

## REVIEW

# A meta-analysis of the combined effects of elevated carbon dioxide and chronic warming on plant %N, protein content and N-uptake rate

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## Abstract

Elevated CO<sub>2</sub> (eCO<sub>2</sub>) and high temperatures are known to affect plant nitrogen (N) metabolism. Though the combined effects of eCO<sub>2</sub> and chronic warming on plant N relations have been studied in some detail, a comprehensive statistical review on this topic is lacking. This meta-analysis examined the effects of eCO<sub>2</sub> plus warming on shoot and root %N, tissue protein concentration (root, shoot and grain) and N-uptake rate. In the analyses, the eCO<sub>2</sub> treatment was categorized into two classes (<300 or ≥300 ppm above ambient or control), the temperature treatment was categorized into three classes (<1.5, 1.5–5 and >5 °C above ambient or control), plant species were categorized based on growth form and functional group and CO<sub>2</sub> treatment technique was also investigated. Elevated CO<sub>2</sub> alone or in combination with warming reduced shoot %N (more so at ≥300 vs. <300 ppm above ambient CO<sub>2</sub>), while root %N was significantly reduced only by eCO<sub>2</sub>; warming alone often increased shoot %N, but mostly did not affect root %N. Decreased shoot %N with eCO<sub>2</sub> alone or eCO<sub>2</sub> plus warming was greater for woody and non-woody dicots than for grasses, and for legumes than non-legumes. Though root N-uptake rate was unaffected by eCO<sub>2</sub>, eCO<sub>2</sub> plus warming decreased N-uptake rate, while warming alone increased it. Similar to %N, protein concentration decreased with eCO<sub>2</sub> in shoots and grain (but not roots), increased with warming in grain and decreased with eCO<sub>2</sub> and warming in grain. In summary, any benefits of warming to plant N status and root N-uptake rate will generally be offset by negative effects of eCO<sub>2</sub>. Hence, concomitant increases in CO<sub>2</sub> and temperature are likely to negate or decrease the nutritional quality of plant tissue consumed as food by decreasing shoot %N and shoot and/or grain protein concentration, caused, at least in part, by decreased root N-uptake rate.

**Keywords:** Climate change; elevated CO<sub>2</sub>; heat stress; meta-analysis; nitrogen metabolism; nitrogen translocation; nitrogen-uptake rate; protein; warming

## Introduction

Present-day atmospheric carbon dioxide (CO<sub>2</sub>) levels (ca. 400 ppm) are unprecedented over the past 420 000 years (Petit *et al.* 1999). With industrialization, and its expansion due to economic and population growth, atmospheric CO<sub>2</sub> levels have increased up to 46 % in the last 170 years. According to low

and intermediate CO<sub>2</sub>-emission scenarios, atmospheric CO<sub>2</sub> is likely to be in the range of 450–1000 ppm by the end of this century (IPCC 2014). Carbon dioxide is a greenhouse gas and its emissions account for ca. two-thirds of current global warming. Due to global warming, the increase in Earth's mean surface

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temperature is likely to be in the range of 1.5–6 °C by 2100 (IPCC 2014). The concomitant increases in CO<sub>2</sub> and temperature are expected to have various impacts on plant species, including those used in agriculture and forestry. Though the interactive effects of CO<sub>2</sub> enrichment and warming on plant growth and function have been studied in some detail (Morison and Lawlor 1999; Wang et al. 2012), nitrogen (N) metabolism in response to concomitant increases in CO<sub>2</sub> and temperature is still poorly understood.

Plant N relations in response to eCO<sub>2</sub> have been extensively studied. Due to natural variation among species and differences among experimental protocols, most plant responses to eCO<sub>2</sub> are highly variable; one exception to this pattern is tissue N concentration (Bassirirad 2000; Ainsworth and Long 2005; Taub and Wang 2008; Ainsworth and Long 2021). Elevated CO<sub>2</sub> stimulates photosynthesis which then stimulates production of non-structural carbohydrates. When the sugar production exceeds the plant sink capacity, it induces a negative feedback on the transcription of Ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), resulting in reduced Rubisco concentrations (and thus leaf N; see also Moore et al. 1999), and thus net CO<sub>2</sub> assimilation (which may still be higher compared to plants grown under ambient CO<sub>2</sub>) (Ainsworth and Long 2005; Taub and Wang 2008). According to the progressive N limitation (PNL) hypothesis, eCO<sub>2</sub> could enhance the sequestration of N into long-lived plant biomass and soil organic matter which in turn reduce the available soil N for plant growth resulting lower tissue N (Luo et al. 2004). In addition, several other mechanisms, such as decreased root N uptake, less efficient root architecture and increased N loss, have been proposed to contribute to lower tissue N concentrations at eCO<sub>2</sub> (Taub and Wang 2008). As with tissue %N, eCO<sub>2</sub> is also likely to reduce tissue protein concentration (Taub et al. 2008; Feng et al. 2015). However, legumes are hypothesized to have an advantage over other C<sub>3</sub> species when grown at eCO<sub>2</sub> due to their ability to exchange carbon for N with their N-fixing symbionts (Rogers et al. 2009). Though eCO<sub>2</sub> tends to decrease root N-uptake rate, especially in plants rooted in solid media, past studies collectively have shown that both N-uptake kinetics and root N-uptake rate in response to eCO<sub>2</sub> can be highly variable (Bassirirad 2000; Taub and Wang 2008).

Plant N relations in response to chronic warming have been studied in some detail. A meta-analysis conducted by Zvereva and Kozlov (2006) found a non-significant negative effect of warming (≥4 or <4 °C above control) on above-ground N concentration. Heat stress is known to cause rates of protein degradation to exceed rates of new protein synthesis (Huang et al. 2012). Huang et al. (2012) further showed that some cultivars were capable of producing more thermostable proteins and maintaining low levels of proteolytic enzyme activities to protect against warming. Therefore, though warming is likely to reduce protein concentration, variation in plant protein levels in response to warming could be either interspecies- or intraspecies-specific. Optimal growth temperature is species-specific, and hence, warming from suboptimal to optimal temperatures is likely to increase root N-uptake rate (Clarkson and Warner 1979; Tindall et al. 1990; Cruz et al. 1993; Atkin and Cummins 1994), while warming (acute or chronic) from optimal to supra-optimal temperatures is likely to decrease this rate (Tindall et al. 1990; Delucia et al. 1992; Bassirirad et al. 1993; Mainali et al. 2014; Giri et al. 2017).

Combined eCO<sub>2</sub> plus warming is likely to decrease above-ground N concentration in plant tissue, and the magnitude of this decrease has been greater for C<sub>3</sub> and woody species than

C<sub>4</sub> and herbaceous species (Zvereva and Kozlov 2006; Wang et al. 2012); however, root %N is likely to be unaffected by eCO<sub>2</sub> plus warming (Wang et al. 2012). A limited number of studies suggests that the effects of eCO<sub>2</sub> plus warming on N-uptake rate can be variable, within and among species (Coleman and Bazzaz 1992; Dijkstra et al. 2010; Arndal et al. 2014; Jayawardena et al. 2017). Few studies have looked at the effects of eCO<sub>2</sub> plus warming on tissue protein concentration (total protein per g dry mass). They suggest that grain protein concentration can vary in response to eCO<sub>2</sub> plus warming (Abebe et al. 2016; Jing et al. 2016; Palacios et al. 2019; Qiao et al. 2019). Previously, we examined the effects of eCO<sub>2</sub> plus warming on total root protein concentration of tomato (*Solanum lycopersicum*) provided either nitrate (NO<sub>3</sub><sup>-</sup>) or ammonium (NH<sub>4</sub><sup>+</sup>) as the sole N source and noted a significant decrease in root protein concentration in both sets of plants (Jayawardena et al. 2017). To the best of our knowledge, total shoot protein concentration in response to eCO<sub>2</sub> plus warming has not been studied before. Collectively, these studies indicate that the effects of eCO<sub>2</sub> plus warming on root N uptake and assimilation are not fully understood.

