

1 **Growth responses of trees and understory plants to nitrogen fertilization in** 2 **a subtropical forest in China**

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19 20 **Abstract**

21 Reactive nitrogen (N) increase in the biosphere has been a noteworthy aspect of global
22 change, producing considerable ecological effects on the functioning and dynamics of the
23 terrestrial ecosystems. A number of observational studies have explored responses of plants to
24 experimentally simulated N enrichment in boreal and temperate forests. Here we asked how
25 the dominant trees and different understory plants respond to experimental N enrichment in a
26 subtropical forest in China. We conducted a 3.4-year N fertilization experiment in an
27 old-aged subtropical evergreen broad-leaved forest in eastern China with three treatment
28 levels applied to nine 20 m×20 m plots and replicated in three blocks. We divided the plants
29 into trees, saplings, shrubs (including tree seedlings), and ground-cover plants (ferns)
30 according to the growth forms, and then measured the absolute and relative basal area
31 increments of trees and saplings and the aboveground biomass of understory shrubs and ferns.
32 We further grouped individuals of the dominant tree species *Castanopsis eyrei* into three size
33 classes to investigate their respective growth responses to the N fertilization. Our results
34 showed that the plot-averaged absolute and relative growth rates of basal area and

35 aboveground biomass of trees were not affected by N fertilization. Across the individuals of
36 *C. eyrei*, the small trees with a DBH (diameter at breast height) of 5-10 cm has declined by
37 66.4% and 59.5%, respectively, in N50 (50 kg N ha⁻¹ yr⁻¹) and N100 fertilized plots (100 kg
38 N ha⁻¹ yr⁻¹), while the growth of median and large trees with a DBH of >10 cm has not
39 significantly changed with the N fertilization. The growth rate of small trees, saplings and the
40 aboveground biomass of understory shrubs and ground-cover ferns decreased significantly in
41 the N fertilized plots. Our findings suggested that N might not be a limiting nutrient in this
42 mature subtropical forest, and the limitation of other nutrients in the forest ecosystem might
43 be aggravated by the enhanced N availability, potentially resulting in an adverse effect on the
44 development of natural subtropical forest.

45

46 **Key-words:** *Castanopsis eyrei*, N fertilization, plant growth, shrub layer, subtropical forest,
47 tree layer, ground-cover fern

48 **1 Introduction**

49

50 Reactive nitrogen (N) increase in the biosphere, especially atmospheric N deposition, is a
51 globally prevalent phenomenon (Galloway *et al.* 2004). It has become a serious
52 environmental issue in China, especially in the southeastern regions, with drastic increase of
53 N entering terrestrial ecosystems which produces considerable ecological effects on the
54 functioning and dynamics of the terrestrial ecosystems (Liu *et al.* 2013; Gu *et al.* 2015). Since
55 the 1990s, the simulated N-fertilization experiments have been conducted in various forest
56 ecosystems to explore the responses of plants and other organisms to the potential high N
57 enrichment and changes of soil N availability (e.g., Wright & Tietema 1995; Bobbink *et al.*
58 2010; Fowler *et al.* 2015). Although a number of studies have reported a general positive
59 effect of N enrichment on plants in N-limited forests and a negative effect of excess N (e.g.,
60 Aber *et al.* 1998; Högberg *et al.* 2006; Gilliam 2006; Thomas *et al.* 2010), specific responses
61 of plants appeared to be highly growth form-dependent and ecosystem-dependent (LeBauer
62 & Treseder 2008; Bedison & McNeil 2009; Dirnböck *et al.* 2014).

63

64 Due to the widespread heavy N deposition in Europe and America, numerous studies that
65 focused on the growth responses of plants to N enrichment have been carried out in boreal
66 and temperate forests during the past several decades (Magill 2000; Högberg *et al.* 2006).
67 These studies showed that most trees have a positive growth response to N fertilization and
68 therefore have higher potential carbon sequestration because the status of N limitation was
69 largely alleviated by the increasing N inputs (e.g., Thomas *et al.* 2010; BassiriRad *et al.*
70 2015). However, the understory plants in these forest ecosystems inconsistently showed
71 general negative responses to N enrichment with declined biomass or shifted community
72 structure (Rainey *et al.* 1999; Du *et al.* 2014; Dirnböck *et al.* 2014). In addition to the opposite
73 responses of trees and understory plants to N enrichment, differences remained in the effects
74 of N enrichment on single plant growth form in these forests. Generally, the limited light
75 availability in these ecosystems with high tree canopy cover was ascribed to the negative
76 effects of N fertilization (Strengbom & Nordin 2008).

77

78 Recently, the effects of N enrichment on tropical forests raised researchers' concern.
79 Fertilization experiments in tropical forests showed different growth responses of trees to
80 nutrient addition among individual size levels, understory shrubs and tree seedlings (Wright
81 *et al.* 2011; Pasquini & Santiago 2012; Santiago *et al.* 2012) which contrasted with the ones

82 found for trees in the previously described experiments. For example, phosphorus (P)
83 fertilization enhanced the growths of small trees and seedlings but had no effect on median
84 and large trees, while N addition did not show any significant effect on plant growth in a
85 lowland tropical forest (Alvarez-Clare *et al.* 2013). In addition to the ubiquitous concept that
86 P was a critical element driving plant growth in tropical forests (Vitousek *et al.* 1991),
87 heterogeneous nutrient limitation that the growths of plants were co-limited by multiple
88 nutrients was further proposed to explain why diverse plants respond differently to N
89 enrichment (Wright *et al.* 2011; Alvarez-Clare *et al.* 2013; Wurzburger & Wright 2015).
90 Nevertheless, the patterns of specific nutrient limitation and responses of plants to N
91 enrichments among diverse forest ecosystems need further exploration.

92
93 As most of the nutrient fertilization experiments have focused on boreal forests, temperate
94 forests and lowland tropical forests, few studies have investigated the effects of N enrichment
95 on subtropical forests despite their broad distribution throughout the world and great
96 contribution to global carbon sink (Zhou *et al.* 2013; Yu *et al.* 2014; Huang *et al.* 2015). With
97 increasing N deposited in the subtropical ecosystems in southeastern China (Du *et al.* 2014),
98 it is important to diagnose the nutrient limitation and evaluate the responses of different plant
99 growth forms to N enrichment in subtropical forests for the assessment of carbon
100 sequestration and community dynamics.

