fungal pathogens, and competition between fixers in low soil nitrogen, to the strong negative density-dependent effects experienced by fixer seedlings. At a mechanistic level, I now know that herbivory governs fixation alongside nitrogen, phosphorus, molybdenum and light (Barron *et al.*, 2009; Batterman *et al.*, 2013c; Taylor and Menge, 2018) and further research is also needed to investigate how these factors interact to determine plant and ecosystem-level fixation rates.

Answering these questions will allow us to determine whether herbivory has a net positive or negative effect on fixation in tropical forests (see Fig. 5.2). Understanding the overall effect of herbivory on tropical nitrogen fixation may also help us to understand why nitrogen stores, turnover and exports are so substantial in tropical forests. The role of herbivory in governing nitrogen fixation has been long established in aquatic ecosystems and long suspected in terrestrial systems (Vitousek and Howarth, 1991; Vitousek and Field, 1999). The findings from this thesis provide clear evidence for the first time that herbivory does govern fixation in tropical ecosystems and should stimulate an exciting new body of research in terrestrial biogeochemistry.

Second, Dynamic Global Vegetation models, the terrestrial component of Earth System Models, currently often overestimate the capacity of biological nitrogen fixation to underpin tropical forest growth, by predominately basing fixation estimates on forest net primary productivity or evapotranspiration (Gerber *et al.*, 2010; Wieder *et al.*, 2015a). More ecologically realistic models of tropical nitrogen fixation incorporate fixation based on plant demand for nitrogen relative to supply that considered the carbon costs and benefits of fixation (Gerber *et al.*, 2010; Wieder *et al.*, 2015a). These models generate more accurate estimates of fixation when compared to field measurements than those used in in current dynamic global vegetation models (Gerber *et al.*, 2010; Wieder *et al.*, 2015a; Levy-Varon *et al.*, 2019). However, importantly, no simulations of tropical nitrogen fixation to date have included the effects of herbivory.

Conceptual figure: Herbivory and tropical nitrogen fixation



Synthesis of results: Herbivory and tropical nitrogen fixation

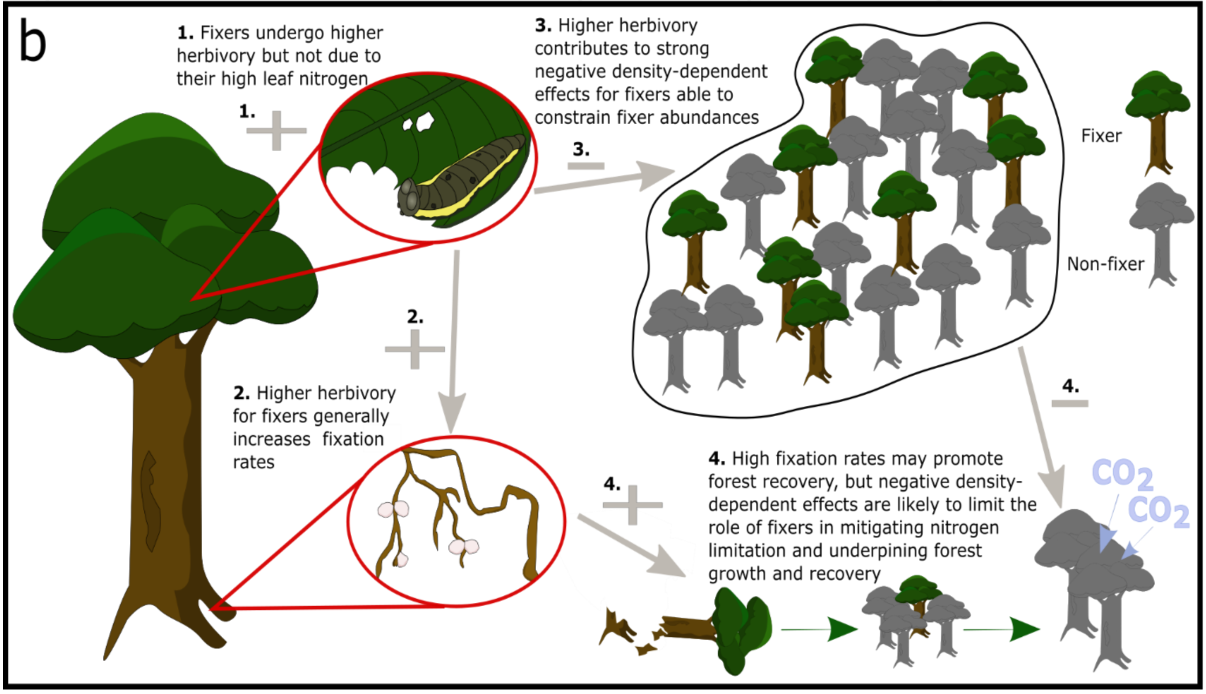


***Figure 5. 3**. **Changes in my understanding of the role of herbivory in governing tropical nitrogen fixation.** Panel a shows how I expected high herbivory for fixers, driven by high leaf nitrogen (1), to reduce the fixation rates of individual nitrogen-fixing trees (2), to constrain the abundance and distribution of fixer individuals (3), and the consequences this will have on forest growth and, in particular, forest recovery after disturbance (4). Panel b reflects how my understanding of the role of herbivory in governing nitrogen fixation has been changed by my findings. I now know that fixers do undergo higher herbivory but find no evidence that this is due to high leaf nitrogen concentrations for fixers (1) and that this high herbivory for fixers increases fixation rates overall, despite some variation in fixation*

responses to herbivory (2). I next find that herbivory contributes to much stronger negative density-dependent effects for fixers than non-fixers, which could constrain fixer abundances and distributions (3). Combined, my findings suggest that herbivory may increase fixation rates in recovering forest, and may upregulate fixation rates of adult trees in mature forests, but that strong negative density dependent effects will limit the number of fixers and the ability of fixers to aggregate at sites with low soil nitrogen, therefore limiting the role of fixation in mitigating nitrogen limitation of tropical forest growth (4). Minus symbols represent a negative effect of each step on the next, whilst a positive symbol represents a positive effect.

My findings (1) that herbivory is a major carbon cost to fixers and may upregulate fixation in certain forest types and (2) that fixers undergo strong negative density dependent effects that are likely to constrain fixer abundances and distributions suggest that both the fixation rates of individual trees and the spread of fixers across ecosystems will vary greatly across tropical forests. Herbivory pressure itself is also likely to vary within and across forests and forest types, as has been found over altitudinal, rainfall and soil nutrient gradients (Metcalfe *et al.*, 2014; Galmán *et al.*, 2018; Weissflog *et al.*, 2018). These differences in herbivory pressure across tropical forests may create further variation in the amount of nitrogen fixed across ecosystems. Some recent Earth Systems Models have begun to incorporate ways of modelling the cost-benefit trade off of nitrogen fixation. For example, the most recent Community Land Model, Version 5, uses the Fixation and Uptake of Nitrogen model which simulates the dynamics and carbon cost of nitrogen fixation for fixing plants by having a carbon cost paid for each gram of nitrogen fixed (Fisher *et al.*, 2019). However, whilst including the carbon economics of nitrogen fixation is a positive step, the majority of Earth Systems Models still do not account for the ability of nitrogen-fixing trees to regulate their fixation rates in response to nitrogen or light and none include herbivory, and so will not capture further variation in fixation rates across tropical forests driven by herbivory (Wieder *et al.*, 2015b; Fisher *et al.*, 2019). It is therefore likely that nitrogen fixation in tropical forests is more heterogenous and plastic in response to changing environmental factors such as atmospheric CO_2 than currently predicted by Dynamic Global Vegetation models.

To realistically simulate fixation in tropical forests both the inherent costs of fixation and the fixation-associated cost of herbivory must be incorporated into Dynamic Global Vegetation Models, as well as the effects of herbivory on plant fixation rates, abundances, and distributions. I therefore suggest, first, that modellers include mechanisms to simulate the carbon costs of fixation, such as by incorporating the Fixation and Uptake of Nitrogen model, and second, that fixers should face an additional, constant, and fixer-specific herbivory cost of ~3.3% of leaf carbon, as I identified in my second chapter. This is similar to the cost of a ~ 4% higher biomass turnover rate for fixers found to constrain fixation in early modelling by Vitousek and Field (1999). This cost of herbivory should increase with fixer density where possible to effectively model the negative-density dependent effects outlined in my fourth chapter. Further work will be needed to establish how strong or weak a negative density-dependent effect is needed to replicate observed fixer abundances across different forests and how these seedling dynamics may drive adult tree abundance. Finally, to incorporate the effects of herbivory on fixation rates, further research is needed. We need to know whether the response of upregulating fixation rates following herbivory, identified in seedlings for my second chapter, is representative of a wider range of fixer species. Also, we will need to determine whether this effect is also consistent for adult trees. Fixers fulfil a critical and well-established role in underpinning tropical forest growth and recovery, so these omissions are likely to confound estimates of carbon uptake rates in tropical forests.

Finally, my findings will be of importance for reforestation programs as a strategy to mitigate climate change. Planting fixers in such programs is likely to support forest growth and recovery, promoting carbon sequestration in young forests and carbon storage once these forests reach maturity (Voigtlaender *et al.*, 2012; Batterman *et al.*, 2013b; Sang *et al.*, 2013). My findings suggest that herbivory on fixer seedlings in secondary forests may upregulate fixation rates and so promote this critical role of fixer seedlings. However, my result of strong negative density-dependent effects for fixer species indicates that fixer seedlings should be well spaced in planting programs, and argues against planting only fixer species in an attempt to rapidly increase carbon uptake or soil fertility on disturbed land. I would therefore recommend that fixers are included in a diverse species mix in tropical reforestation programmes and that deterrents against herbivory are not used, to prevent high fixer seedling mortality at high conspecific densities and to allow for herbivory to facilitate fixation and distribute fixed leaf nitrogen across the site.

5.7 Conclusion

My findings highlight a critical role for herbivory in governing tropical nitrogen fixation by first, affecting the fixation rates of individual trees, and second, in driving density-dependent mechanisms that can explain why the basal area of nitrogen-fixing trees is capped at ~15% across the neotropics (Ter Steege *et al.*, 2006; Gei *et al.*, 2018). My findings may also explain why fixer abundances are limited in Asian mature tropical forests and in temperate forests (Menge *et al.*, 2017, 2019). The effects of herbivory here identified are only likely to become stronger as herbivory pressure increases under continuing climate and land use change (Hahn *et al.*, 2015; Morante-Filho *et al.*, 2016; Zavala *et al.*, 2017; Hall *et al.*, 2020). The role of abiotic factors like soil nitrogen in governing tropical nitrogen fixation is well established (Barron *et al.*, 2011; Batterman *et al.*, 2013c, 2013b; Wurzburger and Hedin, 2016; Brookshire *et al.*, 2019; Levy-Varon *et al.*, 2019). The work carried out in my thesis now expands my understanding of the essential ecosystem function of nitrogen fixation to also include biotic interactions - I find that plant-herbivore interactions likely govern the prevalence and the role of fixation in tropical forests. My findings are therefore of vital importance for understanding tropical biodiversity, nitrogen cycling and nitrogen limitation of the tropical terrestrial carbon sink in a changing climate (Janzen, 1970; Hedin *et al.*, 2009; Townsend *et al.*, 2011; Vitousek *et al.*, 2013; Brienen *et al.*, 2015; Terrer *et al.*, 2019).

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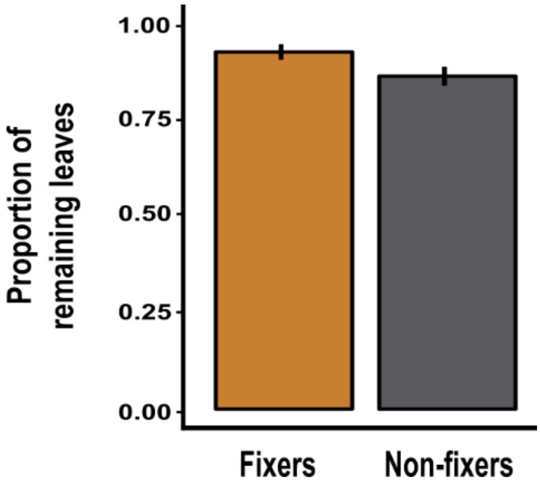
Appendices

Appendix 1

The differences in leaf retention between fixer and non-fixer seedlings over three months in the 50-ha plot on BCI

I explored the role of leaf retention in driving higher herbivory measurements for fixers than non-fixers. Theoretically, if non-fixers drop leaves more readily after herbivory it could reduce average herbivory measurements for the non-fixer group, as I did not measure herbivory on fallen leaves. Accordingly, I recorded leaf retention for 226 young leaves over three months in 2017. I found no significant difference in leaf turnover rate between fixers ($p > 0.05$, $R^2 = 0.16$) and non-fixers, suggesting that differences in herbivory between the two functional groups are not driven by differences in leaf retention, but are governed by other leaf and species traits (See supplemental Fig. 1).



A1. Supplemental Figure 1. No difference in leaf retention between fixer and non-fixer seedlings from over three months. Error bars represent standard error.

Appendix 2

Supplemental methods for chapter two

Nutrient and physical defence traits

For nutrient and physical defence traits, three leaves were sampled from the highest point of the crown for the largest six and smallest six individuals of each species in the 50-ha plot on BCI and then stored on ice until they could be oven dried at 60 C. All samples were measured within 2-24 hours after sampling[1].This work was carried out between July 2007 and January 2008. Leaves were sampled across species and light environment, with two observers estimating a crown exposure index for each individual[2]. The crown exposure index contains five values, from 1 (no exposure), 2 (lateral light only), 3 (partial exposure to overhead light), 4 (full exposure to overhead light) or 5 (emergent). Crown exposure values 1 and 2 were counted as shade leaves whilst 3-5 were recorded as sun leaves[2,3]. In my survey, I collected herbivory data for seedlings under the canopy, so I only used shade leaf trait data. To measure the species averages for leaf nutrient and defensive fibre concentrations, composite samples were made by mixing the same amount (mass) of ground leaf tissues from the individuals sampled across each species and light environment. Thus, each value is a mean calculated from three to six individuals for each species in each light level environment. An adapted previous method of Van Soest et al. (1991) was chosen to calculate the cellulose, hemicellulose, silicon and lignin concentrations per unit leaf dry mass (see Appendix A of Westbrook et al., 2011)[2].

Measure of leaf chemical profile similarity

In addition, metrices of chemical similarity were included that allow differences in secondary metabolites to be compared between species and species groups. These metrics distinguish molecular networks of unidentified compounds, methanol extracted from homogenized leaf tissue. Molecules are identified using ultra high-performance liquid chromatography, electrospray ionization and molecular fragmentation, and tandem mass spectrometry of molecular fragments (see Sedio et al., 2017)[4]. Networks of these molecules can be constructed that portray the structural similarity of unknown compounds, as molecules with similar structures fragment into many of the same substructures. By then comparing the mass to charge ratio of the fragments of two molecules, one can compare the similarity in the molecule structures. Structural similarity can then be quantified for every pair of compounds as the cosine of the angle between vectors that comprise the mass to charge ratio of their constituent fragments, with a cosine of over 0.6 showing a meaningful similarity. All pairwise combinations of said compounds were then used to calculate Compositional Structural Chemical Similarity (CSCS) for each pairwise combination of species sampled. From this, two variables were derived, first, the nearest neighbour Chemical Structural and Compositional Similarity (nnCSCS), which measures the similarity in leaf secondary metabolites between the two most chemically related species in the plot, and second, the mean Chemical Structural and Compositional Similarity (mCSCS), which measures the mean similarity across all pairs of species and acts to show the similarity between the focal species and the entire species group.

Direct measures of leaf toughness

Measurements of leaf toughness were also included as explanatory variables, as greater leaf toughness makes it more difficult for insect herbivores to damage leaves. Leaf toughness is derived from a combination of leaf lamina thickness and density and the concentration of the various microfibrils described above (cellulose, hemicellulose, lignin). Fundamentally, toughness is measured as resistance to fracture, either per unit dry mass (density corrected fracture toughness), unit volume (fracture toughness (J cm^{-2})) or unit cut length (Work to shear (J cm^{-1})). These measures, alongside lamina density (g cm^{-3}) and Leaf Mass per Area (LMA, g cm^{-2}) were also included as they have been previously found to correlate with increased leaf lifespan and reduced herbivory (for protocol, see Westbrook et al., 2011, Kitajima et al., 2012, Kitajima et al., 2016), but these variables did not differ between fixers and non-fixers, nor explain what drove herbivory across species, and so are not included in my main analyses[2,5,6].