Free-air CO<sub>2</sub> enrichment (FACE) experiments are thought to provide the most realistic measures of the effects of eCO<sub>2</sub> on crop yields (Ainsworth et al. 2008; Ainsworth and Long 2021). However, the high cost associated with FACE experiments (ca. US\$1 million in maintenance per year plus investigation costs) (DaMatta et al. 2010) makes it inaccessible to many researchers who are interested in investigating environmental variables such as CO<sub>2</sub> and temperature on plant responses. Though many available enclosure techniques produce a ‘chamber effect’ (Ainsworth et al. 2008), they have often produced results similar to those of FACE experiments (e.g., see Taub et al. 2008). This comparison has mostly been assessed only for eCO<sub>2</sub> and not eCO<sub>2</sub> plus other factors, so it would be useful to compare plant responses (e.g. plant %N) to eCO<sub>2</sub> plus warming using different eCO<sub>2</sub> treatment delivery techniques.

Our current understanding of the effects of eCO<sub>2</sub> plus warming on plant N metabolism has important knowledge gaps. Therefore, the main objective of this study was to narrow these knowledge gaps using a comprehensive meta-analysis of the effects of eCO<sub>2</sub> plus warming on variables related to plant N metabolism, such as shoot and root %N, tissue protein concentration and root N-uptake rate. This includes a subgroup analysis of the effects of eCO<sub>2</sub> plus warming on different growth forms, functional groups and eCO<sub>2</sub> treatment techniques. Results of this study will help crop scientists, plant breeders and molecular biologists better understand how plant N metabolism will likely respond to future predicted climate conditions and provide targets for developing new genotypes with improved N-relation traits suited for future climate conditions.

## Methods

### Data collection

This meta-analysis mostly followed methods described by Wang et al. (2012). A literature search was conducted between November 2019 and March 2020 using the search engines PubMed and Google Scholar to construct the database for this meta-analysis (Table 1). The obtained peer-reviewed research or review papers were cross-referenced to help ensure the inclusion of all relevant articles. Research papers published in English which met the following criteria were included in the meta-analysis: (i) CO<sub>2</sub> × temperature treatment interaction (full

**Table 1.** Plant species, response variables extracted, subgroup of the response variable extracted and references used in the meta-analysis.

Species	Response variable extracted				Subgroup of the response variable extracted			References
	Shoot %N	Root %N	Root N-uptake rate	Protein concentration	Growth form	Functional group	Treatment technique	
<i>Abutilon theophrasti</i>	*	*			NWD	NL	GC	<a href="#">Coleman and Bazzaz (1992)</a>
<i>Acer rubrum</i>	*				W	NL	OTC	<a href="#">Norby et al. (2000)</a>
<i>Acer rubrum</i>		*			W	NL	OTC	<a href="#">Wan et al. (2004)</a>
<i>Acer rubrum</i>	*				W	NL	OTC	<a href="#">Williams et al. (2003)</a>
<i>Acer rubrum</i>	*				W	NL	OTC	<a href="#">Williams et al. (2000)</a>
<i>Acer saccharum</i>	*				W	NL	OTC	<a href="#">Norby et al. (2000)</a>
<i>Acer saccharum</i>		*			W	NL	OTC	<a href="#">Wan et al. (2004)</a>
<i>Acer saccharum</i>	*				W	NL	OTC	<a href="#">Williams et al. (2000)</a>
<i>Alliaria petiolata</i>	*				NWD	NL	GC	<a href="#">Anderson and Cipollini (2013)</a>
<i>Amaranthus retroflexus</i>	*	*			NWD	NL	GC	<a href="#">Coleman and Bazzaz (1992)</a>
<i>Betula pendula</i>			*		W	NL	GC	<a href="#">Kellomaki and Wang (2001)</a>
<i>Betula pendula</i>	*				W	NL	CTC	<a href="#">Kuokanen et al. (2001)</a>
<i>Betula pendula</i>	*				W	NL	CTC	<a href="#">Kuokanen et al. (2003)</a>
<i>Betula pendula</i>	*				W	NL	CTC	<a href="#">Lavola et al. (2013)</a>
<i>Brassica juncea</i>	*				NWD	NL	GC	<a href="#">Seth and Misra (2014)</a>
<i>Calluna vulgaris</i>	*	*			W	NL	FACE	<a href="#">Andresen et al. (2009)</a>
<i>Calluna vulgaris</i>	*	*			W	NL	FACE	<a href="#">Andresen et al. (2010)</a>
<i>Calluna vulgaris</i>			*		W	NL	FACE	<a href="#">Arndal et al. (2014)</a>
<i>Coffea arabica</i>	*				G	NL	GC	<a href="#">Ramalho et al. (2018)</a>
<i>Deschampsia flexuosa</i>	*	*			W	NL	FACE	<a href="#">Andresen et al. (2009)</a>
<i>Deschampsia flexuosa</i>	*	*			G	NL	FACE	<a href="#">Andresen et al. (2010)</a>
<i>Deschampsia flexuosa</i>			*		G	NL	FACE	<a href="#">Arndal et al. (2014)</a>
<i>Echinium plantagineum</i>	*				NWD	NL	GC	<a href="#">Johns and Hughes (2002)</a>
<i>Eucalyptus globulus</i>	*				W	NL	CTC	<a href="#">Crous et al. (2013)</a>
<i>Eucalyptus globulus</i>	*				W	NL	OTC	<a href="#">Sharwood et al. (2017)</a>
<i>Eucalyptus robusta</i>	*				W	NL	GH	<a href="#">Gherlenda et al. (2015)</a>
<i>Eucalyptus saligna</i>	*				W	NL	GH	<a href="#">Ayub et al. (2011)</a>
<i>Eucalyptus saligna</i>	*				W	NL	GH	<a href="#">Ghannoum et al. (2010a)</a>
<i>Eucalyptus saligna</i>	*	*			W	NL	GH	<a href="#">Ghannoum et al. (2010b)</a>
<i>Eucalyptus sideroxylon</i>	*				W	NL	GH	<a href="#">Ghannoum et al. (2010a)</a>
<i>Eucalyptus sideroxylon</i>	*	*			W	NL	GH	<a href="#">Ghannoum et al. (2010b)</a>
<i>Eucalyptus tereticornis</i>	*				W	NL	GH	<a href="#">Gherlenda et al. (2015)</a>
<i>Eucalyptus tereticornis</i>	*				W	NL	GH	<a href="#">Gherlenda et al. (2016)</a>
<i>Eucalyptus tereticornis</i>	*				W	NL	GH	<a href="#">Murray et al. (2013)</a>
<i>Geum vernum</i>	*				NWD	NL	GC	<a href="#">Anderson and Cipollini (2013)</a>
<i>Glycine max</i>				*	NWD	L	OTC	<a href="#">Palacios et al. (2019)</a>
<i>Glycine max</i>				*	NWD	L	OTC	<a href="#">Qiao et al. (2019)</a>
<i>Glycine max</i>	*				NWD	L	FACE	<a href="#">Rosenthal et al. (2014)</a>
<i>Gossypium hirsutum</i>	*				W	NL	GC	<a href="#">Zhang et al. (2017)</a>
Grasses	*				G	NL	GC	<a href="#">Johnson and Hartley (2018)</a>

