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INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA  
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**Respostas *In Situ* de plantas de sub-bosque à concentração elevada de CO<sub>2</sub>  
em uma floresta de terra firme na Amazônia Central**

Amanda Rayane Damasceno Macambira

**Manaus – Amazonas**

**Novembro/2021**

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Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Ecologia do Instituto Nacional de Pesquisas da Amazônia – INPA, como parte dos requisitos para obtenção do título de Mestre em Ecologia.

**Manaus – Amazonas**

**Novembro/2021**

**PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA**

**ATA DA DEFESA PÚBLICA DA DISSERTAÇÃO DE MESTRADO DO PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA DO INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA.**

Aos 30 dias do mês de Novembro do ano de 2021, às 14h00min, por videoconferência, reuniu-se a Comissão Examinadora de Defesa Pública, composta pelos seguintes membros: o Dr. **Giordane Martins**, do Instituto Nacional de Pesquisas da Amazônia – INPA, a Dra. **Flavia Delgado Santana**, do Instituto Nacional de Pesquisas da Amazônia - INPA e o Dr. **Bart Kruijt**, da Universidade de Wageningen, tendo como suplentes o Dr. Marciel José Ferreira, da Universidade Federal do Amazonas - UFAM e a Dra. Izabela Fonseca Aleixo, Pós-Doc da Amazon Face, do Instituto Nacional de Pesquisas da Amazônia, sob a presidência do orientador, a fim de proceder a arguição pública do trabalho de DISSERTAÇÃO DE MESTRADO da **AMANDA RAYANE DAMASCENO MACAMBIRA**, intitulado: "RESPOSTAS IN SITU DE PLANTAS DE SUB-BOSQUE À CONCENTRAÇÃO ELEVADA DE CO2 EM UMA FLORESTA DE TERRA FIRME NA AMAZÔNIA CENTRAL", orientada pelo Dr. Carlos Alberto Nobre Quesada, do Instituto Nacional de Pesquisas da Amazônia – INPA e Co-orientada pelos Drs. Sabrina Garcia e Tomas Ferreira Domingues.

Após a exposição, o discente foi arguido oralmente pelos membros da Comissão Examinadora, tendo recebido o conceito final:

APROVADO (A)                       REPROVADO (A)  
 POR UNANIMIDADE                       POR MAIORIA

Nada mais havendo, foi lavrada a presente ata, que, após lida e aprovada, foi assinada pelos membros da Comissão Examinadora.

DR. GIORDANE MARTINS  
 DRA. FLAVIA DELGADO SANTANA  
 DR. BART KRUIJT



(Coordenação PPG-ECO/INPA)

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### **Sinopse:**

Foi investigado respostas fotossintéticas sob concentração elevada de dióxido de carbono de uma comunidade de plantas de sub-bosque da Amazônia Central. Resultados relacionados ao aparato fotossintético (trocas gasosas) e parâmetros de crescimento (índices biométricos) foram avaliados.

Palavras-chave: mudanças climáticas, fotossíntese, eCO<sub>2</sub>, câmaras de topo aberto, floresta tropical

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## RESUMO

A Amazônia detém um grande estoque de carbono e desempenha um papel importante no ciclo do carbono (C), atuando como sumidouro de dióxido de carbono (CO<sub>2</sub>) e ajudando a mitigar os efeitos do aumento exponencial de CO<sub>2</sub> na atmosfera. No entanto, nenhuma planta *in situ* foi submetida a um experimento de CO<sub>2</sub> elevado (eCO<sub>2</sub>) na Amazônia, restringindo a capacidade dos modelos de vegetação de capturar o efeito do eCO<sub>2</sub> à luz das mudanças climáticas. Aqui, pela primeira vez, foram instaladas câmaras de topo aberto (CTAs) no sub-bosque de uma floresta (*in situ*) na Amazônia Central, para avaliar a resposta das plantas ao aumento iminente de CO<sub>2</sub>. Após 120 dias de fertilização com CO<sub>2</sub>, observamos um aumento significativo de 35% nas taxas de assimilação de CO<sub>2</sub> (*A*), 32% na eficiência do uso da água (*EUA*), 18% na taxa máxima de transporte de elétrons (*Jmax*) e 32% no rendimento quântico fotossintético aparente ( $\Phi$ ). Embora não tenha havido diferença estatística na condutância estomática (*gs*) entre os tratamentos, foi observada uma redução de 6% nas plantas sob e[CO<sub>2</sub>]. Após 266 dias de fertilização com CO<sub>2</sub>, os índices biométricos (Diâmetro e Altura) e o fluxo foliar não apresentaram alterações significativas, mas um aumento significativo na área foliar foi observado sob e[CO<sub>2</sub>]. O sub-bosque da floresta amazônica, apesar de seu ambiente com pouca luz e heterogeneidade vegetal, responde positivamente ao enriquecimento de CO<sub>2</sub>. É necessário considerar como os possíveis efeitos do e[CO<sub>2</sub>] podem afetar a floresta amazônica, juntamente com seus serviços ecossistêmicos, e entender cada compartimento e estrato dessa floresta é importante para tentar prever a magnitude desses impactos.

**Palavras-chave:** Mudanças climáticas, Floresta Tropical, fotossíntese, área foliar, eficiência do uso da água, rendimento quântico fotossintético aparente.

## ABSTRACT

The Amazon holds a large carbon stock and plays an important role in the carbon cycle (C), acting as a carbon dioxide (CO<sub>2</sub>) sink and helping to mitigate the effects of the exponential increase of CO<sub>2</sub> in the atmosphere. However, no plant *in situ* has ever been subjected to experimental elevated CO<sub>2</sub> (eCO<sub>2</sub>) in Amazonia, constraining the ability of vegetation models to capture the eCO<sub>2</sub> effect in light of climate change. Here, for the first time, open-top chambers (OTCs), with a CO<sub>2</sub> enrichment atmosphere of 200 ppm above the ambient concentration, were installed in the understory of a forest (*in situ*) in the central Amazon, to evaluate plants' response to the imminent CO<sub>2</sub> increase. After 120 days of CO<sub>2</sub> fertilization, we observed a significant increase of 35% in the CO<sub>2</sub> assimilation rates (*A*), 32% in the water use efficient (*WUE*), 18% in the maximum electron transport rate (*Jmax*), and 32% in the apparent photosynthetic quantum yield ( $\Phi$ ). Although there was no statistical difference in stomatal conductance (*g<sub>s</sub>*) between the treatments, it was observed a reduction of 6% in plants under e[CO<sub>2</sub>]. After 266 days of CO<sub>2</sub> fertilization, the biometric indexes (*Diameter and Height*) and leaf flushing showed no significant changes, but a significant increase in leaf area was observed under e[CO<sub>2</sub>]. The Amazon Forest understory, despite its light limited environment and plant heterogeneity, respond positively to CO<sub>2</sub> enrichment. It is necessary to consider how the possible effects of e[CO<sub>2</sub>] could affect the Amazon forest, along with its ecosystem services, and understanding each compartment and stratum of this forest is important to try to predict the magnitude of these impacts.

**Keywords:** Climate change, Tropical Forest, photosynthesis, leaf area, water use efficiency, apparent photosynthetic quantum yield.



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## LISTA DE ABREVIACES E SIGLAS

Variable (abbreviation)	Definition	Unit
A	Net CO <sub>2</sub> assimilation	$\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$
Asat	Net CO <sub>2</sub> assimilation at saturating light	$\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$
ATP	Adenosina triphosphate	-
DB	Diameter Base	mm
E	Leaf transpiration	$\text{mmol H}_2\text{O m}^2 \text{ s}^{-1}$
<i>gs</i>	Stomatal conductance to water vapor	$\text{mmol H}_2\text{O m}^2 \text{ s}^{-1}$
Ht	Total height	cm
Ic	Light compensation point	$\mu\text{mol m}^2 \text{ s}^{-1}$
<i>Jmax</i>	Maximum rate of transport of electrons	$\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$
PPFD	Photosynthetic photon flux density	$\mu\text{mol photon m}^{-2}\text{s}^{-1}$
RuBP	Rubisco's substrate, stands for ribulose-1,5-biphosphate	-
<i>Vcmax</i>	Maximum carboxylation rate	$\mu\text{mol m}^2 \text{ s}^{-1}$
<i>WUE</i>	Water use efficiency	$\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1} / \text{mmol H}_2\text{O m}^2 \text{ s}^{-1}$
( $\Phi$ )	Apparent photosynthetic quantum yield	$\mu\text{mol m}^2 \text{ s}^{-1}$

Rubisco: Ribulose-1,5-biphosphate carboxylase/oxygenase

## INTRODUÇÃO GERAL

Os efeitos do atual aumento do dióxido de carbono (CO<sub>2</sub>) atmosférico causam incerteza sobre como as florestas tropicais, e especialmente a floresta amazônica, responderão a essas mudanças (Cernusak et al., 2013). A floresta amazônica é um grande sumidouro de carbono (Pan et al., 2011), e usa o CO<sub>2</sub> como base de seu metabolismo, armazenando quantidades substanciais de carbono em sua estrutura (Brienen et al., 2015). No entanto, o aumento de CO<sub>2</sub> não é necessariamente consistente com a resposta síncrona de sua absorção, uma vez que outros fatores como luz, água e disponibilidade de nutrientes também influenciam o processo de fixação de CO<sub>2</sub> das plantas (Fleischer et al., 2019; Mendes et al., 2013; Chazdon, 1988).

A Amazônia detém cerca de 20% do carbono armazenado nas florestas mundiais (Nogueira et al., 2018; Saatchi et al., 2011) e é caracterizada por uma alta diversidade de espécies, que compõe um ecossistema heterogêneo (Fauset et al., 2015; Steege et al., 2013), com um dossel denso e contínuo, e um perfil vertical com gradiente de luminosidade decrescente, do topo do dossel até o sub-bosque da floresta (Wright & Van Schaik, 1994). A quantidade de radiação solar que atinge a camada inferior da floresta varia entre 2 a 7% daquela que atinge a parte superior, tornando a luz um fator limitante para plantas de sub-bosque (Baldocchi e Collineau, 1994; Chazdon, 1988). A luz é essencial para a fixação de CO<sub>2</sub> das plantas, e esta variação vertical resulta em uma diferença na assimilação de CO<sub>2</sub> através dos estratos da floresta, com um sub-bosque dependente de períodos breves e instáveis de alta densidade de luz (*Sunflecks*) para manter um balanço de carbono positivo. Apesar da importância do sub-bosque para a diversidade vegetal das florestas tropicais (Memiaghe et al., 2016), esse estrato é muitas vezes negligenciado devido à quantidade relativamente menor de biomassa armazenada em sua estrutura (Bastin et al., 2017). No entanto, um estudo recente mostrou uma contribuição de sub-bosque de 20% para o sumidouro de carbono em uma floresta tropical da África Central (Hubau et al., 2019) e efeitos positivos da concentração elevada de CO<sub>2</sub> (e[CO<sub>2</sub>]) em plantas sob ambientes de luz limitantes já foram observados (Wurth et al., 1998; Kubiske & Pregitze, 1996), demonstrando a importância de estudos futuros observarem a floresta como um todo, considerando todos os estratos e sua contribuição para a ciclagem do carbono florestal.