101
102 To better predict the responses of subtropical forests and different plant growth forms to N
103 enrichment, we carried out a 3.4-year N fertilization experiment with three treatment levels
104 applied to nine 20 m × 20 m plots and replicated in three blocks in a subtropical forest in
105 south-eastern China. We attempt to explore whether N is a limiting element in the old-aged
106 evergreen broad-leaved subtropical forest. We hypothesize a positive response of trees to N
107 fertilization, but a negative response of understory growth forms to N fertilization due to the
108 expansion of canopy crown and consequent reduction of light availability.

109 110 **2 Materials and methods**

111 112 **2.1 Study site and experimental design**

113 The N fertilization experiment site was located at 30°01'47" N latitude and 117°21'23" E
114 longitude at an altitude of 375 metres in the natural conservation zone of Guniujiang in Anhui
115 Province, eastern China. As a commendable representative of the typical subtropical

116 broadleaved evergreen forest, the Guniujiang experimental site is an important part of the
117 NEECF (Network of Nutrient Enrichment Experiments in China's Forests) project (Du *et al.*
118 2013), because of its representativeness in both species composition and landscape structure
119 in the subtropical evergreen forest region. The study area has a humid climate with strong
120 summer monsoons with an annual average precipitation of 1,700 mm and an average annual
121 temperature of 14.9 °C. The amount of wet N deposition in this region was 5.9-7.3 kg N
122 ha⁻¹ yr⁻¹. The soil in this area has been classified as yellow brown earth (Chinese Soil
123 Taxonomic Classification), and the pH_{H2O} value at 0-10 cm soil depth was 4.58±0.05
124 (mean±SE). The total N, P, NH₄⁺-N and NO₃-N content in the soil at 0-10 cm depth were 3.23
125 (0.37), 0.32 (0.02), 0.012 (0.001), and 0.002 (0.0006) mg g⁻¹, respectively (Li *et al.* 2015).

126

127 The study was conducted in a well-protected, mature subtropical evergreen forest (>300 year
128 age) with a three-layered vertical structure: the canopy tree layer (DBH>5 cm and height>5
129 m); the understory layer of saplings, shrubs and seedlings (DBH<5 cm and height<5 m); and
130 the ground-cover layer (ferns and herbs). The average density and basal area of trees were
131 1,219 trees ha⁻¹ and 36.35 m² ha⁻¹, respectively; *Castanopsis eyrei* was the dominant species
132 (which was also an important species at some other sites in subtropical forests) and accounted
133 for 87% of the total aboveground biomass of trees. The understory saplings and shrubs
134 contained several species, including *Cleyera japonica*, *Camellia cuspidata*, *Rhododendron*
135 *ovatum*, *Eurya muricata*, *Cinnamomum japonicum*, *Cinnamomum subavenium*, *Sarcandra*
136 *glabra*, and *C. eyrei*, and other native subtropical evergreen species (Table 1). Two fern
137 species (*Woodwardia japonica* and *Dryopteris hwangshanensis*) and an orchid (*Cymbidium*
138 *tortisepalum* var. *longibracteatum*) appeared on the floor layer, while *W. japonica* exclusively
139 dominated the floor layer with a coverage of 10%-20%.

140

141 We began N fertilization in March 2011. A randomized block design was used to avoid spatial
142 heterogeneity. We chose three blocks with similar stand growth, species composition and site
143 condition to establish three N treatments in each block: CK (0 kg N ha⁻¹ yr⁻¹), N50 (50 kg N
144 ha⁻¹ yr⁻¹), and N100 (100 kg N ha⁻¹ yr⁻¹). In total, nine 20 m × 20 m plots were established
145 with a 5-10 m buffer zone between each plot. The total NH₄NO₃ was divided into 12 dosages
146 and applied to the forest in each month at regular intervals. NH₄NO₃ in dosages of 0.48
147 kg/plot and 0.95 kg/plot were dissolved in 15 L of fresh water, respectively, and then sprayed
148 uniformly in N50 and N100 plots using a back-hatch sprayer. The unfertilized plots (controls)
149 were similarly treated with 15 L of fresh water without NH₄NO₃.

150

151 **2.2 Sampling and measurement**

152 In March 2011, the species of all trees higher than 2 m in each plot were labelled and their
153 initial DBH (1.3 m) was measured. Then, autonomous band dendrometers made of
154 aluminium tape and springs were installed on trees with a DBH greater than 5 cm. After one
155 month to allow the tapes and springs on the trees to become stable, we began to measure the
156 changes in the gaps on the tapes using vernier callipers (measured in July 2014) and then
157 calculated tree DBH according to the following equation:

$$158 \quad \text{DBH} = \text{DBH}_1 + \frac{X_2 - X_1}{3.14 \times 10}$$

159 where DBH_1 represents the initial DBH (cm) of trees measured in March 2011, and X_2 and X_1
160 (mm) represent the widths of gaps on the tapes measured in July 2014 and at the beginning of
161 the experiment, respectively.
162

163

164 The basal area is a common indicator for weighing the biomass of trees. Therefore, tree basal
165 area increments were calculated to indicate the responses of tree biomass to the N fertilization.
166 First, to test community-level responses of tree layer to N fertilization, we calculated the sum
167 of total basal area increase ($\text{m}^2 \text{ha}^{-2} \text{year}^{-1}$) of all trees in a plot after 3.4 years of N
168 fertilization and divided this value by the period of N fertilization (3.4 years) to obtain the
169 annual basal area increase rate of the trees (dead trees were not included). Second, relative
170 annual basal area growth rate (RGR, $\text{m}^2 \text{m}^{-2} \text{year}^{-1}$) was used to eliminate the conceivable
171 interferential effects resulting from the differences in the number and size of original
172 individuals among plots according to the following equation, similar to Alvarez-Clare et al.'s
173 method (2013):

$$174 \quad \text{RGR} = \frac{\ln(2014 \text{ BA}) - \ln(2011 \text{ BA})}{3.4}$$

175 where RGR represents the relative annual basal area growth rate ($\text{m}^2 \text{m}^{-2} \text{year}^{-1}$), BA indicates
176 the sum of basal area of all trees in each plot, and 3.4 (years) is the N fertilization period.
177

178

179 Because *C. eyrei* was the only dominant species in the tree layer, we separated it from other
180 tree species and grouped its individuals into three classes based on their DBH values (i.e.
181 5-10 cm, 10-30 cm and >30 cm) to investigate the effects of N fertilization on the growth of
182 trees after removing the plant species and original size factors. During the monitoring of tree
183 growth, dead trees were recorded. Then, we calculated the aboveground biomass increments

184 of trees and the proportion of dead biomass using allometric equations (see Table S1).

