Elevated CO$_2$ concentration induces photosynthetic down-regulation with changes in leaf structure, non-structural carbohydrates and nitrogen content of soybean

Yunpu Zheng$^1$, Fei Li$^1$, Lihua Hao$^{1,*}$, Jingjin Yu$^2$, Lili Guo$^1$, Haoran Zhou$^3$, Chao Ma$^1$, Xixi Zhang$^1$ and Ming Xu$^{4,5,*}$

Abstract

**Background:** Understanding the mechanisms of crops in response to elevated CO$_2$ concentrations is pivotal to estimating the impacts of climate change on the global agricultural production. Based on earlier results of the “doubling-CO$_2$ concentration” experiments, many current climate models may overestimate the CO$_2$ fertilization effect on crops, and meanwhile, underestimate the potential impacts of future climate change on global agriculture ecosystem when the atmospheric CO$_2$ concentration goes beyond the optimal levels for crop growth.

**Results:** This study examined the photosynthetic response of soybean (*Glycine max* (L.) Merr.) to elevated CO$_2$ concentration associated with changes in leaf structure, non-structural carbohydrates and nitrogen content with environmental growth chambers where the CO$_2$ concentration was controlled at 400, 600, 800, 1000, 1200, 1400, 1600 ppm. We found CO$_2$-induced down-regulation of leaf photosynthesis as evidenced by the consistently declined leaf net photosynthetic rate ($A_n$) with elevated CO$_2$ concentrations. This down-regulation of leaf photosynthesis was evident in biochemical and photochemical processes since the maximum carboxylation rate ($V_{c_{\text{max}}}$) and the maximum electron transport rate ($J_{\text{max}}$) were dramatically decreased at higher CO$_2$ concentrations exceeding their optimal values of about 600 ppm and 400 ppm, respectively. Moreover, the down-regulation of leaf photosynthesis at high CO$_2$ concentration was partially attributed to the reduced stomatal conductance ($G_s$) as demonstrated by the declines in stomatal density and stomatal area as well as the changes in the spatial distribution pattern of stomata. In addition, the smaller total mesophyll size (palisade and spongy tissues) and the lower nitrogen availability may also contribute to the down-regulation of leaf photosynthesis when soybean subjected to high CO$_2$ concentration environment.

**Conclusions:** Down-regulation of leaf photosynthesis associated with the changes in stomatal traits, mesophyll tissue size, non-structural carbohydrates, and nitrogen availability of soybean in response to future high atmospheric CO$_2$ concentration and climate change.

**Keywords:** CO$_2$ enhancement, Down regulation, Non-structural carbohydrates, N availability, Stomatal traits, Soybean crops
Background

It is well known that human activities have dramatically increased atmospheric concentrations of greenhouse gases [1, 2], particularly the elevated atmospheric carbon dioxide concentration due to fossil fuel combustion and land use change following the nineteenth century industrial revolution [3–5]. The most recently released report by the Inter-governmental Panel on Climate Change (IPCC) [6] showed that global atmospheric CO2 concentration has been dramatically increased from 280 ppm (the pre-industrial level) to over 400 ppm (the present level) with the growth rate of CO2 concentration by ~1.0 ppm per year [6], and may even be over 1000 ppm at the end of this century [7]. The elevated atmospheric CO2 concentration may lead to drastic impacts on the structure and function of natural and managed ecosystems [8–12].

Plant responses to elevated CO2 concentration are fundamentally mediated by leaf photosynthesis, which is closely associated with the changes in leaf structure, chemical composition and carbon balance depending on plant species and/or functional types [13–15]. Many previous studies have shown that elevated CO2 generally stimulated the net photosynthetic rate (A0) of plants, namely “CO2 fertilization effect”, especially for the C3 species, because the ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) of C3 plants is not CO2-saturated at the current atmospheric CO2 concentration [14, 16–21]. Meanwhile, the enhanced A0 may also be resulted from the reduced photorespiration and dark respiration and enhanced carboxylation efficiency under high CO2 concentrations [22–25]. However, other studies reported that the A0 was not marginally enhanced and even declined when plants exposed to long-term elevated CO2 concentrations [26–28]. For example, Kane moto [29] found that leaf photosynthesis of soybean plants was substantially decreased with elevating CO2 concentration from about 400 ppm to 1000 ppm for 27 days of treatment. This down-regulation of A0 may be attributed to the lower Rubisco concentration and activity [30–34] or/and the source-sink imbalance due to leaf carbohydrates accumulation under elevated CO2 concentration [29, 35–38]. In addition, the down-regulation of A0 at high CO2 concentration may also be caused by the decline of stomatal conductance [4, 39–46]. Xu [47] found that the decline in biomass of winter wheat at high CO2 concentration might be attributed to the decrease of Gs mainly due to the reduction in stomatal length and stomatal density.

In addition to physiological traits, leaf structural and biochemical characteristics may also play a pivotal role in plant response to high CO2 concentration [48–50]. Elevated CO2 concentration usually generates greater leaf thickness and total mesophyll size, which closely correlated to leaf photosynthetic rate [51–54]. Previous studies have shown that elevated CO2 concentration increased the leaf thickness and mesophyll cross-section area, which was mainly attributable to greater cell expansion rather than enhanced cell division due to the increase of carbohydrate substrate availability [55–57]. The thicker mesophyll tissue and larger cell volume may provide more space for accommodating chloroplasts and more intercellular surface area for leaf gas exchange [42, 58–60]. Meanwhile, elevated CO2 concentration may also change leaf biochemical compositions including the non-structural carbohydrates and nitrogen concentration (N), which play an important role in controlling over the responses of plants and/or ecosystems to rising atmospheric CO2 levels [17, 61, 62]. Understanding the mechanisms of leaf structure and biochemistry in response to high CO2 concentration is critical for assessing the changes in leaf functional traits and thus ecosystem functioning under future global change.