Atualmente, a assimilação do carbono é limitada pela capacidade da enzima ribulose 1,5-bisfosfato carboxilase / oxigenase (RuBisCO), uma vez que o ambiente atual [CO<sub>2</sub>] não é suficiente para saturar o RuBisCO (Wullschleger, 1993; Sharkey, 1985). O aumento de CO<sub>2</sub>, além

do potencial para estimular a assimilação de carbono, pode tornar as plantas mais eficientes fotossinteticamente, diminuindo a taxa máxima de carboxilação ( $V_{cmax}$ ), que é uma proxy para a quantidade e atividade do RuBisCO, uma vez que as folhas podem otimizar suas taxas de carboxilação sem a necessidade de aumentar a síntese e produção de RuBisCO (Drake et al., 1997). No entanto, o aumento da assimilação de carbono aumenta a demanda por cofatores produzidos por reações fotossintéticas de luz (ATP e NADPH), que podem alterar a fotossíntese de ser limitada por Rubisco para ser limitada pela capacidade de regeneração de RUBP (Drake et al., 1997). Uma vez que a limitação pela regeneração RUBP é mais provável de ocorrer em ambientes com pouca luz (Sharkey, 1985), e a limitação da luz restringe a assimilação de carbono, o  $e[CO_2]$  pode compensar a limitação da fotossíntese pela regeneração RUBP aumentando a assimilação de carbono em plantas de sub-bosque. Além disso, para compensar a baixa disponibilidade de luz, plantas do sub-bosque das florestas tropicais tendem a investir recursos em estratégias para otimizar a captura de luz, como maior área foliar e maiores razões de fotossistema II: I (Gommers et al., 2013; Valladares & Niinemets, 2008).

Em geral, pesquisas anteriores que estudaram a resposta das plantas sob  $e[CO_2]$  observaram um aumento nas taxas de assimilação de  $CO_2$  ( $A$ , fotossíntese líquida) e uma diminuição na condutância estomática ( $g_s$ ), como os primeiros efeitos fisiológicos de  $e[CO_2]$  (Ellsworth et al., 2017; Ainsworth & Long, 2004). O aumento de  $A$  é esperado, uma vez que o  $CO_2$  é o substrato primário para o processo fotossintético (Long, 1991), e a redução da  $g_s$  ocorre porque as trocas de água e  $CO_2$  ocorrem pelo mesmo poro (estomático) e, pelo fato de haver mais  $CO_2$  disponível no ambiente, as folhas podem reduzir a condutância para minimizar a perda de água sem interferir na captação de  $CO_2$  por difusão (Responorth et al., 2008; Drake et al., 1997).

Uma das principais questões sobre como a floresta amazônica responderá ao aumento do  $CO_2$  atmosférico refere-se à diminuição da transpiração foliar ( $E$ ) como consequência da diminuição da  $g_s$ . Isso ocorre porque a floresta amazônica recicla cerca de 10 a 30% de sua água por meio do processo de  $E$ , dependendo da estação das chuvas (Sampaio et al., 2021; Satyamurty et al., 2013; Eltahir & Bras, 1994), e as projeções futuras sugerem que o aumento de  $CO_2$ , e a consequente diminuição de  $E$ , pode resultar em uma redução de 12% na precipitação local precipitação (Sampaio et al., 2021), alterando os serviços ecossistêmicos e a composição florística regional, onde espécies mais adaptadas a menores quantidades de precipitação poderiam substituir a fitofisionomia atual (Lapola et al., 2009).

O aumento exponencial do CO<sub>2</sub> atmosférico pode causar efeitos positivos ou negativos na floresta, aumentando a absorção de carbono (Poulter et al., 2010), ou aumentando a limitação de CO<sub>2</sub> por outros fatores, como nutrientes (Fleischer et al., 2019), ou mesmo mudando sua composição e estrutura florística por meio da seleção diferencial de espécies com estratégias funcionais mais vantajosas do que outras (Lapola et al., 2009). Seja qual for a resposta, é difícil prever com precisão como uma floresta hiperdiversa, como a floresta amazônica, responderá a essa mudança ambiental sem estudos prévios *in situ*.

Para melhor compreender a resposta das florestas tropicais ao aumento do CO<sub>2</sub> atmosférico, é necessário realizar estudos que simulem cenários previstos em condições naturais e, embora existam vários experimentos com e[CO<sub>2</sub>] (Fauset et al., 2019; Norby et al., 2015; Drake et al., 1997), nenhum ainda foi realizado em uma floresta tropical hiperdiversa. Aqui nós investigamos o efeito de e[CO<sub>2</sub>] nos parâmetros fotossintéticos relacionados à assimilação de carbono e uso de luz, e no crescimento das plantas, em uma comunidade hiperdiversa de plantas sub-bosque em uma floresta natural antiga da Amazônia Central. Portanto, testamos a hipótese de que ambientes de sub-bosque tropical serão afetados pelo e[CO<sub>2</sub>]. Esta hipótese foi avaliada em um conjunto de câmaras de topo aberto (CTAs), instaladas em uma comunidade de plantas de sub-bosque *in situ*, medindo a assimilação líquida de CO<sub>2</sub> na luz de saturação (*Asat*), capacidade fotossintética (*Vcmax* e *Jmax*), condutância estomática (*gs*), transpiração (*E*), ponto de compensação de luz (*Ic*), eficiência quântica aparente ( $\Phi$ ) e crescimento da planta (lançamento foliar – *Lff*, área foliar – *Lfarea*, altura total – *Ht*, diâmetro da base – *BD*).

### **Objetivo Geral:**

Investigar os efeitos do e[CO<sub>2</sub>] no aparato fotossintético e índices biométricos (crescimento) de uma comunidade de plantas no sub-bosque de uma floresta tropical.

### **Objetivos Específicos:**

- Investigar como o e[CO<sub>2</sub>] afeta a assimilação de carbono (*A*), a capacidade fotossintética (*Vcmax* e *Jmax*) e a condutância estomática (*gs*) em plantas sob e[CO<sub>2</sub>];
- Investigar os efeitos de e[CO<sub>2</sub>] nos parâmetros de resposta à luz: ponto de compensação de luz (*Ic*) e rendimento quântico aparente ( $\Phi$ );



- Avaliar a dinâmica da liberação de folhas (*Flushing*) em plantas sob ambiente de  $e[\text{CO}_2]$ , e observar se o  $e[\text{CO}_2]$  interfere em sua magnitude ou padrão após o início da fertilização com  $\text{CO}_2$ ;
- Avaliar as mudanças nos índices biométricos de crescimento foliar e vegetal (área foliar, altura total e diâmetro da base) sob  $e[\text{CO}_2]$ .

## CAPÍTULO ÚNICO

Este artigo será submetido à revista *Acta Amazônica*, classificação B1.

### **In situ responses of Amazonian understory plants to elevated CO<sub>2</sub>**

**Abstract:** The Amazon holds a large carbon stock and plays an important role in the carbon cycle (C), acting as a carbon dioxide (CO<sub>2</sub>) sink and helping to mitigate the effects of the exponential increase of CO<sub>2</sub> in the atmosphere. However, no plant in situ has ever been subjected to experimental elevated CO<sub>2</sub> (eCO<sub>2</sub>) in Amazonia, constraining the ability of vegetation models to capture the eCO<sub>2</sub> effect in light of climate change. Here, for the first time, open-top chambers (OTCs), with a CO<sub>2</sub> enrichment atmosphere of 200 ppm above the ambient concentration, were installed in the understory of a forest (*in situ*) in the central Amazon, to evaluate plants' response to the imminent CO<sub>2</sub> increase. After 120 days of CO<sub>2</sub> fertilization, we observed a significant increase of 35% in the CO<sub>2</sub> assimilation rates (*A*), 32% in the water use efficient (*WUE*), 18% in the maximum electron transport rate (*Jmax*), and 32% in the apparent photosynthetic quantum yield ( $\Phi$ ). Although there was no statistical difference in stomatal conductance (*g<sub>s</sub>*) between the treatments, it was observed a reduction of 6% in plants under e[CO<sub>2</sub>]. After 266 days of CO<sub>2</sub> fertilization, the biometric indexes (*Diameter and Height*) and leaf flushing showed no significant changes, but a significant increase in leaf area was observed under e[CO<sub>2</sub>]. The Amazon Forest understory, despite its light limited environment and plant heterogeneity, respond positively to CO<sub>2</sub> enrichment. It is necessary to consider how the possible effects of e[CO<sub>2</sub>] could affect the Amazon forest, along with its ecosystem services, and understanding each compartment and stratum of this forest is important to try to predict the magnitude of these impacts.

**Keywords:** Climate change, Tropical Forest, photosynthesis, leaf area, water use efficiency, apparent photosynthetic quantum yield.