Table 1. Continued

Species	Response variable extracted				Subgroup of the response variable extracted			References
	Shoot %N	Root %N	Root N-uptake rate	Protein concentration	Growth form	Functional group	Treatment technique	
<i>Lantana camara</i>	*				W	NL	GC	Johns et al. (2003)
<i>Lolium perenne</i>	*	*			G	NL	CTC	Soussana et al. (1996)
<i>Lolium perenne</i>	*				G	NL	CTC	Zavalloni et al. (2012)
<i>Lotus corniculatus</i>	*				NWD	L	CTC	Zavalloni et al. (2012)
<i>Medicago lupulina</i>	*				NWD	L	CTC	Zavalloni et al. (2012)
<i>Medicago sativa</i>	*				NWD	L	TGT	Aranjuelo et al. (2005)
<i>Medicago sativa</i>		*			NWD	L	TGT	Aranjuelo et al. (2008)
<i>Medicago sativa</i>	*				NWD	L	GH	Ariz et al. (2015)
<i>Oryza sativa</i>				*	G	NL	FACE	Jing et al. (2016)
<i>Oryza sativa</i>	*				G	NL	TGT	Kim et al. (2011)
<i>Oryza sativa</i>	*	*			G	NL	FACE	Li et al. (2017)
<i>Oryza sativa</i>	*				G	NL	OTC	Liu et al. (2019)
<i>Panicum maximum</i>	*				G	NL	FACE	de Assis Prado et al. (2016)
<i>Phalaris aquatica</i>	*				G	NL	TGT	Lilley et al. (2001)
<i>Phalaris aquatica</i>	*				G	NL	TGT	Volder et al. (2015)
<i>Phaseolus vulgaris</i>	*				NWD	L	CTC	Prasad et al. (2004)
<i>Pinus ponderosa</i>		*			W	NL	GH	King et al. (1997)
<i>Pinus sylvestris</i>	*				W	NL	CTC	Luomala et al. (2003)
<i>Pinus taeda</i>		*			W	NL	GH	King et al. (1997)
<i>Plantago lanceolata</i>	*				G	NL	CTC	Zavalloni et al. (2012)
<i>Poa pratensis</i>	*				G	NL	CTC	Zavalloni et al. (2012)
<i>Pseudotsuga menziesii</i>		*			W	NL	CTC	Chen et al. (2008)
<i>Pseudotsuga menziesii</i>	*				W	NL	CTC	Hobbie et al. (2001)
<i>Quercus robur</i>	*				W	NL	GH	Dury et al. (1998)
<i>Rumex acetosa</i>	*				NWD	NL	CTC	Zavalloni et al. (2012)
<i>Salix myrsinifolia</i>	*				W	NL	CTC	Veteli et al. (2002)
Semi-arid grasses	*	*			G	NL	FACE	Dijkstra et al. (2010)
<i>Solanum lycopersicum</i>	*	*	*	*	NWD	NL	GC	Jayawardena et al. (2017)
<i>Solanum lycopersicum</i>	*	*	*	*	NWD	NL	GC	Jayawardena et al. (2021)
Species mix		*			N/A	NL	CTC	Kandeler et al. (1998)
Species mix	*				N/A	NL	FACE	Mueller et al. (2016)
Species mix		*			G	NL	CTC	Zavalloni et al. (2012)
<i>Trifolium subterraneum</i>	*				NWD	L	TGT	Lilley et al. (2001)
<i>Triticum aestivum</i>	*	*	*	*	G	NL	GC	Jayawardena et al. (2020)
<i>Triticum durum</i>	*				NWD	NL	TGT	Jauregui et al. (2015)
<i>Vitis vinifera</i>	*	*			W	NL	GH	Salazar-Parra et al. (2015)
<i>Zea mays</i>				*	G	NL	OTC	Abebe et al. (2016)
<i>Zea mays</i>	*				G	NL	CTC	Kim et al. (2007)
<i>Zea mays</i>				*	G	NL	OTC	Qiao et al. (2019)

References included in the meta-analysis are available in [Supporting Information—Appendix S2](#).

\* denotes the response variable extracted from each reference. Different growth forms are denoted as woody, W; grassy, G; and non-woody dicot, NWD. Different functional groups are denoted as legume, L; and non-legume, NL. Different treatment techniques are denoted as open-top chambers, OTC; closed-top chambers, CTC; greenhouses, GH; free-air CO<sub>2</sub> enrichment, FACE; temperature-gradient tunnels, TGT; and growth chambers, GC.

factorial or at least the interaction and the control treatment), (ii) warming treatment that was chronic (i.e. growing plants for a longer period of time ranging from weeks to years at higher than ambient/near-optimal temperatures; abrupt or short-term heating was not considered here, i.e. heat-shocking plants with supra-optimal temperatures for hours to a few days), (iii) whole-plant warming throughout the day (soil or night-time-only warming was excluded) and (iv) reporting of standard error or standard deviation and number of replicates. For all studies considered, ambient or control CO<sub>2</sub> concentration (aCO<sub>2</sub>) ranged between 350 and 400 ppm, while eCO<sub>2</sub> ranged between 490 and 800 ppm. If aCO<sub>2</sub> was not reported for a study, mean annual CO<sub>2</sub> concentration for the year in which the study was conducted was estimated using CO<sub>2</sub>.earth (<https://www.co2.earth/monthly-co2>). The lowest elevated temperature was ambient temperature ( $T_{amb}$ ) + 0.6 °C, while the highest elevated temperature was  $T_{amb} + 19$  °C. However, there were only four experimental observations with elevated temperatures >10 °C above ambient. Response variables extracted for analysis included shoot and root %N (per unit dry mass), root N-uptake rate (rate per unit dry mass per unit time) and tissue protein concentration (total protein per unit dry mass). Shoot %N data had four experimental observations with partial irrigation, eight experimental observations with insect feeding and one experimental observation with elevated Ultraviolet-B light treatment. Root %N data had one experimental observation with partial irrigation. When taking these observations into account, the non-CO<sub>2</sub> or non-temperature-stress treatment alone was used as the control. These observations were not excluded from the database in order to increase sample size (and response patterns did not change with the exclusion of these observations). Root N-uptake rate included total N, NO<sub>3</sub><sup>-</sup> or NH<sub>4</sub><sup>+</sup> uptake rates of intact or excised roots. Graphically presented data were extracted using the data extraction software WebPlotDigitizer version 4.2 (Rohatgi 2017). Standard errors of the mean (SEM) were converted to standard deviations (SDs) using the equation;  $SD = SEM \times \sqrt{n}$ , where  $n$  is the number of replicates [see Supporting Information—Appendix S1].

### Categorization of data

The increase in global mean surface temperature is likely to exceed 1.5 °C by 2100 under all emission scenarios. It is also likely to be in the range of 1.5–4.5 °C, and very unlikely to be greater than 6 °C, by 2100 (IPCC 2014). Based on these predictions, the warming treatments were categorized into three temperature classes as ambient or control plus: <1.5 °C ( $T_L$ ), 1.5–5 °C ( $T_M$ ) and >5 °C ( $T_H$ ). According to the low and intermediate CO<sub>2</sub> emission scenarios, atmospheric CO<sub>2</sub> is likely to increase between 450 and 1000 ppm by 2100 (IPCC 2014). Therefore, a breakpoint for categorization of eCO<sub>2</sub> treatments into two levels was arbitrarily selected as <300 ppm or ≥300 ppm above ambient or control (350–400 ppm CO<sub>2</sub>). Plant species were categorized based on growth form (woody, grassy or non-woody dicots), functional group (legumes or non-legumes) and treatment technique (open-top chambers, OTC; closed-top chambers, CTC; greenhouses, GH; FACE; temperature-gradient tunnels, TGT; or growth chambers, GC). Only shoot and root %N data were categorized into temperature or CO<sub>2</sub> subclasses (growth form, functional group and treatment technique) due to the high availability of experimental observations for these two response variables. Protein concentration was categorized based on tissue type (grain, shoot or root).

