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Analyse von Wachstum und Qualität von Weizen unter ansteigender CO_2 Konzentration als Folge des Klimawandels

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Inhalt

Relevante Abkürzungen

a[CO_2]:	Umgebungs-Kohlenstoffdioxid-Konzentration
AR:	über die Vegetationsperiode akkumulierte absorbierte photosynthetisch aktive Strahlung
C:	Kohlenstoff
CO_2	Kohlenstoffdioxid
e[CO_2]:	erhöhte atmosphärische Kohlenstoffdioxid-Konzentration
FACE:	Free Air CO_2 Enrichment
GOGAT:	Glutamin-Oxoglutarat-Aminotransferase
GS:	Glutamin-Synthetase
HI:	Ernteindex
HMW-GS:	hochmolekulare Glutenin-Untereinheiten
LAI:	grüner Blattflächenindex
LMW-GS:	niedermolekulare Glutenin-Untereinheiten
N:	Stickstoff
NADH:	Nicotinamidadenindinukleotid
Nabs:	postanthetische N-Aufnahme
NH_4^+:	Ammonium
NO_3^-:	Nitrat
NO_2^-:	Nitrit
Nrem:	N-Remobilisierung
NR:	Nitratreduktase
NRA:	Nitratreduktase-Aktivität
NRE:	Effizienz der N-Remobilisierung
O_2	Sauerstoff
ppm:	Parts per Million
RuBisCO:	Ribulose-1,5,-bisphosphat Reduktase/Oxygenase
RUE:	Strahlungsnutzungseffizienz
S	Schwefel

1. Allgemeine Einleitung

1.1 Bedeutung von Weizen

Weizen (*Triticum aestivum* L.) ist global die zweitwichtigste Nutzpflanze, die etwa 30 % des Energie-, 20 % des Proteinbedarfs sowie 20–40 % des Bedarfs wichtiger Mineralien der Weltbevölkerung deckt (Shewry und Hey, 2015; FAOSTAT, 2017). Zudem hat Weizenmehl einzigartig gute Backeigenschaften, die wesentlich auf der Zusammensetzung des Kornproteins basieren (Belitz et al., 2009). Dieses kann chemisch in eine Albumin/ Globulin-, Gliadin- und Glutenin-Fraktion aufgetrennt werden. Die letzten beiden Fraktionen werden als Gluten zusammengefasst und bestimmen die Backqualität durch Bildung eines viskoelastischen Protein-Netzwerks beim Teigrühren. Gliadine sind Einzelproteine, die in ω-, α- und γ-Gliadine unterteilt werden und die Viskosität des Teiges bestimmen. Glutenine sind Proteinaggregate aus hoch- (HMW-GS) und niedermolekularen Untereinheiten (LMW-GS), die für die Elastizität und Stabilität des Teigs verantwortlich sind. Das Gluten-Netzwerk sorgt dafür, dass der Teig CO_2-Moleküle aus der Hefegärung halten kann und dadurch „aufgeht“.

1.2 Ertragsbildung

Bei Weizen (und anderen Getreiden) kann der Kornertrag (Y) als Funktion dreier Komponenten beschrieben werden: (1.) Photosynthetisch aktive Strahlung die über die Vegetationsperiode vom Weizenbestand absorbiert und akkumuliert wird (AR), (2.) Umwandlungseffizienz der AR in Biomasse (Strahlungsnutzungseffizienz: RUE) sowie (3.) Biomasseanteil der den Körnern zugewiesenen wird (Ernteindex: HI).

$$Y = AR * RUE * HI$$

AR hängt von der zeitlichen Entwicklung und Größe des grünen Blattflächenindexes (LAI) ab, wobei AR kurvenförmig mit zunehmendem LAI bis zu einem kritischen Wert (LAI_{krit}) ansteigt, ab dem keine weitere AR-Steigerung mehr erfolgt. Dagegen nehmen mit ansteigendem LAI Beschattungseffekte zwischen den Blättern zu, die mit einer Abnahme der Blatt-Stickstoff (N)-Konzentration verbunden sind (Bertheloot et al., 2008). RUE hängt wesentlich vom Gehalt und der Aktivität des Enzyms

Ribulose-1,5,-bisphosphat Reduktase/Oxygenase (RuBisCO) ab, das durch dessen Reduktase-Funktion die CO_2-Fixierung und durch dessen Oxygenase-Funktion die Photorespiration einleitet.

Eine Erhöhung der N-Versorgung steigert den Kornertrag wesentlich durch Steigerung der AR bis der kritische LAI-Wert erreicht ist. RUE wird durch die N-Versorgung kaum beeinflusst, aber nimmt bei starkem N-Mangel deutlich ab (Sinclair und Horie, 1989). RUE wird stark durch die CO_2-Konzentration durch Änderung der RuBisCO-Aktivität beeinflusst. Ein Anstieg des Quotienten der CO_2- und O_2-Konzentration steigert die CO_2-Fixierung und dadurch RUE, wohingegen eine Abnahme dieses Quotienten die Photorespiration steigert und so die RUE reduziert. Ein Anstieg der Temperatur bewirkt eine Abnahme der Affinität von RuBisCO zu CO_2 und fördert so die Reaktivität der CO_2-Fixierung auf eine erhöhte atmosphärische CO_2-Konzentration.

1.3. N-Aneignung des Korns

Bei Weizen und anderen Getreiden hängt der N-Gehalt im Korn wesentlich von drei Prozessen ab: (1.) N-Aufnahme der vegetativen Organe bis zur Anthese, (2.) Verlagerung von diesem aufgenommenen N in die Körner während der Kornfüllungsphase (N-Remobilisierung) und (3.) N-Aufnahme der Körner während der Kornfüllung (postanthetische N-Aufnahme).

Der 1. und 3. Prozess beinhaltet wesentlich die Aufnahme von Nitrat (NO_3^-) durch die Wurzel, dessen Xylem-Transport sowie Assimilation. Bei geringen NO_3^--Konzentrationen im Boden sind die Hauptorte der NO_3^--Assimilation die Wurzeln und bei hohen Konzentrationen wie in Agrarsystemen sind die Hauptorte die Blätter (Andrews et al., 2013). Der erste Schritt der NO_3^--Assimilation erfolgt durch das Enzym Nitratreduktase (NR) das Nicotinamidadenindinukleotid (NADH)-abhängig NO_3^- zu Nitrit (NO_2^-) reduziert. Die beiden weiteren Schritte sind die Reduktion von NO_2^- zu Ammonium (NH_4^+) und dessen Assimilation zu Glutamin und Glutaminsäure durch die Enzyme Glutamin-Synthetase (GS) und Glutamin-Oxoglutarat-Aminotransferase (GOGAT). Eine weitere wichtige N-Quelle für Weizen ist NH_4^+. Jedoch wird dieses verglichen mit NO_3^- in geringeren Mengen aufgenommen was wesentlich auf Nitrifikation zurückzuführen ist. Dessen Assimilation erfolgt hauptsächlich in den Wurzeln und beinhaltet die NH_4^+-Assimilation zu Glutamin und Glutaminsäure durch die Enzyme GS und GOGAT.

Das für die Aktivität der Nitratreduktase (NRA) notwendige NADH stammt aus der Photorespiration (Bloom et al., 2015) sowie der CO_2-Fixierung (Foyer et al., 2009). Die Photorespiration treibt den Malat-Transport aus den Chloroplasten ins Cytoplasma an, wobei durch die anschließende Oxidation des Malats NADH generiert wird. Die CO_2-Fixierung fördert die Synthese von Glycerinaldehyd-3-phoshat und dessen Transport ins Cytoplasma, in dem durch die Oxidation von Glycerinaldehyd-3-phoshat NADH entsteht.

Die N-Remobilisierung (Nrem) beginnt direkt nach dem Wachstumsstadium der Anthese und lässt sich in eine Prä-Seneszenz (bis 8–16 Tage nach der Anthese) und Seneszenz-abhängige Phase einteilen (Kong et al., 2016). Über 80 % des remobilisierten N wird in Form von Aminosäuren (größtenteils Glutamin) über das Phloem angeliefert. Diese entstehen wesentlich aus dem Proteinabbau durch Proteasen und GS-abhängigem Recycling (Kong et al., 2016). Die übrigen 20 % des remobilisierten N werden in Form von kleinen Peptiden, NO_3^-, NH_4^+ oder Harnstoff angeliefert (Have et al., 2016). Die Nrem erfolgt wesentlich aus den Blättern, Halm und Spelzen, wobei bei ersterem die Effizienz am größten ist. Die Effizienz der N-Remobilisierung (NRE) ist definiert als der Anteil des bis zur Anthese aufgenommenen N, der in das Korn remobilisiert wurde.

Von Sorte und Umweltbedingungen abhängig stammen 40–95 % des Korn-N aus der Nrem und dementsprechend 5–60 % aus der postanthetische N-Aufnahme (Nabs) (Kong et al., 2016). Die beiden Prozesse sind negativ korreliert (Bogard et al., 2010).

1.4 Anthropogener Klimawandel

Die atmosphärische CO_2-Konzentration lag vor der Industrialisierung bei etwa 280 Parts per Million (ppm). Durch die massive Verbrennung fossiler Brennstoffe ist diese auf gegenwärtig 414 ppm angestiegen und könnte bis 2100 weiter auf 730–1020 ppm zunehmen (IPCC, 2013). Eine erhöhte atmosphärische CO_2-Konzentration (e[CO_2]) ist wesentlich für die globale Klimaerwärmung verantwortlich, die zukünftig die Intensität und Dauer von Hitze- und Trockenperioden stark erhöhen könnte (IPCC, 2013). Jedoch soll nach dem Übereinkommen von Paris der Anstieg der globalen Durchschnittstemperatur auf 1.5–2 °C begrenzt werden und nach Betts und McNeall (2018) würde das einem CO_2-Anstieg in der Atmosphäre zwischen 500–620 ppm entsprechen.

1.5 Wirkung von e[CO_2]

Eine entscheidende Wirkung von e[CO_2] ist die Steigerung der Photosynthese (CO_2-Fixierung) sowie von Wachstum und Ertrag von C3-Nutzpflanzen (Ainsworth und Long, 2005). Nach einer Meta-Analyse könnte bei Weizen der Kornertrag um 15 % unter der e[CO_2] von 550 ppm zunehmen (Ainsworth und Long, 2005). Zudem vermindert e[CO_2] die Transpiration durch Verringerung der Öffnungsweite der Stomata (Ainsworth und Rogers, 2007) was zu einer Verminderung der Evapotranspiration und dadurch von Trockenstress führen kann (Manderscheid et al., 2018). Das Ausmaß der Ertragssteigerung durch e[CO_2] ist von Standortfaktoren abhängig, insbesondere der Temperatur (Long, 1991) sowie der Wasser- und N-Verfügbarkeit (Kimball et al., 2002). Wegen der abnehmenden Affinität von RuBisCO zu CO_2 mit ansteigender Temperatur, verstärkt ein Temperaturanstieg die Photosynthese- und Wachstumssteigerung durch e[CO_2]. Weiterhin wurde festgestellt dass die Ertragssteigerung durch e[CO_2] bei Trockenstress verstärkt, aber unter starkem N-Mangel vermindert ist (Kimball et al., 2002).

Neben diesen positiven Effekten führt e[CO_2] zu einer Reduktion der N- bzw. Protein-Konzentration, insbesondere im Blatt und Korn. Meta-Analysen zeigten, dass bei Weizen die Blatt N-Konzentration zwischen 9 bis 16 % (Cotrufo et al., 1998; Wang et al., 2013) und eine Modellierungsstudie, dass die Kornprotein-Konzentration um 9 % unter der e[CO_2] von 550 ppm abnehmen könnte (Asseng et al., 2019). Mehr als 25 % des Blattproteins ist RuBisCO und deshalb könnte eine Reduktion des Blattproteins durch e[CO_2] zu einer Abnahme der Photosynthesekapazität führen, was in einer Meta-Analyse bei verschiedenen Pflanzenarten festgestellt wurde (Ainsworth und Long, 2005). Die Reduktion der Kornprotein-Konzentration durch e[CO_2] war wesentlich mit einer Reduktion von backrelevanten Gluten-Proteinen verbunden (Wieser et al., 2008; Högy et al., 2013; Fernando et al., 2015). Dementsprechend wurde auch eine Reduktion der Backqualität durch e[CO_2] festgestellt (Panozzo et al., 2014).

Für die Reduktion der Protein-Konzentration durch e[CO_2] bei Getreide gibt es nach Pleijel et al. (2019) vier Haupthypothesen. Eine ist ein Verdünnungseffekt, der besagt dass die N-Aufnahme mit einer gesteigerten Biomasse- und Ertragsbildung durch e[CO_2] nicht Schritt halten kann (Loladze, 2002). Die zweite ist eine e[CO_2]-induzierte Hemmung der Genexpression von RuBisCO im Blatt

(Stitt und Krapp, 1999). Die beiden letzten Hypothesen beinhalten eine Reduktion des Nährstoffflusses zur Wurzel durch die Abnahme der Transpirationsrate durch e[CO_2] (McGrath und Lobell, 2013) und eine Hemmung der NO_3^--Assimilation in den Blättern durch e[CO_2], welche mit einer Reduktion der Rate der Photorespiration verbunden ist (Bloom et al., 2010).

Neben der N-Konzentration kann e[CO_2] auch die Konzentration anderer Makro- und Mikronährstoffe im Korn, insbesondere die von Eisen (Fe) und Zink (Zn), reduzieren (Myers et al., 2014). Diese Reduktionen könnten zusammen mit der Reduktion der Korn-N-Konzentration die menschliche Ernährung, insbesondere in den Entwicklungsländern, gefährden (Myers et al., 2014; Smith und Myers, 2018).

1.6 Free Air CO_2 Enrichment

Eine Wachstumssteigerung durch e[CO_2] ist seit etwa 200 Jahren bekannt. CO_2-Anreicherungsstudien wurden vermehrt ab den 1960er Jahren durchgeführt; angefangen mit Klimakammerstudien über Tunnel- und Open-Top-Kammer-Versuchen bis hin zu Free Air CO_2 Enrichment (FACE)-Experimenten (Ziska und Bunce, 2007). FACE-Versuche wurden zum ersten Mal 1989 durchgeführt (Kimball et al., 2002). Diese sind die realistischste CO_2-Anreicherungsmethode, da das Pflanzenwachstum nicht durch das Versuchssystem beeinträchtigt wird (Long et al., 2005). Hier werden Pflanzen unter Feldbedingungen innerhalb ringförmiger Anordnungen mit Durchmesser von etwa 20 m durch senkrecht stehende Pfeifen mit CO_2 angereicherter Luft angereichert (**Abb. 1**). Die CO_2-Konzentration sowie Windrichtung und -stärke wird innerhalb der FACE-Ringe etwa sekündlich gemessen. Darauf basierend wird die Öffnungsweite von Düsen in den Pfeifen angepasst um eine konstante CO_2-Konzentration im Ringinneren zu gewährleisten.

1.7 Offene Fragen und Ziele der Dissertation

Das erste Hauptziel dieser Doktorarbeit war die Prozessanalyse der Reduktion der Kornprotein-Konzentration durch e[CO_2] (**Kapitel 3 und 4**) und das Zweite die Analyse der e[CO_2]-Wirkung auf die Kornprotein-Zusammensetzung und die Backqualität bei Winterweizen der Sorte „Batis“ (**Kapitel 5**). Dies erfolgte im Rahmen eines zwei-jährigen FACE-Versuchs mit zwei CO_2-Konzentrationen (~393 und 600 ppm) und vier N-Düngebehandlungen (**Abb. 1**). Bei diesen handelt es sich um drei

Stufen einer herkömmlichen NO_3^- -betonten N-Düngung mit kg N ha^{-1}: 40/35 (Mangel), 180/200 (praxisüblich) und 320/320 (Überschuss) und um eine Stufe einer NH_4^+ betonten N-Düngung mit 180/200 kg N ha^{-1}. Des Weiteren wurde der Einfluss der Quantität der N-Düngung auf die Bildung von Kornertrag unter e[CO_2] analysiert (**Kapitel 2**). Es folgt eine Übersicht über die wichtigsten Hypothesen der vorliegenden Arbeit:

Kapitel 2: Nach einer Meta-Analyse nimmt die Ertragsseigerung durch e[CO_2] bei N-Mangel ab (Pleijel et al., 2019). Jedoch sind die Mechanismen für eine solche Abnahme unklar. Ein möglicher Prozess dafür könnte eine Reduktion der AR durch eine e[CO_2]-induzierte Beschleunigung der Seneszenz sein (Brooks et al., 2000; Jamieson et al. 2000). Ein weiterer Prozess könnte eine stärkere Reduktion der Blatt N-Konzentration verbunden mit einer stärkeren Reduktion der Photosynthesekapazität durch e[CO_2] bei N-Mangel als bei hoher N-Versorgung sein (Sinclair et al., 2000). Hinweise für beide Prozesse konnte in einem FACE-Versuch in Arizona gefunden werden.

Die Hypothesen für **Kapitel 2** sind:

- Die Ertragssteigerung durch e[CO_2] nimmt bei starkem N-Mangel ab
- Ein Grund hierfür ist eine Reduktion der AR durch e[CO_2] in der Kornfüllungsphase bei starkem N-Mangel
- Ein weiterer Grund ist eine verminderte Steigerung der RUE wegen starker Reduktion der Blatt-N-Konzentration durch e[CO_2] bei starkem N-Mangel

Kapitel 3: Es wurde analysiert wie e[CO_2] die Nrem von Blatt, Halm und Ähre sowie die Nabs beeinflusst, wovon es bisher keine Daten aus FACE-Versuchen gibt. In einem FACE-Versuch wurde eine starke Reduktion der N-Konzentration im Blatt und Korn durch e[CO_2] bei N-Mangel, aber nur eine geringe Reduktion durch e[CO_2] bei hoher N-Versorgung festgestellt (Kimball et al. 2001, 2002). Dies zeigt, dass eine Abnahme der N-Konzentration im Blatt durch e[CO_2] zur Anthese die Nrem reduzieren könnte. Die Nabs könnte durch e[CO_2] über eine Hemmung der NO_3^--Assimilation oder Beschleunigung der Seneszenz (Brooks et al., 2000; Fangmeier et al., 2000) reduziert werden.

Die Hypothesen für **Kapitel 3** sind:

- e[CO_2] reduziert die Nrem durch Reduktion der N-Konzentration in den vegetativen Organen zum Wachstum-Stadium der Anthese
- e[CO_2] reduziert die Nabs aufgrund einer Beschleunigung der Seneszenz
- Die Reduktion der Korn-N-Konzentration durch e[CO_2] ist stärker bei N-Mangel als bei hoher N-Versorgung

Kapitel 4: Es wurde unter Feldbedingungen untersucht ob e[CO_2] die Blatt NO_3^--Assimilation hemmt und so die Reduktion der N-Konzentration durch e[CO_2] erklärt. Eine Hemmung der NO_3^--Assimilation konnte in Hydrokulturversuchen festgestellt werden (Bloom et al., 2002, 2010). Jedoch haben die Pflanzen in solchen Versuchen ein stark beschränktes Wurzelvolumen, das die e[CO_2]-Wirkung auf Wachstum und N-Aneignung modifiziert (Arp, 1991; Long et al., 2005). Doch wurden auch Hinweise einer Hemmung der NO_3^--Assimilation durch e[CO_2] in einem FACE-Versuch gefunden, aber in diesem gab es keine Reduktion der N-Konzentration durch e[CO_2] (Bloom et al., 2014).

Die Hypothesen für **Kapitel 4** sind:

- e[CO_2] hemmt die NO_3^--Assimilation im Blatt durch Hemmung der NRA
- Durch Hemmung der NO_3^--Assimilation gibt es eine stärkere N-Aneignung und Wachstumssteigerung durch e[CO_2] bei NH_4^+- als bei NO_3^--betonter N-Düngung
- Aufgrund einer möglichen Abhängigkeit der NRA von der Photorespiration (Bloom 2015) ist eine Hemmung der NRA stärker bei hohen (> 25 °C) als bei niedrigen Temperaturen

In **Kapitel 5** wurde untersucht ob e[CO_2] Gluten-Proteine beeinträchtigt und dadurch die Backqualität vermindert. In vorherigen FACE-Studien war eine Abnahme der Kornprotein-Konzentration durch e[CO_2] eng mit einer Abnahme von Gluten-Proteinen verbunden (Wieser et al., 2008; Högy et al., 2013). Auch konnte eine Reduktion des Backvolumens durch e[CO_2] beobachtet werden (Panozzo et al., 2014). Insgesamt sind aber die Ergebnisse von FACE-Studien bezüglich des e[CO_2] Effekts auf die verschiedenen Gluten-Fraktionen uneinheitlich. Zudem ist die Anzahl solcher Versuche mit gleichzeitiger Untersuchung des e[CO_2]-Effekts auf das Korn-Proteom und die Backqualität sehr gering.

Die Hypothesen für **Kapitel 5** sind:

- e[CO_2] beeinträchtigt Gluten-Proteine und reduziert dadurch die Backqualität
- Der Gesamtgehalt von Albumin/Globulin bleibt durch e[CO_2] unbeeinträchtigt.

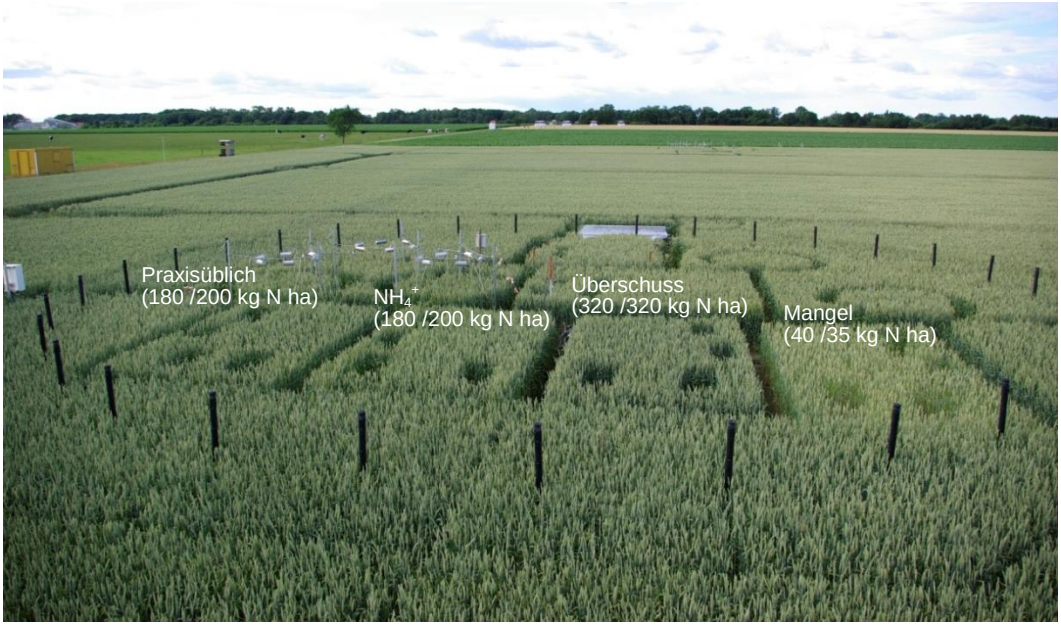

Abb. 1: e[CO_2] Parzelle des Braunschweiger FACE-Versuchs: In dem Versuch gab es jeweils drei FACE- sowie a[CO_2]-Ringe mit bzw. ohne CO_2-Anreicherung. Innerhalb der Versuchsringe wurden die vier verschiedenen N-Düngebehandlungen (s. Abbildung) auf Parzellen mit 5 x 3 m zufällig verteilt.

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2. Kapitel: Einfluss von Quantität der N-Düngung auf die Bildung des Kornertrags unter e[CO_2]

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Decreased wheat grain yield stimulation by free air CO_2 enrichment under N deficiency is strongly related to decreased radiation use efficiency enhancement

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ABSTRACT

Uncertainty still exists about the extent and mechanisms of yield stimulation by elevated atmospheric CO_2 (e[CO_2]) in wheat. Particularly, data of the e[CO_2] effect under severe N deficiency from field experiments are scarce. To investigate the interaction of e[CO_2] and N fertilization on important variables that determine grain yield and are often used in crop simulation models, e.g. radiation absorption by the canopy (AR), radiation use efficiency (RUE) and specific leaf N weight (SLNW), a two-year Free Air CO_2 Enrichment (FACE) experiment was conducted with two [CO_2] (393 and 600 ppm) and three N levels (severe N deficiency (Nd) with 40 (1st year) and 35 kg N ha^{-1} (2nd year); adequate N supply with 180 (1st year) and 200 kg N ha^{-1} (2nd year); and excess N supply with 320 kg N ha^{-1} (1st and 2nd year).

Final aboveground biomass ranged from 816 to 2012 g m^{-2} and grain yield from 417 to 973 g m^{-2}. e[CO_2] increased aboveground biomass by 13, 18 and 14% and grain yield by 10, 17 and 17% under Nd, Nad and Nex, respectively. Yield stimulation was primarily due to enhanced grain number. With increasing N supply, peak values of green area index were increased under e[CO_2] by 4 up to 22%, while AR was unaffected. RUE was increased by both rising SLNW, which depended on N supply, and e[CO_2] and the RUE increase was larger under Nad (+20%) and Nex (+18%) than under Nd (+6%). SLNW was decreased by e[CO_2] and this decrease was very similar among N levels (~ −6%). However, if leaf area index was included as covariable, then a e[CO_2] induced decrease of SLNW was only found under Nd.

The present study demonstrates that yield stimulation by e[CO_2] is smaller under severe N deficiency compared to high N supply in wheat. In contrast to the results of another FACE study, this decrease was not due to reduced AR but reduced RUE, which might be attributed to both restrictions on source activity, i.e. photosynthetic capacity and sink size, i.e. ear growth.

1. Introduction

Atmospheric CO_2 concentration is continuing to rise from current 400 up to 730–1020 ppm by the end of this century (IPCC, 2013). Elevated CO_2 concentration (e[CO_2]) will increase air temperature and thus possibly the frequency and severity of weather extremes that could reduce future crop yields (IPCC, 2013). On the other hand, e[CO_2] increases photosynthesis in C_3 plants (Ainsworth and Long, 2005) and reduces stomatal conductance in C_3 and C_4 plants (Ainsworth and Rogers, 2007; Wang et al., 2013), which often lead to stimulation of biomass production and grain yield (Ainsworth and Long, 2005; Manderscheid et al., 2014).

Wheat is the third most important crop in terms of global production and its range of cultivation plays a key role for food security (Shewry and Hey, 2015). Crop models predict that growth stimulation of C_3 crops by e[CO_2] will compensate for yield losses due to rising air temperature up to 2 °C, but the extent of this compensation might be different depending on the region and its related agricultural conditions such as N fertilizer availability (Rosenzweig et al., 2014). Therefore, uncertainty still exists about the extent of wheat yield stimulation by future e[CO_2]. Free Air CO_2 Enrichment (FACE) experiments can help to overcome this uncertainty and were conducted with wheat in

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Arizona (Kimball et al., 2002), Australia (Tausz-Posch et al., 2012), China (Kou et al., 2007; Ma et al., 2007; Lam et al., 2012; Han et al., 2015; Cai et al., 2016) and Germany (Högy et al., 2009; Weigel and Manderscheid, 2012). However, comprehensive growth data from such studies, especially those on the interactions between e[CO_2] and N fertilization, are still scarce (Rosenzweig et al., 2014; Vanuytrecht and Thorburn, 2017).

Grain yield (Y) can be described as a function of three factors describing fundamental processes: (i) absorbed photosynthetic active radiation (PAR) accumulated over the growing season (AR), (ii) conversion efficiency of AR to biomass (radiation use efficiency: RUE) and (iii) share of produced biomass allocated to the grains (harvest index: HI):

$$Y = AR * RUE * HI \quad (1)$$

AR is determined by the size and evolution of the green area index (GAI), the sum of the green surfaces of leaves, stems and ears. RUE increases with rising light-saturated net photosynthetic rate in a curvelinear manner (Sinclair and Horie, 1989) and both variables depend on both the leaf biochemical composition such as level and activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and the internal structure of the leaf. Easily determinable proxies for the variables describing the leaf biochemical composition and internal structure are green leaf N concentration, leaf mass per unit leaf area (specific leaf weight) and their composite variable leaf N mass per unit leaf area (specific leaf N weight). Increase in soil N availability stimulates crop growth primarily by increasing the size and duration of GAI and thus AR (Garcia et al., 1988; Jamieson et al., 2000). RUE is not influenced over a broad range of leaf and soil N levels, but declines sharply when these N levels become very low (Sinclair and Horie, 1989). Effects of e[CO_2] are expressed primarily through effects on RUE via influencing the activity of RuBisCO (Long, 1991; Jamieson et al., 2000).

Variation in grain yield due to e[CO_2] is primarily due to variation in grain number (Högy et al., 2009; Cai et al., 2016), but some studies also found e[CO_2] effects on individual grain weight (Fernando et al., 2014; Tausz-Posch et al., 2015). Grain number is strongly associated with growth between flag leaf emergence and anthesis (Bindraban et al., 1998), the period in which radiation supply (Fischer, 1975) and e[CO_2] (Fischer and Aguilarm, 1976) have their greatest influence on grain number.

CO_2 enrichment studies with chambers (Sionit et al., 1981; Wolf, 1996) and FACE (Kimball et al., 2002; Weigel and Manderscheid, 2012) showed that growth stimulation of wheat by e[CO_2] declined when N supply decreased. Kimball et al. (2002) reported for the Arizona FACE study, the only previous FACE study that had a severe N deficiency treatment, that the grain yield increase under e[CO_2] was only half as much under severe N deficiency compared to ample N supply. Nevertheless, FACE studies showed similar biomass and grain yield increases under e[CO_2] in plants grown under low and high N supply (Ma et al., 2007; Han et al., 2015). In the two-year FACE study of Weigel and Manderscheid (2012) lower grain yield stimulation under moderate N deficiency as compared to adequate N supply was only observed in one year.

While AR, RUE and harvest index are important parameters used in many crop models, only little experimental data from FACE experiments related to these parameters exists (Vanuytrecht et al., 2012), which might be responsible for the poor representation of CO_2 x N interactions in crop growth models (Rosenzweig et al., 2014; Vanuytrecht and Thorburn, 2017). According to Jamieson et al. (2000), the smaller grain yield stimulation of e[CO_2] under severe N deficiency in the Arizona FACE experiment was due to reduced AR because of accelerated canopy senescence under e[CO_2]. Moreover, in the same experiment e[CO_2] reduced leaf N concentration by up to -25% (Kimball et al., 2002) under severe N deficiency when leaf N levels were low (Sinclair et al., 2000), but there was hardly any reduction of leaf N concentration by e[CO_2] under ample N supply (Kimball et al., 2002). Based on these findings, it was concluded that photosynthetic capacity is primarily reduced by e[CO_2] under very low N availability (Rubio-Asensio and Bloom, 2016), which might result in smaller stimulation of RUE by e[CO_2] under such N levels compared to high N supply. However, there is no field study available for wheat that has investigated in wheat the CO_2 x N interaction on RUE. Because limited N fertilizer levels are affordable in many developing countries, investigating e[CO_2] effects on yield formation under severe N deficiency is of overall importantance to provide more accurate predictions of future global food security.

In the present study a two-year FACE experiment was conducted with winter wheat (*Triticum aestivum* L.) under irrigated agriculture to avoid interactive effects with water stress supplied with three levels (severe deficiency, adequate, excess) of N fertilizer. The main objective was to analyze how e[CO_2] influences growth and yield formation under a broad range of N fertilizer levels, and in particular whether and to what extent growth and yield stimulation by e[CO_2] is lower under severe N deficiency compared to high N supply. Moreover, the specific question was addressed: is lower grain yield stimulation by e[CO_2] under severe N deficiency associated with (i) decreased AR under e[CO_2] due to accelerated canopy senescence or (ii) reduced RUE stimulation by e[CO_2] compared to high N supply due to a strong decrease of leaf N concentration?

2. Material and methods

2.1. Study site and experimental design

The experiment was conducted on a field site (52°18′N, 10°26′E, 79 m.a.s.l.) at the Thünen-Institute in Braunschweig, Germany in 2014 and 2015. The soil profile has a depth of about 60 cm (30 cm Ap, 15 cm Al, 15 cm Bt, and > 60–70 cm CII) and the lower layers are almost pure sand. The soil in the plough horizon (0–40 cm) is a luvisol of loamy sand texture (69% sand, 24% silt and 7% clay) with a pH of 6.88, and a carbon and nitrogen (N) content of 1.00% and 0.09%, respectively. Soil N levels at the beginning of the main growing season (mid-March) were 14.2 and 22.4 kg N ha^{-1}. The lower (−1.5 MPa soil water tension) and upper limit (0.01 MPa soil water tension) of plant available soil water are a volumetric soil water content of 5 and 23%, respectively. The soil water amount in the 0–40 cm soil profile at the beginning of the main growing season was 92 mm. Altogether, the soil has low to intermediate fertility with a shallow rooting zone.

Winter wheat (*Triticum aestivum* L. variety "Batis") was grown at ambient [CO_2] and e[CO_2] in circular plots (diameter 20 m), in which three N subplots (3 m × 5 m) were randomly established. Overall the experiment consisted of six different CO_2 x N treatments that were replicated three times. The CO_2 and N treatments had the same position on the field site in 2014 and 2015.

2.2. CO_2 enrichment

CO_2 enrichment was carried out with a Free Air CO_2 Enrichment (FACE) system with blowers constructed according to the Brookhaven National Laboratory design (Lewin et al., 1992). CO_2 enrichment started from the four leaf stage on March 31 in 2014 and the three leaf stage on March 12 in 2015. CO_2 enrichment took place during the day and was interrupted when wind speed exceeded 6 m s^{-1} or air temperature fell below 5 °C. The 1 min average [CO_2] in the e[CO_2] plots was within 600 ppm ± 10% for 95.6% of the operation time in 2014 and 95.7% for the one in 2015. During the CO_2 enrichment period, average [CO_2] in the ambient plots was 394 in 2014 and 392 ppm in 2015.

2.3. Crop management

Winter wheat was sown with a density of 380 kernels per m^{-2} on October 29 in 2014 and on November 4 in 2015. Crop management measures were performed according to local farm practice with

Table 1
Important cultivation measures and growth stages.

Event		Date	
Management	Phenological phase	2014	2015
Sowing		Oct 29	Nov 4
	Emergence	Nov 18	Nov 19
Start CO_2 enrichment	4 leaf/3 leaf stage	Mar 31	Mar 12
	1st node stage	Apr 18	Apr 30
1st fungicide treatment		Apr 29	Apr 31
	Flag leaf stage	May 14	May 21
2nd fungicide treatment		–	June 4
	Anthesis	June 6	June 12
	Milk ripe stage	June 23	June 30
End of CO_2 enrichment		July 21	July 24
	Grain maturity	July 21	July 27

pesticide applications and adequate nutrient supply based on analysis of soil nutrient content (Ca, K, Mg, P, S) determined in early springtime. Table 1 presents important cultivation measures and plant developmental stages of the experiment. In both years, N fertilization was implemented by manual application of calcium ammonium nitrate (CAN, 27% N). The experiment had three N fertilizer levels: severe deficiency (Nd) with 40 kg N ha^{-1} in 2014 and 35 kg N ha^{-1} in 2015, adequate (Nad) with 180 kg N ha^{-1} in 2014 and 200 kg N ha^{-1} in 2015, and excess N supply (Nex) with 320 kg N ha^{-1} per year. The adequate N fertilizer level corresponds to the amount commonly applied in German agriculture. Because of low grain protein concentration in 2014, the last N dose was increased by 20 kg N ha^{-1} in 2015 under Nad. To prevent drought stress plant available volumetric soil water content was kept in the range of 50 up to 90% of field capacity by manual irrigation. Irrigation and precipitation as well as monthly mean temperature and irradiance over the main growth season are presented in Table 2. These data measured at 2 m height near to the field site of the experiment (< 500 m) were provided by the German Weather Service. Volumetric soil water content in the 40 cm soil profile was measured twice a week with time domain reflectometry (TDR) sensors. Irrigation requirements were forecast by means of the AMBAV model (Kersebaum et al., 2005) by the German Weather Service.

Heavy rainfall caused lodging of the plants grown under excess N supply on July 5 in 2015. To minimize lodging effects on grain growth, these plants were erected by means of a rope net until final harvest.

2.4. Analysis of crop growth

Samplings were conducted at 1st node stage, flag leaf stage, anthesis, milk-ripe stage and final harvest at grain maturity. The sampling area was 1.8 m^2 at final harvest and 0.5 m^2 at the four samplings. At each sampling a subsample (25% of the harvest) was separated into stem (including leaf sheath), leaf lamina and ear (if present). The green surface area of these subsample fractions was determined with a leaf area meter (Model LI-3100, LICOR, USA) followed by biomass determination after drying at 105 °C. The remaining sample was dried at 105 °C followed by biomass determination. Green area index of leaf (LAI), stem (SAI) and ear (EAI) and its sum green area index (GAI) were calculated on the basis of the green surface area and biomass of the plant fractions of the subsample and biomass of the main fraction. Stand height was measured at anthesis by putting a polystyrene disc on top of the plant stand and measuring the distance between polystyrene disc and soil surface. In each subplot, stand height was measured at three random positions.

At grain maturity ears from the 1.8 m^2 sampling area were counted, weighed and stored at room temperature before threshing and determination of yield components. After threshing, the grain and chaff fraction were determined by weight and dry weight determined from a 100 g grain and 200 g chaff subsample after drying at 105 °C. Grain number of the 100 g subsample was determined with a seed counter (model CONTADOR, PFEUFFER, Germany). 1000 grain weight (TGW) was calculated by means of the grain number and the subsample biomass. Grain number per m^2 ground area was calculated on the basis of TGW and grain yield.

Plants were regularly examined for fungal infestations. At the milk-ripe stage in 2015, infestations with *Gaeumannomyces graminis* were detected. To overcome this problem, infested plants were sorted out followed by counting the infested and healthy plant fractions. The maximum share of infested plants of a subplot was less than 15%. Yield variables were determined on the basis of the fraction of healthy plants and were then normalized to total plant number (healthy + infested plants).

2.5. Determination of photosynthetic active radiation absorption, radiation use efficiency and harvest index

The fraction of photosynthetic active radiation (PAR) absorbed by the green canopy (f_{ar}) was measured with a line quantum sensor (model SUNSCAN, DELTA-T-DEVICES, USA). Measurements were conducted every week on sunny days from 1st node stage up to grain maturity. Each measurement included the PAR irradiance above the green canopy (I_o), the PAR irradiance below the green canopy (I_c) and the PAR irradiance reflected by the canopy (I_r) and this was repeated three times per plot. Based on the measured parameters, f_{ar} was calculated by the following equation:

$$f_{ar} = \frac{(I_o - I_c - I_r)}{I_o} \quad (2)$$

The f_{ar} value for the days in-between two consecutive measurements were obtained by linear interpolation. Absorbed global radiation accumulated from 1st node stage to grain maturity (AR) was calculated by the sum of the daily values of absorbed global radiation, the product of the daily values of f_{ar} and global radiation.

Radiation use efficiency (RUE) was calculated as the slope of the regression of AR on above-ground biomass. Harvest index was calculated by dividing grain yield by aboveground biomass at grain maturity.

Table 2
Monthly means of temperature, global radiation, precipitation and irrigation over the main growing season in 2014, 2015 and the period between 1980 and 2010.

	Temperature (°C)			Global radiation (W m^{-2})			Precipitation (mm)			Irrigation[1] (mm)	
	2014	2015	1980–2010	2014	2015	1980–2010	2014	2015	1980–2010	2014	2015
March	7.6	5.9	4.3	118	99	97	7	42	51	0	0
April	11.5	8.8	8.9	162	192	162	41	43	43	11	8
May	12.9	12.5	13.4	195	213	210	94	15	52	19	43
June	16.0	15.7	16.0	230	223	223	60	37	68	38	73
July	20.3	19.2	18.2	236	220	213	27	104	63	18	38

[1] Water use differed considerably among plants grown under the different CO_2 and N levels. Therefore, irrigation was adapted to the differing water demands and only the irrigation means over the two CO_2 and three N levels are presented.

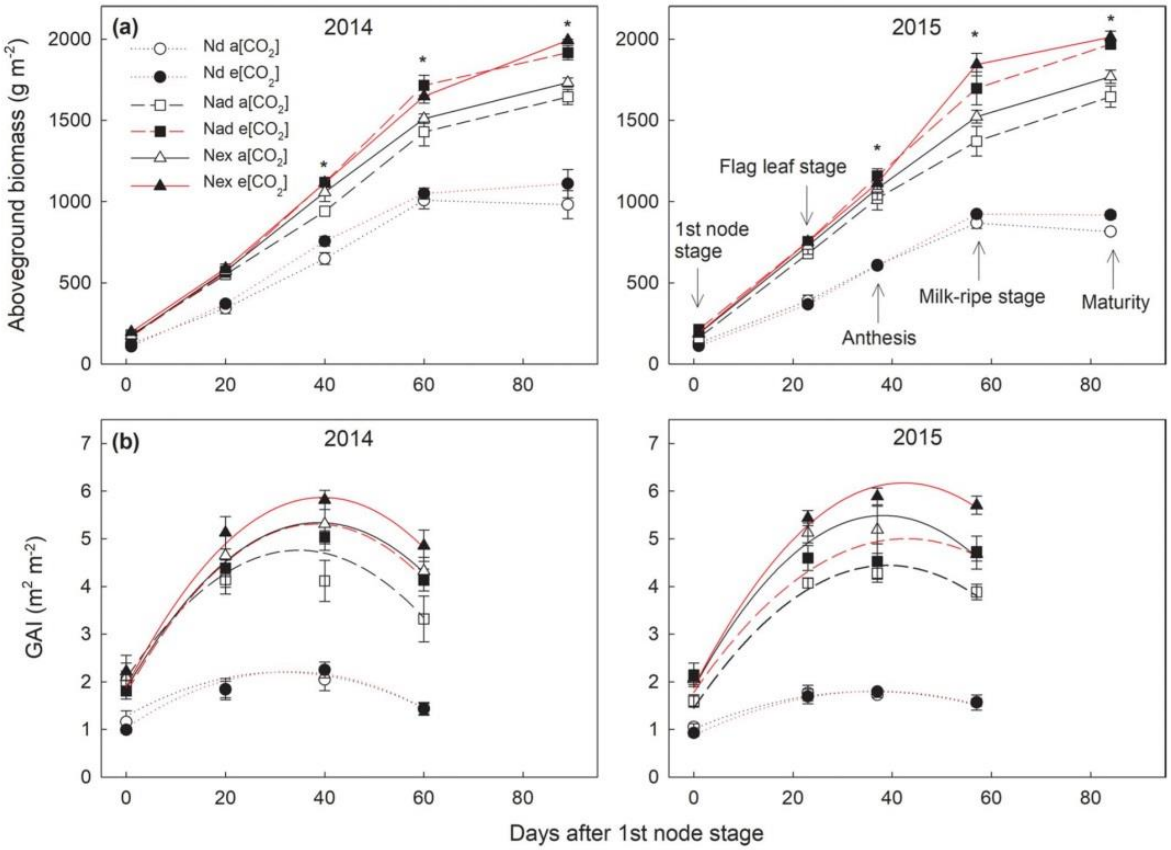

Fig. 1. Time course of aboveground biomass (AGBM) (a) and green area index (GAI) (b) of the two CO_2 and three N levels in 2014 and 2015. Time course of AGBM and GAI is shown from 1st node-stage up to maturity and milk-ripe stage, respectively. Presented are mean values ± standard error (n = 3). Table S1 shows the statistical analysis of the time courses and the asterisks point to the samplings at which significant differences between the marginal CO_2 means (CO_2 means across over all N treatments) ($^*P < 0.05$) were observed. Nd = severe N deficiency; Nad = adequate; and Nex = excess N level. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.6. Determination of specific leaf weight, green leaf N concentration and specific leaf N weight

Specific leaf weight (SLW) was determined as the quotient from biomass and area of the green leaf blade fraction of the harvest subsamples described in Section 2.4. N concentration was determined via grinding dried leaf blades to a fine powder in a rotor mill (Brabender, Germany) followed by N concentration determination using an element analyzer (Leco TruSpec CNS, USA). Specific leaf N weight (SLNW) was determined by multiplying SLW with leaf blade N concentration.