## Introduction

### Title: "In situ responses of Amazonian understory plants to elevated CO<sub>2</sub>"

**Abstract:** The Amazon holds a large carbon stock and plays an important role in the carbon cycle (C), acting as a carbon dioxide (CO<sub>2</sub>) sink and helping to mitigate the effects of the exponential increase of CO<sub>2</sub> in the atmosphere. However, no plant *in situ* has ever been subjected to experimental elevated CO<sub>2</sub> (eCO<sub>2</sub>) in Amazonia, constraining the ability of vegetation models to capture the e[CO<sub>2</sub>] effect in light of climate change. Here, for the first time, open-top chambers (OTCs) with a CO<sub>2</sub> enrichment atmosphere of 200 ppm above the ambient concentration, were installed in the understory of a forest (*in situ*) in the central Amazon, to evaluate plants' response to the imminent CO<sub>2</sub> increase. After 120 days of CO<sub>2</sub> fertilization, we observed a significant increase of 36% in the CO<sub>2</sub> assimilation rates ( $A_{sat}$ ), 33% in the water use efficient ( $WUE$ ), 19% in the maximum electron transport rate ( $J_{max}$ ), and 33% in the apparent photosynthetic quantum yield ( $\Phi$ ). Although there was no statistical difference in stomatal conductance ( $g_s$ ) between the treatments, it was observed a reduction of 7% in plants under e[CO<sub>2</sub>]. The biometric indexes (*Base diameter and Height*) and leaf flushing showed no significant changes, but a significant increase in leaf area was observed under e[CO<sub>2</sub>]. The Amazon forest understory, despite its light limited environment and great diversity of species, responds positively to CO<sub>2</sub> enrichment. It is necessary to consider how the possible effects of e[CO<sub>2</sub>] could affect the Amazon forest, along with its ecosystem services, and understanding each compartment and stratum of this forest is important to try to predict the magnitude of these impacts.

**Keywords:** Climate change, Tropical forest, photosynthesis, leaf area, water use efficiency, apparent photosynthetic quantum yield.

## 1- Introduction

The effects of the current atmospheric carbon dioxide (CO<sub>2</sub>) increase cause uncertainty about how tropical forests, and especially the Amazon forest, will respond to this change (Cernusak et al., 2013). The Amazon forest is a large carbon sink (Pan et al., 2011), and uses CO<sub>2</sub> as the basis of its metabolism, storing substantial amounts of carbon in its structure (Brienen et al.,

2015). However, the CO<sub>2</sub> increase is not necessarily consistent with the synchronous response of its absorption, since other factors such as light, water, and nutrient availability also influence plants' CO<sub>2</sub> fixation process (Fleischer et al., 2019; Mendes et al., 2013; Chazdon, 1988).

The Amazon holds about 20% of the carbon stored in the world's forests (Nogueira et al., 2018; Saatchi et al., 2011) and is characterized by a high species diversity, which composes a heterogeneous ecosystem (Fauset et al., 2015; Steege et al., 2013), with a dense and continuous canopy, and a vertical profile with a decreasing luminosity gradient, from the top of the canopy to the forest understory (Wright & Van Schaik, 1994). The amount of solar radiation that reaches the lower layer of the forest ranges between 2 to 7% of that reaches the upper, turning light a limiting factor for understory plants (Baldocchi and Collineau, 1994; Chazdon, 1988). The light is essential for plants' CO<sub>2</sub> fixation, and this vertical variation results in a difference in CO<sub>2</sub> assimilation through the forest strata, with an understory dependent on brief and unstable periods of high light density (*sunflecks*) to maintain a positive carbon balance.

Despite the importance of the understory for the plant diversity of tropical forests (Memiaghe et al., 2016), this stratum is often neglected due to the relatively smaller amount of biomass stored in its structure (Bastin et al., 2017). Nonetheless, a recent study showed an understory contribution of 20% to the carbon sink in a Central African tropical forest (Hubau et al., 2019), and positive effects of elevated CO<sub>2</sub> concentration (e[CO<sub>2</sub>]) in plants under limiting light environments have already been observed (Wurth et al., 1998; Kubiske & Pregitze, 1996), demonstrating the importance of future studies observe the forest as a whole, considering all strata and their contribution to forest carbon cycling.

At present, carbon assimilation is limited by the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) capacity, once the current ambient [CO<sub>2</sub>] is not sufficient to saturate the RuBisCO (Wullschleger, 1993; Sharkey, 1985). The CO<sub>2</sub> increase, besides the potential to stimulate carbon assimilation, can turn the plants more photosynthetically efficient, decreasing the maximum carboxylation rate ( $V_{cmax}$ ), which is a proxy to the amount and activity of the RuBisCO, since the leaves can optimize their carboxylation rates without the need to increase RuBisCO synthesis and production (Drake et al., 1997). However, increased carbon assimilation increases the demand for cofactors produced by photosynthetic light reactions (ATP and NADPH), which can shift photosynthesis from being limited by Rubisco to being

limited by the regeneration capacity of RUBP (Drake et al., 1997). Since limitation by RUBP regeneration is most likely to occur in low light environments (Sharkey, 1985), and light limitation constrains carbon assimilation, the  $e[\text{CO}_2]$  can compensate the photosynthesis limitation by RUBP regeneration by increasing carbon assimilation in understory plants. In addition, to offset the low light availability, plants from the tropical forests' understory tend to invest resources in strategies to optimize light capture, such as larger leaf area and higher photosystem II:I ratios (Gommers et al., 2013; Valladares & Niinemets, 2008).

In general, previous research that studied plants' response under  $e[\text{CO}_2]$  observed an increase in  $\text{CO}_2$  assimilation rates ( $A$ , net photosynthesis) and a decrease in stomatal conductance ( $g_s$ ), as the first physiological effects of  $e[\text{CO}_2]$  (Ellsworth et al., 2017; Ainsworth & Long, 2004). The increase on  $A$  is expected, once  $\text{CO}_2$  is the primary substrate for the photosynthetic process (Long, 1991), and the reduction on  $g_s$  occurs because the water and  $\text{CO}_2$  exchanges occur through the same pore (stomatic) and, since there is more  $\text{CO}_2$  availability in the environment, the leaves can reduce the conductance to minimize water loss without interfering in the  $\text{CO}_2$  uptake by diffusion (Answorth et al., 2008; Drake et al., 1997).

One of the main questions about how the Amazon forest will respond to the atmospheric  $\text{CO}_2$  increase refers to the decrease in leaf transpiration ( $E$ ) as a consequence of the decrease in  $g_s$ . That is because the Amazon forest recycles about 10 to 30% of its water through  $E$ , depending on the rainfall season (Sampaio et al., 2021; Satyamurty et al., 2013; Eltahir & Bras, 1994), and future projections suggest that the  $\text{CO}_2$  increase, and the consequent  $E$  decrease, can result in a reduction of 12% in local precipitation (Sampaio et al., 2021), changing the provision of ecosystem services and the regional floristic composition, where species more adapted to lower precipitation amounts could replace the current phytophysionomy (Lapola et al., 2009).

The exponential increase of atmospheric  $\text{CO}_2$  may cause positive or negative effects on the forest, increasing the carbon uptake (Poulter et al., 2010), or increasing  $\text{CO}_2$  limitation by other factors, such as nutrients (Fleischer et al., 2019), or even changing its floristic composition and structure through the differential selection of species with advantageous functional strategies rather than others (Lapola et al., 2009). Whatever the answer, it is hard to accurately predict how a hyperdiverse forest, like the Amazon forest, will respond to this environmental change without prior *in situ* studies.

To better understand the response of tropical forests to atmospheric CO<sub>2</sub> increase, it is necessary to conduct studies that simulate predicted scenarios under natural conditions and, although there are several experiments with e[CO<sub>2</sub>] (Fauset et al., 2019; Norby et al., 2015; Drake et al., 1997), none have yet been carried out in a hyperdiverse tropical forest. Here we investigate the effect of e[CO<sub>2</sub>] on the photosynthetic parameters related to carbon assimilation and light use, and on plant growth, in a hyperdiverse community of understory plants on an old-growth natural forest of central Amazon. Therefore, we tested the hypothesis that tropical understory environments will be affected by e[CO<sub>2</sub>]. This hypothesis was evaluated in a set of open-top chambers (OTCs), installed in a community of *in situ* understory plants, by measuring net CO<sub>2</sub> assimilation at saturating light ( $A_{sat}$ ), photosynthetic capacity ( $V_{cmax}$  and  $J_{max}$ ), stomatal conductance ( $g_s$ ), transpiration ( $E$ ), light compensation point ( $I_c$ ), apparent quantum yield ( $\Phi$ ), and plant growth (leaf flushing –  $Lf_f$ , leaf area –  $Lf_{area}$ , total tree height –  $Ht$ , base diameter –  $BD$ ).

## 2- Materials and Methods

### 2.1 AmazonFACE project and site description

The AmazonFACE (*Free-Air CO<sub>2</sub> Enrichment* - <https://amazonface.inpa.gov.br>) is the world's first FACE experiment in an old-growth tropical forest. Located at the Experimental Station of Tropical Forestry (EEST/ ZF-2), in Central Amazon (2°35'39" S, 60°12'29"W), and maintained by the National Institute of Amazonian Research (INPA), the project aims to find out how the ecosystem is likely to respond to rising levels of carbon dioxide (Lapola & Norby, 2014). The site is situated on a highly weathered deep soil, rich in clay (76%), with 15% nitrogen, 2.25% carbon, and 0.01% phosphorus (101.78 mg Kg<sup>-1</sup>) (Quesada et al., 2010). The vegetation of this site is classified as *terra firme* (upland) evergreen forest, characterized by the high diversity of species, with an average canopy height of 30 m and canopy tree crowns close to each other (Pereira et al., 2019), resulting in reduced light incidence in the lowest forest stratum (understory). The climate of the region is classified as a rainy tropical climate according to Köppen-Geiger (Peel et al., 2007). The mean annual temperature in the site is 26.7 °C with low seasonal variation (24.5 - 27.5 °C - min and max, respectively), and mean annual precipitation of 2400 mm, with a drier period between July and September when monthly precipitation can reach less than 100 mm (Tanaka et al., 2014; Ferreira et al., 2009).

## 2.2 Experimental design

Eight steel-polypropylene open top chambers (OTCs), with 2.5 m diameter and 3 m height each, designed to increase the CO<sub>2</sub> concentration inside them (Supplementary Figure 1), were installed in the experimental site. The chambers were set up in pairs, with four control (ambient – a[CO<sub>2</sub>], n = 4) and four treatment (+200 ppmv – e[CO<sub>2</sub>], n = 4) chambers. Each pair of OTC has a CO<sub>2</sub> sensor (Li-Cor 840A, Li-Cor Inc., Lincoln, NE, USA), installed in a nearby central system that measures the CO<sub>2</sub> inside the chambers and controls the CO<sub>2</sub> injection in the e[CO<sub>2</sub>] ones, whenever the difference between the pair of OTCs falls below 200 ppm. The CO<sub>2</sub> is injected inside the e[CO<sub>2</sub>] chambers through a gas line, connected to a central cylinder system, and spread by fans installed close to the CO<sub>2</sub> injection hose. CO<sub>2</sub> injectors are switched on at 6 am and off at 6 pm. Besides the eight OTCs installed, there are four outside control areas (no chamber – OC, n = 4), of the same size that the OTCs, that are monitored. All the 12 areas (4-a[CO<sub>2</sub>], 4-e[CO<sub>2</sub>], 4-OC) were trenched (30 cm wide by 50 cm deep) to isolate them from the surrounding forest. The number of trees varies from 9 to 18 between the 12 plots (OTCs and outside control areas) and, due to the high local biodiversity, there is not a species that occurs in all of them (Supplementary - Tabela 1). The CO<sub>2</sub> injection in the e[CO<sub>2</sub>] chambers started in November 2019.