Several previous studies have documented that different plants features with different optimal CO2 concentrations for plant growth [47, 63] and thus plants with high optimal CO2 concentrations will suffer less from climate change and meanwhile benefit the most from the CO2 fertilization effect due to high nitrogen and water use efficiency [24]. Exploring the mechanisms of CO2 fertilization effect on crops is critical to estimating global agriculture yield under climatic change [64]. Numerous studies have investigated CO2 fertilization effect primarily focusing on the impact of twofold current [CO2] on plants with doubling CO2 concentration experiment [64–66], which normally increased CO2 concentration from 300 to 400 ppm to the projected atmospheric CO2 concentration of 600–800 ppm at the end of the next century [6, 67]. However, the atmosphere CO2 concentration has covered a much wider range throughout geological time scales with an estimated value of 6000 ppm during the Paleozoic Era about 500 million years ago [24]. To our knowledge, few studies have examined the responsible mechanism of A0 associated with changes in leaf structure, non-structural carbohydrates and nitrogen content of soybean (Glycine max (L.) Merr.), the fourth important crop species in the world under higher CO2 concentrations beyond the twofold current CO2 concentration of 800 ppm. Therefore, we conducted this experiment with environmental growth chambers controlling multiple high CO2 levels from 400 ppm to 1600 ppm to test the following hypotheses:

1. Leaf photosynthesis is down-regulated at higher CO2 concentrations beyond the optimal atmospheric CO2 concentration for the growth of soybean (HY1).

2. This down-regulation of leaf photosynthesis may attribute to the declines in biochemical and photo-chemical efficiency such as the maximum
carboxylation rate \( (V_{\text{cmax}}) \) and the maximum electron transport rate \( (J_{\text{max}}) \) (HY2).

(3) The CO\(_2\)-induced stomatal closure and irregular distribution pattern of stomata on soybean leaves will partially explain the down-regulation of leaf photosynthesis under high CO\(_2\) concentrations (HY3).

(4) Changes in leaf mesophyll anatomy and chemical composition may also play essential roles in the down-regulation processes of leaf photosynthesis when soybean subjected to elevated atmospheric CO\(_2\) concentrations (HY4).

**Results**

**CO\(_2\) effects on leaf photosynthesis, stomatal conductance, water use efficiency, and dark respiration**

We found a negative quadratic relationship between leaf photosynthesis and CO\(_2\) concentration \( (R^2 = 0.83) \) with the minimum leaf photosynthesis occurred at the CO\(_2\) concentration of 1200 ppm (Fig. 1a). Similar with the leaf photosynthesis, elevated CO\(_2\) concentrations resulted in non-linear decrease in stomatal conductance, which followed a quadratic relationship \( (R^2 = 0.91) \) with the minimum value occurring around 1200 ppm (Fig. 1b). Meanwhile, a quadratic equation can also be used to describe the relationship \( (R^2 = 0.51) \) between the leaf-level water use efficiency \( (WUE) \) and the CO\(_2\) concentration (Fig. 1c). However, the leaf dark respiration rate demonstrated a bell-shaped curve \( (R^2 = 0.60) \) peaking at 900 ppm in relation to CO\(_2\) concentration (Fig. 1d).

**CO\(_2\) effects on \( V_{\text{cmax}}, J_{\text{max}}, \) and the \( V_{\text{cmax}} / J_{\text{max}} \) ratio**

Both the maximum carboxylation rate \( (V_{\text{cmax}}) \) and the \( V_{\text{cmax}} / J_{\text{max}} \) ratio in response to increasing CO\(_2\) concentration featured bell-shaped curves, peaking at 592.5 ppm (Fig. 2a) and 666.7 ppm (Fig. 2c), respectively. However, the increase in CO\(_2\) concentration led to a non-linear decline in the maximum electron transport rate \( (J_{\text{max}}) \) with the maximum value occurring around 390 ppm (Fig. 2b). These relationships of \( V_{\text{cmax}}, J_{\text{max}}, \) and \( V_{\text{cmax}} / J_{\text{max}} \) ratio in relation to CO\(_2\) concentration could be described by quadratic equations with \( R^2 \) values of 0.85, 0.76, and 0.74, respectively (Fig. 2).

**CO\(_2\) effects on morphological traits and spatial distribution pattern of stomata**

We found that the stomatal area was substantially enhanced by 37% on the adaxial surfaces enhancing CO\(_2\) concentration from 400 to 1200 ppm \( (p = 0.03) \), although stomatal length, width, perimeter and shape index were barely affected by elevated CO\(_2\) concentration \( (p > 0.05; \) Table 1; Fig. 3). Our results also showed that elevated CO\(_2\) concentration significantly decreased stomatal area index on both the adaxial and abaxial leaf surfaces.
except for increasing CO\(_2\) concentration from 400 to 600 ppm, where the stomatal area index on the abaxial side was marginally increased by 15% and reached its maximum value at 600 ppm (Table 1; Fig. 3). In contrast, the stomatal area index on the adaxial surface was significantly decreased by about 60% with the increase of CO\(_2\) concentration from 400 to 600 ppm and reached its minimum value at 600 ppm (Table 1; Fig. 3). Moreover, our results also showed that the stomatal density on the adaxial side was decreased by about 57% \((p = 0.013)\), 61% \((p = 0.025)\), 32% \((p = 0.026)\) and 48% \((p = 0.003)\) with increasing CO\(_2\) concentration to 600, 800, 1200, 1400, and 1600 ppm, respectively (Table 1; Fig. 3). However, elevating CO\(_2\) concentration from 400 to 600 ppm made the stomatal density on the abaxial sides increased 21% (Table 1; Fig. 3). In addition, we also found the interactive effect of leaf surface and CO\(_2\) concentration on the stomatal density \((p = 0.009)\) and stomatal area \((p = 0.006; \text{ Table 2})\).

Elevated CO\(_2\) concentration not only changed the morphological traits of individual stoma but also affected stomatal distribution on soybean leaves. We found that the spatial distribution pattern of stomata was highly scale-dependent with regular patterns at small scales of about 70–170 \(\mu\)m (below the lower 95% envelope) and random patterns at larger scales up to 200 \(\mu\)m (between the upper and lower 95% envelope) on both leaf surfaces (Fig. 4). Increasing CO\(_2\) concentration from 400 to 600 ppm caused the stomatal distribution to become more regular at small scales on the adaxial surface as evidenced by the decrease of Lhat \((d)\) value from 1.69 to 12.00. However, the stomata on the adaxial surfaces tend to be less regular than those on the adaxial surface because the abaxial surface had higher Lhat \((d)\) values at the same scale (Fig. 4). In addition, elevated CO\(_2\) concentration increased the scale range of regular distribution from 50 \(\mu\)m to 180 \(\mu\)m on the adaxial surface (Fig. 4a), while the scale range of regular distribution on the abaxial surface was decreased from 160 \(\mu\)m to 100 \(\mu\)m (Fig. 4b). In general, this enhanced CO\(_2\) concentration effect on the spatial distribution pattern of stomata was greater on the adaxial surface than the abaxial surface of soybean leaves.

