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Different-Sized Vessels of *Quercus variabilis* Blume Respond Diversely to Six-Year Canopy and Understory N Addition in a Warm-Temperate Transitional Zone

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Abstract: Nitrogen is a necessary macroelement in plant growth and is usually considered a limiting factor in many forest ecosystems. Increasing N deposition has been reported to affect tree growth. However, the effects still remain controversial due to variable N fertilization methods used. In order to study the realistic responses of tree growth to increasing N deposition, we investigated effects of canopy and understory N addition on tree-ring growth and vessel traits of *Quercus variabilis* Blume. Since 2013, 50 kg N ha^{-1} year was applied monthly from April to December to either the canopy (CN) or understory (UN) of trees in a warm-temperate forest in Central China. During 2013–2018, tree-ring growth and vessel-related traits (mean vessel area, theoretical xylem hydraulic conductivity (KH), relative ratio of KH, etc.) were analyzed. Tree rings were negatively impacted by both CN and UN treatments, but only the effect of UN was significant. Neither CN nor UN significantly impacted the detected vessel traits. However, some diverging influencing trends were still showed in some vessel traits. Both CN and UN treatments positively affected the percentage of annual total vessel area and vessel density, with the effect of UN on vessel density being more severe. All the detected vessel traits of the large vessels formed at the beginning of the tree-ring responded positively to CN, whereas the opposite response to UN was showed on mean vessel area and the relative ratio of KH. All these diverging responses in different vessel traits likely reflected the compensation and trade-off between maximizing growth and adapting to CN and UN treatments. Six-year long N addition negatively and positively affected tree-ring growth and vessel traits of *Q. variabilis* in Central China, respectively. UN treatment could not fully simulate the real effect on tree growth, especially on the hydraulic architecture.

Keywords: canopy nitrogen addition; understory nitrogen addition; vessel; theoretical hydraulic conductivity; tree-ring

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1. Introduction

Nitrogen (N) deposition caused by anthropogenic activities has been dramatically enhanced in recent decades and is predicted to increase continuously to more than 50 kg N ha^{-1} $year^{-1}$ by 2050 in Central and Eastern China [1–3]. N is one of the most important macroelements in plant growth and is usually considered a limiting factor in many territorial ecosystems [4]. Therefore, understanding the effects of increased N deposition on tree growth is crucial for improving the prediction of responses of tree growth and carbon sequestration by forest ecosystems in terms of future global climate change.

Tree-ring growth and vessel-related features (e.g., vessel size, percentage of total vessel area of the xylem, etc.) are often investigated to interpret tree growth responses to environmental changes [5–7]. Tree-ring growth is also an important direct indicator of carbon fixation by trees [8], and vessel-related traits reflect water transport availability, which could affect photosynthesis and thereby ultimately determine carbon fixation during tree growth [9,10]. Moreover, vessels are also the vital regulator of the carbon and water balance during tree growth under changing and stressful environments [11,12]. Therefore, the ongoing increase in N deposition has great potential to impact tree growth and subsequent ecosystem carbon and water fluxes [13,14].

Many studies have reported the effects of simulated N deposition on tree growth, but most N treatments were applied directly to the soil or understory of the canopy, ignoring N retention by the canopy in a natural atmospheric deposition situation [15,16]. In the field or in a natural atmospheric N deposition situation, N would first contact the canopy, where it could be intercepted and absorbed by leaves or fixed by canopy biota before reaching the forest floor [17–19]. Thus, applying N to the canopy of trees is generally and theoretically considered a more realistic method for simulating natural N deposition in the field [20,21].