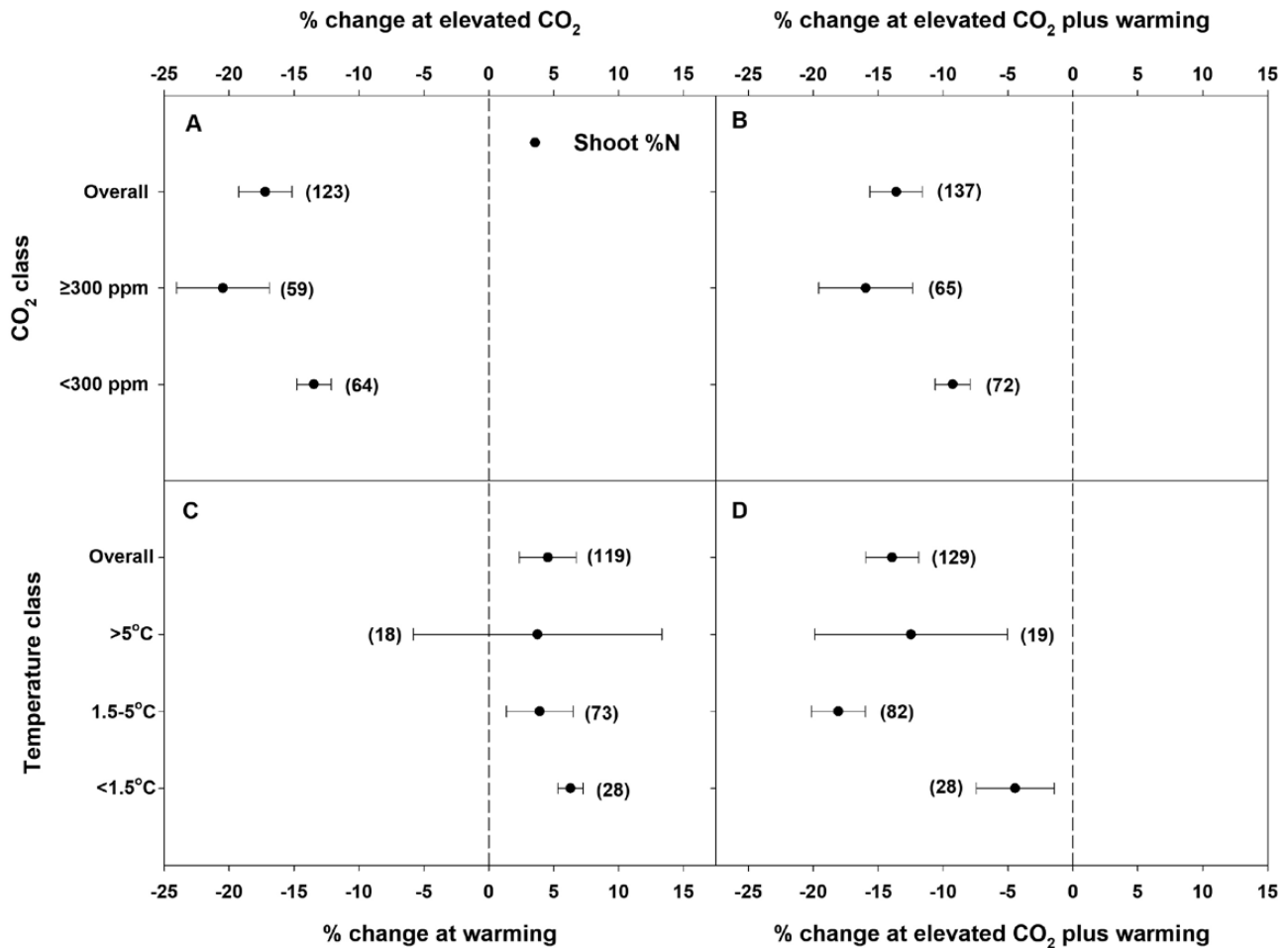
### Meta-analytic method

In this meta-analysis, the natural-log response ratio  $[\ln(r)]$  between the means of experimental and control groups was used as the metric of the effect size (Hedges et al. 1999). The effect size was graphically presented as the mean % change  $[(r - 1) \times 100]$  (Ainsworth et al. 2002) with its 95 % confidence interval (CI). The meta-analysis was performed using OpenMEE an open-source software for meta-analysis in ecology and evolutionary biology (Wallace et al. 2017). A continuous random-effects model with Hedges–Olkin method that relies on inverse-variance weighting to account for variation in precision (sampling error) within and between studies was used (Hedges and Olkin 1985; Wallace et al. 2017). The independent variables (eCO<sub>2</sub>, warming and eCO<sub>2</sub> plus warming) were considered to have a significant effect on the dependent variables if CIs did not overlap the reference line at 0 % change. The outcome was considered significant if  $P < 0.05$ . Normality assumption was checked using normal quantile–quantile plots. Publication bias was checked using Rosenthal's fail-safe number and funnel plots. Rosenthal's fail-safe number was calculated using OpenMEE software. This number indicates the number of non-significant and unpublished studies required for the meta-analysis to change the statistical significance of the meta-analytic result to a non-significant result (Rosenthal 1979). If this number was greater than  $5n + 10$ , where  $n$  is the number of experimental observations, publication bias could be safely ignored (Rosenberg 2005). In addition, if data were symmetrically distributed in the funnel plot, publication bias was safely ignored.

### Results

Elevated CO<sub>2</sub> alone significantly reduced both shoot and root %N, by 17 and 10 % overall, respectively. The magnitude of the decrease was greater at high eCO<sub>2</sub> (≥300 ppm above control) than at low eCO<sub>2</sub> (<300 ppm above control), although it was significant only for shoots (Figs 1A and 2A). Warming alone significantly increased shoot %N by 5 % and non-significantly increased root %N by 2 %. The magnitude of a warming-driven increase in shoot %N decreased as temperature increases from  $T_L$  to  $T_H$ , although CIs overlapped for all three temperature classes. Notably, the increase in shoot %N at  $T_H$  was non-significant (Fig. 1C). Meanwhile, root %N neither increased nor decreased at  $T_L$ , but significantly increased at  $T_M$  and non-significantly decreased at  $T_H$  (Fig. 2C). There was a publication bias for the effect of temperature on root %N. Irrespective of the CO<sub>2</sub> or temperature classes, eCO<sub>2</sub> plus warming significantly reduced shoot %N by 14 %. The magnitude of decrease was greater at high eCO<sub>2</sub> or  $T_M$  (1.5–5 °C above control) than at low eCO<sub>2</sub> or  $T_L$  (<1.5 °C above control) (Fig. 1B and D). Elevated CO<sub>2</sub> plus warming non-significantly decreased root %N by 3 % and trended towards a greater decrease at high eCO<sub>2</sub> or  $T_H$  (>5 °C above control) than at low eCO<sub>2</sub> or  $T_M$  (Fig. 2B and D). There was a publication bias for the effects eCO<sub>2</sub> plus warming on root %N.

Elevated CO<sub>2</sub> alone or in combination with warming significantly reduced shoot %N in all woody, grassy and non-woody dicot growth forms (Fig. 3A and C). Grasses had the smallest decrease in shoot %N in response to eCO<sub>2</sub> (11 %) and to eCO<sub>2</sub> plus warming (3 %), while non-woody dicots had the largest decrease in shoot %N in response to eCO<sub>2</sub> (21 %) and eCO<sub>2</sub> plus warming (19 %). Woody species had an intermediate decrease in shoot %N in response to eCO<sub>2</sub> (19 %) and eCO<sub>2</sub> plus warming (13 %) (Fig. 3A and C). Warming significantly increased shoot %N



**Figure 1.** Percent change (compared to ambient or controls) in shoot %N in response to elevated CO<sub>2</sub> (eCO<sub>2</sub>) (A) or eCO<sub>2</sub> plus warming (B) at different eCO<sub>2</sub> classes (ambient + <300 or ≥300 ppm) and warming (C) or eCO<sub>2</sub> plus warming (D) at different temperature classes (ambient + <1.5, 1.5–5 or >5 °C). Each data point represents the mean ± 95 % CI. Numbers within parentheses represent the number of experimental observations. The dashed vertical line is the reference line at 0 % change. Treatment effects are non-significant at  $P < 0.05$  if CIs overlap the zero line, and differences among treatments are non-significant if CIs overlap.

in both grasses (3 %) and non-woody dicots (5 %), while it non-significantly increased shoot %N in woody species (3 %) (Fig. 3B). Elevated CO<sub>2</sub> (significantly, 12 % in woody; 15 % in grassy; and 8 % in non-woody dicot) and eCO<sub>2</sub> plus warming (non-significantly, 5 % in woody; 7 % in grassy; and 1 % in non-woody dicot) reduced root %N in all three growth forms (Fig. 4A and C). Warming non-significantly increased root %N in woody species by 6 %, but it did not influence root %N in grasses or non-woody dicots (Fig. 4B).