**CO\(_2\) effects on leaf anatomic characteristics**

Elevated CO\(_2\) concentration significantly increased cell length, whereas decreased cell width of palisade mesophyll (PM) \((p < 0.05; \text{ Table 3; Fig. 5})\). Elevating CO\(_2\) concentration from 400 ppm to 1000 ppm made the cell length of palisade layer increased from about 36 \(\mu\)m to 42 \(\mu\)m (Table 3). Relative to ambient CO\(_2\) concentration, the cell size of palisade layer was also significantly affected by elevated CO\(_2\) concentration (Fig. 5). Enhancing CO\(_2\) concentration from 400 ppm to 1000 ppm resulted in increases of cell area and cell perimeter by about 10 and 20%, mainly due to the larger cell length of PM. Elevated CO\(_2\) concentration from 400 ppm to 1000 ppm caused a decrease in the cell width of palisade by 23% (Table 3). Moreover, the cell length of spongy mesophyll (SM) was substantially enhanced by 32%, and thus the cell area was increased by 25% with elevating CO\(_2\) concentration of 1000 ppm. In addition, elevated CO\(_2\) concentration significantly affected both the thickness (LT) and the palisade/spongy ratio of soybean leaves \((p < 0.05; \text{ Fig. 5})\).

**CO\(_2\) effects on tissue carbon and nitrogen**

Elevated CO\(_2\) concentration dramatically affected tissue carbon (C) and nitrogen (N) as well as C/N of soybean plants (Table 4). Specifically, increasing CO\(_2\) concentration
| Stomatal parameters | CO₂ concentration (ppm) | 400 | 600 | 800 | 1000 | 1200 | 1400 | 1600 | p value |
|---------------------|-------------------------|-----|-----|-----|------|------|------|------|--------|
| Adaxial surface     |                         |     |     |     |      |      |      |      |        |
| Stomatal length (μm)| 94 ± 13 (a)             | 9.7 ± 16 (a) | 9.6 ± 0.6 (a) | 9.6 ± 0.8 (a) | 9.0 ± 20 (a) | 9.50 ± 0.5 (a) | 9.8 ± 1.6 (a) | p > 0.05 |
| Stomatal width (μm) | 22 ± 09 (a)             | 1.8 ± 04 (a) | 2.6 ± 0.5 (a) | 1.7 ± 0.2 (a) | 2.7 ± 05 (a) | 2.4 ± 0.5 (a) | 2.5 ± 0.7 (a) | p > 0.05 |
| Stomatal area (μm²) | 926 ± 16.2 (b)          | 887 ± 96 (b) | 1054 ± 5.2 (ab) | 833 ± 90 (b) | 126 ± 241 (a) | 100.3 ± 172 (b) | 103.9 ± 110 (ab) | p = 0.03 |
| Stomatal perimeter (μm) | 407 ± 0.5 (ab)        | 45.0 ± 10 (ab) | 41.0 ± 0.1 (ab) | 37.9 ± 0.2 (b) | 46.2 ± 0.4 (a) | 40.5 ± 0.3 (ab) | 41.2 ± 0.27 (ab) | p > 0.05 |
| Stomatal density (N/mm²) | 168 ± 89 (a)          | 7.3 ± 49 (c) | 6.6 ± 47 (bc) | 17.8 ± 53 (a) | 10.4 ± 64 (bc) | 11.4 ± 6.3 (b) | 8.7 ± 3.5 (b) | p = 0.04 |
| Stomatal shape index (%) | 240 ± 13 (a)          | 204 ± 47 (b) | 25.0 ± 0.2 (a) | 24.1 ± 0.2 (a) | 24.3 ± 24 (a) | 24.6 ± 0.6 (a) | 24.7 ± 0.7 (a) | p > 0.05 |
| Stomatal area index (%) | 94 ± 17 (a)           | 3.9 ± 04 (d) | 4.2 ± 0.3 (d) | 9.0 ± 1.0 (a) | 7.9 ± 15 (ab) | 6.9 ± 1.2 (bc) | 5.4 ± 0.6 (cd) | p < 0.001 |
| Abaxial surface     |                         |     |     |     |      |      |      |      |        |
| Stomatal length (μm)| 96 ± 1.7 (ab)           | 9.8 ± 06 (ab) | 9.5 ± 0.8 (ab) | 10.2 ± 07 (a) | 8.9 ± 06 (b) | 9.4 ± 0.2 (ab) | 9.2 ± 0.1 (ab) | p > 0.05 |
| Stomatal width (μm) | 3.1 ± 09 (a)            | 3.1 ± 03 (a) | 3.2 ± 0.3 (a) | 2.9 ± 0.4 (a) | 3.0 ± 04 (a) | 3.1 ± 0.2 (a) | 2.8 ± 0.1 (a) | p > 0.05 |
| Stomatal area (μm²) | 1331 ± 0.5 (a)          | 1239 ± 165 (ab) | 1089 ± 13.8 (bc) | 1023 ± 61 (c) | 1093 ± 75 (bc) | 1139 ± 92 (abc) | 1101 ± 178 (bcd) | p > 0.05 |
| Stomatal perimeter (μm) | 428 ± 5.5 (a)          | 446 ± 28 (a) | 41.1 ± 31 (a) | 407.1 ± 14 (a) | 40.7 ± 20 (a) | 42.6 ± 1.7 (a) | 41.8 ± 3.3 (a) | p > 0.05 |
| Stomatal density (N/mm²) | 1013 ± 20.1 (abc)      | 1283 ± 18 (a) | 95.7 ± 10.8 (bc) | 92.0 ± 17.0 (c) | 1048 ± 97 (abc) | 84.2 ± 9.2 (c) | 111.4 ± 17.8 (cd) | p > 0.05 |
| Stomatal shape index (%) | 253 ± 0.1 (a)          | 249 ± 0.1 (a) | 25.2 ± 0.4 (a) | 24.9 ± 01 (a) | 25.1 ± 05 (a) | 25.0 ± 04 (a) | 25.0 ± 0.0 (a) | p > 0.05 |
| Stomatal area index (%) | 819 ± 0.3 (b)          | 963 ± 128 (a) | 63.2 ± 8.0 (cd) | 57.1 ± 34 (d) | 69.5 ± 48 (bcd) | 58.2 ± 4.72 (d) | 74.8 ± 12.1 (bc) | p < 0.001 |
| Adaxial/abaxial ratio | 0.23 ± 0023 (ab)       | 0.12 ± 0022 (c) | 0.13 ± 0042 (c) | 0.26 ± 0002(a) | 0.21 ± 0018 (b) | 0.20 ± 0013 (b) | 0.09 ± 0024 (c) | p < 0.001 |
from 400 ppm to 1000 ppm substantially decreased C concentrations of leaf and stem \((p < 0.001)\), whereas the root C content was significantly increased by 5% from 354.8 mg g\(^{-1}\) to 371.2 mg g\(^{-1}\) with further increasing CO\(_2\) concentration from 1000 ppm to 1600 ppm \((p < 0.001)\). Moreover, elevated CO\(_2\) concentration enhanced the N content of stem and root (Table 4), while the leaf N was significantly decreased from 32.0 mg g\(^{-1}\) to 30.8 mg g\(^{-1}\) with increasing CO\(_2\) concentration from 400 ppm to 1200 ppm \((p < 0.001;\) Table 4). Elevated CO\(_2\) concentration decreased the tissue C/N ratio due mainly to the increased N and decreased C in stems and roots \((p < 0.001;\) Table 4). In addition, enhancing CO\(_2\) concentration from 400 ppm to 800 ppm slightly increased the leaf, stem, and total TNC by 12.8, 4.9, and 5.9%, whereas the TNC in leaves and stems were dramatically reduced with further increasing CO\(_2\) concentration from 800 ppm to 1600 ppm (Table 5).