2.7. Statistics

The experiment was analyzed as split plot design with the CO_2 treatment as main plot factor and the N treatment as sub plot factor and the year effect with repeated measurements because the CO_2 and N treatments had the same position on the field in both years. F-tests were carried out with SAS (version 9.4) proc mixed with the following mixed model:

$$y = \mu + CO_2 + N + Y + CO_2\,x\,N + CO_2\,x\,Y + N\,x\,Y + CO_2\,x\,N\,x\,Y + R + R\,x\,N \quad (3)$$

where y is the dependent variable; μ the overall mean; CO_2 the CO_2 effect; N the N fertilizer effect; Y the year effect; CO_2 x N, CO_2 x Y, N x Y and CO_2 x N x Y the interaction effects; R the main plot error and R x N the residual error. To model the variance/covariance between both years of the main plot and residual error, the UN(1) model was used. This model applies no covariance but allows different error variances for each year.

For the investigation of the CO_2 and N effect on aboveground biomass, GAI, SLW, green leaf N concentration and SLNW over the main growing season in each year the year effect of Eq. (3) was replaced by the effect of sampling. To model the variance/covariance of the main plot and residual error, the best model with regard to the lowest Akaike Information Criterion (AIC) value was selected from six candidate models. Table S1 contains a brief description of the used models.

Least square difference (LSD) tests were carried out with SAS (version 9.4) proc glimmix and means were regarded as different if $P < 0.05$.

Analysis of covariance of the effect of CO_2 and leaf area index (LAI) on SLNW was implemented by sequential F-tests with SAS proc mixed and F-tests were adjusted for the effect of LAI and CO_2 to test the CO_2 and LAI effect, respectively.

The relation of SLNW and leaf area index and of grain yield and level of N fertilizer was reported by quadratic regression (SAS proc mixed) with the following mixed model:

$$y = \mu + CO_2 + \gamma_{co_2 1}\,x + \gamma_{co_2 2}\,x^2 + Y + R + R\,x\,N + Y\,x\,R + Y\,x\,R\,x\,N \quad (4)$$

where y is the dependent variable; μ the overall intercept; CO_2 the parameter for the two CO_2 treatments altering the intercept; γ_{CO2} the parameters for the linear and quadratic term of the two CO_2 treatments; x the quantitative covariable; Y the random year effect, R the random main plot error; R x N the random subplot error; Y x R the random year

effect main plot error interaction; and Y x R x N the residual error.

3. Results

3.1. Growing conditions

Monthly mean temperature and global radiation were in the range of normal variation in both years and did hardly differ from the long-term average (Table 2). Nevertheless, monthly average temperature in April 2014 (11.5 °C) was somewhat higher compared to 2015 (8.8 °C) and the long-term average (8.9 °C). The amount of precipitation in May and June was considerably lower in 2015 compared to 2014 and the long-term average, respectively, but this water deficit was compensated by manual irrigation.

3.2. Vegetative growth of winter wheat

Fig. 1 shows the evolution of aboveground biomass (AGBM) and green area index (GAI) over the growing season. Both variables were strongly influenced by the level of N fertilizer, because for AGBM and GAI there were considerable differences between plants grown under severe N deficiency (Nd), adequate (Nad) and excess N supply (Nex). AGBM was significantly increased by e[CO_2] from anthesis onwards in both years, except for Nd in 2015 where a significant increase was only detected at maturity. GAI was increased under e[CO_2] primarily from flag leaf emergence onwards in both years, but this was only significant under Nad and Nex in 2015. Thus, in 2015 the CO_2 x N interaction was significant for AGBM and GAI. Across all N levels, peak GAI values were in the range of 2.0 to 5.8 in 2014 and 1.8 to 5.9 in 2015 and were increased by e[CO_2] by 10 to 22% in 2014 and 4 to 19% in 2015 (Fig. 1).

The increase of AGBM and GAI by e[CO_2] was associated with enhanced stem, ear and leaf growth (Table 3). At anthesis in both years, e[CO_2] significantly increased the variables AGBM, stem biomass, GAI, leaf area index, stem area index and stand height in all N treatments. Ear biomass was significantly increased by e[CO_2] only in plants grown under Nad and ear area index was significantly increased by e[CO_2] in plants grown under Nad and Nex but not under Nd. Averaged over both years, e[CO_2] increased stem biomass by 9, 17 and 5%, ear biomass by 4, 17 and 8%, and leaf biomass by 6, 7 and 5% in plants grown under Nd, Nad and Nex, respectively.

3.3. Wheat growth and yield variables at grain maturity

Table 4 presents the mean values of variables determining growth and yield of winter wheat at maturity. All variables were significantly influenced by the N level as well as experimental year as demonstrated by a significant year effect or N x year interaction (Table S2). AGBM as well as stem and leaf biomass were significantly increased by e[CO_2] in all N treatments in both years. Averaged over both years, AGBM was increased by 13, 18 and 14%, stem biomass by 18, 21 and 14% and leaf biomass by 8%, 17% and 4% in plants grown under Nd, Nad and Nex, respectively.

With respect to yield variables, significant CO_2 x N interactions were detected for ear biomass, grain yield and grain number per m^2 ground area (Table 4). These interactions were because e[CO_2] increased these variables significantly under Nad and Nex, but not under Nd. Averaged over both years, grain yield was greater by 10, 17 and 17%, grain number per m^2 ground area by 8, 12 and 12%, and ear biomass by 10, 17 and 16% in plants grown under the Nd, Nad and Nex level, respectively (Table 4). The rise of grain number per m^2 was due to increased ear number per m^2 ground area, whereas e[CO_2] did not influence grain number per ear. 1000 grain weight was not significantly influenced by e[CO_2].

3.4. Radiation absorption, radiation use efficiency and harvest index

Absorbed radiation accumulated over the main growing season (AR), radiation use efficiency (RUE) and harvest index were significantly influenced by the level of N supply (Table 5). AR increased with rising N supply. RUE was similar under Nad and Nex but significantly higher compared to Nd. Harvest index was highest under Nad, but decreased with increasing and decreasing N level.

e[CO_2] did not significantly influence AR, although e[CO_2] increased AR slightly by 4% in 2014 and 10% in 2015 (Table 5). RUE was significantly increased by e[CO_2] in plants grown under Nad (22% in 2014 and 18% in 2015) and Nex (15% in 2014 and 20% in 2015) but not under Nd. Harvest index was slightly but significantly ($P < 0.01$) decreased by e[CO_2] in plants grown under Nd (-1 in 2014 and -5% in 2015).

3.5. Specific leaf weight, green leaf N concentration and specific leaf N weight

Fig. S1 shows the time course of specific leaf weight (SLW), green leaf N concentration and specific leaf N weight (SLNW) and Table S1 the corresponding statistical analysis. SLW was significantly higher in plants grown under Nd compared to the other N levels, however, SLW was not influenced by e[CO_2]. Green leaf N concentration and SLNW declined strongly with progressing growth stage and decreasing N supply. However, green leaf N concentration and SLNW were only slightly decreased by e[CO_2]. Averaged over all samplings, e[CO_2] decreased green leaf N concentration by −8, −3 and −4% in 2014 and −6, −9 and −3% in 2015 under Nd, Nad and Nex, respectively. SLNW was decreased by −6% under each N treatment in 2014, and by −7, −11, −4% under Nd, Nad and Nex, respectively, in 2015. The reduction of green leaf N concentration and SLNW by e[CO_2] were only significant in 2015.

3.6. Relation between leaf area index and specific leaf N weight

Fig. 2 shows the linear regression between leaf area index (LAI) and SLNW based on data of the harvests taken at 1st node stage, flag leaf stage and anthesis while Table S3 shows the results of analysis of covariance of the CO_2 and LAI effect on SLNW within each year x N combination. The variation of LAI explained a large part of the variation of SLNW ($0.29 \leq r^2 \leq 0.71$) and SLNW declined significantly with increasing LAI in all six CO_2 x N combinations in both years. This decline occurred under relative low SLNW values (1.04 to 2.25 g N m^{-2}) under Nd and under higher SLNW values under Nad and Nex ranging from 1.43 to 3.10 g N m^{-2} (Nad) and from 1.66 to 3.33 g N m^{-2} (Nex). With respect to the CO_2 effect, no significant CO_2 x LAI interaction was detected by means of analysis of covariance, indicating the same slope of the regression lines of ambient [CO_2] and e[CO_2]. SLNW was significantly reduced by e[CO_2] regardless of LAI under Nd in 2015 but not in all other year x N combinations.

3.7. Relation between specific leaf N weight and radiation use efficiency

In both CO_2 treatments, RUE increased with rising SLNW in a quadratic manner (Fig. 3). The slope of the RUE x SLNW regression was highest at low SLNW values and declined under increasing SLNW. The initial increase of RUE with rising SLNW values was markedly higher under e[CO_2] compared to ambient [CO_2] which also means much stronger reduction of RUE with decreasing SLNW under e[CO_2].

4. Discussion

The objective of the present FACE study was to detect interactions between e[CO_2] and widely differing levels of N fertilization on wheat growth and yield as well as on important growth parameters, which are

Table 3
Effect of the two CO_2 and three N levels on wheat growth at anthesis. Shown are mean values (n = 3) and the percentage effect of e[CO_2] (Δ (%)). Different small letters indicate significant differences among the marginal means of the N treatments. Asterisks next to the e[CO_2] symbol indicate that the F-test (Table S2) resulted in a significant CO_2 effect, but no significant CO_2 x N and CO_2 x year interaction. If the F-test resulted in a significant CO_2 x N interaction, then different capital letters indicate significant differences for CO_2 means separate for each N treatment and different small letters show significant differences for N means separate for each CO_2 treatment (letters are in bold for e[CO_2]). All letters refer to the means over both years.

	2014			2015		
	Nd	Nad	Nex	Nd	Nad	Nex
AGBM (g m^{-2})						
a[CO_2]	649 c	941 a	1058 a	609 b	1019 a	1080 a
e[CO_2]*	756 c	1118 a	1116 a	606 b	1158 a	1113 a
Δ (%)	17	19	5	−1	14	3
Stem BM (g m^{-2})						
a[CO_2]	452 c	600 a	644 a	407 b	663 a	670 a
e[CO_2]*	528 c	721 a	690 a	413 b	767 a	693 a
Δ (%)	17	20	7	2	16	3
Leaf BM (g m^{-2})						
a[CO_2]	100 c	188 b	238 a	96 c	187 b	224 a
e[CO_2]	116 c	210 b	237 a	94 c	196 b	240 a
Δ (%)	16	12	0	−3	5	7
Ear BM (g m^{-2})						
a[CO_2]	97 A c	154 B b	176 A a	106 A c	169 B b	186 A a
e[CO_2]	113 A **b**	186 A **a**	189 A **a**	99 A **b**	195 A **a**	203 A **a**
Δ (%)	16	21	7	−7	15	9
GAI (m^2 m^{-2})						
a[CO_2]	2.04 c	4.12 b	5.31 a	1.73 c	4.28 b	5.19 a
e[CO_2]*	2.25 c	5.03 b	5.82 a	1.79 c	4.52 b	5.89 a
Δ (%)	10	22	10	4.	6	13
LAI (m^2 m^{-2})						
a[CO_2]	1.21 c	2.61 b	3.46 a	1.03 c	2.90 b	3.49 a
e[CO_2]	1.38 c	3.35 b	3.95 a	1.10 c	2.96 b	3.93 a
Δ (%)	15	28	14	6	2	12
SAI (m^2 m^{-2})						
a[CO_2]	0.65 c	1.17 b	1.46 a	0.52 c	1.08 b	1.35 a
e[CO_2]*	0.66 c	1.30 b	1.45 a	0.54 c	1.23 b	1.55 a
Δ (%)	1	11	−1	3	14	15
EAI (m^2 m^{-2})						
a[CO_2]	0.185 A c	0.328 B b	0.398 B a	0.172 A c	0.294 B b	0.352 B a
e[CO_2]	0.203 A **c**	0.382 A **b**	0.422 A **a**	0.162 A **c**	0.337 A **b**	0.389 A **a**
Δ (%)	10	16	6	−6	15	11
Stand height (m)						
a[CO_2]	0.812 c	0.950 b	0.980 a	0.706 c	0.866 b	0.908 a
e[CO_2]*	0.863 c	0.983 b	1.02 a	0.760 c	0.934 b	0.959 a
Δ (%)	6	4	4	8	8	6

also used in crop growth models (i.e. absorbed radiation by the green canopy accumulated over the growing season (AR), radiation use efficiency (RUE) and harvest index). Also, the CO_2 x N interaction on green leaf N concentration, which influences photosynthesis and RUE (Sinclair and Horie, 1989) and is altered by N supply and e[CO_2] (Kimball et al., 2002), was analyzed.

4.1. Biomass production and grain yield

Starting from anthesis aboveground biomass (AGBM) was increased by e[CO_2]. From the 1st node stage up to flag leaf emergence, average air temperature was low (below 12 °C) but then it exceeded 15 °C. This coincidence of growth stimulation by e[CO_2] with higher temperature is consistent with other experimental data (Shimono et al., 2008) and the theoretical prediction that e[CO_2] increases photosynthesis only when temperatures exceed 10 °C (Long, 1991).

The increase of AGBM by e[CO_2] at grain maturity (13, 18 and 14% under Nd, Nad and Nex) is in agreement with other FACE studies. For example, if the e[CO_2] effect is recalculated for the [CO_2] increase of 210 ppm, then AGBM in spring wheat was increased under adequate N supply by 17% (Högy et al., 2009) as well as by 16–19% in winter wheat (Kou et al., 2007; Weigel and Manderscheid, 2012). For winter wheat grown under moderate N deficiency, an increase of 9 and 14% was found in FACE studies in China (Kou et al., 2007) and on the same field site of the present study (Weigel and Manderscheid, 2012). Increases of 6% (severe N deficiency) and 9% (ample N supply) were reported by Kimball et al. (2002) for spring wheat in Arizona and an increase of 28% was reported for winter wheat grown under ample N supply in China (Ma et al., 2007).

The growth data at anthesis and grain maturity show that while stem and ear growth were similarly enhanced by e[CO_2] this effect was larger than the stimulation of leaf growth. These results are in agreement with other FACE studies (Liu et al., 2008; Manderscheid et al., 2009) and are probably due to the different temperature conditions at these growth stages and the resulting CO_2 x temperature interaction on photosynthesis (Long, 1991).

In the present study, grain yield under ambient [CO_2] and adequate N supply corresponded to empirical data for the local wheat growing area and was higher compared to other FACE studies (Fig. 4), whereas the relative and absolute yield reduction by N deficiency was much stronger than in other studies. The significant N x year interaction on grain number might be partly explained by different climatic conditions. The photothermal quotient 30 days before anthesis that is important to determine grain number (Fischer, 1985) was about 10%

Table 4
Effect of the two CO_2 and three N levels on growth and yield variables at maturity. Shown are mean values (n = 3) and the percentage effect of e[CO_2] (Δ (%)). Different small letters indicate significant differences among the marginal means of the N levels. Asterisks next to the e[CO_2] symbol indicate that the F-test (Table S2) resulted in a significant CO_2 effect, but no significant CO_2 x N and CO_2 x year interaction. If the F-test resulted in a significant CO_2 x N interaction, then different capital letters indicate significant differences for CO_2 means separate for each N treatment and different small letters show significant differences for N means separate for each CO_2 treatment (letters are in bold for e[CO_2]). All letters refer to the means over both years.

	2014			2015		
	Nd	Nad	Nex	Nd	Nad	Nex
AGBM (g m^{-2})						
a[CO_2]	981 B c	1644 B b	1732 B a	816 B c	1646 B b	1769 B a
e[CO_2]	1110 A **b**	1917 A **a**	1993 A **a**	918 A **b**	1968 A **a**	2012 A **a**
Δ (%)	13	17	15	13	20	14
Stem BM (g m^{-2})						
a[CO_2]	340 B c	553 B b	598 B a	265 B c	541 B b	616 B a
e[CO_2]	400 A **b**	657 A **a**	680 A **a**	316 A **b**	666 A **a**	704 A **a**
Δ (%)	18	19	14	19	23	14
Leaf BM (g m^{-2})						
a[CO_2]	68 c	112 b	142 a	46 c	97 b	123 a
e[CO_2]*	68 c	125 b	144 a	54 c	118 b	132 a
Δ (%)	1	12	2	18	22	8
Ear BM (g m^{-2})						
a[CO_2]	574 A b	979 B a	993 B a	505 A b	1008 B a	1030 B a
e[CO_2]	642 A **b**	1134 A **a**	1169 A **a**	548 A **b**	1185 A **a**	1175 A **a**
Δ (%)	12	16	18	9	18	14
Grain yield (g m^{-2})						
a[CO_2]	472 A b	817 B a	818 B a	417 A b	831 B a	829 B a
e[CO_2]	528 A **b**	949 A **a**	971 A **a**	446 A **b**	973 A **a**	948 A **a**
Δ (%)	12	16	19	7	17	14
Grain number (m^{-2})						
a[CO_2]	10248 A c	17827 B b	18977 B a	9342 A c	19236 B b	21873 B a
e[CO_2]	11488 A **c**	20032 A **b**	21416 A **a**	9691 A **c**	21607 A **b**	24254 A **a**
Δ (%)	12	12	13	4	12	11
Ear number (m^{-2})						
a[CO_2]	314 c	430 b	451 a	292 c	420 b	529 a
e[CO_2]*	323 c	461 b	502 a	313 c	476 b	559 a
Δ (%)	3	7	11	7	13	6
Grain number (ear^{-1})						
a[CO_2]	32.8 b	41.5 a	42.8 a	32.0 b	45.8 a	41.4 a
e[CO_2]	35.4 b	43.5 a	42.7 a	31.0 b	45.4 a	43.5 a
Δ (%)	8	5	0	−3	−1	5
TGW (g)						
a[CO_2]	46.1 a	45.9 a	43.1 b	44.7 a	43.2 a	37.9 b
e[CO_2]	45.9 a	47.4 a	45.4 b	46.1 a	45.0 a	39.1 b
Δ (%)	0	3	5	3	4	3

Table 5
Effect of the two CO_2 treatments and three N levels on radiation absorption accumulated from 1st node stage up to maturity (AR), radiation use efficiency (RUE) and harvest index. Presented are mean values (n = 3) and the percentage effect of e[CO_2] (Δ (%)). Different small letters indicate significant differences among the marginal means of the N levels. If the F-test (Table S2) resulted in a significant CO_2 x N interaction, then different capital letters indicate significant differences for CO_2 means separate for each N treatment and different small letters show significant differences for N means separate for each CO_2 treatment (letters are in bold for e[CO_2]). All letters refer to the means over both years.

	2014			2015		
	Nd	Nad	Nex	Nd	Nad	Nex
AR (MJ m^{-2})						
a[CO_2]	826 c	1205 b	1293 a	682 c	1141 b	1293 a
e[CO_2]	863 c	1216 b	1295 a	748 c	1160 b	1296 a
Δ (%)	4	1	0	10	2	0
RUE (g MJ^{-1})						
a[CO_2]	1.09 A b	1.21 B a	1.21 B a	1.08 A b	1.29 B a	1.24 B a
e[CO_2]	1.17 A **b**	1.47 A **a**	1.39 A **a**	1.12 A **b**	1.52 A **a**	1.48 A **a**
Δ (%)	8	22	15	4	18	20
Harvest index						
a[CO_2]	0.481 A a	0.497 A a	0.472 A b	0.511 A a	0.505 A a	0.469 A b
e[CO_2]	0.475 B **b**	0.495 A **a**	0.487 A **b**	0.487 B **b**	0.494 A **a**	0.471 A **b**
Δ (%)	−1	0	3	−5	−2	1

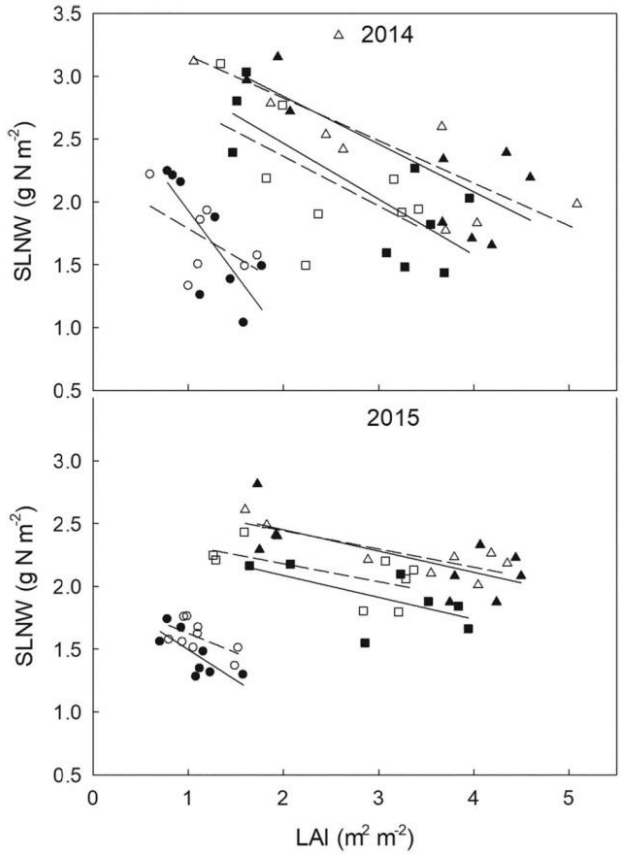

Fig. 2. Linear regression of leaf area index (LAI) on specific leaf N weight (SLNW) among the values of the harvests taken at 1st node stage, flag leaf stage and anthesis in 2014 (top) : Nd and a[CO_2] (○) $r^2 = 0.29$, $P = 0.21$; Nd and e[CO_2] (●) $r^2 = 0.61$, $P < 0.05$; Nad and a[CO_2] (□) $r^2 = 0.33$, $P = 0.14$; Nad and e[CO_2] (■) $r^2 = 0.61$, $P < 0.01$; Nex and a[CO_2] (Δ) $r^2 = 0.58$, $P < 0.05$; Nex and e[CO_2] (▲) $r^2 = 0.58$, $P < 0.01$; and in 2015 (bottom): Nd and a[CO_2] (○) $r^2 = 0.36$, $P < 0.10$; Nd and e[CO_2] (●) $r^2 = 0.56$, $P < 0.05$; Nad and a[CO_2] (□) $r^2 = 0.38$, $P = 0.11$; Nad and e[CO_2] (■) $r^2 = 0.38$, $P = 0.14$; Nex and a[CO_2] (Δ) $r^2 = 0.71$, $P < 0.01$; Nex and e[CO_2] (▲) $r^2 = 0.48$, $P < 0.05$.

larger in 2015 compared to 2014 (data not shown) and thus was possibly responsible for the larger grain number under adequate (8%) and excess N supply (14%) in 2015. The grain yield stimulation by e[CO_2] in the present study (10, 17 and 17% under Nd, Nad and Nex) is in agreement with other FACE studies (Kimball et al., 2002; Weigel and Manderscheid, 2012; Han et al., 2015). However, higher yield stimulation up to 35% and 47% were found at FACE sites in semi-arid regions in Australia (Tausz-Posch et al., 2012, 2015) and China (Lam et al., 2012), respectively where N availability was unlimiting.

The regression of N fertilizer level on grain yield (Fig. 5) suggests that the yield stimulation by e[CO_2] in the present study increased with N supply up to a certain N amount. This is in line with the two-year Arizona FACE study, where e[CO_2] hardly increased grain yield (5%) under severe deficiency (15 kg N ha^{-1}) in the 2nd year, but the yield increase by e[CO_2] was considerably stronger (12%) under less severe N deficiency (70 kg N ha^{-1}) in the 1st year (Kimball et al., 2002).

4.2. Radiation absorption and radiation use efficiency

Green area index (GAI) and AR were markedly influenced by the N level as was observed in many other studies (e.g. Garcia et al., 1988; Sieling et al., 2016). Previous FACE experiments with wheat reported various effects of e[CO_2] on the size of GAI: a decrease under N deficiency (Brooks et al., 2000; Jamieson et al., 2000), an increase and

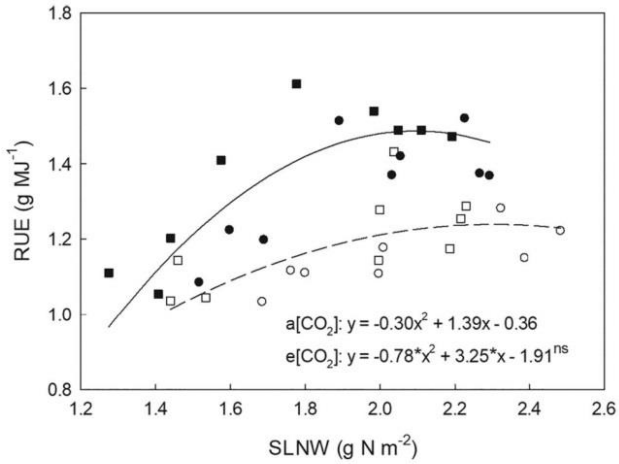

Fig. 3. Regression of radiation use efficiency (RUE) on specific N weight of green leaves (SLNW). To implement this regression the means of SLNW over the three and four harvests in 2014 and 2015, respectively were used. The circles and squares indicate the data of 2014 and 2015, respectively. Open symbols and dashed line = ambient [CO_2] and closed symbols and solid line = e[CO_2]. The equations describe the regression curves, where y = RUE and x = SLNW. Asterisks indicate significant differences ($P < 0.05$) between the parameters of the ambient [CO_2] and e[CO_2] treatment.

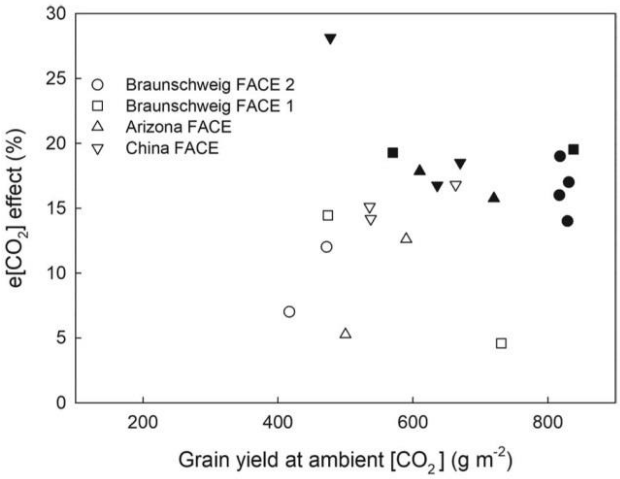

Fig. 4. Overview of the effect of e[CO_2] on grain yield of different FACE studies with several N fertilization levels and sufficient water supply. The percentage effect of e[CO_2] was normalized to the [CO_2] increase of 210 ppm. White symbols = N deficiency and black symbols = ample N supply. The data sources are the following: Braunschweig FACE 2 = data of the present study; Braunschweig FACE 1 = Weigel and Manderscheid (2012); Arizona FACE = Jamieson et al. (2000) and Kimball et al. (2002); China FACE = Kou et al. (2007)*, Ma et al. (2007)* and Han et al. (2015). *Grain yield was calculated from biomass, assuming a harvest index of 0.5.

decrease under adequate N supply (Bunce, 2016; Cai et al., 2016), and an increase under excess N supply (Brooks et al., 2000; Jamieson et al., 2000). In the present study, GAI was increased by e[CO_2] under all N levels, while AR was not influenced, but tended to be increased by e[CO_2] under N deficiency. For the adequate and excess N level, the increases in GAI under e[CO_2] became apparent only after flag leaf emergence when the soil was already completely covered by the canopy so that the rise of GAI hardly altered AR but increased light attenuation within the canopy. However, under severe N deficiency where GAI was very low (≤2.2) the slight increase of GAI under e[CO_2] resulted in a slight increase of AR. These results are in contrast with those obtained in the Arizona FACE experiment where GAI was much higher (≤3.8)

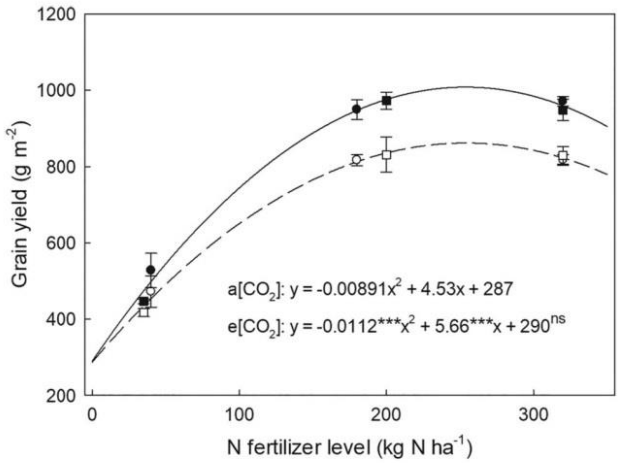

Fig. 5. Regression of grain yield on total amount of N fertilizer. Presented are the mean values (n = 3; ± S.E.M) and corresponding regression curves for the ambient [CO_2] (dashed line) and e[CO_2] treatment (solid line). Circles and squares indicate the data of 2014 and 2015, respectively. Open symbols = ambient [CO_2] and closed symbols = e[CO_2]. The integrated equations describe the regression curves, whereby y = grain yield and x = level of N fertilizer. Asterisks indicate significant differences ($P < 0.001$) between the parameters of the ambient [CO_2] and e[CO_2] treatment.

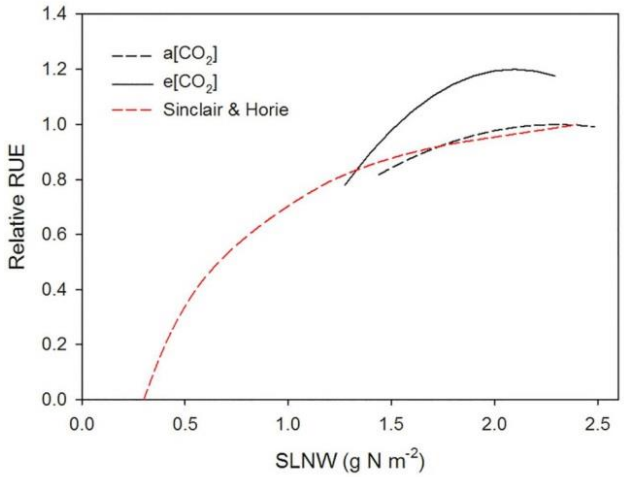

Fig. 6. Relation between relative radiation use efficiency (RUE) and specific N weight of green leaves (SLNW). The ambient [CO_2] (a[CO_2]) and e[CO_2] curves were derived from the regression of RUE on SLNW (Fig. 3). Additionally, the rice data of the relation between RUE and SLNW of Sinclair and Horie (1989) were included. Relative RUE was derived by normalization with the maximum RUE at ambient [CO_2] and of Sinclair and Horie (1989), respectively.

under comparable severe N deficiency (70 and 15 kg N ha^{-1}) and e[CO_2] reduced both the size and duration of GAI and thus AR, leading to the reduction of grain yield stimulation by e[CO_2] (Jamieson et al., 2000).

Photosynthetic capacity and RUE are closely associated variables that are stable over a broad range of leaf and soil N levels, but decline sharply when these become very low (Sinclair and Horie, 1989). In the present study, RUE did not differ between plants grown under adequate and excess N supply but declined under severe N deficiency, which is in agreement with other studies (e.g. Garcia et al., 1988; Sieling et al., 2016) and suggests that photosynthetic capacity was also reduced under N deficiency. RUE was strongly increased by e[CO_2] by 20% under adequate and 18% under excess N supply, which is consistent with the value (17%) used in the SIRIUS growth model (Jamieson et al., 2000), but hardly under severe N deficiency (6%).

Fig. 6 compares the relationship between RUE and SLNW of the present study and the one of Sinclair and Horie (1989). At ambient [CO_2], there was a good correspondence of relative RUE in the overlaying SLNW area where RUE was hardly affected by decreasing SLNW. However, at e[CO_2] RUE declined significantly stronger with decreasing SLNW compared to ambient [CO_2]. Thus, crop models using RUE to calculate biomass production should include the different dependence of RUE on SLNW under ambient [CO_2] and e[CO_2].

The CO_2 x N interaction on RUE of the present study indicates that the smaller yield stimulation by e[CO_2] under N deficiency compared to high N was associated with a smaller effect of e[CO_2] on RUE, but not on AR as was observed in the Arizona FACE experiment (Jamieson et al., 2000).

4.3. Green leaf N concentration

In the Arizona FACE study a strong decline in leaf N concentration by e[CO_2] was detected under severe N deficiency (Sinclair et al., 2000) which was on average −25% in the 1st and −19% in the 2nd year, but leaf N concentration was not affected under ample N supply (Kimball et al., 2002). Based on those and other results of several enclosure studies (Stitt and Krapp, 1999), it was concluded that downregulation of photosynthetic capacity by e[CO_2] in wheat primarily occurs under N deficiency (Wall et al., 2000; Rubio-Asensio and Bloom, 2016). However, in contrast to Sinclair et al. (2000), but in agreement with Tausz et al. (2017), in the present study the reduction of leaf N concentration was very similar among N levels with values of −7, −6, −4% under severe N deficiency, adequate and excess N supply, respectively. Because specific leaf weight (SLW) was not influenced by e[CO_2] in the present study, the reduction of SLNW corresponded to the reduction of leaf N concentration.

SLNW decreases within the canopy from the top to the bottom because of light attenuation (Hirose, 2005; Bertheloot et al., 2008). In the present study, the effect of e[CO_2] on LAI corresponded to the one on GAI with an increase from flag leaf emergence under all N levels in 2014 and a general increase under adequate and excess N supply in 2015 (data not shown). In the present study, SLNW decreased with increasing LAI and this relation was not influenced by e[CO_2] under adequate and excess N supply, but only under severe N deficiency. These results suggest that the decline in SLNW under e[CO_2] and high N supply was primarily due to the increase of GAI and resulting adaption of the leaves down in the canopy to higher light attenuation under e[CO_2]. Nie et al. (1995) reported similar results based on a FACE study with ample N supply where leaf RuBisCO content was not declined by e[CO_2] until LAI was increased by e[CO_2] (Kimball et al., 1995). Also, another FACE study showed no influence of e[CO_2] on the relation between LAI and leaf N content per m^2 ground area under ample N supply (Cai et al., 2016). The herein observed reduction of SLNW under e[CO_2] regardless of the effect of LAI under severe N deficiency suggests that a direct e[CO_2] induced reduction of leaf N concentration occurs in field grown wheat primarily only under very low soil N availability.

4.4. Mechanism of the lower RUE associated yield stimulation of e[CO_2] under severe N deficiency

As shown above, the stimulation of RUE as well as ear growth and related variables (grain number and grain yield) of e[CO_2] were lower under severe N deficiency compared to high N supply. These CO_2 x N interactions might be partly explained by the fact that the small reduction of SLNW by e[CO_2] under severe N deficiency brought about a reduction in photosynthetic capacity. However, there is indication that insufficient N supply for ear growth was also involved. The regression of SLNW on RUE (Fig. 3) shows that RUE was elevated under e[CO_2] but declined stronger with decreasing SLNW under e[CO_2] compared to ambient [CO_2], which is indicative of increasing N shortage with

respect to plant growth under rising N deficiency. Given that RuBisCO content would only limit RUE stimulation under e[CO_2] one would rather expect parallel curves for both CO_2 treatments. Moreover, in the present study, harvest index was reduced by e[CO_2] under severe N deficiency because of the strong stimulation of stem growth by e[CO_2] and the CO_2 x N interaction on ear growth and related variables. According to van Kraalingen (1990), additional assimilates from growth under e[CO_2] are primarily allocated to organs with low N contents when soil N supply is deficient. Because ear N concentration is about twice as much than the one of stem, this concept might explain the decrease of harvest index. This decrease of harvest index indicates that under e[CO_2] severe N deficiency induced an imbalance between photosynthetic capacity (source) and ear growth (sink capacity), where the latter seems to be more restrictive with respect to the grain yield stimulation by e[CO_2]. The competiton for N between stems and ears could be mitigated by changing the temporal pattern of N fertilization by adding more fertilizer around booting and less before this stage. However, as water availability is critical for the effect of N fertilization but is often strongly limited in developing countries, this strategy could there not be readily feasible.

In conclusion, the present field study shows that only a small grain yield stimulation by e[CO_2] can be expected for wheat grown under severe N deficiency but adequate water supply. Moreover, Walker et al. (2017) showed that grain yield stimulation was not present at all under N deficiency and limited water availability. Hence, CO_2 fertilization might not be an important factor in counteracting negative climate change effects on wheat yields primarily in developing countries where high N fertilizer levels are hardly affordable. This reduced yield reaction to e[CO_2] was not due to a decreased AR caused by accelerated canopy senescence under e[CO_2] as was observed in the Arizona FACE study (Jamieson et al., 2000). Rather, it was due to a decreased RUE stimulation later in the season, which was attributed to a deficiency of N for ear growth and a reduction of photosynthetic capacity by e[CO_2] where the former seemed to have a larger impact. It remains to be investigated whether grain yield stimulation by e[CO_2] under severe N deficiency can be increased by adjusting the timing of N fertilization, i.e. applying more fertilizer at booting and less before.

Conflict of interest

None of the authors has any conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.eja.2018.08.007.

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2.6 Anhang

Table S1: Result of the repeated measurements analysis of the effect of the two CO_2 and three N treatments on aboveground biomass (AGBM) and green area index (GAI) as well as specific weight (SLW), N concentration (N conc GL) and specific N weight of green leaves (SLNW).

Variable	Year	Covariance model	CO_2	N	Harvest	CO_2 x N	CO_2 x H	N x H	CO_2 x N x H
AGBM (g m^{-2})	2014	CSH	**	***	***	ns	**	***	ns
	2015	ARH(1)	**	***	***	**	*	***	ns
GAI (m^2 m^{-2})	2014	ARH(1)	ns	***	***	ns	ns	***	ns
	2015	CS	**	***	***	**	ns	***	ns
SLW (g m^{-2})	2014	CSH	ns	**	ns	ns	ns	ns	ns
	2015	AR(1)	ns	***	**	ns	ns	*	ns
N conc GL (mg g^{-1})	2014	ARH(1)	ns	***	***	ns	ns	*	ns
	2015	UN(1)	*	***	***	ns	ns	**	ns
SLNW (g m^{-2})	2014	CSH	ns	***	***	ns	ns	ns	ns
	2015	AR(1)	(*)	***	***	ns	ns	*	ns

AR(1): Correlation among sampling time of the main and residual error drops with time lag; error variances are the same for each sampling

ARH(1): Correlation among sampling time of the main and residual error drops with time lag; error variances are different for each sampling

CS: Correlation among sampling time of the main and residual error is independent of time lag; error variances are the same for each sampling

CSH: Correlation among sampling time of the main and residual error is independent of time lag; error variances are different for each sampling

UN(1): No correlation among sampling time; error variances are different for each sampling

$^{(*)}P < 0.1$; $^{*}P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$.

Table S2: F-test result of the effect of the two CO_2 and three N levels on growth and yield variables at anthesis and maturity, and on important yield determinants. Aboveground biomass = AGBM; green area index = GAI; stem area index = SAI; leaf area index = LAI; ear area index = EAI; 1000 grain weight = TGW; absorbed PAR accumulated from 1st node stage up to maturity = AR; radiation use efficiency =RUE.

Variable	CO_2	N	Year	CO_2 x N	CO_2 x Y	N x Y	CO_2 x N x Y
Growth up to anthesis							
AGBM (g m^{-2})	*	***	ns	ns	ns	ns	ns
Stem BM (g m^{-2})	**[1]	***[1]	ns[1]	ns[1]	ns[1]	**[1]	ns[1]
Leaf BM (g m^{-2})	ns	***	ns	ns	ns	ns	ns
Ear BM (g m^{-2})	**	***	(*)	(*)	ns	ns	ns
GAI (m^2 m^{-2})	*	***	ns	ns	ns	ns	ns
SAI (m^2 m^{-2})	(*)	***	ns	ns	ns	ns	ns
LAI (m^2 m^{-2})	*	***	ns	ns	ns	ns	ns
EAI (m^2 m^{-2})	**	***	***	(*)	ns	ns	ns
Stand height (m)	***	***	***	ns	ns	(*)	ns
Growth up to maturity							
AGBM (g m^{-2})	**	***	ns	*	ns	**	ns
Stem BM (g m^{-2})	***	***	(*)	*	ns	***	ns
Leaf BM (g m^{-2})	**	***	***	ns	ns	ns	ns
Ear BM (g m^{-2})	**	***	ns	*	ns	*	ns
Grain yield (g m^{-2})	**	***	ns	*	ns	(*)	ns
Grain number (m^{-2})	**	***	(*)	(*)	ns	***	ns
Ear number (m^{-2})	*	***	ns	ns	ns	*	ns
Grain number (ear^{-1})	ns	***	ns	ns	ns	*	ns
TGW (g)	ns	***	**	ns	ns	**	ns
Yield determinants							
AR (MJ m^{-2})	ns	***	*	ns	ns	*	ns
RUE (g MJ^{-1})	**	***	ns	**	ns	ns	ns
Harvest index	ns	***	ns	**	ns	**	ns

$^{(*)}P < 0.1$; $^{*}P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$.

The index 1 indicates log-transformation of the respective variable to comply with variance homogeneity of the residual error

Table S3: Result of analysis of covariance of the effect of the two CO_2 levels and LAI on SLNW. Analysis was conducted separately for each year x N level combination.

		Effect		
Year	N level	CO_2	LAI	CO_2 x LAI
2014	Nd	ns	**	ns
	Nad	ns	***	ns
	Nex	ns	**	ns
2015	Nd	*	**	ns
	Nad	ns	*	ns
	Nex	ns	**	ns

$^{*}P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$.

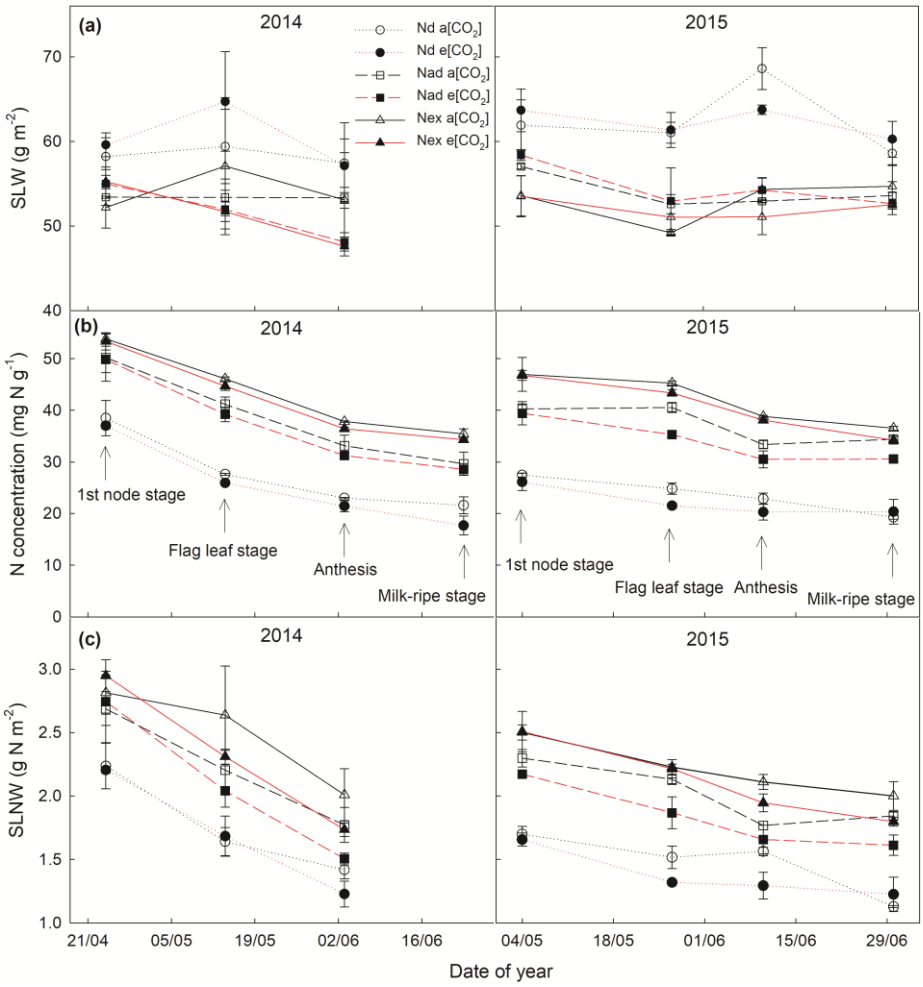

Fig. S1: Effect of the two CO_2 and three N treatments on specific weight (SLW) (a), N concentration (b) and specific N weight (SLNW) (c) of green leaves from the 1st node stage up to the milk-ripe stage. Presented are mean values (n=3; ±S.E.M) and the statistical analysis is shown in **Table S1**. Nd = severe N deficiency; Nad = adequate; and Nex = excess N level. Note that no measurement of SLW was conducted at the milk-ripe stage in 2014 and thus SLNW was not calculated.