185

186 We examined the effects of N fertilization on understory tree saplings distributed in the plots
187 according to their sizes and characteristics. For small trees with DBH<5 cm and height>2 m
188 (defined as “saplings”), DBH was measured at the beginning of N fertilization and in July
189 2014. Then, annual basal area growth rate and RGR of saplings were calculated based on
190 DBH changes. For very small trees or shrubs with DBH<5 cm and height<2 m (defined as
191 “shrubs/seedlings”), we set two 5 m × 5 m subplots in each plot along a diagonal direction
192 and investigated the abundance, dominance, basal diameter (diameter at 10 cm above the
193 ground), height and crown diameters of all shrubs/seedlings inside the subplots at two
194 specific times. The first time was at the beginning of N fertilization (March 2011), and the
195 second was in July 2014. The length, width and number of fern leaves were measured
196 carefully in the above-mentioned subplots, and the allometric equations for seven dominant
197 species were then obtained (Table S1). Because the average aboveground biomass of
198 shrubs/seedlings and ferns showed no significant differences across three N treatments before
199 N fertilization in March 2011, we regarded the distribution of these understory
200 shrubs/seedlings and ferns to be homogeneous among the three treatments. Then we
201 identified the effects of N fertilization by comparing the aboveground biomass of
202 shrubs/seedlings and ferns in 2014 among the different treatments. Meanwhile, to investigate
203 the canopy cover and understory light availability, we used a digital camera (Canon, Japan)
204 with a fisheye lens (Sigma circular fisheye) to take photographs of canopy. In each subplot,
205 we put the camera at 1m above ground and took 5 photos upwards from understory.

206

207 In addition, to further explore the influences of N fertilization on plants’ growth from
208 biogeochemical aspect, we measured soil N, P content and pH. Specifically, we set three
209 subplots randomly within each plot and collected three subsamples of 0-10 cm soil for each
210 subplot using a hand-held steel soil borer (3 cm in diameter), during investigation of the
211 understory plants. Then, the three subsamples were mixed together to form one sample per
212 plot and transported to a laboratory and air dried naturally. After air-dried, soil samples were
213 ground with a ball mill (NM200, Retsch, Haan, Germany) and screened through a 100 mesh
214 sieve. The N concentration of soil was measured using an elemental analyser (2400 Series2
215 CHNS/O Elemental Analyzer, Perkin-Elmer, USA). After acid digestion of the samples, soil P
216 concentrations were measured using a flow injection analysis instrument (AutoAnalyser3,
217 Bran+Lubbe City, Germany). Soil pH was measured by dry soil in water suspension with a

218 water:soil ratio of 1:2.5.

219

220 **2.3 Statistical analysis**

221 We used an analysis of variance (ANOVA) to evaluate the effects of N fertilization on soil N
222 and P content, soil pH, tree basal area increments, RGR, aboveground biomass increments,
223 proportion of dead trees, and aboveground biomass of shrubs/seedlings and ferns. Block and
224 N treatment were both regarded as fixed factors in the statistical model. We excluded the
225 interactions between block and N treatment from the model because they do not have
226 ecological meaning. Tukey's honest significant difference (HSD) tests were used to conduct
227 the multi-comparisons among the three N treatments. For the estimation of canopy cover, we
228 followed the detailed procedures of weighted ellipsoidal method using the software of
229 Hemisfer (version 2.16.6) to obtain values of vertical total gap fraction (Fmv) which indicate
230 the proportion of projected light spots to the total projected area (Thimonier *et al.* 2010).
231 Then we obtained the values of [1-Fmv] to indicate canopy cover. All statistical analyses
232 were performed in R.3.2 (R Development Core Team, 2010), and all figures were drawn in
233 SigmaPlot 12 (Systat, 2010).

234

235 **3 Results**

236

237 **3.1 Effects of N fertilization on canopy cover, soil N and P contents and pH**

238 The indicator of forest canopy (i.e. [1-Fmv]) showed no significant differences between
239 unfertilized and fertilized plots with 3.4 years of N fertilization (Table 2). Although the fish
240 eye measurements did not provide evidence for the changes in total forest cover with the
241 effects of N fertilization, there still may be a shift between the contribution of overstory and
242 understory trees to the total forest cover.

243

244 3.4 years of N fertilization significantly increased the N content of 0-10 cm soil ($p=0.03$),
245 especially in N100 plots (Fig. 1a), but showed no significant effect on soil P content (Fig. 1b,
246 $p>0.05$), thus leading to a significant increase in soil N:P ratio (Fig. 1c, $p=0.02$). Additionally,
247 the N fertilization also decreased soil pH and aggravated soil acidification (Fig. 1d, $p=0.05$).

248

249 **3.2 Growth responses of trees to N fertilization**

250 The increments of absolute basal area, aboveground biomass and RGR of all trees at plot
251 level showed no significant response to N fertilization during 3.4-year N fertilization (Fig.
252 2a~2c). Compared with the unfertilized plots, N50 and N100 fertilized plots showed a

253 tendency toward higher averaged proportions of dead trees' aboveground biomass despite no
254 significant difference between them (Fig. 2d).