### Relationships among photosynthesis, leaf structure, non-structural carbohydrates, and nitrogen content

We estimated the relationships between photosynthesis and stomatal conductance as well as photosynthesis and stomatal area and found that leaf photosynthesis was increased linearly by the enhancement of stomatal conductance and stomatal area on the adaxial surface with R\(^2\) values of 0.81 \((p = 0.01)\) and 0.67 \((p = 0.02)\), respectively (Fig. 6a-b). In contrast to the stomatal area on the adaxial surface, we found no linear relationship between leaf photosynthesis and stomatal area on the abaxial surface of soybean plants \((R^2 = 0.07, p = 0.60;\) Fig. 6c). Moreover, we also found that leaf photosynthesis was linearly increased by the cell enlargement of spongy and palisade tissues with R\(^2\) values of 0.74 \((p = 0.01)\) for spongy cell area and 0.72 \((p = 0.02)\) for palisade cell area, respectively (Fig. 7). In addition to leaf structure, we also evaluated the relationship among leaf photosynthesis, carbohydrates and nitrogen content. We found a positive but not significative relationship between leaf photosynthesis and non-structural carbohydrate content following a linear equation \((R^2 = 0.44, p = 0.11;\) Fig. 8).
**Discussion**

**Down-regulation of leaf photosynthesis under elevated atmospheric CO₂ concentrations**

It is demonstrated that elevated CO₂ concentration generally stimulates plant growth and enhanced crop yield through the CO₂ fertilization effect [17, 18], whereby augmented atmosphere CO₂ concentration can directly boost carboxylation in the Calvin-Benson-Bassham cycle and competitively inhibit dark respiration and photorespiration [13, 16]. By contrast, several studies claim that some plants may develop an adverse response through a process known as “down-regulation” of photosynthesis when plants exposed to higher CO₂ concentration beyond certain thresholds [4, 26, 28]. We also found a negative
| Parameters          | CO₂ concentrations (ppm) | p value |
|---------------------|---------------------------|---------|
|                     | 400  | 600  | 800  | 1000 | 1200 | 1400 | 1600 |         |
| Palisade layer      |      |      |      |      |      |      |      |         |
| Cell length (μm)    | 35.69 ± 3.04 (c)          | 39.14 ± 3.43 (ab) | 38.59 ± 34.46 (b) | 42.03 ± 5.23 (a) | 33.8 ± 6.24 (d) | 29.76 ± 4.47 (e) | 41.15 ± 7.35 (a) | p < 0.001 |
| Cell width (μm)     | 9.35 ± 1.76 (b)           | 8.93 ± 1.78 (b) | 7.90 ± 31.22 (de) | 9.00 ± 1.50 (bc) | 7.25 ± 1.29 (e) | 10.40 ± 1.18 (a) | 8.13 ± 2.00 (cd) | p < 0.001 |
| Cell area (μm²)     | 303.3 ± 39.6 (b)          | 348.7 ± 39.4 (a) | 2750 ± 76.2 (c) | 328.0 ± 809 (ab) | 213.1 ± 46.3 (d) | 232.1 ± 605 (d) | 303.5 ± 88.9 (bc) | p < 0.001 |
| Cell perimeter (μm) | 83.0 ± 6.4 (c)            | 95.1 ± 12.6 (b) | 806 ± 99 (c) | 99.3 ± 12.7 (ab) | 71.4 ± 9.4 (d) | 67.6 ± 102 (d) | 100.9 ± 19.3 (a) | p < 0.001 |
| Spongy layer        |      |      |      |      |      |      |      |         |
| Cell length (μm)    | 20.72 ± 2.43 (cd)         | 2485 ± 4.00 (ab) | 2261 ± 5.45 (bc) | 27.36 ± 7.75 (a) | 19.87 ± 3.11 (d) | 20.15 ± 2.87 (d) | 20.07 ± 5.11 (d) | p = 0.009 |
| Cell width (μm)     | 11.91 ± 3.13 (b)          | 1204 ± 3.75 (a) | 939 ± 5.44 (d) | 10.54 ± 3.71 (ac) | 8.28 ± 2.14 (e) | 12.68 ± 2.15 (ad) | 8.46 ± 2.40 (bef) | p < 0.001 |
| Cell area (μm²)     | 207.8 ± 57.6 (b)          | 265.3 ± 88.4 (a) | 1920 ± 64.5 (b) | 258.9 ± 24.5 (a) | 150.0 ± 43.8 (c) | 215.6 ± 530 (b) | 177.2 ± 67.7 (b) | p = 0.010 |
| Cell perimeter (μm) | 86.2 ± 13.2 (bc)          | 7688 ± 13.8 (a) | 606 ± 13.3 (c) | 71.4 ± 21.7 (ab) | 55.5 ± 12.0 (d) | 55.6 ± 13.7 (d) | 63.1 ± 15.3 (bc) | p = 0.020 |
| Palisade/Spongy ratio | 1.88 ± 0.68 (b)          | 252 ± 0.23 (a) | 259 ± 0.64 (ab) | 27.7 ± 0.54 (a) | 2.13 ± 0.60 (abc) | 1.90 ± 0.49 (bc) | 1.75 ± 0.25 (c) | p < 0.031 |
| Leaf thickness (μm) | 103.5 ± 11.0 (c)          | 1269 ± 9.9 (a) | 1215 ± 9.9 (b) | 125.4 ± 12.7 (b) | 108.7 ± 14.1 (c) | 108.0 ± 12.2 (c) | 106.9 ± 11.1 (c) | p = 0.001 |
quadratic relationship between leaf photosynthesis and CO$_2$ concentration ($R^2 = 0.83$; Fig. 1), indicating down-regulation of leaf photosynthesis did occur when soybean plants subjected to enhanced CO$_2$ concentrations. This down-regulation of leaf photosynthesis may be caused by various limiting factors such as lower Rubisco concentration and activity [29, 30, 32, 34] reduced stomatal conductance [15, 68, 69], and excessive carbohydrates accumulation in leaves [29, 36–38].