However, the difficulties of applying N to the canopy of tall mature trees in a forest hampered the determination of the effects of canopy N addition on tree growth and xylem hydraulic traits in forests. Therefore, the effects of canopy and understory N addition on tree growth require further evaluation to obtain a thorough understanding of the actual responses of tree growth to N deposition. Increasing N deposition might be beneficial for tree growth with the production of wider tree-ring and larger xylem vessels that would enhance the water transportation capability, thereby improving photosynthesis and carbon fixation [9,10]. Xylem vessels, as water conduction elements in deciduous trees, are a highly efficient water conduction organ, a permanent and easy-to-measure trait for water transport availability, and thus is vital for tree growth. For instance, a greenhouse experiment on hybrid poplar saplings confirmed that N addition increased the area of large xylem vessels, suggesting that N addition might be advantageous to tree growth [15]. However, large vessels would increase the risk of vessel cavitation and loss of water transport ability, which would pose negative effects on photosynthesis and tree growth [22,23]. These potentially divergent impacts of N deposition on tree growth pose great challenges for understanding the mechanism underlying tree adaptation to future global change (e.g., especially through regulation of vessel-related hydraulic traits). The ongoing intensification of N deposition requires a further understanding of how tree growth responds to increasing N availability.

The aim of the present study was therefore to investigate how trees adjust their xylem traits in response to the ongoing increasing N deposition. Since April 2013, we have conducted an experiment in a warm-temperate forest in Central China involving the application of 2.5 times the current natural N deposition of inorganic N either to the canopy or to the understory at the study sites. An oak species, *Q. variabilis*, was selected, as it is one of the most widespread temperate deciduous tree species in eastern Asia and also an important species for afforestation [24]. *Q. variabilis* is a ring-porous species that is characterized by the formation of different-sized vessels along the tree-ring to satisfy the required water transport in the process of tree growth during growing season [25]. Generally, a small number of large-sized vessels are usually formed at the beginning of tree-ring growth, followed by abundant, but smaller vessels in the later tree-ring growth [26].

We analyzed tree-ring widths and vessel-related traits along tree rings in trees exposed to canopy and understory N addition treatments from 2013 to 2018. Based on previous studies conducted at the same experimental site, the understory N addition caused a more serious soil acidification and nutrient imbalance than that observed with canopy N addition [27,28]. *Q. variabilis* is a non-leguminous species and lacks the ability to perform N_2 fixation; therefore, this oak was expected to be sensitive to N deposition [29]. Therefore, we hypothesized that tree-ring width and vessel-related traits would be stimulated by canopy and understory N addition, but that understory N addition might be less effective.

2. Materials and Methods

2.1. Study Site

This study was conducted in a natural mixed deciduous forest in the Jigongshan National Nature Reserve (31°51′ N, 114°05′ E) of Henan Province, Central China. This area is located in the transitional zone between subtropical and warm-temperate climates, where *Q. variabilis* is one of the dominant woody species in this region [21]. This forest is 49 years old and grows in a soil classified as yellow-brown loam [21,28]. Based on 60-year climate data (http://data.cma.cn/site/index.html accessed on 1 January 2015), the mean annual temperature of this area is 15.2 °C and the total mean annual precipitation is 1119 mm, with 80% of the precipitation falling between April and October. Rain provides a natural source of N deposition, estimated at an average of 19.6 kg N ha^{-1} $year^{-1}$ [21]. During the detected years (2013–2018), mean annual temperature was above 16 °C, with variation less than 1 °C (Figure 1). Mean annual precipitation was 1108 mm, and the precipitation differed a lot between the years. The smallest amount of precipitation was 952 mm in 2013, the largest amount of precipitation was 1326 mm in 2017 (Figure 1).

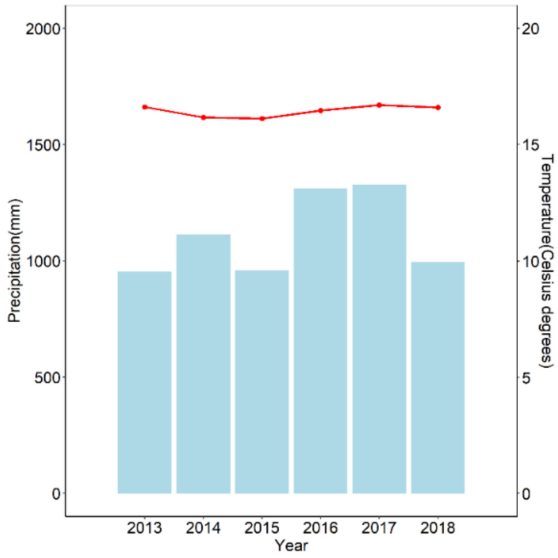

Figure 1. Annual mean temperature and total precipitation of the study site during the detected year (2013–2018).