Elevated CO<sub>2</sub> alone or in combination with warming significantly decreased shoot %N in legumes (by 27 and 22 %, respectively) and non-legumes (16 and 12 %, respectively). However, warming by itself significantly increased shoot %N in non-legumes by 5 % but did not influence shoot %N of legumes (Fig. 3D–F).

In each eCO<sub>2</sub> treatment technique, eCO<sub>2</sub> significantly reduced both shoot and root %N, and this decrease was greatest for plants grown in GH (Figs 3G and 4D). Excluding GH, the magnitude of eCO<sub>2</sub>-driven decreases in shoot %N was similar across the different eCO<sub>2</sub> techniques. Meanwhile, eCO<sub>2</sub>-driven decreases in root %N were similar for FACE (13 %) and GC (11%), both of which were similar to the overall decrease in root %N (11%). Except for plants grown in OTC, eCO<sub>2</sub> plus warming significantly reduced shoot %N in plants grown using all eCO<sub>2</sub> treatment techniques (Fig. 3I). Similar to eCO<sub>2</sub> alone, this decrease in shoot %N was

also greatest when plants were grown in GH. Likewise, similar decreases in shoot %N in response eCO<sub>2</sub> plus warming were observed for plants grown using FACE (14 %), TGT (14 %) and GC (16 %), which were also similar to the overall decrease in shoot %N (14 %). A significant decrease in root %N in response to eCO<sub>2</sub> plus warming was found only in plants grown in GH; all other treatment techniques were not significantly affected by eCO<sub>2</sub> plus warming (Fig. 4F). Plants grown using FACE and growth-chamber techniques had similar decreases in root %N (4 %) in response to eCO<sub>2</sub> plus warming which were similar to the overall decrease in root %N (3 %). Warming alone significantly (FACE and GC) or non-significantly (all other eCO<sub>2</sub> treatment techniques) increased shoot %N, while, except for GH, warming did not influence root %N in plants grown using any other technique (Fig. 4E).

Specific root N-uptake rate was not influenced by eCO<sub>2</sub>, but it did significantly increase by 13 % in response to warming and significantly decrease by 15 % in response to eCO<sub>2</sub> plus warming (Fig. 5). The publication bias could not be ignored for the individual effect of eCO<sub>2</sub> on root N-uptake rate.

Elevated CO<sub>2</sub> alone or in combination with warming significantly or non-significantly decreased protein concentration in shoots, roots and grains, while warming alone significantly or non-significantly increased protein concentrations in all three tissue types (Fig. 6).

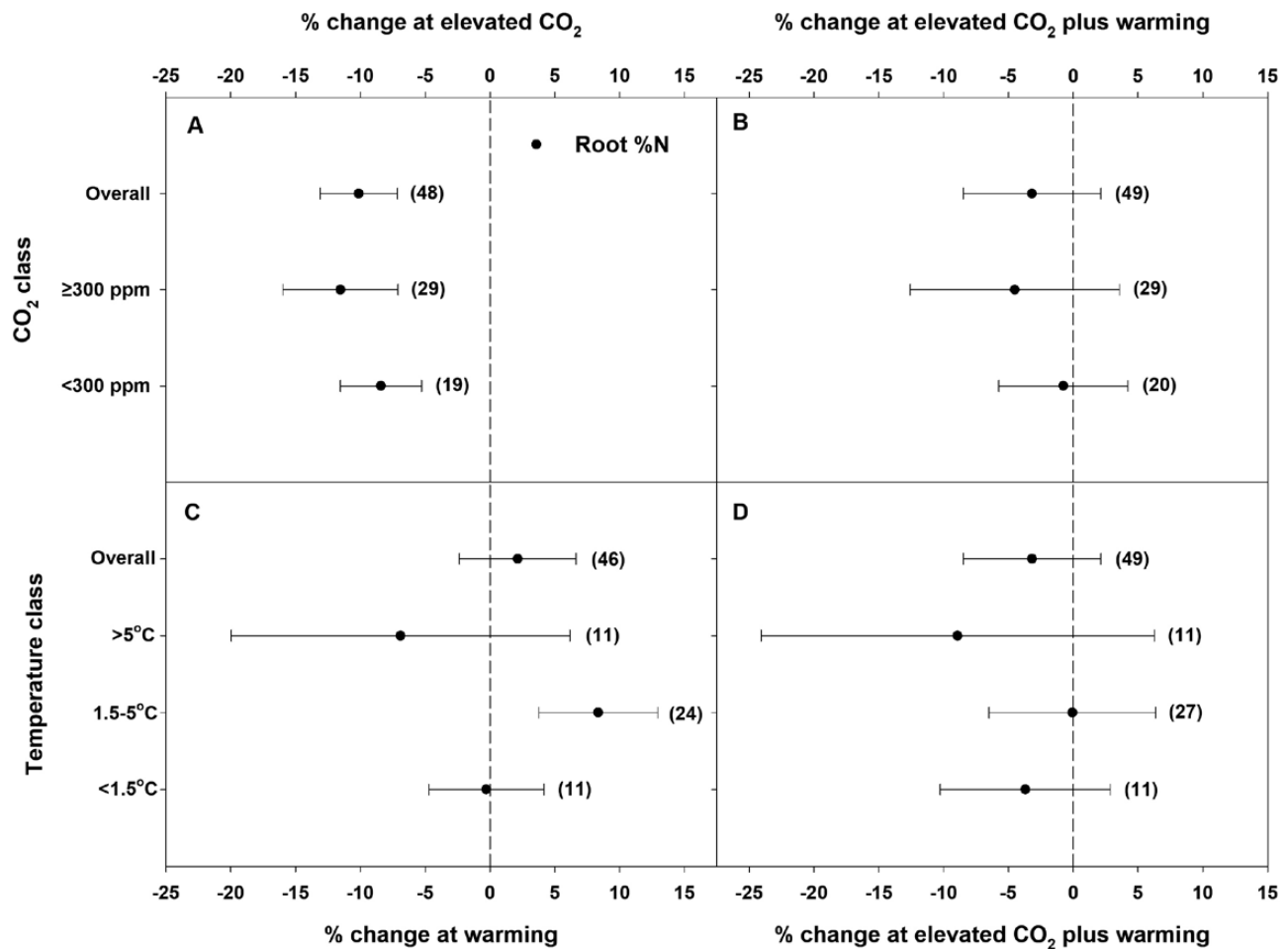


Figure 2. Percent change (compared to ambient or controls) in root %N in response to elevated CO<sub>2</sub> (eCO<sub>2</sub>) (A) or eCO<sub>2</sub> plus warming (B) at different eCO<sub>2</sub> classes (ambient + <300 or ≥300 ppm) and warming (C) or eCO<sub>2</sub> plus warming (D) at different temperature classes (ambient + <1.5, 1.5–5 or >5 °C). Each data point represents the mean ± 95% CI. Numbers within parentheses represent the number of experimental observations. The dashed vertical line is the reference line at 0% change. Treatment effects are non-significant at  $P < 0.05$  if CIs overlap the zero line, and differences among treatments are non-significant if CIs overlap.