Methods references

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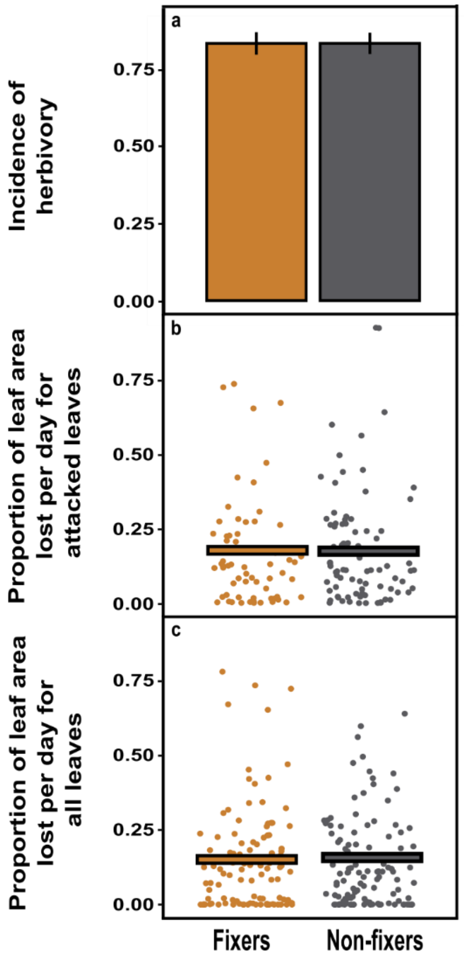
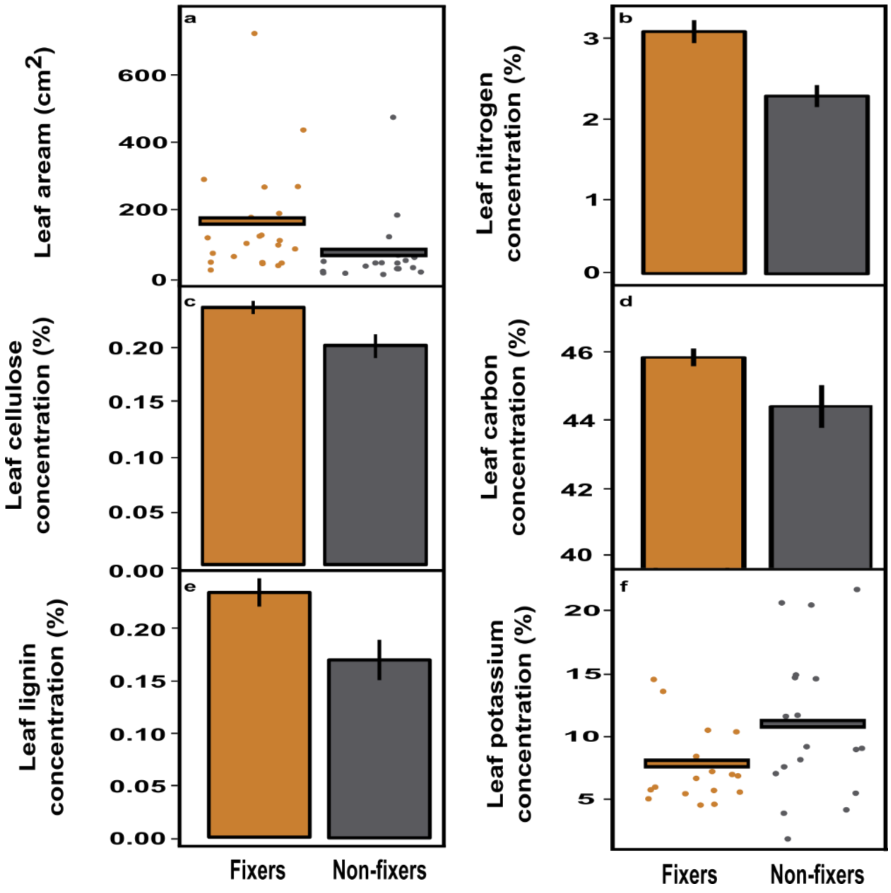
Appendix 3

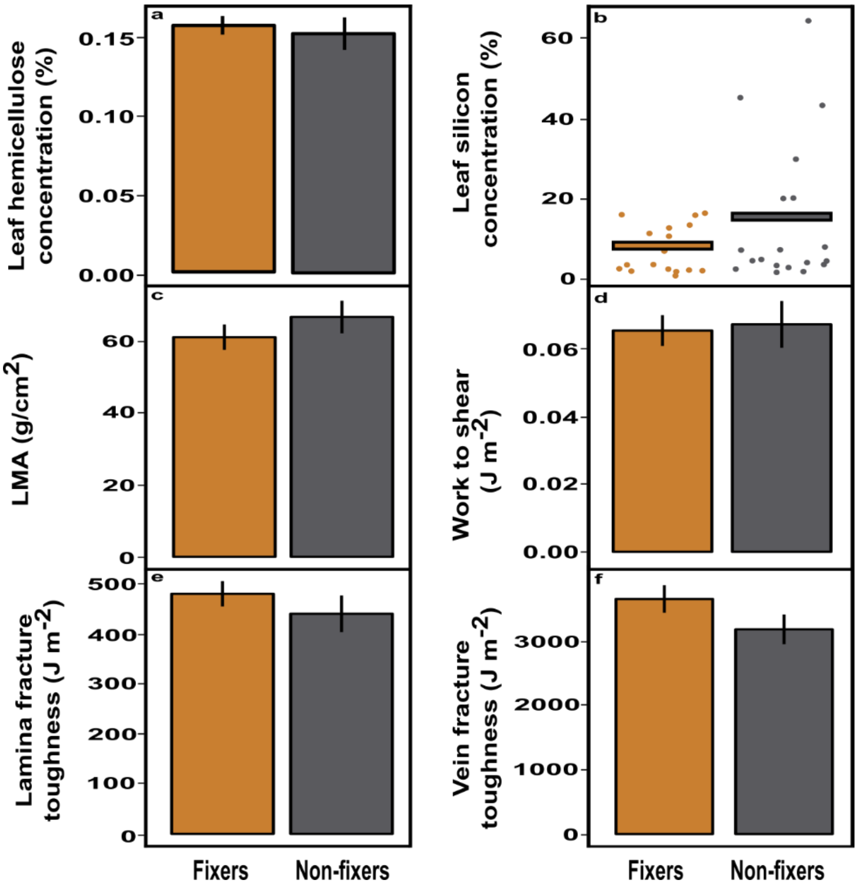
Supplemental figures for chapter two



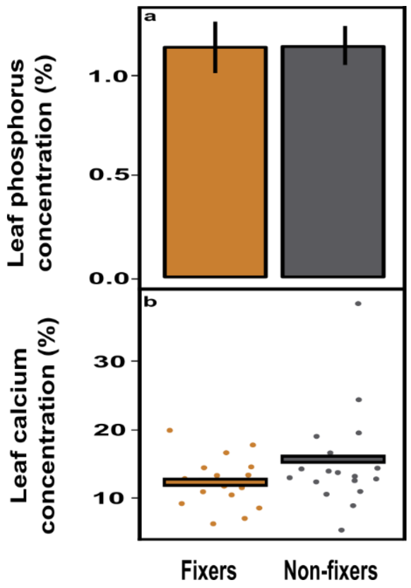
A3. Supplemental Figure 1. Differences in herbivory on young leaves between fixers and non-fixers. For young leaves, the Incidence of herbivory (a), the proportion of leaf area lost to herbivory per day for damaged leaves (Proportion^{damaged}) from each seedling (b) and the proportion of leaf area lost to herbivory per day for all leaves (Proportion^{all}) from each per seedling (c), for fixers (orange) and non-fixers (grey). Bars represent mean +/- standard error.



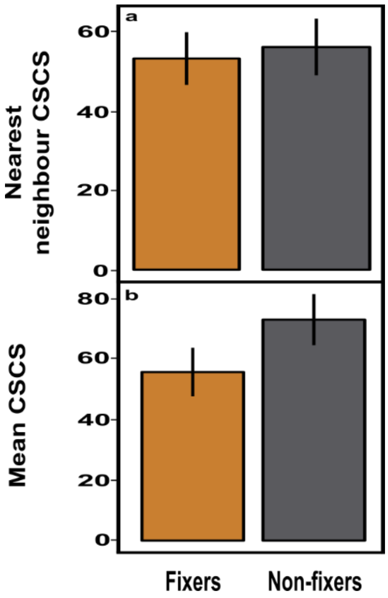
A3. Supplemental Figure 2. Leaf traits that vary between fixer and non-fixer species. The difference in average leaf area (a), leaf nitrogen concentration (b), leaf cellulose concentration (c), leaf carbon concentration (d), leaf lignin concentration (e) and leaf potassium concentration (f). Bars represent mean +/- standard error. All differences are significant. P values are derived from Wilcoxon rank test.



A3. Supplemental Figure 3. Defensive leaf traits that did not vary between fixer and non-fixer species. The average leaf hemicellulose concentration (a), leaf silicon concentration (b), Leaf Mass per Area (LMA, c), work to shear (d), lamina facture toughness (e) and vein fracture toughness (f). Bars represent mean +/- standard error. There are no significant differences between fixers and non-fixers for these variables. P values are derived from Wilcoxon rank test.



A3. Supplemental Figure 4. Nutrient leaf traits that did not vary between fixer and non-fixer species. The average leaf phosphorus concentration (a) and leaf calcium concentration (b). Bars represent mean +/- standard error. There are no significant differences between fixers and non-fixers for these variables. P values are derived from Wilcoxon rank test.



A3. Supplemental Figure 5. Measures of leaf chemical similarity between species that did not vary between fixer and non-fixer species. The average nearest neighbour Compositional Structural Chemical Similarity (CSCS) (a) and mean CSCS (b). These are measures of chemical similarity between a focal species and its most chemically similar species in the 50-haplot (a) and of the average chemical similarity between all pairwise combinations of species (b). Bars represent mean +/- standard error. There are no significant differences between fixers and non-fixers for these variables. P values are derived from Wilcoxon rank test.

Appendix 4: <u>Supplemental tables 1 for chapter two: Leaf traits expected to govern herbivory</u>

A4. Supplemental Table 1. The variables included in my linear mixed effects modelling approach, with the appropriate units, the hypothetical mechanism by which they would affect herbivory, predicted effect on herbivory and reference.

Variable	**Variable category**	**Units**	**Mechanism**	**Expected effect**	**Reference**
Fixation status	Species attributes	A categorical variable where species were split into two groups, those either able or unable to fix atmospheric nitrogen	Herbivory has been suggested as an explanation for low fixer abundances across the tropics, despite the advantage of the trait of nitrogen fixation. Fixers may undergo high herbivory due to higher average leaf nitrogen concentrations than non-fixers, as insect herbivores have been previously shown to preferentially target nitrogen rich leaf tissue.	Positive	(Vitousek and Howarth, 1991; Vitousek and Field, 1999, Menge et al., 2008; Ritchie and Tilman, 1995; Ritchie and Raina, 2016)
Nitrogen concentration	Leaf nutrients	Dry mass mg*100 g^{-1} leaf tissue	Herbivores target leaf tissue high in nitrogen, as nitrogen is a limiting nutrient for insect growth and metamorphosis.	Positive	(Matson Jr, 1980; Kursar and Coley, 1991, 2003)
Carbon concentration	Leaf nutrients	Dry mass mg*100 g^{-1} leaf tissue	Carbon is an important macronutrient needed by insects for growth but is also found in other chemical forms used for herbivore defence traits (such as cellulose).	Positive or negative	(Kursar and Coley, 1991; Coley et al., 2005)
Phosphorus concentration	Leaf nutrients	Dry mass mg*100 g^{-1} leaf tissue	An additional key/limiting nutrient needed by insect herbivores for growth.	Positive	(Matson Jr, 1980; Perkins et al., 2004)
Potassium concentration	Leaf nutrients	Dry mass mg*100 g^{-1} leaf tissue	An additional key/limiting nutrient needed by insect herbivores for growth.	Positive	(Matson Jr, 1980; Perkins et al., 2004)

Appendix 4

Variable	**Variable category**	**Units**	**Mechanism**	**Expected effect**	**Reference**
Calcium concentration	Leaf nutrients	Dry mass mg*100 g^{-1} leaf tissue	An additional key/limiting nutrient needed by insect herbivores for growth, but also a component of plant cell walls that may deter chewing insect herbivores.	Positive or negative	(Demarty et al., 1984; Perkins et al., 2004)
Leaf area	Leaf physical traits	Total leaf area (m^{-2})	Larger leaves may be easier to locate, land on, lay eggs on and may also take longer to expand through the early vulnerable stage of leaf development.	Positive	(Feeny, 1976; Ribeiro et al., 1994; Moles and Westoby, 2000)
LMA	Leaf physical traits	Mean leaf mass per unit area measured for the entire leaf including the petiole (g m^{-2})	Low LMA usually corelates to high growth rates, and therefore increased susceptibility to herbivory.	Negative	(Kitajima and Poorter, 2010; Westbrook et al., 2011; Kitajima et al., 2012, 2016; Osnas et al., 2018)
Work to shear	Leaf physical traits	Structural toughness of leaf tissue (J m^{-1})	A measure of the mechanical toughness of leaf tissue (toughness per unit cut length), and therefore difficulty in chewing, that reflects leaf thickness and material strength.	Negative	(Kitajima and Poorter, 2010; Westbrook et al., 2011; Kitajima et al., 2012, 2016; Osnas et al., 2018)
Vein toughness	Leaf physical traits	Mean fracture toughness of central vein (J m^{-2})	A measure of the material toughness, and therefore difficulty in chewing, of leaf vein tissue. Material toughness is driven by lamina density and cellulose concentrations.	Negative	(Kitajima and Poorter, 2010; Westbrook et al., 2011; Kitajima et al., 2012, 2016; Osnas et al., 2018)

Variable	**Variable category**	**Units**	**Mechanism**	**Expected effect**	**Reference**
Lamina toughness	Leaf physical traits	Mean fracture toughness of leaf lamina (J m^{-2})	A measure of the material toughness, and therefore difficulty in chewing, of leaf lamina tissue. Material toughness is driven by lamina density and cellulose concentrations.	Negative	(Kitajima and Poorter, 2010; Westbrook et al., 2011; Kitajima et al., 2012, 2016; Osnas et al., 2018)
Cellulose concentration	Leaf physical traits	Dry mass mg g^{-1} leaf tissue	A key defensive microfibril found to toughen cell walls and prevent chewing by insect herbivores. May also hinder digestion of leaf material.	Negative	(Westbrook et al., 2011; Kitajima et al., 2012, 2016)
Hemicellulose concentration	Leaf physical traits	Dry mass mg g^{-1} leaf tissue	A key defensive microfibril that cross links cellulose fibres within the cell wall. However, recent work suggests that higher hemicellulose concentrations may reduce leaf fracture toughness. Higher hemicellulose concentrations may be found in species specializing in growth rather than defence. Hemicellulose may act to fortify veins within leaves, and greater venation is found in sun adapted, high growth species.	Negative	(Westbrook et al., 2011; Kitajima et al., 2012, 2016)
Lignin concentration	Leaf physical traits	Dry mass mg g^{-1} leaf tissue	A key defensive microfibril that cross links cellulose fibres within the cell wall; however, recent work suggests that lignin may reduce fracture toughness of leaves.	Negative	(Westbrook et al., 2011; Kitajima et al., 2012, 2016)

Appendix 4

Variable	**Variable category**	**Units**	**Mechanism**	**Expected effect**	**Reference**
Silicon concentration	Leaf physical traits	Dry mass mg g^{-1} leaf tissue	Silicon has been shown to deter feeding by insect and mammalian herbivores and to reduce digestibility of leaf tissue.	Negative	(Massey et al., 2007; Hartley and Degabriel, 2016; Schaller et al., 2018)
Nearest neighbour Chemical Structural and Compositional Similarity (nnCSCS)	Leaf chemical profile	Derived metric representing the similarity in secondary metabolites between a focal species and its nearest chemical neighbour in the 50ha plot, BCI	Whilst physical defences are the immediate deterrent to herbivores, chemical defences can also play a role. Species reliant on chemical defences throughout evolution should be more chemically distinct from their neighbours in order to escape specialist herbivores.	Positive	(Sedio et al., 2017; Coley et al., 2018; Endara et al., 2018)
Mean Chemical Structural and Compositional Similarity (mCSCS)	Leaf chemical profile	Derived metric representing the similarity in secondary metabolites between a focal species and the species community in the 50ha plot, BCI	Whilst physical defences are the immediate deterrent to herbivores, chemical defences can also play a role. Species reliant on chemical defences throughout evolution should be more chemically distinct from their neighbours in order to escape specialist herbivores.	Positive	(Sedio et al., 2017; Coley et al., 2018; Endara et al., 2018)

Appendix 5

Supplemental tables 2 for chapter two: the difference in herbivory between fixers and non-fixers

The effect of fixation in driving measures of herbivory across mature and young leaves.