3. Kapitel: Wirkung von e[CO_2] auf N-Aneignung, N-Remobilisierung und postanthetische N-Aufnahme

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Positive effects of free air CO_2 enrichment on N remobilization and post-anthesis N uptake in winter wheat

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ABSTRACT

Elevated atmospheric CO_2 concentration (e[CO_2]) often increases cereal yield, but can also decrease vegetative and grain tissue nitrogen (N) concentration that might affect future food and feed quality. However, data about CO_2 x N interactions on key processes determining grain N yield and concentration, which are remobilization of vegetative N taken up before anthesis (Nrem) and post-anthesis N uptake (Nabs), are scarce. Therefore, a two-year Free Air CO_2 Enrichment (FACE) experiment was conducted with winter wheat grown under two CO_2 (~393 and 600 ppm) and three N levels (severe deficiency with N nutrition index (NNI) of 0.4, adequate with NNI of 0.8 and excess with NNI of 1.1).

e[CO_2] did not influence the allometric relation between aboveground N concentration and biomass up to anthesis. At anthesis, e[CO_2] increased N acquisition of stem and ear, but not of leaf. Correspondingly, e[CO_2] increased Nrem of stem and chaff. Moreover, e[CO_2] enhanced the efficiency of Nrem of stem and aboveground plant in the first year, indicating increased N mobilization from vegetative tissue. Nabs tended to be increased by e[CO_2], especially in the second year. Finally, e[CO_2] increased grain N yield (8 to 12%), N use efficiency (13 to 18%) and N uptake efficiency (10 to 12%). Grain N concentration was slightly decreased by e[CO_2] in both years (-1 to -6%), while grain N concentration was considerably larger (9 to 19%) in the second year compared to the first year. There was a strong linear relation between grain N yield and grain number (r^2 = 0.98) that was not influenced by e[CO_2], suggesting grain number as important factor determining the grain N yield increase under e[CO_2]. Grain N concentration was more strongly affected by e[CO_2] than mean N content per grain.

1. Introduction

Wheat (*Triticum aestivum* L.) is an important basic food of a large part of the world population, with annual production has increased during the last half-century (Simoni, 2009) and is expected to further rise in future (Shewry and Hey, 2015). The atmospheric CO_2 concentration is predicted to continue to rise from current 408 to 730–1020 ppm by the end of this century (IPCC, 2013). Elevated atmospheric CO_2 concentration (e[CO_2]) stimulates photosynthesis in C_3 crops, which is often associated with biomass and yield increases (Ainsworth and Long, 2005). However, these positive e[CO_2] effects are often accompanied by a decrease of tissue nitrogen (N) concentration. For wheat, meta-analyses found reductions of -9 to -16% of vegetative tissue (Cotrufo et al., 1998; Wang et al., 2013) and -6 to -16% of grain (Taub et al., 2008; Myers et al., 2014). Moreover, a modelling study, taking climate change adaptions concerning grain yield into account, suggested global mean decrease of grain protein concentration of -9% under e[CO_2] in 2050 (Asseng et al., 2019). There is concern that those reductions will result in poor food (Myers et al., 2014), feed (Sinclair et al., 2000) and baking quality (Wieser et al., 2008; Panozzo et al., 2014).

In wheat, the highest proportion of grain N originates from remobilization of N acquired by the vegetative organs before anthesis to the grains during grain filling (Barbottin et al., 2005). Leaf and stem are the most important N sources of N remobilization (Nrem), contributing about 75% of the N originating from Nrem, whereas the residual share of Nrem is provided by chaff and roots (Gaju et al., 2014). Another important N source of the grains is post-anthesis N uptake (Nabs). Depending on environmental conditions, 40 to 90% of the grain N originates from Nrem and thus 10 to 60% from Nabs (Kong et al., 2016). Nabs depends on soil mineral N availability at early grain filling

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(Bancal, 2009). Many studies (e.g. Ma et al., 1995; Jamieson and Semenov, 2000) indicated that grain N acquisition is determined by the N source, i.e. N acquisition before anthesis and soil N availability at early grain filling. However, a strong inverse relationship between Nrem and Nabs exists (Barbottin et al., 2005; Kong et al., 2016), indicating a certain degree of sink regulation of grain N acquisition. According to Martre et al. (2003), accumulation of structural and metabolic proteins, making up about 30% of the grain protein in wheat, is sink regulated, whereas accumulation of storage protein (gluten) is source regulated. In addition to grain N acquisition, individual grain growth, i.e. starch acquisition, can be important in determining grain N concentration. The processes controlling grain N and starch acquisition act partly independently (Jenner et al., 1991).

The mechanism by which e[CO_2] decreases tissue N concentration is still elusive. Several mechanisms have been hypothesized, which could occur simultaneously or interact with each other. A hypothesis is growth dilution, implying that plant N acquisition is not directly influenced by e[CO_2] but cannot fully keep pace with the enhanced growth under e[CO_2] (Loladze, 2002). This hypothesis implies increased N acquisition to some extent in plants grown under e[CO_2], which was observed in wheat in several Free Air CO_2 Enrichment (FACE) experiments (e.g. Han et al., 2015; Cai et al., 2016; Tausz et al., 2017). However, Pleijel and Uddling (2012) and Feng et al. (2015) found that e[CO_2] can also decrease tissue N concentration under conditions of no growth stimulation by e[CO_2]. A potential mechanism for the decrease of N concentration is inhibition of NO_3^- assimilation by e[CO_2] (Bloom et al., 2010), which could explain a decrease of N concentration without enhanced growth. Another hypothesis is that e[CO_2] decreases leaf N concentration because of downregulation of ribulose-1,5-bisphosphat carboxylase/oxygenase (RuBisCO) gene expression, especially under N deficiency (Stitt and Krapp, 1999).

There are also other e[CO_2] induced processes that could enhance N acquisition and thus tissue N concentration. These include stimulation of root growth (Pacholski et al., 2015) and increase of whole-crop N sink strength (Feng et al., 2015).

Plant N concentration declines as biomass increases during crop development because of shading effects and change of the leaf: stem ratio (Justes et al., 1994; Sadras and Lemaire, 2014). Therefore, the decrease of N concentration under e[CO_2] can be, at least partly, explained by increased biomass according to the negative allometric relation between N concentration and biomass. Some crop models assume a reduction of critical N concentration, i.e. the concentration that is required to ensure potential production, when wheat is grown under e[CO_2] (Vanuytrecht and Thorburn, 2017). However, few experimental studies (e.g. Coleman et al., 1993) have considered the allometry between N concentration and biomass in their comparison of the effect of CO_2 on N concentration.

It was proposed that a decrease of tissue N concentration by e[CO_2] at anthesis results in a decrease of Nrem (Rubio-Asensio and Bloom, 2016). In line with this, the extent of decrease of leaf and grain N concentration by e[CO_2] was strongly correlated in a FACE study (Kimball et al., 2001, 2002). It is also possible that while Nrem is unaffected the efficiency of Nrem, which is the proportion of the N taken up before anthesis that is remobilized, is decreased under e[CO_2]. This might occur when e[CO_2] enhances tillering (Cai et al., 2016), but decreases Nrem per tiller. However, regarding Nrem, processes possibly exist that could compensate for reduced vegetative tissue N concentration under e[CO_2]. For instance, studies showed that e[CO_2] enhances the grain N sink strength (Lam et al., 2012) and under ambient [CO_2] a large amount of N that is potentially available for Nrem, i.e. that is not structural N, remains in vegetative tissue at maturity (Pask et al., 2012).

Studies found that canopy senescence was accelerated under e[CO_2] at grain filling (Osborne et al., 1998; Brooks et al., 2000; Fangmeier et al., 2000) and thus, the time interval of Nabs might be reduced under e[CO_2]. Moreover, Nabs could be reduced by e[CO_2] because of inhibition of NO_3^- assimilation (Bloom et al., 2010), even if indication of such inhibition was not found in the present experiment (Dier et al., 2018a).

The decrease in tissue N concentration by e[CO_2] is stronger under low compared to high N supply in wheat (Kimball et al., 2002; Taub et al., 2008). The meta-analysis of Taub et al. (2008), which included chamber and field studies, showed for grain N concentration a decrease of -10% under high and of -16% under low N supply. The FACE study of Kimball et al. (2002) revealed a strong decrease of leaf and grain N concentration under severe N deficiency, but no decrease under ample N supply. However, other FACE studies showed no difference in the e[CO_2] effect on N concentration between low and high N supply (Ma et al., 2007; Erbs et al., 2010; Lam et al., 2012; Han et al., 2015) and little difference between adequate and excess N supply (Tausz et al., 2017). Therefore, the CO_2 x N interaction on grain N concentration under field conditions is still unclear.

In the present study, a two-year FACE experiment was conducted with winter wheat under well-watered conditions, comprising two CO_2 (393 and 600 ppm) and three N fertilizer levels (severe deficiency, adequate and excess). The main objective was to investigate CO_2 x N interactions on Nrem, considering different vegetative organs, and Nabs. The following specific questions were addressed: (i) can the decrease of N concentration by e[CO_2] before anthesis be explained by the negative allometric relation between biomass and N concentration (ii) does decrease of N concentration by e[CO_2] at anthesis result in decreased Nrem and (iii) does e[CO_2] decrease Nabs because of accelerated canopy senescence and/or inhibited NO_3^- assimilation.

2. Material and methods

2.1. Study site and experimental design

The experiment was conducted on a field site (52°18′N, 10°26′E, 79 m a.s.l.) at the Thünen-Institut in Braunschweig, Germany in 2014 and 2015. Winter wheat (*Triticum aestivum* L. Variety "Batis") was grown under ambient [CO_2] and e[CO_2] of 600 ppm and three levels of nitrogen (N) fertilizer. The CO_2 treatments were conducted on circular plots with a diameter of 20 m and the N fertilizer treatments on rectangular subplots (3 m × 5 m), which were randomly established within the CO_2 plots. Altogether, the experiment consisted of six different CO_2 x N treatments that were replicated three times. The position of the CO_2 and N treatments on the field site did not differ between the two years.

The soil in the 0–40 cm plough horizon is a luvisol of loamy sand texture consisting of 69% sand, 24% silt and 7% clay. The lower (-1.5 MPa soil water tension) and upper limit (-0.01 MPa soil water tension) of plant available soil water is a volumetric water content of 5% and 23%, respectively. Important soil parameters were measured of the 0–40 cm soil profile in each N subplot in March 2015. Soil was dried at 105 °C and passed through a 2 mm sieve before carbon (C) and N determination with an element analyzer (Leco TruSpec CNS, USA). Soil pH was measured in a soil suspension with water. The results were (mean ± SD; n = 18): pH, 6.83 ± 0.26; C content, 0.98 ± 0.05%; and N content, 0.09 ± 0.00%. Mineral N in the 0–40 cm soil profile measured in middle of March was 14.2 ± 2.4 kg N ha^{-1} in 2014 and 22.4 ± 5.8 kg N ha^{-1} in 2015. With respect to soil mineral N content, statistical analysis did not result in significant CO_2, N and CO_2 x N effects.

2.2. Crop management and CO_2 enrichment

Wheat was sown with a density of 380 kernels per m^2 on October 29 in 2014 and November 4 in 2015. Crop management was conducted according to local farm practice with sufficient pesticide application and nutrient supply. N fertilization was conducted with calcium ammonium nitrate (CAN; 27% N), which was scattered by hand. A severe deficient N level with 40 (2014) /35 kg N ha^{-1} (2015) (*Nd*), an adequate with 180 (2014) /200 kg N ha^{-1} (2015) (*Nad*) and an excess with 320 kg N ha^{-1} (2014 and 2015) (*Nex*) were used. Table 1 presents an overview of the application dates with the corresponding N fertilizer doses. At anthesis in 2015, an aqueous solution of CAN labelled with

Table 1
N fertilizer treatments with application dates and quantities. *Nd* refers to the severe deficiency, *Nad* to the adequate and *Nex* to the excess N level. At mid-anthesis on June 11 in 2015 ^{15}N labeled N fertilizer with 5% ^{15}N excess was applied.

	2014					2015				
	Quantity (kg N ha^{-1})					Quantity (kg N ha^{-1})				
N level	Mar 19th	Apr 14th	May 4th	June2nd	Total	Mar 18th	Apr 28th	May11th	June 11th	Total
Nd	20	20			40	15	15		5 (^{15}N)	35
Nad	70	35	35	40	180	70	35	35	60 (^{15}N)	200
Nex	120	60	60	80	320	120	60	60	80 (^{15}N)	320

^{15}N (5% excess) was applied, whose amounts are shown in Table 1. The solution was prepared by mixing of a solution of CAN with a solution of ^{15}N labelled ammonium nitrate ($^{15}NH_4^+$ $^{15}NO_3^-$; 98% excess). This solution was then carefully applied with a watering can. Manual irrigation was implemented to keep volumetric soil water content in the range of 14–21% (50–90% of field capacity) and irrigation was adapted to the differing water demand of the different CO_2 x N treatments (for details see: Manderscheid et al., 2018).

CO_2 enrichment was conducted with a FACE system constructed according to the Brookhaven National Laboratory design (Lewin et al., 1992). CO_2 enrichment started at the four and three leaf stage on March 31 in 2014 and March 12 in 2015, respectively. CO_2 enrichment took place during the daytime hours and was interrupted when air temperature fell below 5 °C or wind speed exceeded 6 m s^{-1}.

2.3. Determination of N concentration, atom% ^{15}N excess and N yield

Plant samples were taken from an area of 0.5 m^2 at three stages (first node stage, flag leaf stage, anthesis) and from an area of 1.8 m^2 at maturity. At the samplings up to anthesis, a small subsample consisting of 30 plants was then separated into stems (including leaf sheaths), leaf blades and ears followed by drying at 105 °C and biomass determination. The remaining subsample was dried at 105 °C followed by biomass determination. Both subsamples were used for biomass determination. At maturity, ears were sampled from the whole (1.8 m^2) sampling area. After threshing, the grain and chaff fraction were determined by weight and dry weight was determined from a grain and chaff subsample after drying at 105 °C. Biomass data have already been published (Dier et al., 2018b).

Tissue N concentration was determined using an element analyzer (Leco TruSpec CNS, USA). Before analysis, the stem, leaf blade and chaff fraction were dried at 105 °C and ground to a fine powder in a rotor mill (Brabender, Germany). The grain samples were ground to pass a 0.75 mm sieve in an ultracentrifugal mill (Retsch type ZM1, Germany) after drying at 105 °C. To determine atom% ^{15}N excess, a subsample of the ground plant material was further ground in a ball mill (MM 400, Retsch, Germany) followed by atom% ^{15}N determination with an isotope ratio mass spectrometer (IRMS) (Delta Plus, Thermo Fisher Scientific, USA). Atom % ^{15}N excess was calculated as atom% ^{15}N in the sample minus ^{15}N natural abundance. To test whether there is a CO_2 or N effect on atom% ^{15}N of unlabelled plants at maturity, a plant sample was taken from the area fertilized with unlabelled CAN. However, neither a significant CO_2 and N effect nor CO_2 x N interaction were detected.

2.4. Determination of soil mineral N and ^{15}N excess

Soil samples of the 0–40 cm soil profile were taken at the beginning and the end of the main growing season in both years, and at anthesis in 2015 (before application of the ^{15}N labelled fertilizer). Subsamples of approx. 40 g soil were taken from six random positions, pooled and extracted with KCl (2 M) followed by photometrical determination of soil mineral N (Nmin) with a Continuous-Flow Analyzer (Model SA3000/5000, Scalar, Netherlands).

^{15}N excess was determined as described in 2.3. Before determination, all visible plant material was removed from the soil samples before drying of the soil at 105 °C and grinding to fine powder in a ball mill (MM 400, Retsch, Germany).

2.5. Determination of N nutrition index

N nutrition index (NNI) was determined as the ratio of the actual aboveground N concentration and critical N concentration belonging to the actual aboveground biomass. The critical N concentration was determined according to the critical dilution curve described for wheat (Justes et al., 1994). NNI was measured at flag leaf stage (DC39) and anthesis (DC65) because of the strong connection between NNI during this period and grain yield (Ravier et al., 2017).

2.6. Determination of N remobilization and post-anthesis N uptake

N remobilization (Nrem) and post-anthesis N uptake (Nabs) were estimated with the apparent method (Kichey et al., 2007) in both years. Nrem was calculated by subtracting N yield of the vegetative plant fractions (stem + leaf sheath, leaf blade and chaff) at maturity from the ones at anthesis. Efficiency of Nrem was calculated as the ratio of Nrem to N yield at anthesis. Nabs was calculated from the difference between N yield of all plant fractions at maturity and at anthesis.

Nrem and Nabs were additionally estimated by ^{15}N isotope labelling in 2015. This was conducted according to Kichey et al. (2007) on the basis of the following equations:

$$N_{rem} = \frac{[N_{ant}(E_{abs} - E_{ant})] - [N_{mat}(E_{abs} - E_{mat})]}{E_{rem} - E_{abs}} \quad (1)$$

$$N_{abs} = \frac{[N_{mat}(E_{mat} - E_{ant})] - [N_{ant}(E_{ant} - E_{rem})]}{E_{abs} - E_{rem}} \quad (2)$$

where N_{ant} is N yield at anthesis; N_{mat}, N yield at maturity; E_{ant}, ^{15}N excess at anthesis; E_{mat}, ^{15}N excess at maturity; E_{rem}, ^{15}N excess derived from Nrem; E_{abs} ^{15}N excess derived from Nabs. Because there was no ^{15}N excess in the plant fractions at anthesis, E_{rem} was regarded as zero. E_{abs} was calculated with the following equation:

$$E_{abs} = \frac{(N_{fer} * E_{fer}) + (N_{soil} * E_{soil}) + (N_{minr} * E_{minr})}{N_{fer} + N_{soil} + N_{minr}} \quad (3)$$

where N_{fer} is the amount of labelled fertilizer applied at anthesis; E_{fer}, ^{15}N excess of the fertilizer (5%); N_{soil}, soil Nmin at anthesis; E_{soil}, ^{15}N excess in the soil at anthesis; N_{minr}, N derived from mineralization from anthesis up to maturity, which was calculated as total N (aboveground plant + Nmin) under *Nd* at maturity minus total N at anthesis under *Nd*; and E_{minr} ^{15}N excess derived from mineralization from anthesis up to maturity. E_{minr} was regarded as zero because of the absence of ^{15}N excess in the soil at anthesis.

2.7. Determination of relative greenness

Relative greenness (SPAD) of the flag leaf was measured with a portable chlorophyll meter (Model SPAD 502, Minolta, Japan) starting at medium milk development (DC75). In each N subplot, 10 randomly chosen plants were used and SPAD measurements were conducted at three leaf positions (distal, medial, and proximal).

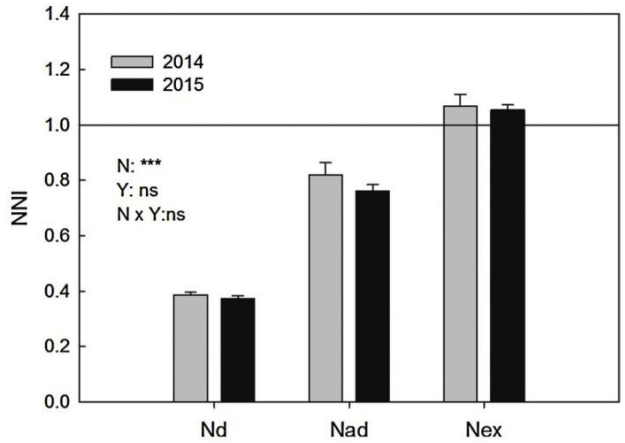

Fig. 1. Nitrogen nutrition index (NNI) of the three N treatments (severe deficiency (*Nd*), adequate (*Nad*) and excess (*Nex*)) in both years of the experiment. NNI was determined at the flag leaf stage and anthesis and shown are the mean values over the two growth stages and CO_2 levels (± S.E.M; n = 12) and the F-test result of the N and year effect. The horizontal line indicates the NNI of one, from which onwards the crop N status can be considered as non-limiting for growth. ***P < 0.001.

2.8. Determination of N use efficiency, its components and N harvest index

N use efficiency (NUE) was calculated by dividing grain biomass by Nmin at the beginning of the main growing season plus fertilizer N (Moll et al., 1982). N uptake efficiency (NUpE) was calculated by dividing total aboveground N yield by the same denominator used to calculate NUE. N utilization efficiency (NUtE) was calculated by dividing grain biomass by total aboveground N yield. N harvest index (NHI) was calculated by dividing grain N yield by total aboveground N yield. Grain specific N uptake efficiency (gNUpE) was calculated by multiplying NUpE with NHI.

2.9. Statistical analysis

The experiment was analyzed with SAS (version 9.4) proc mixed as split plot design with the CO_2 treatment as main plot factor and the N treatment as sub plot factor. If the year was added as third factor to the mixed model, then statistical analysis was conducted as repeated measurements. The year was treated as fixed effect and the variance/covariance between the two years of the random main plot and residual error was modelled with the UN (1) covariance model. This model applies no covariance between both years, but different error variances between the two years. Least square difference tests were implemented with SAS proc glimmix and mean values were regarded as significantly different if $P < 0.05$.

The CO_2 effect on the allometric relation between aboveground N concentration (N%) and aboveground biomass (W), which follows the equation:

$$N\,\% = aW^{-b} \quad (4)$$

(Sadras and Lemaire, 2014), was examined by transforming Eq. (4) to:

$$\log(N\%) = \log(a) - b\log(W) \quad (5)$$

and subjecting this linear relation to analysis of covariance.

Analysis of covariance of the effect of CO_2 and aboveground biomass on aboveground N concentration as well as the effect of CO_2 and grain number on variables describing N acquisition at maturity (e.g. Nrem and Nabs) was implemented by sequential F-tests with SAS proc mixed. The interaction effect was analyzed by testing the full model against the model without the interaction effect. The main effects were analyzed by testing the model without the interaction against the model further reduced by the main effect to be tested.

3. Results

3.1. CO_2 enrichment and climatic conditions

CO_2 enrichment took place during 99.0% of the target time in 2014 and 97.4% in 2015. The one minute average [CO_2] was within ± 10% of the target concentration of 600 ppm for 95.6% of the operating time in 2014 and for 95.7% in 2015. Monthly mean temperature and global radiation as well as rainfall were in the range of normal variation in both years. In 2014, temperature in March and April was warmer compared to 2015 and the long term mean, respectively. A detailed description of the environmental conditions is shown elsewhere (Dier et al., 2018a,b). In 2015 rainfall was low in May and June and thus intensive irrigation was necessary (Manderscheid et al., 2018).

3.2. Characterization of the N treatments

Fig. 1 shows the N nutrition index (NNI) measured at the flag leaf stage and anthesis of the three N levels (Table 1). Rising N supply significantly increased NNI and over both growth stages, CO_2 levels and years NNI was 0.4 under the N deficiency (*Nd*), 0.8 under the adequate (*Nad*) and 1.1 under the excess N level (*Nex*). Neither a significant CO_2 and year effect nor a CO_2 x N, N x year and CO_2 x N x year interaction on NNI were detected.

3.3. Allometric relation between plant N concentration and biomass

Fig. 2 shows the allometric relation between aboveground biomass and aboveground N concentration from 1 st node stage up to anthesis for each CO_2 x N combination comprising the data of both years. Rising N supply shifted the allometric curves to generally larger N concentration, but e[CO_2] did not significantly influence the allometric relation under any N level (Table S1).

3.4. Tissue N concentration at anthesis and maturity and mean N content per grain

N concentration of all wheat fractions were strongly increased by increasing N level at anthesis and maturity (Tables 2, 3 and S2). Most of these N concentrations were influenced by the year, especially grain N concentration was considerably larger in 2015 compared to 2014 (16%

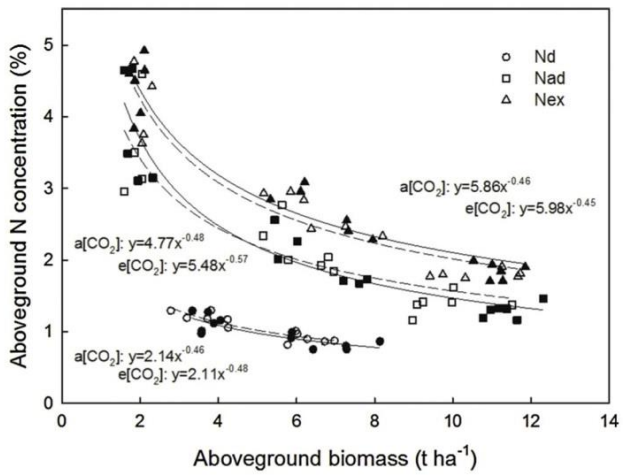

Fig. 2. Allometric relation between plant N concentration and biomass for each CO_2 x N combination comprising the data of both years. Open symbols and dashed regression line = ambient [CO_2]; closed symbols and solid regression line = e[CO_2]. Result of analysis of covariance of the linearized allometric relation is shown in Table S1. Only data of the biomass range of 1.55–12 t ha^{-1} were considered for analysis in accordance with Justes et al. (1994).

Table 2
F-test result of the effect of the two CO_2 and three N levels on tissue N concentration (Nconc); N yield; mean N content per grain; percentage of N in stem, leaf and ear as a proportional of total aboveground N yield (%N); N use efficiency (NUE); N uptake efficiency (NUpE); N utilization efficiency (NUtE); N harvest index (NHI) and N uptake efficiency of grain (NUpEg). A refers to the sampling at anthesis and M to the one at maturity.

Variable	Growth stage	CO_2	N	Year	CO_2 x N	CO_2 x Y	N x Y	CO_2 x N x Y
Nconc stem (mg N g^{-1})	A	ns	***	*	ns	ns	(*)	ns
	M	*	***	***	ns	(*)	***	ns
Nconc leaf (mg N g^{-1})	A	ns	***	(*)	ns	ns	ns	ns
	M	*	***	**	(*)	ns	**	(*)
Nconc ear/chaff (mg N g^{-1})	A	ns	***	ns	*	ns	ns	ns
	M	ns	***	ns	ns	ns	ns	ns
Nconc grain (mg N g^{-1})	M	(*)	***	***	*	ns	***	ns
N content per grain (mg)	M	ns	***	*	ns	ns	***	ns
N yield stem (g N m^{-2})	A	*	***	*	ns	ns	ns	ns
	M	(*)[1]	***[1]	*[1]	ns[1]	ns[1]	ns[1]	ns[1]
N yield leaves (g N m^{-2})	A	ns	***	ns	ns	ns	ns	ns
	M	ns	***	***	ns	ns	**	ns
N yield chaff (g N m^{-2})	A	*	***	ns	ns	ns	ns	ns
	M	ns	***	(*)	ns	ns	**	ns
N yield total (g N m^{-2})	A	ns	***	ns	ns	ns	ns	ns
	M	(*)	***	***	ns	ns	**	ns
N yield grain (g N m^{-2})	M	*	***	***	(*)	ns	***	ns
%N of stem	A	***[2]	***[2]	***[2]	ns[2]	(*)[2]	ns[2]	ns[2]
%N of leaf	A	(*)[2]	***[2]	(*)[2]	ns[2]	ns[2]	ns[2]	ns[2]
%N of ear	A	ns[2]	***[2]	ns[2]	ns[2]	ns[2]	ns[2]	ns[2]
NUE (g g^{-1} N)	M	**[1]	***[1]	*[1]	ns[1]	ns[1]	ns[1]	ns[1]
NUpE (g N g^{-1} N)	M	*	***	ns	ns	ns	ns	ns
NUtE (g g^{-1} N)	M	ns	***	**	ns	ns	**	ns
NHI	M	ns	***	**	ns	(*)	ns	ns
NUpEg (g N g^{-1} N)	M	*[1]	***[1]	ns[1]	ns[1]	ns[1]	ns[1]	ns[1]

(*)$P < 0.1$ *$P < 0.05$ **$P < 0.01$ ***$P < 0.001$.
[1] Prior to analysis, data were log-transformed to ensure variance homogeneity and normal distribution of the residual error.
[2] Data were square root-transformed to ensure variance homogeneity and normal distribution of the residual error.

at *Nd*, 19% at *Nad*, 9% at *Nex*). Additionally, mean N content per grain N was larger under *Nd* (14%) and *Nad* (13%) in 2015 (Tables 2 and 3).

e[CO_2] did not affect stem N concentration at anthesis, but significantly ($P < 0.1$) reduced it under all N levels by -9 to -17% at maturity in 2014 (Tables 2 and S2). Leaf N concentration was not significantly affected by e[CO_2] at anthesis, but was significantly ($P < 0.05$) reduced at maturity in both years (-7% (*Nd*), -16 (*Nad*), -10% (*Nex*)). Ear N concentration was significantly ($P < 0.05$) reduced under *Nad* (-6%) at anthesis in both years.

Grain N concentration was slightly reduced by e[CO_2] in both years (-1% (*Nd*), -6% (*Nad*) and -4% (*Nex*)), which was significant ($P < 0.05$) only under *Nad* (Tables 2 and 3). e[CO_2] did not significantly affect mean N content per grain.

3.5. N yield at anthesis and maturity and N allocation at anthesis

Total aboveground N yield ranged from 5.3 to 21.6 at anthesis and from 7.9 to 28.3 g N m^{-2} at maturity (Table 4). Grain N yield ranged from 6.5 to 21.5 g N m^{-2}. Rising N level strongly increased N yield of all wheat fractions at anthesis and maturity and most of these variables were significantly different in both years (Tables 2 and 4 and S3).

At anthesis, e[CO_2] did not significantly influence total aboveground N yield (Tables 2 and 4), but increased N yield of stem (5–12%) and ear (12%) under *Nad* and *Nex* in both years and these variables under *Nd* in 2014 (Tables 2 and S3). At maturity, e[CO_2] increased aboveground N yield on average by 8% (*Nd*), 9% (*Nad*) and 9% (*Nex*) and grain N yield by 8% (*Nd*), 10% (*Nad*) and 12% (*Nex*) (Table 4) where the former effect was significant for all N levels ($P < 0.1$) and the latter only under *Nad* and *Nex* ($P < 0.1$) (Table 2). Stem N yield was increased by e[CO_2] primarily in 2015, although no significant CO_2 x year interaction was detected (Table 2 and S3).

Rising N supply increased the relative contribution of N in leaf as a proportional of the total N contained in the aboveground compartment whereas the relative contribution of N contained in the ear was decreased (Table 2 and Fig. S1). The relative contribution of N contained in the stem was largest under *Nd* and smallest under *Nad*. e[CO_2]

Table 3
Effect of the two CO_2 and three N levels on grain N concentration and mean N content per grain. Shown are mean values (n = 3) and the percentage effect of e[CO_2] (Δ (%)). Different small letters indicate significant differences among the marginal means of the N treatments. If the F-test resulted in a significant CO_2 x N interaction (Table 2), then different capital letters indicate significant differences for CO_2 means separate for each N level and different small letters significant differences for N means separate for each CO_2 treatment (letters are in bold for e[CO_2]). All letters refer to the mean over both years.

	2014			2015		
	Nd	Nad	Nex	Nd	Nad	Nex
Nconc grain (mg N g^{-1})						
a[CO_2]	13.9 A c	18.6 A b	21.7 A a	16.0 A c	22.1 A b	23.7 A a
e[CO_2]	13.6 A **c**	17.5 B **b**	20.9 A **a**	15.9 A **c**	20.9 B **b**	22.7 A **a**
Δ (%)	−2	−6	−4	−1	−6	−4
N content per grain (mg)						
a[CO_2]	0.642 b	0.852 a	0.934 a	0.716 b	0.955 a	0.899 a
e[CO_2]	0.625 b	0.828 a	0.947 a	0.731 b	0.939 a	0.887 a
Δ (%)	−3	−3	1	2	−2	−1

Table 4
Effect of the two CO_2 and three N levels on total aboveground (AGN) and grain N yield. Shown are mean values (n = 3) and the percentage effect of e[CO_2] (Δ (%)). Different small letters indicate significant differences among the marginal means of the N treatments. If the F-test resulted in a significant CO_2 x N interaction (Table 2), then different capital letters indicate significant differences for CO_2 means separate for each N treatment and different small letters significant differences for N means separate for each CO_2 treatment (letters are in bold for e[CO_2]). All letters refer to the mean over both years.

		2014			2015		
		Nd	*Nad*	*Nex*	*Nd*	*Nad*	*Nex*
AGN yield (g N m^{-2})							
Anthesis	a[CO_2]	5.50 c	13.2 b	18.7 a	5.83 c	14.2 b	19.8 a
	e[CO_2]	6.11 c	14.7 b	19.6 a	5.34 c	14.8 b	21.6 a
	Δ (%)	11	11	4	−8	4	9
Maturity	a[CO_2]	7.92 c	19.1 b	24.8 a	7.85 c	22.1 b	26.1 a
	e[CO_2]	8.60 c	20.3 b	27.2 a	8.48 c	24.5 b	28.3 a
	Δ (%)	9	7	10	8	11	9
Grain N yield (g N m^{-2})							
	a[CO_2]	6.55 A c	15.2 B b	17.7 B a	6.68 A c	18.4 B b	19.7 B a
	e[CO_2]	7.18 A **c**	16.6 A **b**	20.3 A **a**	7.08 A **c**	20.3 A **b**	21.5 A **a**
	Δ (%)	10	9	14	6	10	9

significantly ($P < 0.1$) decreased the relative contribution of N in the leaf in both years on average by -4, -6 and -7% but significantly ($P < 0.001$) increased the one of stem by 4, 4 and 5% under *Nd*, *Nad* and *Nex*, respectively. There was a significant ($P < 0.1$) CO_2 x Y interaction on the relative contribution of N in the stem because of a slightly stronger increase by e[CO_2] in 2015.

3.6. N use efficiency, its components and N harvest index

N use efficiency (NUE) ranged from 24.1 to 100 g g^{-1} N, N uptake efficiency (NUpE) from 0.74 to 1.64 g N g^{-1} N, N utilization efficiency (NUtE) from 31.8 to 61.5 g g^{-1} N, N harvest index (NHI) from 0.714 to 0.851 and grain specific N uptake efficiency (NUpEg) from 0.53 to 1.37 g N g^{-1} N (Table 5). These variables were significantly decreased by rising N fertilization (Tables 2 and 5). Regarding year, NUE under all N levels and NUtE under *Nd* and *Nad* were larger in 2014, whereas NHI was larger under all N levels in 2015.

e[CO_2] significantly increased NUE ($P < 0.01$), NUpE ($P < 0.05$) and gNUpE ($P < 0.05$) in both years on average by 13, 18 and 17% (NUE); 12, 11 and 10% (NUpE); and 11, 12, 13% (gNUpE) under *Nd*, *Nad* and *Nex*, respectively (Tables 2 and 5). NHI was slightly but significantly ($P < 0.1$) increased by e[CO_2] in 2014 (1–4%). NUtE was not significantly influenced by e[CO_2].

3.7. N remobilization and N remobilization efficiency

Fig. 3 shows the effect of CO_2, N and year on N remobilization (Nrem) and N remobilization efficiency (NRE) for stem, leaf, chaff and aboveground plant based on the apparent method. Over both years and all CO_2 and N levels, Nrem was 3.47, 4.10 and 2.07 g N m^{-2} and NRE 61, 76 and 73% for stem, leaf and chaff, respectively. Nrem was strongly correlated with N yield at anthesis with r^2 values of 0.92 (stem), 0.98 (leaf) and 0.96 (chaff).

Rising N fertilization strongly increased Nrem of all wheat fractions. NRE of stem and total aboveground plant were decreased by rising N supply and NRE of leaf and chaff were similar under *Nd* and *Nad*, but were larger compared to *Nex*. Nrem and NRE were significantly influenced by year; in particular leaf NRE was considerably larger in 2015.

e[CO_2] increased Nrem of stem and chaff on average by 15–23% and 13–16%, respectively under *Nad* and *Nex* in both years and by 11% and 10% under *Nd* in 2014. However, e[CO_2] did not significantly influence Nrem of leaf and aboveground plant. Determination of Nrem by ^{15}N labelling, which was only conducted in 2015, showed no influence of e[CO_2] on total plant Nrem (Fig. S2). Linear regression comparing the apparent with the ^{15}N labelling method yielded a slope of 1.03 ($r^2 = 0.97$), indicating similar values for total plant Nrem (Fig. S3).

Table 5
Effect of the two CO_2 and three N levels on N use efficiency (NUE), N uptake efficiency (NUpE), N utilization efficiency (NUtE), N harvest index (NHI) and N uptake efficiency of grain (NUpEg). Shown are mean values (n = 3) and the percentage effect of e[CO_2] (Δ (%)). Different small letters indicate significant differences among the marginal means of the N treatments. All letters refer to the means over both years.

	2014			2015		
	Nd	*Na*	*Ne*	*Nd*	*Na*	*Ne*
NUE (g g^{-1} N)						
a[CO_2]	84.7 a	41.7 b	24.3 c	75.8 a	37.2 b	24.1 c
e[CO_2]	100 a	49.3 b	29.2 c	81.5 a	44.2 b	27.6 c
Δ (%)	18	18	20	7	19	15
NUpE (g N g^{-1} N)						
a[CO_2]	1.42 a	0.97 b	0.74 c	1.42 a	0.99 b	0.76 c
e[CO_2]	1.64 a	1.06 b	0.82 c	1.54 a	1.11 b	0.82 c
Δ (%)	15	8	11	9	13	9
NUtE (g g^{-1} N)						
a[CO_2]	59.5 a	43.1 b	33.0 c	53.2 a	37.7 b	31.8 c
e[CO_2]	61.5 a	46.8 b	35.7 c	52.7 a	39.7 b	33.5 c
Δ (%)	3	8	8	−1	5	5
NHI						
a[CO_2]	0.827 a	0.798 b	0.714 c	0.851 a	0.833 b	0.755 c
e[CO_2]	0.835 a	0.816 b	0.744 c	0.835 a	0.829 b	0.761 c
Δ (%)	1	2	4	−2	−1	1
NUpEg (g N g^{-1} N)						
a[CO_2]	1.17	0.77	0.53	1.21	0.82	0.57
e[CO_2]	1.37	0.86	0.61	1.29	0.92	0.63
Δ (%)	16	11	16	6	12	10

In 2014, e[CO_2] significantly increased NRE of stem ($P < 0.1$) by 2, 7 and 10% and of aboveground plant ($P < 0.05$) by 2, 6 and 4% under *Nd*, *Nad* and *Nex*, respectively (Fig. 3). However, under *Nex*, stem NRE was significantly ($P < 0.1$) increased in both years on average by 10%.

3.8. Flag leaf senescence, post-anthesis N uptake and soil mineral N at maturity

In both years, relative greenness of the flag leaf increased with rising N supply (Fig. 4). Starting from milk-ripe stage, flag leaf greenness declined with progressing growth stage, whereby this decline was delayed by rising N supply. Flag leaf greenness was not affected by e[CO_2].

The apparent (Fig. 5) and the ^{15}N labelling method (Fig. S2) indicate that post-anthesis N uptake (Nabs) was increased by rising N fertilization from *Nd* to *Nad*, but further increases in *Nex* did not result in increased Nabs. Nabs considerably exceeded the amount of N fertilizer applied at anthesis under *Nd* and *Nad* (Figs. 5 and S2). Regarding year, Nabs was significantly ($P < 0.05$) larger under *Nad* in 2015 than

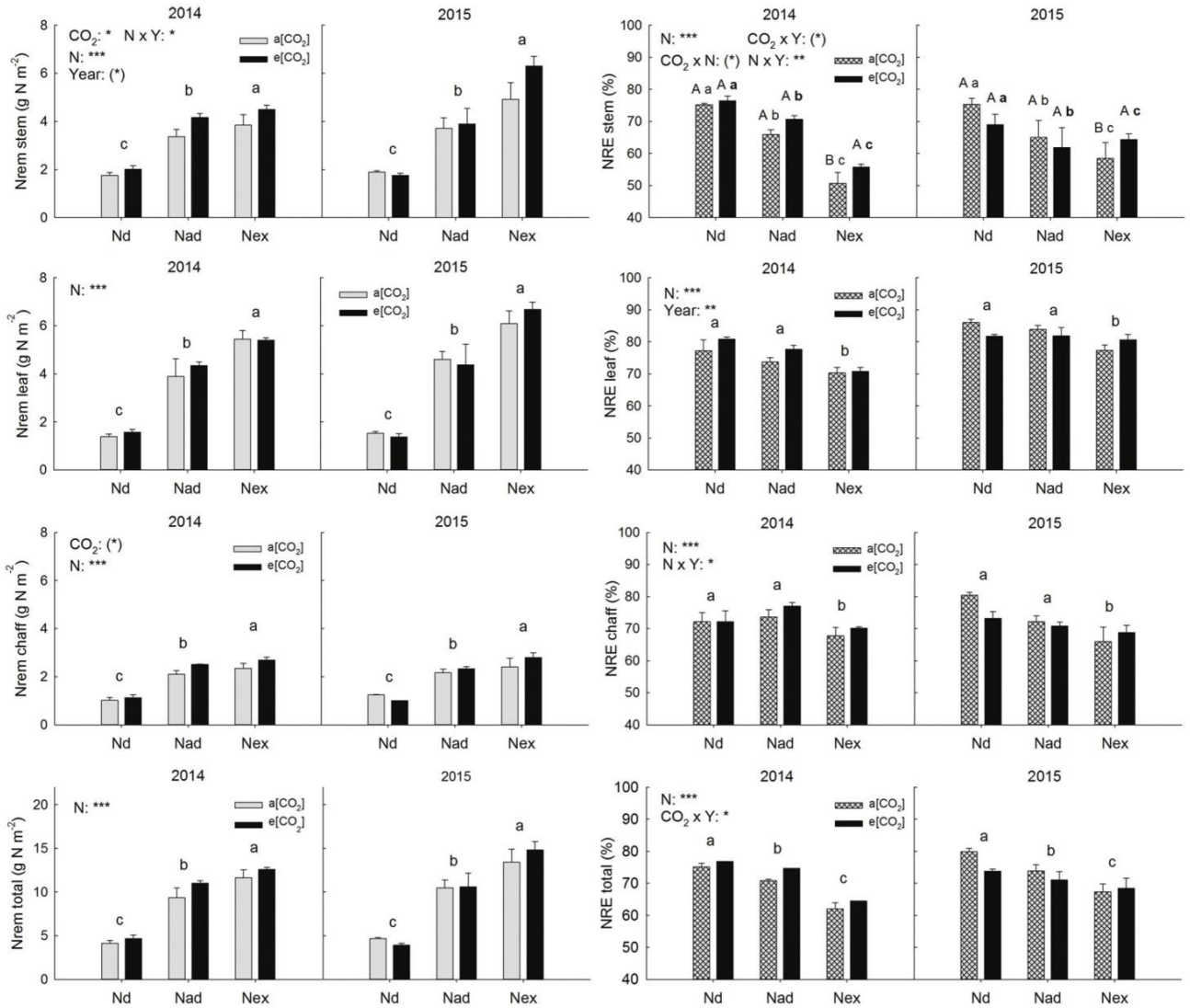

Fig. 3. Effect of the two CO_2 and three N levels on N remobilization (Nrem) and N remobilization efficiency (NRE) of stem, leaf, chaff and aboveground plant based on the apparent method. Shown are the means values (± S.E.M; n = 3) and the F-test result of the significant effects. Different small letters indicate significant differences among the marginal N means (mean over both CO_2 treatments and years). With a significant CO_2 x N interaction: different capital letters indicate significant differences between the CO_2 means separate for each N level and different small letters significant differences among the N means separate for each CO_2 treatment (letters are in bold for e[CO_2]). $^{(*)}P < 0.1$ $^{*}P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$.

in 2014. Both the apparent method and ^{15}N labelling, which showed a strong correlation ($r^2 = 0.94$) with respect to Nabs (Fig. S3), indicate a trend of increased Nabs under e[CO_2] at *Nad* in 2015, even if a significant interaction with CO_2 was not detected.