## Discussion

The effects of eCO<sub>2</sub> on N concentration of plant tissues have been extensively studied and the findings often show a negative effect (Cotrufo et al. 1998; Curtis and Wang 1998; Ainsworth and Long 2005; Taub and Wang 2008). In agreement with previous reports, in this meta-analysis, eCO<sub>2</sub> significantly reduced both shoot and root %N and the decrease was greater for shoots (17%) than roots (10%). These results are consistent with Cotrufo et al. (1998), who also found a 14 and 9% decrease in above- and below-ground tissue N concentrations, respectively, in response to eCO<sub>2</sub>. As with the effects of eCO<sub>2</sub> on %N, eCO<sub>2</sub> plus warming also decreased %N in shoots (significantly) and roots (non-significantly). However, the magnitude of the negative effect of eCO<sub>2</sub> plus warming on tissue %N was smaller than that of eCO<sub>2</sub> alone. Previously, with smaller sample sizes, Zvereva and Kozlov (2006) and Wang et al. (2012) also reported negative effects of eCO<sub>2</sub> plus warming on above-ground %N. The current meta-analysis further revealed that tissue quality (i.e. %N) can be persistently decreased with continuous exposure to eCO<sub>2</sub>, regardless of the temperature. Therefore, in the future, eCO<sub>2</sub> is likely to reduce shoot %N regardless of the temperature, and, as a result, herbivores are likely to be N-limited and so would be required to consume more leaf tissues in order to meet their

N requirement. This would eventually reduce photosynthetic rate followed by plant growth. One of the widely accepted hypotheses for low tissue %N at eCO<sub>2</sub> is the dilution of N by increased photosynthetic assimilation of carbon (Ainsworth and Long 2005; Taub and Wang 2008). In addition, decreased root N-uptake rate has also been hypothesized as a potential cause of low tissue %N in plants grown at eCO<sub>2</sub> (Taub and Wang 2008; Feng et al. 2015). Though the results of this meta-analysis do not support this hypothesis (Fig. 5A), it cannot be completely ruled out due to the large variation observed for N-uptake rate in response to eCO<sub>2</sub> alone. However, in this meta-analysis, root N-uptake rates showed a positive relationship with shoot or root %N with warming or eCO<sub>2</sub> plus warming, suggesting a greater dependence of N-uptake rate on temperature than CO<sub>2</sub>. Previously, Zvereva and Kozlov (2006), using 42 experimental observations, showed a negative but non-significant effect of warming on above-ground %N. In contrast, based on 119 experimental observations, the current meta-analysis reports a significant increase in above-ground %N in response to warming. Warming with <1.5 °C ( $T_L$ ) significantly increased shoot %N but this increase was neutralized as the magnitude of temperature elevation increased from  $T_L$  to  $T_M$  to  $T_H$  (<1.5, 1.5–5 or >5 °C above control), suggesting the inability of plants to maintain tissue quality (i.e. %N) at higher than optimal temperatures. Likewise,

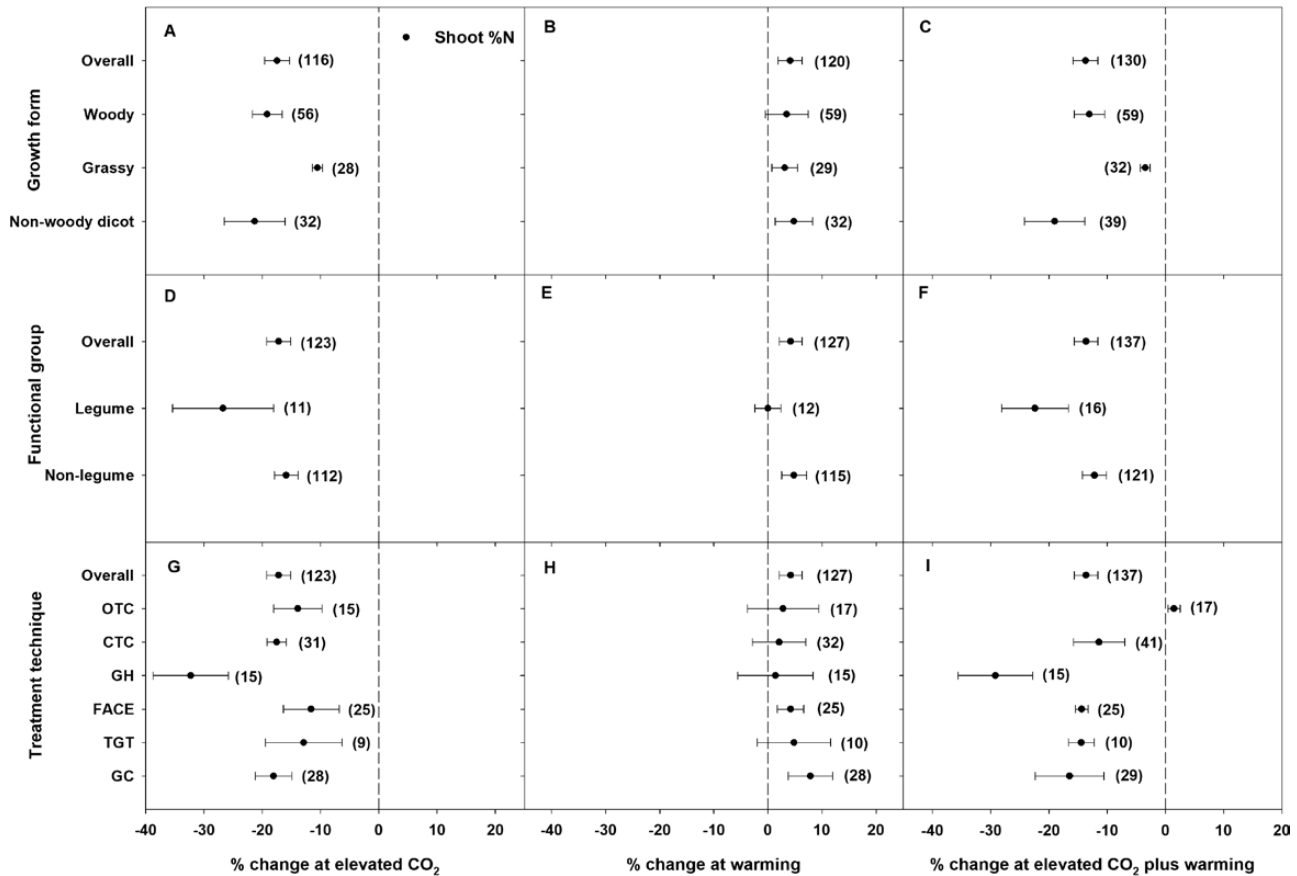


Figure 3. Percent change (compared to ambient or controls) in shoot %N in different growth forms (A–C), functional groups (D–F) and elevated CO<sub>2</sub> (eCO<sub>2</sub>) treatment techniques (G–I, OTC = open-top chambers; CTC = closed-top chambers; GH = greenhouses; FACE = free-air CO<sub>2</sub> enrichment; TGT = temperature-gradient tunnels; GC = growth chambers) in response to eCO<sub>2</sub> (A, D, G), warming (B, E, H) and eCO<sub>2</sub> plus warming (C, F, I). Each data point represents the mean ± 95% CI. Numbers within parentheses represent the number of experimental observations. The dashed vertical line is the reference line at 0% change. Treatment effects are non-significant at  $P < 0.05$  if CIs overlap the zero line, and differences among treatments are non-significant if CIs overlap.

the magnitude of the negative effect of eCO<sub>2</sub> plus warming on shoot %N further increased as temperature increased from  $T_L$  to  $T_M$ , suggesting an inability of plants to maintain tissue quality at higher temperatures, even when combined with eCO<sub>2</sub>.  $T_M$  alone or in combination with eCO<sub>2</sub> showed a tendency to increase root %N relative to the effect of  $T_L$  or its combination with eCO<sub>2</sub> on root %N. However,  $T_H$  alone or in combination with eCO<sub>2</sub> showed a tendency to decrease root %N relative to the effect of  $T_L$  or its combination with eCO<sub>2</sub> on root %N. These results suggest that although shoot or root %N respond to different levels of CO<sub>2</sub> similarly, irrespective of the temperature, they may not respond to different temperatures in a similar way, irrespective of the CO<sub>2</sub> level.