## 2.3 Leaf level gas exchange measurements

Leaf level gas exchange parameters were determined through light-saturated CO<sub>2</sub> assimilation vs intercellular CO<sub>2</sub> concentration ( $A/C_i$ ) curves in up to three leaves from three individuals per OTC (1-3<sub>leaves</sub> x 3<sub>i</sub> x 4 a-e[CO<sub>2</sub>], n<sub>total</sub> = 70), and light response curves in one leaf from three individuals per OTC (1<sub>leaf</sub> x 3<sub>i</sub> x 4 a-e[CO<sub>2</sub>], n<sub>total</sub> = 24), during March 2020, 120 days after CO<sub>2</sub> fertilization starts. From each  $A/C_i$  response curve, the net CO<sub>2</sub> assimilation at saturating light ( $A_{sat}$ ), stomatal conductance ( $g_s$ ), leaf transpiration ( $E$ ), and water use efficiency ( $WUE$ ) parameters were measured, and the apparent maximum rate of Rubisco carboxylation ( $V_{cmax}$ ) and the apparent maximum rate of electron transport for RuBP regeneration under saturating light ( $J_{max}$ ) were quantified using the biochemical model of Farquhar-von Caemmerer-Berry (1980) and subsequent modifications (Sharkey et al., 2007), using the photosynthetic kinetic

parameters proposed by Bernacchi et al., 2001. From the light response curves, the light compensation point ( $I_c$ ) and the apparent quantum yield ( $\Phi$ ) were calculated. These measurements were taken using a portable infrared gas analyzer (IRGA - LI-6400XT; Li-Cor® Biosciences, Lincoln, NE, USA).

For all measurements, the standardized set conditions inside the IRGA chamber were: airflow of  $400 \mu\text{mol s}^{-1}$ , relative humidity between 60 and 70%, and leaf temperature of  $30 \text{ }^\circ\text{C}$ . The  $A/C_i$  curves were performed at a saturated photosynthetic photon flux density (PPFD) of  $1000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , and the reference  $[\text{CO}_2]$  were controlled as follows: 400, 300, 200, 75, 50, 400, 600, 800, 1000, 1200 and  $1500 \mu\text{mol mol}^{-1}$ . The  $A_{sat}$ ,  $g_s$ ,  $E$ , and  $WUE$  parameters were taken from these curves according to the  $\text{CO}_2$  treatment, at  $400 \mu\text{mol mol}^{-1}$  for  $a[\text{CO}_2]$ , and  $600 \mu\text{mol mol}^{-1}$  for  $e[\text{CO}_2]$ . The photosynthetic capacity parameters ( $V_{cmax}$  and  $J_{max}$ ) were calculated by a curve-fitting routine based on minimum least squares (Domingues et al., 2010) and adjusted to the standard temperature of  $25 \text{ }^\circ\text{C}$  (Bernacchi et al., 2001). The light response curves were performed at  $[\text{CO}_2]$  of  $400 \mu\text{mol mol}^{-1}$  for  $a[\text{CO}_2]$ , and  $600 \mu\text{mol mol}^{-1}$  for  $e[\text{CO}_2]$ , using a PPFD sequence of 250, 500, 750, 1000, 1500, 500, 250, 100, 75, 50, 25, 10, 5 and  $0 \mu\text{mol m}^{-2} \text{ s}^{-1}$ . The  $I_c$  was calculated by the equation:

$$I_c = \frac{R_d}{\alpha} \quad (1)$$

where  $R_d$  is the dark respiration rate (PPFD = 0) and  $\alpha$  is the light response curve initial slope, between 0 and  $75 \mu\text{mol m}^{-2} \text{ s}^{-1}$  PPFD. The  $\Phi$  was determined as the light response curve initial slope above  $I_c$ .

The measurements were carried out on fully expanded leaves, between 8:00 am and 3:00 pm (local time), in individuals selected through the criteria of occurrence of the species among chambers and the size of the individual's leaf area.

#### 2.4 Leaf flushing

We measured leaf flushing (production) of all individuals from the three treatments ( $a[\text{CO}_2]$ ,  $e[\text{CO}_2]$ , and OC,  $n_{\text{total}} = 158$ ) from November 2019 to October 2020. The leaf flushing dynamic was monitored by following the leaf demography methodology described by Menezes et al., 2021 where, at the first time point, all leaves were included as initial stock, and the leaves from



both ends of each branch were tagged to monitor changes in leaf demography dynamics. Thereafter, the flushed leaves were included in the demographic censuses when its leaf blade was nearly expanded, while leaves that had abscised petiole were considered dead. Leaves demographic censuses were performed in November 2019, January, March, July, and October 2020. The cumulative leaf flushing was calculated by the sum of total leaf flushing during the sampled period.

### 2.5 Leaf area and biometric indexes of plant growth

We measured the leaf area in 121 of the 166 monitored individuals ( $n_{e[CO_2]} = 41$ ;  $n_{a[CO_2]} = 42$  and  $n_{OC} = 38$ )

We quantify changes in leaf area by measuring two fully expanded leaves from the same branch, one that flushed before and the other that flushed after the beginning of the experiment (November 2019). The measurements of both leaves were taken in the same campaign, in July 2020. Leaf area was determined from photographs taken from leaves on graph paper and processed in the ImageJ software (<https://imagej.nih.gov/ij/>).

The percentage change in leaf area ( $L_{farea}\%$ ) was calculated as the difference between the leaves that flushed before and after the CO<sub>2</sub> fertilization starts, in the same branch, by the following equation:

$$L_{farea}\% = [(L_f - L_0)/L_0 * 100] \quad (3)$$

where  $L_f$  is the sum of the leaf area of leaves that flushed after CO<sub>2</sub> fertilization and  $L_0$  is the sum of the areas from leaves that flushed before that.

We also measure the total tree height ( $n = 148$ ) and the increment of stem base diameter ( $n = 156$ ) between November 2019 and September 2020. The total tree height ( $Ht$ ) was measured with a millimeter measuring tape, from the base of the stem to the higher plant shoot apex. We divided the trees in three height classes:  $20 < 80$ ,  $80 < 140$ , and  $\geq 140$  cm. The base diameter ( $BD$ ) point measure was set in the first measure campaign, at 5 cm from the ground. Thereafter, subsequent measurements were taken at the point marked. The  $BD$  measurements were taken using a digital caliper (Digital Caliper - Mitutoyo/Absolute).

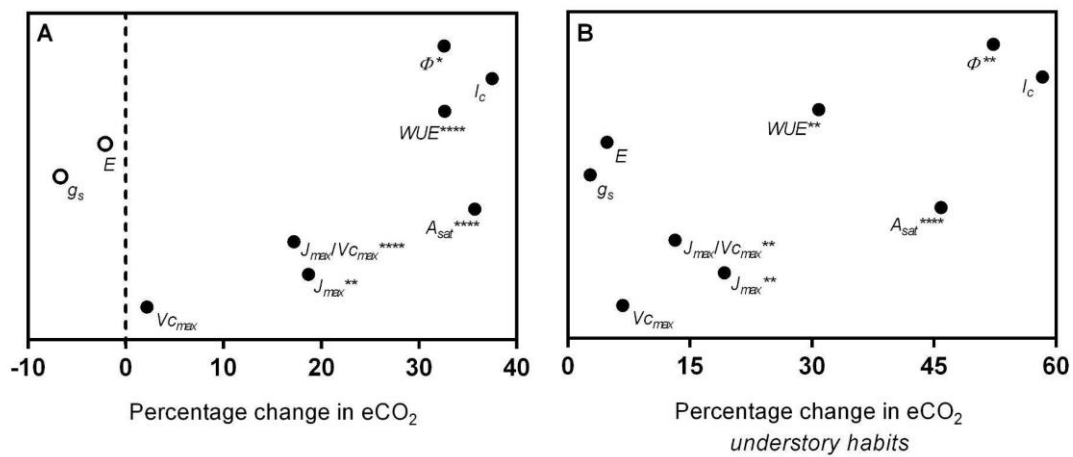
### 3 - Statistical analyses

We tested for normal distributions and equal variances of residuals with *Shapiro-Wilk* and *Levene's* tests, to meet the assumptions for the following tests. Data on gas exchange and leaf area parameters were analyzed by two-tailed unpaired *t-test* for parametric data, and *Mann Whitney t-test* for nonparametric data. Ordinary one-way ANOVA and *Kruskal Wallis* tests were used to test whether there is a difference between the biometric indexes, *Ht* and *BD*. These leaf gas exchange results are reported as the mean percentage change  $[(r - 1) \times 100]$  at elevated  $[CO_2]$ . Significant differences were regarded at  $p < 0.05$ . All analyses were performed with Graphpad - Prism program (version 8.0.1).

### 4 - Results

#### 4.1 $CO_2$ enrichment effects on leaf gas exchange

We analyzed the effects of  $e[CO_2]$  (environment + 200 ppmv) on *Asat*, *gs*, *E*, *WUE*, *Vcmax*, *Jmax*, *Jmax : Vmax*, *Ic*, and  $\Phi$ , in an understory tree community, using two different approaches: (1) considering the effect on all individuals measured; and (2) considering the effect only on understory habit trees. Furthermore, we analyzed whether the habit of the trees (canopy and understory) influences the variables analyzed under ambient conditions –  $a[CO_2]$ , without  $CO_2$  fertilization.



**Figure 1.** Percent change of *Asat*, *gs*, *E*, *WUE*, *Vcmax*, *Jmax*, *Jmax : Vmax*,  $\Phi$  and *Ic* to  $e[CO_2]$ . The dashed line represents no change, black circle (●) an increase, and open circle (○) a decrease in relation

to e[CO<sub>2</sub>], to (A) all trees ( $n = 70$ ), and (B) to understory trees ( $n = 36$ ). The asterisks indicate significant treatment effect ( $*p < 0.05$ ,  $***p < 0.001$  and  $****p < 0.0001$ ;  $t$ -test).