Further analysis showed that leaf biochemical and photochemical efficiency might play a pivotal role in the down-regulation of leaf photosynthesis in the current study. Our results showed that the maximum carboxylation rate of Rubisco ($V_{\text{cmax}}$) and the maximum capacity of electron transport RuBP regeneration ($J_{\text{max}}$) were dramatically decreased by elevated CO$_2$ concentrations, suggesting that enhanced CO$_2$ concentrations may affect both the light and dark reactions of photosynthesis.
| Element (mg g$^{-1}$ DW) | CO$_2$ concentrations (ppm) |   |   |   |   |   |   |
|------------------------|----------------------------|---|---|---|---|---|---|
|                        | 400 | 600 | 800 | 1000 | 1200 | 1400 | 1600 |
| **Leaf**               |     |     |     |     |     |     |     |
| C                      | 403.6 ± 0.33(a) | 386.14 ± 0.23(d) | 390.54 ± 0.24 (d) | 373.66 ± 12.73 (c) | 393.30 ± 0.70 (bd) | 392.07 ± 0.50 (bd) | 395.71 ± 0.37(ab) |
| N                      | 32.04 ± 0.12 (bc) | 30.56 ± 0.22 (d) | 33.56 ± 0.16 (a) | 31.61 ± 1.00 (c) | 34.04 ± 0.12 (a) | 32.61 ± 0.07 (b) | 30.83 ± 0.2 (d) |
| C/N                    | 12.60 ± 0.05 (b) | 12.64 ± 0.10 (b) | 11.64 ± 0.06 (e) | 11.82 ± 0.03 (d) | 11.55 ± 0.03 (e) | 12.02 ± 0.04 (c) | 12.84 ± 0.08 (a) |
| **Stem**               |     |     |     |     |     |     |     |
| C                      | 375.71 ± 0.66 (a) | 358.33 ± 0.04 (f) | 348.84 ± 0.17 (d) | 347.86 ± 0.20 (e) | 335.51 ± 0.05 (g) | 339.93 ± 1.01 (f) | 359.40 ± 0.30 (c) |
| N                      | 26.47 ± 0.14 (g) | 32.58 ± 0.19 (f) | 43.24 ± 0.25 (b) | 45.85 ± 0.41 (a) | 37.51 ± 0.10 (d) | 42.24 ± 0.12 (c) | 36.94 ± 0.20 (e) |
| C/N                    | 14.19 ± 0.09 (a) | 10.10 ± 0.06 (b) | 8.07 ± 0.05 (e) | 7.59 ± 0.07 (f) | 8.95 ± 0.023.6 (d) | 8.05 ± 0.04 (e) | 9.73 ± 0.06 (c) |
| **Root**               |     |     |     |     |     |     |     |
| C                      | 352.38 ± 0.13 (cd) | 347.81 ± 0.42 (d) | 350.68 ± 0.18 (cd) | 354.78 ± 0.52 (c) | 376.80 ± 0.62 (ab) | 378.59 ± 2.97 (a) | 371.19 ± 8.64 (b) |
| N                      | 26.08 ± 0.29 (c) | 34.40 ± 0.19 (b) | 38.35 ± 0.09 (a) | 37.70 ± 0.10 (a) | 36.79 ± 0.25 (a) | 37.57 ± 0.33 (a) | 33.59 ± 3.33 (b) |
| C/N                    | 13.51 ± 0.15 (a) | 10.11 ± 0.06 (c) | 9.14 ± 0.02 (d) | 9.41 ± 0.03 (d) | 10.24 ± 0.08 (c) | 10.07 ± 0.01 (c) | 11.10 ± 0.81 (b) |