255

256 Individuals of the dominant species *C. eyrei* with different initial DBH showed divergent
257 responses of absolute basal area increments and RGR to N fertilization (Fig. 3a-3f). The
258 small trees with a DBH of 5-10 cm growing under unfertilized plots showed greater basal
259 area increments than those growing under N fertilized plots (Fig. 3a, $p=0.02$). Specifically,
260 the N50 and N100 fertilization decreased the absolute basal area increments of small
261 individual trees at rates of $2.2 \text{ cm}^2 \text{ tree}^{-1} \text{ year}^{-1}$ and $1.98 \text{ cm}^2 \text{ tree}^{-1} \text{ year}^{-1}$, respectively, which
262 indicated that the decreasing degrees of the absolute basal area of small trees reached 66.4%
263 and 59.5% in N50 and N100 plots. The small individual trees also showed a tendency toward
264 lower averaged RGR in N fertilized plots although no significant difference was detected
265 between them (Fig. 3d, $p>0.05$). As opposed to the negative responses of small trees to N
266 fertilization, the basal area increment and RGR of median *C. eyrei* individuals (DBH of 10-30
267 cm) and large *C. eyrei* individuals (DBH of $>30\text{cm}$) showed no significant response to N
268 fertilization, but the averaged growth rate of large *C. eyrei* individuals in N50 plots almost
269 doubled the value of the corresponding large individuals in unfertilized plots (Fig. 3b-3c and
270 3e-3f, $p>0.05$ in all cases).

271

272 **3.3 Growth responses of understory saplings, shrubs/seedlings, and ferns to N** 273 **fertilization**

274 Responses of understory saplings to N fertilization were similar to those of small dominant
275 trees. Although the annual absolute increments of basal area increments of saplings showed
276 no significant response to N fertilization (Fig. 4a, $p>0.05$), the RGR of sapling growing in
277 N50 and N100 plots showed a substantial decrease at rates of $0.021 \text{ m}^2 \text{ m}^{-2} \text{ yr}^{-1}$ and 0.019 m^2
278 $\text{m}^{-2} \text{ yr}^{-1}$, respectively, compared to sapling growing in unfertilized plots (Fig. 4b, $p<0.001$). In
279 addition, a general negative effect of N fertilization also occurred on understory shrubs and
280 ground-cover ferns. The aboveground biomass of seven predominant shrubs/seedlings was
281 drastically decreased by 69.4% and 79.1% in N50 and N100 fertilized plots, respectively,
282 compared with those in the unfertilized plots (Fig. 5a, $p<0.01$). Remarkably, the aboveground
283 biomass of ground-cover ferns significantly declined by 92.4% and 93.4% in N50 and N100
284 fertilized plots (Fig. 5b, $p<0.05$).

285

286 **4 Discussion**

287

288 **4.1 Growth responses of trees to N fertilization**

289 Nutrient limitation was generally determined through evaluating ecosystem feedbacks to
290 nutrient addition (Vitousek 1991; Santiago *et al.* 2012; Alvarez-Clare *et al.* 2013). When the
291 forest ecosystems showed a positive response to added nutrient, e.g., plant growth or rates of
292 physiological processes were promoted, the added nutrient then could be interpreted as
293 limiting to the ecosystem, otherwise, as not limiting to the ecosystem (Santiago 2015). We
294 initially expected positive growth responses of trees exposed to N fertilization in this
295 subtropical forest because N availability in the soil would be enhanced by N fertilization and
296 the potential N limitation of plants in the forest ecosystem could be alleviated. However,
297 contrary to our expectation, we did not observe strong positive growth responses of trees to N
298 fertilization (Figs. 2 and 3). Across individual trees of different sizes and plant growth forms,
299 we only observed substantial negative responses of small trees (5-10 cm DBH; Fig. 3a and 3d)
300 and saplings (Fig. 4a and 4b) and weak responses of median and large trees (>10 cm DBH) to
301 N fertilization (Fig. 3b-3c and 3e-3f), which further demonstrated that the growth of trees in
302 this old-aged subtropical forest was not essentially limited by N as hypothesized.

303

304 Contrasted with previous positive responses of trees to N fertilization in boreal and temperate
305 forests which were considered as N limited ecosystems (Högberg *et al.* 2006; Thomas *et al.*
306 2010; BassiriRad *et al.* 2015), our finding of the unchanged responses of trees to N
307 fertilization was partly consistent with observations of trees from tropical forests (e.g.,
308 Santiago *et al.* 2012; Alvarez-Clare *et al.* 2013). Studies from mature tropical forests have
309 revealed that P availability was a critical element shaping tree species distribution and
310 productivity (Santiago 2016; Dalling *et al.* 2016). Given the similar high-weathered soil
311 properties, humid climatic conditions and dominant evergreen broadleaf trees in mature
312 subtropical forest as those in wet tropical forest, we speculated that P limitation, rather than N
313 limitation, might have played a key role in influencing growth of plants in subtropical forest.

314

315 The N and P stoichiometry of soil might have objectively provided indicators of P limitation
316 with the effects of N fertilization in this subtropical forest, because soil N contents and N:P
317 ratio in N fertilized plots were remarkably higher than those in unfertilized plots (Fig. 1).
318 Additionally, limitation of other nutrients, such as K (potassium) which was highlighted in
319 tropical forests, and their combination as well as heterogeneous nutrient limitation of specific
320 species, plant growth forms and individuals in different sizes may warrant further

321 consideration in subtropical forests (Wright *et al.* 2011; Santiago *et al.* 2012; Alvarez-Clare *et*
322 *al.* 2013).

323

324 Moreover, the high spatial heterogeneity in old-aged subtropical forest, similar to tropical
325 forests, could be a possible explanation for the lack of significant responses of plot-averaged
326 basal area growth, RGR, aboveground biomass of trees with a DBH of >5 cm and the
327 proportion of dead trees to N fertilization. In eastern China, the distributions of subtropical
328 forest stands are quite topographically fragmented, while relative flat stands are required to
329 avoid N losses and minimize spatial heterogeneity among experimental treatments. The
330 actual distribution and topography of the subtropical forests limited the number of
331 replications in the N fertilization experiment. This limitation might reduce the statistic power
332 of N treatment on plot-averaged plant growth rate which has been pointed out in previous
333 studies (Wright *et al.* 2011; Alvarez-Clare *et al.* 2013). Furthermore, our observation of large
334 trees with DBH >30 cm showed that the averaged growth rate of large *C. eyrei* individuals in
335 N50 plots almost doubled the value of the corresponding large individuals in unfertilized
336 plots. Nevertheless, the results of ANOVA showed that the effect was not significant. As the
337 number of large trees in the experiment was relatively less than the small trees, the low
338 replication and high spatial site heterogeneity might have reduced the statistical power of N
339 fertilization on the large trees. Thus, fertilization experiments with more homogeneous plots
340 and more replicates are warranted to further strengthen these findings. Overall, given the
341 negative and potential positive effects of N fertilization on small and large trees, it is of
342 urgent necessity to conduct long-term monitoring of the trees which would provide
343 alternatives for accurately evaluating the forest dynamics under the enhanced global N
344 deposition.