A5. Supplemental Table 1: The effect of fixation, leaf area and stem length in driving **the incidence of herbivory** across all species (R^2c: 0.34, R^2m: 0.08, AIC: 1766.46, number of observations: 1626 (862 fixer leaves, 764 non-fixer leaves)). Leaf area was standardized within species. Seedling, species and plot identity were included as random effects.

Variable	**Estimate**	**Z value**	**p value**
Fixation	1.1	3.5	**0.00051**
Leaf area	0.26	3.5	**0.00044**
Stem length	0.09	0.98	0.33

A5. Supplemental Table 2: The effect of fixation, leaf area and stem length in driving **the proportion of leaf area lost to herbivory for damaged leaves (Proportion^{damaged})** across all species (R^2c: 0.21, R^2m: 0.02, AIC: -349.65, number of observations: 326 (177 fixer seedlings, 149 non-fixer seedlings)). Leaf area was standardized within species. Species and plot identity were included as random effects.

Variable	**Estimate**	**Z value**	**p value**
Fixation	0.039	1.8	0.078
Leaf area	-0.00027	-0.033	0.97
Stem length	0.0017	0.21	0.84

A5. Supplemental Table 3: The effect of fixation, leaf area and stem length in **the proportion of leaf area lost for all leaves (Porportion^{all})** to herbivory across species (R^2c: 0.22, R^2m: 0.04, AIC: -1070.9, number of observations: 350 (184 fixer seedlings, 166 non-fixer seedlings)). Leaf area was standardized within species. Species and plot identity were included as random effects.

Variable	**Estimate**	**Z value**	**p value**
Fixation	0.31	2.1	**0.04**
Leaf area	0.057	1.1	0.28
Stem length	0.026	0.47	0.64

A5. Supplemental Table 4: The effect of fixation, leaf area and stem length in **driving the incidence of herbivory** observed over three months for young leaves across all species (R^2c: 0.22, R^2m: 0.01 AIC: 207.51, number of observations: 226 (119 fixers, 107 non-fixers)). Leaf area was standardized within species. Seedling, species and plot identity were included as random effects.

Variable	**Estimate**	**Z value**	**p value**
Fixation	0.047	0.11	0.92
Leaf area	0.18	0.77	0.44
Stem length	-0.19	-0.9	0.37

A5. Supplemental Table 5: The effect of fixation, leaf area and stem length in driving **the proportion of leaf area lost to herbivory per day for damaged young leaves** across all species (R^2c: 0.05, R^2m: 0.03, AIC: -20.07, number of observations: 149 (82 fixer leaves, 67 non-fixer leaves). Leaf area was standardized within species. Species and plot identity were included as random effects.

Variable	**Estimate**	**Z value**	**p value**
Fixation	0.0027	0.073	0.94
Leaf area	0.035	1.8	0.073
Stem length	0.003	0.17	0.86

A6. Supplemental Table 6: The effect of fixation, leaf area and stem length in driving **the proportion of leaf area lost per day for all young leaves** over three months across all species (R^2c: 0.02, R^2m: 0.NA, AIC: -432.77, number of observations: 226 (119 fixers, 107 non-fixers)). Leaf area was standardized within species. Species and plot identity were included as random effects

Variable	**Estimate**	**Z value**	**p value**
Fixation	-0.044	-0.32	0.75
Leaf area	0.13	1.5	0.13
Stem length	-0.1	-1.2	0.21

Appendix 6

Supplemental tables 3 for chapter two: the role of leaf traits in driving differences in herbivory between fixers and non-fixers

Tables showing the output from my models comparing the relationship between leaf traits and measures of herbivory across fixer species, non-fixer species and all species grouped together. These models were run to determine if each leaf trait explained higher herbivory for fixers - I would expect a trait that drives high herbivory for fixers to have a consistent relationship across all species groups. The models were run for leaf nitrogen concentration, leaf area, leaf carbon concentration, leaf potassium concentration, leaf cellulose concentration and leaf lignin concentration.

Leaf nitrogen concentration

A6. Supplemental Table 1: The effect of leaf nitrogen concentration, leaf area and stem length in driving the **incidence of herbivory** across all species, fixer species alone and non-fixer species alone (number of observations: 350 (184 fixers, 166 non-fixers)). All three fixed effects were included in each model, only changing the data set between models. Leaf area was standardized within species. Seedling, species and plot identity were included as random effects.

Dataset	Variables	Estimate	Z Value	p value	R^2m	R^2c
All species	Leaf nitrogen concentration	0.37	2.2	**0.029**	0.041	0.33
	Leaf area	0.24	3.2	**0.0014**		
	Stem length	0.071	0.74	0.46		
Fixers species	Leaf nitrogen concentration	-0.18	-0.81	0.42	0.047	0.26
	Leaf area	0.42	3.3	**0.00081**		
	Stem length	0.049	0.33	0.74		
Non-fixer species	Leaf nitrogen concentration	0.45	1.6	0.11	0.033	0.3
	Leaf area	0.13	1.3	0.18		
	Stem length	0.083	0.67	0.5		

A6. Supplemental Table 2: The effect of leaf nitrogen concentration, leaf area and leaf number in driving **the proportion of leaf area lost to herbivory for damaged leaves (Proportion$^{\mathbf{damaged}}$)** across all species, fixer species alone and non-fixer species alone (number of observations: 350 (184 fixers, 166 non-fixers)). All three fixed effects were included in each model, only changing the data set between models. Leaf area was standardized within species. Seedling and plot identity were included as random effects.

Dataset	Variables	Estimate	Z Value	p value	R^2m	R^2c
All species	Leaf nitrogen concentration	0.013	1.2	0.25	0.0093	0.22
	Leaf area	-0.0011	-0.13	0.89		
	Stem length	0.002	0.23	0.82		
Fixers species	Leaf nitrogen concentration	-0.018	-0.97	0.35	0.022	0.32
	Leaf area	0.013	1.1	0.25		
	Stem length	0.00084	0.065	0.95		
Non-fixer species	Leaf nitrogen concentration	0.024	1.5	0.18	0.037	0.24
	Leaf area	-0.017	-1.4	0.18		
	Stem length	-0.0033	-0.29	0.77		

A6. Supplemental Table 3. The effect of leaf nitrogen concentration, leaf area and leaf number in driving **the proportion of leaf area lost to herbivory for all leaves (Proportion^{all})** across all species, fixer species alone and non-fixer species alone (number of observations: 350 (184 fixers, 166 non-fixers)). All three fixed effects were included in each model, only changing the data set between models. Leaf area was standardized within species. Seedling, species and plot identity were included as random effects

Dataset	**Variables**	**Estimate**	**Z Value**	**p value**	**R^{2}m**	**R^{2}c**
All species	Leaf nitrogen concentration	0.1	1.3	0.2	0.018	0.21
	Leaf area	0.053	0.97	0.33		
	Stem length	0.034	0.6	0.55		
Fixers species	Leaf nitrogen concentration	-0.074	-0.58	0.56	0.022	0.31
	Leaf area	0.13	1.6	0.1		
	Stem length	-0.037	-0.42	0.68		
Non-fixer species	Leaf nitrogen concentration	0.12	0.87	0.38	0.016	NA
	Leaf area	-0.00021	-0.0027	1		
	Stem length	0.069	0.9	0.37		

Leaf carbon concentration

A6. Supplemental Table 4. The effect of leaf carbon concentration, leaf area and leaf number in driving **the incidence of herbivory** across all species, fixer species alone and non-fixer species alone (number of observations: 350 (184 fixers, 166 non-fixers)). All three fixed effects were included in each model, only changing the data set between models. Leaf area was standardized within species. Seedling, species and plot identity were included as random effects.

Dataset	**Variables**	**Estimate**	**Z Value**	**p value**	**R^2m**	**R^2c**
All species	Leaf carbon concentration	0.49	3	**0.0026**	0.064	0.33
	Leaf area	0.25	3.3	**0.0011**		
	Stem length	0.05	0.53	0.6		
Fixers species	Leaf carbon concentration	0.17	0.43	0.67	0.044	0.26
	Leaf area	0.41	3.3	**0.00091**		
	Stem length	0.064	0.43	0.67		
Non-fixer species	Leaf carbon concentration	0.4	2.3	**0.02**	0.055	0.3
	Leaf area	0.14	1.4	0.16		
	Stem length	0.056	0.45	0.65		

A6. Supplemental Table 5. The effect of leaf carbon concentration, leaf area and leaf number in driving **the proportion of leaf area lost to herbivory for damaged leaves (Proportion^{damaged})** across all species, fixer species alone and non-fixer species alone (number of observations: 350 (184 fixers, 166 non-fixers)). All three fixed effects were included in each model, only changing the data set between models. Leaf area was standardized within species. Seedling, species and plot identity were included as random effects.

Dataset	**Variables**	**Estimate**	**Z Value**	**p value**	**R^{2}m**	**R^{2}c**
All species	Leaf carbon concentration	0.013	1	0.3	0.0076	0.21
	Leaf area	-0.00055	-0.065	0.95		
	Stem length	0.00058	0.066	0.95		
Fixers species	Leaf carbon concentration	-0.023	-0.69	0.5	0.017	0.33
	Leaf area	0.013	1.1	0.27		
	Stem length	0.0024	0.19	0.85		
Non-fixer species	Leaf carbon concentration	0.011	1	0.33	0.027	0.24
	Leaf area	-0.016	-1.3	0.2		
	Stem length	-0.0048	-0.41	0.68		

A6. Supplemental Table 6. The effect of leaf carbon concentration, leaf area and leaf number in driving **the proportion of leaf area lost to herbivory for all leaves (Proportion^{all})** across all species, fixer species alone and non-fixer species alone (number of observations: 350 (184 fixers, 166 non-fixers)). All three fixed effects were included in each model, only changing the data set between models. Leaf area was standardized within species. Seedling, species and plot identity were included as random effects.

Dataset	**Variables**	**Estimate**	**Z Value**	**p value**	**R^2m**	**R^2c**
All species	Leaf carbon concentration	0.097	1.2	0.24	0.018	0.21
	Leaf area	0.057	1	0.3		
	Stem length	0.026	0.46	0.65		
Fixers species	Leaf carbon concentration	-0.14	-0.62	0.54	0.024	0.31
	Leaf area	0.12	1.6	0.11		
	Stem length	-0.031	-0.36	0.72		
Non-fixer species	Leaf carbon concentration	0.08	1.3	0.2	0.017	NA
	Leaf area	0.00024	0.0029	1		
	Stem length	0.033	0.42	0.67		

Leaf potassium concentration

A6. Supplemental Table 7. The effect of leaf potassium concentration, leaf area and leaf number in driving **the incidence of herbivory** across all species, fixer species alone and non-fixer species alone (number of observations: 350 (184 fixers, 166 non-fixers)). All three fixed effects were included in each model, only changing the data set between models. Leaf area was standardized within species. Seedling, species and plot identity were included as random effects.

Dataset	**Variables**	**Estimate**	**Z Value**	**p value**	**R^2m**	**R^2c**
All species	Leaf potassium concentration	0.044	0.23	0.82	0.014	0.33
	Leaf area	0.24	3.2	**0.0013**		
	Stem length	0.057	0.59	0.55		
Fixers species	Leaf potassium concentration	-0.13	-0.37	0.71	0.043	0.26
	Leaf area	0.42	3.3	**0.00086**		
	Stem length	0.056	0.38	0.71		
Non-fixer species	Leaf potassium concentration	0.32	1.6	0.1	0.036	0.3
	Leaf area	0.13	1.3	0.18		
	Stem length	0.082	0.66	0.51		

A6. Supplemental Table 8. The effect of leaf potassium concentration, leaf area and leaf number in driving **the proportion of leaf area lost to herbivory for damaged leaves (Proportion^{damaged})** across all species, fixer species alone and non-fixer species alone (number of observations: 350 (184 fixers, 166 non-fixers)). All three fixed effects were included in each model, only changing the data set between models. Leaf area was standardized within species. Seedling, species and plot identity were included as random effects.

Dataset	**Variables**	**Estimate**	**Z Value**	**p value**	**R^{2}m**	**R^{2}c**
All species	Leaf potassium concentration	0.002	0.18	0.86	0.00029	0.21
	Leaf area	-0.00082	-0.097	0.92		
	Stem length	0.0011	0.13	0.9		
Fixers species	Leaf potassium concentration	-0.038	-1.5	0.16	0.038	0.33
	Leaf area	0.014	1.2	0.24		
	Stem length	0.00029	0.022	0.98		
Non-fixer species	Leaf potassium concentration	0.019	1.8	0.11	0.047	0.23
	Leaf area	-0.017	-1.4	0.17		
	Stem length	-0.0029	-0.25	0.8		

A6. Supplemental Table 9. The effect of leaf potassium concentration, leaf area and leaf number in driving **the proportion of leaf area lost to herbivory for all leaves (Proportion^{all})** across all species, fixer species alone and non-fixer species alone (number of observations: 350 (184 fixers, 166 non-fixers)). All three fixed effects were included in each model, only changing the data set between models. Leaf area was standardized within species. Seedling, species and plot identity were included as random effects.

Dataset	**Variables**	**Estimate**	**Z Value**	**p value**	**R^{2}m**	**R^{2}c**
All species	Leaf potassium concentration	0.028	0.34	0.73	0.0081	NA
	Leaf area	0.055	1	0.31		
	Stem length	0.029	0.52	0.6		
Fixers species	Leaf potassium concentration	-0.21	-1.1	0.26	0.038	0.31
	Leaf area	0.13	1.6	0.1		
	Stem length	-0.04	-0.45	0.65		
Non-fixer species	Leaf potassium concentration	0.15	1.8	0.075	0.045	NA
	Leaf area	-0.0019	-0.025	0.98		
	Stem length	0.063	0.83	0.41		

Leaf cellulose concentration

A6. Supplemental Table 10. The effect of leaf cellulose concentration, leaf area and leaf number in driving **the incidence of herbivory** across all species, fixer species alone and non-fixer species alone (number of observations: 350 (184 fixers, 166 non-fixers)). All three fixed effects were included in each model, only changing the data set between models. Leaf area was standardized within species. Seedling, species and plot identity were included as random effects.

Dataset	**Variables**	**Estimate**	**Z Value**	**p value**	**R^2m**	**R^2c**
All species	Leaf cellulose concentration	0.086	0.51	0.61	0.017	0.34
	Leaf area	0.26	3.4	**0.00064**		
	Stem length	0.049	0.51	0.61		
Fixers species	Leaf cellulose concentration	0.023	0.089	0.93	0.06	0.26
	Leaf area	0.48	3.9	**0.00011**		
	Stem length	0.1	0.73	0.47		
Non-fixer species	Leaf cellulose concentration	-0.13	-0.59	0.55	0.0067	0.32
	Leaf area	0.098	0.98	0.33		
	Stem length	0.027	0.21	0.83		

A6. Supplemental Table 11. The effect of leaf cellulose concentration, leaf area and leaf number in driving **the proportion of leaf area lost to herbivory for damaged leaves (Proportion^{damaged})** across all species, fixer species alone and non-fixer species alone (number of observations: 350 (184 fixers, 166 non-fixers)). All three fixed effects were included in each model, only changing the data set between models. Leaf area was standardized within species. Seedling, species and plot identity were included as random effects.