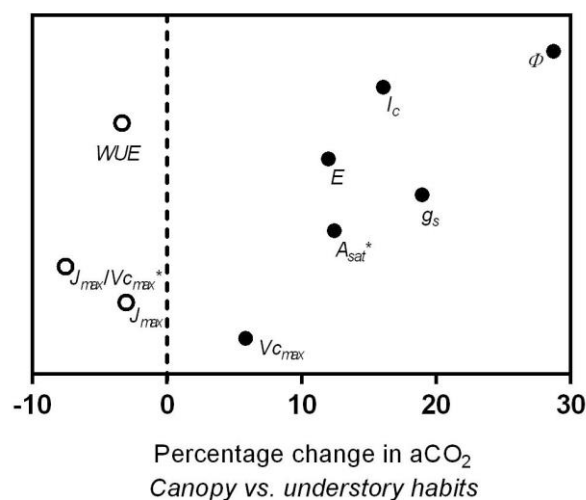
$A_{sat}$  was 36% higher under e[CO<sub>2</sub>] in all trees and 46% when we observed understory trees ( $p < 0.0001$  for both). The  $WUE$  was 33% ( $p < 0.0001$ ) and 31% ( $p = 0.0024$ ) higher in all and understory trees, respectively. The  $J_{max}$  and the  $J_{max} : V_{cmax}$  ratio were 19% and 17% higher in all trees ( $p = 0.0014$  and  $p < 0.0001$ ) and 19% and 13% in understory trees ( $p < 0.0025$  and  $p < 0.0019$ ). Although there was no significant difference on  $g_s$ , there was a decrease of 7% in trees under e[CO<sub>2</sub>]. The values of  $\Phi$  were 33% higher under e[CO<sub>2</sub>] in all trees, and 52% in understory trees ( $p = 0.0215$  and  $p = 0.005$ ), while  $I_c$  did not show significant difference (Figure 1, Table 1).

**Table 1.** Gas exchange parameters of trees under ambiente (a[CO<sub>2</sub>]) and elevated (e[CO<sub>2</sub>]) CO<sub>2</sub> concentration. Mean  $\pm$  SE, number of trees ( $n$ ), and  $p$ -value for each parameter analyzed between treatments.

Variable	a[CO <sub>2</sub> ]	$n$	e[CO <sub>2</sub> ]	$n$	$p$
$A_{sat}$	4.419 $\pm$ 0.1829	36	5.998 $\pm$ 0.3003	34	< 0.0001
$g_s$	0.09529 $\pm$ 0.00646	36	0.08891 $\pm$ 0.004435	34	0.4189
$E$	1.306 $\pm$ 0.07833	36	1.279 $\pm$ 0.05598	34	0.7759
$WUE$	3.661 $\pm$ 0.1754	36	4.856 $\pm$ 0.2443	34	< 0.0001
$V_{Cmax}$	18.41 $\pm$ 0.6141	36	18.81 $\pm$ 0.8756	34	0.7106
$J_{max}$	26.11 $\pm$ 0.7683	36	31.00 $\pm$ 1.235	34	0.0014
$J_{max} : V_{Cmax}$	1.437 $\pm$ 0.03367	36	1.684 $\pm$ 0.04444	34	< 0.0001
$\Phi$	0.05381 $\pm$ 0.00414	12	0.07134 $\pm$ 0.00568	12	0.0215
$I_c$	5.13 $\pm$ 0.933	12	7.053 $\pm$ 1.566	12	0.3053

Gas exchange parameters  $A_{sat}$  = assimilation at saturating light ( $\mu\text{mol m}^2 \text{s}^{-1}$ ),  $g_s$  = stomatal conductance ( $\text{mmol H}_2\text{O m}^2 \text{s}^{-1}$ ),  $E$  = transpiration ( $\text{mmol H}_2\text{O m}^2 \text{s}^{-1}$ ),  $WUE$  = water use efficiency ( $\mu\text{mol CO}_2 \text{ m}^2 \text{s}^{-1} / \text{mmol H}_2\text{O m}^2 \text{s}^{-1}$ ),  $V_{cmax}$  = apparent maximum rate of Rubisco carboxylation,  $J_{max}$  = apparent maximum rate of electron transport for RuBP regeneration under saturating light,  $J_{max} : V_{cmax}$  = ratio,  $\Phi$  = apparent quantum yield ( $\mu\text{mol m}^2 \text{s}^{-1}$ ),  $I_c$  = light compensation point ( $\mu\text{mol m}^2 \text{s}^{-1}$ ).  $p$ -value calculated by parametric and non-parametric  $t$ -test (Mann Whitney) depending on normal distributions and equal variances of residuals (see the Materials and Methods section).

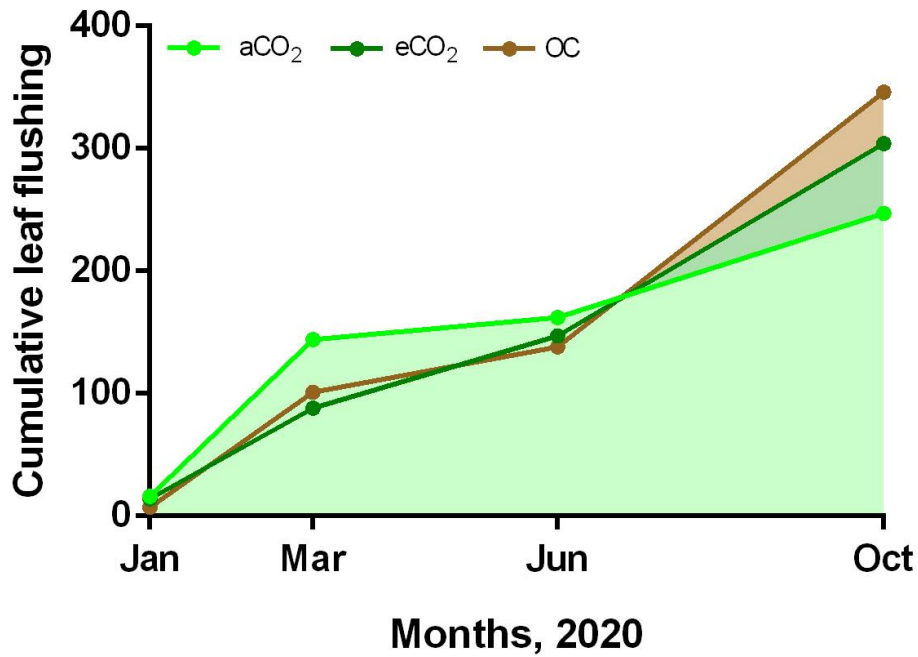
Furthermore, we performed analyzes between trees with canopy and understory habits under  $a[\text{CO}_2]$ . Only  $A_{sat}$  and  $J_{max} : V_{cmax}$  showed significant changes, where  $A_{sat}$  was 11% higher ( $p = 0.0043$ ), and  $J_{max} : V_{cmax}$  was 8% lower ( $p = 0.0211$ ) in canopy trees compared to understory (Figure 2).



**Figure 2.** Percent change of  $A_{sat}$ ,  $g_s$ ,  $E$ ,  $WUE$ ,  $V_{cmax}$ ,  $J_{max}$ ,  $J_{max} : V_{cmax}$ ,  $\Phi$  and  $I_c$  of canopy trees ( $n = 15$ ) to understory ( $n = 21$ ) under  $a[\text{CO}_2]$ . The dashed line represents no change, black circle (●) an increase, and open circle (○) a decrease. The asterisks indicate significant treatment effect (\* $p < 0.05$ , \*\*\* $p < 0.001$  and \*\*\*\* $p < 0.0001$ ;  $t$ -test).

#### 4.2 $\text{CO}_2$ enrichment effects on leaf flushing

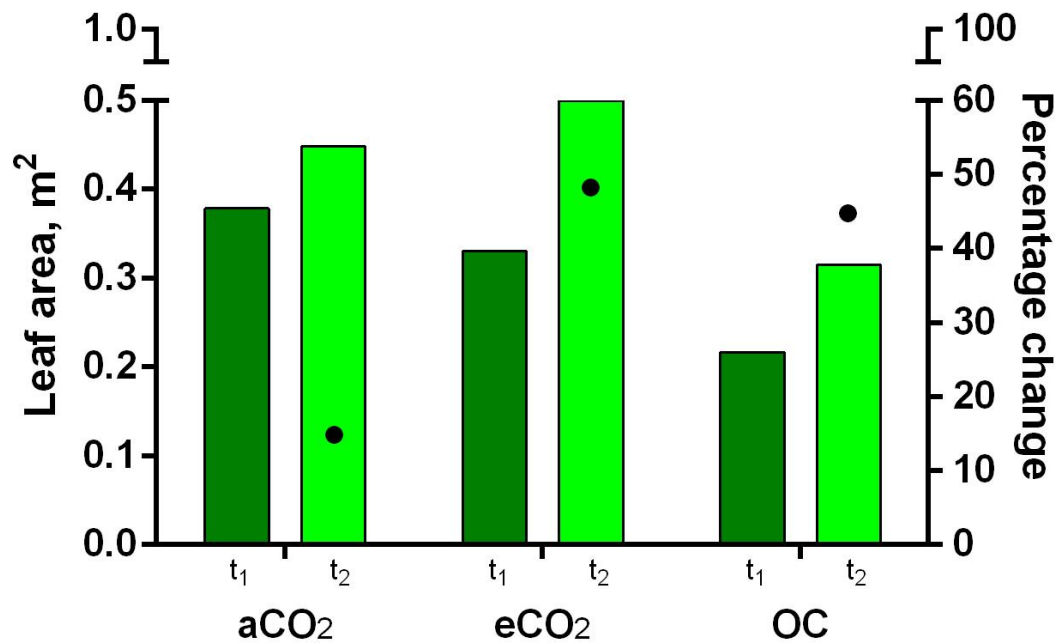
Across four campaigns, it was not possible to observe significant differences in leaf flushing between the three treatments ( $a[\text{CO}_2]$ ,  $e[\text{CO}_2]$ , and OC). However, when we analyzed the difference just between the trees under  $a[\text{CO}_2]$  and  $e[\text{CO}_2]$ , the final cumulative leaf flushing was significantly higher ( $p = 0.0219$ , Mann Whitney  $t$ -test) in trees under  $e[\text{CO}_2]$ . All in all, the final cumulative leaf flushing of trees under  $e[\text{CO}_2]$  was 23% higher than trees under  $a[\text{CO}_2]$  and 14% higher in trees from OC areas compared to those under  $e[\text{CO}_2]$ .