345

346 **4.2 Growth responses of small trees, understory saplings, shrubs/seedlings and ferns to** 347 **N fertilization**

348 Although the positive responses of small or juvenile trees to nutrient fertilization has been
349 reported in boreal, temperate and tropical forest (e.g., Högberg *et al.* 2006; Bedison &
350 McNeil 2009; Alvarez-Clare *et al.* 2013), our results showed a remarkable negative effect of
351 N fertilization on small-sized plants including trees, understory saplings, shrubs/seedlings and
352 ferns. During our field investigation, we also found that the average proportion of dead trees
353 (Fig. 2d) tended to increase in N fertilized plots although the result was not statistically
354 significant ($p=0.50$). Additionally, the ground-cover ferns in N100 plots almost disappeared

355 after 3.4-year N fertilization (personal observation). Given the high stand density in this
356 mature subtropical forest, we suggest that N fertilization might potentially lead to increased
357 self- and alien-thinning of individuals through decreasing understory light availability.

358

359 The pivotal role of light availability in the eco-physiological processes of understory growth
360 forms has been widely recognized (Santiago 2015). Due to the limited light availability,
361 understory plants may not be able to incorporate the added nutrient and promote their
362 photosynthetic rates (Alvarez-Clare *et al.* 2013). However, a study conducted in tropical
363 forest with thick canopy showed that photosynthetic process could be enhanced by nutrient
364 addition even under low light availability (Pasquini & Santiago 2012). In a sharp contrast, the
365 study conducted in an Australian rainforest revealed that understory seedlings increased
366 growth when the light availability was high, but showed no significant response to nutrient
367 fertilization in low lights (Thompson *et al.* 1988). These studies, together with our field
368 observations, suggest that the growth of understory plants is largely co-limited by nutrient
369 and light availability in the local environment. Further, our results of forest canopy cover
370 estimated by photographic fisheye showed no significant differences between unfertilized and
371 N fertilized plots, which was consistent with the findings of Lu *et al.* (2010). Although the
372 understory light irradiance fluctuated largely during a day and was very hard to detect
373 precisely, our measurements of forest canopy cover provided a rough evaluation for light
374 availability and a potential shift between the contribution of overstory and understory trees to
375 the total forest cover which could partly explain the differences in the responses of trees with
376 different sizes (i.e. different DBH classes). The results might indicate that other factors in
377 addition to the low light availability in this old-aged forest had also played a crucial role in
378 influencing understory plants during 3.4 years' N fertilization.

379

380 **4.3 Potential N saturation and plant growth**

381

382 The striking biomass reduction of the understory plants, especially ferns, in response to N
383 fertilization in our study well corroborated the similar findings in an old-aged tropical forest
384 at Mt. Dinghushan in China (Lu *et al.*, 2010). Also, consistent with previous studies obtained
385 from boreal, temperate and tropical forests (Rainey *et al.* 1999; Alvarez-Clare *et al.* 2013;
386 Dirnböck *et al.* 2014), our experiment revealed that understory small-sized plants responded
387 sensitively to nutrient fertilization, which might indicate a possibility of N saturation in the
388 subtropical forest. According to the definition of N saturation addressed by Aber *et al.* (1998)

389 (i.e. N availability in the forest ecosystem exceeded the demand of plants and microbes), the
390 drastic decrease of understory ferns, shifted composition of understory plant community, and
391 cation imbalances of understory species after 7 years' chronic N fertilization at Harvard
392 Forest, USA, could be interpreted as useful indicators of N saturation (Rainey *et al.* 1999).
393 Moreover, a 6-year N fertilization experiment in an old-aged tropical forest at Mt.
394 Dinghushan also showed signs of N saturation, such as significant increases in nitrate (NO₃-)
395 leaching, inorganic N concentration and N₂O emissions of soils, and soil acidification (Lu *et*
396 *al.* 2014; Chen *et al.* 2015). In our experiment, the soil acidification and increased soil N
397 concentration in high N fertilized plots (Fig. 1) combined with the negative responses of
398 understory plants suggest that the 3.4-year N fertilization in this mature subtropical forest site
399 has potentially caused N saturation. Nevertheless, further observations are still required to
400 explore the mechanisms underlying the changes of different growth forms with the effects of
401 N enhancement in the subtropical forests.

402

403 **5. Conclusion**

404

405 Contrasting growth responses among plant growth forms to N fertilization were present in the
406 mature subtropical evergreen forest in this study. Overall growth of trees at the plot level
407 showed no significant response to the N fertilization; however, if the dominant tree species *C.*
408 *eyrei* was grouped into three DBH classes, the basal area increment of small trees with a
409 DBH of 5-10 cm declined 66.4% and 59.5% in N50 and N100 fertilized plots, respectively,
410 while the growth of median and large trees with a DBH of >10 cm showed weak responses to
411 N fertilization. The growths of understory saplings, shrubs/seedlings, and ground-cover ferns
412 showed a negative response to N fertilization. Our results indicated that N might not be a
413 limited nutrient in this subtropical forest and that other nutrient and light availability may
414 potentially co-limit growth of plants with different growth forms. Our data also suggested
415 that even short-term N fertilization might have caused N saturation in this mature subtropical
416 forest and the limitation of other nutrients might be amplified with increasing N addition.

417 *Funding:* This study was funded by the National Natural Science Foundation of China
418 (31321061 and 31330012).

419

420 *Acknowledgements:* We wish to thank Bernhard Schmid, Gianalberto Losapio, Lilian Dutoit,
421 Peter Schmid and Jessica Baby for their helpful suggestions on the manuscript, and the editor
422 and two anonymous reviewers for their insightful comments that greatly improved this
423 manuscript. We also thank the Sino-German Center for Research Promotion for the
424 participation in a summer school in Jingdezhen (GZ1146).