Dataset	**Variables**	**Estimate**	**Z Value**	**p value**	**R^2m**	**R^2c**
All species	Leaf cellulose concentration	-0.00081	-0.074	0.94	0.00057	0.19
	Leaf area	0.0016	0.19	0.85		
	Stem length	-0.0034	-0.38	0.7		
Fixers species	Leaf cellulose concentration	0.0032	0.15	0.89	0.0087	0.32
	Leaf area	0.013	1.2	0.24		
	Stem length	-0.00032	-0.025	0.98		
Non-fixer species	Leaf cellulose concentration	-0.013	-1.1	0.31	0.029	0.13
	Leaf area	-0.013	-1	0.31		
	Stem length	-0.0084	-0.68	0.5		

A6. Supplemental Table 12. The effect of leaf cellulose concentration, leaf area and leaf number in driving **the proportion of leaf area lost to herbivory for all leaves (Proportion^{all})** across all species, fixer species alone and non-fixer species alone (number of observations: 350 (184 fixers, 166 non-fixers)). All three fixed effects were included in each model, only changing the data set between models. Leaf area was standardized within species. Seedling, species and plot identity were included as random effects.

Dataset	**Variables**	**Estimate**	**Z Value**	**p value**	**R^{2}m**	**R^{2}c**
All species	Leaf cellulose concentration	0.022	0.29	0.77	0.0048	0.21
	Leaf area	0.062	1.1	0.26		
	Stem length	-0.0026	-0.045	0.96		
Fixers species	Leaf cellulose concentration	0.013	0.087	0.93	0.017	0.31
	Leaf area	0.13	1.7	0.098		
	Stem length	-0.043	-0.5	0.62		
Non-fixer species	Leaf cellulose concentration	-0.055	-0.62	0.54	0.0048	0.15
	Leaf area	-0.0017	-0.02	0.98		
	Stem length	0.021	0.22	0.82		

Leaf lignin concentration

A6. Supplemental Table 13. The effect of leaf lignin concentration, leaf area and leaf number in driving **the incidence of herbivory** across all species, fixer species alone and non-fixer species alone (number of observations: 350 (184 fixers, 166 non-fixers)). All three fixed effects were included in each model, only changing the data set between models. Leaf area was standardized within species. Seedling, species and plot identity were included as random effects.

Dataset	**Variables**	**Estimate**	**Z Value**	**p value**	**R^2m**	**R^2c**
All species	Leaf lignin concentration	0.38	2.3	0.021	0.045	0.33
	Leaf area	0.26	3.4	**0.00062**		
	Stem length	0.044	0.46	0.64		
Fixers species	Leaf lignin concentration	0.38	1.8	0.074	0.083	0.26
	Leaf area	0.49	3.8	**0.00015**		
	Stem length	0.093	0.64	0.52		
Non-fixer species	Leaf lignin concentration	0.19	0.84	0.4	0.011	0.32
	Leaf area	0.099	0.99	0.32		
	Stem length	0.02	0.15	0.88		

A6. Supplemental Table 14. The effect of leaf lignin concentration, leaf area and leaf number in driving **the proportion of leaf area lost to herbivory for damaged leaves (Proportion^{damaged})** across all species, fixer species alone and non-fixer species alone (number of observations: 350 (184 fixers, 166 non-fixers)). All three fixed effects were included in each model, only changing the data set between models. Leaf area was standardized within species. Seedling, species and plot identity were included as random effects.

Dataset	**Variables**	**Estimate**	**Z Value**	**p value**	**R²m**	**R²c**
All species	Leaf lignin concentration	0.027	2.6	**0.012**	0.039	0.19
	Leaf area	0.0027	0.31	0.75		
	Stem length	-0.0057	-0.65	0.51		
Fixers species	Leaf lignin concentration	0.05	3.1	**0.0048**	0.097	0.36
	Leaf area	0.015	1.3	0.19		
	Stem length	-0.0036	-0.31	0.76		
Non-fixer species	Leaf lignin concentration	0.008	0.59	0.57	0.02	0.13
	Leaf area	-0.012	-0.96	0.34		
	Stem length	-0.0094	-0.75	0.45		

A6. Supplemental Table 15. The effect of leaf lignin concentration, leaf area and leaf number in driving **the proportion of leaf area lost to herbivory for all leaves (Proportion^{all})** across all species, fixer species alone and non-fixer species alone (number of observations: 350 (184 fixers, 166 non-fixers)). All three fixed effects were included in each model, only changing the data set between models. Leaf area was standardized within species. Seedling, species and plot identity were included as random effects.

Dataset	**Variables**	**Estimate**	**Z Value**	**p value**	**R^2m**	**R^2c**
All species	Leaf lignin concentration	0.2	2.7	**0.0079**	0.052	0.22
	Leaf area	0.066	1.2	0.23		
	Stem length	-0.014	-0.24	0.81		
Fixers species	Leaf lignin concentration	0.33	3	**0.0028**	0.11	0.34
	Leaf area	0.14	1.8	0.067		
	Stem length	-0.064	-0.77	0.44		
Non-fixer species	Leaf lignin concentration	0.067	0.69	0.49	0.0067	0.15
	Leaf area	0.0021	0.025	0.98		
	Stem length	0.019	0.21	0.84		

A6. Supplemental Table 16. The difference between fixers and either non-fixers or non-fixing members of the Fabaceae in the incidence of herbivory and the Proportion^{all} of leaf area lost to herbivory: 350 (184 fixers, 166 non-fixers, 3 non-fixing Fabaceae)). Seedling, species and plot identity were included as random effects for the incidence model, species and plot identity for the Proportion^{all} model.

Dataset	**Variables**	**Estimate**	**Z Value**	**p value**	**R^2m**	**R^2c**
Incidence of herbivory	Non-fixers	-1.4	-4.6	3.60E-06	0.11	0.34
	Non-fixing Fabaceae	0.55	0.97	0.33		
(Proportion^{all}) of leaf area lost to herbivory	Non-fixers	-0.37	-2.3	0.021	0.04	0.24
	Non-fixing Fabaceae	-0.21	-0.79	0.43		

Appendix 7

Supplemental methods for chapter three

A7. Supplemental Table 1. The factorial combination of nitrogen and herbivory treatments across seedling of *I. bella*, showing the number of seedlings surviving in each treatment.

Herbivory treatment	**No nitrogen added**	**Moderate nitrogen addition (3M solution)**	**High nitrogen addition (10M nitrogen solution)**
No damage (no leaf area removed)	10 seedlings	11 seedlings	11 seedlings
Moderate damage (8.5% of leaf area removed)	10 seedlings	11 seedlings	11 seedlings
High damage (20% of leaf area removed)	11 seedlings	11 seedlings	11 seedlings

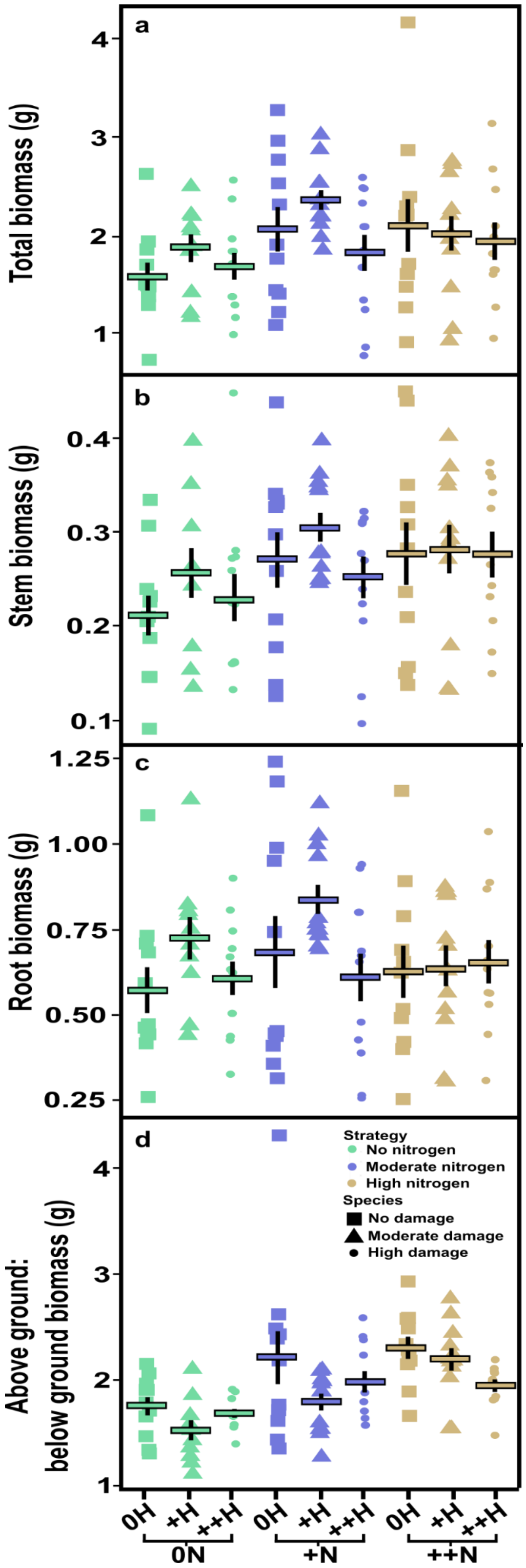
A7. Supplemental Table 2. The three herbivory treatments across the four species selected from the two antiherbivore strategy groups, with the number of seedlings under each factorial treatment.

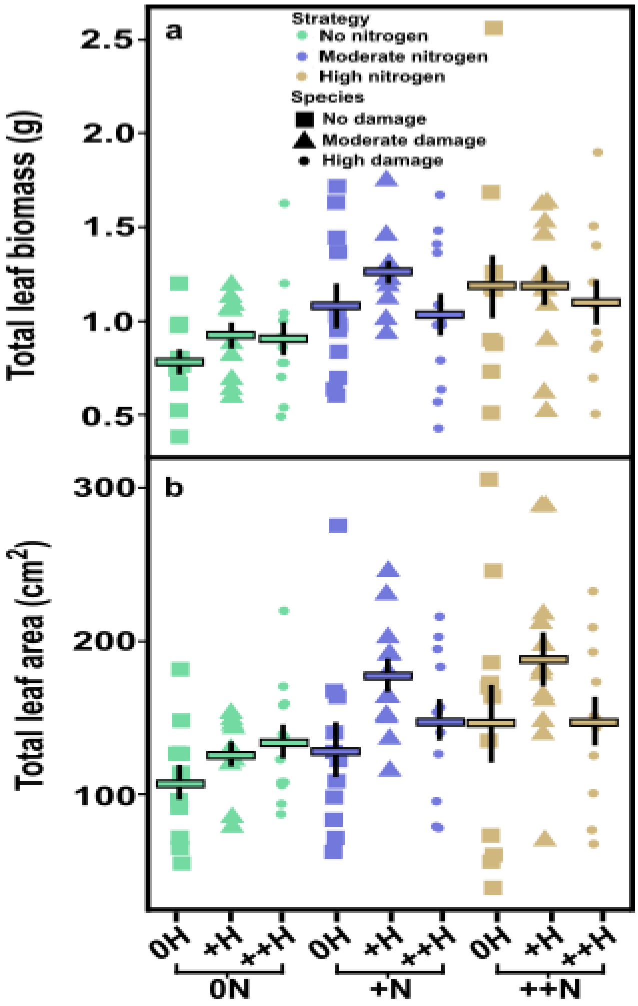
Herbivory treatment	**Escape**		**Defence**	
	Inga alba	***Inga laurina***	***Inga vera***	***Inga spectabilis***
No leaf area removed	10 seedlings	10 seedlings	10 seedlings	10 seedlings
20% of leaf area removed	10 seedlings	10 seedlings	10 seedlings	10 seedlings
20% of leaf area removed and Methyl-Jasmonate	0 seedlings	10 seedlings	10 seedlings	0 seedlings

Appendix 8

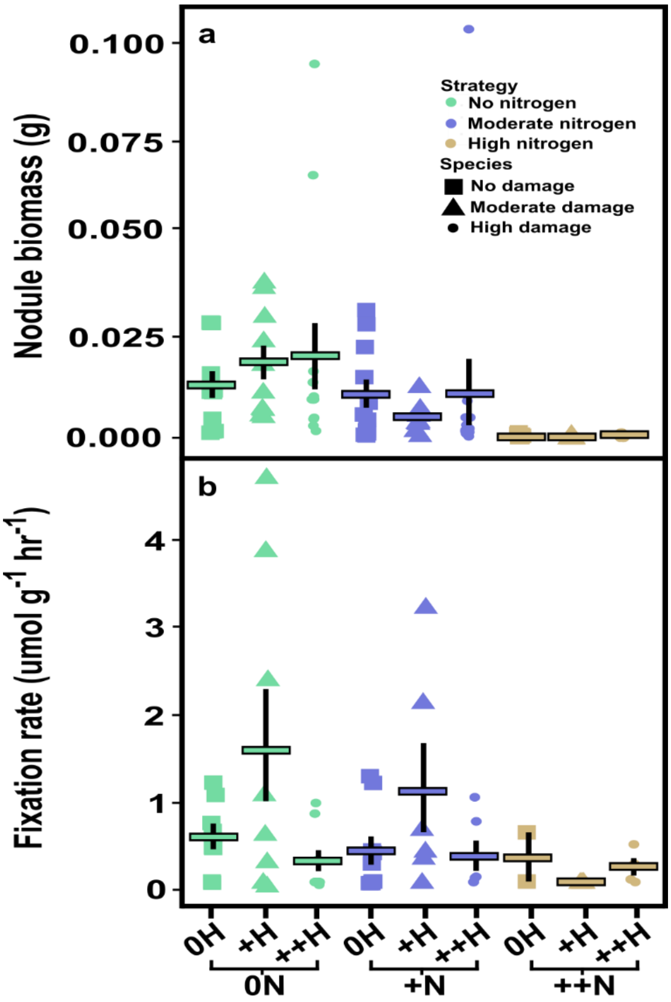
Supplemental figures for the chapter three

A8. Supplemental Figure 1. The effect of each factorial treatment combination in my nitrogen and herbivory level experiment on total plant biomass (a), stem biomass (b), root biomass (c) and above ground to below ground biomass (d). *No nitrogen addition is shown as 0N (in green), the addition of a 3µM nitrogen solution is represented by +N (in blue) and the 10µM nitrogen solution is represented by ++N (in gold). Seedlings that underwent no simulated herbivory treatment are denoted as 0H (square data points), those from which I removed 8.5% of leaf area as +H (triangular data points) and those which lost 20% of leaf area as ++H (circular data points). Bars represent the mean with standard error for each treatment combination.*