**Figure 3.** Cumulative leaf flushing measured from 158 trees over four field campaigns (January, March, June, and October 2020) across treatments: light green is a[CO<sub>2</sub>], dark green is e[CO<sub>2</sub>], and brown is OC.

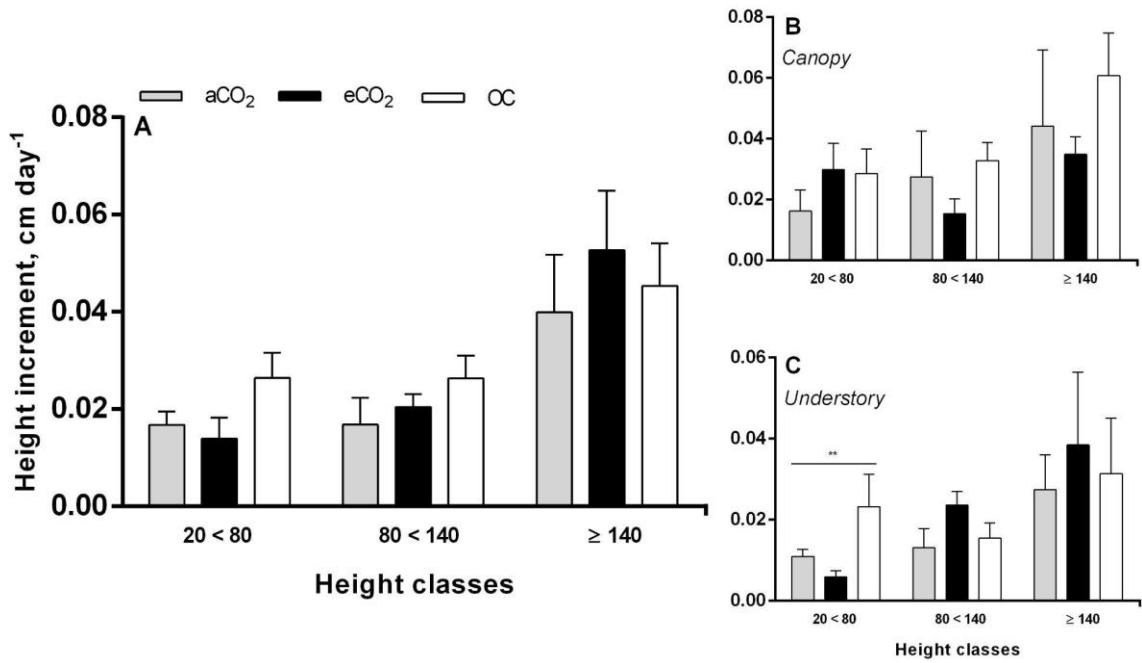
#### 4.3 CO<sub>2</sub> enrichment effects on leaf area and plant growth

There was an increase in the  $L_{farea}$  of leaves that flushed after the beginning of CO<sub>2</sub> fertilization (November 2019) compared to those that flushed after the fertilization started. The  $L_{farea}$  of trees under e[CO<sub>2</sub>] increased 51% ( $p = 0.0004$ ), while in trees under a[CO<sub>2</sub>] and OC the increase was of 45% ( $p = 0.0063$ ) and 18% ( $p = 0.0699$ ), respectively (Figure 4).

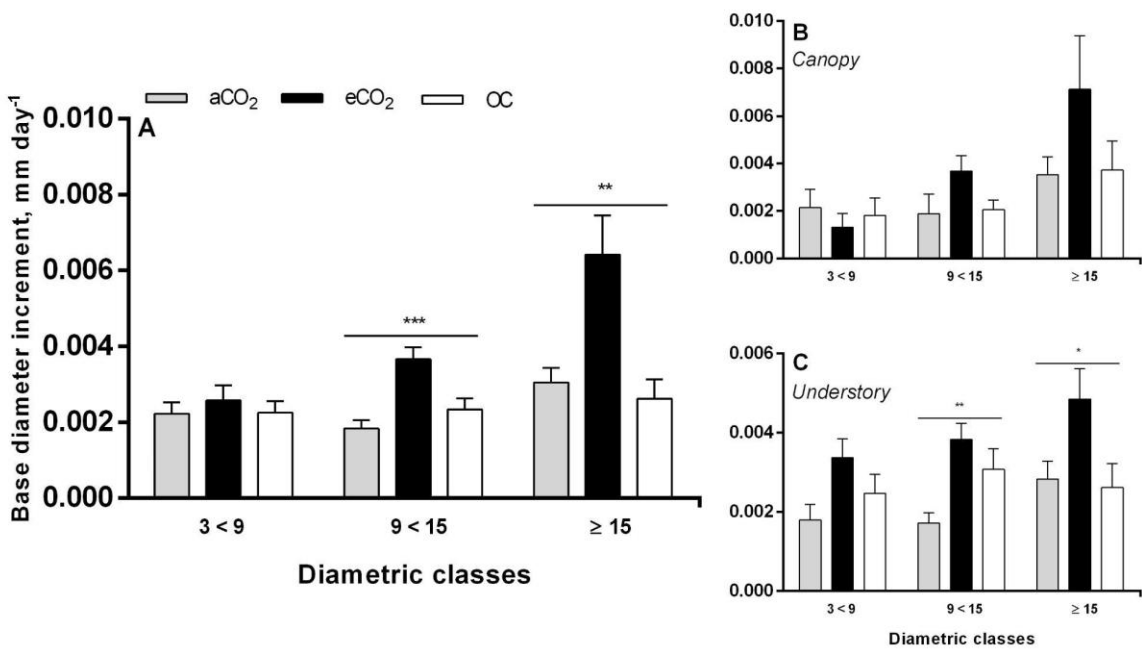


**Figure 4.** Leaf area flushed before (dark green bars - t<sub>1</sub>) and after (light green bars - t<sub>2</sub>) the CO<sub>2</sub> fertilization starts (November 2019). The left y-axis shows leaf area increase given in absolute number (m<sup>2</sup>) and the right y-axis shows leaf area increase in percentage (%). The black circle (●) is the percentage value of leaf area increase related to the right y-axis.

There was no e[CO<sub>2</sub>] effect detected for *Ht* increment when analyzing all trees, disregarding the habit (Figure 5A). However, the *Ht* increment of understory trees, belonging to the 20 < 80 cm height class, that were in the OC areas were significantly higher than the trees in the chambers (Figure 5C,  $p_{20 < 80 \text{ cm}} = 0.0026$ ). By contrast, the *BD* increment under e[CO<sub>2</sub>] was significantly higher in the 9 < 15 and  $\geq 15$  mm diameters classes when all trees, from canopy and understory, were analyzed together (Figure 6A,  $p_{9 < 15 \text{ mm}} = 0.0001$  and  $p_{\geq 15} = 0.0011$ ), as well as when only the understory trees were analyzed (Figure 6C,  $p_{9 < 15 \text{ mm}} = 0.0013$  and  $p_{\geq 15} = 0.0493$ ). There was no e[CO<sub>2</sub>] effect detected for *Ht* and *BD* increments when analyzing only the canopy trees (Figures 5B and 6B).



**Figure 5.** Daily mean height increment (cm day<sup>-1</sup>) of trees under a[CO<sub>2</sub>], e[C] and OC, divided into three height classes (20 < 80, 80 < 140 and ≥ 140 cm) for (A) all trees, (B) canopy habit trees, and (C) understory habit trees. Error bars indicate the standard error of the mean. The asterisks indicate significant treatment effect (\**p* < 0.05, \*\*\**p* < 0.001 and \*\*\*\**p* < 0.0001).



**Figure 6.** Daily mean base diameter increment (mm day<sup>-1</sup>) of trees under a[CO<sub>2</sub>], e[CO<sub>2</sub>] and OC, divided into three diameter classes (3 < 9, 9 < 15 and ≥ 15 mm) for (A) all trees, (B) canopy habit trees,

and (C) understory habit trees. Error bars indicate the standard error of the mean. The asterisks indicate significant treatment effect ( $*p < 0.05$ ,  $***p < 0.001$  and  $****p < 0.0001$ ).

## 5 - Discussion

### 5.1 Leaf carbon and water exchange response to elevated $CO_2$

Generally, the first physiological effects of  $e[CO_2]$  are increased photosynthetic rates ( $A_{sat}$ ) and decreased stomatal conductance ( $g_s$ ) (Cernusak et al., 2013; Ainsworth & Long, 2004). Here, a significant increase in  $A_{sat}$  was observed in trees under  $e[CO_2]$  however, no significant changes in  $g_s$  were observed between treatments, although there is a possible trend towards a reduction in  $g_s$ . The lack of  $g_s$  response to  $e[CO_2]$  may have been due to the non-variation of other environmental forcings, such as humidity and temperature (and consequently the vapor pressure deficit - VPD), since the stomata are extremely sensitive to environmental changes and these variables are, in general, constant in the understory of tropical forests (Mendes & Marengo, 2017).

Stomatal opening controls water vapor and  $CO_2$  exchanges between plants and the surrounding environment, allowing plants' transpiration ( $E$ ) and photosynthesis. In this study,  $E$  and  $g_s$  did not change under  $e[CO_2]$ , which, together with the  $A_{sat}$  increase, resulted in an 32% increase in plants' water use efficiency ( $WUE$ ) as they increased  $CO_2$  assimilation without changing water loss. Under  $e[CO_2]$ , the  $g_s$  decrease is expected to be stronger in sun-exposed leaves, due to the higher amounts of radiation and temperature in this environment (Ref), which also contribute to the  $g_s$  decrease. In the Amazon forest, where temperature and incident radiation are high and relatively constant, the decrease in  $g_s$  (and, consequently,  $E$ ) can reduce the amount of evaporated water into the atmosphere and alter rainfall patterns, both locally and over much South America (Sampaio et al., 2021; Eltahir & Bras, 1994). However, we did not observe a reduction in  $E$ , suggesting that, in this understory community, the  $E$  was not sensitive to the  $CO_2$  environmental change.