Soil mineral N at maturity was significantly ($P < 0.01$) decreased under e[CO_2] under all N levels in both years (Fig. 5) on average by -22, -22 and -26% under *Nd*, *Nad* and *Nex*, respectively.

3.9. Correlation between grain number and variables describing grain N acquisition

Fig. 6 shows linear regression of grain number on aboveground and grain N yield, grain N concentration, mean N content per grain, Nrem and Nabs. In both years, e[CO_2] increased grain number on average by 8% under *Nd* and 12% under *Nad* and *Nex*, respectively (Dier et al., 2018b). All variables were strongly positive correlated with grain number, whereby r^2 values were highest for grain N yield (0.98, ambient [CO_2]; 0.97, e[CO_2]) and lowest for Nabs (0.86, ambient [CO_2]; 0.71, e[CO_2]). Analysis of covariance (Fig. 6) resulted in significant grain number effects on all variables ($P < 0.001$) and significant CO_2 effects on aboveground N yield ($P < 0.1$), grain N concentration ($P < 0.001$) and mean N content per grain ($P < 0.1$). Significant CO_2 effects imply that the regression line with respect to e[CO_2] runs below the one regarding ambient e[CO_2]. This was also the case for Nrem, although no significant CO_2 effect was detected.

4. Discussion

The main objective of the present FACE study was to investigate in winter wheat grown under well-watered conditions the CO_2 x N interactions on key processes determining grain N acquisition and thus grain N concentration, which are N remobilization (Nrem) and post-anthesis grain N acquisition (Nabs). The data show that under widely differing N levels, comprising a N nutrition index (NNI) between 0.4 and 1.1, e[CO_2] improved Nrem and tended to increase Nabs that resulted in increased grain N yield and only slightly reduced grain N concentration.

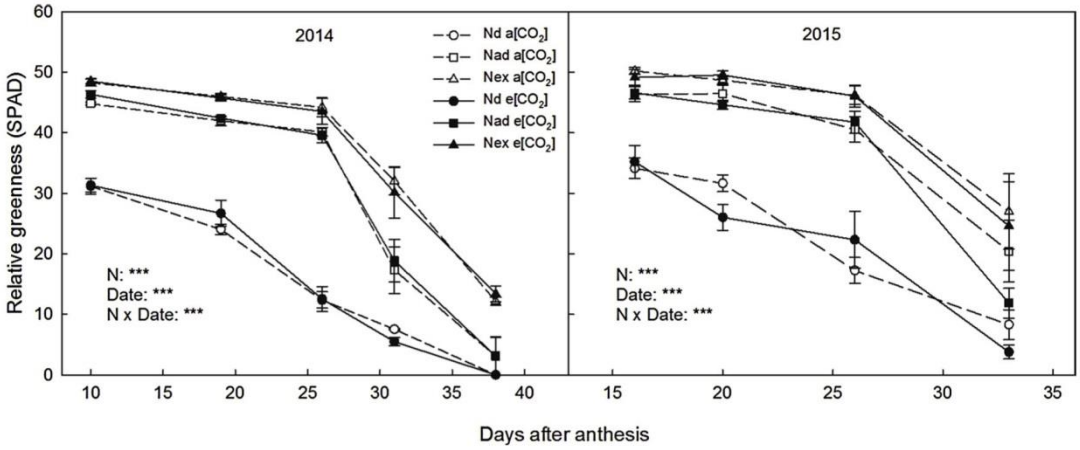

Fig. 4. Effect of the two CO_2 and three N levels on relative greenness (SPAD) of the flag leaf during grain filling. Shown are mean values (± S.E.M; n = 3) and the F-test results of the significant effects.

4.1. Allometry between plant N concentration and biomass

In the present study, tissue N concentration at anthesis was slightly reduced by e[CO_2] which corresponds to previous FACE studies (Han et al., 2015; Tausz et al., 2017). If the negative allometric relation between N concentration and biomass (Justes et al., 1994; Sadras and Lemaire, 2014) is considered, where plants are compared at common biomass, then N concentration was unaffected by e[CO_2]. Consistent results were also found in a growth chamber study with annual C_3 weed (*Abutilon theophrasti*) where decrease of N concentration by e[CO_2] disappeared when N concentration was compared at common biomass with regard to CO_2 level (Coleman et al., 1993). Similarly, in the present experiment green leaf N concentration was not decreased by e[CO_2] when this variable was compared at common leaf area index (Dier et al., 2018b). Thus, there is indication that the decrease of N concentration by e[CO_2] before anthesis results only from increased growth under e[CO_2], but not from direct physiological effects on N acquisition such as downregulation of RuBisCO gene expression in leaf (Stitt and Krapp, 1999). Consequently, it might not be necessary to adjust the critical N concentration to e[CO_2] in wheat growth models as it was previously discussed by Vanuytrecht and Thorburn (2017).

4.2. N acquisition

Aboveground plant N acquisition up to anthesis was not significantly increased by e[CO_2]. This result contrasts with other FACE studies where significant increases of 13–17% were found (Han et al., 2015; Tausz et al., 2017). In the present study, N allocation to the different organs was altered by e[CO_2] because e[CO_2] increased the proportion of stem in aboveground N but decreased that of leaf. This effect is consistent with Han et al. (2015) and could be explained by the larger stimulation of stem compared to leaf growth by e[CO_2] (Dier et al., 2018b).

At maturity, a FACE study conducted under subtropical climate and well-watered conditions found increased grain N yield of 17% under e[CO_2] at ample N supply (about 460 kg N ha^{-1}), but grain N yield was

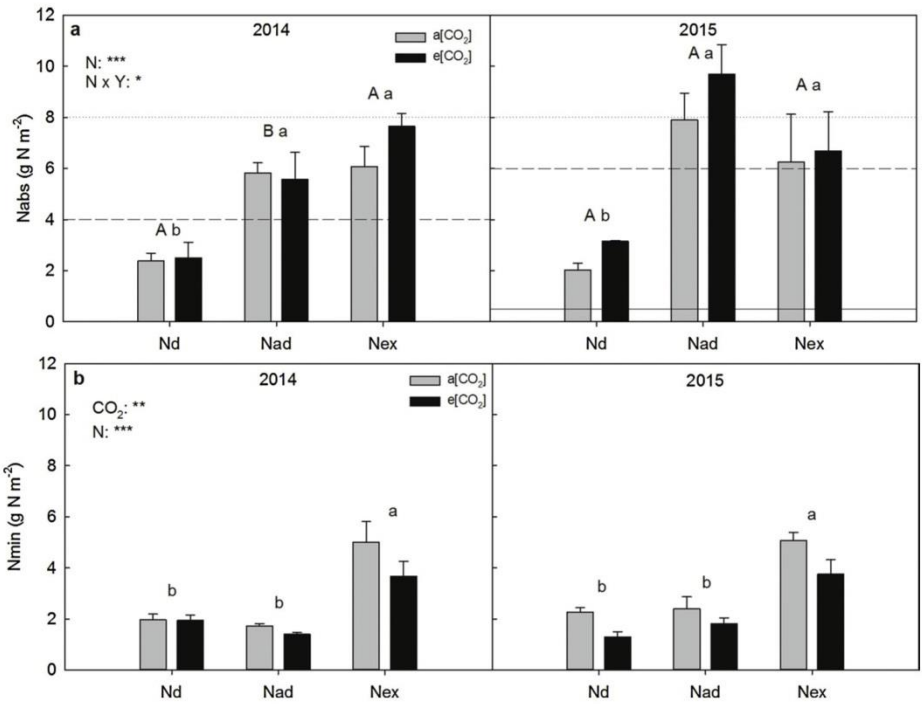

Fig. 5. Effect of the two CO_2 and three N levels on (a) post-anthesis N uptake (Nabs) based on the apparent method and (b) mineral N in the 0–40 cm soil layer (Nmin) at maturity. Shown are mean values (± S.E.M; n = 3) and the F-test result of the significant effects. The lines indicate the amount of N applied at anthesis (solid = *Nd*; dashed = *Nad*; dotted = *Nex*). Different small letters indicate significant differences between the marginal N means (mean over both CO_2 treatments). With a significant Y x N interaction different capital letters indicate significant differences between both years of the marginal N means within each N level and different small letters significant differences between the marginal N means within each year. $^{*}P < 0.05$ $^{***}P < 0.001$.

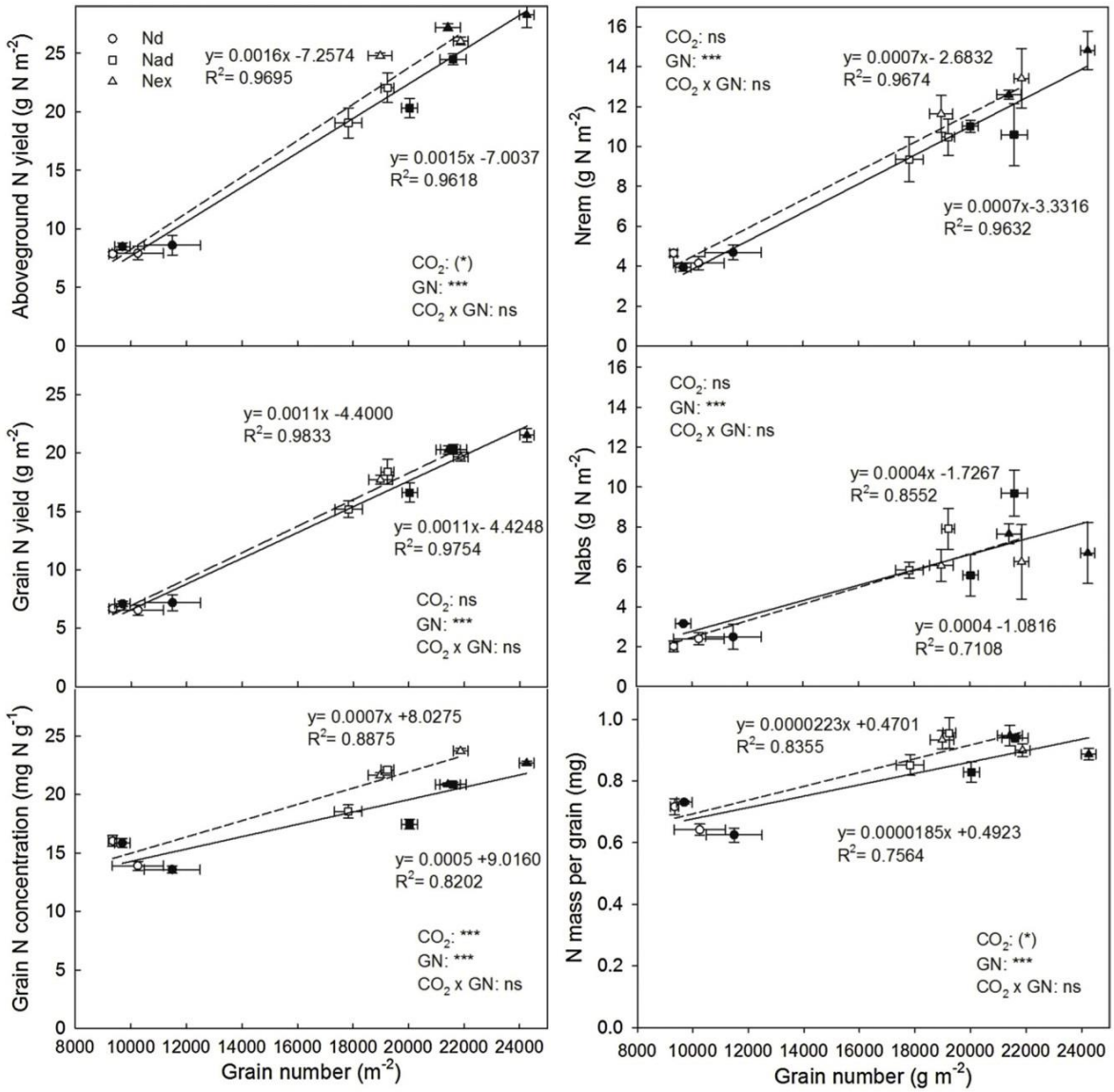

Fig. 6. Linear regression of grain number on variables describing N acquisition at maturity (aboveground and grain N yield; grain N concentration; Nrem; Nabs and; mean N content per grain). Shown are the mean values (± S.E.M; n = 3) for each CO_2 x N x year combination. Open symbols and dashed regression line = ambient [CO_2]; closed symbols and solid regression line = e[CO_2]. Each diagram includes the result of the analysis of covariance. $^{(*)}P < 0.1$ $^{***}P < 0.001$.

uninfluenced under N deficiency (about 100 kg N ha^{-1}) (Sinclair et al., 2000; Kimball et al., 2001). However, other FACE studies conducted under similar conditions (Ma et al., 2007; Han et al., 2015) and in a semi-arid environment where water availability is particularly low at grain filling (Tausz et al., 2017) showed increased aboveground N yield of 9–21% under a low (100–180 kg N ha^{-1}) and 9–22% under a high N treatment (170–240 kg N ha^{-1}). In the present study under temperate climate and well-watered conditions, e[CO_2] increased grain and total aboveground N yield under all N levels, but the e[CO_2] effect on grain N yield was slightly less pronounced under *Nd* (8%) compared to *Nad* (10%) and *Nex* (12%). The very strong linear relation (r^2 = 0.98) between grain number and grain N yield (Fig. 6) indicates that increase of grain number by e[CO_2], which was 8% under *Nd* and 12% under *Nad* and *Nex*, respectively (Dier et al., 2018b), was the reason for the enhanced grain N yield.

The finding that e[CO_2] increased N use efficiency primarily through the increase of N uptake efficiency, being consistent with the FACE study of Tausz et al. (2017), can be attributed to the enhanced N acquisition of stem and grain.

4.3. Nitrogen sources contributing to the increased grain N acquisition under e[CO_2]

4.3.1. Nitrogen remobilization

In the present study, rising N supply increased Nrem but decreased the efficiency of Nrem (NRE). While the former effect is consistent with other studies (Barbottin et al., 2005; Gaju et al., 2014), various effects of rising N supply on NRE were found (Barbottin et al., 2005; Gaju et al., 2011, 2014). It is established that a high post-anthesis plant N status delays senescence, being a major N source for Nrem (Kong et al., 2016), which might explain the decrease of NRE by rising N supply. Moreover, grain sink strength might be a reason because N yield at anthesis per individual grain decreased with decreasing N supply and linear regression resulted in a stronger negative correlation between this variable and NRE (r^2 = –0.76) compared to N yield at anthesis and NRE (r^2 = –0.67) (data not shown). Consistent with other studies (Pask et al., 2012; Gaju et al., 2014) leaves had the highest NRE (76%) followed by chaff (73%) and stems (61%).

It was suggested that the decrease of vegetative tissue N concentration by e[CO_2] at anthesis result in a decrease of Nrem (Kimball et al.,

2001; Rubio-Asensio and Bloom, 2016). However, a FACE study showed no e[CO_2] effect on total plant Nrem (Tausz et al., 2017). In the present study, e[CO_2] did not affect leaf Nrem and enhanced Nrem of stem and chaff as well as NRE of stem and aboveground plant. While the increase of Nrem is consistent with the increased stem and ear N yield at anthesis, the e[CO_2] effect on NRE of stem and total aboveground plant indicate that e[CO_2] enhanced mobilization of vegetative N during grain filling. Indication that e[CO_2] increased the rate of mobilization of N from vegetative tissue was found in other FACE studies, where e[CO_2] accelerated the decline of leaf proteins (Nie et al., 1995; Osborne et al., 1998) and of N in stem tissue during grain filling (Lam et al., 2012) and stimulated the gene expression of glutamine synthetase (Buchner et al., 2015), whose activity correlates with Nrem (Kichey et al., 2007).

Under *Nd* and *Nad*, stem and aboveground plant NRE as well as NHI were increased by e[CO_2] only in 2014, while NHI and mean N content per grain were generally larger in 2015. These effects could be associated with larger post-anthesis soil N availability in 2015, whereby in 2014 it was possibly insufficient to serve an increased grain N sink strength due to the grain number increase by e[CO_2]. Reasons for increased post-anthesis soil N availability per grain in 2015 are the increased N fertilizer supply at anthesis under *Nad* (Table 1) and the lower grain number in 2015 (Dier et al., 2018b) but similar Nabs between both years under *Nd*.

4.3.2. Post-anthesis N uptake

Judging from relative greenness of the flag leaf during grain filling, senescence was not accelerated by e[CO_2], which contrasts with previous studies (Osborne et al., 1998; Brooks et al., 2000; Fangmeier et al., 2000) possibly due to differences in climatic conditions and the effect on leaf temperature. Correspondingly, Nabs was not significantly affected, but tended to be increased by e[CO_2], which is consistent with the significantly lower soil mineral N content at maturity under e[CO_2] compared to ambient [CO_2]. Moreover, e[CO_2] enhanced N acquisition more at maturity compared to anthesis, indicating that Nabs was important to cover an increased grain N sink demand under e[CO_2]. The fact that Nabs considerably exceeded the amount of N applied at anthesis under *Nd* and *Nad* suggests that there was a source limit of post-anthesis soil N availability under these N levels. It is difficult to conclude, therefore, whether higher N application at anthesis would have led to a significant increase of Nabs by e[CO_2] under *Nd* and *Nad*. However, there is indication from a glasshouse study that e[CO_2] can strongly increase Nabs, leading to no decrease of grain N concentration under e[CO_2], when wheat is grown under relatively low pre-anthesis, but high post-anthesis N availability (Fernando et al., 2017). Because water availability is critical for N uptake, Nabs might be unimportant to serve an increased grain N demand under e[CO_2] in a semi-arid environment despite sufficient N availability in the soil at grain filling.

Under *Nex*, the relative low Nabs despite non-limiting N availability might be explained by decreased post-anthesis root N uptake. It is established that root NO_3^- uptake is decreased under a high plant N status (Glass et al., 2002; Barneix, 2007). That the plant N status was high under *Nex* is indicated by the NNI of 1.1. Moreover, individual grain weight was smaller under *Nex* compared to *Nd* and *Nad* (Dier et al., 2018b), suggesting limited C assimilation per grain and thus limited energy supply for this process. Energy costs are higher for N than C assimilation (Munier-Jolain and Salon, 2005) and thus increased competition between N and C assimilation for energy might also be a reason for the relatively low Nabs.

The high Nabs under *Nd* and *Nad* suggest that NO_3^- assimilation was not affected by e[CO_2], which is consistent with the finding that e[CO_2] did not reduce gene expression and activity of NO_3^- reductase before and at grain filling (Dier et al., 2018a).

4.4. Grain N concentration

In previous FACE studies under subtropical and semi-arid conditions, e[CO_2] decreased grain protein concentration by -9 and -10% under N deficiency (Kimball et al., 2001; Walker et al., 2017). Those results contrast with the present study, in which hardly any reduction of grain N concentration (-1%) was found under *Nd* (NNI: 0.4). The decrease under *Nad* (NNI: 0.8) (-6%) is consistent with other FACE studies using adequate N levels in which reductions were found to be -5 to -9% (Kimball et al., 2002; Erbs et al., 2010; Lam et al., 2012; Tausz et al., 2017). The decrease under *Nex* (NNI: 1.1) (-4%) contrasts with the FACE study of Kimball et al. (2001) where no reduction was found under ample N supply.

Grain N concentration was considerably larger in 2015 than 2014, which strongly exceeded the e[CO_2] effect. This could be partly explained under *Nd* and *Nad* by the increased post-anthesis N availability per grain due to the smaller grain number under *Nd* (Dier et al., 2018b) and the higher N fertilizer supply at anthesis under *Nad* in 2015. Moreover, under *Nad* and *Nex*, grain number was increased but individual grain weight was decreased in 2015 (Dier et al., 2018b). Therefore, dilution of N content per grain by grain growth was decreased in 2015 compared to 2014.

The smaller relative reduction of grain N concentration by e[CO_2] under *Nd* compared to high N supply could be attributed to the smaller grain yield stimulation under *Nd* (Dier et al., 2018b) and that grain N accumulation is partly sink regulated, especially under low grain N concentration (Martre et al., 2003). The contrasting results of grain N concentration under N deficiency between the present (-1%) and the Kimball et al. (2001) study (-9%) could be explained by differences in post-anthesis soil N availability. While a large proportion of grain N originated from Nabs in the present study, post-anthesis soil N availability was possibly deficient in the study of Kimball et al. (2001) as, unlike the present study, flag leaf senescence was accelerated under e[CO_2] (Brooks et al., 2000).

The decrease of grain N concentration by e[CO_2] could be attributed to several factors. As indicated by the regression of grain number on aboveground and grain N yield (Fig. 6), the N source per grain (i.e. vegetative N) was reduced under e[CO_2]. Secondly, the finding that grain N concentration was more strongly affected by e[CO_2] than mean N content per grain while individual grain weight was increased (Dier et al., 2018b) indicate that growth dilution was another reason. Moreover, the e[CO_2] induced increase of the proportion of stem in aboveground N at the expense of leaf, having much larger NRE than stem, possibly led to a decreased whole-plant Nrem potential.

4.5. Recommendation of N fertilization in future

The results herein show that grain N yield increased with rising grain number and this relation was not affected by e[CO_2] (Fig. 6). Hence, increased N fertilization in the phase between flag leaf emergence and anthesis, when NNI (Ravier et al., 2017) and e[CO_2] (Fisher and Aguilar, 1976) have their greatest influence on grain number, might enhance the grain number and thus N yield increase by e[CO_2]. Moreover, as indicated by the positive e[CO_2] effect on Nabs, supply of additional N at anthesis could enable the plant to increase grain N concentration under e[CO_2], provided that the plant N status before anthesis is not excessive.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.fcr.2019.02.013.

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3.6 Anhang

Table S1: Result of analysis of covariance of the effect of CO_2 levels and aboveground biomass (W) on aboveground N concentration (N%). Analysis was conducted with the data of both years.

	Effect		
N level	CO_2	W	CO_2 x W
Nd	ns	***	ns
Nad	ns	***	ns
Nex	ns	***	ns

Analysis is based on the linearized relation of $N\% = aW^{-b}$ that is $logN\% = log(a) - b\ log(W)$. a= parameter corresponding to the CO_2 effect; b= parameter corresponding to the W and CO_2 x W effect.

The significance threshold is at P =0.1; ***P <0.001.

Table S2: Effect of the two CO_2 and three N levels on tissue N concentration at anthesis and maturity. Shown are mean values (n=3) and the percentage effect of e[CO_2] (Δ (%)). Different small letters indicate significant differences among the marginal means (mean over both CO_2 levels) of the N treatments. If the F-test (**Table 2**) resulted in a significant CO_2 x N interaction, then different capital letters indicate significant differences for CO_2 means separate for each N treatment and different small letters significant differences for N means separate for each CO_2 treatment (letters are in bold for e[CO_2]). All letters refer to the mean over both years.

		2014			2015		
		Nd	*Nad*	*Nex*	*Nd*	*Nad*	*Nex*
Nconc stem (mg N g^{-1})							
Anthesis	a[CO_2]	5.15 c	8.53 b	11.7 a	6.18 c	8.57 b	12.4 a
	e[CO_2]	4.98 c	8.16 b	11.7 a	5.89 c	8.09 b	13.9 a
	Δ (%)	-3	-4	0	-5	-6	12
Maturity	a[CO_2]	1.70 c	3.16 b	6.19 a	2.33 c	3.64 b	5.57 a
	e[CO_2]	1.55 c	2.63 b	5.27 a	2.37 c	3.49 b	5.51 a
	Δ (%)	-9	-17	-15	2	-4	-1
Nconc leaf (mg N g^{-1})							
Anthesis	a[CO_2]	18.0 c	27.6 b	32.5 a	18.5 c	29.3 b	35.2 a
	e[CO_2]	16.8 c	26.6 b	32.3 a	16.5 c	26.7 b	34.5 a
	Δ (%)	-7	-4	-1	-11	-9	-2
Maturity	a[CO_2]	6.10 A c	12.1 A b	16.2 A a	5.40 A c	9.10 A b	14.4 A a
	e[CO_2]	5.47 A **c**	10.0 B **b**	15.5 B **a**	5.20 A **c**	7.77 B **b**	12.1 B **a**
	Δ (%)	-10	-18	-4	-4	-15	-16
Nconc ear,chaff (mg N g^{-1})							
Anthesis	a[CO_2]	14.3 A c	18.6 A b	19.6 A a	14.6 A c	17.7 A b	19.3 A a
(Ear)	e[CO_2]	13.6 A **c**	17.5 B **b**	20.4 A **a**	13.9 A **c**	16.8 B **b**	19.9 A **a**
	Δ (%)	-5	-6	4	-4	-5	3
Maturity	a[CO_2]	3.79 c	4.67 b	6.33 a	3.45 c	4.67 b	5.99 a
(Chaff)	e[CO_2]	3.70 c	4.07 b	5.80 a	3.61 c	4.51 b	5.69 a
	Δ (%)	-2	-13	-8	5	-4	-5

Table S3: Effect of the two CO_2 and three N levels on N yield of the vegetative organs. Shown are mean values (n=3) and the percentage effect of e[CO_2] (Δ (%)). Different small letters indicate significant differences among the marginal means of the N treatments. If the F-test (**Table 2**) resulted in a significant CO_2 x N interaction, then different capital letters indicate significant differences for CO_2 means separate for each N treatment and different small letters significant differences for N means separate for each CO_2 treatment (letters are in bold for e[CO_2]). All letters refer to the mean over both years.

		2014			2015		
		Nd	*Nad*	*Nex*	*Nd*	*Nad*	*Nex*
N yield stem (g N m^{-2})							
Anthesis	a[CO_2]	2.33 c	5.13 b	7.55 a	2.51 c	5.69 b	8.35 a
	e[CO_2]	2.63 c	5.89 b	8.09 a	2.42 c	6.22 b	9.80 a
	Δ (%)	13	15	7	-4	9	17
Maturity	a[CO_2]	0.58 c	1.76 b	3.70 a	0.62 c	1.97 b	3.43 a
	e[CO_2]	0.62 c	1.72 b	3.58 a	0.75 c	2.32 b	3.89 a
	Δ (%)	8	-2	-3	21	18	13
N yield leaf (g N m^{-2})							
Anthesis	a[CO_2]	1.80 c	5.24 b	7.73 a	1.77 c	5.49 b	7.86 a
	e[CO_2]	1.94 c	5.59 b	7.63 a	1.55 c	5.29 b	8.29 a
	Δ (%)	8	7	-1	-12	-4	5
Maturity	a[CO_2]	0.42 c	1.36 b	2.28 a	0.25 c	0.88 b	1.77 a
	e[CO_2]	0.37 c	1.25 b	2.23 a	0.28 c	0.92 b	1.61 a
	Δ (%)	-10	-8	-2	14	4	-9
N yield ear,chaff (g N m^{-2})							
Anthesis	a[CO_2]	1.40 c	2.87 b	3.46 a	1.55 c	2.98 b	3.60 a
(Ear)	e[CO_2]	1.54 c	3.26 b	3.85 a	1.37 c	3.28 b	4.06 a
	Δ (%)	10	14	11	-11	10	13
Maturity	a[CO_2]	0.38 c	0.76 b	1.11 a	0.30 c	0.83 b	1.20 a
(Chaff)	e[CO_2]	0.43 c	0.75 b	1.15 a	0.37 c	0.96 b	1.29 a
	Δ (%)	11	-2	4	22	16	8

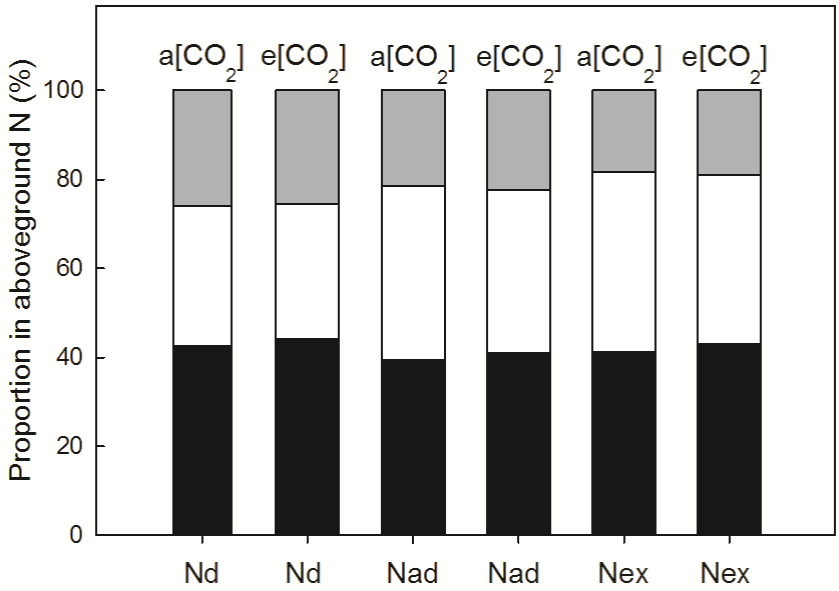

Fig. S1: Effect of the two CO_2 and three N levels on the proportion of stem (black), leaf (white) and ear (grey) in total aboveground plant N at anthesis. Shown are the mean values pooled across both years. e[CO_2] significantly increased the proportion in aboveground N of stem by 4, 4 and 5%, but decreased the one of leaf by -4, -6 and -7% under *Nd*, *Nad* and *Nex*, respectively.

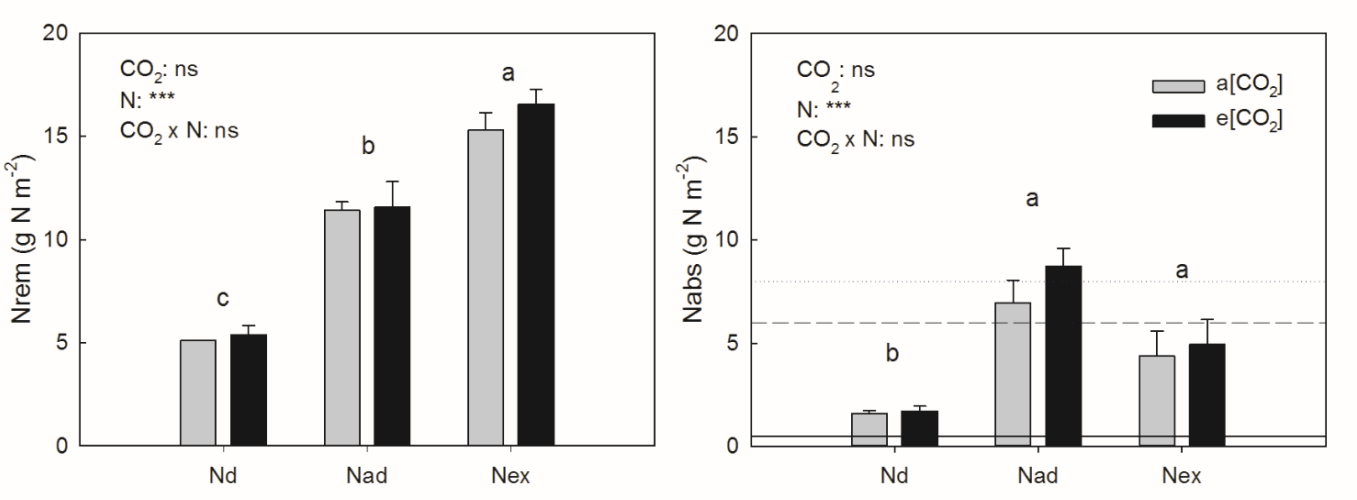

Fig. S2: Effect of the two CO_2 and three N levels on total plant N remobilization (Nrem) and post-anthesis N uptake (Nabs) based on ^{15}N labelling that was only conducted in 2015. Shown are the mean values (± S.E.M; n=3) and F-test results. Different letters indicate significant differences between the marginal N means. The lines indicate the amount of N fertilizer applied at anthesis (solid = *Nd*, dashed = *Nad* and dotted =*Nex*).

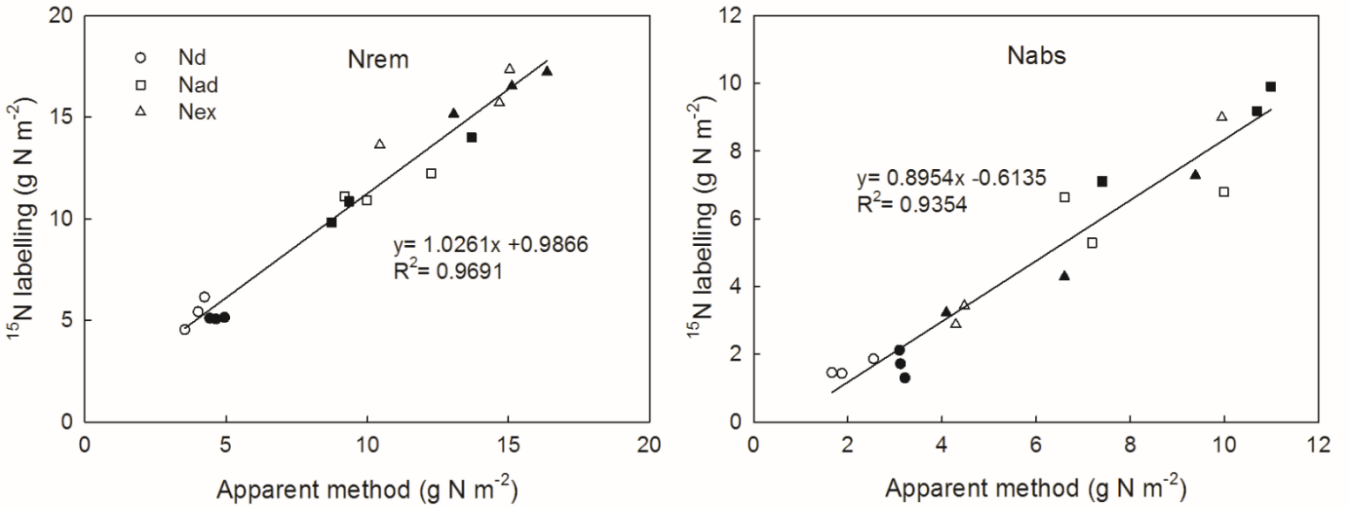

Fig. S3: Comparison of N remobilization (Nrem) and post-anthesis N uptake (Nabs) determined either by the apparent method or by ^{15}N labelling. Open symbols = ambient [CO_2]; closed symbols = e[CO_2].

4. Kapitel: Wirkung von e[CO_2] auf NO_3^--Assimilation und Wachstum bei NO_3^-- und NH_4^+-basierter N-Düngung

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PRIMARY RESEARCH ARTICLE

WILEY Global Change Biology

Effects of free air carbon dioxide enrichment (FACE) on nitrogen assimilation and growth of winter wheat under nitrate and ammonium fertilization

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Abstract

A 2-year **F**ree **A**ir **C**O_2 **E**nrichment (FACE) experiment was conducted with winter wheat. It was investigated whether elevated atmospheric CO_2 concentration (e[CO_2]) inhibit nitrate assimilation and whether better growth and nitrogen acquisition under e[CO_2] can be achieved with an ammonium-based fertilization as it was observed in hydroponic culture with wheat. Under e[CO_2] a decrease in nitrate assimilation has been discussed as the cause for observed declines in protein concentration in C_3 cereals. Wheat was grown under ambient [CO_2] and e[CO_2] (600 ppm) with three levels (deficiency, optimal, and excessive) of nitrate-based fertilization (calcium ammonium nitrate; CAN) or with optimal ammonium-based fertilization. Ammonium fertilization was applied via injection of an ammonium solution into the soil in the 1st year and by surface application of urea combined with nitrification inhibitors (UNI) in the 2nd year. Results showed that ammonium-based fertilization was successfully achieved in the 2nd year with respect to nitrification control, as soil ammonium concentration was considerably higher over the growing season for UNI fertilized plots compared to optimal CAN plots. Also, stem nitrate concentration, flag leaf nitrate reductase activity, and transcript levels were lower in UNI fertilized plants compared to optimal CAN. Regarding the e[CO_2] effect on nitrate reductase activity and transcript levels, no alteration could be observed for any nitrogen fertilizer treatment. Flag leaf growth was stimulated under e[CO_2] leading to an enhanced nitrate reductase activity referred to m^2 ground area at late flowering being in line with a higher nitrogen acquisition under e[CO_2]. Moreover, nitrogen acquisition was considerably higher in nitrate fertilized plants compared to ammonium fertilized plants under e[CO_2]. Our results obtained under field conditions show that a change from nitrate- to ammonium-based fertilization will not lead to a better growth and nitrogen acquisition of winter wheat under future e[CO_2].

KEYWORDS
ammonium fertilization, climate change, free air CO_2 enrichment, N acquisition, nitrate assimilation, nitrate fertilization, nitrate reductase, *Triticum aestivum*

*Both authors contributed equally to this work.

1 | INTRODUCTION

Atmospheric carbon dioxide concentration ([CO_2]) is predicted to continuously rise from currently 400 ppm [CO_2] up to a level of 790–1020 ppm by the end of this century (IPCC, 2013). C_3 cereals react to elevated CO_2 concentration (e[CO_2]) with an increase in (i) photosynthetic CO_2 fixation (Ainsworth & Long, 2005), (ii) above- and below-ground biomass production (Ma et al., 2007; Pacholski, Manderscheid, & Weigel, 2015; Ziska, Morris, & Goins, 2004), and (iii) grain yield (Han et al., 2015; Weigel & Manderscheid, 2012). However, CO_2 stimulation effects are generally accompanied by a considerable decrease in nitrogen (N) concentrations of plant tissue (Ainsworth & Long, 2005; Cotrufo, Ineson, & Scott, 1998; Taub, Miller, & Allen, 2008). The possible mechanisms behind this decrease remain elusive but have been linked to an altered nitrate (NO_3^-) assimilation (Bloom, Burger, Rubio-Asensio, & Cousins, 2010; Pleijel & Uddling, 2012).

NO_3^- is the main N form taken up by cereals from agricultural soils (Andrews, Raven, & Lea, 2013). At low soil NO_3^- concentrations, NO_3^- is primarily assimilated in the roots, but with increasing soil NO_3^- concentration, the NO_3^- reduction is shifted toward the leaves (Andrews, Morton, Lieffering, & Bisset, 1992). Ammonium (NH_4^+) is another important N source for cereals, however, with lower uptake and assimilation compared to NO_3^- due to rapid nitrification in the soil which converts NH_4^+ into NO_3^- (Andrews et al., 2013; Subbarao et al., 2006). Nitrification occurs in two consecutive steps, whereby the first step is the reduction in NH_4^+ to nitrite (NO_2^-) that acidifies the soil solution triggered by bacteria from the *Nitrosomonas* genera and the second step is the reduction in NO_2^- to NO_3^- triggered by bacteria from the *Nitrobacter* genera. High nitrification rates occur at a neutral soil solution pH (Suzuki, Dular, & Kwok, 1974). As cereal NH_4^+ uptake acidifies the soil solution and NO_3^- uptake alkalifies it, nitrification is therefore decreased by NH_4^+ but stimulated by plant NO_3^- uptake. NH_4^+ is mainly assimilated in roots via the glutamine synthetase/glutamate synthase pathway and incorporated into amino acids. NO_3^- assimilation follows the same pathway, but requires two consecutive upstream reactions. The first one is the reduction in NO_3^- to NO_2^- catalyzed by the enzyme nitrate reductase (NR) in the cytoplasm. The second reaction is the reduction in NO_2^- to NH_4^+ via nitrite reductase inside the chloroplasts.

Recent evidence indicates that e[CO_2] might interfere with NO_3^- assimilation by impeding NO_3^- and NO_2^- reduction in C_3 plants (Bloom, 2015a). For instance, it has been observed in wheat and *Arabidopsis thaliana* that e[CO_2] lowered the incorporation of ^{15}N labeled NO_3^- into organic N compounds (Bloom et al., 2010) and decreased the NR activity (NRA) of wheat leaves (Bloom, Smart, Nguyen, & Searles, 2002). Reduced NO_3^- assimilation of C_3 plants is thought to be strongly linked to a lower photorespiration rate under e[CO_2] (Bloom, 2015a; Rachmilevitch, Cousins, & Bloom, 2004) and a subsequent decreased supply of the reductant nicotinamide adenine dinucleotide (NADH) which powers the NO_3^- reduction (Bloom, 2015b). Photorespiration drives the malate transport from the chloroplast into the cytoplasm where it is oxidized to oxalacetic acid for the generation of NADH (Bloom, 2015b; Rachmilevitch et al., 2004). This "malate valve" represents an important player in the complex network between N and C metabolism and is involved in supplying energy for NR (Scheibe, 2004; Taniguchi & Miyake, 2012).

The process of photorespiration is favored by the oxygenase function of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) under ambient [CO_2] and high temperatures. Because e[CO_2] shifts the activity of RuBisCO toward CO_2 fixation at high temperatures, inhibition of NO_3^- assimilation by e[CO_2] is thought to take place primarily at elevated temperatures (Bloom, 2015b; Jauregui et al., 2015). However, the oxidation of triose phosphates, derived from CO_2 fixation, might also increase NADH availability and is therefore an important energy source for NO_3^- assimilation (Foyer, Bloom, Queval, & Noctor, 2009; Kaiser, Kandlbinder, Stoimenova, & Glaab, 2000). Consequently, the inhibition of NO_3^- assimilation induced by e[CO_2] as cited above is assumed to be the reason for a decrease in plant N and in particular declining leaf and cereal grain protein concentrations (Bloom, 2015a; Bloom et al., 2010). A decline in leaf protein could negatively affect animal feedstuff quality and hence animal production including changes in livestock grazing patterns (Sinclair et al., 2000). Furthermore, a reduction in cereal grain protein might decrease flour baking quality (Wieser, Manderscheid, Erbs, & Weigel, 2008) and, particularly, could exacerbate protein deficiency in developing countries (Myers et al., 2014). Therefore, food and feed qualities are predicted to be severely affected by e[CO_2] if cereals are continuously fertilized with NO_3^--dominated N fertilizer regimens (Bloom et al., 2010). To prevent these adverse effects of e[CO_2] on NO_3^- assimilation and its resulting negative consequences for crop growth and food as well as feed quality it has been proposed to use NH_4^+-based N fertilizers as an alternative. This recommendation is based on laboratory experiments in hydroponics under e[CO_2] conditions where several C_3 species showed enhanced growth and N acquisition when they received NH_4^+ instead of NO_3^- as a sole N source (Bloom et al., 2002, 2012; Carlisle, Myers, Raboy, & Bloom, 2012).

Thus far, nearly all experiments investigating e[CO_2] effects on crop NO_3^- assimilation were conducted in hydroponic cultures under laboratory conditions with a restricted rooting volume (Bloom et al., 2002, 2010, 2012). These restrictions are known to strongly alter e[CO_2] responses on growth and N acquisition (Arp, 1991; Long, Ainsworth, Leakey, & Morgan, 2005), which limits the reliability of predictions of e[CO_2] effects on N assimilation in field-grown cereals based on such experiments. While there is only one field experiment under free air CO_2 enrichment (FACE) where an inhibition of NO_3^- assimilation by e[CO_2] has been shown (Bloom, Burger, Kimball, & Pinter, 2014), there are no FACE experiments where NO_3^--based fertilization has been compared directly with NH_4^+-based N fertilization under e[CO_2].