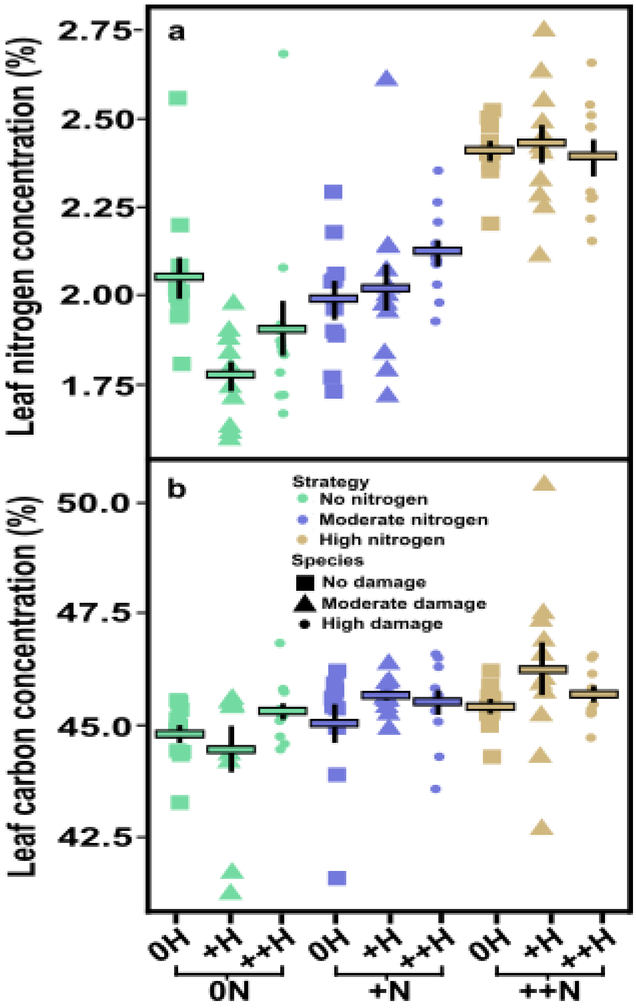




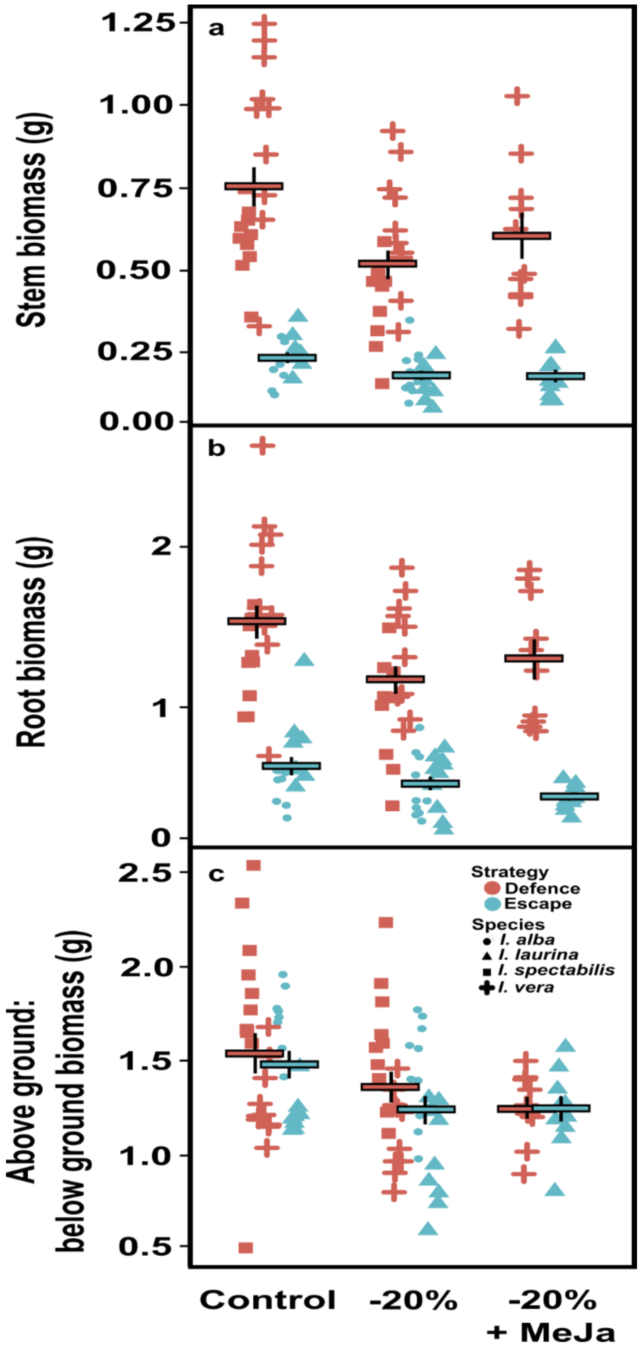
A8. Supplemental Figure 2. The effect of each factorial treatment combination in my nitrogen and herbivory level experiment on total leaf biomass (a) and total leaf area (b), when accounting for leaf biomass and area removed in herbivory treatments. *No nitrogen addition is shown as 0N (in green), the addition of a 3µM nitrogen solution is represented by +N (in blue) and the 10µM nitrogen solution is represented by ++N (in gold). Seedlings that underwent no simulated herbivory treatment are denoted as 0H (square data points), those from which I removed 8.5% of leaf area as +H (triangular data points) and those which lost 20% of leaf area as ++H (circular data points). Bars represent the mean with standard error for each treatment combination.*



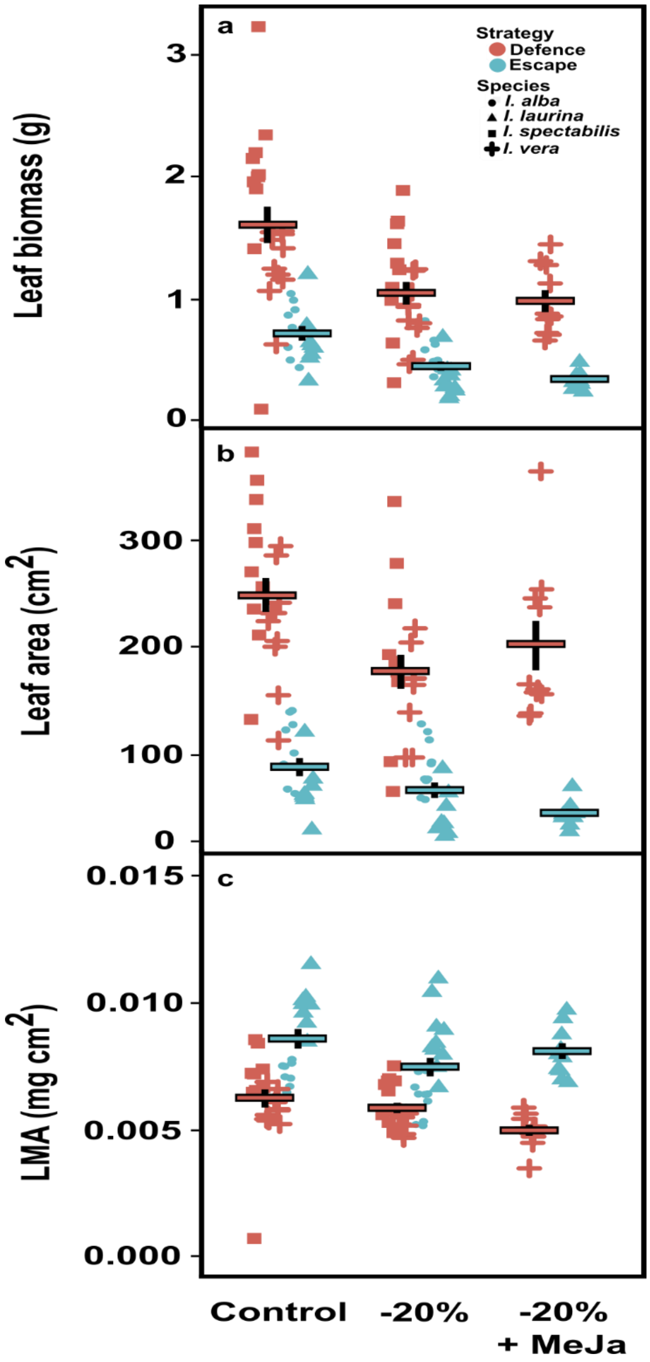
A8. Supplemental Figure 3. The effect of each factorial treatment combination in my nitrogen and herbivory level experiment on plant nodule biomass (a) and fixation rate per nodule (b). *No nitrogen addition is shown as 0N (in green), the addition of a 3µM nitrogen solution is represented by +N (in blue) and the 10µM nitrogen solution is represented by ++N (in gold). Seedlings that underwent no simulated herbivory treatment are denoted as 0H (square data points), those from which I removed 8.5% of leaf area as +H (triangular data points) and those which lost 20% of leaf area as ++H (circular data points). Bars represent the mean with standard error for each treatment combination.*



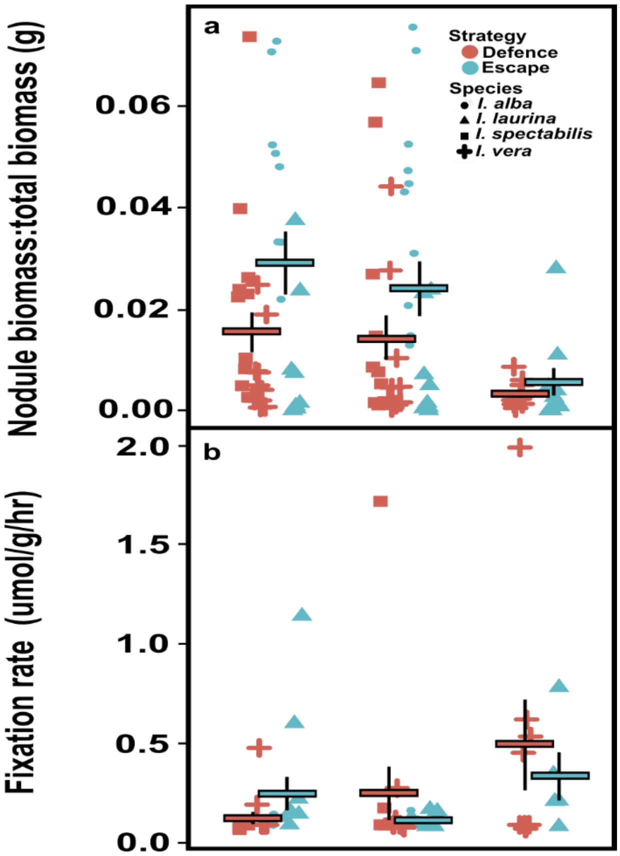
A8. Supplemental Figure 4. The effect of each factorial treatment combination in my nitrogen and herbivory level experiment on leaf nitrogen concentration (a) and leaf carbon concentration (b). *No nitrogen addition is shown as 0N (in green), the addition of a 3µM nitrogen solution is represented by +N (in blue) and the 10µM nitrogen solution is represented by ++N (in gold). Seedlings that underwent no simulated herbivory treatment are denoted as 0H (square data points), those from which I removed 8.5% of leaf area as +H (triangular data points) and those which lost 20% of leaf area as ++H (circular data points). Bars represent the mean with standard error for each treatment combination.*



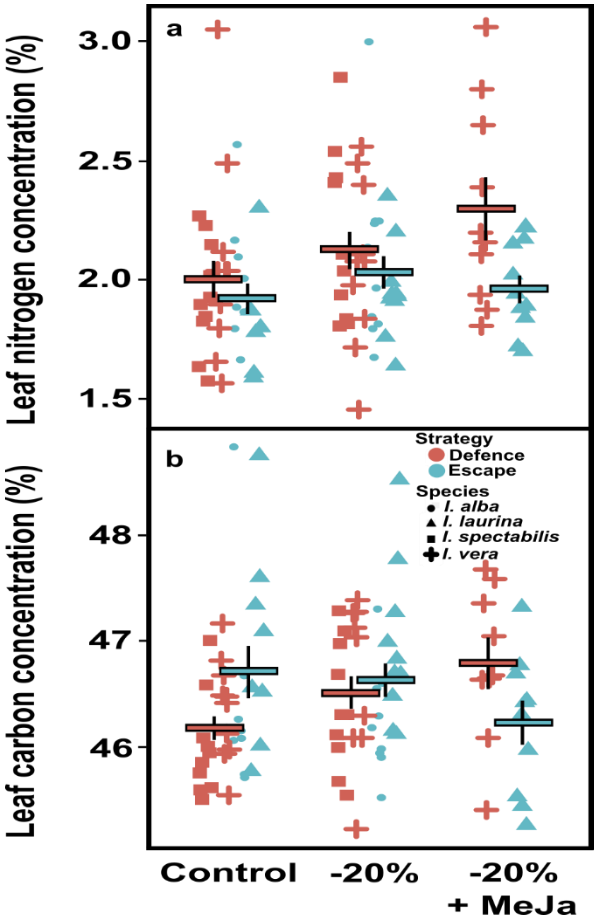
A8. Supplemental Figure 5. The effect of herbivory treatment and species antiherbivore strategy on the stem biomass (a), root biomass (b) and above ground to below ground biomass ratio (c). *Antiherbivore strategy for each seedling is represented by colour, with defence specialists in red and escape specialists in blue. Individual seedlings from each species are represented by shape with I. alba shown as circles, I. laurina represented by triangles, I. spectabilis represented by squares and I. vera represented by crosses. Bars represent the mean with standard error for each treatment combination.*



A8. Supplemental Figure 6. The effect of herbivory treatment and species antiherbivore strategy on leaf biomass (a), leaf area (b) and LMA (c). *Antiherbivore strategy for each seedling is represented by colour, with defence specialists in red and escape specialists in blue. Individual seedlings from each species are represented by shape with I. alba shown as circles, I. laurina represented by triangles, I. spectabilis represented by squares and I. vera represented by crosses. Bars represent the mean with standard error for each treatment combination.*



A8. Supplemental Figure 7. The effect of herbivory treatment and species antiherbivore strategy on the ratio of nodule to total plant biomass (a) and fixation rate per nodule biomass (b). *Antiherbivore strategy for each seedling is represented by colour, with defence specialists in red and escape specialists in blue. Individual seedlings from each species are represented by shape with I. alba shown as circles, I. laurina represented by triangles, I. spectabilis represented by squares and I. vera represented by crosses. Bars represent the mean with standard error for each treatment combination.*

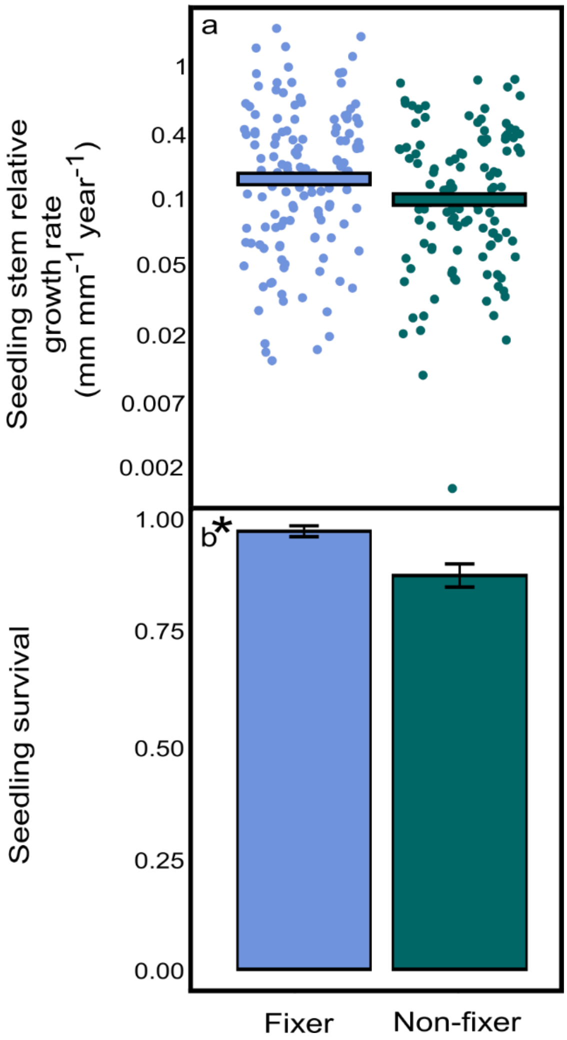


A8. Supplemental Figure 8. The effect of herbivory treatment and species antiherbivore strategy on leaf nitrogen concentration (a) and leaf carbon concentration (b).