**Table 4** Effects of elevated CO$_2$ concentrations on carbon and nitrogen contents of soybean

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| CO₂ concentrations (ppm) | p value |
|---------------------------|---------|
| 400 | 600 | 800 | 1000 | 1200 | 1400 | 1600 |
| Root Soluble sugar | 58.4 ± 5.0(bc) | 96.9 ± 5.7(a) | 61.2 ± 5.3(b) | 40.1 ± 2.5(d) | 63.4 ± 2.0(b) | 65.2 ± 3.3(b) | 52.1 ± 5.0(c) | p < 0.001 |
| Starch | 26.8 ± 3.3(a) | 17.2 ± 6.9(b) | 25.6 ± 2.3(a) | 18.1 ± 2.3(b) | 184 ± 1.1(b) | 20.16 ± 0.96(ab) | 13.3 ± 1.5(b) | p = 0.014 |
| TNC | 85.3 ± 7.8(b) | 114.1 ± 14.8(a) | 86.8 ± 6.5(b) | 58.1 ± 2.6(c) | 818 ± 30(b) | 85.4 ± 3.9(b) | 654 ± 6.5(c) | p < 0.001 |
| Stem Soluble sugar | 51.1 ± 6.4(a) | 59.7 ± 4.9(a) | 55.8 ± 4.3(a) | 64.5 ± 7.4(a) | 542 ± 3.5(a) | 47.2 ± 1.2(a) | 53.3 ± 5.1(a) | p > 0.05 |
| Starch | 16.2 ± 5.8(bc) | 20.2 ± 2.7(ab) | 20.2 ± 4.1(ab) | 11.7 ± 0.3(c) | 232 ± 41(a) | 16.2 ± 0.7(bc) | 109 ± 3.9(c) | p < 0.007 |
| TNC | 67.3 ± 8.3(a) | 79.9 ± 7.5(a) | 75.9 ± 4.7(a) | 76.2 ± 7.6(a) | 774 ± 36(a) | 63.4 ± 1.7(a) | 642 ± 8.7(a) | p > 0.05 |
| Leaf Soluble sugar | 65.5 ± 3.9(a) | 30.5 ± 5.0(b) | 72.6 ± 13.8(a) | 59.0 ± 13.3(a) | 274 ± 1.7(b) | 29.7 ± 7.6(b) | 226 ± 2.7(b) | p < 0.001 |
| Starch | 17.9 ± 7.8(c) | 26.0 ± 5.3(ab) | 14.9 ± 2.0(c) | 27.5 ± 4.4(a) | 169 ± 5.0(c) | 15.8 ± 1.7(c) | 194 ± 1.9(bc) | p = 0.022 |
| TNC | 83.4 ± 7.1(a) | 56.5 ± 1.5(b) | 87.5 ± 15.5(a) | 86.5 ± 9.5(a) | 443 ± 64(b) | 45.5 ± 5.9(b) | 420 ± 3.3(b) | p < 0.001 |
| Total Soluble sugar | 182 ± 3(abc) | 196 ± 27(ab) | 212 ± 41 (a) | 164 ± 11(bcd) | 145 ± 30(c-d) | 142 ± 10(c-d) | 128 ± 9(d) | p = 0.004 |
| Starch | 61.0 ± 11.3(a) | 63.4 ± 10.4(a) | 60.7 ± 4.4(a) | 57.3 ± 6.5(a) | 585 ± 8.2(a) | 52.2 ± 2.4(ab) | 435 ± 6.4(b) | p < 0.001 |
| TNC | 236 ± 7(ab) | 250 ± 19(a) | 250 ± 16(a) | 221 ± 5(bc) | 203 ± 27(c) | 194 ± 8(c-d) | 171 ± 15(d) | p > 0.05 |
Moreover, our results also suggested that elevated CO₂ may have greater impacts on carboxylation processes than the photochemical processes as indicated by the rapidly decreased $V_{\text{cmax}}/J_{\text{max}}$ ratio beyond the optimal CO₂ concentration of about 670 ppm (Fig. 2). Therefore, the lower carboxylation and photochemical efficiency as evidenced by the declines of the $V_{\text{cmax}}$ and $J_{\text{max}}$ values as well as the $V_{\text{cmax}}/J_{\text{max}}$ ratio at high CO₂ concentrations may explain the negative CO₂ effects on leaf photosynthesis of soybean plants as observed in the current study.
Additionally, it is important to note that dark respiration increased with the change of CO₂ from 400 ppm to 900 ppm, which may also contribute to the down-regulation of leaf photosynthesis. However, dark respiration started to decrease when the CO₂ concentration is beyond 900 ppm which offsets the effects of $V_{\text{cmax}}$ and $J_{\text{max}}$ in the down-regulation of photosynthesis.

**Stomatal diffusion processes explain the down-regulation of leaf photosynthesis**

In addition to biochemical and photochemical processes, our results also showed that enhancing CO₂ concentrations generally decreased stomatal density on both leaf surface, especially the stomatal density on the adaxial leaf surface was substantially decreased by about 50% with increasing CO₂ concentration from 400 ppm to 1600 ppm (Table 1). This CO₂-induced decrease of stomatal density may explain the down-regulation of leaf photosynthesis because stomatal density partially determines CO₂ diffusion efficiency from atmosphere to mesophyll tissues [52–54] and well correlates with stomatal conductance [47], which is closely associated with leaf photosynthesis [42, 58, 59]. Meanwhile, elevated CO₂ concentrations significantly decreased the total stomatal area per unit leaf area (stomatal area index) on both leaf sides, suggesting the CO₂-induced stomatal closure may also contribute to the decline of leaf photosynthesis through reducing stomatal conductance at high CO₂ concentration. Previous studies have claimed that elevated CO₂ can reduce stomatal openness by changing concentrations of ion and organic solutes and depolarizing the water potential of cell membrane [47, 54, 59].

In the current study, we also found well-correlated relationships among leaf photosynthesis, stomatal conductance, and stomatal area (Fig. 6), confirming that this down-regulation of leaf photosynthesis may be attributed to the decline of stomatal conductance through reducing stomatal openness when soybean plants exposed to high CO₂ concentrations. Additionally, the less regular stomatal distribution pattern on the adaxial leaf surface of soybean plants as evidenced by the larger Lhat (d) at higher CO₂ concentrations may contribute to the decline of stomatal conductance through increasing the average distance of CO₂ diffusion from stomata to chloroplasts [47, 63]. Overall, the fewer stomata and smaller stomatal pore aperture, as well as the more irregular spatial distribution patterns at high CO₂ concentrations may partially explain the decline of stomatal conductance in the current study. Also, several recent studies have claimed that down-regulation of leaf photosynthesis is well associated with the declined stomatal conductance, which is mainly attributed to decreases of stomatal density and stomatal openness [21, 70, 71]. It should be noted that the declined $G_s$ does not necessarily reduce leaf photosynthesis when plants exposed to elevated CO₂ concentrations [24]. Nevertheless, the leaf photosynthesis-$G_s$ relationship did follow a linear equation in the current study ($R^2 = 0.81$), indicating that the decreased stomatal conductance under high CO₂ concentrations contributed to leaf photosynthesis down-regulation of soybean plants.