To test whether e[CO_2] decreases NO_3^- assimilation in the field, a 2-year FACE experiment was carried out with winter wheat (*Triticum aestivum*) supplied with three levels of a NO_3^- and one level of

an NH_4^+-based fertilization. The NH_4^+-based fertilization was applied in the 1st year by **C**ontrolled **U**ptake **L**ong **T**erm **A**mmonium **N**utrition (CULTAN) fertilization, a method where nitrification immune NH_4^+ depots are injected into the root space at the beginning of the growing season (Petersen, Hansen, & Sorensen, 2004; Wetselaar, Passioura, & Singh, 1972). However, it is still a matter of discussion whether nitrification is effectively inhibited with CULTAN (Deppe et al., 2016). Therefore, in the 2nd year a NH_4^+-based fertilization was carried out by applying N as urea combined with nitrification inhibitors directly into the soil. There, urea is quickly transformed to NH_4^+, but the nitrification inhibitors impede the first nitrification step (Subbarao et al., 2006) and additionally prevent toxic NO_2^- accumulation in the soil (Ma, Shan, & Yan, 2015). Based on a sound field trial with three different quantities of NO_3^--based fertilization (deficiency, optimal, and excessive) together with an optimal quantity of NH_4^+ based fertilization the following hypotheses were tested: (i) Does e[CO_2] inhibit leaf NO_3^- assimilation in the field by decreasing NR activity and thus influencing NR transcript levels? and (ii) Is there an intensified growth stimulation without a decline in N acquisition if plants take up N primarily in form of NH_4^+ instead of NO_3^-?

2 | MATERIALS AND METHODS

2.1 | Experimental design and crop management

The experiment was conducted on a field site (52°18′N, 10°26′E, 79 ma.s.l.) at the Thünen Institute in Braunschweig, Germany, in 2014 and 2015. The mean annual temperature is 9.1°C and the mean annual precipitation is 617 mm. The soil profile has a depth of about 60 cm (−30 cm Ap, −15 cm Al, −15 cm Bt, and >60–70 cm CII). The lower layers, in particular >70 cm, are characterized by a coarser soil texture (almost pure sand) and are structured by the succession of thin silt/clay layers. The soil in the plough horizon (0–40 cm) is a luvisol of loamy sand texture (69% sand, 24% silt, and 7% clay). Measuring of soil variables in each subplot (mean±*SD*; $n = 24$) in March 2015 resulted in a pH of 6.88 ± 0.39 and a carbon and nitrogen content of 1.00 ± 0.04% and 0.09 ± 0.00%, respectively. The lower (−1.5 MPa) and upper limits (−0.01 MPa soil water tension) of plant-available soil water are a volumetric soil water content of 5 and 23%, respectively. Altogether, the soil has low-to-intermediate fertility with a shallow rooting zone.

Winter wheat (*Triticum aestivum* L. variety "*Batis*") was grown at ambient [CO_2] of about 390 ppm and e[CO_2] of 600 ppm. The CO_2 treatments were carried out on circular plots with a diameter of 20 m, in which four subplots (3 m × 5 m) as N treatments were randomly established. Altogether, the experiment consisted of eight different CO_2 × N fertilization combinations, which were replicated three times. The CO_2 plots were placed at the same position each year.

CO_2 enrichment was carried out by a FACE system constructed according to the Brookhaven National Laboratory design (Lewin, Hendrey, & Kolber, 1992). CO_2 enrichment started at the four leaf stage on March the 31st in 2014 and at the three leaf stage on March the 12th in 2015. CO_2 enrichment took place during the daytime hours and was interrupted if wind speed exceeded 6 m/s or if air temperature fell below 5°C. The 1 min average [CO_2] was within the range of 600 ppm ± 10% for 95.6% and 95.7% for the operation time in 2014 and 2015, respectively. The average daytime [CO_2] at the ambient air plots was 394 ppm in 2014 and 392 ppm in 2015.

Winter wheat was sown at the end of October with a density of 380 kernels/m^2. Crop management measures were performed according to local farm practice with adequate nutrient supply and pesticide applications. N fertilizer was applied as summarized in Table 1. Three different NO_3^--based N fertilization regimes were implemented with calcium ammonium nitrate (CAN, 27% N) with a deficient (CAN40/ CAN35), an optimal (CAN180/ CAN200) and an excessive (CAN320/ CAN320) quantity for 2014 and 2015, respectively. The optimal level corresponds to the present common agricultural practice. For CULTAN fertilization in 2014 (CUL180), a water solution of ammonium sulfate (8% N) was manually injected into the soil in a depth of 7 cm with a density of 24 injection holes per m^2. For the combined urea (46.5% N) and nitrification inhibitors treatment in 2015 (UNI200) the commercial urea fertilizer Alzon M+ (SKW Stickstoffwerke Piesteritz, Germany) was applied, containing 1.4% nitrification inhibitors (dicyandiamide + triazole). Supplementary nitrification inhibitors (5% triazole + methylpyrazole) were applied on three other dates to control nitrification (April 29th, May 11th, and June 15th 2015). The used amounts were equivalent to the quantity of inhibitors contained in Alzon M+ for the application of 10, 35, and 40 kg N/ha, respectively. To prevent drought stress and nitrate leaching, soil water content was kept in the range of 50–90% of plant-available water content by manual irrigation. Figure 1 presents daily mean temperature, precipitation, and irrigation over the main

TABLE 1 N fertilizer treatments with application dates and quantities. CAN refers to fertilization with calcium ammonium nitrate, CUL to CULTAN fertilization, and UNI to the application of urea + nitrification inhibitors

2014							2015					
	Quantity (kg N/ha)							Quantity (kg N/ha)				
	Mar 17th	Mar 19th	Apr 14th	May 4th	June 2nd	Total		Mar 18th	Apr 28th	May 11th	June 11th	Total
Treatment							Treatment					
CAN40		20	20			40	CAN35	15	15		5	35
CAN180		70	35	35	40	180	CAN200	70	35	35	60	200
CAN320		120	60	60	80	320	CAN320	120	60	60	80	320
CUL180	180					180	UNI200	70	35	35	60	200

growing season. Average temperature of the main growing season (April–July) in 2014 (15.2°C) and in 2015 (14.0°C) was similar to the long-term mean (1980–2010: 14.1°C). For monitoring plant water supply, volumetric soil water content in the 40 cm soil profile was measured twice a week with time domain reflectometry (TDR) sensors.

2.2 | Determination of soil NO_3^--N and NH_4^+-N and stem NO_3^- concentration

For winter wheat growth at this site, it was found that 90% of the root biomass was located in the 0–30 cm soil profile (Pacholski et al., 2015). Therefore, soil samples from the 30 cm soil profile were taken on a weekly basis from CAN200- and UNI200-treated subplots in 2015. At each sampling, subsamples with approx. 40 g soil were taken from six random positions of each subplot and the subsamples were pooled for further analysis. Soil samples were extracted with $CaCl_2$ (0.01 M) and NO_3^--N and NH_4^+-N determinations were carried out photometrically with a Continuous-Flow Analyzer (Model SA3000/5000, Scalar, Netherlands).

Stems from 30 plants were randomly chosen from four destructive harvests (0.5 m^2 ground area) at early stem elongation, early heading, flowering, and milk-ripe stage. Stem NO_3^- concentration was determined according to Padgett and Leonard (1993) with some modifications. Stem material was ground to a fine powder in a rotor mill (Brabender, Duisburg, Germany) after drying at 105°C. For each sample, 50 mg stem material was mixed with 25 ml distilled water and incubated in a 45°C water bath for 4 hr. The extracts and water blanks were gravity filtered through Whatman No.1 filters and analyzed photometrically as described above.

2.3 | Flag leaf sampling for nitrate reductase activity and growth parameter determination

Flag leaves were sampled twice before flowering in 2014. In 2015 flag leaves were sampled before flowering and at late flowering. The sampling dates and the related environmental conditions are shown in Table 2. At each sampling 10 flag leaf blades were randomly selected from all experimental plots and N treatments, respectively. They were immediately frozen and ground to a fine powder in liquid N_2 for further analyses. In addition, 20 flag leaf blades were sampled to measure fresh weight, leaf area with a leaf area meter (Model LI-3100, LICOR, USA), and dry weight after drying at 105°C. With these data dry weight/fresh weight ratio, individual flag leaf area (FLA), individual flag leaf biomass, and specific flag leaf weight (SFLW) were calculated.

Flag leaf number (FLN) was derived from ear number counted in destructive plant harvest of 0.5 m^2 ground area at flowering and milk-ripe stage and of 1.8 m^2 ground area at grain maturity. Flag leaf biomass per m^2 ground area (FLB) was determined by multiplying individual flag leaf biomass with FLN. Flag leaf area index (FLAI) was calculated as the quotient from FLB and SFLW.

2.4 | Total canopy sampling and green area index determination of ear and residual leaves

Three destructive harvests were taken at early heading, flowering, and milk-ripe stage (0.5 m^2 ground area). Green area of leaf blades and ears was determined with a leaf area meter (Model LI-3100

TABLE 2 Dates of flag leaf sampling for NRA determination and environmental conditions during sampling. Values are indicated as daily average and for the hour of sampling. Samples were always taken at midday between 1 and 2 p.m.

	2014		2015	
Date	May 19th	May 22th	May 29th	June 15th
Daily mean temperature (°C)	14.4	22.8	13.2	13.5
Temperature at sampling (°C)	16.7	27.6	16.5	18.2
Daily mean irradiance (W/m^2)	214	290	223	322
Irradiance at sampling (W/m^2)	791	762	383	898

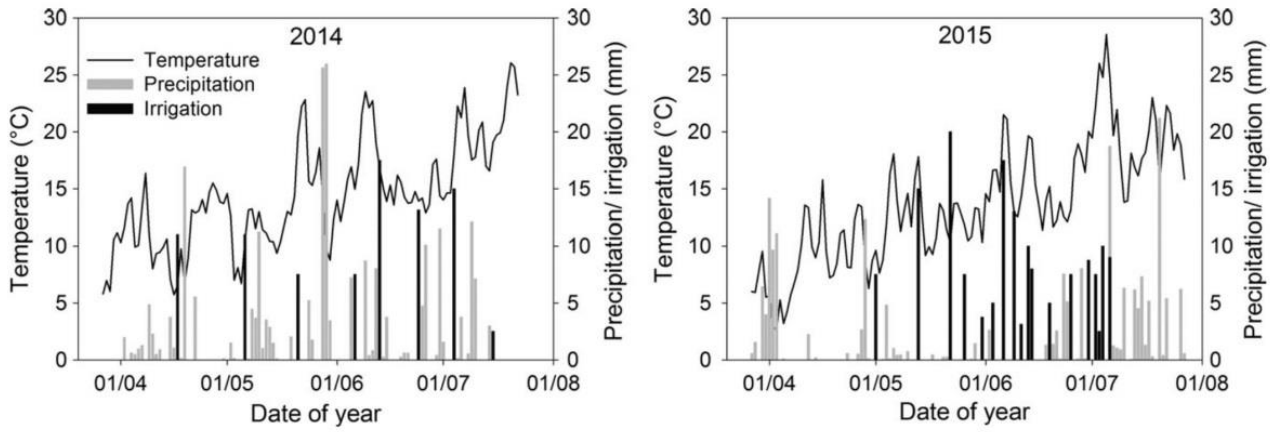

FIGURE 1 Course of daily mean temperature, precipitation, and irrigation over the main growing season

from LICOR) of subsamples of 25% of the harvests. In addition, the same subsample was used to determine biomass partitioning to leaf blades, ears, and stems after drying at 105°C. The rest of the sample was directly dried at 105°C followed by dry weight determination. Green area indices were determined on the basis of the measured green areas and dry weights. Leaf area index (LAI) was calculated as the mean over the three harvests and ear area index as the mean over the two final harvests. Residual leaf area index (RLAI) was computed by subtracting FLAI from LAI.

2.5 | Calculation of radiation-adjusted flag leaf and residual leaf area index

Irradiance (% of value at the top of canopy) that reached a certain canopy fraction (*I*) was calculated with the equation of Monsi and Saeki (2005):

$$I = e^{-k*\mathrm{GAI}}$$

where *k* is the extinction coefficient and GAI the cumulative green area index at the bottom of the considered canopy fraction. The extinction coefficient was assumed to be 0.53 prior and 0.58 from flowering stage onward according to Shearman, Sylvester-Bradley, Scott, and Foulkes (2005). The plant canopy was divided into three layers (ear, flag leaf, and residual leaves). Irradiance (relative irradiance with regard to the value at the top of the canopy) on a certain canopy layer was calculated by the mean of the value on top (i.e., the bottom of the overlying canopy layer) and on the bottom of the considered canopy layer. A value was calculated for the time before (minus ear) and after the flowering stage (plus ear) and both values were averaged. Radiation-adjusted flag leaf and residual leaf area indices were calculated by multiplying the irradiance on the flag leaf and residual leaf fraction with FLAI and RLAI, respectively.

2.6 | Measurement of aboveground biomass and N acquisition

Data of leaf blade, ear, stem, and total aboveground biomass at the milk-ripe stage were collected as described in 2.4. Dried plant material of leaf blades, ears, and stems was ground to a fine powder in a rotor mill (Brabender, Germany) and this material was analyzed for its N concentration (N conc) using an element analyzer (Leco TruSpec CNS, USA). N acquisition was calculated for each plant fraction by multiplying the N conc with the aboveground biomass. Total aboveground N acquisition was calculated by adding up the N acquisition of leaf blade, ear, and stem.

2.7 | Determination of flag leaf nitrate reductase activity (NRA)

Flag leaf NRA per gram fresh weight was measured with an in vitro assay according to Scheible, Lauerer, Schulze, Caboche, and Stitt (1997) with modifications. One hundred milligram of the liquid N_2 powdered material was thoroughly mixed with four volumes of ice-cold extraction buffer (100 mM HEPES-KOH (pH 7.5), 5 mM magnesium acetate, 1 mM EDTA, 10% (v/v) glycerol, 0.5% (w/v) BSA, 0.1% Triton X-100, 1% polyvinylpolypyrrolidone (PVPP), 5 μM $NaMoO_4$, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM DTT, 20 μM FAD, and 25 μM leupeptin).

Only the active state of NR was measured, so one volume of extract was mixed with five volumes of prewarmed (25.5°C) assay buffer in a heat block (100 mM HEPES-KOH, 6 mM KNO_3, 12 mM $MgOAc_2$, 0.6 mM NADH, 20 μM leupeptin, 12 μM FAD, 0.3 mM DTT, 6 μM $NaMoO_4$). After 5, 10, and 15 min at 25.5°C, respectively, the reaction was stopped by removing a 300 μl aliquot of the assay mixture and adding each aliquot to 25 μl 600 mM zinc acetate. To remove unreacted NADH, 75 μl 0.25 mM phenazine methosulfate was added and incubated for 15 min in the dark. Formed NO_2^- was determined colorimetrically as described by Scheible et al. (1997). Each sample was run in triplicates.

NRA per gram fresh weight was referred to m^2 ground area basis by using the dry weight/fresh weight ratio and FLB.

2.8 | Nitrate reductase gene expression analysis

Total wheat RNA of flag leaves from the second sampling at late flowering in 2015 (Table 2) was isolated using the innuPREP Plant RNA Kit (Analytik Jena, Germany) according to the manufacturer`s protocol with an additional DNase I digest (innuPREP DNase I-Mix (Analytik Jena, Germany)). Two hundred nanogram of total RNA was reverse transcribed via the innuSCRIPT reverse transcriptase kit (Analytik Jena, Germany). Quantitative reverse transcription PCR reactions were performed using the TOptical Thermocycler (Analytik Jena, Germany) and the innuMIX qPCR SyGreen MasterMix (Analytik Jena, Germany) with 1:50 cDNA dilutions. Nitrate reductase (*nia)* gene sequences on chromosomes 6AS, 6DS, and 7DS were identified via BLAST analysis of the International Wheat Genome Sequencing Consortium and Ensembl plants database. Primer sequences and gene accessions are listed in Table S1 in the supporting information. ADP-ribosylation factor and translation elongation factor 1 alpha were used as reference genes (Gimenez, Piston, & Atienza, 2011; Paolacci, Tanzarella, Porceddu, & Ciaffi, 2009). Primer efficiency was analyzed in cDNA dilution series and primer combinations with efficiencies between 95% and 105% were used. For calculation of relative fold changes and to test the statistical significance of the gene expression ratios, the **R**elative **E**xpression **S**oftware **T**ool 2009 (REST 2009) (Pfaffl, Horgan, & Dempfle, 2002) was used.

2.9 | Statistics

Each $CO_2 \times N$ combination was replicated three times. As an exception to this procedure for the measurement of NRA the samples in 2014 CUL180 ambient, CAN180 e[CO_2], and CAN320 e[CO_2] were tested in duplicates, and both CAN40 and CUL180 e[CO_2] as a singular probe due to tube breakage.

Wald *F*-tests were done with SAS (version 9.4) proc mixed using the following mixed model:

$$y = \mu + CO_2 + N + CO_2 \times N + R + e$$

where y is the dependent variable, μ the overall mean, CO_2 the CO_2 effect, N the N fertilizer effect, $CO_2 \times N$ the interaction effect, R the main plot error, and e the residual error. When sampling time was added as a third factor to the mixed model, *F*-tests were carried out as repeated measurements. In this case, sampling time was treated as a fixed effect and the UN(1) covariance model was used to model the correlation among samplings at different time points for the random main plot and residual error. The UN(1) model applies no covariance for sampling time, but allows different error variances for the different samplings. Least square difference (LSD) tests were carried out with SAS pro glimmix after removing the nonsignificant effects from the model. Mean values were regarded as significantly different if $p < .05$.

Each year was considered as an individual experiment. To take possible legacy effects with respect to soil mineral N availability into account, total soil mineral N in the 30 cm plough horizon was measured in all plots in March 2014 and 2015. Statistical analysis did not show a significant CO_2, N, and $CO_2 \times N$ effect on total soil mineral N as well as the NO_3^--N and NH_4^+-N content. Averaged over all CO_2 and N treatments the total soil mineral N was 14 kg N/ha in 2014 and 22 kg N/ha in 2015.

3 | RESULTS

3.1 | Pool size of mineral N in soil and wheat stem NO_3^- concentration

In 2015, NO_3^--N and NH_4^+-N were monitored over the whole growing season in the uppermost 30 cm soil profile of the NO_3^--based fertilization (CAN200) and the NH_4^+-based fertilization plots (UNI200). Statistical analysis showed neither a significant CO_2 effect nor an interaction with CO_2 for all tested parameters (Table 3). Therefore, mean values of NO_3^--N, NH_4^+-N, and total soil mineral N over both CO_2 treatments were used and are depicted in Figure 2. Statistical analysis comparing the time course of NO_3^--N as well as NH_4^+-N between the CAN200 and the UNI200 plots showed a significant N and N × sampling effect (Table 3). As depicted in Figure 2a soil NO_3^--N concentration of the CAN200 plots was higher compared to soil NH_4^+-N concentration over the whole growing season in 2015. In the UNI200 plots soil NH_4^+-N concentration was higher compared to soil NO_3^--N concentration over the growing season, except for one sampling at the end of April (Figure 2b). Averaged over the two CO_2 treatments and all samplings the soil NO_3^--N concentration was 21.3 kg/ha in the CAN200 plots and 9.0 kg/ha in the UNI200 plots. The soil NH_4^+-N concentration was 8.6 kg/ha in the CAN200 plots and 17.0 kg/ha in the UNI200 plots. However, no significant differences in total soil mineral N between the CAN200 and the UNI200 plots were observed (Table 3, Figure 2c).

Stem NO_3^- concentration was significantly lower in the UNI200 compared to the CAN200 treatment at two of four samplings in 2015 (Table 3, Figure 3). In contrast, no significant differences in stem NO_3^- concentration between the NO_3^--based CAN180 treatment and the CUL180 treatment were observed in 2014. Furthermore, CO_2 enrichment had no significant effect on stem NO_3^- concentration in both years.

3.2 | Leaf and ear growth

Table 4 presents the percentage effect of e[CO_2] and the statistics of the CO_2, N, and $CO_2 \times N$ effect on variables describing leaf and ear growth and Tables S2 and S3 the corresponding mean values. e[CO_2] significantly increased the following variables: flag leaf biomass per m^2 ground area, flag leaf area index, and radiation-adjusted flag leaf area index in all N treatments in both years; flag leaf number per m^2 ground area and ear area index in all N treatments in 2014; and flag leaf number per m^2 ground area and ear area index in all N treatments except under N deficiency (CAN35) in 2015. No significant CO_2 and $CO_2 \times N$ effect was observed for specific flag leaf

TABLE 3 Result of *F*-tests comparing the time course of stem NO_3^- concentration and of soil NO_3^--N, NH_4^+-N, and total mineral N concentration in the uppermost 30 cm soil layer. Results show stem NO_3^- concentration under two N (CAN180/CUL180) and two CO_2 treatments (ambient [CO_2]/e[CO_2]) in 2014 and soil NO_3^--N, NH_4^+-N, and total mineral N as well as stem NO_3^- concentration under two N (CAN200/UNI200) and two CO_2 treatments (ambient [CO_2]/ e[CO_2]) in 2015. Prior to analysis the data were log-transformed to comply with variance homogeneity

	2014	2015			
	Stem NO_3^- (mg/g)	Soil NO_3^--N (kg/ha)	Soil NH_4^+-N (kg/ha)	Soil total N (kg/ha)	Stem NO_3^- (mg/g)
CO_2	ns	ns	ns	ns	ns
N	ns	**	**	ns	**
$CO_2 \times N$	ns	ns	ns	ns	ns
Sampling (S)	**	**	**	**	**
$CO_2 \times S$	ns	ns	ns	ns	ns
$N \times S$	ns	**	*	ns	ns
$CO_2 \times N \times S$	ns	ns	ns	ns	ns

ns, not significant; *$p < .01$; **$p < .001$.

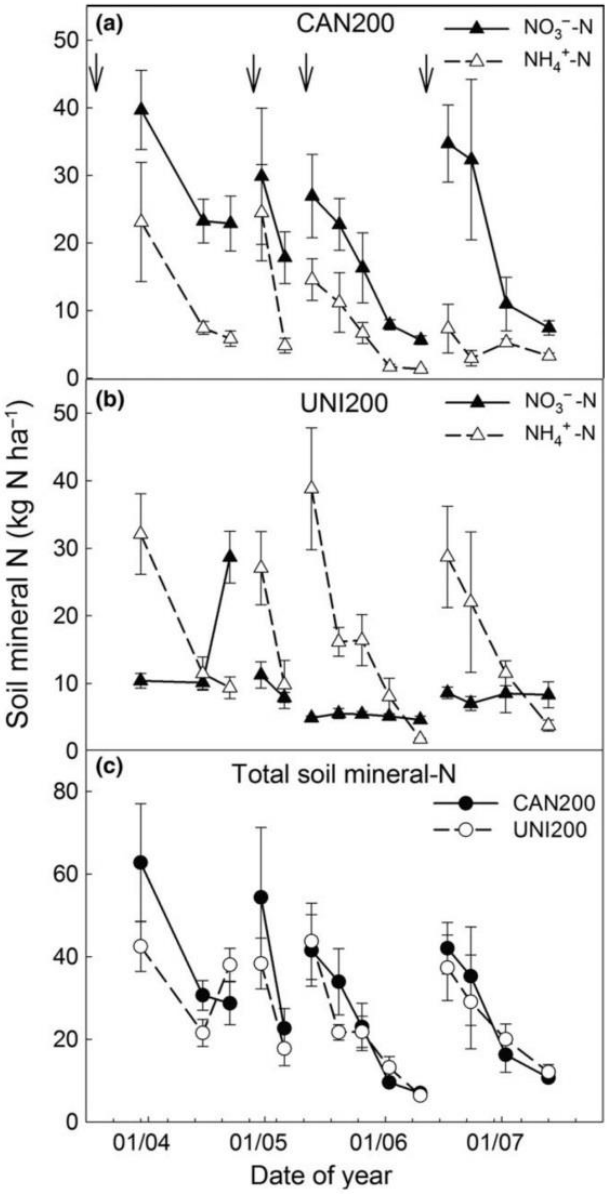

FIGURE 2 Time course of soil NO_3^--N and NH_4^+-N in the uppermost 30 cm soil profile of the CAN200 (a) and UNI200 treatment (b) during winter wheat growth. In (c), the amount of total soil mineral N in the CAN200 and UNI200 treatment is shown. Dates of N fertilization are indicated by arrows and data points around the N fertilizer applications are not interconnected. The data points represent mean values (± standard error of mean) over the two CO_2 treatments (n = 6)

weight, individual flag leaf area, residual leaf area index, and radiation-adjusted residual leaf area index.

3.3 | Flag leaf nitrate reductase activity and gene expression

Flag leaf nitrate reductase activity (NRA) referred to gram fresh weight was strongly influenced by N fertilization throughout all samplings in both 2014 and 2015 (Table 5). It was observed that NRA increased with increasing N fertilization (Figure 4), but neither a significant CO_2 × N interaction nor a significant CO_2 effect was observed in both years. Statistical analysis comparing flag leaf NRA between the two samplings within a particular year revealed no significant CO_2 × sampling and no CO_2 × N × sampling interaction in both years. Despite the large temperature difference of 11°C between the first and second sampling in 2014 the measured NRA of the ambient and e[CO_2] treatments of each N fertilizer treatment showed no significant difference between the two samplings (Table 2, Figure 4). The calculation of flag leaf NRA per m^2 ground area revealed no e[CO_2] effect for the time before flowering in both years (Figure 5a,b). However, a higher NRA under e[CO_2] was observed at late flowering in 2015 (Figure 5c).

To test whether there are differences in flag leaf NRA between NO_3^-- and NH_4^+-based fertilization marginal N means (mean over both CO_2 treatments) between the CUL180 and the CAN180 treatment in 2014 as well as the UNI200 and the CAN200 treatment in 2015 were compared. No significant difference for the marginal N means of flag leaf NRA between the CUL180 and the CAN180 treatment was detected for both samplings in 2014 (Figure 4a). In 2015 on the other hand, the marginal N means of flag leaf NRA of the UNI200 and CAN200 treatments were similar at the first sampling, but were significantly lower for the UNI200 compared to the CAN200 treatment at the second sampling (Figure 4b). Furthermore, when referred to m^2 ground area significant differences in flag leaf NRA between the CAN200 and the UNI200 treatment were detected for both samplings in 2015 (Figure 5b,c), but no differences between the CAN180 and the CUL180 treatment were observed in 2014 (Figure 5a). Averaged over the two CO_2 treatments and both samplings flag leaf NRA referred to m^2 ground area of the CUL180 treatment was 98% of the NRA of the CAN180 treatment in 2014. In 2015, flag leaf NRA per m^2 ground area of the UNI200 treatment was 70% of the NRA of the CAN200 treatment.

Investigation of flag leaf NR (*nia*) gene expression of three NR genes localized on chromosome 6AS, 6DS, and 7DS revealed a considerable influence of N fertilization with significantly lower *nia* transcript levels for plants grown under CAN35 and UNI200 compared to plants grown under CAN200 and CAN320 (Fig. S1). However, no e[CO_2] effect on *nia* transcription was observed for any N fertilization treatments (Fig. S2).

3.4 | Total aboveground biomass and N acquisition

Aboveground biomass was increased by e[CO_2] with relative effects of 4 up to 20% in 2014 and 6 up to 24% in 2015, respectively (Figure 6a). Tissue N concentrations of stem, leaf, ear, and total plant were not significantly influenced by e[CO_2] in 2014 (Tables S5 and S6). In 2015, e[CO_2] significantly reduced N concentration of leaf (−15%) and total plant (−9%) of the CAN200 treatment as well as N concentration of leaf (−20%), ear (−11%), and total plant (−19%) of the UNI200 treatment. In 2014, N acquisition of the CAN180, CAN320, as well as CUL180 treatment was increased by e[CO_2]

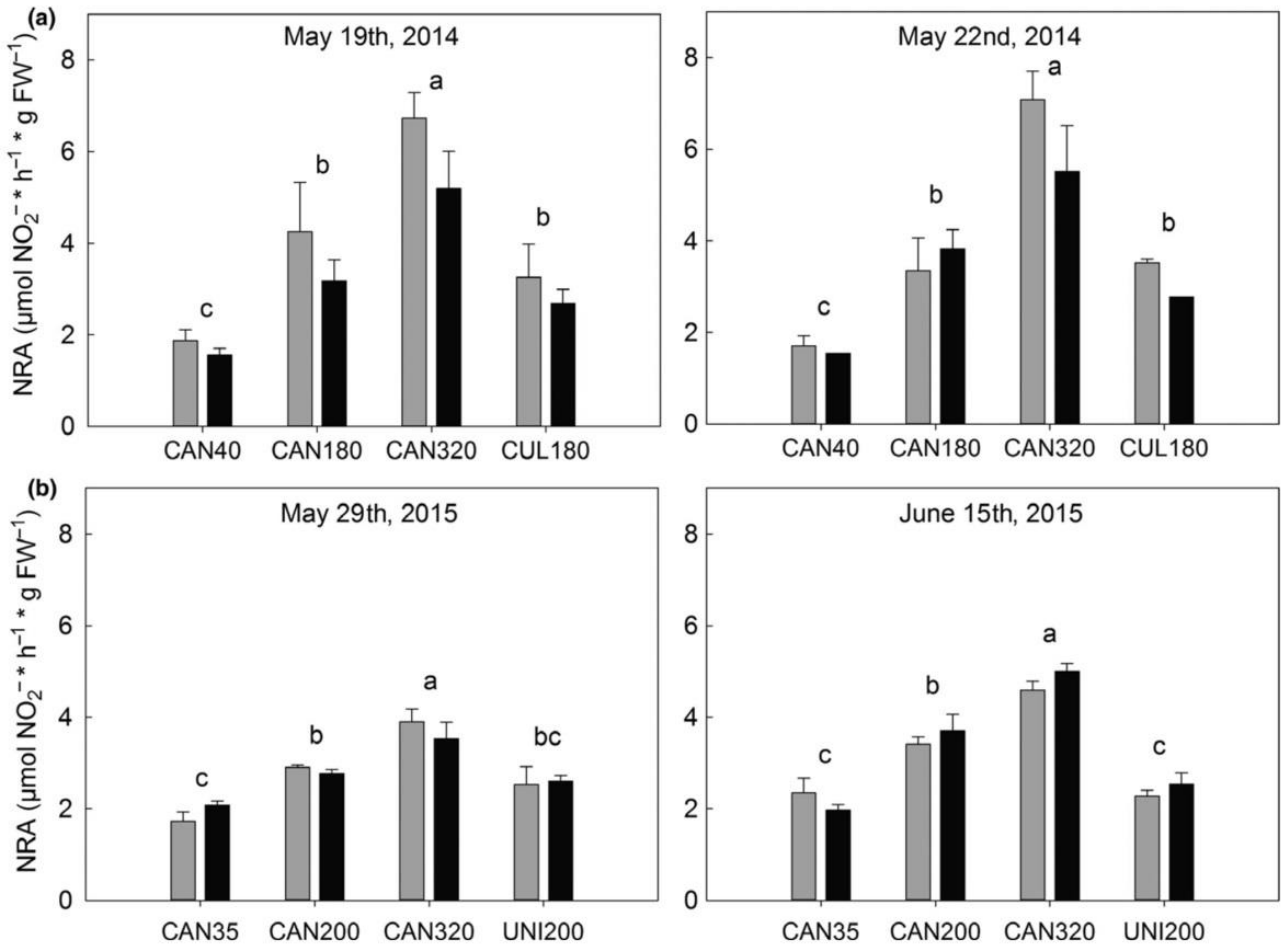

FIGURE 4 Effect of two CO_2 and four N treatments on flag leaf NRA referred to gram fresh weight. (a) Samplings in 2014 and (b) samplings in 2015 (mean± standard error of mean; for number of replicates see 2.9). Grey bars represent ambient [CO_2] and black bars e [CO_2]. Different letters indicate significant differences among the marginal means of the N treatments

with relative effects of 4 up to 13%, but this increase was not significant (Figure 6b). A significant $CO_2 \times N$ interaction was only observed in 2015. Here, e[CO_2] significantly increased N acquisition of the CAN200 and the CAN320 treatment by 14 and 16%, respectively. However, there was no e[CO_2] effect on N acquisition of the CAN35 and the UNI200 treatment (Figure 6b).

4 | DISCUSSION

A decline in NO_3^- assimilation and consequently lower protein concentrations in cereals under e[CO_2] would have unpredictable effects on future human and animal nutrition. Therefore, the effect of rising [CO_2] concentrations on N status of crops has been studied intensively (Ainsworth & Long, 2005; Taub & Wang, 2008). NO_3^- is the major plant-available N form in agricultural soils due to nitrification (Andrews et al., 2013; Subbarao et al., 2006) and consequently, the well-studied enzyme NR is of high interest with respect to its response to e[CO_2]. However, its regulation under these conditions is not completely understood as transcriptional regulation as well as posttranslational inhibition play important roles in the highly connected N and C metabolism (Stitt & Krapp, 1999). In this study it was tested whether NO_3^- assimilation is inhibited by e[CO_2] and whether an intensified growth and a higher N acquisition can be achieved with an NH_4^+-based fertilization in field-grown wheat.

4.1 | Leaf growth and estimation of flag leaf contribution to NO_3^- assimilation

The leaf canopy provides the main contribution to NO_3^- assimilation of crops (Andrews et al., 1992). In order to infer the e[CO_2] effect on the leaf canopy's NO_3^- assimilation the e[CO_2] effect on leaf growth has to be considered. In previous studies with wheat plants grown in pots with an artificial belowground environment, for instance, a restricted rooting volume or changed soil temperature as compared to the field (Arp, 1991; Long et al., 2005) was observed that specific leaf weight tended to be higher under e[CO_2] (Thilakarathne et al., 2013; Ziska et al., 2004). Furthermore, a slight increase in individual leaf area has been detected under restricted rooting volume (Seneweera & Conroy, 2005). In contrast, in the

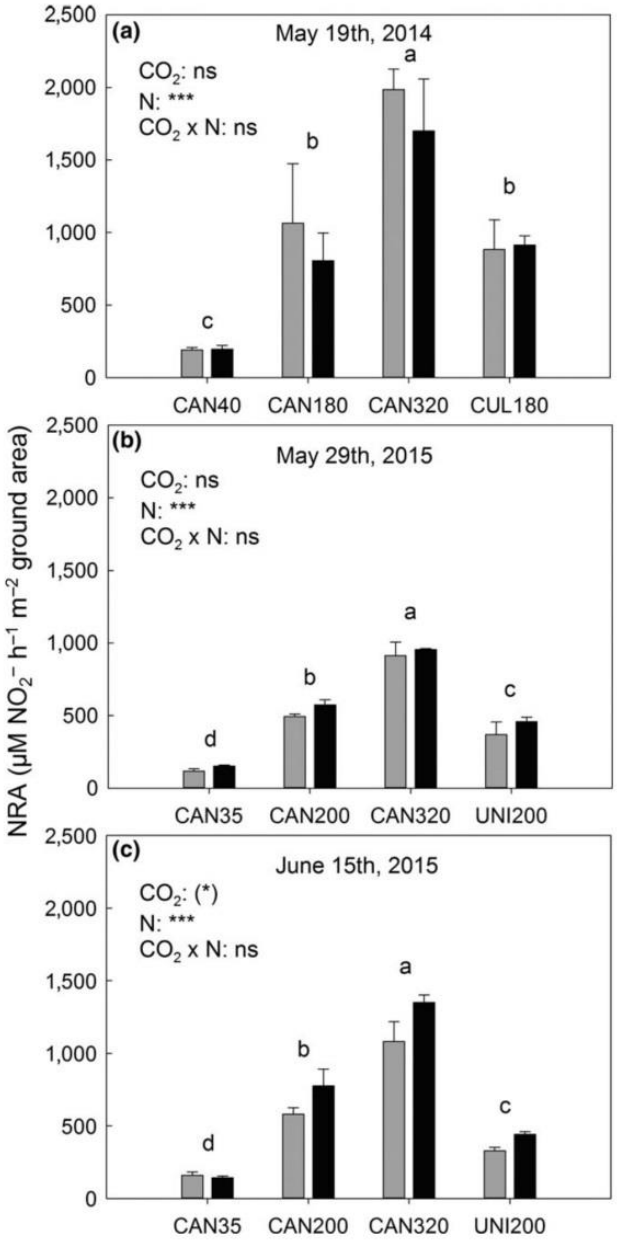

FIGURE 5 Effect of two CO_2 and four N treatments on flag leaf NRA referred to m^2 ground area. (a) Sampling before flowering in 2014, (b) sampling before flowering in 2015, (c) sampling at late flowering in 2015 (mean± standard error of mean; $n = 3$). Grey bars represent ambient [CO_2] and black bars e[CO_2]. *F*-test results are included in each diagram: ns: not significant; (*)$p < .10$; *$p < .05$; ***$p < .001$. Different letters indicate significant differences among the marginal means of the N treatments

present field study specific flag leaf weight as well as individual flag leaf area were not significantly altered by e[CO_2]. The observed significant increase in flag leaf biomass and flag leaf area per m^2 ground area under e[CO_2] resulted from a higher number of leaves as a result of an increased tiller number per plant. Higher tiller number per plant has also been found in another FACE study (Cai et al., 2016) as well as in a growth chamber study (Ziska et al., 2004).

NO_3^- assimilation and in particular nitrate reductase activity (NRA) depend on photosynthetic photon flux density (PPFD). The importance of flag leaves for NO_3^- assimilation was estimated on the basis of the PPFD distribution within the canopy because in this study only the NRA of the flag leaf was measured. Flag leaf area index accounted for only 28 up to 45% of the total leaf area index (Table S3) which might indicate a minor importance of the flag leaf for NO_3^- assimilation compared to the residual leaves. However, by taking into account that flag leaves were exposed to a higher PPFD, the radiation-adjusted flag leaf area index (raFLAI) was calculated. Based on this calculation flag leaves accounted for 38% at N deficiency (CAN40/35) up to 65% at N excess (CAN320) of the total leaf radiation-adjusted area index. Consequently, the flag leaf layer may constitute the major part of leaf NO_3^- assimilation under optimal and excess N fertilization. The importance of flag leaves is likely to increase until late grain filling as senescence proceeds from the bottom to the top of the canopy (Bertheloot, Martre, & Andrieu, 2008).

4.2 | Nitrate reductase activity and gene expression

In the present 2-year FACE experiment no significant inhibition of NRA under e[CO_2] was observed at four sampling dates. Therefore, the hypothesized decrease in NRA in wheat from other e[CO_2] studies conducted in hydroponic cultures (Bloom et al., 2002, 2010) cannot be supported with these results gained in this field experiment. Other FACE experiments with different plant species showed variable results regarding NRA. Natali, Sanudo-Wilhelmy, and Lerdau (2009) observed an inhibition of NRA in Loblolly pine (*Pinus taeda*), but no e[CO_2] effect for American sweetgum (*Liquidambar styraciflua*). Hu, Wang, Yang, Zhou, and Zhu (2006) found consistently higher NRA under e[CO_2] over the growing season for rice (*Oryza sativa*).

The NO_3^- assimilation is hypothesized to be inhibited under e[CO_2] due to a lower photorespiration rate and a subsequent lower supply of the reductant NADH for the NR caused by a decreased malate export from the chloroplast (Bloom, 2015a; Rachmilevitch et al., 2004). The malate valve represents an important player in the complex network between N and C metabolism and is involved in supplying energy in form of NADH for nitrate reduction (Scheibe, 2004). However, oxidation from triose phosphate sugars also increases cytosolic NADH availability and was long thought to be the ultimate energy source for NR as several intermediates and photosynthetic metabolites migrate from chloroplasts into the cytosol (Foyer et al., 2009; Kaiser et al., 2000). As cytosolic NADH concentration is generally estimated as very low it is uncertain to which amount the NADH for nitrate reduction is supplied by malate and to which amount from triose phosphate sugars originating from Calvin cycle. To address the dependency of NRA on photorespiration NRA was measured at moderate (16.7°C) and warm (27.6°C) temperature conditions before flowering in 2014. Between both temperatures no difference in NRA was observed. If this dependency would have been supported with this experiment, an inhibition of NRA by

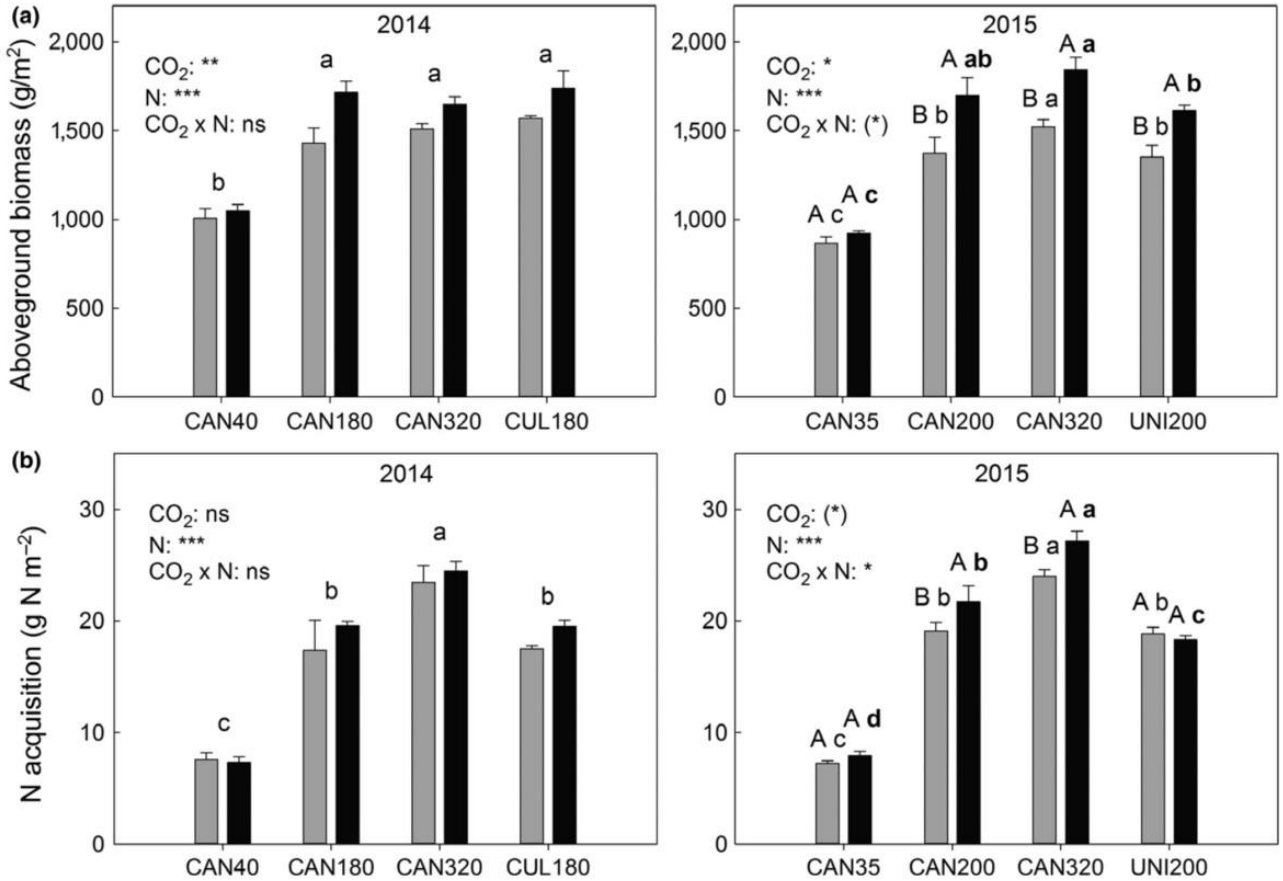

FIGURE 6 Effect of two CO_2 and four N treatments on aboveground biomass (a) and aboveground N acquisition (b) at the milk-ripe stage (mean± standard error of mean; $n = 3$). Grey bars represent ambient [CO_2] and black bars e[CO_2]. *F*-test results are included in each diagram: ns: not significant; (*)$p < .1$; *$p < .05$; **$p < .01$; ***$p < .001$. With no significant interaction from the *F*-test different lower case letters indicate significant differences among the marginal means of the N treatments. With significant interaction from the *F*-test different capital letters indicate significant differences for CO_2 means separate for each N treatment and different lower case letters show significant differences for N means separate for each CO_2 treatment (letters are in bold for e[CO_2])

e[CO_2] should be detectable at warm temperatures. This has been previously observed in a growth chamber study where e[CO_2] inhibited both photorespiration and NRA in wheat only at temperatures above 24°C (Jauregui et al., 2015). Nevertheless, the results of this study indicate no dependency of NRA on photorespiration in the field and suggest that rising temperatures will not induce or exacerbate inhibition of NO_3^- assimilation in wheat under future e[CO_2].