### 5.2 Photosynthetic capacity response to elevated $CO_2$



$V_{cmax}$  data showed no significant difference between treatments and remained without major variations. After a while of  $e[CO_2]$  exposure, is often observed a decrease in  $V_{cmax}$  values related to the acclimation of the photosynthetic apparatus, known as "down-regulation" (Ainsworth et al., 2004). The photosynthesis acclimation may be caused by several mechanisms, such as the accumulation of non-structural carbohydrates, as a result of increased photosynthetic rates, which can inhibit photosynthesis-related gene expression, leading to feedback inhibition of photosynthesis (Thompson et al. 2017; Ainsworth & Long, 2005; Drake et al., 1997). The absence of  $V_{cmax}$  variation under  $e[CO_2]$  may indicate that the period that the trees were under elevated  $CO_2$  was not enough for them to acclimate the photosynthetic apparatus.

On the other hand, *the*  $J_{max}$  and the  $J_{max}:V_{cmax}$  ratio showed a significant increase under  $e[CO_2]$ . These increases may be related to the increased demand for ATP and NADPH to regenerate RUBP, due to the increase in  $A_{sat}$ , as a consequence of  $e[CO_2]$  (Zhang et al. 2008).

### 5.3 Effects of $e[CO_2]$ on photosynthetic light response

The higher  $\Phi$  observed under  $e[CO_2]$  showed that these plants are being more efficient in fixing carbon than plants under  $a[CO_2]$ , in the same light environment. Under  $e[CO_2]$  an increase in photosynthetic rates is expected. RuBisco binds  $CO_2$  stronger than it does  $O_2$  and, with a higher  $CO_2$  concentration in the environment, the photorespiration rates (oxygenase) decrease, as a consequence of the increase in the  $CO_2:O_2$  ratio (Lambers et al., 2008). Here, the plants under  $e[CO_2]$  exhibited a significant  $\Phi$  increase, showing the ability of these plants to take advantage and improve the light use efficiency of the understory community.

Although a decrease in the  $I_c$  is also expected under  $e[CO_2]$ , due to the decrease in photorespiration rates, we didn't observe a significant difference between the treatments ( $a[CO_2]$  and  $e[CO_2]$ ). Indeed, the variation in the direction of  $I_c$  response, under  $e[CO_2]$ , are higher than  $\Phi$ , and depend on the species functional group and habit (Kubiske & Pregitze, 1996), which can explain the greater variation observed in the  $I_c$  range compared to  $\Phi$ .

### 5.3 Tree grown response to elevated $CO_2$

Regarding phenological measures, at the end of the last monitoring (October-2021) the accumulated amount of leaves flushed from trees in the e[CO<sub>2</sub>] chambers was significantly higher compared to the a[CO<sub>2</sub>] chambers. This greater leaf production may be related to the increase in photosynthesis (*Asat*) for biomass production consequently (Ainsworth & Long, 2004).

The increase in leaf area observed under e[CO<sub>2</sub>] (51%) may indicate an investment of the extra carbon assimilated in tissues for light capture. Previous studies showed an increase in aboveground biomass allocation in plants under e[CO<sub>2</sub>] (Norby et al., 2005), however, this response can vary depending on the species, functional group, and environment in which the plants are, such as forests or crops (Ainsworth & Long, 2004). The a[CO<sub>2</sub>] chambers showed an increase of 18% in leaf area, and the OC areas an increase of 45%. The higher leaf area values observed in the OC areas, compared to a[CO<sub>2</sub>], may be because the OC areas do not have the physical barrier imposed by the chambers, which may have influenced plant development, even under the same [CO<sub>2</sub>].

Although this period has a common release of leaves in the forest, especially described for canopy species ([citar artigo de fenologia](#)), the amount of leaves released from individuals under e[CO<sub>2</sub>] was higher than under a[CO<sub>2</sub>].

The biometric indices of *Ht* and *DB* showed different responses depending on the approach with which we analyzed. Although *Ht* was significant, only by habit, individuals belonging to the e[CO<sub>2</sub>] chambers did not show the greatest increments. However, both the larger diametric classes and the variation by functional group, specifically the understory, of the classes (9 < 15 and > 15) had positive increases under e[CO<sub>2</sub>], which leads us to consider that plants with understory habits under a high concentration of CO<sub>2</sub> prefers to allocate this available carbon in diameter, which can be a strategy at that time for these individuals to establish in the environment and not preferring to grow in height, since both leaf release and leaf area in exposed individuals when e[CO<sub>2</sub>] showed a positive effect, these results together reinforce the behavior of the functional group (understory). Ainsworth & long (2004), also found significant results of growth under e[CO<sub>2</sub>] for diameter, branch and number of leaves and reinforced that the responses depend on variation between species and environmental conditions.

Previous studies find distinct responses regarding tree growth and biomass accumulation in mature forests. In a eucalyptus forest was not observed a significant biomass increase after three years of CO<sub>2</sub> fumigation (Ellsworth et al., 2017), but a significant increase was observed in a loblolly pine forest stand during eight years of experiment (Moore et al., 2006), and in a deciduous forest only in the first year of exposure (Norby et al., 2002). The extra carbon absorbed by plants in an e[CO<sub>2</sub>] environment can be used or stored in different compartments to improve water and nutrient absorption (roots), light absorption (leaves), or support (trunks and branches) (Ainsworth & Long., 2004). In general, the results of these studies, including this one, indicate that such effects vary depending on the species, functional groups, and their different habits. However, it is the first time that the e[CO<sub>2</sub>] effect has been evaluated in an understory hyperdiverse old-growth tropical forest. Here, we show an increase in net CO<sub>2</sub> assimilation in saturated light (*Asat*) and *Lf<sub>area</sub>*, however, this effect may not have been reflected in an increase in height growth, probably due to the short time under e[CO<sub>2</sub>] (eight months) and possible preference for the allocation of extra carbon obtained with e[CO<sub>2</sub>] in other tissues, such as leaves and roots.

## 6 - Conclusion

We investigate the e[CO<sub>2</sub>] effects on the photosynthetic process and plant growth, in a heterogeneous community of understory plants on an old-growth forest of central Amazon. Our results showed an increase in CO<sub>2</sub> assimilation rates and a leaf area increase of 51% under e[CO<sub>2</sub>]. Although the stomatal conductance and the transpiration rates did not show any change, the water use efficiency was higher under e[CO<sub>2</sub>], driven by the increase in assimilation. The increase in leaf area, without changes in transpiration rates, observed in this understory community, can indicate an increase in the amount of water released into the atmosphere by this stratum, which could compensate the decrease in transpiration rates observed in species under direct light.

We showed that the e[CO<sub>2</sub>] increases the CO<sub>2</sub> assimilation rate in an old-grown forest. It is known that the understory of tropical forests are light-limited environments and, despite this limitation, the increase in CO<sub>2</sub> assimilation, and in its quantum yield, showed that plants in the understory light ambient can be positively influenced by the e[CO<sub>2</sub>]. However, this extra

absorbed carbon has not been converted to increased trunk productivity (height or diameter), indicating that, in short term, it may be being invested in other less permanent plant tissues (as leaves and fine roots), resulting in a faster cycle of C through the system rather than increasing the storage of C in the wood. In the current scenario of atmospheric CO<sub>2</sub> increase, and its impact on global climate change, this study is fundamental to understanding how biodiverse tropical forests respond to eminent CO<sub>2</sub> increases.

The results shown here are important for us to understand that the fact that tropical forests harbor a high diversity of species, there is still great complexity to understand the consequences and how these changes will actually affect this large ecosystem. Although, what the results of this study indicate, is that different forest strata may respond differently to high CO<sub>2</sub>, and considering all forest strata within this scenario, as well as their different responses, could be the key to mitigating these global changes that are already happening.

Our results indicate that the understory of tropical forests, by not decreasing transpiration, may not contribute to decreasing evapotranspiration or release of water in the atmosphere and, together with the increase in leaf area, could compensate for the decrease in *E* observed in the canopy.

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## Tables

Variable	a[CO <sub>2</sub> ]	<i>n</i>	e[CO <sub>2</sub> ]	<i>n</i>	<i>p</i>
<i>A<sub>sat</sub></i>	4.419 ± 0.1829	36	5.998 ± 0.3003	34	< 0.0001
<i>g<sub>s</sub></i>	0.09529 ± 0.00646	36	0.08891 ± 0.004435	34	0.4189
<i>E</i>	1.306 ± 0.07833	36	1.279 ± 0.05598	34	0.7759
<i>WUE</i>	3.661 ± 0.1754	36	4.856 ± 0.2443	34	< 0.0001
<i>V<sub>Cmax</sub></i>	18.41 ± 0.6141	36	18.81 ± 0.8756	34	0.7106
<i>J<sub>max</sub></i>	26.11 ± 0.7683	36	31.00 ± 1.235	34	0.0014
<i>J<sub>max</sub>:V<sub>Cmax</sub></i>	1.437 ± 0.03367	36	1.684 ± 0.04444	34	< 0.0001
<i>Φ</i>	0.05381 ± 0.00414	12	0.07134 ± 0.00568	12	0.0215
<i>I<sub>c</sub></i>	5.13 ± 0.933	12	7.053 ± 1.566	12	0.3053

**Table 1.** Gas exchange parameters *A<sub>sat</sub>* = assimilation at saturating light ( $\mu\text{mol m}^2 \text{s}^{-1}$ ), *g<sub>s</sub>* = stomatal conductance ( $\text{mmol H}_2\text{O m}^2 \text{s}^{-1}$ ), *E* = transpiration ( $\text{mmol H}_2\text{O m}^2 \text{s}^{-1}$ ), *WUE* = water use efficiency ( $\mu\text{mol CO}_2 \text{ m}^2 \text{s}^{-1} / \text{mmol H}_2\text{O m}^2 \text{s}^{-1}$ ), *V<sub>Cmax</sub>* = maximum rubisco carboxylation rate, *J<sub>max</sub>* = rate maximum electron transport, *J<sub>max</sub> : V<sub>Cmax</sub>* = ratio, *Φ* = apparent quantum yield ( $\mu\text{mol m}^2 \text{s}^{-1}$ ), *I<sub>c</sub>* = light compensation point ( $\mu\text{mol m}^2 \text{s}^{-1}$ ). Analyzed by treatment where a[CO<sub>2</sub>] represents chambers with ambient CO<sub>2</sub> and e[CO<sub>2</sub>] chambers with high CO<sub>2</sub>, each variable has its respective *n* of individuals. For the nine variables, the values are mean ± SE, *p*-value analyzed using the parametric and non-parametric *t*-test (Mann Whitney) depending on the normality of the data (described in section 3).