2.2. Experimental Design and Tree Selection

In this experiment, four blocks were established in the study area. In each block, three circled plots were randomly assigned and subjected to different treatments: (1) control (C): nothing was applied; (2) canopy nitrogen addition treatment (CN): nitrogen applied to the tree canopy at the amount of 50 kg N ha^{-1} $year^{-1}$; and (3) understory nitrogen addition treatment (UN): nitrogen applied to the understory at the amount of 50 kg N ha^{-1} $year^{-1}$ (Supplementary Figure S1). Each plot was 907 m^2 in area. More than 20 m of buffer zone and polyvinylchloride (PVC) boards were installed at the edge of each plot to avoid contamination by the N solution and interactive influences between the plots.

The N fertilizer in this experiment was ammonium nitrate, as the local naturally deposited N is mainly NH_4^+ and NO_3^- at a ratio close to 1. The treatments consisted of sprinkling the ammonium nitrate solution as homogeneously as possible from the top of the tree canopy (CN) or from 1.5 m above the ground (UN) (for more details, please see reference [21]). Since 2013, the equivalent of 3 mm of rain containing the N solution was applied every year to the treated plots once a month during the growing season (from April to October). Overall, the treatment provided approximately 21 mm of additional precipitation per year, representing <1% of the total annual precipitation at the site. Any

confounding effect of water addition by the N addition treatments is therefore considered marginal in this experiment.

In each plot, three healthy mature and similar sized *Q. variabilis* with an upright stem were selected for sampling (Table S1). Overall, the analysis involved twelve CN-treated trees (CN-trees), twelve UN-treated trees (UN-trees) and twelve control trees (C-trees).

2.3. Sampling and Section Preparation

2.3.1. Sampling

In May 2019, wood microcores containing at least six recent tree rings were collected from the southern exposure of each stem at breast height using a Trephor [30]. These microcores were immediately put into Eppendorf microtubes containing 50% aqueous ethanol solution and stored at 4 °C as soon as possible.

2.3.2. Section Preparation

The microcores were successively immersed in ethanol, D-limonene solutions and liquid paraffin, and then embedded in paraffin [31]. Transverse sections of 8–12 µm thickness were cut from the paraffin-fixed sample blocks using a rotary microtome (Leica RM2235). The sections were stained with cresyl violet acetate (0.05% in water) and observed under visible light at magnifications of 40× Images of the sections were captured using LAS software and a camera fixed on the microscope (V4.6, Leica Microsystems, Wetzlar, Germany). Tree-ring width and vessel diameters along the long and short axes (Supplementary Figure S2) were measured for each year (2013–2018) using the LAS software.

2.3.3. Calculation of Vessel-Related Traits

In this study, the shape of the vessel was treated as an ellipse. Every vessel area was calculated using the mathematical formula of ellipse area as: $V = \pi\frac{ab}{4}$, where V is the vessel area, a and b are the diameters along long and short axis of vessel, respectively (Supplementary Figure S2).

We also calculated the theoretical xylem hydraulic conductivity (KH; kg m^{-1}s^{-1}MPa^{-1}) of the observed xylem area in year t based on the Hagen–Poiseuille (HP) law [32]:

$$KH_t = \left(\pi\rho/128\eta\ A_{image}\right)\sum_1^n d_i^4 \quad (1)$$

where π is the ratio of the circumference of a circle to its diameter (π = 3.14), ρ is the density of water at 20 °C (998.2 kg m^{-3}), η is the viscosity of water at 20 °C (1.002 × 10^{-9} MPa), A_{image} is the analyzed xylem area in year t and d_i is the diameter of each of the vessels measured in year t (d_i is calculated as the mean diameter of the long and short axis of the ith vessel in year t). According to the HP law, the KH value could represent the hydraulic conductivity efficiency of the analyzed xylem area, where a larger KH value indicates a stronger hydraulic conductivity per unit xylem area [32].