A FACE study indicated an inhibition of NO_3^- assimilation based on data of increased ratio of NO_3^- to total N concentration due to accumulation of unassimilated NO_3^- in leaf tissue (Bloom et al., 2014). In addition, less ^{15}N-enriched organic N and NO_3^- in leaf tissue under e[CO_2] was observed which the authors have claimed to result from declined NO_3^- assimilation relative to replenishment. Although no similar measurements were conducted in this study and vice versa, this study disagrees with the study of Bloom et al. (2014) because no declines in NRA as well as nitrate reductase (*nia)* transcript levels under e[CO_2] were observed. An explanation for the differing results might be that the experiment of Bloom et al. (2014) was conducted under subtropical climate and excess N fertilization with 350 kg N/ha. Under these conditions NRA might have been very high due to (i) the excess N fertilization as it was observed in this study and (ii) the theoretically high NADH supply from very high photorespiration rates owing to high daytime temperatures. Consequently, the described and hypothesized e[CO_2]-induced NADH shortage for NR due to reduced photorespiration (Bloom, 2015a; Bloom, 2015b) might therefore occur in the field only under extreme conditions that facilitate very high NRA, namely, very high photorespiration rates and excess N fertilization, but not under moderate climate and adequate N supply. Moreover, in the study of Bloom et al. (2014) e[CO_2] did not decrease N concentrations implying that despite inhibition of NO_3^- assimilation N acquisition of wheat was not strongly affected.

The regulation of the nitrate reductase (*nia)* transcript is rather complex but follows a light-dependent diurnal expression pattern and is generally positively influenced by increasing cellular NO_3^- and repressed by high glutamine levels (Stitt & Krapp, 1999). In this

study, the levels of *nia* transcript were significantly lower in the deficient NO_3^- (CAN35)- and optimal NH_4^+ (UNI200)-based fertilization treatments compared to the optimal (CAN200) and excessive (CAN320) NO_3^--based ones. These results support that increasing NO_3^- concentrations induced the *nia* transcription and suggest that an adaption to the lower NO_3^- status under N deficiency and NH_4^+-based fertilization took place. Regarding the e[CO_2] effect on flag leaf *nia* transcription Buchner et al. (2015) observed 1.6- to 1.8-fold higher *nia* transcript levels under e[CO_2] in spring wheat flag leaves at flowering. In contrast, in this study no significant differences in *nia* transcript levels were observed which is consistent with no e[CO_2] effect on flag leaf NRA. This discrepancy could be attributed to the different growing conditions between the two study sites. For example, in this study the irrigation was rigorously controlled, whereas in the study of Buchner et al. (2015) wheat was grown in dry-land agriculture.

4.3 | Establishment of an NH_4^+-based fertilization

In order to establish an NH_4^+-dominated fertilization, CULTAN fertilization was used in the 1st year and surface application consisting of a combination of urea with nitrification inhibitors in the 2nd year. Particularly in the first two growing stages, stem NO_3^- concentration was considerably higher in 2014 compared to 2015. This could indicate generally higher nitrification rates in spring 2014, which might be connected with the higher average temperature in April in 2014 (11.5°C) compared to 2015 (8.2°C). Net mineralization, which is another important NH_4^+ source, was estimated based on total N fertilizer applied and aboveground N acquisition in the N deficiency treatment (CAN40/35). This estimation resulted in no large difference between 2014 (35 kg N/ha) and 2015 (41 kg N/ha).

With CULTAN, depots containing high concentrations of NH_4^+ are injected into the soil next to the plants. The high NH_4^+ concentration of these depots is assumed to be toxic for microorganisms thus preventing nitrification and forcing plants to take up NH_4^+ (Petersen et al., 2004; Wetselaar et al., 1972). Consequently, rhizospere azidification by plant NH_4^+ uptake should further decrease nitrification because high nitrification rates occur at a neutrale soil pH (Suzuki et al., 1974). In this study in 2014, however, no differences in stem NO_3^- concentration as well as flag leaf NRA were observed, suggesting similar soil NO_3^--N supply for the CUL180 and CAN180 fertilization. Inadequate control of nitrification with CULTAN was also observed in another study because soil NO_3^--N content did not differ between CULTAN and surface spreading of ammonium sulfate (Deppe et al., 2016).

In contrast, in 2015, soil NO_3^--N concentration, stem NO_3^- concentration, flag leaf NRA, as well as flag leaf *nia* transcript levels were lower for plants grown under UNI200 compared to plants grown under CAN200, whereas total soil mineral N supply was similar in both N treatments. This result indicates lower NO_3^- but similar mineral N availability in the UNI200 compared to the CAN200 plots and suggests that nitrification was substantially controlled over the growing season in the UNI200 plots. Nevertheless, soil pH did not differ significantly between the CAN200 and UNI200 plots (data not shown), even if the soil pH decreased by 0.5 units from March until middle of June probably due to nitrification and/or plant NH_4^+ uptake. At the end of April 2015, a short-term increase in soil NO_3^--N occurred in the UNI200 plots which can be explained by the short half-live of nitrification inhibitors (Subbarao et al., 2006). Furthermore, unusual heavy rainfalls with 50 mm occurred between the end of March and the beginning of April. This may have caused leaching of nitrification inhibitors, which could have further decreased the inhibition efficiency. Apart from that, soil NO_3^--N concentration in the UNI200 plots remained constantly at a low level, but never fell to zero suggesting a base NO_3^--N level even under good nitrification control.

Averaged over CO_2 treatments and samplings, the soil NO_3^--N concentration in the UNI200 plots was only 40% of the soil NO_3^--N concentration in the CAN200 plots with similar soil mineral N supply for both N treatments. However, flag leaf NRA per m^2 ground area of plants grown with UNI200 fertilization was 70% compared to CAN200 fertilization. This indicates that wheat strongly favors NO_3^- over NH_4^+ assimilation even at high soil NH_4^+-N and low NO_3^--N supply. However, an ultimate proof which N form is preferred by the plant might provide the application of ^{15}N-labeled NO_3^- or NH_4^+ fertilizers and the subsequent tracing of the ^{15}N-labeled N compounds. In addition, the high NRA in the UNI200 treatment indicates that the largest portion of the urea applied in the UNI200 plots was nitrified.

Based on the preference of wheat to take up NO_3^- and the difficulty of keeping high NH_4^+-N concentrations in the soil, it can be predicted that the establishment of an agricultural system based on primary uptake of NH_4^+ as major N form is not readily manageable.

4.4 | Growth and N acquisition under NO_3^-- and NH_4^+-based fertilization

In this study aboveground biomass was significantly increased by e[CO_2] under optimal (CAN180/200) and excessive (CAN320) NO_3^--based fertilization as well as under CUL180 and NH_4^+-based fertilization (UNI200), but not under the severe N deficiency treatment (CAN40/35). Dependency on N availability and in particular declining growth stimulation by e[CO_2] under N deficiency has been reported for wheat (Wolf, 1996) and other species in growth chamber (Stitt & Krapp, 1999) and FACE studies (Ainsworth & Long, 2005). However, a NH_4^+-based fertilization did not enhance the stimulating effect of e[CO_2] on aboveground biomass any further compared to NO_3^--based fertilization as it was proposed by Bloom et al. (2002).

Previous meta-analyses that are primarily based on enclosure studies reported tissue N concentration decreases under e[CO_2] in wheat with an average of −16% for vegetative tissue (Cotrufo et al., 1998) and −10% for grain (Taub et al., 2008). However, previous FACE studies with wheat showed no decrease in N concentration of ear (Tausz-Posch et al., 2015) and only small decreases in stem and

leaf (Bloom et al., 2014; Tausz-Posch et al., 2015). In this FACE study under NO_3^--based fertilization, e[CO_2] did not significantly reduce stem and ear N concentration and the reduction in leaf N concentration was only moderate. For example, e[CO_2] decreased total plant tissue N concentration by only −5% in 2014 and −9% in 2015 in plants grown under optimal NO_3^--based fertilization. In addition to the milk-ripe stage, which is presented herein, four other growth stages analyzed in this study showed similarly moderate e[CO_2] effects on tissue N concentration (data not shown). It can therefore be concluded that the reduction in tissue N concentrations in wheat grown under e[CO_2] is generally lower in FACE compared to enclosure studies. In this study in plants grown under optimal NH_4^+-based fertilization (UNI200), e[CO_2] decreased total plant tissue N concentration by −19% and this reduction is considerably stronger compared to one observed in plants grown under NO_3^--based fertilization.

Due to the growth stimulation and only a slight decrease in tissue N concentration of plants grown under e[CO_2], the effect of e[CO_2] did not lead to a decrease but to an increase in aboveground N acquisition under NO_3^--based fertilization. This result is well reflected by the positive effects of e[CO_2] on NRA per m^2 ground area. The results with regard to the e[CO_2] effect on NRA, biomass, and aboveground N acquisition were similar between both years. Therefore, these results can be considered as representative at least for locations with similar conditions with regard to climate and soil. Higher N acquisition under e[CO_2] was also observed in other FACE experiments with NO_3^--based fertilization using the same wheat cultivar (Weigel & Manderscheid, 2012) as well as with other winter wheat cultivars (Cai et al., 2016; Han et al., 2015; Ma et al., 2007). Hence, a stimulation of NRA per m^2 ground area by e[CO_2] might have occurred in these studies. However, e[CO_2] did not increase N acquisition with an optimal NH_4^+-based fertilization (UNI200) and this result disagrees with the results of hydroponic experiments of Bloom et al. (2002) and Carlisle et al. (2012). Besides the physiological differences in terms of plant NO_3^- and NH_4^+ acquisition, it is important to consider the differences between a NO_3^-- and NH_4^+-based fertilization in terms of N losses to the environment (Masclaux-Daubresse et al., 2010). In this study under all N treatments but excess fertilization (CAN320), the plants acquired about 90% of the soil mineral N deriving from fertilization and mineralization. This indicates generally high nitrogen use efficiency in our study as well as no difference between the N fertilizer forms (CAN180/200 vs. CUL180/UNI200) in terms of N losses to the environment.

In conclusion, our results suggest that NO_3^--based fertilization is superior to an NH_4^+-based one under e[CO_2] for field-grown wheat. Therefore, a change from NO_3^-- to NH_4^+-based fertilization might not be beneficial under future e[CO_2]. Furthermore, our results suggest that an inhibition of NO_3^- assimilation by e[CO_2], as postulated in prior research, is not the reason for the decrease in tissue N concentration under e[CO_2] and that the reduction in e[CO_2] on tissue N concentration in wheat is much smaller in field studies with FACE compared to enclosure studies.

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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4.6 Anhang

Table S1: Listed target genes for quantitative reverse transcription PCR gene expression analysis with forward and reverse oligonucleotide primer sequences (*NCBI GeneBank accession, ** EnsemblPlants database)

Gene	Source	Forward primer	Reverse primer
Nitrate reductase 6AS	TRIAE_CS42_6AS_TGACv1_486190_AA1557980**	GCCGAGTCCGACAACTACTA	TATGCGTATCCCTTGATGGT
Nitrate reductase 6DS	TRIAE_CS42_6DS_TGACv1_543770_AA1744010**	GTACTGGTGCTGGTGCTTCT	ACGTTCACCTTGACCTTGAA
Nitrate reductase 7DS	TRIAE_CS42_7DS_TGACv1_622737_AA2044470**		
ADP-ribosylation factor	AY736124*, AB050957*	ACCATCGGGTTTAATGTTGA	TTCAACAACACGGTCCCTAT
Translation elongation factor 1 alpha	M90077*	CCGCTGAGATGAACAAGAGGT	CGATGATGAGAACAGCACAG

Table S2: Effects of four N and two CO_2 treatments on flag leaf growth variables. Shown are mean values (±standard error of mean; n=3) and the percentage effect (Δ) of elevated [CO_2]. Amb [CO_2] stands for the ambient CO_2 of about 390 ppm and e[CO_2] for the elevated [CO_2] of 600 ppm. With no significant interaction from the F-test different lower case letters indicate significant differences among the marginal means (mean values over both CO_2 treatments) of the N treatments. With a significant interaction from the F-test different capital letters indicate significant differences for CO_2 means separate for each N treatment and different lower case letters show significant differences for N means separate for each CO_2 treatment (letters for e[CO_2]) are in bold). For explanation of the abbreviations see legend to Table 4.

	2014				2015			
	CAN40	CAN180	CAN320	CUL180	CAN35	CAN200	CAN320	UNI200
SFLW (g m^{-2})								
Amb [CO_2]	59.3±2.3a	55.5±1.9b	54.9±1.2b	54.4±1.4b	70.9±1.5a	67.6±0.1b	66.9± 2.0b	66.5±1.2b
e[CO_2]	63.0±1.5a	55.5±2.8b	53.3±1.3b	55.1 ± 1.3b	70.1± 0.5a	66.5±0.7b	64.5±1.0b	66.2±0.7b
Δ	6	0	-3	1	-1	-2	-4	-1
FLA (cm^2 $leaf^{-1}$)								
Amb [CO_2]	17.2±1.4c	26.1±3.5b	31.5±2.5a	26.2±3.3ab	11.6±1.0c	21.0±1.8b	23.0±1.5a	19.4±1.2b
e[CO_2]	20.4±1.4c	25.0±2.5b	33.0±1.9a	29.8±2.5ab	13.2±1.7c	22.2±1.8b	24.5 ±1.4a	21.3±1.7b
Δ	19	-4	5	14	14	6	6	9
FLN (m^{-2})								
Amb [CO_2]	294±23c	432±22 b	470±30b	522±14a	282±13A c	393±5B b	489±17B a	378±13B b
e[CO_2]	299±20c	473±23b	492±7b	571±29a	267±7A **c**	453±22A **b**	534±8A **a**	419±4A **b**
Δ	2	9	5	9	-5	15	9	11
FLB (g m^{-2})								
Amb [CO_2]	29.7±1.7c	62.4±9.4b	80.4±1.6a	73.8±1.7a	23.3±2.2d	54.3±1.1b	73.9±5.0a	47.2±2.0c
e[CO_2]	38.2±1.9c	65.9±7.9b	87.2±8.9a	93.2±3.2a	24.7±0.9d	66.0±3.8b	82.3± 1.1a	56.4±2.0c
Δ	29	6	8	26	6	22	11	20

Table S3: Effects of four N and two CO_2 treatments on green area index of flag leaves, residual leaves and ears as well as radiation adjusted green area index of flag and residual leaves. Shown are mean values (±standard error of mean; n=3) and the percentage effect (Δ) of e[CO_2]. Different letters indicate significant differences among the marginal means of the N treatments. For explanation of the abbreviations see legend to Table 4.

	2014				2015			
	CAN40	CAN180	CAN320	CUL180	CAN35	CAN200	CAN320	UNI200
FLAI (m^2 m^{-2})								
Amb [CO_2]	0.502±0.036c	1.14±0.17b	1.47±0.01a	1.36±0.03a	0.329±0.028c	0.798±0.022b	1.11±0.10a	0.713±0.039c
e[CO_2]	0.610±0.036c	1.19±0.10b	1.63±0.11a	1.69±0.06a	0.353±0.012c	0.993±0.066b	1.29±0.02a	0.861±0.036c
Δ	21	4	11	24	7	24	16	21
RLAI (m^2 m^{-2})								
Amb [CO_2]	0.842±0.110b	1.85±0.17a	2.15±0.55a	2.13±0.45a	0.771±0.075d	2.05±0.07b	2.42±0.11a	1.67±0.05c
e[CO_2]	0.812±0.093b	2.30±0.18a	2.45±0.14a	2.04±0.17a	0.737±0.073d	2.21±0.05b	2.41± 0.22a	1.72±0.14c
Δ	-4	24	14	-5	-4	8	0	3
EAI (m^2 m^{-2})								
Amb [CO_2]	0.207±0.007c	0.350±0.031b	0.413±0.007a	0.399±0.003a	0.191±0.005c	0.338±0.012b	0.401±0.009a	0.315±0.012b
e[CO_2]	0.217±0.008c	0.413±0.010b	0.438±0.014a	0.449±0.026a	0.189±0.003c	0.389±0.016b	0.446±0.027a	0.362±0.005b
Δ	5	18	6	13	-1	15	11	15
raFLAI								
Amb [CO_2]	0.42±0.03c	0.79±0.11b	0.95±0.08a	0.90±0.07b	0.20 ±0.02d	0.60 ± 0.04 b	0.76±0.08a	0.55±0.04c
e[CO_2]	0.49±0.03c	0.80±0.08b	1.02±0.10a	1.04±0.10b	0.30±0.01d	0.70 ± 0.06 b	0.85± 0.08a	0.63±0.05c
Δ	18	2	7	16	7	18	11	16
raRLAI								
Amb [CO_2]	0.49±0.05b	0.62±0.11a	0.55±0.1ab	0.59±0.11b	0.50±0.05c	0.80±0.07a	0.75±0.0ab	0.72±0.06b
e[CO_2]	0.44± 0.05b	0.69±0.10a	0.56±0.0ab	0.47±0.07b	0.48±0.04c	0.74±0.07a	0.66±0.0ab	0.67±0.06b
Δ	-8	11	2	-19	-5	-6	-11	-7

Table S4: Effects of four N and two CO_2 treatments on mean irradiance (% of value at top of canopy) on the flag leaf (% irr on FL) and residual leaf fraction (% irr on RL). Shown are mean values (±standard error of mean; n=3) and the percentage effect (Δ) of e[CO_2].

	2014				2015			
	CAN40	CAN180	CAN320	CUL180	CAN35	CAN200	CAN320	UNI200
% irr on FL								
Amb [CO_2]	82.9± 0.9	69.9 ± 3.4	64.5 ± 0.3	66.0 ± 0.4	86.9 ± 0.6	74.9 ± 0.5	69.2 ± 1.4	76.7 ± 0.9
e[CO_2]	80.6 ± 0.8	68.0 ± 1.6	62.6 ± 1.4	61.7 ± 0.6	86.4 ± 0.3	71.0 ± 1.2	66.1 ± 0.6	73.4 ± 0.5
Δ	-3	-3	-3	-6	-1	-5	-4	-4
% irr on RL								
Amb [CO_2]	58.3 ± 2.5	33.3 ± 3.7	26.6 ± 2.3	28.0 ± 1.3	65.3 ± 0.1	38.8 ± 0.6	30.8 ± 1.4	43.3 ± 1.2
e[CO_2]	55.1 ± 1.8	29.9 ± 1.5	22.9 ± 1.8	23.2 ± 0.4	65.0 ± 1.0	33.8 ± 1.6	27.7 ± 1.2	39.1 ± 1.4
Δ	-6	-10	-14	-17	-1	-13	-10	-10

Table S5: F-test results of the effect of four N and the two CO_2 treatments on stem, leaf, ear and total plant N concentration at the milk-ripe stage of winter wheat. ns: not significant; *: p<0.05; **: p<0.01; ***:p<0.001

	2014			2015		
	CO_2	N	CO_2 x N	CO_2	N	CO_2 x N
N conc stem (mg g^{-1})	ns	***	ns	ns	***	ns
N conc leaf (mg g^{-1})	ns	***	ns	**	***	*
N conc ear (mg g^{-1})	ns	***	ns	ns	***	*
N conc total (mg g^{-1})	ns	***	ns	*	***	**

Table S6: Effects of four N and two CO_2 treatments on stem, leaf, ear and total plant N concentration at the milk-ripe stage of winter wheat. Shown are mean values (±standard error of mean; n=3) and the percentage effect (Δ) of e[CO_2]. With no significant interaction from the F-test different lower case letters indicate significant differences among the marginal means (mean values over both CO_2-treatments) of the N treatments. With a significant interaction from the F-test different capital letters indicate significant differences for CO_2 means separate for each N treatment and different lower case letters show significant differences for N means separate for each CO_2-treatment (letters for e[CO_2]) are in bold). For explanation of the abbreviations see legend to Table 4.

	2014				2015			
	CAN40	CAN180	CAN320	CUL180	CAN35	CAN200	CAN320	UNI200
N conc stem (mg g^{-1})								
Amb [CO_2]	3.4±0.2c	6.4±1.0b	10.3±0.1a	6.0±0.1b	3.7±0.3c	8.0±0.5b	10.2±0.3a	7.7±0.6b
e[CO_2]	2.8±0.2c	6.3±0.3b	9.6±0.5a	6.2±0.2b	4.0±0.5c	7.6±0.8b	10.2±0.2a	6.0±0.2b
Δ	-17	-2	-6	3	10	-5	0	-22
N conc leaf (mg g^{-1})								
Amb [CO_2]	13.1±0.3c	23.0±2.6b	29.7±2.2a	23.0±0.8b	13.3±0.1A b	29.9±0.5A a	31.9±0.7A a	29.2±0.5A a
e[CO_2]	10.4±0.5c	23.0±0.2b	28.4±0.8a	22.9±0.0b	14.8±2.4A **c**	25.3±0.7B **b**	30.0±1.0A **a**	23.3±1.3B **b**
Δ	-20	0	-4	-1	11	-15	-6	-20
N conc ear (mg g^{-1})								
Amb [CO_2]	11.8±1.2d	16.3±1.0b	17.1±1.0a	14.2±0.3c	14.3±0.4A b	17.6±0.3A a	17.8±0.6A a	18.5±0.3A a
e[CO_2]	11.8±0.4d	15.4±0.4b	17.1±0.4a	14.7±0.3c	14.4±0.3A **c**	17.0±0.5A **ab**	17.5±0.3A **a**	16.4±0.4B **b**
Δ	0	-5	0	4	1	-3	-2	-11
N conc total (mg g^{-1})								
Amb [CO_2]	7.5±0.6c	12.0±1.2b	15.4±0.6a	11.1±0.2b	8.4±0.2A c	14.0±0.4A b	15.8±0.1A a	14.0±0.3A b
e[CO_2]	7.0±0.3c	11.4±0.5b	14.9±0.1a	11.2±0.4b	8.6±0.5A **d**	12.8±0.5B **b**	15.2±0.2A **a**	11.3±0.1B **c**
Δ	-7	-5	-3	1	3	-9	-4	-19

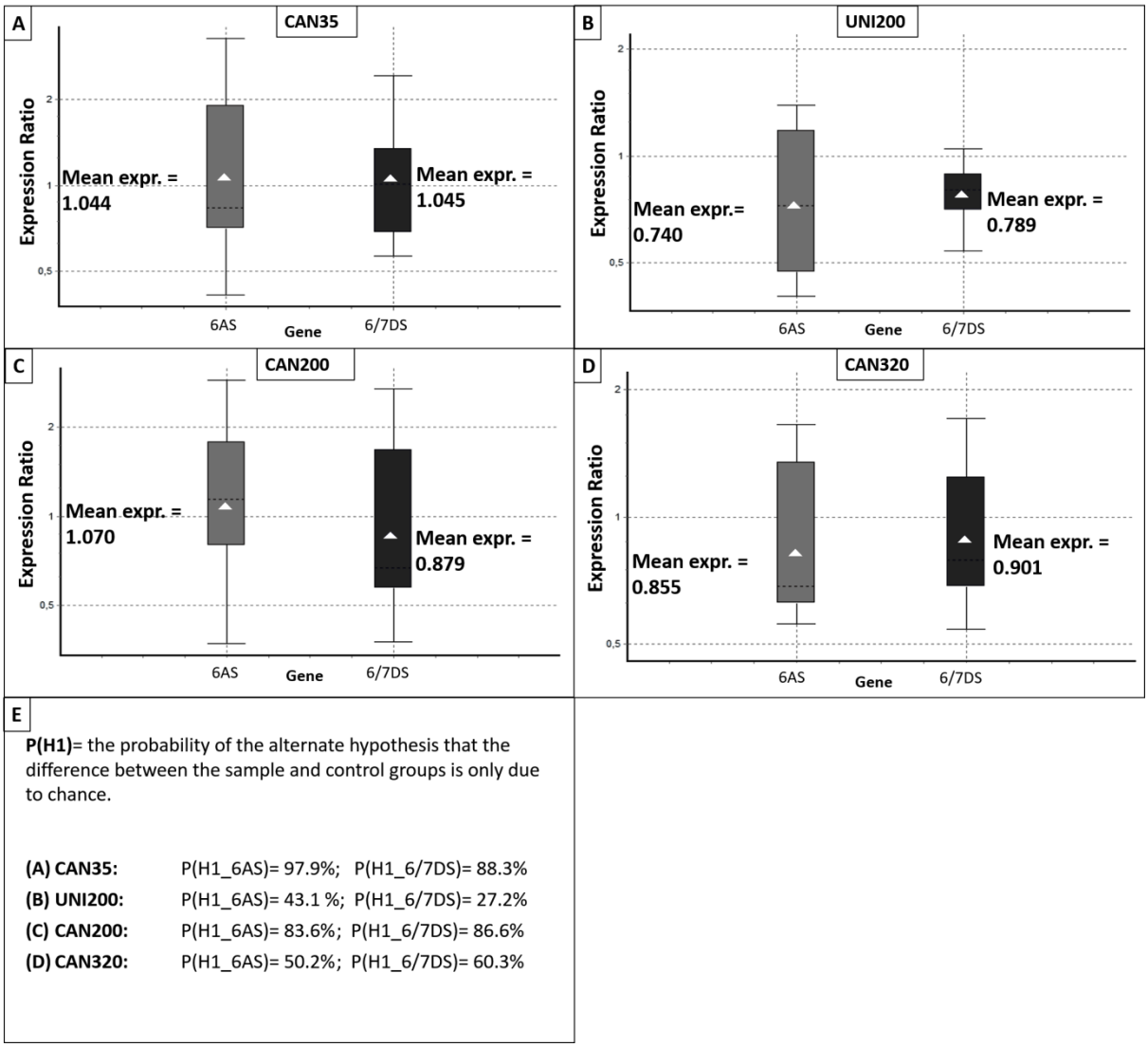

Fig. S1: Expression ratio results given in whisker-box plots regarding the influence of e[CO_2] on nitrate reductase 6AS (light gray box plot), 6DS and 7DS (dark gray box plot) mRNA levels from the 2nd sampling of 2015 in winter wheat. Colored box area encompass 50% of all observations, the whiskers represent the outer 50% of observations, the dotted line represents the sample median and the mean expression ratio is depicted as a white triangle. A) CAN35. B) UNI200 C) CAN200. D) CAN320. Listed probabilities of the hypothesis that differences in expression levels are due to chance.

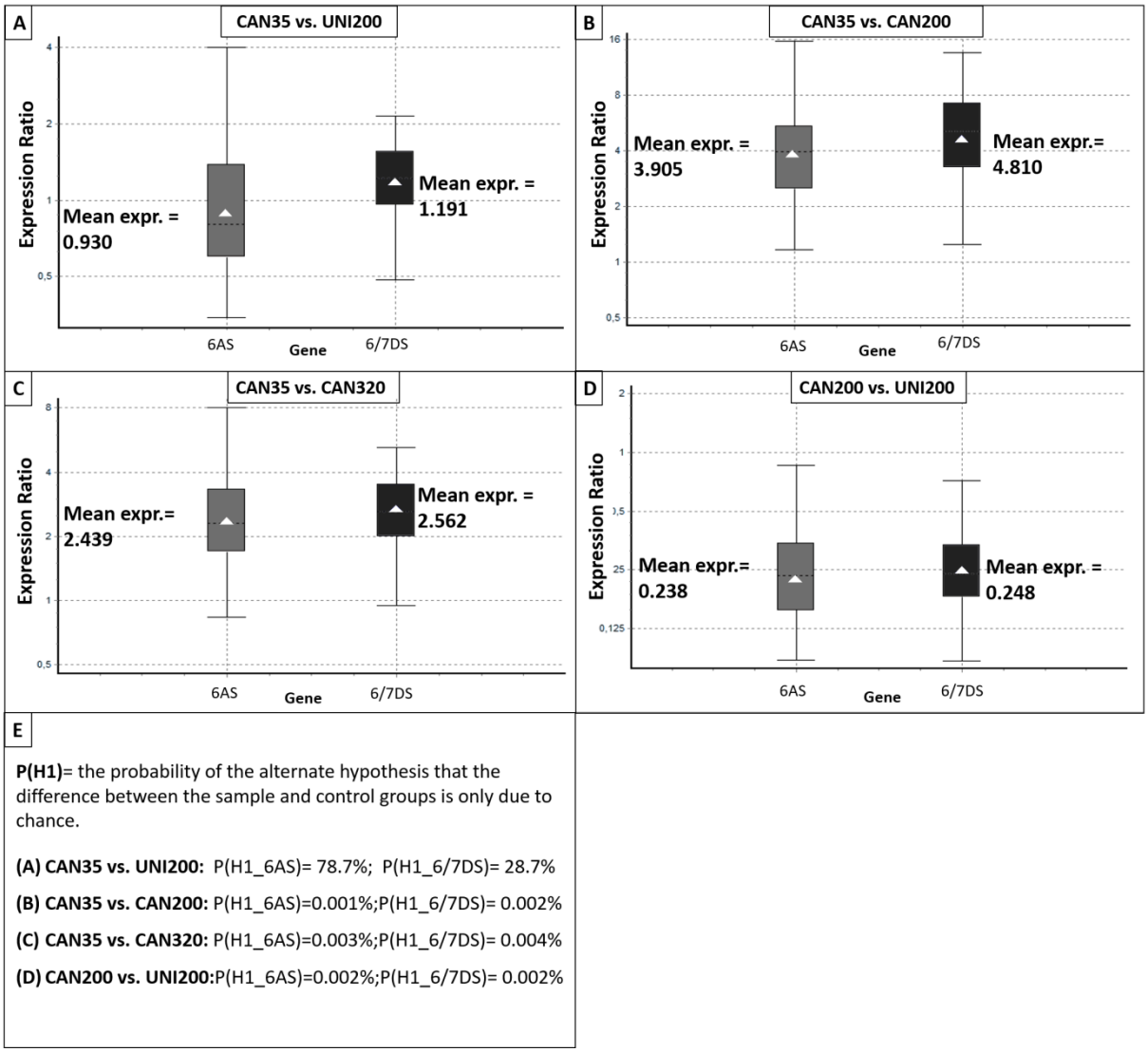

Fig. S2: Expression ratio results given in whisker-box plots regarding the influence of N fertilization treatment and N amount on nitrate reductase 6AS (light gray box plot), 6DS and 7DS (dark gray box plot) mRNA levels in winter wheat. Colored box area encompass 50% of all observations, the whiskers represent the outer 50% of observations, the dotted line represents the sample median and the mean expression ratio is depicted as white triangle. A) CAN35 compared to UNI200. B) CAN35 compared to CAN200. C) CAN35 compared to CAN320. D) UNI200 compared to CAN200. Listed probabilities of the hypothesis that differences in expression levels are due to chance.

Kapitel 5: Wirkung von e[CO_2] auf die Kornprotein-Zusammensetzung und Backqualität

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Elevated Atmospheric CO_2 Concentration Has Limited Effect on Wheat Grain Quality Regardless of Nitrogen Supply

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ABSTRACT: Elevated atmospheric CO_2 concentrations (e[CO_2]) can decrease the grain quality of wheat. However, little information exists concerning interactions between e[CO_2] and nitrogen fertilization on important grain quality traits. To investigate this, a 2-year free air CO_2 enrichment (FACE) experiment was conducted with two CO_2 (393 and 600 ppm) and three (deficiency, adequate, and excess) nitrogen levels. Concentrations of flour proteins (albumins/globulins, gliadins, and glutenins) and key minerals (iron, zinc, and sulfur) and baking quality (loaf volume) were markedly increased by increasing nitrogen levels and varied between years. e[CO_2] resulted in slightly decreased albumin/globulin and total gluten concentration under all nitrogen conditions, whereas loaf volume and mineral concentrations remained unaffected. Two-dimensional gel electrophoresis revealed strong effects of nitrogen supply and year on the grain proteome. Under adequate nitrogen, the grain proteome was affected by e[CO_2] with 19 downregulated and 17 upregulated protein spots. The downregulated proteins comprised globulins but no gluten proteins. e[CO_2] resulted in decreased crude protein concentration at maximum loaf volume. The present study contrasts with other FACE studies showing markedly stronger negative impacts of e[CO_2] on chemical grain quality, and the reasons for that might be differences between genotypes, soil conditions, or the extent of growth stimulation by e[CO_2].

KEYWORDS: *baking quality, free-air CO_2 enrichment, grain quality, iron concentration, nitrogen fertilization, proteomics, Triticum aestivum, zinc concentration*

1. INTRODUCTION

Wheat (*Triticum aestivum* L.) is the second most important staple crop in the world, providing globally about 20% of protein and 20−40% of minerals for human nutrition.[1,2] Grains of wheat are mostly processed into baked products, and thus, chemical grain quality is important for both nutritional value and end use quality. The atmospheric CO_2 concentration is predicted to continue to rise from currently 408 to 730−1020 μmol mol^{-1} (=ppm) by the end of this century.[3] Free-air CO_2 enrichment (FACE) studies of wheat and other C_3 crops have shown that elevated atmospheric CO_2 concentrations (e-[CO_2]) increase photosynthesis and grain yield,[4−7] while water use is reduced by decreased stomatal conductance.[8,9] However, these crops often exhibit decreased grain nutrient concentrations, especially of crude protein and of sulfur (S), iron (Fe), and zinc (Zn) under e[CO_2].[4,10−13] For wheat, a meta-analysis and a modeling study have suggested that crude protein, Fe, and Zn concentrations will decrease globally on average by 6−9% under e[CO_2] of 550 ppm.[14,15] In view of current Fe and Zn deficiencies in human diets and of high grain quality requirements for baking, negative consequences for human health[16,17] and for the baking industry[18,19] are therefore expected in the future.

Baking quality strongly depends on the concentrations and composition of grain proteins. Based on their solubility in various solvents, wheat grain proteins are classified into three main groups: albumins/globulins (ALGL), gliadins (GLIA), and glutenins (GLUT).[20] Whereas ALGL often have structural and metabolic functions, GLIA and GLUT are storage proteins that are responsible for baking quality. GLIA are monomers and are subdivided into S-poor ω- and S-rich α-, and γ-GLIA, which are responsible for dough viscosity.[21] GLUT are protein aggregates of disulfide-linked subunits of high (HMW-GS) and low (LMW-GS) molecular weight and are responsible for the elasticity and strength of the dough. The concentrations and composition of GLIA and GLUT, which are summarized as gluten, strongly depend on the genotype and growth conditions, especially the N and S availability in the soil. According to Martre et al.,[22] the accumulation of gluten proteins is regulated by the N source (i.e., N availability for the grains), whereas the accumulation of ALGL is regulated by the N sink (i.e., grains).

A decrease of grain protein concentration by e[CO_2] has been associated with a decrease of gluten concentration, with the concentration of ALGL being unaffected, in previous FACE studies.[4,23,24] However, results with regard to the e[CO_2] effect on gluten subfractions are inconsistent, possibly attributable to genotype[25] or environmental conditions such as soil N availability.[26,27] In Australian wheat cultivars grown

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under semi-arid conditions, e[CO_2] strongly decreases HMW-GS contents,[19] but in another study, γ-GLIA concentrations have been shown to decrease in three cultivars with gluten proteins being unaffected in one cultivar.[25] In German wheat cultivars grown under well-watered conditions and a temperate climate,[23] studies have found strong reductions (by 12−35%) of all GLIA (ω, α, and γ) and of GLUT fractions (HMW-GS and LMW-GS) in winter wheat. However, in spring wheat grown under these conditions, only a significant decrease of the GLIA content occurs.[4,24]

Loaf volume, being the most integrative factor of baking quality, has been found to be decreased under e[CO_2] (by 5−17%) in Australia.[12,18,19,28] The strong decrease of all gluten fractions observed by Wieser et al.[23] suggests that e[CO_2] might also reduce the loaf volume under Central European growth conditions. However, apart from one study in which no significant effects have been demonstrated,[29] no information is available on the e[CO_2] effect on the loaf volume under such conditions.

Despite the lack of an e[CO_2] effect on the concentration of ALGL,[4,23,24] e[CO_2] induces changes in the composition of albumins and globulins. These changes include the up- and downregulation of proteins with metabolic and stress-related functions and of storage globulins.[19,30,31]

The S, Fe, and Zn acquisitions of the grains are complex processes involving root uptake and subsequent xylem translocation, remobilization from vegetative organs, and deposition in the grains.[32] The mechanism and environmental conditions leading to the reductions of grain S, Fe, and Zn concentrations by e[CO_2] are unclear. Two essential mechanisms have been hypothesized: (i) growth dilution, implying that grain nutrient acquisition cannot keep pace with the grain yield increase by e[CO_2][33] and (ii) reduced mass flow of nutrients to the roots because of reduced stomatal conductance.[34] Moreover, in wheat, S is an important component of gluten proteins,[21] and at least in durum wheat, grain protein is a potential sink for Fe and Zn,[35] and Fe and Zn remobilization to the grains might be linked to that of N.[36,37] Thus, in wheat, a decrease of S, Fe, and Zn concentrations in grains could be a consequence of an e[CO_2]-induced decrease of grain protein concentration.

Based on the open questions addressed above and the lack of field studies simultaneously investigating the e[CO_2] effect on the wheat grain proteome and baking quality, a two-year FACE experiment was conducted. In this experiment, in which winter wheat was grown under well-watered conditions and two CO_2 concentrations (393 and 600 ppm) and three N supply levels ranging from 35 to 320 kg N ha^{-1}, e[CO_2] led to decreased grain N concentrations by 1−6%.[38] Based on this result, we asked the following questions: (i) does e[CO_2] affect gluten proteins, and does this result in diminished loaf volume, (ii) does e[CO_2] decrease Fe and Zn concentration, and in this regard, is there a correlation between mean protein content per grain and that of Fe and Zn, and (iii) is there a $CO_2 \times N$ interaction with respect to the chemical quality traits of the grains?

2. MATERIAL AND METHODS

2.1. Experimental Design and Crop Management. The experiment was conducted on a field site (52°18′N, 10°26′E, 79 m a.s.l.) at the Thünen-Institut in Braunschweig, Germany, during 2014 and 2015. Winter wheat (*T. aestivum* L., variety "Batis") was grown under the ambient CO_2 concentration (a[CO_2]) (393 ppm) and under e[CO_2] (600 ppm) and under three N levels comprising deficient, adequate, and excess treatments. The CO_2 treatments were conducted in circular plots (diameter of 20 m), in which the N treatments were randomly established at three rectangular subplots (3 m × 5 m). In total, the experiment comprised six different $CO_2 \times N$ treatments that were replicated three times each in both years. Wheat was grown under well-watered conditions and, according to local farm practice, with adequate crop management and nutrient supply. Detailed descriptions of the study site, climatic conditions, crop management, CO_2 enrichment, N fertilization, and irrigation can be found elsewhere.[7,9,38] The amount of N fertilizer was 40 and 35 kg N ha^{-1} for the N deficiency level (Nd), 180 and 200 kg N ha^{-1} for the adequate N level (Nad), and 320 kg N ha^{-1} for the excess N level (Nex) during 2014 and 2015. Additionally, S (20 kg S ha^{-1}) fertilization occurred on March 20 in 2014 and April 7 in 2015. A specific Zn fertilization was not conducted. Based on the measurements from previous years, the Zn concentration in the topsoil (0−30 cm) amounted to 7 mg Zn kg^{-1}.

2.2. Determination of Crude Protein, S, Fe, and Zn Concentrations in Grain. Grain samples were ground to pass through a 0.75 mm sieve in an ultracentrifugal mill (type ZM1, Retsch) after being dried at 105 °C. The N and S concentrations of the ground samples were determined with an element analyzer (TruSpec CNS, Leco), and the crude protein concentration was determined by multiplying the N concentration with a conversion factor of 5.7. Concentrations of Fe and Zn were determined by optical emission spectrometry with inductively coupled plasma after incineration, followed by disintegration with hydrochloric acid according to method number 10.8.2 of VDLUFA.[39]

2.3. Determination of ALGL, GLIA, and GLUT Concentrations. Grains were ground in a falling number mill (Laboratory Mill 120, Perten) and then in a ball mill (MM 400, Retsch) to a fine powder. ALGL, GLIA, and GLUT were stepwise extracted from 100 mg of whole grain flour according to Wieser and Seilmeier.[20] After extraction, the three different fractions were centrifuged (15 min, 15,000*g*, 20 °C), and the upper 1 ml was used for the photometric determination of the protein concentration according to Bradford.[40] Isolated fractions of ALGL, GLIA, and GLUT were used as protein references. ALGL were isolated according to Pflaum et al.,[41] and GLIA and GLUT were isolated according to Thanhaeuser et al.[42] References and samples were diluted 1:5 with H_2O, and 10 μL of the mixture was mixed with 200 μL of 1:5-diluted Bradford solution (Roti-Quant, Roth) followed by measurement of the extinction at 595 nm and 25 °C with a microplate reader (TriStar^2 LB942, Berthold). Calibration curves were prepared with five different concentrations that ranged from 0.5 to 4 $\mu g\ \mu L^{-1}$. Six technical replications were used for each sample and reference, and the measurements were repeated if the coefficient of variation was above 5%.

2.4. Proteomics of Wheat Grain. *2.4.1. Protein Extraction.* Proteins were extracted by a dithiothreitol (DTT)−trichloroacetic acid (TCA)−acetone precipitation method according to Zörb et al.[43] with some modifications. Whole kernel flour (100 mg) was suspended in 1.6 mL of acetone (chilled to 4 °C) containing 10% (w/v) TCA and 50 mM DTT and was incubated at −20 °C overnight. After centrifugation (15 min, 15,000*g*, 4 °C), the pellet was resuspended in 1.5 mL of 90% acetone (chilled to 4 °C) containing 50 mM DTT and incubated at −20 °C for 2 h followed by centrifugation (15 min, 15,000*g*, 4 °C). Both precipitation steps were initiated by vortexing (1 min), sonication (15 min, 4 °C), and re-vortexing (1 min). The pellet was then washed with 1.5 mL of 90% acetone (chilled to 4 °C) containing 50 mM DTT, centrifuged (15 min, 15,000*g*, 4 °C), and dried in a speedvac (SPD121P, Thermo Fisher). The pellet was resuspended in an aqueous solution containing 7 M urea, 2 M thiourea, 0.5% IPG-buffer (Serva Servalyt 3−10 Isodalt), 3% (w/v) CHAPS, 30 mM DTT, and 0.1% (v/v) protease inhibitor followed by magnetic stirring for 2 h at 30 °C. After sonication for 10 min and centrifugation (30 min, 15,000*g*, 10 °C), the upper 0.5 mL of the supernatant was used for two-dimensional gel electrophoresis. The protein concentration was determined by means of the 2D Quant Kit (GE-Healthcare) according to the instruction of the manufacturer.