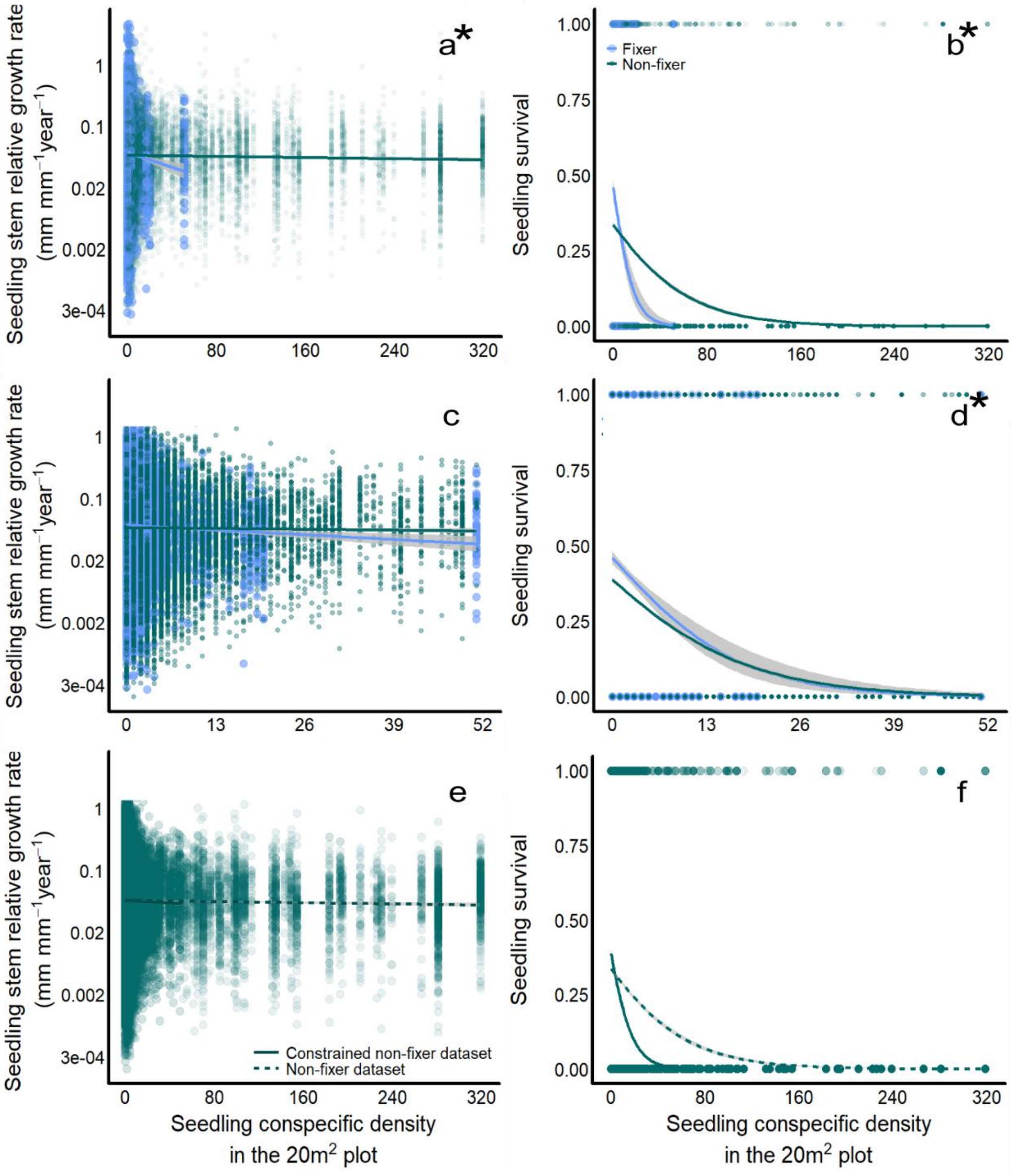
Antiherbivore strategy for each seedling is represented by colour, with defence specialists in red and escape specialists in blue. Individual seedlings from each species are represented by shape with I. alba shown as circles, I. laurina represented by triangles, I. spectabilis represented by squares and I. vera represented by crosses. Bars represent the mean with standard error for each treatment combination.

Appendix 9

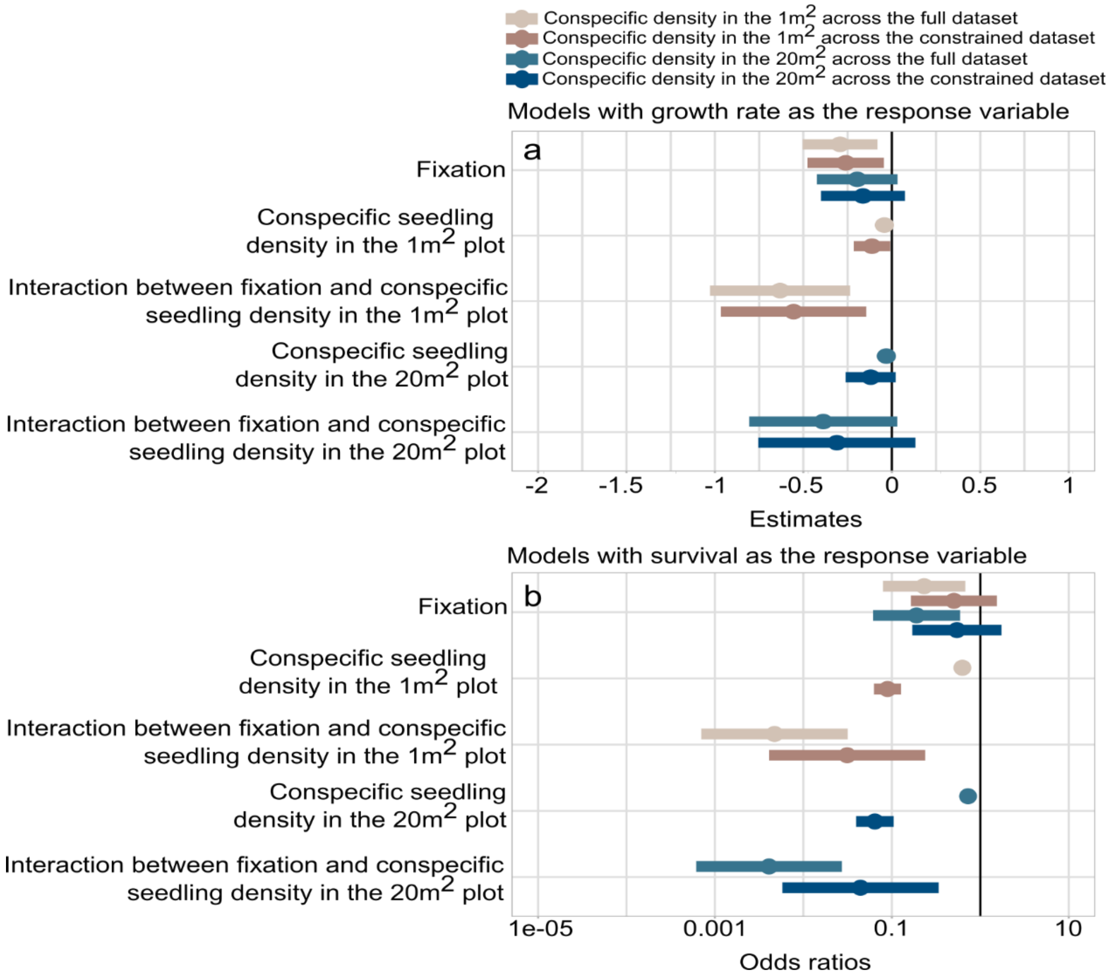
Supplemental figures for chapter four



A9. Supplemental Figure 1. Difference in growth and survival from 2017-2018 for fixer and non-fixer seedlings. *The difference in seedling stem relative growth rate (a) and seedling survival (b) between fixers (blue) and non-fixers (dark green) from 2017-2018 for the 350 seedlings for which I had herbivory data. Asterisks indicate a statistically significant difference between the two groups in my mixed effect model (a), or in a non-parametric Wilcoxon rank test (b).*



A9. Supplemental Figure 2. The difference in the strength of negative density-dependent effects on growth and survival for fixer and non-fixer seedlings. *Showing the relationship between conspecific density in the* $20m^2$ *plot and seedling stem relative growth rate (panels a, c, e) and seedling survival (b, d, f) from 2001-2014 for fixers (blue) and non-fixers (green) across the whole dataset (a and b), the dataset when constrained to conspecific densities at which fixers were observed (c and d), and for non-fixers across both the whole dataset and the constrained dataset. Seedling stem relative growth rate is shown on the log scale. Lines are fitted using a linear model with a Gaussian distribution (a, c, e) and a general linear model with a binomial distribution (b, d, f) between the two variables in each panel. Grey* *shading on lines represents standard error and asterisks represent significant differences between the relationships for fixers and non-fixers from my mixed effects models including species and plot identity as random effects (no statistical comparison was made between lines in panels e and f).*



A9. S**upplemental Figure 3.** ***The strength of negative density-dependent effects on survival and growth rate.*** *Coefficient estimates from models regressing seedling growth rate (a) and seedling survival (b) from 2001-2014 against either conspecific density in the 1m^2 plot or the 20m^2 plot****.*** Models were run using either the full dataset or a constrained version of the dataset in which conspecific density was limited to the max value for any fixer species. *In panel a, the X axis represents changes in stem relative growth rate (in mm). The X axis is panel b is the log odds ratio of survival from my binomial regression (in other words, the odds of survival), where >1 represents an increased chance of survival and <1 represents a reduced chance of survival. The effect of conspecific density in interaction with fixation represents the negative conspecific density-dependent effect for fixers, whilst conspecific density alone represents the size of the effects for non-fixers. Models included the random effects of species identity and 20m^2 plot to control for variation across species and autocorrelation. The black line marks zero on the X axis*

Appendix 10

Supplemental tables 1 for chapter four: growth, survival and density dependence

Differences in survival and growth between fixers and non-fixers.

A10. Supplemental Table 1. Differences in seedling growth and survival between fixers and non-fixers. The effect of fixation on seedling stem relative growth rate and survival from 2001-2014 in the 50-ha plot on BCI. Models included species and plot identity as random effects.

Variable	**Estimate**	**Z value**	**p value**	**R^2m**	**R^2c**	**AIC**
The effect of fixation on growth rate	-0.01	-0.35	0.73	0.0004	0.17	97112.44
The effect of fixation on survival	0.85	2.3	**0.023**	0.01	0.43	32942.69

Differences in the strength of conspecific density-dependent effects between fixers and non-fixers.

A10. Supplemental Table 2. How conspecific density affects the growth of fixer and non-fixer seedlings. The effect of fixation and conspecific seedling at the 1m^2 and the 20m^2 scale and adult density at the 20m^2 scale on seedling stem relative growth rate from 2001-2014 in the 50-ha plot on BCI. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	Variables	Estimate	Z Value	p value	R^{2}m	R^{2}c	AIC
In the **1m^2** plot	Fixation	-0.29	-2.7	**0.0069**	0.0007	0.17	216367.5
	Conspecific **seedling** density	-0.042	-3.2	**0.0012**			
	Heterospecific **seedling** density	-0.0081	-1.5	0.12			
	The interaction between fixation and conspecific **seedling** density	-0.63	-3.1	**0.0018**			
In the **20m^2** plot	Fixation	-0.2	-1.7	0.092	0.0006	0.08	217628.2
	Conspecific **seedling** density	-0.031	-1.9	0.062			
	Heterospecific **seedling** density	-0.013	-1.6	0.11			
	The interaction between fixation and conspecific **seedling** density	-0.39	-1.8	0.069			
In the **20m^2** plot	Fixation	-0.095	-1.4	0.16	0.001	0.08	217599.7
	Conspecific **adult** density	-0.006	-1	0.32			
	Heterospecific **adult** density	0.037	4.7	**2.90E-06**			
	The interaction between fixation and conspecific **adult** density	-0.15	-3.7	**0.00023**			

A10. Supplemental Table 3. How conspecific density affects the survival of fixer and non-fixer seedlings. The effect of fixation and conspecific seedling at the 1m^2 and the 20m^2 scale and adult density at the 20m^2 scale on seedling survival from 2001-2014 in the 50-ha plot on BCI. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	**Variables**	**Estimate**	**Z Value**	**p value**	**R^{2}m**	**R^{2}c**	**AIC**
In the **1m^2** plot	Fixation	-1.5	-2.7	0.0076	0.05	0.45	32785.77
	Conspecific **seedling** density	-0.47	-10	**1.90E-23**			
	Heterospecific **seedling** density	-0.033	-2.3	**0.022**			
	The interaction between fixation and conspecific **seedling** density	-5.4	-5.5	**3.60E-08**			
In the **20m^2** plot	Fixation	-1.7	-2.9	**0.0041**	0.03	0.44	32845.98
	Conspecific **seedling** density	-0.014	-0.73	0.47			
	Heterospecific **seedling** density	-0.32	-6.7	**2.60E-11**			
	The interaction between fixation and conspecific **seedling** density	-5.5	-5.7	**1.30E-08**			
In the **20m^2** plot	Fixation	0.75	2	0.05	0.01	0.44	32937.71
	Conspecific **adult** density	0.056	3	**0.0025**			
	Heterospecific **adult** density	-0.0047	-0.23	0.82			
	The interaction between fixation and conspecific **adult** density	-0.21	-1.5	0.13			

A10. Supplemental Table 4. How density of seedlings in the same functional group (fixer or non-fixer) affects the growth of fixer and non-fixer seedlings. The effect of fixation and functional seedling density (where confunctional density represents the density of seedlings from the same functional group) at the 1m^2 and the 20m^2 scale on seedling stem relative growth rate from 2001-2014 in the 50-ha plot on BCI. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	Variables	Estimate	Z Value	p value	R^{2}m	R^{2}c	AIC
In the **1m^2** plot	Fixation	-0.33	-2.9	**0.004**	0.0007	0.08	217611
	Confunctional seedling density	-0.037	-3.6	**0.00034**			
	Heterofunctional seedling density	-0.0057	-1.1	0.27			
	The interaction between fixation and confunctional seedling density	-0.51	-3.3	**0.00086**			
In the **20m^2** plot	Fixation	-0.34	-1.8	0.069	0.0006	0.08	217628.4
	Confunctional seedling density	-0.031	-2	**0.048**			
	Heterofunctional seedling density	-0.00094	-0.15	0.88			
	The interaction between fixation and confunctional seedling density	-0.35	-1.8	0.08			

A10. Supplemental Table 5. How density of seedlings in the same functional group (fixer or non-fixer) affects the survival of fixer and non-fixer seedlings. The effect of fixation and functional seedling density (where confunctional density represents the density of seedlings from the same functional group) at the $1m^2$ and the $20m^2$ scale on seedling survival from 2001-2014 in the 50-ha plot on BCI. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	Variables	Estimate	Z Value	p value	R^2m	R^2c	AIC
In the **$1m^2$** plot	Fixation	-0.93	-1.7	0.091	0.03	0.44	32832.44
	Confunctional seedling density	-0.32	-9	**3.30E-19**			
	Heterofunctional seedling density	-0.03	-1.8	0.068			
	The interaction between fixation and confunctional seedling density	-2.9	-4.2	**2.60E-05**			
In the **$20m^2$** plot	Fixation	-2.3	-3.2	**0.0016**	0.02	0.44	32905.98
	Confunctional seedling density	-0.14	-3.4	**0.00057**			
	Heterofunctional seedling density	-0.0038	-0.19	0.85			
	The interaction between fixation and confunctional seedling density	-3.6	-5	**6.80E-07**			

Comparing the model fit when using conspecific and heterospecific density or overall seedling density as an explanatory variable.

A10. Supplemental Table 6. A model comparison between models including conspecific and heterospecific density or functional density as explanatory variables, and those including only overall seedling density. All models included species and plot identity as random effects.

Growth rate model	**Growth rate AIC**	**Survival Model**	**Survival AIC**
Conspecific seedling densities in the 1m^2 plot	216367	Conspecific seedling densities in the 1m^2 plot	32785.8
Overall seedling densities in the 1m^2 plot	216370	Overall seedling densities in the 1m^2 plot	32851.4
Conspecific seedling densities in the 20m^2 plot	217628	Conspecific seedling densities in the 20m^2 plot	32846
Overall seedling densities in the 20m^2 plot	217626	Overall seedling densities in the 20m^2 plot	32933.7
Conspecific adult densities in the 20m^2 plot	217600	Conspecific adult densities in the 20m^2 plot	32937.7
Overall adult densities in the 20m^2 plot	217611	Overall adult densities in the 20m^2 plot	32945.1
Functional seedling density in the 1m2	217611	Overall seedling densities in the 1m2 plot	32851.4
Functional seedling density in the 20m2	217628	Conspecific seedling densities in the 20m2 plot	32846

Differences in the strength of conspecific density-dependent effects between fixers and non-fixers, when limiting the range of conspecific densities to those at which fixers were found. This approach allowed us to account for how large discrepancies in observed densities between the two groups may have influenced the results.