**Down-regulation of leaf photosynthesis associates with anatomical structure of mesophyll tissues**

In addition to stomatal traits, the down-regulation of leaf photosynthesis is also associated with changes in the anatomical structure of mesophyll tissues at the high CO₂ concentration [72–74]. Our results showed that the cell area of palisade and spongy tissues were increased by 15% and 28% as CO₂ concentration increased from 400 ppm to 600 ppm, while the cell area of both the palisade and spongy tissues were marginally declined with further increase of CO₂ concentration (Table 3). This decreased cell area of mesophyll tissues is likely to explain the down-regulation of leaf photosynthesis, because the smaller mesophyll cells at higher CO₂ concentration may lead to narrow space for accommodating fewer chloroplasts through constraining the cell expansion, and thus limit the carbon gain efficiency of plants [52–54]. Xu also found that the average cell area of mesophyll tissue was decreased by about 30% at higher CO₂ concentration [60]. Interestingly, we also found linearly positive relationships between leaf photosynthesis and mesophyll cell area, confirming that the down-regulation of leaf photosynthesis may be partially due to the smaller total mesophyll size (palisade and spongy tissues) of soybean plants under high CO₂ environments.

**Changes in leaf non-structural carbohydrates and nitrogen attribute to down-regulation of photosynthesis**

It is well documented that the down-regulation of photosynthesis is usually associated with changes in leaf chemical composition such as the N availability deficit [32–34], the lower Rubisco concentration and activity [33, 34] as well as the source-sink imbalance due to carbohydrates accumulation in leaves under high CO₂ concentration [29, 36–38]. Previous studies have demonstrated that elevated CO₂ concentration enhances leaf C/N ratio mainly due to the decline of N concentration through a process known as “N dilution” [61]. Our results showed that the leaf N was significantly decreased with increasing CO₂ concentration from 400 ppm to 1200 ppm (Table 4), which may also attribute to the down-regulation of leaf photosynthesis, because leaf N is closely related to photosynthetic enzymes such as Rubisco [17]. However, several previous studies have claimed that the Rubisco concentration and activity of plants were substantially reduced at high CO₂ concentration, because leaf N was prior to enzymes relating to
the metabolic processes of carbohydrates than invested to Rubisco when plants were exposed to high CO2 environments [75]. Furthermore, it is important to note that hexokinase is a key functional enzyme for mediating sugar sensing, and thus may contribute the down-regulation of photosynthesis through decreasing the Rubisco concentration with inhibiting the expression of photosynthetic genes [38, 62].

In addition to leaf N, the down-regulation of photosynthesis induced by elevated CO2 is also possibly attributed to the accumulation of carbohydrates in leaves when plants subjected to high CO2 environments for a long time period [29, 46, 63, 64]. Our results showed that the total non-structural carbohydrates in leaves (TNC) was dramatically declined at higher CO2 concentrations (Table 5), suggesting that the source-sink imbalance of carbohydrates should not be a limiting factor for the down-regulation of photosynthesis in the current study. Moreover, we also found a positive linear relationship between leaf photosynthesis and TNC (Fig. 8), which directly supported the above conclusion that the imbalance of carbohydrate concentration in the source and sink contributed little to the leaf photosynthesis down-regulation of soybean plants subjected to high CO2 concentrations.

Conclusions
We found that the net photosynthesis rate of soybean was dramatically declined with elevated CO2 concentration from 400 ppm to 1600 ppm following a typical quadratic relationship. This down-regulation of leaf photosynthesis at higher CO2 concentrations can be attributed to the limiting effects on stomatal diffusion processes and nitrogen availability as well as the changes in the biochemical and photosynthetic efficiency of photosynthesis. Overall, our results suggest that the continuously increasing CO2 concentration in the future may lead to negative impacts on agricultural production through hurting crop growth and/or reducing crop yield. Nevertheless, most of the projections estimated the plant growth and crop production according to the earlier results from “doubling-CO2 experiments” with strong CO2 fertilization effect. Therefore, many current climate change models may underestimate the potential risk of climate change on agricultural production mainly due to the overestimated strong CO2 fertilization effect on plant growth and crop yield under future elevated atmospheric CO2 concentration and climate change.

Methods
Growth chamber experiments
We bought soybean seeds from the Wotu seed company in Hebei Province of China. We grew three plants in each pot (30 cm diameter × 50 cm long), then set up five pots in each of the seven walk-in environmental growth chambers for 90 days CO2 treatment, where the CO2 concentration was regulated to ambient concentration (400 ppm) or elevated concentrations (600, 800, 1000, 1200, 1400 and 1600 ppm). The ambient and elevated CO2 concentrations within the chambers were maintained through a CO2 tank containing high purity CO2 gas (99.99%) to avoid any hurt or pollution on winter wheat plants. All of the seven growth chambers were maintained with the same other environmental factors including relative humidity of 65%, photosynthetic photon flux density (PPFD) of 1000 μmol m–2 s–1, temperature of 25/21 °C (day/night), and 12-h photoperiod for the 90 days treatment. These winter wheat plants were fertilized with half-strength Hoagland’s solution twice weekly (150 mL per pot) and irrigated once daily with plain tap water (200 mL per pot) during the establishment and treatment periods of soybean plants under elevated CO2 concentrations.

Measuring leaf gas exchange
We performed the measurements of leaf gas exchange at the end of the CO2 treatment period. We randomly selected one fully expanded leaf from each pot for leaf gas exchange measurement (n = 5) with a portable photosynthesis system (LI-6400XT; LICOR, Inc.). These selected leaves were firstly equilibrated at the corresponding growth CO2 levels with saturating PPFD of 1500 μmol photon m–2 s–1 and growth temperature of 25°C. The portable photosynthesis system automatically controlled the CO2 concentrations in the cuvette using an injector system combined with a CO2 mixer. All of the measurements on leaf gas exchange were performed with the vapor pressure deficit (VPD) lower than 1.5 kPa to avoid moisture limitation. Then, the photosynthesis vs intercellular CO2 (A–C) curves were measured at cuvette chamber CO2 of 50, 100, 150, 200, 300, 400, 600, 800, 1000, 1200, 1400, and 1600 ppm. Data from A–C curves were used to compare treatment effects on the light-saturated net photosynthetic rates (An) at ambient or elevated CO2 of their growing condition. An estimation method was used to obtain the maximum carboxylation rate of Rubisco (Vmax), and the maximum capacity of electron transport mediated ribulose bisphosphate (RuBP) regeneration (Jmax) for each observed A–C curve. Meanwhile, stomatal conductance (Gs), intercellular CO2 concentration (Ci), transpiration rate (Tr), and dark respiration rate (Rd) were also determined with the portable photosynthesis system (LI-6400XT; LICOR, Inc.). In addition, the leaf-level water use efficiency (WUE) was determined by the values of the net photosynthetic rate (An) and transpiration rate (Tr) according to the formula $WUE = An / Tr$.