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Table 1. *F*-Test Result of the Effect of the Two CO_2 and Three N Levels on Grain Elemental Concentrations; Grain N-to-S Ratio; Grain Albumin/Globulin (ALGL), Gliadin (GLIA), Glutenin (GLUT), and Total Gluten Concentrations; Dough Water Absorption, Development Time, and Loaf Volume[a]

variable	CO_2	N	year (Y)	$CO_2 \times N$	$CO_2 \times Y$	$N \times Y$	$CO_2 \times N \times Y$
crude protein conc (%)	(*)	***	***	*	ns	***	ns
S conc (mg g^{-1})	ns	***	*	ns	ns	ns	ns
Fe conc (mg kg^{-1})	ns	***	***	*	ns	***	*
Zn conc (mg kg^{-1})	ns	***	ns	ns	(*)	ns	ns
N/S ratio	ns	***	ns	ns	ns	ns	ns
ALGL conc (mg g^{-1})	(*)[b]	***[b]	ns[b]	ns[b]	ns[b]	***[b]	ns[b]
GLIA conc (mg g^{-1})	ns	***	***	ns	ns	ns	ns
GLUT conc (mg g^{-1})	ns[c]	***[c]	***[c]	ns[c]	ns[c]	**[c]	ns[c]
gluten conc (mg g^{-1})[d]	(*)	***	***	ns	ns	**	ns
GLIA/GLUT ratio	ns	ns	ns	(*)	ns	(*)	ns
dough water absorption (mL)	(*)	***	(*)	ns	(*)	***	ns
dough development time (min)	ns[b]	***[b]	*[b]	ns[b]	ns[b]	**[b]	ns[b]
loaf volume (mL)	ns	***	ns	ns	ns	***	ns

[a](*)$P < 0.1$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. [b]Data were log-transformed to ensure variance homogeneity and normal distribution of the residual error. [c]Data were square root-transformed to ensure variance homogeneity and normal distribution of the residual error. [d]Sum of GLIA and GLUT concentrations.

2.4.2. Two-Dimensional Gel Electrophoresis. IPG strips (11 cm, pH 3−10, Serva) were placed into a 200 μL protein solution containing 50 μg of protein and were incubated for 14 h at ~20 °C. Isoelectric focusing (IEF) was conducted with an IEF system (Protean i12, Bio-Rad) under the following conditions: 250 V for 0.5 h, 1000 V for 1 h, and 6000 V for 3 h at 20 °C. After IEF, the strips were incubated stepwise in equilibration buffer [30% (v/v) glycerol, 50 mM Tris−HCl, pH 8.8; 6 M urea, 2% (w/v) SDS] containing 1% DTT and then in equilibration buffer containing 4% (w/v) iodoacetamide, under slight shaking for 15 min each.

SDS-PAGE was conducted in a vertical second dimension electrophoresis system (SE900, Hoefer) by using 12.5% (v/v) polyacrylamide gels. The strips were briefly incubated in running buffer [25 mM Tris, 192 mM glycin, 0.1% (w/v) SDS], and after application onto the gel, the strips were sealed with 0.5% (w/v) molten agarose containing a small amount of bromophenol blue. The running conditions of each gel were 20 °C at 20 mA for the first 2 h and at 80 mA until the dye front left the gel. After the run, gels were fixed with 40% ethanol and 10% acetic acid overnight followed by staining with hot (80−90 °C) Coomassie R 250 for 10 min. Gels were destained with 10% acetic acid for 24 h.

2.4.3. Image and Data Analysis. Gels were scanned in a transmission and grayscale mode (300 dpi, 16 bits per pixel), analyzed with the software Delta2D (version 4.4, Decodon), and warped with a group warping strategy to generate a fusion gel by means of average fusion algorithms. The fusion gel was used to detect and edit spots and to delete artefacts. The same set of spots was used across all analyzed $CO_2 \times N \times$ year treatments. Spot intensity was regarded as significantly different between treatments if the pairwise *t*-test resulted in $P < 0.05$. Spots were disregarded if average relative spot intensity was below 0.02 or the coefficient of variation was above 40%. One technical gel replication was used.

2.4.4. Protein Identification. Spots were manually excised from the gels and were subjected to in-gel trypsin digestion according to Olsen et al.[44] with modifications. The gel pieces were incubated stepwise in an aqueous solution with 20 mM NH_4HCO_3 and in acetonitrile, each for 5 min, and this was repeated at least twice. The samples were then stepwise incubated for 20 min in an aqueous solution with 20 mM NH_4HCO_3 and 10 mM DTT at 56 °C and in a solution with 20 mM NH_4HCO_3 and 55 mM iodoacetamide at about 20 °C. Trypsin (modified sequencing grade, Promega) digestion was conducted overnight at a concentration of 12.5 ng μL^{-1}, and the tryptic peptides were extracted from the gels by acidification with trifluoroacetic acid to the concentration of 0.1%. The supernatant was concentrated in a speedvac (SPD121P, Thermo Fisher) to a volume of 20−40 μL and desalted by using reversed-phase C_{18} tips (Thermo Scientific) according to the instruction of the manufacturer.

Tryptic peptide mixtures were analyzed by LC/MS/MS by using a nanoflow Easy-nLC1000 (Thermo Scientific) as a high-performance liquid chromatography system and a Quadrupole-Orbitrap hybrid mass spectrometer (Q-Exactive Plus, Thermo Scientific) as a mass analyzer. Peptides were eluted from a 75 μm × 50 cm analytical column (Thermo Scientific) on a linear gradient running from 4 to 64% acetonitrile over 240 min and were sprayed directly into an LTQ-Orbitrap mass spectrometer. Proteins were identified by MS/MS by using information-dependent acquisition of fragmentation spectra of multiple charged peptides. Up to 12 data-dependent MS/MS spectra were acquired for each full-scan spectrum acquired at 60,000 full-width half-maximum resolution. Overall cycle time was approximately 1 s.

Protein identification within each spot of interest was carried out by MaxQuant version 1.4.0.1.[45] Spectra were matched against the wheat proteome (Taestivum_296_v2.2, 293053 entries) by using Andromeda.[46] Thereby, carbamidomethylation of cysteine was set as a fixed modification; oxidation of methionine was set as a variable modification. Mass tolerance for the database search was set to 20 ppm on full scans and 0.5 Da for fragment ions. Multiplicity was set to 1. For label-free quantitation, retention time matching between runs was chosen within a time window of 2 min. Peptide false discovery rate (FDR) and protein FDR were set to 0.01, whereas site FDR was set to 0.05. Hits with regard to contaminants (e.g., keratins) and reverse hits identified by MaxQuant were excluded from further analysis. Spot intensity quantitation was performed by image analysis.

2.5. Micro-Scale Baking Test. Grains were ground in a falling number mill (Laboratory Mill 120, Perten). Before usage, the whole grain flour was stored for 2 weeks at ~20 °C. Dough water absorption and development time were determined for each sample by mixing 10 g of flour, 0.2 g of NaCl, and water at 22 °C for 20 min in a micro-farinograph (Brabender). For the baking test, 10 g of whole grain flour, 0.7 g of yeast, 0.2 g of NaCl, 0.2 g of sucrose, 0.1 g of coconut fat, 0.3 mL of ascorbic acid solution (0.67 g L^{-1}), and water according to the determined water absorption were mixed in the micro-farinograph, and the dough was kneaded according to the determined dough development time.[47] The dough was then weighed and allowed to ferment at 100% relative humidity at 30 °C for 20 min. After rounding, the dough was again allowed to ferment at 100% relative humidity at 29 °C for 35 min followed by baking at 120, 180, and 250 °C for 10 min. Loaf volume was measured in a Volscan Profiler 600 (Stable Micro Systems) 1 h after baking.

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2.6. Statistical Analysis. The *F*-test was implemented with SAS (version 9.4) proc mixed and mean comparison with proc glimmix as previously described.[7,38]

The relationship between loaf volume and concentration of crude protein, GLIA, or GLUT was calculated by quadratic regression with SAS proc mixed under the following mixed model

$$v = \mu + CO_2 + Pp + CO_2 \times Pp + P \times Pp^2 + CO_2 \times P \times Pp^2 + Y + R + N \times R + Y \times R + N \times Y \times R$$

where v is loaf volume; p is crude protein, GLIA, or GLUT concentration; μ is the overall y-intercept; CO_2 is the parameter of CO_2 treatment altering the y-intercept; P and $P \times P$ are the general parameters of crude protein, GLIA, or GLUT concentration; $CO_2 \times P$ and $CO_2 \times P \times P$ are the parameters of CO_2 treatment altering the concentration parameters; Y, R, $N \times R$, and $Y \times R$ are the random effect of year, main plot, subplot, and year × main plot interaction, respectively; and $N \times Y \times R$ is the residual error.

3. RESULTS

3.1. Concentration of Nutrients and N-to-S Ratio of Wheat Grain. Grain crude protein concentrations ranged from 7.7 to 13.5%, S concentrations from 1.03 to 1.44 g kg^{-1}, Fe concentrations from 29.4 to 45.0 mg kg^{-1}, Zn concentrations from 19.9 to 26.6 mg kg^{-1}, and N-to-S ratios from 13.1 to 16.7. Crude protein and Zn concentration and N-to-S ratio significantly increased with increasing N supply in both years (Tables 1 and 2). The S concentration increased from the N deficiency (Nd) to the adequate N level (Nad), but further rises in excess N supply (Nex) did not result in further increases. Fe concentration was increased under Nex compared with Nd and Nad in 2014. Crude protein and S concentrations were significantly increased by 14 and 9%, respectively, in 2015 compared with 2014, whereas Fe concentration was increased (23%) in 2014.

A correlation was found between mean crude protein content per grain and that of S ($r^2 = 0.62$, $P < 0.001$) but not between mean protein content per grain and that of Fe or Zn (Figure S1).

e[CO_2] significantly ($P < 0.05$) decreased crude protein concentration in both years on average by 6% under Nad (Table 2). Fe concentration was significantly ($P < 0.05$) increased by e[CO_2] (18%) under Nd in 2015, but otherwise, it was unaffected. A significant ($P < 0.1$) CO_2 × year interaction was detected for Zn concentration; this was attributable to increased values in 2014 compared with 2015 under e[CO_2] and to no difference between the two years under ambient [CO_2]. Neither S concentration nor N-to-S ratio was significantly influenced by e[CO_2].

The grain yields of S, Fe, and Zn were increased by e[CO_2] under all N levels by 7−37% (Table S1), but these increases were only significant for Fe (Table S2).

Table 2. Effect of the Two CO_2 and Three N Levels on Grain Crude Protein, S, Fe, and Zn Concentrations and on Grain N-to-S Ratio[a,b]

	2014			2015		
	Nd	Na	Ne	Nd	Na	Ne
			Crude Protein Conc (%)			
a[CO_2]	7.9 c	10.6 A b	12.3 a	9.1 c	12.6 A b	13.5 a
e[CO_2]	7.7 c	10.0 B b	11.9 a	9.0 c	11.9 B b	12.9 a
Δ (%)	−2	−6	−4	−1	−6	−4
			S Conc (mg g^{-1})			
a[CO_2]	1.07 b	1.29 a	1.37 a	1.16 b	1.41 a	1.44 a
e[CO_2]	1.03 b	1.26 a	1.32 a	1.17 b	1.38 a	1.40 a
Δ (%)	−3	−3	−4	1	−2	−2
			Fe Conc (mg kg^{-1})			
a[CO_2]	35.4 b	35.9 b	44.7 a	29.4 B	33.4	34.1
e[CO_2]	36.8 b	39.7 b	45.0 a	34.7 A	30.5	32.7
Δ (%)	4	11	1	18	−9	−4
			Zn Conc (mg kg^{-1})			
a[CO_2]	19.9 c	21.0 b	23.1 a	20.0 c	21.5 b	25.0 a
e[CO_2]	19.9 c	23.1 b	26.6 a	19.9 c	21.1 b	24.2 a
Δ (%)	0	10	15	0	−2	−3
			N/S Ratio			
a[CO_2]	13.1 c	14.4 b	15.8 a	13.9 c	15.7 b	16.7 a
e[CO_2]	13.2 c	13.9 b	15.8 a	13.5 c	15.2 b	16.2 a
Δ (%)	1	−3	0	−3	−4	−3

[a]Mean values ($n = 3$) and the percentage effect of e[CO_2] [Δ (%)] are shown. Different small letters indicate significant differences among the marginal means (mean over the two CO_2 treatments) of the N levels within each year. Different capital letters indicate significant differences between the means of the CO_2 treatments within each N level. [b]N treatments: Nd = N deficiency, Nad = adequate N supply, Nex = excess N supply.

3.2. Concentration of Protein Fractions of Wheat Grain. ALGL concentrations ranged from 37.7 to 52.3 mg g^{-1}, GLIA concentrations from 15.3 to 37.6 mg g^{-1}, GLUT concentrations from 17.3 to 43.2 mg g^{-1}, total gluten concentrations from 32.6 to 75.5 mg g^{-1}, and GLIA-to-GLUT ratios from 0.8 to 1.1 (Table 3). The ALGL concentration was significantly increased by Nex in 2014 and increased with rising N supply in 2015 (Tables 1 and 3). The GLIA concentration increased with rising N supply in both years. The GLUT and total gluten concentrations increased with rising N level in 2014 but only increased for Nd to Nad in 2015. Over both years, the concentration of ALGL respectively increased by 16 and 10%, GLIA by 65 and 11%, and GLUT by 56 and 10% for Nd to Nad and from Nad to Nex. The GLIA (16%) and GLUT (25%) concentrations were increased in 2015 compared with 2014.

e[CO_2] significantly ($P < 0.1$) decreased the ALGL concentration in both years on average by 1, 3, and 3% and total gluten concentration by 7, 7, and 3% under Nd, Nad, and Nex, respectively (Tables 1 and 3). The GLIA-to-GLUT ratio was significantly ($P < 0.1$) increased in both years on average by 16% under Nad because of the decrease of the GLUT concentration.

3.3. Baking Quality. Dough water absorption and loaf volume significantly increased with rising N supply in 2014 and increased only from Nd to Nad in 2015 (Tables 1 and 4). The largest difference in loaf volume was detected between the Nd and Nad levels in 2014: loaf volume was increased by 51% under Nad. Dough development time was significantly increased under Nd compared with Nad and Nex for both years.

e[CO_2] slightly but significantly ($P < 0.1$) decreased dough water absorption under all N levels (by 1−3%) in 2015 (Table 4). However, dough development time and loaf volume were unaffected.

3.4. Proteomics of Wheat Grain. The Nd and a[CO_2] treatment of 2014 and the Nad treatment with a[CO_2] and e[CO_2] of both years were used further, and 453 common protein spots were analyzed. The comparison between Nd and Nad in 2014 when loaf volume showed the largest difference

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Table 3. Effect of the Two CO_2 and Three N Levels on Grain Albumin/Globulin (ALGL), Gliadin (GLIA), Glutenin (GLUT), and Total Gluten Concentrations and on GLIA-to-GLUT Ratio[a,b]

	2014			2015		
	Nd	Na	Ne	Nd	Na	Ne
			ALGL Conc (mg g^{-1})			
a[CO_2]	41.9 b	44.5 b	49.7 a	37.9 c	48.9 b	52.3 a
e[CO_2]	41.2 b	43.8 b	49.2 a	37.7 c	46.6 b	50.0 a
Δ (%)	−2	−2	−1	0	−5	−4
			GLIA Conc (mg g^{-1})			
a[CO_2]	18.1 c	28.6 b	33.3 a	21.1 c	32.3 b	37.6 a
e[CO_2]	15.3 c	28.0 b	31.0 a	19.6 c	33.6 b	34.5 a
Δ (%)	−16	−2	−7	−7	4	−8
			GLUT Conc (mg g^{-1})			
a[CO_2]	18.7 c	30.0 b	37.2 a	25.6 b	43.2 a	37.3 a
e[CO_2]	17.3 c	26.4 b	36.5 a	25.8 b	36.5 a	39.3 a
Δ (%)	−8	−12	−2	1	−16	5
			Gluten Conc (mg g^{-1})[c]			
a[CO_2]	36.8 c	58.5 b	70.5 a	46.7 b	75.5 a	74.9 a
e[CO_2]	32.6 c	54.3 b	67.4 a	45.4 b	70.0 a	73.8 a
Δ (%)	−12	−7	−4	−3	−7	−2
			GLIA/GLUT Ratio			
a[CO_2]	0.969	0.964 B	0.901	0.824	0.765 B	1.02
e[CO_2]	0.879	1.06 A	0.861	0.765	0.934 A	0.883
Δ (%)	−9	10	−4	−7	22	−14

[a]Mean values (n = 3) and the percentage effect of e[CO_2] [Δ (%)] are shown. Different small letters indicate significant differences among the marginal means of the N levels within each year. Different capital letters indicate significant differences between the means of the CO_2 treatments within each N level. [b]N treatments: Nd = N deficiency, Nad = adequate N supply, Nex = excess N supply. [c]Sum of GLIA and GLUT concentration.

Table 4. Effect of the Two CO_2 and Three N Levels on Dough Water Absorption and Development Time[a,b]

	2014			2015		
	Nd	Na	Ne	Nd	Na	Ne
			Water Absorption (mL)			
a[CO_2]	5.67 c	6.54 b	6.80 a	5.84 b	6.66 a	6.74 a
e[CO_2]	5.64 c	6.45 b	6.79 a	5.78 b	6.53 a	6.57 a
Δ (%)	0	−1	0	−1	−2	−3
			Development Time (min)			
a[CO_2]	10.9 a	6.23 b	4.70 b	7.33 a	5.03 b	5.47 ab
e[CO_2]	9.30 a	6.87 b	5.07 b	7.00 a	5.70 b	5.80 ab
Δ (%)	−15	10	8	−5	13	6
			Loaf Volume (mL)			
a[CO_2]	23.3 c	35.4 b	37.2 a	27.7 b	35.1 a	36.2 a
e[CO_2]	22.8 c	34.3 b	38.3 a	27.5 b	35.8 a	35.1 a
Δ (%)	−2	−3	3	−1	2	−3

[a]Mean values (n = 3) and the percentage effect of e[CO_2] [Δ (%)] are shown. Different small letters indicate significant differences among the marginal means of the N levels within each year. [b]N treatments: Nd = N deficiency, Nad = adequate N supply, Nex = excess N supply.

revealed that the increased N supply resulted in the increased expression of 48 protein spots (20 with an increase of >50%) (Figure 1) and decreased the expression of 117 spots (54 with a decrease of >50%). Under Nad, 127 protein spots were found with increased intensity (71 with an increase of >50%)

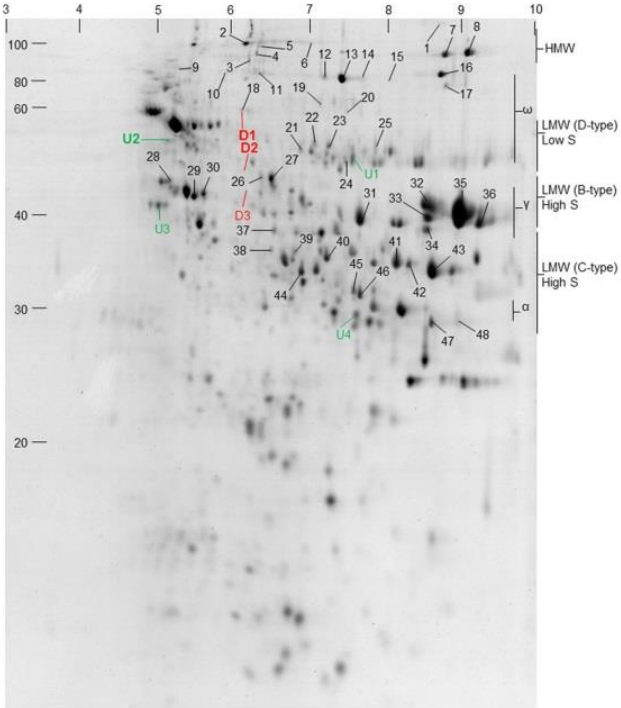

Figure 1. Exemplary two-dimensional gel image of proteins from plants grown under the adequate N level (Nad) in 2014. Black lines indicate the protein spots that were increased by increasing N supply from Nd to Nad. Red lines indicate the spots that were downregulated by e[CO_2], and green lines indicate those that were upregulated. Spot descriptions for which the e[CO_2] effect was >50% are in bold. The numbers from the left to the right indicate pH values of the isoelectric point, and numbers from top down indicate molecular mass (kDa). The descriptions on the right indicate the positions at which gliadins (ω, α, and γ) and glutenins (HMW-glutenin subunits and LMW-glutenin subunits) are expected to lie according to Belitz et al.[21]

and 62 spots showed decreased expression (8 with a decrease of >50%) in 2015 compared with 2014.

e[CO_2] increased the expression of 4 protein spots (1 with an increase of >50%) and decreased that of 3 spots (2 with a decrease of >50%) in 2014 (Figure 1 and Table 5). In 2015, e[CO_2] increased 13 protein spots and decreased 16 spots (5 with a decrease of >50%) (Figure 2 and Table 5). Seven out of the 36 protein spots that were influenced by e[CO_2] also increased under an increased N supply from Nd to Nad (Table 5) and, thus, might have an impact on baking quality. These spots comprised D1 (unidentified), D10, D11, D14 (identified as protein with cupin domain), D13, and D18 (identified as 12 S seed storage protein) that decreased (by 5−71%) and U10 (identified as ATP synthase subunit) that increased under e[CO_2] (Table 5). Nineteen out of the 36 spots altered by e[CO_2] were also influenced by the year of the experiment. Thereby, 10 of these proteins were increased and 9 were decreased in 2015 compared with 2014.

Proteins with functions in storage, metabolism, and stress response were detected in 28 out of the 36 protein spots that were influenced by e[CO_2] (Table 5). As storage proteins, cupin-domain-containing proteins were detected in 12 protein spots with decreased intensity (D4−D12, D14−D16) and in two spots with increased intensity (U1 and U16). 12S seed storage protein was detected in 3 spots with decreased intensity (D13, D17, and D18). The cupin-domain-containing

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Table 5. Effect of CO_2 and N Level and Year on Wheat Grain Proteome[a,d]

spot ID	year	relative volume a[CO_2]	% e[CO_2]	% N	% year	detected protein	possible function
D1	2014	0.103	−71*	152*** (18)		not identified[b]	
D2		0.032	−55*		32*	not identified[c]	
D3		0.054	−46*		34*	not identified[c]	
U1		0.156	23**			cupin-domain-containing protein	storage
U2		0.060	56**			ATP synthase subunit β	metabolism
U3		0.286	15*		−25***	serine protease inhibitor	stress response
U4		0.281	31**		−71***	not identified[b]	
D4	2015	0.089	−68*			cupin-domain-containing protein	storage
D5		0.037	−75*			cupin-domain-containing protein	storage
D6		0.035	−127*			cupin-domain-containing protein	storage
D7		0.066	−45*			cupin-domain-containing protein	storage
D8		0.022	−121*			cupin-domain-containing protein	storage
D9		0.023	−97*			cupin-domain-containing protein	storage
D10		0.192	−43**	54*** (22)	−39***	cupin-domain-containing protein	storage
D11		0.258	−34**	21* (23)	−19**	cupin-domain-containing protein	storage
D12		0.191	−8*		−27***	cupin-domain-containing protein	storage
D13		0.198	−25**	34*** (21)	−18*	12S seed storage protein	storage
D14		0.221	−19*	23* (24)	22**	cupin-domain-containing protein	storage
D15		0.257	−13*		33***	cupin-domain-containing protein	storage
D16		0.472	−16*		20**	cupin-domain-containing protein	storage
D17		0.567	−9*			12S seed storage protein	storage
						heat shock protein 70kDa	stress response
						elongation factor 1-γ	protein synthesis
D18		1.178	−5*	20* (40)		12S seed storage protein	storage
D19		1.095	−13*			ATP synthase β subunit	metabolism
						mitochondrial carrier protein	metabolism
U5		0.104	35**			β amylase	metabolism
U6		0.042	63**		53**	cot identified[c]	
U7		0.206	19*		−29***	stress responsive protein	stress response
U8		0.038	40*			not identified[c]	
U9		0.186	16*		−29***	glucose and ribitol dehydrogenase homolog 1-related	metabolism
U10		1.053	10*	26*** (47)	24***	ATP synthase α subunit	metabolism
U11		0.042	29*			ATP synthase β subunit	metabolism
U12		0.242	24*		−16*	triosephosphate isomerase, cytosolic	metabolism
U13		0.063	38***		102***	not identified[c]	
U14		0.284	21**			chitinase family protein precursor	stress response
U15		0.115	20*			ATP synthase β subunit	metabolism
U16		0.388	18*		38***	ATP synthase β subunit	metabolism
						ATP synthase α subunit	metabolism
						cupin-domain-containing protein	storage
U17		0.090	26*		47*	not identified[c]	

[a]Protein spots that were found to be influenced by e[CO_2] under Nad with mean relative spot volume at ambient [CO_2] and the percentage effect of e[CO_2] (% e[CO_2]), increasing N supply from Nd to Nad in 2014 (% N) and year (% year) are shown. The numbers in brackets in the fifth column are the numbers of the upregulated protein spots by increased N supply shown in Figure 1. Stars indicate the *t*-test result with regard to mean relative spot volume. Proteins detected by means of tandem mass spectrometry are shown. [b]Not identified by tandem mass spectrometry. [c]Protein spot was not excised [d]*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

proteins showed most similarity with globulin-3 (80%) and the 12S seed storage proteins with globulin-1 (99%) based on a BLAST search of the NCBI database. ATP synthase was detected in 5 spots with increased intensity (U2, U10, U11, U15, and U16). Other metabolic proteins affected were β amylase (U5), glucose and the ribitol dehydrogenase homolog (U9), and triose phosphate isomerase (U12). A mitochondrial carrier protein was found in a spot with decreased intensity (D19). Serine protease inhibitor (U3), stress responsive protein (U7), chitinase family protein precursor (U14), and heat shock protein 70 kDa (D17) were detected as proteins related to stress response.

3.5. Relationship between Protein Concentration of Grain and Baking Quality. Figures 3 and 4 show the regression of loaf volume on crude protein concentration and the regression of loaf volume on GLIA and GLUT concentrations, respectively. These relationships corresponded more to a quadratic function (crude protein, $r^2 = 0.92$; GLIA, $r^2 = 0.87$; GLUT, $r^2 = 0.83$), with the increase of loaf volume at a maximum level and a subsequent decline, rather than to a linear function (crude protein, $r^2 = 0.77$; GLIA, $r^2 = 0.75$; GLUT, $r^2 = 0.67$).

e[CO_2] significantly ($P < 0.1$) altered the course of the regression curve of loaf volume on crude protein concentration (Figure 3) by decreasing the protein concentration for

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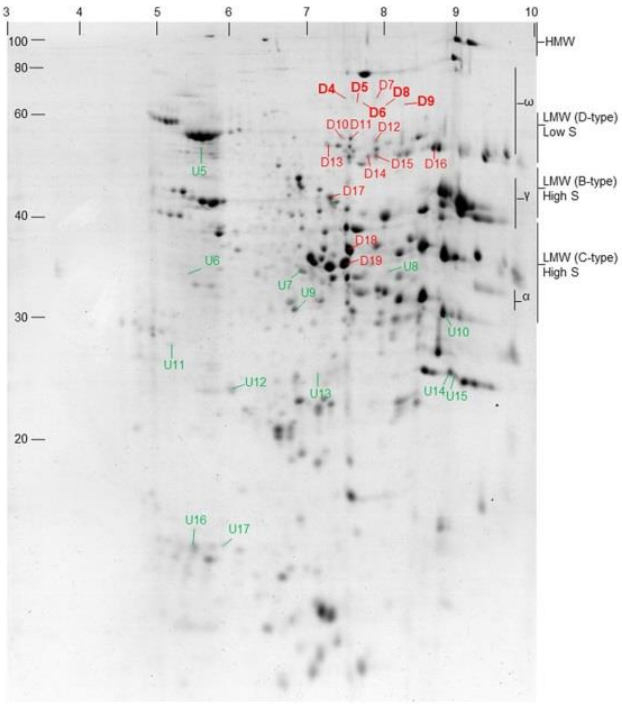

Figure 2. Exemplary two-dimensional gel image of proteins in plants grown under Nad in 2015. Red lines indicate the protein spots that were downregulated by e[CO_2], and green lines indicate those that were upregulated. Spot descriptions for which the e[CO_2] effect was >50% are in bold. Numbers from the left to the right indicate pH values of the isoelectric point, and numbers from top down indicate molecular mass (kDa). The descriptions on the right indicate the positions where gliadins (ω, α, and γ) and glutenins (HMW-glutenin subunits and LMW-glutenin subunits) are expected to lie according to Belitz et al.[21]

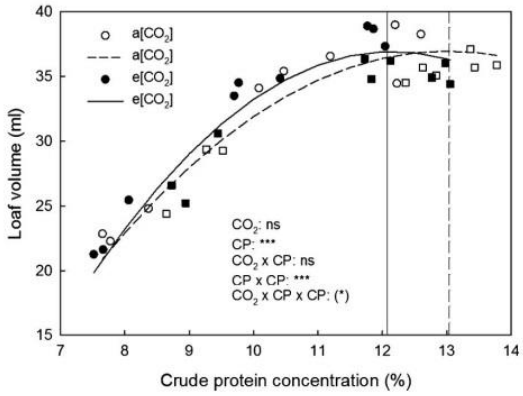

Figure 3. Relationship between loaf volume and crude protein concentration. The curves derive from the quadratic regression model described in 2.6. Circles indicate the values of 2014, and squares indicate those of 2015. The vertical lines indicate maximum loaf volume derived from the regression. The diagram includes the statistical results of the parameters of the regression model; CP stands for crude protein concentration. $^{(*)}P < 0.1$ $^{***}P < 0.001$.

maximum loaf volume from 13.0 to 12.1%. e[CO_2] did not influence the course of the regression curve of loaf volume on GLIA or GLUT concentrations (Figure 4).

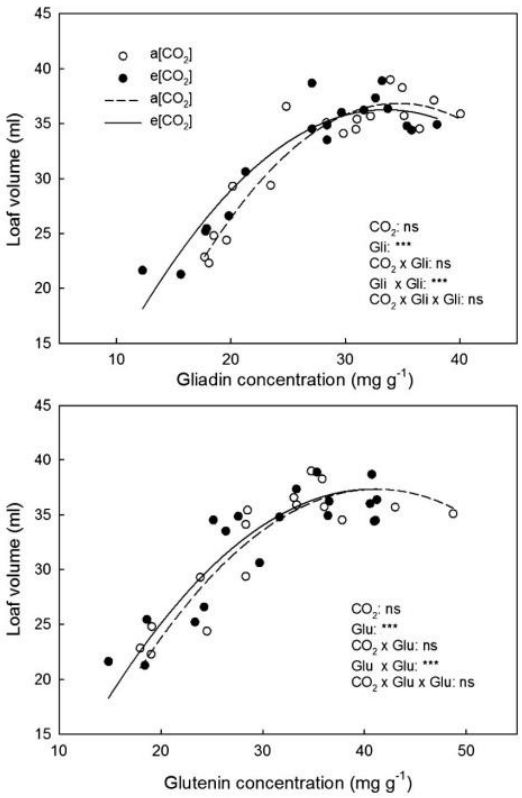

Figure 4. Relationship between loaf volume and gliadin and glutenin concentrations, respectively. The curves derive from the quadratic regression model described in 2.6. The diagram includes the statistical results of the parameters of the regression model; Gli and Glu stand for gliadin and glutenin concentrations, respectively. $^{***}P < 0.001$.

4. DISCUSSION

In the present study, interactions between e[CO_2] and widely differing N fertilizer levels on the important chemical grain quality traits of gluten concentration and composition, baking quality, and Fe and Zn concentrations were investigated in winter wheat grown under German conditions. Furthermore, the e[CO_2] effect on the grain proteome was analyzed under an adequate N supply. Specifically, it was tested whether e[CO_2] affected gluten proteins and thereby decreased baking quality and whether e[CO_2] decreased grain Fe and Zn concentrations.

4.1. Grain Proteome. Increasing N supply increased ALGL, GLIA, and GLUT concentrations with a stronger effect on the gluten fractions than on ALGL. This result agrees with those of Triboi et al.[48] and partly with those of Martre et al.[22] who have found that the grain accumulation of gluten proteins is regulated by the N source size (i.e., vegetative N at anthesis and availability of N in soil at grain filling), but that the grain accumulation of ALGL is regulated by the N sink size of the grains. However, the ALGL concentration and proteins with a cupin domain showing most similarity with globulin-3 were strongly increased by increased N supply. This result suggests a strongly increased sink demand for N by increased N supply or source regulation for some globulin-like proteins. The high ALGL concentration under Nex might also be associated with the lower individual grain weight under this N level compared with Nd and Nad,[7] implying a smaller dilution of ALGL by grain growth.

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e[CO_2] reduced crude protein concentration in grain by 6% under Nad and by 4% under Nex, results that are consistent with those from other FACE studies (e.g.[10,24,49]). However, the slight decrease under Nd (by 1%) contrasts with FACE studies under subtropical and semiarid conditions in which a strong decrease of the protein concentration (by 9−10%) was found under N deficiency.[26,50] A possible reason for the differing results is larger soil N availability per grain during grain filling in the present study because of a different timing in the main N supply from mineralization. Mineralization depends on temperature, and under a warm climate, main mineralization very likely occurs before anthesis mainly influencing growth, but under moderate climate, it occurs after anthesis influencing grain N supply.

The decrease of crude protein concentration by e[CO_2] was associated with a decrease of gluten proteins, although the ALGL concentration was shown to be unaffected in previous FACE studies.[4,19,23,24,29,30] For instance, a strong decrease of HMW-GS,[19,30] which is particularly important for baking quality,[51] of all GLIA fractions (by 8−26%),[24] and all GLIA types (ω, α and γ) and GLUT subunits (HMW-GS and LMW-GS) (by 12−35%)[23] have been found. In the present study, however, apart from a slight decrease of total gluten concentration under all N levels and that of GLUT under Nad, no e[CO_2] effects on concentration and composition of gluten proteins were detected. Nevertheless, an unidentified protein spot (D1) that might be important for baking quality was strongly decreased by e[CO_2]. This protein spot might be important because of its increased expression under the increased N supply from Nd to Nad and its closest link to loaf volume by cluster analysis (Figure S2). e[CO_2] affected the total amount of ALGL as indicated by the decrease of the ALGL concentration and the decrease of several proteins that showed most similarity to globulin-1 or globulin-3. This result contrasts with previous FACE studies.[4,23,24]

The contrasting results between the present study and that of Wieser et al.[23] in which e[CO_2] strongly affected gluten proteins in the same cultivar grown at the same field site as in the present study might be explained by differences in soil S availability. Whereas the grain N-to-S ratio ranged from 13 to 17 in the present study, it was 23−26 in our former FACE experiment.[10] An N-to-S ratio above 17 indicates an S deficiency in the wheat plant and can lead not only to a strong decrease of S-rich α- and γ-GLIA and LMW-GS but also to a decrease of S-poor HMW-GS.[52] Thus, S deficiency possibly exacerbated the decline of gluten proteins by e[CO_2] in the former FACE experiment.[23]

The number of protein spots that were decreased under e[CO_2] differed between the two years, indicating a rather unspecific proteome response to e[CO_2], and thus a large environmental influence on this response. This CO_2 × year interaction might be associated with increased mean N content per grain (by 13%) in 2015 compared with 2014.[38] Nevertheless, the proteins affected by e[CO_2] were mainly GLUT and proteins with a cupin domain, all of which preferentially accumulate at late grain filling.[48,53] Thus, a reducing effect of e[CO_2] on the grain proteome might primarily occur at late grain filling.

Among the proteins that increased under e[CO_2], subunits of ATPase were most frequently detected (in one protein spot in 2014 and in 5 spots in 2015). Subunits of ATPase and enzymes of glycolysis are involved in the energy supply for starch synthesis.[54] In the present experiment, e[CO_2] increased individual grain weight[7] and the rate of grain filling (data not shown). Thus, the increased abundance of ATPase subunits and the enzyme triosephosphate isomerase of glycolysis might be associated with the e[CO_2] effect on individual grain growth. Consistent with other FACE studies,[19,30] the expression of stress-related proteins such as serine protease inhibitor and chitinase-related protein was increased under e[CO_2], although the extent of the increase was much lower in the present study. For instance, the increase of the serine protease inhibitor was more than 10 times greater in the study of Högy et al.[30] than in the present study.

4.2. Baking Quality. Loaf volume increased with increasing crude protein concentration in a curvilinear manner with a maximum at a concentration of 13% at a[CO_2]. This result is consistent with the study of Gabriel et al.[55] who found similar curvilinear trends for 7 out of 13 German wheat cultivars, of which some showed relative large loaf volume at low protein concentration. However, the relationship was linear for the other cultivars. The curvilinear relationship between loaf volume and protein concentration can be attributed to the curvilinear relationships between loaf volume and GLIA or GLUT concentrations (Figure 4), indicating a change of GLIA and GLUT compositions with increasing concentration.

e[CO_2] did not affect loaf volume, despite decreased crude protein and gluten concentrations. This result is consistent with another German FACE study,[29] but contrasts with Australian FACE studies under semiarid conditions in which decreases of loaf volume from 5 to 17% were found.[12,18,19,28] These contrasting results might be explained by a strong linear relationship existing between loaf volume and protein concentration for Australian cultivars compared with the curvilinear relationship found in the present study. Different water availability seems unlikely to be a reason for the different e[CO_2] effect on baking quality between the present and Australian studies, as loaf volume was also unaffected by e[CO_2] in a drought stress treatment in the present study (data not shown). Interestingly, the relationship between loaf volume and protein concentration was slightly influenced by e[CO_2], decreasing the crude protein concentration for maximum loaf volume from 13 to 12%. An explanation might be the increased GLIA-to-GLUT ratio toward the value of one under Nad. In summary, the use of cultivars showing relatively large loaf volume at low protein concentration and a curvilinear relationship between these variables might be a measure for maintaining baking quality under future e[CO_2].

4.3. Grain Iron, Zinc, and Sulfur Concentrations. Grain Fe, Zn, and S concentrations increased with an increasing N level, a result that is consistent with those of other studies.[35,56] These increases might be attributed to several factors, including increased levels of enzymes and chelates involved in mineral uptake and transport, increased grain sink size for minerals, or association with N remobilization.[35,37,56] However, no correlation between mean protein content per grain and that of Fe and Zn was detected, indicating no necessary association between the remobilization of N and these minerals in winter wheat. A reason for the year effect on grain Fe concentration might be increased bioavailability of Fe in the soil during 2014 compared with 2015.

e[CO_2] did not affect grain Fe, Zn, and S concentrations but increased the Fe concentration under Nd. In other European FACE studies, Fe,[31] Fe and Zn,[10] and Zn and S concentrations[4,24] were also unaffected, but occasional

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decreases of Fe (6%),[24] Zn (11%),[31] and S (5%) concentrations[10] were found. In contrast, consistent decreases of Fe, Zn, and S concentrations in wheat (4−17%) were detected in Australian FACE studies.[11−14]

The present study indicates that decreases of Fe, Zn, and S concentrations are not a general response to e[CO_2]. Moreover, the absence of a correlation between mean protein content per grain and that of Fe and Zn suggests no association between e[CO_2]-induced decline in grain protein content and these minerals. However, a decline in grain S content could be associated with an e[CO_2] induced decline in the protein content because of the correlation between the S and protein content per grain. The more consistent decrease of Fe, Zn, and S concentrations in Australian FACE studies under semi-arid conditions compared with the European studies without severe drought stress might be explained by the stronger grain yield increase in the former (38−59%)[6,11] than in the latter studies (10−21%).[4,5,7,31] Moreover, decreases of Fe and Zn concentrations often seemed to occur at high Fe and Zn contents. For instance, Verrillo et al.[31] found a reduction of Zn concentration of 18% at 35 mg kg^{-1} but only of 3% at 22 mg kg^{-1}, and Högy et al.[24] found a decreased Fe concentration (6%) at 45 mg kg^{-1}. In comparison, mean Fe and Zn concentrations were 36 and 22 mg kg^{-1}, respectively, in the present study.

Fe and Zn yields were increased by e[CO_2] in the present (Table S1) and in another FACE study.[12] Thus, besides possibly decreased concentrations, increased grain Fe and Zn yields and N yields[38,49] should be taken into account in assessing the impacts of future e[CO_2] on human nutrition.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.9b07817.

> Regression of mean content per grain of S, Fe, and Zn on mean crude protein content per grain based on all $CO_2 \times N \times$ year combinations; effect of the two CO_2 and three N levels on grain yield of S, Fe, and Zn and their *F*-test result; and cluster analysis based on the relative protein spot volume of the protein spots increased by the rising N level from Nd to Nad in 2014 and the loaf volume of the Nd and Nad N level of 2014 (PDF)

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Notes

The authors declare no competing financial interest.

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5.6 Anhang

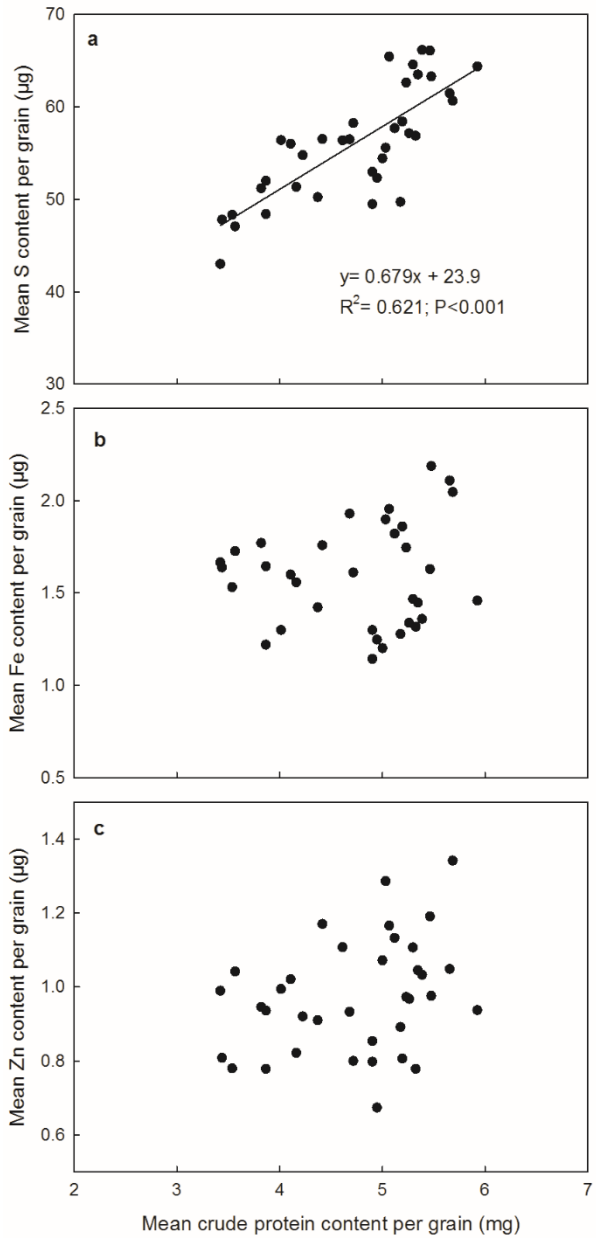

Figure S1: Regression of mean content per grain of S (a), Fe (b), and Zn (c) on mean crude protein content per grain. The values of all CO_2 × N × year treatments are shown. The diagram of the relationship between Fe and Zn content, respectively, and that of crude protein does not contain a regression line because of the lack of significant correlation.