OTC	OTC	Family	Species	n_tree_sp
1	a[CO <sub>2</sub> ]	Annonaceae	Duguetia flagellaris	7
1	a[CO <sub>2</sub> ]	Bignoniaceae	Memora flaviflora	2
1	a[CO <sub>2</sub> ]	Burseraceae	Protium sp.	1
1	a[CO <sub>2</sub> ]	Chrysobalanaceae	Licania gracilipes	1
1	a[CO <sub>2</sub> ]	Connaraceae	Rourea krukovii	2
1	a[CO <sub>2</sub> ]	Convolvulaceae	Dicranostyles sp.	1
1	a[CO <sub>2</sub> ]	Lecythidaceae	Couratari stellata	1
1	a[CO <sub>2</sub> ]	Moraceae	Helianthostylis sprucei	1
1	a[CO <sub>2</sub> ]	Violaceae	Paypayrola grandiflora	1
2	e[CO <sub>2</sub> ]	Não identificada	Não identificada	1
2	e[CO <sub>2</sub> ]	Annonaceae	Annona ambotay	1
2	e[CO <sub>2</sub> ]	Annonaceae	Duguetia flagellaris	1
2	e[CO <sub>2</sub> ]	Burseraceae	Protium spruceanum	1
2	e[CO <sub>2</sub> ]	Celastraceae	Salacia multiflora	1
2	e[CO <sub>2</sub> ]	Euphorbiaceae	Hevea guianensis	1
2	e[CO <sub>2</sub> ]	Fabaceae	Bocoa viridiflora	1
2	e[CO <sub>2</sub> ]	Fabaceae	Inga huberi	1
2	e[CO <sub>2</sub> ]	Fabaceae	Peltogyne paniculata	1
2	e[CO <sub>2</sub> ]	Lauraceae	Ocotea tabacifolia	1
2	e[CO <sub>2</sub> ]	Rubiaceae	Coussarea ampla	1
2	e[CO <sub>2</sub> ]	Sapotaceae	Chrysophyllum sanguinolentum ssp spurium	1
2	e[CO <sub>2</sub> ]	Violaceae	Rinorea amapensis	1
3	OC	Não identificada	Não identificada	1
3	OC	Annonaceae	Unonopsis duckei	1
3	OC	Apocynaceae	Tabernaemontana angulata	3
3	OC	Bignoniaceae	Memora adenophora	1
3	OC	Burseraceae	Protium amazonicum	1
3	OC	Euphorbiaceae	Anomalocalyx uleanus	1
3	OC	Euphorbiaceae	Micrandropsis scleroxylon	1
3	OC	Fabaceae	Clathrotropis flava	2
3	OC	Fabaceae	Tachigali micropetala	1
3	OC	Fabaceae	Machaerium verrucosum	1
3	OC	Lacistemataceae	Lacistema aggregatum	1
3	OC	Lecythidaceae	Gustavia elliptica	1

3	OC	Menispermaceae	Abuta panurensis	1
3	OC	Siparunaceae	Siparuna decipiens	2
3	OC	Violaceae	Paypayrola grandiflora	1
4	e[CO2]	Celastraceae	Cheiloclinium hippocrateoides	1
4	e[CO2]	Chrysobalanaceae	Licania macrophylla	1
4	e[CO2]	Rubiaceae	Coussarea ampla	1
4	e[CO2]	Rubiaceae	Alibertia myrciifolia	1
4	e[CO2]	Siparunaceae	Siparuna guianensis	1
4	e[CO2]	Violaceae	Paypayrola grandiflora	6
4	e[CO2]	Violaceae	Rinorea amapensis	1
5	e[CO2]	Não identificada	Não identificada	1
5	e[CO2]	Anisophylleaceae	Anisophyllea manausensis	1
5	e[CO2]	Burseraceae	Protium altsonii	1
5	e[CO2]	Fabaceae	Tachigali setifera	1
5	e[CO2]	Lauraceae	Mezilaurus duckei	1
5	e[CO2]	Myrtaceae	Eugenia pallidopunctata	1
5	e[CO2]	Rubiaceae	Psychotria pacimonica	1
5	e[CO2]	Rubiaceae	Amaioua guianensis	1
5	e[CO2]	Solanaceae	Markea coccinea	1
5	e[CO2]	Violaceae	Rinorea amapensis	2
5	e[CO2]	Violaceae	Paypayrola grandiflora	1
8	a[CO2]	Bignoniaceae	Pleonotoma jasminifolia	1
8	a[CO2]	Bignoniaceae	Memora adenophora	1
8	a[CO2]	Burseraceae	Protium hebetatum	1
8	a[CO2]	Connaraceae	Rourea paraensis	1
8	a[CO2]	Dilleniaceae	Davilla rugosa	2
8	a[CO2]	Erythroxylaceae	Erythroxylum macrophyllum	1
8	a[CO2]	Menispermaceae	Abuta rufescens	1
8	a[CO2]	Nyctaginaceae	Neea oppositifolia	1
8	a[CO2]	Siparunaceae	Siparuna cuspidata	1
8	a[CO2]	Urticaceae	Pourouma villosa	1
8	a[CO2]	Violaceae	Paypayrola grandiflora	2
9	a[CO2]	Não identificada	Não identificada	1
9	a[CO2]	Anacardiaceae	Astronium lecointei	1
9	a[CO2]	Annonaceae	Duguetia flagellaris	3
9	a[CO2]	Annonaceae	Duguetia ulei	1
9	a[CO2]	Celastraceae	Cheiloclinium hippocrateoides	1
9	a[CO2]	Convolvulaceae	Maripa scandens	1
9	a[CO2]	Dilleniaceae	Davilla rugosa	1
9	a[CO2]	Lauraceae	Ocotea cujumary	1
9	a[CO2]	Lecythidaceae	Eschweilera pedicellata	1
9	a[CO2]	Melastomataceae	Bellucia grossularioides	3
9	a[CO2]	Rubiaceae	Capirona decorticans	1
9	a[CO2]	Siparunaceae	Siparuna decipiens	1

9	a[CO2]	Violaceae	Rinorea guianensis	1
9	a[CO2]	Violaceae	Paypayrola grandiflora	1
10	OC	Primulaceae	Clavija lancifolia	1
10	OC	Euphorbiaceae	Senefeldera macrophylla	1
10	OC	Violaceae	Rinorea guianensis	1
10	OC	Chrysobalanaceae	Licania micrantha	1
10	OC	Violaceae	Rinorea guianensis	1
10	OC	Apocynaceae	Odontadenia puncticulosa	1
10	OC	Violaceae	Rinorea guianensis	1
10	OC	Myrtaceae	Eugenia omissa	1
10	OC	Celastraceae	Cheiloclinium hippocrateoides	1
10	OC	Violaceae	Paypayrola grandiflora	1
10	OC	Connaraceae	Rourea paraensis	2
10	OC	Lecythidaceae	Eschweilera pedicellata	1
11	OC	Annonaceae	Duguetia flagellaris	5
11	OC	Annonaceae	Fusaea longifolia	1
11	OC	Apocynaceae	Odontadenia puncticulosa	1
11	OC	Bignoniaceae	Fridericia chica	1
11	OC	Burseraceae	Protium hebetatum	1
11	OC	Elaeocarpaceae	Sloanea latifolia	1
11	OC	Euphorbiaceae	Micrandropsis scleroxylon	1
11	OC	Euphorbiaceae	Senefeldera macrophylla	1
11	OC	Fabaceae	Machaerium caudatum	1
11	OC	Violaceae	Paypayrola grandiflora	1
X	a[CO2]	Lauraceae	Ocotea puberula	1
X	a[CO2]	Moraceae	Sorocea ilicifolia	1
X	a[CO2]	Moraceae	Sorocea pubivena	1
X	a[CO2]	Rubiaceae	Alibertia myrciifolia	1
X	a[CO2]	Violaceae	Paypayrola grandiflora	3
X	a[CO2]	Não identificada	Não identificada	1
Y	e[CO2]	Bignoniaceae	Memora flavida	1
Y	e[CO2]	Burseraceae	Protium tenuifolium	1
Y	e[CO2]	Celastraceae	Cheiloclinium hippocrateoides	1
Y	e[CO2]	Violaceae	Paypayrola grandiflora	6

**Table 2.** Description of the species that are within the OTCs with the respective treatments that each one belongs to and the number of individuals within the chambers in which we monitor.

## Supporting Information



**(Figure-1)** Structure of Open Top Chambers. Images from two of the eight chambers installed inside the forest.

## CONCLUSÃO

Investigamos os efeitos do  $e[\text{CO}_2]$  no processo fotossintético e no crescimento das plantas, em uma comunidade heterogênea de plantas de sub-bosque em uma floresta antiga da Amazônia central. Nossos resultados mostraram um aumento nas taxas de assimilação de  $\text{CO}_2$  e um aumento na área foliar de 51% sob  $e[\text{CO}_2]$ . Embora a condutância estomática e as taxas de transpiração não tenham apresentado alteração, a eficiência do uso da água foi maior sob  $e[\text{CO}_2]$ , impulsionada pelo aumento da assimilação. O aumento da área foliar, sem alterações nas taxas de transpiração, observado nesta comunidade de sub-bosque, pode indicar um aumento na quantidade de água lançada na atmosfera por este estrato, o que poderia compensar a diminuição nas taxas de transpiração observada em espécies sob luz direta.

Mostramos que o  $e[\text{CO}_2]$  aumenta a taxa de assimilação de  $\text{CO}_2$  em uma floresta antiga. Sabe-se que os sub-bosques de florestas tropicais são ambientes com limitação de luz e, apesar dessa limitação, o aumento na assimilação de  $\text{CO}_2$ , e em seu rendimento quântico, mostrou que plantas no sub-bosque em ambientes claros podem ser influenciados positivamente pelo  $e[\text{CO}_2]$ . Porém, esse carbono extra absorvido não foi convertido em aumento da produtividade do tronco (altura ou diâmetro), indicando que, em curto prazo, pode estar sendo investido em outros tecidos vegetais menos permanentes (como folhas e raízes finas), resultando em um processo mais rápido ciclo de C através do sistema em vez de aumentar o armazenamento de C na madeira. No cenário atual de aumento do  $\text{CO}_2$  atmosférico, e seu impacto nas mudanças climáticas globais, este estudo é fundamental para entender como as florestas tropicais biodiversas respondem a aumentos eminentes de  $\text{CO}_2$ .

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