Elevated CO<sub>2</sub> is likely to reduce the protein concentration of many plant species, including those grown for human consumption. The C<sub>3</sub> grasses such as wheat and rice are more likely to be negatively affected by eCO<sub>2</sub> than legumes (Myers et al. 2014). Though the mechanism by which eCO<sub>2</sub> decreases tissue protein concentration is not well understood, one possible explanation could be the increased concentration of non-structural carbohydrates relative to protein when plants are grown under eCO<sub>2</sub> (Taub et al. 2008). In this meta-analysis, eCO<sub>2</sub> alone or in combination with warming significantly or non-significantly reduced total protein concentrations in all shoots, roots and grains. These results are in conformity with the results observed for C<sub>3</sub> grasses by Myers et al. (2014). Decreased protein concentration in edible portions of the crops can cause malnutrition among

humans (Myers et al. 2014). In contrast, warming alone significantly or non-significantly increased the total protein concentration in all three tissue types. Interestingly, the variation in shoot or root protein concentration in response to eCO<sub>2</sub> and/or warming scaled with the variation in shoot or root %N in response to these independent variables, suggesting a dependence of tissue protein concentration and, hence the nutritional quality, on tissue %N. At warming alone or eCO<sub>2</sub> plus warming, tissue protein concentration also showed a positive relationship with root N-uptake rate, suggesting the dependence of nutritional quality on N-uptake rate when temperature is involved.

Grasses had the smallest decrease in shoot %N, but the largest decrease in root %N, in response to both eCO<sub>2</sub> and eCO<sub>2</sub> plus warming. In contrast, non-woody dicots had the largest decrease in shoot %N, but the smallest decrease in root %N, in response to both eCO<sub>2</sub> and eCO<sub>2</sub> plus warming. These results suggest a potential enhancement of net N translocation from roots-to-shoots in grasses, while a potential inhibition of net N translocation in non-woody dicots, in response to both eCO<sub>2</sub> and eCO<sub>2</sub> plus warming. Based on shoot and root %N data of woody species, both eCO<sub>2</sub> and eCO<sub>2</sub> plus warming are also likely to inhibit net N translocation in woody species. In addition, based on shoot and root %N data of grasses and non-woody dicots, warming is likely to enhance net N translocation in both growth forms. Collectively, these results suggest that the net translocation of N from roots-to-shoots will respond differently among



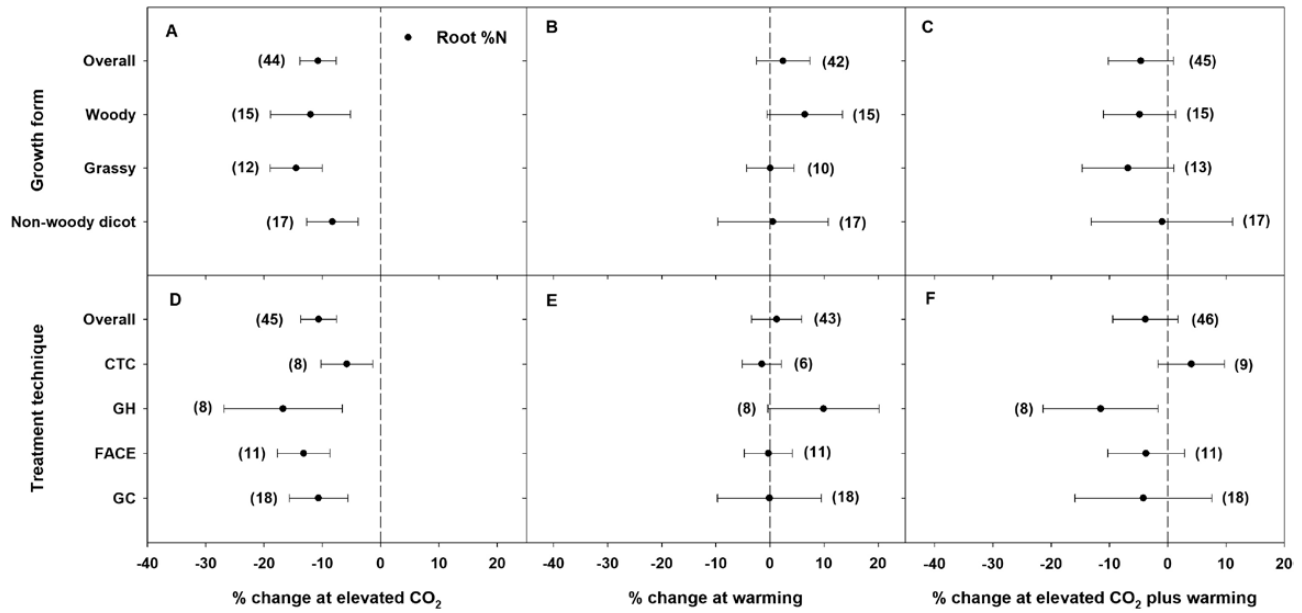


Figure 4. Percent change (compared to ambient or controls) in root %N in different growth forms (A–C) and elevated CO<sub>2</sub> (eCO<sub>2</sub>) treatment techniques (D–F, CTC = closed-top chambers; GH = greenhouses; FACE = free-air CO<sub>2</sub> enrichment; GC = growth chambers) in response to eCO<sub>2</sub> (A, D), warming (B, E) and eCO<sub>2</sub> plus warming (C, F). Each data point represents the mean  $\pm$  95 % CI. Numbers within parentheses represent the number of experimental observations. The dashed vertical line is the reference line at 0 % change. Treatment effects are non-significant at  $P < 0.05$  if CIs overlap the zero line, and differences among treatments are non-significant if CIs overlap.

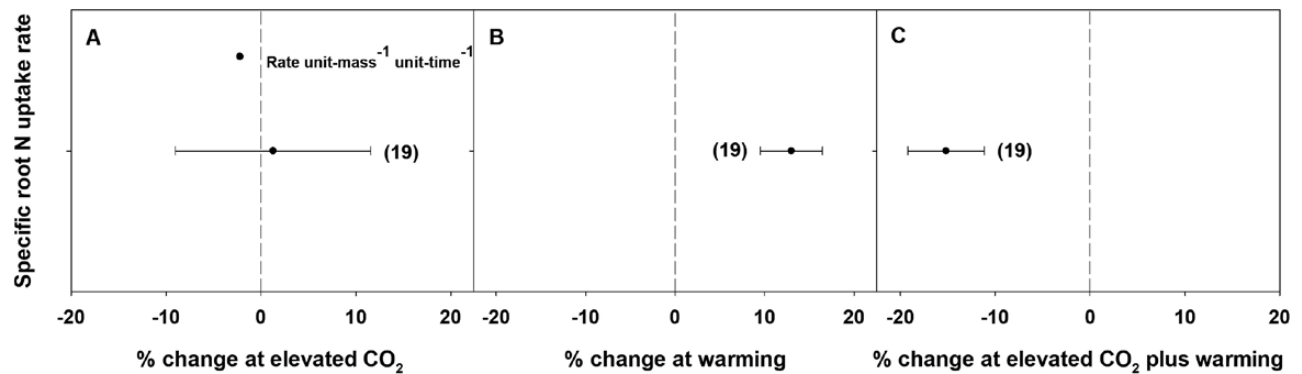


Figure 5. Percent change (compared to ambient or controls) in root N-uptake rate in response to elevated CO<sub>2</sub> (A), warming (B) and elevated CO<sub>2</sub> plus warming (C). Each data point represents the mean  $\pm$  95 % CI. Numbers within parentheses represent the number of experimental observations. The dashed vertical line is the reference line at 0 % change. Treatment effects are non-significant at  $P < 0.05$  if CIs overlap the zero line.