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531 **Table 1** Growth measurements for four plant growth forms in this study before N fertilization.
 532 Numbers in the tables represent means (or mean \pm (standard error), $n=9$) of plants across all
 533 plots. TBA: total basal area of trees; DBH: diameter at breast height (1.3 m); Basal diameter:
 534 diameter at 10 cm above the ground.

Growth forms	Species	Growth variable		
		TBA ($\text{m}^2 \text{ha}^{-1}$)	DBH (cm)	Height (m)
Trees	<i>Castanopsis eyrei</i>	32.5 \pm 2.7	15.7 \pm 3.6	11.8 \pm 2.1
Saplings	<i>C. eyrei</i>	0.61 \pm 0.10	3.81 \pm 0.04	2.59 \pm 0.06
		Coverage (%)	Basal diameter (mm)	Height (cm)
Shrubs & Seedlings	<i>Cleyera japonica</i>	2.89	9.24 \pm 5.13	79.8 \pm 40.82
	<i>Camellia cuspidata</i>	8.60	7.01 \pm 0.62	60.1 \pm 4.37
	<i>Rhododendron ovatum</i>	5.97	16.81 \pm 8.91	167.5 \pm 65.02
	<i>Eurya muricata</i>	3.04	7.00 \pm 1.57	111.0 \pm 38.16
	<i>Cinnamomum japonicum</i>	2.85	4.44 \pm 1.46	51.1 \pm 26.59
	<i>Cinnamomum subavenium</i>	5.03	2.77 \pm 0.64	29.9 \pm 7.54
	<i>Sarcandra glabra</i>	2.92	3.60 \pm 0.11	35.7 \pm 3.69
		Density (shoots m^{-2})		
Ferns	<i>Woodwardia japonica</i>	1.19 \pm 0.23		

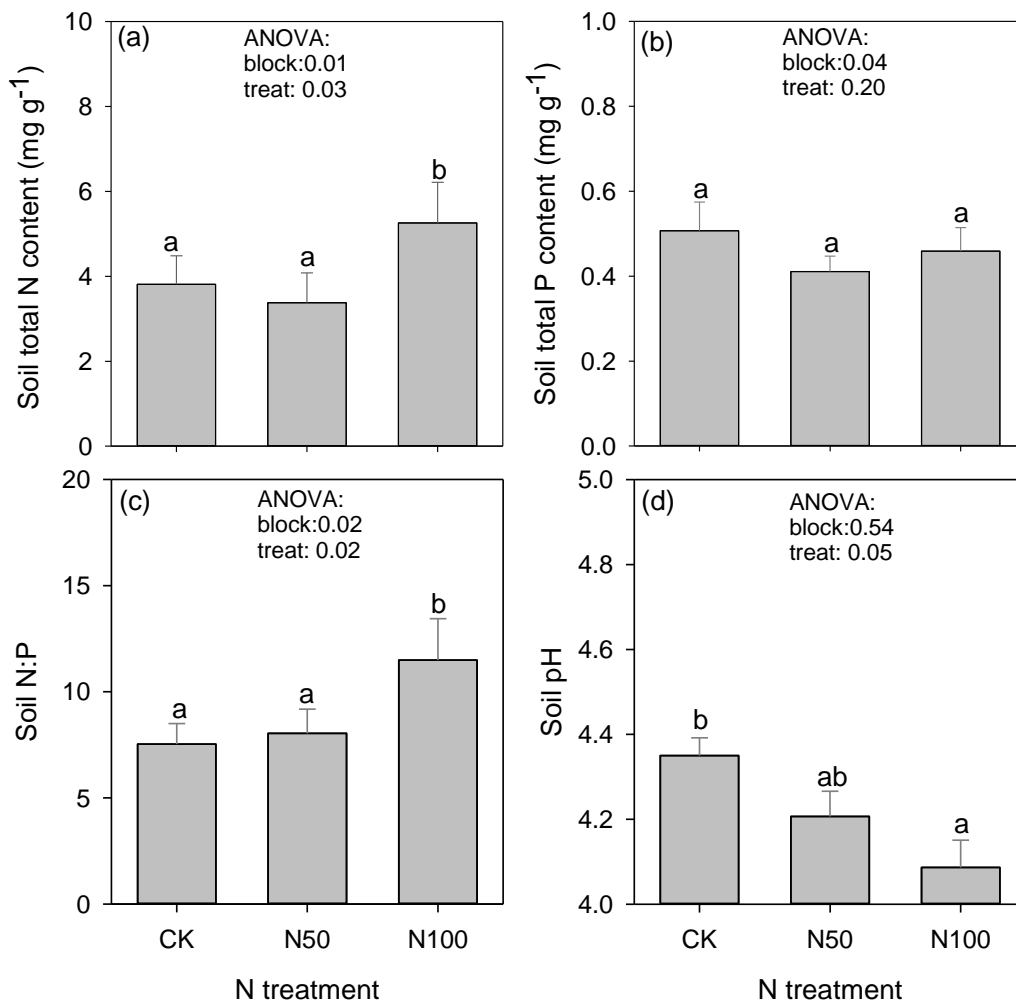
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536 Table 2 The indicator of canopy cover (i.e. [1-Fmv]) of the three treatments in our
537 experiments. *n* indicates the number of replicates. *SE* indicates the standard error.