Based on GAM-modeled curves of vessel size along the tree-rings, vessel size in all treatments sharply decreased from the start of the tree-ring, i.e., the 0th% of the tree-ring, to 30th% of the tree-ring, followed by a slow decrease from 30th% to 100th% along the tree-ring (Figure 2). Meanwhile, in order to have a comparable understanding of the first 3/10 and last 3/10 vessels along tree-ring, the vessels between 70th% and 100th% along tree-ring was also selected for our analysis. Therefore, the vessels in each tree ring were divided into three groups, which were group I (i.e., 0th%–30th% along tree-ring), group II (i.e., 30th%–70th% along tree-ring) and group III (i.e., 70th%–100th% along tree-ring).

Moreover, annual mean vessel area (V_{mean_t}), annual percentage of total vessel area (Per_V), annual KH, mean vessel area of each group, percentage of the total vessel area of each group and KH of each group were also calculated as follows:

$$V_{mean_t} = \frac{\sum_1^n V_i}{n} \quad (2)$$

$$Per_V = \frac{\sum_1^n V_i}{A_{image}} \quad (3)$$

where V_{mean} is mean vessel area, V_i is the vessel area of the ith vessel in year t, n is the number of vessels in year t, Per_V represents the percentage of total vessel area and A_{image} is the analyzed xylem area in year t. The V_{mean} and Per_V functions could also be applied to the calculation of the mean vessel area and percentage of total vessel area in each group, in which case the n is the number of vessels of each group in year t.

$$KH_{t_g} = \left(\pi\rho/128\eta\ A_{image}\right)\sum_1^m d_i^4 \quad (4)$$

$$Per_KH = \frac{KH_{t_g}}{KH_t} \quad (5)$$

The percentage of the KH of each group (Per_KH) in each year was also calculated, where KH_{t_g} is the theoretical xylem hydraulic conductivity of each group in year t and m is the number of vessels in each group.