A10. Supplemental Table 7. How conspecific density affects the growth of fixer and non-fixer seedlings. The effect of fixation and conspecific seedling at the $1m^2$ and the $20m^2$ scale and adult density at the $20m^2$ scale on seedling stem relative growth rate from 2001-2014 in the 50-ha plot on BCI, when excluding non-fixer seedlings observed at conspecific densities greater than the maximum conspecific density for fixer seedlings. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	**Variables**	**Estimate**	**Z Value**	**p value**	**R^2m**	**R^2c**	**AIC**
In the **$1m^2$** plot	Fixation	-0.26	-2.4	**0.018**	0.0005	0.17	207934
	Conspecific **seedling** density	-0.11	-2.1	**0.033**			
	Heterospecific **seedling** density	-0.0076	-1.4	0.16			
	The interaction between fixation and conspecific **seedling** density	-0.56	-2.7	**0.008**			
In the **$20m^2$** plot	Fixation	-0.16	-1.3	0.18	0.0005	0.08	207324.3
	Conspecific **seedling** density	-0.12	-1.7	0.099			
	Heterospecific **seedling** density	-0.012	-1.5	0.14			
	The interaction between fixation and conspecific **seedling** density	-0.31	-1.4	0.17			
In the **$20m^2$** plot	Fixation	-0.1	-1.5	0.14	0.001	0.08	186180.3
	Conspecific **adult** density	0.0086	0.56	0.58			
	Heterospecific **adult** density	0.037	4.6	**5.90E-06**			
	The interaction between fixation and conspecific **adult** density	-0.16	-3.3	**0.00092**			

A10. Supplemental Table 8. How conspecific density affects the survival of fixer and non-fixer seedlings. The effect of fixation and conspecific seedling at the 1m^2 and the 20m^2 scale and adult density at the 20m^2 scale on seedling survival from 2001-2014 in the 50-ha plot on BCI, when excluding non-fixer seedlings observed at conspecific densities greater than the maximum conspecific density for fixer seedlings. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	Variables	Estimate	Z Value	p value	R^{2}m	R^{2}c	AIC
In the **1m^2** plot	Fixation	-0.69	-1.2	0.23	0.06	0.45	31273.8
	Conspecific **seedling** density	-2.4	-13	**7.20E-41**			
	Heterospecific **seedling** density	-0.023	-1.6	0.11			
	The interaction between fixation and conspecific **seedling** density	-3.5	-3.3	**0.00083**			
In the **20m^2** plot	Fixation	-0.61	-1	0.3	0.05	0.44	30971.5
	Conspecific **seedling** density	0.0073	0.37	0.71			
	Heterospecific **seedling** density	-2.7	-11	**4.60E-28**			
	The interaction between fixation and conspecific **seedling** density	-3.1	-3	**0.0027**			
In the **20m^2** plot	Fixation	0.75	2	0.05	0.01	0.44	27998.08
	Conspecific **adult** density	0.056	3	0.0027			
	Heterospecific **adult** density	0.0012	0.057	0.95			
	The interaction between fixation and conspecific **adult** density	-0.2	-1.5	0.13			

Seedling density models using the number of conspecific and heterospecific seedlings within 5m, 10m or 20m from the focal seedling.

A10. Supplemental Table 9. How conspecific seedling density affects the growth rate of fixer and non-fixer seedlings. The effect of fixation and conspecific **seedling** density within 5m, 10m or 20m from each focal seedling on seedling stem growth rate from 2001-2014 in the 50-ha plot on BCI. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	Variables	Estimate	Z Value	p value	R^2m	R^2c	AIC
Within **5m** from focal seedling	Fixation	-0.19	-1.8	0.078	0.0006	0.17	216378.8
	Conspecific seedling density	-0.022	-2.1	**0.035**			
	Heterospecific seedling density	-0.013	-2.2	**0.028**			
	The interaction between fixation and conspecific seedling density	-0.48	-2	**0.049**			
Within **10m** from focal seedling	Fixation	-0.11	-1.2	0.22	0.0005	0.17	216382.4
	Conspecific seedling density	-0.026	-2.3	**0.021**			
	Heterospecific seedling density	-0.0093	-1.4	0.15			
	The interaction between fixation and conspecific seedling density	-0.25	-1.4	0.16			
Within **20m** from focal seedling	Fixation	-0.069	-0.85	0.4	0.0007	0.17	216381
	Conspecific seedling density	-0.03	-2.6	**0.0086**			
	Heterospecific seedling density	-0.015	-2.1	**0.037**			
	The interaction between fixation and conspecific seedling density	-0.12	-0.92	0.36			

A10. Supplemental Table 10. How conspecific seedling density affects the survival of fixer and non-fixer seedlings. The effect of fixation and conspecific **seedling** density within 5m, 10m or 20m from each focal seedling on seedling survival from 2001-2014 in the 50-ha plot on BCI. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	Variables	Estimate	Z Value	p value	R^2m	R^2c	AIC
Within **5m** from focal seedling	Fixation	-2.9	-3.7	**0.00019**	0.04	0.44	32808.4
	Conspecific seedling density	-0.34	-8.2	**1.70E-16**			
	Heterospecific seedling density	-0.00024	-0.016	0.99			
	The interaction between fixation and conspecific seedling density	-7.9	-5.6	**2.40E-08**			
Within **10m** from focal seedling	Fixation	-2.1	-3.8	**0.00012**	0.03	0.44	32823.5
	Conspecific seedling density	-0.24	-5.8	**6.50E-09**			
	Heterospecific seedling density	0.032	1.9	0.057			
	The interaction between fixation and conspecific seedling density	-6.1	-7.1	**1.30E-12**			
Within **20m** from focal seedling	Fixation	-1.3	-2.7	**0.0069**	0.02	0.44	32824.1
	Conspecific seedling density	-0.22	-5	**4.60E-07**			
	Heterospecific seedling density	0.06	3.3	**0.0011**			
	The interaction between fixation and conspecific seedling density	-3.9	-7.5	**4.50E-14**			

A10. Supplemental Table 11. How conspecific adult density affects the growth rate of fixer and non-fixer seedlings. The effect of fixation and conspecific **adult** density within 5m, 10m or 20m from each focal seedling on seedling stem growth rate from 2001-2014 in the 50-ha plot on BCI. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	**Variables**	**Estimate**	**Z Value**	**p value**	**R^2m**	**R^2c**	**AIC**
Within **5m** from focal seedling	Fixation	-0.029	-0.44	0.66	0.0003	0.17	216384.5
	Conspecific seedling density	-0.011	-2.2	**0.03**			
	Heterospecific seedling density	0.0099	1.8	0.073			
	The interaction between fixation and conspecific seedling density	-0.044	-1.4	0.15			
Within **10m** from focal seedling	Fixation	-0.03	-0.45	0.65	0.00008	0.17	216393.9
	Conspecific seedling density	-0.004	-0.74	0.46			
	Heterospecific seedling density	-0.00028	-0.046	0.96			
	The interaction between fixation and conspecific seedling density	-0.028	-0.96	0.34			
Within **20m** from focal seedling	Fixation	-0.039	-0.58	0.57	0.00008	0.17	216393.4
	Conspecific seedling density	0.00081	0.14	0.89			
	Heterospecific seedling density	-0.00013	-0.018	0.99			
	The interaction between fixation and conspecific seedling density	-0.041	-1.3	0.21			

A10. Supplemental Table 12. How conspecific adult density affects the survival of fixer and non-fixer seedlings. The effect of fixation and conspecific **adult** density within 5m, 10m or 20m from each focal seedling on seedling survival from 2001-2014 in the 50-ha plot on BCI. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	Variables	Estimate	Z Value	p value	R^2m	R^2c	AIC
Within **5m** from focal seedling	Fixation	0.85	2.3	**0.023**	0.01	0.44	32948.07
	Conspecific adult density	0.0055	0.4	0.69			
	Heterospecific adult density	0.0095	0.62	0.54			
	The interaction between fixation and conspecific adult density	-0.037	-0.38	0.7			
Within **10m** from focal seedling	Fixation	0.87	2.3	**0.019**	0.01	0.44	32946.55
	Conspecific adult density	-0.014	-0.93	0.35			
	Heterospecific adult density	0.014	0.84	0.4			
	The interaction between fixation and conspecific adult density	0.081	0.85	0.4			
Within **20m** from focal seedling	Fixation	0.86	2.3	**0.023**	0.01	0.44	32941.72
	Conspecific adult density	-0.0088	-0.55	0.59			
	Heterospecific adult density	0.048	2.6	0.01			
	The interaction between fixation and conspecific adult density	0.0097	0.094	0.93			

Appendix 11

Supplemental tables 2 for chapter four: relationships between herbivory, density and demographic traits

The effect of herbivory measures in driving seedling survival and growth for seedlings sampled from 2017-2018.

A11. Supplemental Table 1. How the incidence of herbivory affects seedling growth rates. The effect of the incidence of herbivory on seedling stem relative growth rate from 2017-2018 in the 50-ha plot on BCI for 350 seedlings for which I had herbivory data. The model included species and plot identity as random effects (R^2m:0.08, R^2c 0.34, AIC: 753.64).

Variable	**Estimate**	**Z value**	**p value**
Incidence of herbivory	0.048	0.65	0.52
Stem length	-0.31	-4.6	**5.40E-06**

A11. Supplemental Table 2. How the incidence of herbivory affects seedling growth. The effect of the incidence of herbivory and conspecific seedling density at the $1m^2$ on seedling stem relative growth rate from 2017-2018 in the 50-ha plot on BCI. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	Variables	Estimate	Z Value	p value	R^2m	R^2c	AIC
In the **$1m^2$** plot	Incidence of herbivory	0.035	0.48	0.63	0.13	0.37	757.78
	Conspecific seedling density	-0.23	-2.8	**0.0082**			
	Heterospecific seedling density	-0.047	-0.78	0.44			
	Stem length	-0.31	-4.7	**4.30E-06**			
	The interaction between Incidence of herbivory and conspecific seedling density	-0.15	-2	**0.049**			
In the **$20m^2$** plot	Incidence of herbivory	0.031	0.43	0.67	0.11	0.34	761.33
	Conspecific seedling density	-0.19	-2.5	**0.014**			
	Heterospecific seedling density	-0.053	-0.88	0.38			
	Stem length	-0.33	-4.9	**2.20E-06**			
	The interaction between Incidence of herbivory and conspecific seedling density	-0.11	-1.4	0.15			

A11. Supplemental Table 3. How the incidence of herbivory affects seedling growth. The effect of the incidence of herbivory and functional seedling density (where confunctional density represents the density of seedlings from the same functional group) at the 1m^2 and the 20m^2 on seedling stem relative growth rate from 2017-2018 in the 50-ha plot on BCI. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	**Variables**	**Estimate**	**Z Value**	**p value**	**R^{2}m**	**R^{2}c**	**AIC**
In the **1m^2** plot	Incidence of herbivory	0.11	1.3	0.18	0.04	0.6	578.0
	Confunctional seedling density	-0.058	-0.55	0.58			
	Heterofunctional seedling density	0.033	0.4	0.69			
	Stem length	-0.14	-1.8	0.073			
	The interaction between Incidence of herbivory and confunctional seedling density	0.11	1.3	0.18			
In the **20m^2** plot	Incidence of herbivory	0.097	1.2	0.25	0.03	0.58	577.05
	Confunctional seedling density	0.057	0.56	0.58			
	Heterofunctional seedling density	0.069	0.71	0.48			
	Stem length	-0.14	-1.6	0.11			
	The interaction between Incidence of herbivory and confunctional seedling density	0.097	1.2	0.25			

A11. Supplemental Table 4. How leaf area loss affects seedling growth rates. The effect of the proportion of leaf area lost to herbivory on seedling stem relative growth rate from 2017-2018 in the 50-ha plot on BCI for 350 seedlings for which I had herbivory data. The model included species and plot identity as random effects (R^2m:0.05, R^2c 0.29, AIC: 787.81).

Variable	**Estimate**	**Z value**	**p value**
Proportion of leaf area lost to herbivory	-0.13	-2	**0.049**
Stem length	-0.3	-4.5	**9.30E-06**

A11. Supplemental Table 5. How the proportion of leaf area lost to herbivory affects seedling growth. The effect of the proportion of leaf area lost to herbivory and conspecific seedling density at the 1m^2 and the 20m^2 scale on seedling stem relative growth rate from 2017-2018 in the 50-ha plot on BCI. The models included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	Variables	Estimate	Z Value	p value	R^{2}m	R^{2}c	AIC
In the **1m^2** plot	Proportion of leaf area lost to herbivory	-0.11	-1.7	0.083	0.12	0.34	758.47
	Conspecific seedling density	-0.22	-2.7	**0.011**			
	Heterospecific seedling density	-0.053	-0.88	0.38			
	Stem length	-0.31	-4.6	**7.40E-06**			
	The interaction between Proportion of leaf area lost to herbivory and conspecific seedling density	-0.055	-0.82	0.41			
In the **20m^2** plot	Proportion of leaf area lost to herbivory	-0.12	-1.8	0.074	0.12	0.34	761.02
	Conspecific seedling density	-0.18	-2.4	**0.02**			
	Heterospecific seedling density	-0.056	-0.92	0.36			
	Stem length	-0.32	-4.7	**3.90E-06**			
	The interaction between Proportion of leaf area lost to herbivory and conspecific seedling density	0.0097	0.15	0.88			

A11. Supplemental Table 6. How the proportion of leaf area lost to herbivory affects seedling growth. The effect of the proportion of leaf area lost to herbivory and functional seedling density (where confunctional density represents the density of seedlings from the same functional group) at the $1m^2$ and the $20m^2$ on seedling stem relative growth rate from 2017-2018 in the 50-ha plot on BCI. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	**Variables**	**Estimate**	**Z Value**	**p value**	**R^2m**	**R^2c**	**AIC**
In the **$1m^2$** plot	Proportion of leaf area lost to herbivory	-0.082	-0.99	0.32	0.03	0.61	577.5
	Confunctional seedling density	-0.13	-1.2	0.25			
	Heterofunctional seedling density	0.034	0.42	0.67			
	Stem length	0.17	2.1	0.041			
	The interaction between Proportion of leaf area lost to herbivory and confunctional seedling density	-0.13	-1.6	0.12			
In the **$20m^2$** plot	Proportion of leaf area lost to herbivory	-0.093	-1.1	0.27	0.01	0.57	579.4
	Confunctional seedling density	0.047	0.45	0.65			
	Heterofunctional seedling density	0.095	0.97	0.33			
	Stem length	-0.054	-0.54	0.59			
	The interaction between Proportion of leaf area lost to herbivory and confunctional seedling density	-0.093	-1.1	0.27			

The role of conspecific and heterospecific density in driving measures of herbivory.

A11. Supplemental Table 7. The role of conspecific density in driving differences in the incidence of herbivory between fixers and non-fixers. The effect of fixation and conspecific seedling density at the 1m^2 and the 20m^2 scale on the incidence of herbivory when surveyed in 2017. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	Variables	Estimate	Z Value	p value	R^{2}m	R^{2}c	AIC
In the **1m^2** plot	Fixation	0.35	2	**0.045**	0.01	0.3	1795.75
	Conspecific seedling density	-0.091	-0.77	0.44			
	Heterospecific seedling density	-0.032	-0.43	0.67			
	The interaction between fixation and conspecific seedling density	-0.21	-1.1	0.27			
In the **20m^2** plot	Fixation	0.36	2.1	**0.035**	0.01	0.3	1795.291
	Conspecific seedling density	0.019	0.17	0.87			
	Heterospecific seedling density	0.094	1.1	0.25			
	The interaction between fixation and conspecific seedling density	-0.22	-1.3	0.19			

A11. Supplemental Table 8. The role of conspecific density in driving differences in the proportion of leaf area lost to herbivory between fixers and non-fixers. The effect of fixation and conspecific seedling density at the 1m^2 and the 20m^2 scale on the proportion of leaf area lost to herbivory when surveyed in 2017. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	Variables	Estimate	Z Value	p value	R^{2}m	R^{2}c	AIC
In the **1m^2** plot	Fixation	0.052	2.1	**0.042**	0.04	0.31	352.81
	Conspecific seedling density	-0.0056	-0.17	0.86			
	Heterospecific seedling density	-0.0069	0.92	0.36			
	The interaction between fixation and conspecific seedling density	0.005	0.13	0.89			
In the **20m^2** plot	Fixation	0.06	2.6	**0.014**	0.04	0.33	344.03
	Conspecific seedling density	0.0043	0.4	0.69			
	Heterospecific seedling density	0.0013	0.16	0.87			
	The interaction between fixation and conspecific seedling density	0.013	0.57	0.57			

The role of conspecific and heterospecific density in driving measures of herbivory.