Treatment	<i>n</i>	Canopy cover	
		mean	SE
CK	3	0.77	0.01
N50	3	0.76	0.04
N100	3	0.72	0.01

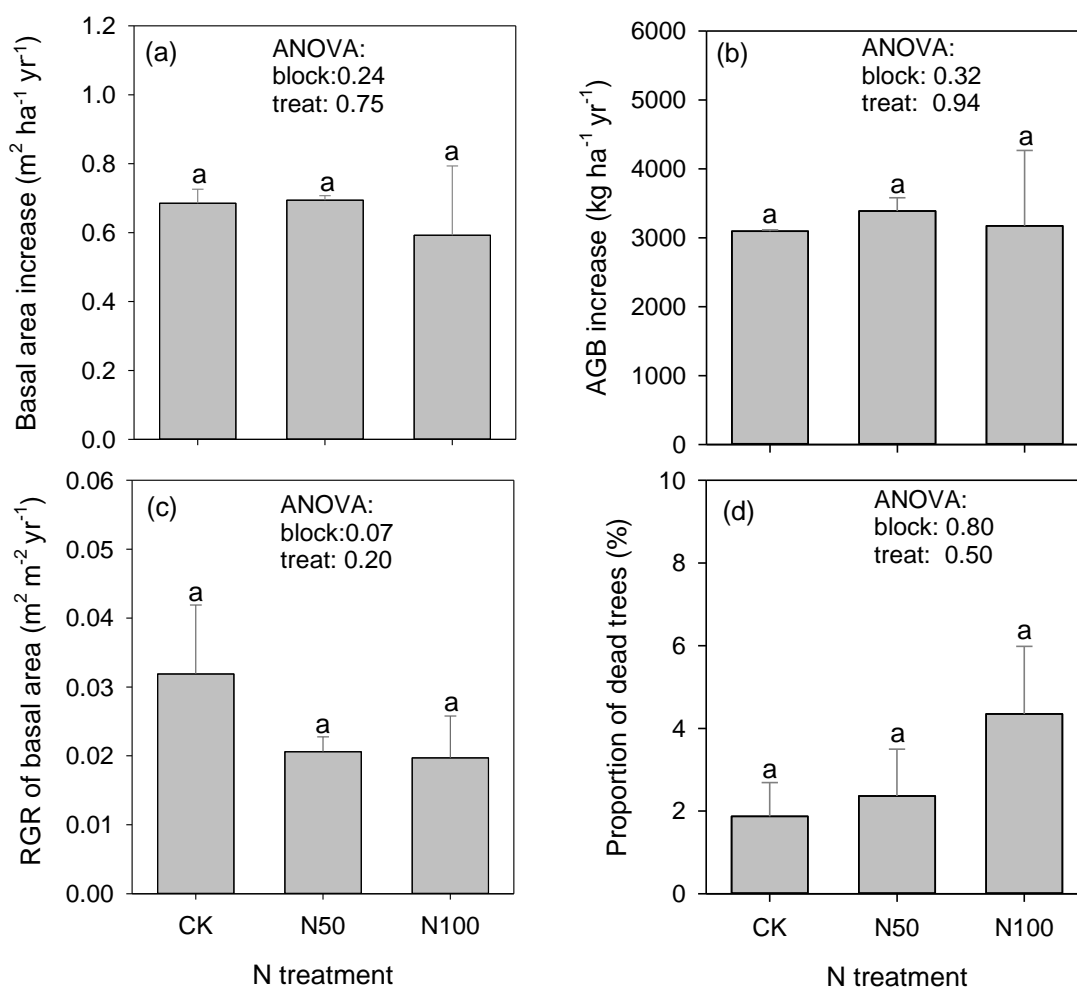
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539 **Figure 1** Effects of N fertilization on soil nutrient content, N:P ratio and pH (mean \pm se, n=3)
 540 at the soil depth of 0-10 cm. (a) Total N content per gram soil; (b) total P content per gram
 541 soil; (c) N:P ratio and (d) soil pH. Numbers in these figures indicate the results of ANOVA .



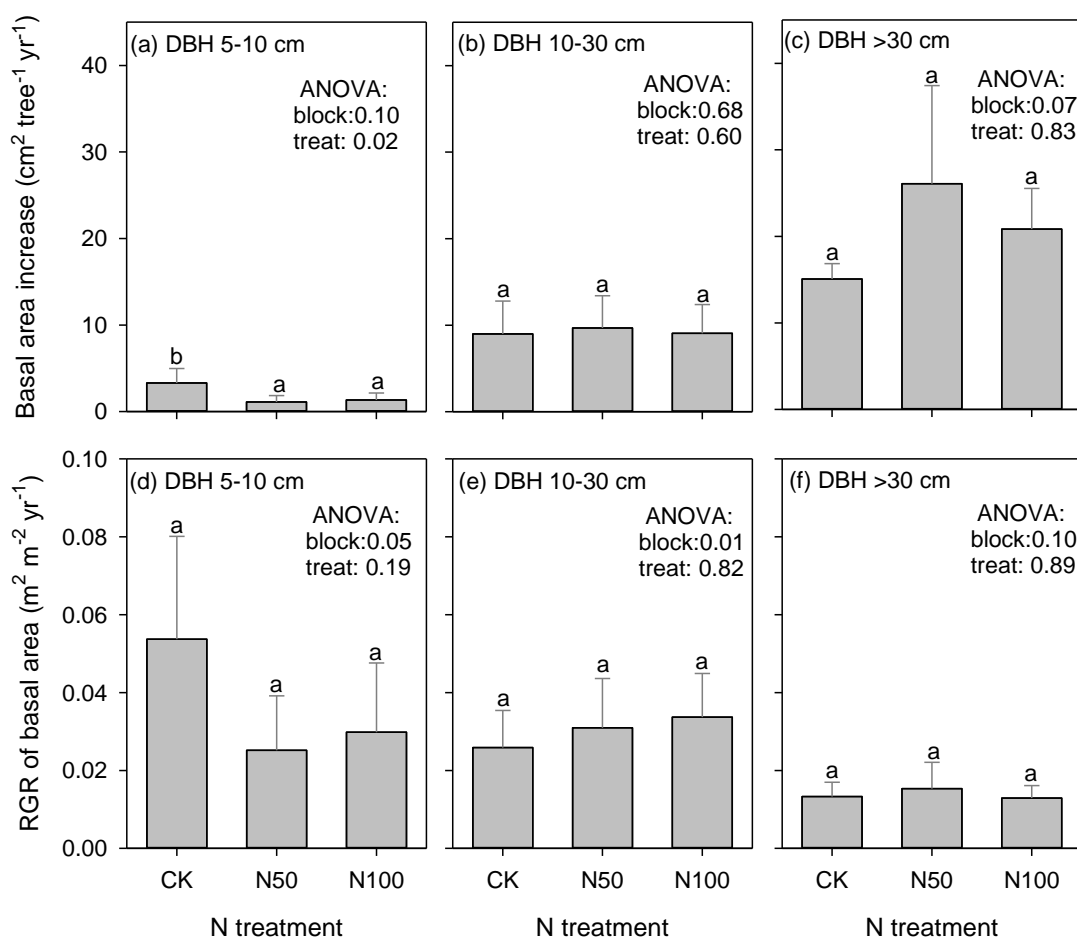
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543 **Figure 2** Effects of N fertilization on the growth and mortality of all trees (mean \pm se). (a)
 544 Absolute basal area increase of all trees; (b) aboveground biomass increase of all trees; (c)
 545 relative growth rate of total tree basal area; and (d) the proportion of all dead trees. The
 546 proportion of dead trees was calculated using the aboveground biomass of all dead trees
 547 during the experiment divided by the total aboveground biomass of all trees in 2014.
 548 Numbers in these figures indicate the results of ANOVA. The N treatment on x-axis
 549 represents three levels of N fertilization: CK (0 kg N ha⁻¹ yr⁻¹), N50 (50 kg N ha⁻¹ yr⁻¹) and
 550 N100 (100 kg N ha⁻¹ yr⁻¹).