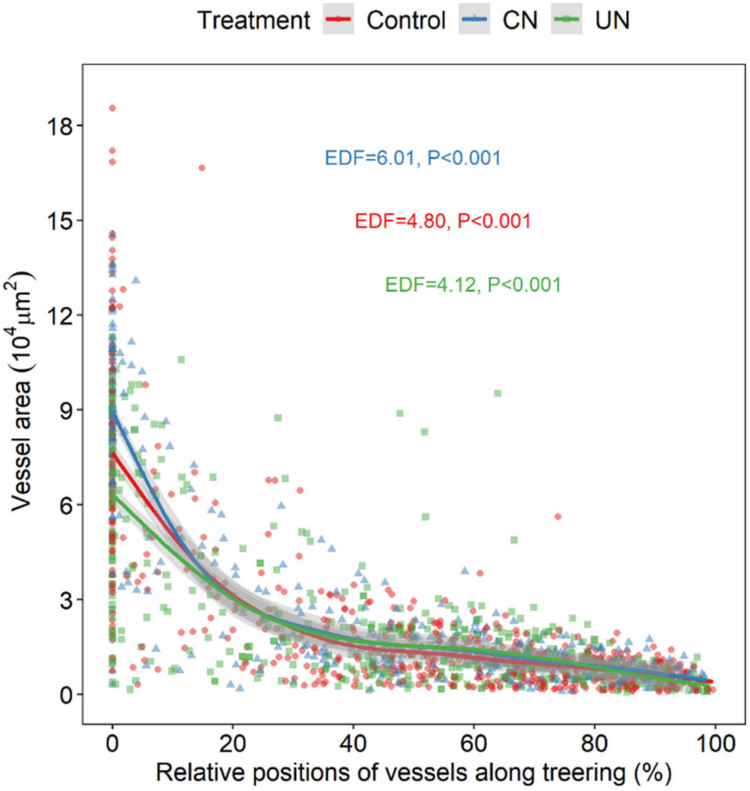

Figure 2. Relative position of vessels in *Quercus variabilis* Bl. along tree-ring in control (Control), canopy N addition (CN) and understory N addition (UN) treatments in 2013–2018. Note: Relative positions of vessels along tree-ring (values of x-axis) = distance of vessel to the start position of tree-ring/tree-ring width of the current year. EDF, the effective degrees of freedom (edf) estimated from generalized additive models.

2.3.4. Data Analysis

During data analysis, autocorrelation of error should be considered in repeated measurement data analysis [33]; therefore, a linear mixed effects model and analysis of variance (ANOVA) from package "nlme" in R were used to analyze the effects of N treatments, year and their interaction on tree-ring growth and vessel-related traits. The linear mixed model was expressed as follows:

$$W_t = \alpha_0 + \alpha_1 T + \alpha_2 Y + \alpha_3 TY + \delta + \varepsilon \quad (6)$$

where W_t is the independent factor (i.e., radial growth and vessel area), T and Y are the fixed effects (i.e., treatment and year, respectively), TY denotes the interactive effect of the two fixed effects, α_0 is the intercept, α_1, α_2 and α_3 are the corresponding fitted parameters, δ is the random intercept across trees and ε is the error in the repeated measurement over time within trees. If the effect had a significant influence on the independent variables, multiple comparisons were used to test the specific significant different groups. All statistical analyses and figure preparation were performed using R 3.6.3.

3. Results

3.1. Distribution of Different-Sized Vessels along Tree-Ring

Vessels of *Q. variabilis* at our experimental site varied from 687 μm^2 to 19 × 10^4 μm^2 along tree-ring. Based on GAM-modeled curves of vessel size along tree-ring, vessel size in all treatments sharply decreased from the start of tree-ring (i.e., the 0th% of the tree-ring) to the 30th% of the tree-ring, followed by a slow decrease until the end of tree-ring (Figure 2). Vessels located before 15th% along tree-ring were generally larger in CN-trees than C-trees, which was larger than UN-trees (Figure 2). However, for the rest of the vessels along tree-ring, there was no clear difference among treatments. These results suggested that N addition treatments probably mainly influenced large vessels formed at the beginning of the growing season and that CN stimulated and UN decreased large vessels.

3.2. Vessel-Related Traits in the Different Groups and Treatments during 2013–2018

For vessels of group I, mean vessel area was larger in CN-trees (51,614–91,841 μm^2) than that in C-trees (53,806–70,638 μm^2), while vessels in C-trees was generally larger than that in UN-trees (40,906–60,022 μm^2) (Figure 3a). On average, mean KH was larger in CN-trees than that in C-trees and UN-trees almost in all years (except 2014), with the mean KH values of 612 kg m^{-1}s^{-1}MPa, 371 kg m^{-1}s^{-1}MPa and 471 kg m^{-1}s^{-1}MPa, respectively, which was in agreement with the patterns of mean vessel area along tree-ring among the treatments (Figure 3d). Average percentage of vessel area in C-trees, CN-trees and UN-trees was 11.6%, 17.2% and 17.0%, respectively (Figure 3a). KH of this group accounted for 89.1%, 93.0% and 85.0% of the total annual KH in C-trees, CN-trees and UN-trees, respectively, indicating a general increase in CN-trees but a slight decrease in UN-trees (Figure 3b).

For vessels of group II, mean vessel areas varied between 7876 μm^2 and 15,069 μm^2 throughout the years, without any obvious difference among treatments (Figure 3b). Mean KH was generally larger in UN-trees (63 kg m^{-1}s^{-1}MPa) than that in C-trees (32 kg m^{-1}s^{-1}MPa) and CN-trees (31 kg m^{-1}s^{-1}MPa). Average percentage of vessel area was generally higher in UN-trees than that in C-trees and CN-trees, with the average values of 7.1%, 4.2% and 4.1%, respectively (Figure 4a). KH of this group accounted for 9.5%, 6.8% and 14.6% of the total annual KH in C-trees, CN-trees and UN-trees, respectively, showing a decrease in CN-trees and an increase in UN-trees in most years (Figure 4b).