A11. Supplemental Table 9. The role of functional seedling density (fixer or non-fixer) in driving differences in the incidence of herbivory between fixers and non-fixers. The effect of fixation and functional seedling density (where confunctional density represents the density of seedlings from the same functional group) at the 1m^2 and the 20m^2 scale on the incidence of herbivory when surveyed in 2017. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	**Variables**	**Estimate**	**Z Value**	**p value**	**R^{2}m**	**R^{2}c**	**AIC**
In the **1m^2** plot	Fixation	0.45	2.1	**0.034**	0.02	0.22	1126.5
	Confunctional seedling density	-0.11	-0.96	0.34			
	Heterofunctional seedling density	-0.052	-0.6	0.55			
	The interaction between fixation and confunctional seedling density	-0.17	-0.67	0.5			
In the **20m^2** plot	Fixation	0.49	2.3	**0.022**	0.02	0.22	1128.3
	Confunctional seedling density	-0.00072	-0.0065	0.99			
	Heterofunctional seedling density	0.051	0.42	0.68			
	The interaction between fixation and confunctional seedling density	-0.026	-0.12	0.9			

A11. Supplemental Table 10. The role of functional seedling density (fixer or non-fixer) in driving differences in the proportion of leaf area lost to herbivory between fixers and non-fixers. The effect of fixation and functional seedling density (where confunctional density represents the density of seedlings from the same functional group) at the 1m^2 and the 20m^2 scale on the proportion of leaf area lost to herbivory when surveyed in 2017. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	Variables	Estimate	Z Value	p value	R^2m	R^2c	AIC
In the **1m^2** plot	Fixation	-0.015	-0.47	0.64	0.04	0.28	-212.3
	Confunctional seedling density	-0.054	-1	0.3			
	Heterofunctional seedling density	0.0015	0.15	0.88			
	The interaction between fixation and confunctional seedling density	0.039	0.73	0.46			
In the **20m^2** plot	Fixation	0.025	0.6	0.55	0.04	0.24	-214.4
	Confunctional seedling density	-0.056	-1.3	0.2			
	Heterofunctional seedling density	0.022	1.8	0.075			
	The interaction between fixation and confunctional seedling density	0.049	1.1	0.28			

The role of overall seedling density in driving measures of herbivory.

A11. Supplemental Table 11. The role of overall density in driving differences in the incidence of herbivory between fixers and non-fixers. The effect of fixation and overall seedling density at the 1m^2 and the 20m^2 scale on the incidence of herbivory when surveyed in 2017. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	Variables	Estimate	Z Value	p value	R^{2}m	R^{2}c	AIC
In the **1m^2** plot	Fixation	0.38	2.2	0.03	0.01	0.3	1792.93
	Overall seedling density	-0.17	-1.7	0.098			
	The interaction between fixation and overall seedling density	0.24	1.7	0.091			
In the **20m^2** plot	Fixation	0.39	2.2	0.026	0.01	0.3	1791.09
	Overall seedling density	-0.071	-0.64	0.52			
	The interaction between fixation and overall seedling density	0.29	2.1	**0.037**			

A11. Supplemental Table 12. The role of overall seedling density in driving differences in the proportion of leaf area lost to herbivory between fixers and non-fixers. The effect of fixation and overall seedling density at the 1m^2 and the 20m^2 scale on the proportion of leaf area lost to herbivory when surveyed in 2017. The model included species and plot identity as random effects. The elements that change between the models are in bold, as are significant p values.

Scale	Variables	Estimate	Z Value	p value	R^2m	R^2c	AIC
In the **1m^2** plot	Fixation	0.033	1.1	0.29	0.04	0.33	347.69
	Overall seedling density	-0.0031	-1.3	0.2			
	The interaction between fixation and overall seedling density	0.0035	0.96	0.34			
In the **20m^2** plot	Fixation	0.054	2.4	**0.022**	0.04	0.31	352.8
	Overall seedling density	-0.0051	-0.45	0.65			
	The interaction between fixation and overall seedling density	0.014	0.93	0.35			

Appendix 12

Species list for chapter four

A12. Table 1. The number of seedlings of each species present at the start of the seedling census in the 50ha plot on BCI, Panama. Showing the species names, family, fixation status and the number of seedlings in the census in 2001.

Species	Family	Fixation status	Seedling abundance
Acalypha Macrostachya	Euphorbiaceae	Non-fixer	6
Adelia Triloba	Euphorbiaceae	Non-fixer	24
Aegiphila Panamensis	Lamiaceae	Non-fixer	6
Alchornea Costaricensis	Euphorbiaceae	Non-fixer	42
Alchornea Latifolia	Euphorbiaceae	Non-fixer	2
Alibertia Edulis	Rubiaceae	Non-fixer	69
Allophylus Psilospermus	Sapindaceae	Non-fixer	43
Alseis Blackiana	Rubiaceae	Non-fixer	1222
Amaioua corymbosa	Rubiaceae	Non-fixer	5
Anacardium Excelsum	Anacardiaceae	Non-fixer	25
Andira Inermis	Fabaceae	Fixer	1
Annona Spraguei	Annonaceae	Non-fixer	21
Apeiba Membranacea	Malvaceae	Non-fixer	39
Apeiba Tibourbou	Malvaceae	Non-fixer	6
Ardisia standleyana	Primulaceae	Non-fixer	7
Aspidosperma spruceanum	Apocynaceae	Non-fixer	184
Astronium Graveolens	Anacardiaceae	Non-fixer	19
Beilschmiedia pendula	Lauraceae	Non-fixer	5611
Brosimum Alicastrum	Moraceae	Non-fixer	358
Calophyllum Longifolium	Calophyllaceae	Non-fixer	235
Casearia Aculeata	Salicaceae	Non-fixer	60
Casearia Arborea	Salicaceae	Non-fixer	11
Casearia Guianensis	Salicaceae	Non-fixer	1
Casearia Sylvestris	Salicaceae	Non-fixer	5
Cassipourea Elliptica	Rhizophoraceae	Non-fixer	99
Cavanillesia Platanifolia	Malvaceae	Non-fixer	3
Cecropia Insignis	Urticaceae	Non-fixer	66
Cecropia Obtusifolia	Urticaceae	Non-fixer	16
Cedrela Odorata	Meliaceae	Non-fixer	5
Ceiba Pentandra	Malvaceae	Non-fixer	2
Celtis Schippii	Cannabaceae	Non-fixer	3
Chamguava Schippii	Myrtaceae	Non-fixer	30
Chrysophyllum Argenteum	Sapotaceae	Non-fixer	174
Chrysophyllum Cainito	Sapotaceae	Non-fixer	283

Cinnamomum Triplinerve	Lauraceae	Non-fixer	82
Coccoloba Coronata	Polygonaceae	Non-fixer	41
Coccoloba Manzinellensis	Polygonaceae	Non-fixer	12
Cordia Alliodora	Cordiaceae	Non-fixer	75
Cordia Bicolor	Cordiaceae	Non-fixer	103
Cordia Lasiocalyx	Cordiaceae	Non-fixer	252
Coussarea Curvigemmia	Rubiaceae	Non-fixer	134
Croton Billbergianus	Euphorbiaceae	Non-fixer	377
Cupania latifolia	Sapindaceae	Non-fixer	8
Cupania Rufescens	Sapindaceae	Non-fixer	18
Cupania Seemannii	Sapindaceae	Non-fixer	49
Dendropanax Arboreus	Araliaceae	Non-fixer	6
Desmopsis Panamensis	Annonaceae	Non-fixer	1081
Diospyros Artanthifolia	Ebenaceae	Non-fixer	35
Dipteryx Oleifera	Fabaceae	Non-fixer	46
Drypetes Standleyi	Putranjivaceae	Non-fixer	614
Erythroxylum Macrophyllum	Erythroxylaceae	Non-fixer	77
Erythroxylum Panamense	Erythroxylaceae	Non-fixer	15
Eugenia Coloradoensis	Myrtaceae	Non-fixer	179
Eugenia Galalonensis	Myrtaceae	Non-fixer	386
Eugenia Nesiotica	Myrtaceae	Non-fixer	433
Eugenia Oerstediana	Myrtaceae	Non-fixer	1895
Faramea Occidentalis	Rubiaceae	Non-fixer	3339
Ficus Costaricana	Moraceae	Non-fixer	1
Garcinia intermedia	Clusiaceae	Non-fixer	515
Garcinia Madruno	Clusiaceae	Non-fixer	30
Genipa Americana	Rubiaceae	Non-fixer	2
Guapira Standleyana	Nyctaginaceae	Non-fixer	27
Guarea fuzzy	Meliaceae	Non-fixer	63
Guarea Grandifolia	Meliaceae	Non-fixer	9
Guarea Guidonia	Meliaceae	Non-fixer	281
Guatteria dumetorum	Annonaceae	Non-fixer	31
Guazuma Ulmifolia	Malvaceae	Non-fixer	7
Guettarda Foliacea	Rubiaceae	Non-fixer	18
Gustavia Superba	Lecythidaceae	Non-fixer	403
Hampea Appendiculata	Malvaceae	Non-fixer	52
Hasseltia Floribunda	Salicaceae	Non-fixer	14
Heisteria Acuminata	Olacaceae	Non-fixer	13
Heisteria Concinna	Olacaceae	Non-fixer	59
Herrania Purpurea	Malvaceae	Non-fixer	25
Hieronyma Alchorneoides	Phyllanthaceae	Non-fixer	11
Hirtella Americana	Chrysobalanaceae	Non-fixer	3
Hirtella Triandra	Chrysobalanaceae	Non-fixer	334
Hura Crepitans	Euphorbiaceae	Non-fixer	9
Inga Acuminata	Fabaceae	Fixer	259

Inga Goldmanii	Fabaceae	Fixer	10
Inga Laurina	Fabaceae	Fixer	19
Inga Marginata	Fabaceae	Fixer	937
Inga Multijuga	Fabaceae	Fixer	31
Inga Nobilis	Fabaceae	Fixer	23
Inga Oerstediana	Fabaceae	Fixer	3
Inga Pezizifera	Fabaceae	Fixer	3
Inga Sapindoides	Fabaceae	Fixer	37
Inga Spectabilis	Fabaceae	Fixer	4
Inga Thibaudiana	Fabaceae	Fixer	16
Inga Umbellifera	Fabaceae	Fixer	42
Inga Vera	Fabaceae	Fixer	7
Jacaranda Copaia	Bignoniaceae	Non-fixer	16
Lacistema Aggregatum	Lacistemataceae	Non-fixer	242
Lacmellea Panamensis	Apocynaceae	Non-fixer	32
Laetia Thamnia	Salicaceae	Non-fixer	25
Licania Hypoleuca	Chrysobalanaceae	Non-fixer	11
Licania platypus	Chrysobalanaceae	Non-fixer	13
Lindackeria Laurina	Achariaceae	Non-fixer	3
Lonchocarpus Heptaphyllus	Fabaceae	Fixer	63
Luehea Seemannii	Malvaceae	Non-fixer	51
Macrocnemum Roseum	Rubiaceae	Non-fixer	1
Maquira Guianensis	Moraceae	Non-fixer	9
Maytenus schippii	Celastraceae	Non-fixer	6
Miconia Affinis	Melastomataceae	Non-fixer	30
Miconia Argentea	Melastomataceae	Non-fixer	99
Miconia Elata	Melastomataceae	Non-fixer	1
Miconia Hondurensis	Melastomataceae	Non-fixer	5
Mosannona Garwoodii	Annonaceae	Non-fixer	32
Myrcia gatunensis	Myrtaceae	Non-fixer	14
Myrospermum Frutescens	Fabaceae	Non-fixer	5
Nectandra Cissiflora	Lauraceae	Non-fixer	20
Nectandra Lineata	Lauraceae	Non-fixer	133
Nectandra purpurea	Lauraceae	Non-fixer	2
Ochroma Pyramidale	Malvaceae	Non-fixer	1
Ocotea Cernua	Lauraceae	Non-fixer	68
Ocotea Oblonga	Lauraceae	Non-fixer	94
Ocotea Puberula	Lauraceae	Non-fixer	34
Ocotea Whitei	Lauraceae	Non-fixer	2407
Ormosia Coccinea	Fabaceae	Fixer	7
Ormosia Macrocalyx	Fabaceae	Fixer	11
Pentagonia Macrophylla	Rubiaceae	Non-fixer	3
Perebea Xanthochyma	Moraceae	Non-fixer	5
Picramnia Latifolia	Picramniaceae	Non-fixer	241
Piper boreum	Piperaceae	Non-fixer	2

Piper Reticulatum	Piperaceae	Non-fixer	10
Platymiscium Pinnatum	Fabaceae	Fixer	226
Platypodium Elegans	Fabaceae	Fixer	26
Posoqueria Latifolia	Rubiaceae	Non-fixer	4
Poulsenia Armata	Moraceae	Non-fixer	7
Pourouma Bicolor	Urticaceae	Non-fixer	22
Pouteria Reticulata	Sapotaceae	Non-fixer	1582
Pouteria Stipitata	Sapotaceae	Non-fixer	9
Prioria Copaifera	Fabaceae	Non-fixer	93
Protium Confusum	Burseraceae	Non-fixer	1
Protium Costaricense	Burseraceae	Non-fixer	28
Protium Panamense	Burseraceae	Non-fixer	120
Protium Tenuifolium	Burseraceae	Non-fixer	449
Protium Tenuifolium	Burseraceae	Non-fixer	449
Pseudobombax Septenatum	Malvaceae	Non-fixer	1
Psidium Friedrichsthalianum	Myrtaceae	Non-fixer	3
Psychotria Grandis	Rubiaceae	Non-fixer	3
Pterocarpus rohrii	Fabaceae	Fixer	62
Quararibea Asterolepis	Malvaceae	Non-fixer	3165
Quararibea Asterolepis	Malvaceae	Non-fixer	3165
Quassia Amara	Simaroubaceae	Non-fixer	2
Randia Armata	Rubiaceae	Non-fixer	752
Sapium Glandulosum	Euphorbiaceae	Non-fixer	3
Simarouba Amara	Simaroubaceae	Non-fixer	249
Siparuna Pauciflora	Siparunaceae	Non-fixer	35
Sloanea Terniflora	Elaeocarpaceae	Non-fixer	7
Solanum Hayesii	Solanaceae	Non-fixer	8
Spondias Mombin	Anacardiaceae	Non-fixer	2
Spondias Radlkoferi	Anacardiaceae	Non-fixer	86
Sterculia Apetala	Malvaceae	Non-fixer	7
Swartzia Simplex	Fabaceae	Fixer	1135
Swartzia Simplex	Fabaceae	Fixer	1135
Symphonia Globulifera	Clusiaceae	Non-fixer	14
Tabebuia guayacan	Bignoniaceae	Non-fixer	15
Tabebuia Rosea	Bignoniaceae	Non-fixer	61
Tabernaemontana Arborea	Apocynaceae	Non-fixer	136
Tachigali versicolor	Fabaceae	Fixer	377
Talisia Nervosa	Sapindaceae	Non-fixer	12
Talisia princeps	Sapindaceae	Non-fixer	76
Terminalia Amazonia	Combretaceae	Non-fixer	1
Terminalia Oblonga	Combretaceae	Non-fixer	1
Terminalia Oblonga	Combretaceae	Non-fixer	1
Tetragastris panamensis	Burseraceae	Non-fixer	1527
Thevetia Ahouai	Apocynaceae	Non-fixer	6

Tocoyena Pittieri	Rubiaceae	Non-fixer	2
Trema Micrantha	Cannabaceae	Non-fixer	1
Trichilia Pallida	Meliaceae	Non-fixer	30
Trichilia Tuberculata	Meliaceae	Non-fixer	1408
Triplaris Cumingiana	Polygonaceae	Non-fixer	26
Trophis Caucana	Moraceae	Non-fixer	80
Trophis Racemosa	Moraceae	Non-fixer	42
Turpinia occidentalis	Staphyleaceae	Non-fixer	8
Unonopsis Pittieri	Annonaceae	Non-fixer	164
Virola Multiflora	Myristicaceae	Non-fixer	2
Virola Sebifera	Myristicaceae	Non-fixer	46
Virola surinamensis	Mysisticaceae	Non-fixer	16
Vismia Baccifera	Hypericaceae	Non-fixer	11
Vochysia Ferruginea	Vochysiaceae	Non-fixer	5
Xylopia Macrantha	Annonaceae	Non-fixer	129
Zanthoxylum Acuminatum	Rutaceae	Non-fixer	4
Zanthoxylum Ekmanii	Rutaceae	Non-fixer	20
Zanthoxylum Panamense	Rutaceae	Non-fixer	24
Zuelania Guidonia	Salicaceae	Non-fixer	3