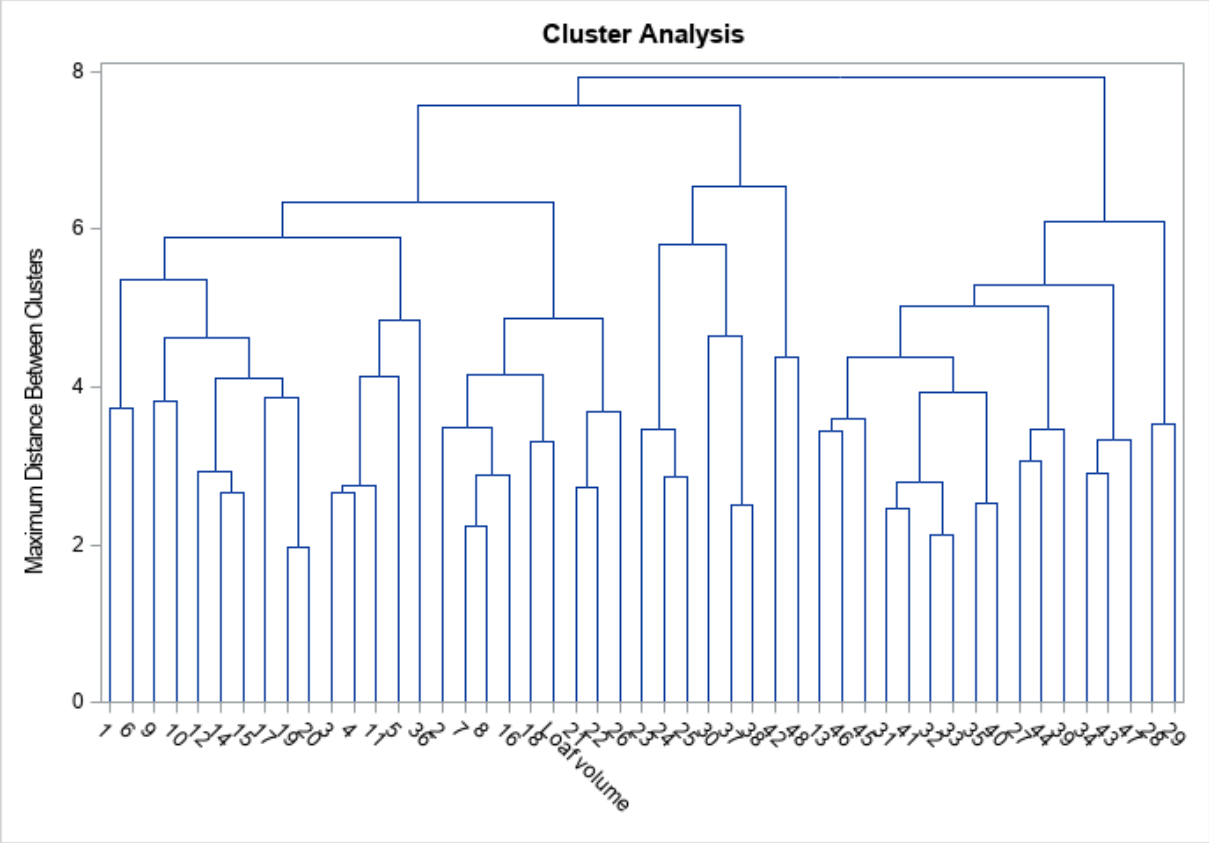

Figure S2: Cluster analysis based on the relative protein spot volume and the loaf volume of the deficient (Nd) and adequate N level (Nad) of 2014. The protein spots that were increased by the rising N level from Nd to Nad were used. The numbers represent the protein spots with the same numbers shown in **Figure 1**. The clustering is based on the complete linkage method.

Table S1: Effect of the two CO_2 and three N levels on grain yield of S, Fe, and Zn. Mean values (n=3) and the percentage effect of e[CO_2] (Δ (%)) are shown.

	2014			2015		
	Nd	Na	Ne	Nd	Na	Ne
S yield (g m^{-2})						
a[CO_2]	0.50	1.05	1.12	0.48	1.17	1.19
e[CO_2]	0.55	1.19	1.28	0.52	1.34	1.33
Δ (%)	9	13	14	8	14	11
Fe yield (mg m^{-2})						
a[CO_2]	16.8	29.3	36.6	12.3	27.8	28.3
e[CO_2]	19.4	37.7	43.7	15.3	29.7	31.0
Δ (%)	16	29	20	25	7	9
Zn yield (mg m^{-2})						
a[CO_2]	9.5	17.2	18.8	8.3	18.0	20.8
e[CO_2]	10.4	21.9	25.8	8.9	20.6	23.0
Δ (%)	10	27	37	7	14	10

Table S 2: F-test result of the effect of the two CO_2 and three N levels on grain yield of S, Fe and Zn

Variable	CO_2	N	Year (Y)	CO_2 × N	CO_2 × Y	N × Y	CO_2 × N × Y
S yield (g m^{-2})	ns	***	ns	ns	ns	ns	ns
Fe yield (mg m^{-2})	*	***	**	ns	ns	**	ns
Zn yield (mg m^{-2})	ns	***	ns	ns	(*)	ns	ns

(*)P <0.1 *P <0.05 **P <0.01 ***P <0.001

6. Kapitel: Abschließende Diskussion

Ein Anstieg der atmosphärischen CO_2-Konzentration auf 500–620 ppm ist vorrausichtlich unvermeidlich und solch eine e[CO_2] könnte das Wachstum und die Qualität von Nutzpflanzen stark verändern (Broberg et al., 2017, 2019). Während für Weizen Ertragssteigerungen erwartet werden, könnte die Nährstoffkonzentration, insbesondere die von N, Fe und Zn, im Korn abnehmen. Jedoch stammen die meisten Daten zur e[CO_2]-Wirkung aus Kammer-Versuchen mit künstlicher Umgebung wie z.B. Klima- oder Open-Top-Kammern, wo ein beschränktes Wurzelwachstum bzw. verändertes Mikroklima eine e[CO_2]-Wirkung stark modifizieren kann (Arp, 1991; Long et al, 2005). Dagegen gibt es immer noch wenige FACE-Versuche. Weiterhin ist der genaue Mechanismus der Abnahme der Konzentration von Nährstoffen durch e[CO_2] unklar. Es könnten mehrere Prozesse gleichzeitig wirken, wobei das Auftreten eines Prozesses stark von Umweltfaktoren abhängen könnte. Auch könnten verschiedene Mechanismen unterschiedliche Auswirkungen auf die Ernährungssicherheit haben. Z.B. könnte ein Verdünnungseffekt (Loladze, 2002) mit höheren N-Erträgen (Fernando et al., 2014), aber eine Hemmung der NO_3^--Assimilation mit geringeren N-Erträgen verbunden sein (Bloom, 2015).

Die N-Versorgung bestimmt maßgebend die Ertragsbildung und Kornqualität von Weizen und beeinflusst die e[CO_2]-Wirkung auf diese Merkmale (Kimball et al., 2001, 2002; Pleijel et al., 2019). Trotzdem gibt es kaum FACE-Versuche in denen CO_2 x N-Interaktionen auf die Bildung von Ertrag und Qualität bei Weizen untersucht wurden. Das erste Hauptziel dieser Doktorarbeit war die Prozessanalyse der Reduktion der Korn-N-Konzentration durch e[CO_2] bei verschiedenen N-Stufen (35–320 kg N ha^{-1}) und N-Formen (NO_3^- und NH_4^+ betonte N-Düngung). Das Zweite Hauptziel war die Analyse der e[CO_2]-Wirkung auf die Kornprotein-Zusammensetzung und auf die Backqualität.

6.1 Einfluss der Quantität der N-Düngung auf die Ertragssteigerung durch e[CO_2]

FACE-Studien mit Weizen und verschiedenen N-Stufen wurden bisher bei gemäßigtem Klima in Deutschland (Weigel und Manderscheid, 2012), bei subtropischem Klima in Arizona und China ohne starken Trockenstress (Kimball et al., 2001; Kou et al., 2007; Ma et al., 2007; Han et al., 2015) und bei semi-ariden Bedingungen in Australien und China (Lam et al., 2012; Fitzgerald et al., 2016) durchgeführt. Wie in diesen Studien steigerte in der vorliegenden Studie e[CO_2] den Kornertrag in

allen N-Stufen (**Kapitel 2**). Jedoch nahm entsprechend unserer Hypothese und der Meta-Analyse von Pleijel et al. (2019), die Kammer- und FACE-Versuche beinhaltet, die Kornertrag-Steigerung bei starkem N-Mangel ab (**Kapitel 2**). Eine Normalisierung des CO_2-Konzentration-Anstiegs auf einen einheitlichen Wert (+ 210 ppm) ergab sehr ähnliche Ertragsanstiege von 15–20 % bei ausreichender Wasser- und N-Versorgung zwischen unserer und den obengenannten FACE-Studien trotz deutlicher Ertragsunterschiede (**Kapitel 2**). Bei semi-ariden Bedingungen wurden dagegen deutlich höhere Ertragssteigerungen durch e[CO_2] von 44–47 % festgestellt (Lam et al., 2012; Fitzgerald et al., 2016). Meta-Analysen entsprechend (Wang et al., 2013; Broberg et al., 2019) basierte die Ertragssteigerung durch e[CO_2] wesentlich auf einer Steigerung der Kornzahl pro Flächeneinheit (8–12 %) und geringfügiger auf einem erhöhten Einzelkorngewicht (2–4 %) (**Kapitel 2**).

Die Kornertrag-Steigerung durch e[CO_2] war auf eine Steigerung der RUE zurückzuführen und diese war gemäß unserer Hypothese bei starkem N-Mangel vermindert (**Kapitel 2**). Jedoch gab es im Gegensatz zu dem Arizona FACE-Versuch (Jamieson et al., 2000) bei N-Mangel keine Verminderung der AR durch e[CO_2] (**Kapitel 2**). Eine Reduktion der N-Konzentration durch e[CO_2] kann eine Abnahme der Photosynthesekapazität bewirken (Ainsworth und Long, 2005). Bei starkem N-Mangel reduzierte e[CO_2] die N-Konzentration ungeachtet des LAI (**Kapitel 2**) und somit möglicherweise die Photosynthesekapazität, was die verminderte RUE-Steigerung durch e[CO_2] bei N-Mangel erklären könnte. Jedoch scheint es vielmehr, dass N-Mangel während des Ährenwachstums zu einer Senken-Limitierung des Kornertrags und dadurch zu der verminderten RUE-Steigerung führte. Hinweise dafür sind der (i.) ungleiche Verlauf der Kurven der Beziehung zwischen RUE und N-Gehalt pro Blattfläche zwischen a[CO_2] und e[CO_2] und (ii.) die Reduktion des HI durch e[CO_2] bei starkem N-Mangel durch deutliche Steigerung der Halm- und geringere Steigerung der Ährenbiomasse durch e[CO_2] (**Kapitel 2**). Nach van Kraalingen (1990) werden bei starkem N-Mangel zusätzliche durch e[CO_2] generierte Assimilate präferenziell in Organe mit geringer N-Konzentration wie dem Halm eingelagert.

6.2 Prozessanalyse der Reduktion der Korn-N-Konzentration durch e[CO_2]

In den meisten FACE-Versuchen steigerte e[CO_2] den N-Ertrag der Körner und der Gesamtpflanze (9–102 %) ungeachtet der N-Verfügbarkeit und klimatischen Bedingungen (z.B. Kimball et al., 2001; Ma et al., 2007; Lam et al., 2012; Han et al., 2015; Tauz et al., 2017). Dies konnte in unserer Studie

bestätigt werden (**Kapitel 3 und 4**). Jedoch gab es Ausnahmen bei dem FACE-Versuch in Arizona bei starkem N-Mangel und im früheren FACE-Versuch in Braunschweig wo keine Veränderung des N-Ertrages durch e[CO_2] beobachtet wurde (Kimball et al., 2001; Weigel und Manderscheid, 2012). Die Meta-Analyse von Pleijel et al. (2019) mit Open-Top-Kammer- und FACE-Versuchen fand eine Verminderung des Korn-N-Ertrags durch e[CO_2] bei der N-Versorgung < 100 kg N ha^{-1}.

Es scheint, dass bei subtropischem Klima die Steigerung des N-Ertrags zum Anthese-Stadium größer ist als zur Kornreife (Ma et al., 2007; Han et al., 2015), aber in unserer Studie war es umgekehrt (**Kapitel 3**). Ursachen dafür könnten (i.) die Temperaturabhängigkeit der Photosynthese-Steigerung durch e[CO_2] (Long, 1991) und (ii.) unterschiedliche N-Verfügbarkeit während der Kornfüllungsphase z.B. durch das Regime der N-Düngung oder verschiedene Umweltbedingungen hinsichtlich der Mineralisierung (z.B. Temperaturunterschiede) sein.

Der Korn N-Ertrag stieg unabhängig von der CO_2-Konzentration linear mit der Kornzahl an (**Kapitel 3**). Dies suggeriert eine enge Verbindung der Steigerung des N-Ertrags mit einer Kornertragssteigerung durch e[CO_2]. Eine Erklärung für diese lineare Beziehung könnte der hohe Albumin/Globulin-Gehalt im Korn (~40 % des Kornproteins) sein (**Kapitel 5**). Nach Martre et al. (2003) reguliert die N-Senke (das sich entwickelnde Korn) die Akkumulation von Albumin/Globulin und somit die Anlieferung von N für die Synthese dieser Proteine.

Eine Steigerung der N-Düngung führt zur Steigerung des N-Ertrags zum Anthese-Stadium und zur Steigerung der Nrem (Barbottin et al., 2005; Gaju et al., 2014). Dies konnte bestätigt werden (**Kapitel 3**). Dagegen kann sich eine Steigerung der N-Düngung unterschiedlich auf die NRE auswirken (Barbottin et al., 2005; Gaju et al., 2011, 2014). In der vorliegenden Studie steigerte zunehmender N-Mangel die NRE (**Kapitel 3**). Eine Quellen-basierte Erklärung dafür wäre, dass ein geringer N-Status in den vegetativen Organen über die Hemmung der Synthese von Phytohormonen (Zytokinine, Auxin) die Seneszenz und dadurch die Nrem beschleunigt (Kong et al., 2016). Eine weitere Erklärung könnte eine Steigerung der Korn-Senkenstärke durch zunehmenden N-Mangel sein. Die Gründe hierfür sind die Erhöhung des Anteils Senken-regulierter Albumine/Globuline (Martre et al., 2003) im Kornprotein (**Kapitel 5**) und die Erhöhung des Verhältnisses von N-Senke (Körner) zu N-Quelle (vegetativer N) durch zunehmenden N-Mangel (Daten nicht dargestellt).

e[CO_2] steigerte die Nrem und NRE und ersteres war mit einer gesteigerte N-Aneignung bis zum Anthese-Stadium und letzteres mit einer verstärkten N-Mobilisierung aus den vegetativen Organen verknüpft (**Kapitel 3**). Dies widerlegt die Hypothese einer Reduktion der Nrem oder der NRE durch e[CO_2] aufgrund einer Reduktion der N-Konzentration zum Anthese-Stadium. Die e[CO_2]-Wirkung auf NRE könnte ähnlich sein wie die von N-Mangel: Durch die Kornzahl-Steigerung (**Kapitel 2**) nimmt die Korn-Senkenstärke zu, dies steigert die Rate der Prä-Seneszenz Nrem, dadurch wird der N-Status im vegetativen Gewebe stärker als bei a[CO_2] reduziert und folglich die Seneszenz und Seneszenz-abhängige Nrem beschleunigt. Eine Beschleunigung der Seneszenz des Fahnenblatts durch e[CO_2] wurde nicht festgestellt (**Kapitel 3**), aber in den Blättern unterhalb des Fahnenblatts gab es Anzeichen einer solchen Beschleunigung (Daten nicht dargestellt).

Die Nabs ist stark von der N-Verfügbarkeit während der Kornfüllungsphase abhängig und beeinflusst negativ die Nrem (Papakosta und Gagianas, 1991). Dies konnte bestätigt werden (**Kapitel 3 und Abb.1**). Eine Ausnahme war jedoch die geringe Abhängigkeit der Nabs von der N-Verfügbarkeit während der Kornfüllung bei übermäßiger N-Düngung.

Die Nabs wurde durch e[CO_2] gesteigert (**Kapitel 3**) und dies widerlegt unsere Eingangshypothese. Im Gegensatz zu anderen Studien (Brooks et al., 2000; Fangmeier et al., 2000), gab es keine Beschleunigung der Fahnenblatt-Seneszenz durch e[CO_2] und dies könnte teilweise mit der hohen Nabs zusammenhängen (**Kapitel 3**). Es ist bekannt, dass Nabs die Biosynthese von Zytokininen fördert, welche wiederum die Seneszenz hemmen (Kong et al., 2016).

Die geringe Abhängigkeit von Nabs von der N-Verfügbarkeit bei der übermäßigen N-Düngung deutet auf eine Hemmung von Nabs durch den hohen N-Status der Pflanze hin (**Kapitel 3**). Es ist bekannt, dass ein hoher N-Status in der Pflanze die NO_3^--Aufnahme der Wurzel hemmt (Glass et al., 2002). Auch könnte Konkurrenz um Energie zwischen C- und N-Assimilation (Munier-Jolain und Salon, 2005) ein Grund sein. Ein Hinweis dafür ist das geringere Einzelkorngewicht bei übermäßiger N-Düngung verglichen mit den anderen N-Stufen (**Kapitel 2**).

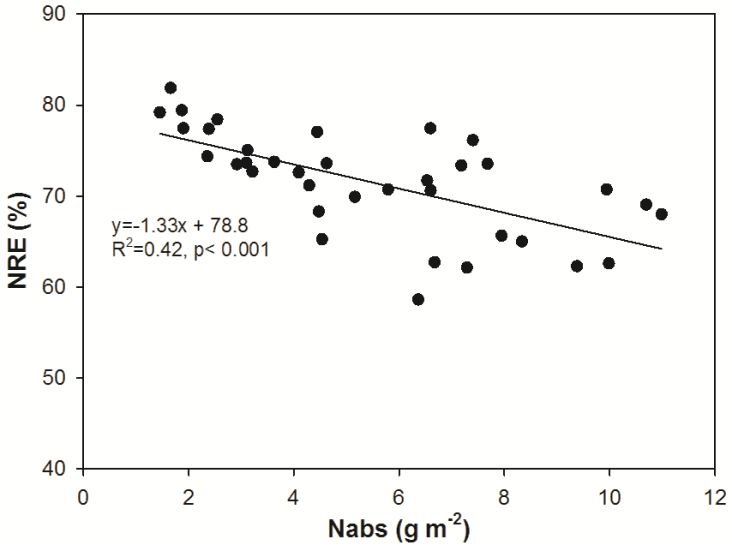

Abb. 2: Beziehung zwischen der NRE und Nabs unter Berücksichtigung aller CO_2 x N x Jahr-Kombinationen.

e[CO_2] reduzierte die N-Konzentration des Blatts um 4 – 7 % (**Kapitel 2**) und die des Korns um 1 – 6 % in allen N-Stufen, wobei letzteres bei starkem N-Mangel lediglich um 1 % reduziert wurde (**Kapitel 3**). Diese Reduktionen sind geringer als jene die von Meta-Analysen, die wesentlich auf Kammer-Studien basieren, beschrieben wurden. Dort reduzierte e[CO_2] die N-Konzentration vegetativer Organe um 16 % (Cotrufo et al., 1998) und die des Korns um 13 % (Taub et al., 2008). Die Reduktion der Korn-N-Konzentration bei praxisüblicher N-Düngung um 6 % entspricht anderen FACE-Studien (Reduktion um 4 – 9 %), die unter verschiedenen klimatischen Bedingungen und Wasserverfügbarkeiten durchgeführt wurden (Kimball et al., 2002; Högy et al., 2009; Erbs et al., 2010; Lam et al., 2012; Han et al., 2015; Tausz-Posch et al., 2015; Tausz et al., 2017). Dagegen wurde bei starkem N-Mangel eine viel stärkere Reduktion der Blatt und Korn-N-Konzentration um 22 % bzw. 9 % bei subtropischen Bedingungen (Kimball et al., 2002) und die des Korns um 10 % bei semi-ariden Bedingungen (Walker et al., 2017) festgestellt.

Gründe für den geringen e[CO_2]-Effekt auf die Korn-N-Konzentration bei starkem N-Mangel in der vorliegenden Studie könnte (i.) die geringe Ertragssteigerung durch e[CO_2] (**Kapitel 2**) und (ii.) das relativ hohe Verhältnis von Senken- (Albumin/Globulin) zu Quellen-regulierten Proteinen (Gliadin und Glutenin) im Korn (Martre et al., 2003) sein (**Kapitel 5**). Dass die Reduktion der Korn-N-Konzentration bei starkem N-Mangel deutlich stärker in den FACE-Versuchen bei subtropischen

(Kimball et al., 2002) und semi-ariden Bedingungen (Walker et al., 2017) war, könnte an einer geringeren N-Verfügbarkeit während der Kornfüllungsphase verglichen mit unserer Studie liegen. In unserer Studie stammte ein erheblicher Teil des durch die Nabs aufgenommenen N aus der Mineralisierung (**Kapitel 3**) und wegen der kontrollierten Bewässerung (Manderscheid et al., 2018) gab es keinen Wassermangel, der eine N-Aufnahme hätte beschränken können.

Wichtige Hypothesen für die Reduktion der N-Konzentration durch e[CO_2] während des vegetativen Wachstums sind eine (i.) Herabregulierung der RuBisCO-Genexpression im Blatt (Stitt und Krapp 1991) und eine (ii.) Hemmung der NO_3^--Assimilation (Bloom et al., 2010). Jedoch fällt die N-Konzentration der Gesamtpflanze generell mit zunehmender Biomasse gemäß einer negativen allometrischen Funktion wegen Abnahme des Blatt/Halm-Verhältnisses und Zunahme von Beschattungseffekten ab (Sadras und Lemaire, 2014). In Ähnlicher Weise gibt es ein Abfall der Blatt-N-Konzentration mit steigendem LAI durch Zunahme von Beschattungseffekten (Bertheloot et al., 2008). Beide negativen allometrischen Beziehungen konnten bestätigt werden und waren durch e[CO_2] unbeeinflusst (**Kapitel 2 und 3**). Dies spricht gegen einen direkten e[CO_2]-Effekt auf die N-Konzentration durch die obigen Hypothesen i. und ii. Eine Ausnahme gab es in einem Versuchsjahr bei starkem N-Mangel wo unter Berücksichtigung des LAI als Kovariable die N-Konzentration durch e[CO_2] reduziert wurde (**Kapitel 2**).

Für die Reduktion der Korn-N-Konzentration durch e[CO_2] waren womöglich mehrere Prozesse verantwortlich. Der Vergleich der N-Konzentration und des durchschnittlichen N-Gehalts pro Korn zeigte, dass ein Verdünnungseffekt durch Steigerung des Einzelkorngewichts ein wichtiger Grund war (**Kapitel 2 und 3**). Des Weiteren reduzierte e[CO_2] das Verhältnis der N-Quelle zur N-Senke durch eine stärkere Steigerung der Kornzahl verglichen mit dem vegetativen N zur Anthese (**Kapitel 3**). Diese Reduktion wurde aber größtenteils durch die Steigerung der NRE durch e[CO_2] kompensiert. Es scheint, dass Weizen bei ausreichender N-Verfügbarkeit über genügend potentiell mobilisierbare N-Reserven verfügt um eine Reduktion der Korn-N-Konzentration durch e[CO_2] vollständig zu kompensieren (**Kapitel 3**; Pask et al., 2012). Jedoch müsste die Mobilisierungsfähigkeit von N aus den vegetativen Organen weiter gesteigert werden.

Durch Hydrokulturversuche wurde festgestellt, dass e[CO_2] die Photorespiration und dadurch die NO_3^- Assimilation hemmt (Bloom et al., 2002, Rachmilevitch et al., 2004; Bloom et al., 2010). Der Grad der Photorespiration-Hemmung durch e[CO_2] steigt mit der Temperatur an und so müsste auch die Hemmung der Blatt NO_3^--Assimilation durch Erhöhung der Temperatur verstärkt werden (Bloom et al., 2015). Beides konnte in der vorliegenden Studie nicht bestätigt werden und die Hinweise dafür sind: (i.) keine Hemmung der NRA durch e[CO_2] bei moderaten (17 °C) und hohen Temperaturen (28 °C) (**Kapitel 4**), (ii.) keine stärkere Steigerung von Wachstum und N-Aneignung sowie ähnliche Reduktion der Blatt- und Korn-N-Konzentration durch e[CO_2] bei NH_4^+- verglichen mit NO_3^--betonter N-Düngung (**Kapitel 4**), (iii.) Steigerung der Nabs durch e[CO_2] obwohl diese bei relativ hohen Temperaturen stattfand (**Kapitel 3**). Jedoch schließen diese Punkte eine Hemmung der NO_3^--Assimilation durch e[CO_2] nicht definitiv aus. Z.B. erfolgte die Messung der NRA bei ausreichender NADH-Versorgung weshalb ein möglicher NADH-Mangel für die NR durch Messung der NRA nicht festgestellt werden konnte. Auch könnte eine Hemmung nachts durch Hemmung der NO_2^--Translokation in den Chloroplasten erfolgen (Rubio-Asensio et al., 2015), aber die CO_2-Anreicherung erfolgte in der vorliegenden Studie nur tagsüber. Dagegen spricht aber die hohe Nacht CO_2-Konzentration (~600 ppm), die in dem vorliegenden Versuch festgestellt wurde (Daten nicht dargestellt).

In zahlreichen FACE-Studien steigerte e[CO_2] den N-Ertrag von Weizen (Ma et al., 2007; Han et al., 2015; Tausz et al., 2017; **Kapitel 3**) und anderen C3-Nutzpflanzen (z.B. Kim et al., 2001; Yang et al., 2007) bei NO_3^--betonter N-Düngung. Deshalb scheint es unwahrscheinlich, dass eine mögliche Hemmung der NO_3^- Assimilation durch e[CO_2] wesentlich die Reduktion der N-Konzentration erklärt und die Ernährungssicherheit gefährden könnte (Bloom, 2015).

6.3 Wirkung von e[CO_2] auf die Kornprotein-Zusammensetzung und Backqualität

In vorherigen FACE-Studien war die Reduktion der Kornprotein-Konzentration durch e[CO_2] mit einer Reduktion von Gluten-Proteinen verbunden, aber der Gehalt von Albumin/Globulin war unbeeinträchtigt (Wieser et al., 2008; Högy et al., 2009, 2013; Fernando et al., 2015). In diesen Studien reduzierte e[CO_2] HMW-GS (Fernando et al., 2015), alle Gliadin-Typen (ω, α und γ) (Högy et al., 2013) oder alle Gliadin-Typen und Glutenin-Fraktionen (HMW-GS und LMW-GS) (Wieser et al.,

2008). In der vorliegenden Studie gab es aber nur eine geringfügige Reduktion der gesamt Gluten-Konzentration und keine bestimmter Gliadine oder Glutenine (**Kapitel 5**). Dagegen reduzierte e[CO_2] auch die Albumin/Globulin -Konzentration sowie die Expression einiger (15) Globulin-verwandter Proteine (**Kapitel 5**). Diese generell verschiedenen Ergebnisse der e[CO_2]-Wirkung auf das Korn-Proteom zwischen den FACE-Studien könnten im Genotyp (z.B. bezüglich eines verschiedenen Albumin/Globulin zu Gluten Verhältnisses) oder in der Nährstoffversorgung begründet sein. Z.B. gab es im ersten Braunschweiger FACE-Versuch eine starke Reduktion aller Gluten-Fraktionen durch e[CO_2] (Wieser et al., 2008) und Schwefel (S)-Mangel könnte der Grund dafür sein. Der Hinweis dafür ist das hohe N zu S Verhältnis im Korn von 25 im ersten Braunschweiger FACE-Versuch (Erbs et al., 2010), wohingegen dieses in der vorliegenden Studie 15 betrug (**Kapitel 5**). S-Mangel, der durch ein N zu S Verhältnis im Korn von ≥ 17 gekennzeichnet ist, kann zu einer starken Reduktion von S-reichen α, γ- Gliadinen und LMW-GS, aber auch zur Reduktion von S-armen HMW-GS führen (Zörb et al., 2009) und die Kornertrag-Steigerung durch e[CO_2] (Weigel und Manderscheid, 2012) könnte den S-Mangel verstärkt haben.

Eine Reduktion des Backvolumens durch e[CO_2] wurde in australischen FACE-Versuchen festgestellt (Fernando et al., 2012; Panozzo et al., 2014; Fernando et al., 2015). Solch eine Reduktion konnte aber nicht bestätigt werden (**Kapitel 5**). Ein Grund hierfür könnte neben einer unterschiedlich starken e[CO_2]-Wirkung auf Gluten-Proteine, ein Sortenunterschied bezüglich der Abhängigkeit des Backvolumens von der Kornprotein-Konzentration sein. Nach einer Studie von Gabriel et al. (2017) zeigen Sorten eine lineare, aber andere eine nichtlineare Beziehung zwischen Backvolumen und Kornprotein-Konzentration, wobei manche ein relativ hohes Backvolumen bei geringer Kornprotein-Konzentration aufwiesen. In unserem Versuch war die Beziehung nichtlinear mit Anstieg des Backvolumens bis zu einem Maximum und anschließendem Abfall. Dies basierte auf einer Änderung der Gliadin- und Glutenin-Zusammensetzung mit steigenden Konzentrationen (**Kapitel 5**). Interessanterweise veränderte e[CO_2] die Beziehung zwischen Kornprotein-Konzentration und Backvolumen durch Reduktion von ersterem bei maximalem Backvolumen. Dies könnte mit der Erhöhung des Gliadin zu Glutenin Verhältnis durch e[CO_2] gegen den Wert von eins bei optimaler N-Versorgung (**Kapitel 5**) zusammenhängen.

6.4 Anpassungsmaßnahmen zur Optimierung des CO_2-Düngeeffekts und Erhalt der Kornqualität unter e[CO_2]

Eine agronomische Maßnahme zur Steigerung des CO_2-Düngeeffekts und des Korn-N-Ertrags könnte eine Steigerung der N-Verfügbarkeit für die Pflanze zwischen Fahnenblatt-Stadium (GS39) und Anthese (GS59) sein. Dies ist die Periode in der der Einfluss von N (Ravier et al., 2017) und e[CO_2] (Fisher und Aguilar, 1976) auf die Kornzahl am größten ist. Aufgrund der starken linearen Abhängigkeit zwischen der Kornzahl und dem Korn-N-Ertrag (**Kapitel 3**) könnte durch die Erhöhung der N-Verfügbarkeit zwischen GS39 und GS59 die Steigerung beider Größen durch e[CO_2] (**Kapitel 2 und 3**) weiter verstärkt werden. In der vorliegenden Studie gab es eine negative Beziehung zwischen der Kornzahl und dem Einzelkorngewicht (**Abb. 2**). So könnte eine Steigerung der Kornzahl auch einen mit dem Einzelkorngewicht verbundenen Verdünnungseffekt reduzieren und so die Korn-N-Konzentration erhöhen. Demgegenüber könnte die N-Düngung zur Bestockung (GS20) wegen ihres relativ geringen Einflusses auf den Kornertrag und -qualität (Ravier et al., 2017) reduziert werden.

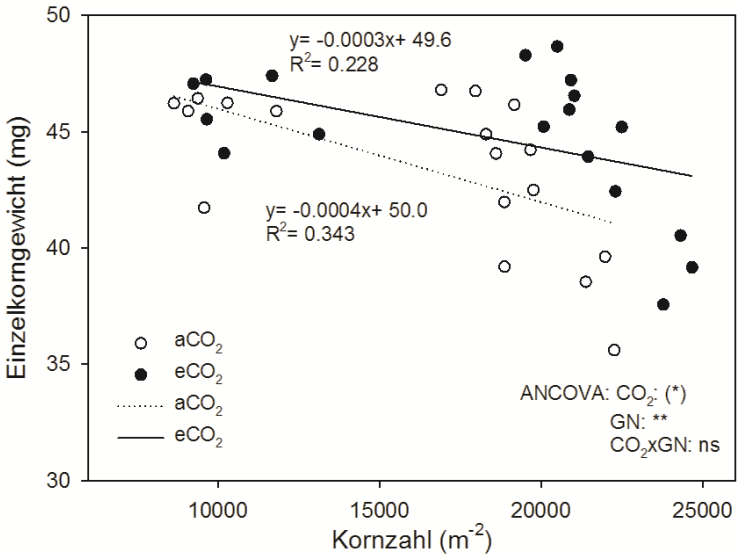

Abb. 3: Beziehung zwischen dem Einzelkorngewicht und Kornzahl pro m^2 Bodenfläche unter Berücksichtigung aller CO_2 x N x Jahr-Kombinationen. Für beide CO_2-Stufen wurde eine Regressionslinie eingezeichnet. Rechts unten ist das Ergebnis der Kovarianzanalyse des Effekts von e[CO_2] und der Kornzahl (GN) dargestellt: (*) $P<0.1$, ** $P<0.01$.

Der positive e[CO_2]-Effekt auf die Nabs bei generell hoher Nabs bei N-Mangel und optimaler N-Versorgung (**Kapitel 3**) deuten hin, dass eine gesteigerte N-Gabe zur Anthese eine Maßnahme ist um die Korn-N-Konzentration unter e[CO_2] zu steigern. Die Voraussetzungen dafür aber wären keine übermäßige N-Versorgung vor der Anthese und eine ausreichende Wasserverfügbarkeit für die N-Aufnahme. Interessanterweise konnte in einem Gewächshausversuch unter diesen Voraussetzungen eine hohe N-Versorgung zur Anthese die Reduktion der Korn-N-Konzentration durch e[CO_2] vollständig kompensieren (Fernando et al., 2017). Die N-Düngung jedoch generell (vor und nach der Anthese) stark zu erhöhen, erscheint nicht sinnvoll da in der vorliegenden (**Kapitel 3**) und einer weiteren FACE-Studie (Tausz et al., 2017) eine übermäßige N-Düngung eine Reduktion der N-Konzentration durch e[CO_2] nicht kompensieren konnte.

Die Hinweise für keine Hemmung der NO_3^--Assimilation durch e[CO_2] (**Kapitel 4**) zeigen, dass eine Umstellung von NO_3^- zu NH_4^+-Düngung in Zukunft nicht sinnvoll ist.

Durch Verwendung von Sorten, die ein relativ hohes Backvolumen bei geringer Kornprotein-Konzentration und eine nichtlineare Beziehung zwischen diesen beiden Größen aufweisen (**Kapitel 5**), könnte eine Reduktion des Backvolumens unter e[CO_2] vermieden werden.

6.5 Ausblick für weitere Forschung

Um vorteilhafte e[CO_2]-Effekte besser ausnutzen zu können und negative weitgehend zu vermeiden, könnte ein Screening von Genotypen mit unterschiedlicher Dauer der Phase zwischen GS39 und GS59 dienen. Da in dieser Periode wesentlich die Kornzahl durch die Umweltbedingungen festgelegt wird, könnte bei Genotypen mit langer Dauer zwischen GS 39 und GS 59 die Kornzahl-Steigerung durch e[CO_2] stärker ausfallen. Durch die enge positive Beziehung zwischen Kornzahl und Korn-N-Ertrag würde die Steigerung von letzterem ebenfalls stärker sein. Gleichzeitig könnte man Genotypen verwenden, die eine lineare oder nichtlineare Beziehung zwischen Kornprotein-Konzentration und Backvolumen aufweisen um zu testen ob Genotypen mit letzterer Beziehung hinsichtlich der Backqualität nur geringfügig negativ oder eher positiv wie in der vorliegenden Studie auf e[CO_2] reagieren.

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7. Kapitel: Zusammenfassung

Die atmosphärische CO_2-Konzentration wird in Zukunft voraussichtlich auf 500–620 ppm ansteigen. Während für Weizen Ertragssteigerungen erwartet werden, könnte unter einer solchen erhöhten atmosphärischen CO_2-Konzentration (e[CO_2]) die N-Konzentration im Gewebe um etwa 9 % abnehmen. Dies könnte die Ernährungssicherheit gefährden. In vorherigen Studien war die Reduktion der Korn-N-Konzentration eng mit der Reduktion von Gluten-Proteinen verbunden, was zudem eine verminderte Backqualität unter e[CO_2] vermuten lässt. Die Mechanismen einer Reduktion der N-Konzentration sind unklar und die Anzahl an FACE-Versuchen mit Untersuchung von CO_2 x N-Interaktionen auf die Bildung von Ertrag- und Qualität bei Winterweizen ist gering.

Das erste Hauptziel dieser Arbeit war die Analyse der Reduktion der Korn-N-Konzentration unter e[CO_2]-Bedingungen im Rahmen eines zweijährigen FACE-Versuchs mit variierenden N-Stufen (35–320 kg N ha^{-1}) und verschiedenen N-Formen (NO_3^- und NH_4^+). Der Fokus lag auf Schlüsselprozesse der Korn-N-Aneignung, bei denen es sich um NO_3^--Assimilation, N-Remobilisierung und postanthetische N-Aufnahme handelt. Zugrundeliegende Hypothese waren: (i) e[CO_2] hemmt die NO_3^--Assimilation, (ii) e[CO_2] reduziert die N-Remobilisierung (Nrem) durch Reduktion der N-Konzentration im Wachstumsstadium der Anthese und (iii) e[CO_2] reduziert die postanthetische N-Aufnahme (Nabs) durch Beschleunigung der Seneszenz oder Hemmung der NO_3^--Assimilation. Das zweite Hauptziel war die kombinierte Analyse der e[CO_2]-Wirkung auf das Korn-Proteom und auf die Backqualität. Hier war die Hypothese, dass e[CO_2] Gluten-Proteine beeinträchtigt und dadurch die Backqualität verringert.

e[CO_2] steigerte den Kornertrag in allen N-Stufen um 10 – 17 %, was hauptsächlich auf der Steigerung der Kornzahl pro m^2 Bodenfläche basierte. Dies war auf eine Zunahme der Strahlungsnutzungseffizienz zurückzuführen (**Kapitel 2**). Jedoch waren diese Steigerungen bei N-Mangel signifikant geringer. Die Gründe hierfür waren eine Reduktion der Photosynthesekapazität durch e[CO_2] und eine Senken-Limitierung des Kornertrags durch N-Mangel während des Ährenwachstums. Die Reduktion der Photosynthesekapazität ließ sich auf die Reduktion der Blatt-N-Konzentration durch e[CO_2] ungeachtet des grünen Blattflächenindexes bei N-Mangel zurückführen. Ein Hinweis für eine Senken-Limitierung des Kornertrags war die Reduktion des Ernteindex durch

e[CO_2] aufgrund einer starken Steigerung der Halm- aber einer geringfügigeren Steigerung der Ährenbiomasse durch e[CO_2].

Der Korn-N-Ertrag wurde durch e[CO_2] in allen N-Stufen gesteigert was auf Steigerungen der Nrem, Effizienz der Nrem und Nabs basierte (**Kapitel 3**). Dies widerlegt die Hypothesen einer Reduktion der Nrem und Nabs durch e[CO_2]. Es gab eine starke lineare Beziehung zwischen dem Korn-N-Ertrag und der Kornzahl, die durch e[CO_2] nicht beeinflusst wurde. Die Korn-N-Konzentration war in allen N-Stufen unter e[CO_2] geringfügig reduziert (1–6 %), wobei diese bei N-Mangel lediglich um 1 % verringert war. Der Hauptgrund für diese Verminderung war ein Verdünnungseffekt durch eine Steigerung des Einzelkorngewichts durch e[CO_2]. Ein weiterer Grund war eine stärkere Steigerung der Kornzahl als des vegetativen N-Ertrags zur Anthese durch e[CO_2], was eine Reduktion der N-Quelle relativ zur N-Senke durch e[CO_2] bedeutet.

Eine Reduktion der NO_3^--Assimilation durch e[CO_2] konnte nicht festgestellt werden (**Kapitel 4**). Die Hinweise dafür sind: keine Hemmung der Blatt Nitratreduktase-Aktivität durch e[CO_2] bei geringen (17 °C) und hohen (28 °C) Temperaturen und keine stärkere Steigerung von Wachstum und N-Aneignung durch e[CO_2] bei NH_4^+ - verglichen mit NO_3^- betonter Düngung.

Die Reduktion der Korn-N-Konzentration durch e[CO_2] war in allen N-Stufen mit einer geringfügigen Reduktion der Albumin/Globulin- und Gluten-Konzentration verbunden (**Kapitel 5**). Bei optimaler N-Versorgung ergab e[CO_2] eine Veränderung des Korn-Proteoms mit insgesamt 19 reduzierten und 17 erhöhten Proteinspots. In 15 der 16 identifizierten verminderten Proteinspots wurden Globuline, aber keine Gluten-Proteine festgestellt. Entsprechend gab es in allen N-Stufen unter e[CO_2] keine Verminderung der Backqualität.

Zusammengefasst, waren unter e[CO_2] die Korn-N-Erträge aufgrund der Zunahme von Nrem und Nabs gesteigert. Die Kornzahl war hierfür die treibende Kraft. Die N-Konzentration war unter e[CO_2] geringfügig reduziert und die zugrundeliegenden Mechanismen waren ein Verdünnungseffekt durch ein gesteigertes Einzelkorngewicht und eine Reduktion der N-Quelle relativ zur N-Senke. Die Reduktion der Korn-N-Konzentration durch e[CO_2] war nicht spezifisch mit einer Reduktion von Gluten-Proteinen assoziiert.

8. Kapitel: Summary

The atmospheric CO_2 concentration is expected to increase to 500–620 ppm in the future. Such an elevated atmospheric CO_2 concentration (e[CO_2]) increases grain yield, but can decrease tissue N concentrations by about 9% in wheat. This could endanger global food security. Moreover, in previous studies, a decrease of grain N concentration by e[CO_2] has closely been associated with that of gluten proteins, indicating a decreased baking quality under e[CO_2]. The mechanisms by which e[CO_2] decreases N concentration are still unclear and FACE studies investigating CO_2 x N interactions on the formation of grain yield and the quality of winter wheat are scarce.

The first main objective was the analysis of a decreased N concentration in the grain by e[CO_2] in winter wheat based on a two-year FACE experiment with widely differing N levels (35 to 320 kg N ha^{-1}) and different N forms (NO_3^- and NH_4^+). The focus was on key processes of grain N acquisition that are leaf NO_3^- assimilation, N remobilization and post-anthesis N uptake. The hypotheses were: e[CO_2] inhibits leaf NO_3^- assimilation, e[CO_2] decreases N remobilization (Nrem) by decreased N concentrations at anthesis and e[CO_2] decreases post-anthesis N uptake (Nabs) by inhibition of leaf NO_3^- assimilation or acceleration of senescence. The second main objective was the simultaneous analysis of the e[CO_2] effect on the grain proteome and baking quality with the hypothesis that e[CO_2] reduces gluten proteins and thereby baking quality.

e[CO_2] increased grain yield in all N levels by 10% to 17% mainly through enhanced grain number per m^2 ground area. This was due to increased radiation use efficiency (**chapter 2**). These increases were smaller under N deficiency compared with high N supply. The reasons were a reduction of photosynthesis capacity by e[CO_2] and a sink limitation concerning grain yield due to N deficiency during ear growth. The indication for the reduction of photosynthesis capacity was a decrease of leaf N concentration under e[CO_2] regardless of green leaf area index under N deficiency. An indication for sink limitation of grain yield was the decrease of harvest index by e[CO_2] because of a strong and small stimulation of stem and ear growth, respectively by e[CO_2].

Grain N yield was increased by e[CO_2] under all N levels (**chapter 3**). There was a strong linear relation between grain N yield and grain number that was unaffected by e[CO_2]. In contrast with the hypotheses of an decreased Nrem and Nabs under e[CO_2], e[CO_2] resulted in an increase of Nrem,

Nrem efficiency and Nabs, causing the increase of grain N yield. Nevertheless, e[CO_2] slightly decreased grain N concentration (by 1 to 6%), whereby the smallest effect of 1% was found under N deficiency. This decrease was primarily related to a growth dilution effect due to an increased individual grain weight under e[CO_2]. A further reason was a stronger increase of grain number than an increase of vegetative N yield at anthesis by e[CO_2] and thereby a decrease of the ratio between the N source and the N sink.

Indication for an e[CO_2] induced inhibition of leaf NO_3^- assimilation was not found as e[CO_2] did not result in a decreased activity of leaf nitrate reductase under all N levels at both cool (17 °C) and warm (28 °C) temperatures (**chapter 4**). Furthermore, the e[CO_2] induced stimulation of growth and N acquisition was not stronger under NH_4^+ compared with NO_3^- based N-fertilization.

Reduction of grain protein concentration by e[CO_2] was associated with reduced albumin/globulin and gluten concentrations under all N levels (**chapter 5**). Under optimal N supply, the grain protein composition was changed by e[CO_2] with altogether 19 decreased and 17 increased protein spots. 15 out of the 16 identified decreased proteins were globulins, whereas specific gluten proteins were not found to be affected by e[CO_2]. Correspondingly, baking quality remained unaffected under e[CO_2] under all N conditions.

In conclusion, grain N yields were increased by e[CO_2] due to an increase of Nrem and Nabs with grain number being the driving force. Grain N concentrations were slightly reduced under e[CO_2] with a growth dilution effect and a changed source to sink ratio as the underlying mechanisms. The reduction of the grain N concentration by e[CO_2] was not specifically associated with a reduction of gluten proteins.

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