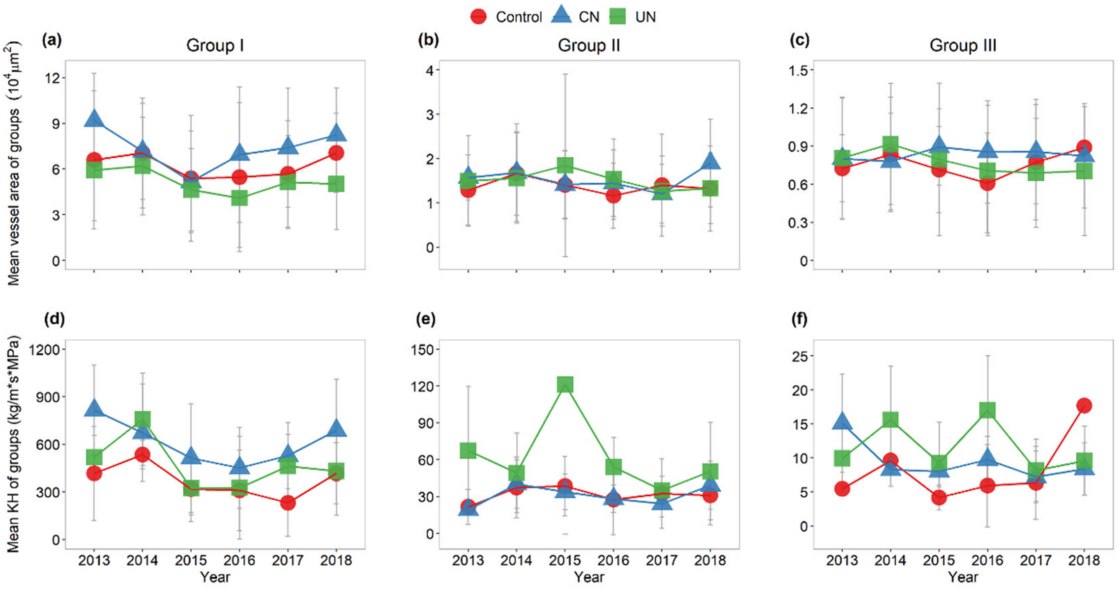

Figure 3. Mean vessel area and mean KH of groups I (**a**,**d**), II (**b**,**e**) and III (**c**,**f**) of *Quercus variabilis* under Control, canopy N addition (CN) and understory N addition (UN) treatments during 2013–2018. Error bars indicate standard deviation.

In group III, mean vessel area and mean KH varied around 7876 μm^2 and 9.9 kg m^{-1}s^{-1}MPa, respectively (Figure 3c,f). As expected, compared to groups I and II, group III had the smallest percentage of vessel area in all treatments, with averaged values of 2.4% (Figure 4a).

Average KH of this group took only 2.0%–3.1% of the total annual KH in all treatments (Figure 4b). None of the vessel-related traits of group III showed any obvious different patterns among treatments. Overall, consistent with the percentage of vessel area, although the total xylem area of group II and III took two-thirds of the tree-ring, the KH was much smaller than that in group I.