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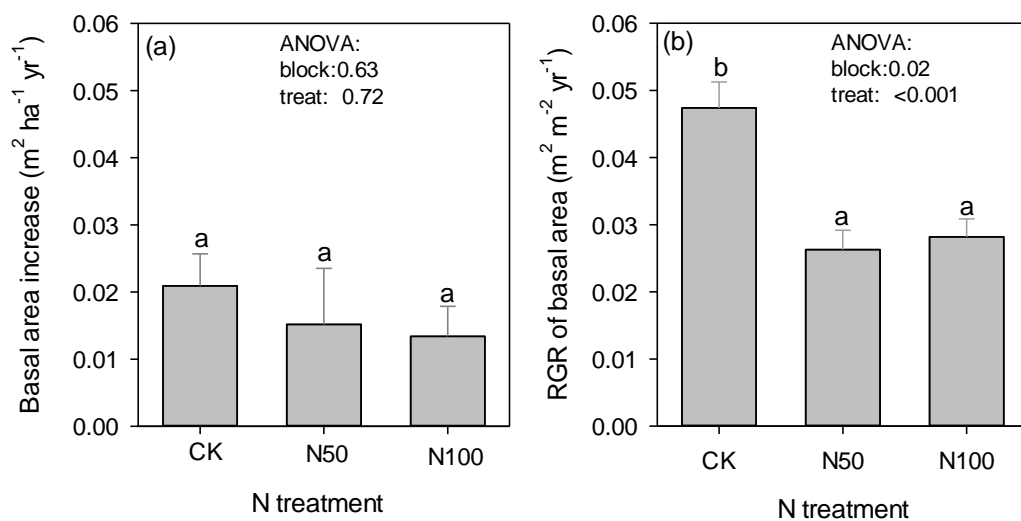
552 **Figure 3** Effects of N fertilization on the growth (mean \pm se) of *C. eyrei* by DBH classes
 553 (5-10 cm, 10-30 cm and >30 cm). (a-c) Absolute basal area increase and (d-f) relative growth
 554 increase rate of basal area. Numbers in these figures indicate the results of ANOVA. The N
 555 treatment on x-axis represents three levels of N fertilization: CK (0 kg N ha⁻¹ yr⁻¹), N50 (50
 556 kg N ha⁻¹ yr⁻¹), and N100 (100 kg N ha⁻¹ yr⁻¹).



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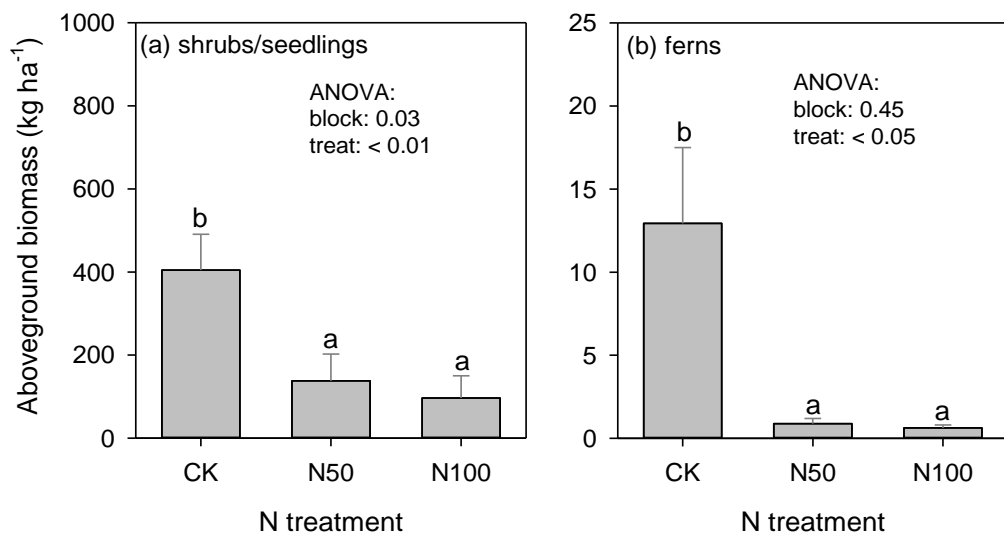
558 **Figure 4** Effects of N fertilization on the growth of saplings (mean \pm se). (a) Absolute basal
559 area increase and (b) the relative growth rate of basal area. Numbers in these figures indicate
560 the results of ANOVA. The N treatment on x-axis represents three levels of N fertilization:
561 CK (0 kg N ha⁻¹ yr⁻¹), N50 (50 kg N ha⁻¹ yr⁻¹) and N100 (100 kg N ha⁻¹ yr⁻¹).

562



563

564 **Figure 5** Effects of N fertilization on the aboveground biomass of shrubs, seedlings and ferns.
565 Bars show the aboveground biomass of (a) shrubs/seedlings and (b) ferns (mean \pm se).
566 Numbers in these figures indicate the results of ANOVA. The N treatment on x-axis
567 represents three levels of N fertilization: CK (0 kg N ha⁻¹ yr⁻¹), N50 (50 kg N ha⁻¹ yr⁻¹) and
568 N100 (100 kg N ha⁻¹ yr⁻¹).
569



570