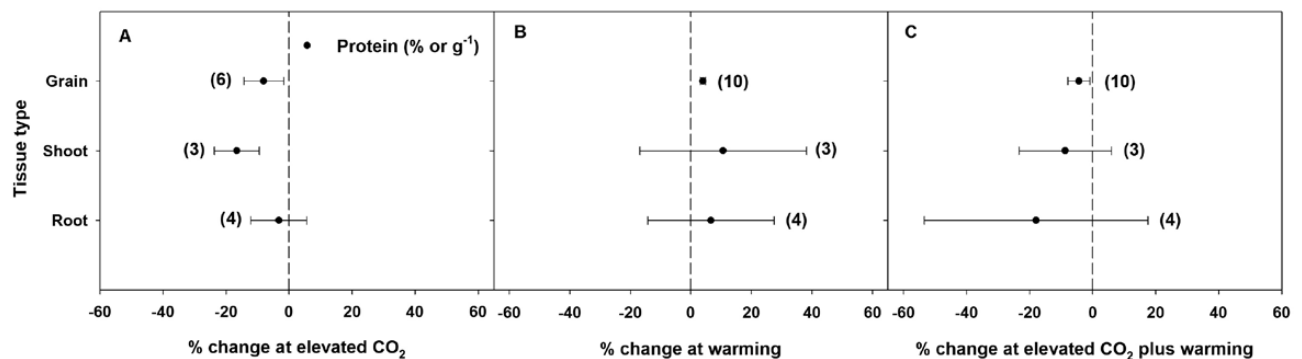


Figure 6. Percent change (compared to ambient or controls) in the concentration of total protein in different tissue types in response to elevated CO<sub>2</sub> (A), warming (B) and elevated CO<sub>2</sub> plus warming (C). Each data point represents the mean  $\pm$  95 % CI. Numbers within parentheses represent the number of experimental observations. The dashed vertical line is the reference line at 0 % change. Treatment effects are non-significant at  $P < 0.05$  if CIs overlap the zero line, and differences among treatments are non-significant if CIs overlap.

plants of different growth forms to future climate conditions. Root-to-shoot translocation in woody species involves long-distance transportation compared to grasses or non-woody dicots. As eCO<sub>2</sub> is likely to reduce xylem volume (Cohen *et al.* 2019), the observed decrease in net N translocation in woody species could be in part due to decreased xylem volume when plants grown at eCO<sub>2</sub>.

Legumes are known to have the ability to withstand the eCO<sub>2</sub>-driven leaf N dilution which is typically observed in C<sub>3</sub> plants grown at eCO<sub>2</sub> (Rogers *et al.* 2009). However, results of this meta-analysis oppose this view as eCO<sub>2</sub> caused a greater decrease in legume shoot %N than non-legume shoot %N, regardless of the temperature. Notably, in this meta-analysis, a greater proportion of experimental observations of legume shoot %N were taken from studies which were conducted under natural soil nutrient conditions (FACE, OTC or TGT techniques with no additional nutrients supplied). As van Groenigen *et al.* (2006) explained, eCO<sub>2</sub> will not have an effect on N<sub>2</sub> fixation when legumes are grown under natural conditions with no fertilizer additions, and this could be one of the potential reasons for the observed result in this study. Meanwhile, though warming significantly increased non-legume shoot %N, it did not have an effect on legume shoot %N. As Hungria and Kaschuk (2014) explained, warming can limit N<sub>2</sub> fixation by inhibiting NH<sub>4</sub><sup>+</sup> assimilation and nitrogenase activity, which could be one of the potential reasons for the observed neutral effect of warming on shoot %N.

In this meta-analysis, a subgroup analysis of different treatment techniques was conducted to find those more suitable for climate studies involving the interaction of CO<sub>2</sub> and temperature. All of these techniques have their own advantages and disadvantages. Both shoot and root %N were investigated here because, apart from biomass measures, these have been widely measured in plants grown with these treatment techniques. Since this meta-analysis focused primarily on the effects of eCO<sub>2</sub> plus warming on plant N relations, the suitability of these techniques is mainly discussed in response to eCO<sub>2</sub> plus warming. The FACE technique is thought to provide the most realistic measure of the effects of eCO<sub>2</sub> on crop yields because enclosure techniques can produce a 'chamber effect' that can exceed the effects of eCO<sub>2</sub> (Ainsworth *et al.* 2005, 2008; Ainsworth and Long 2021). However, in this meta-analysis, some enclosure studies produced results similar to those of FACE studies (e.g. shoot %N in TGT and root %N in GC). Previously, a meta-analysis conducted by Taub *et al.* (2008) also reported similar protein concentrations in response to eCO<sub>2</sub> in crops grown using either FACE or other techniques (OTC, CTC, GH, GC). In the current meta-analysis, the overall decreases in shoot and root %N in response to eCO<sub>2</sub> plus warming were 14 and 3 %, respectively. Interestingly, FACE and TGT studies also showed 14 % decreases in shoot %N, and GC and CTC studies showed 16 and 11 % decreases in shoot %N, respectively, in response to eCO<sub>2</sub> plus warming. Meanwhile, the 4 % decrease in root %N observed for FACE and GC was similar to the overall decrease in root %N in response to eCO<sub>2</sub> plus warming. These results suggest that in addition to FACE technique, other enclosed techniques such as GC, TGT and CTC can produce reliable results when studying the effects of eCO<sub>2</sub> plus warming. Additionally, these three enclosed techniques produced similar results to those of the FACE technique in response to warming alone. This meta-analysis further suggests that the GH technique is likely unsuitable for studies involved with eCO<sub>2</sub> plus warming, due to its overestimation of the negative impacts of eCO<sub>2</sub> plus warming on plant %N.

In the future, global environmental changes such as CO<sub>2</sub> enrichment, warming, drought, N deposition, etc. will occur concomitantly. Therefore, multi-factor manipulation approaches will be necessary to understand the combined

effects of these various factors on plant growth, metabolism and production. This meta-analytic review was designed to improve understanding of the effects of eCO<sub>2</sub> plus warming on plant N metabolism as this area of research has important knowledge gaps. However, one of the limitations of this study was the analysis of the effects of only two predictor variables on plant N metabolism-related response variables. Therefore, future research should focus on incorporating more predictor variables, such as drought, when investigating the impacts of environmental change on plant N metabolism. In addition, it will be interesting to see how experimental duration and the level and form of N affect plant N metabolism under these conditions, and how plant N metabolism-related variables respond to different levels of CO<sub>2</sub> and temperature.

## Conclusions

In the future, concomitant increases in CO<sub>2</sub> and temperature are likely to affect plant N metabolism by lowering plant %N, root N-uptake rate and tissue protein concentration. Therefore, when developing plants for future climates, plant-improvement efforts should focus on generating new genotypes with more-resilient N metabolism.

## Supporting Information

The following additional information is available in the online version of this article—

**Appendix S1.** Database.

**Appendix S2.** References included in the meta-analysis.

## Sources of Funding

None declared.

## Conflict of Interest

None declared.

## Contributions by the Authors

D.M.J. and S.A.H. were involved in the conceptual design of the study. D.M.J. constructed the database, conducted the meta-analysis, generated graphs and wrote the draft manuscript. S.A.H. is the faculty advisor of D.M.J., and was involved in data interpretation and manuscript revision. J.K.B. was involved in manuscript revision and provided valuable feedback.

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## Data Availability

The data are available as [Supporting Information](#).

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