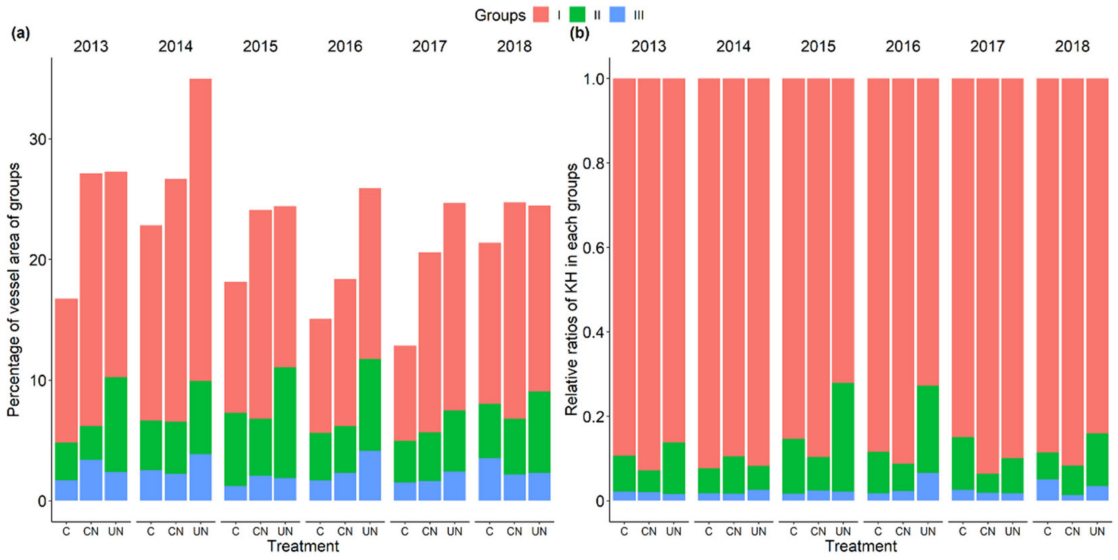

Figure 4. Percentage of vessel area (**a**) and KH (**b**) of group I, II and III of *Q. variabilis* under control (C), canopy N addition (CN) and understory N addition (UN) treatments conditions during 2013–2018. Error bars indicate standard deviation.

3.3. Tree-Ring and Annual Vessel-Related Traits in Treatments

Generally, tree-ring width was smaller in CN-trees and UN-trees than that in C-trees, with averages of 1456.4 μm, 1072.2 μm and 2049.0 μm, respectively (Figure 5). Mean annual vessel area was less varied in all the treatments, at around 2391–24,900 μm^2 during 2013–2018 (Figure 5). For most of the detected years (except 2014), annual KH was higher in CN-trees (597.9 kg $m^{-1}s^{-1}MPa^{-1}$) and UN-trees (519.5 kg $m^{-1}s^{-1}MPa^{-1}$) than that in C-trees (385.8 kg $m^{-1}s^{-1}MPa^{-1}$). Generally, percentage of annual total vessel area was higher in UN-trees than that in CN-trees, which was slightly higher than that in C-trees, with average percentage values of 25.2%, 21.6% and 16.8%, respectively (Figure 5).