Measuring morphological traits of individual stoma and spatial distribution pattern of stomata
We randomly selected five fully expanded ear leaves at the heading stage in each of the ambient and elevated CO2
We photographed the stomatal features with a cover slip and lightly pressured with a fine-point tweezer. The thin film with stomatal impression (approximately 5 mm × 15 mm) was peeled off from the leaf surface and mounted on a glass slide, and immediately covered with a cover slip and lightly pressured with a fine-point tweezer [47, 63]. We photographed the stomatal features with a microscope (DM2500, Leica Corp, Germany) equipped with a digital camera (DFC 300-FX, Leica Corp, Germany), and then analyzed thirty separate fields of 0.16 mm in each leaf section. We also combined and counted the stomata on each surface for calculating stomatal density (SD) of the adaxial and abaxial surface, respectively [47]. Moreover, we randomly selected six digital photographs of the adaxial and abaxial surfaces to measure the stomatal length (SL), stomatal width (SW), stomatal area (SA) and stomatal perimeter (SP) using AutoCAD 2010 software. In addition, we calculated stomatal shape index (SSI), which is calculated by the function that shape index = \( \frac{SA}{SP} \times 100\% \), where SA is the stomatal area and SP is the stomatal perimeter. The stomatal area index (SAI) is defined as the total stomatal area per unit leaf area calculating as stomatal average density × stomatal area per stoma × 100%. In addition to stomatal density and pore traits, we also characterized the spatial distribution pattern of stomata for each image by digitizing the stomatal positions into a shape file in GIS with the ArcMap software [47]. The spatial distribution pattern of stomata on leaves was quantified using the Ripley’s K-function with generating the x and y coordinates of stomata for each image in GIS and then calculating the Lhat (d) value (the transformed K value) based on these stomatal coordinates using the R statistic software. We compared the Lhat (d) values at different scales (distances) for detecting the spatial distribution pattern of stomata with the upper and lower boundaries generated by the 95% confidence level with the Monte Carlo simulations of 100 replicates [47, 76]. In the current study, we only reported the spatial distribution patterns of stomata on the middle section of the leaves due to the large number of stomatal images of winter wheat leaves.

We snapped three leaf pieces (2 mm × 2 mm) from the middle section of each leaf and fixed them with 2.5% (v/v) glutaraldehyde (0.1 M phosphate buffer, pH 7.0) to visualize the changes in stomatal morphology among different CO₂ concentrations. Firstly, we washed these leaf samples several times with buffer and fixed them in 1% (v/v) osmium tetroxide for three hours and these samples were dehydrated with an ethanol series. Then, these leaf samples were carefully coated with gold in a high-vacuum evaporation unit. Finally, we examined and photographed the morphological traits of stomata with a scanning electron microscopy (FEI Corp, USA).

### Measuring leaf anatomical structures

Changes in the leaf internal anatomy of the winter wheat plants exposed to different CO₂ concentrations were examined with leaf cross-sections under a light microscopy [77]. These images of leaf cross-sections were collected from the middle section of leaves to observe and measure leaf anatomical features using Image J software (NIH, USA). We estimated leaf mesophyll thickness between epidermal layers at five points in each cross-section [78]. We also randomly selected 20 clear palisade layer cells and 20 sponge layer cells from each leaf cross-section image to measure cell length, cell width, cell area, and cell perimeter with an Auto CAD software.

### Analyzing leaf non-structural carbohydrates and nitrogen

We collected leaf samples from each pot as a replicate (n = 5 pots) for analyzing the non-structural carbohydrates. These sampled leaves were dried with an oven at 75 °C for 48 h to consistent weight, and then these samples were ground to fine powder for spectrophotometrically analyzing glucose, fructose, sucrose, and starch with a glucose kit [79]. Similarly, we also sampled plant tissues from each pot (n = 5 pots) for analyzing the total carbon (C) and nitrogen (N) in different plant tissues (leaf, stem, and root) with an elemental analyzer [80]. All of the analyses were expressed on a percentage dry matter basis.

### Analyzing data

We used the one-way ANOVA to analyze the effects of CO₂ on the stomatal traits, soluble sugar and starch concentrations, carbon and nitrogen contents, as well as morphological and anatomical features. Two-way ANOVA was employed to test the effects of CO₂ concentration and leaf surface position (abaxial vs. adaxial) on the morphological traits of stomata with statistically significant differences at \( p < 0.05 \) level. We also employed linear and non-linear regressions for estimating the relationships between CO₂ concentration and other variables. The raw data from the leaf photosynthesis measurements were processed in Excel spreadsheets where the non-linear \( A_\text{n-C}_\text{i} \) curve fitting was performed [81]. The net assimilation rate (\( A_\text{n} \)) versus intercellular CO₂ concentration (\( A_\text{n-C}_\text{i} \) curve), was fitted to estimate the maximum carboxylation rate (\( V_{\text{cmax}} \)), maximum electron transport rate (\( I_{\text{max}} \)) based on the measurements of \( A_\text{n-C}_\text{i} \) curves. In addition, linear and non-linear (quadratic equations) regressions were employed to examine the relationships between CO₂ concentration and other variables.
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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

YZ, MX, LH, XZ and FL designed the experiments. LH, JY, LG, HZ, CM, and XZ performed the experiments and analyzed the data. FL, JY, YZ, HZ, XZ, CM, and MX analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no competing interests.

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Author details

1. School of Water Conservancy and Hydropower, Hebei University of Engineering, Handan 056038, China. 2. School of Agro-Grassland Science, Nanjing Agricultural University, Nanjing 210095, People’s Republic of China. 3. Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA. 4. Key Laboratory of Geospatial Technology for the Middle and Lower Yellow River Regions, College of Environment and Planning, Henan University, Kaifeng 475004, China. 5. Center for Remote Sensing and Spatial Analysis, Department of Ecology, Evolution and Natural Resources, Rutgers University, 14 College Farm Road, New Brunswick, NJ 08901, USA.

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