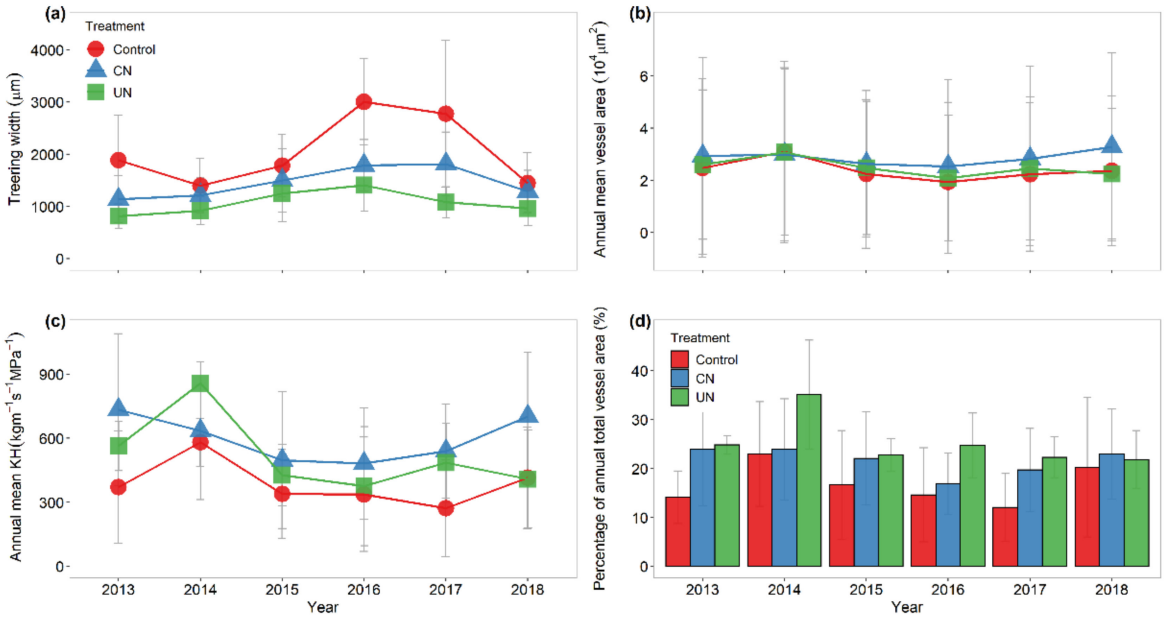

Figure 5. Tree-ring width (**a**), annual mean vessel area (**b**), annual mean KH (**c**) and percentage of annual total vessel area (**d**) of Quercus variabilis under control, canopy N addition (CN) and understory N addition (UN) treatments during 2013–2018. Error bars indicate standard deviation.

3.4. Effects of N Addition on Vessel-Related Traits and Tree-Ring Width

No significant effect of treatment, year or their interaction was found for any of the vessel-related traits of groups I and II, suggesting that neither CN nor UN had any significant effect on mean vessel area, percentage of total vessel area, KH or the percentage of KH of group I and II (Table 1). These vessel-related traits in group III showed no significant effect for treatment and year, but their interaction (Treatment × Year) was significant (Table 1), suggesting that, on average, effect of N addition treatments on these vessel-related traits was not significant, however, it varied with years.

Effect of treatment on tree-ring width was significant, suggesting that N addition impacted tree-ring width of *Q. variabilis* (Table 2). Multiple comparison (Tukey test) result showed that effect of UN was significantly different from that of control ($p < 0.05$, Table S2), while effect of CN was not significantly different from control. This result indicated that UN significantly decreased the tree-ring width, but not CN. For annual vessel-related traits (i.e., annual mean vessel area, annual KH and percentage of annual total vessel area), treatment, year and their interaction (Treatment × Year) had no significant effect (Table 2), suggesting that these vessel-related traits were not significantly different between all treatments and years.

Table 1. ANOVA results showing effects of nitrogen (N) addition treatments, year and their interactive effects on different groups of vessel-related traits in *Quercus variabilis* Blume during 2013–2018 in Central China.

Vessel Groups	Vessel-Related Traits	Source of Variation	*F*-Value	*p*-Value
Group I	Mean vessel area	Treatment	1.94	0.17
		Year	0.22	0.64
		Treatment × Year	0.95	0.39
	Percentage of total vessel area	Treatment	3.24	0.06
		Year	2.17	0.14
		Treatment × Year	0.40	0.67
	Grouped KH	Treatment	1.62	0.23
		Year	2.23	0.14
		Treatment × Year	0.44	0.65
	Percentage of grouped KH	Treatment	0.66	0.53
		Year	0.38	0.54
		Treatment × Year	1.30	0.28
Group II	Mean vessel area	Treatment	0.35	0.71
		Year	0.15	0.70
		Treatment × Year	0.65	0.53
	Percentage of total vessel area	Treatment	0.47	0.63
		Year	0.23	0.63
		Treatment × Year	1.40	0.25
	Grouped KH	Treatment	1.21	0.32
		Year	0.88	0.35
		Treatment × Year	1.26	0.29
	Percentage of grouped KH	Treatment	0.77	0.48
		Year	0.02	0.90
		Treatment × Year	0.28	0.76
Group III	Mean vessel area	Treatment	0.14	0.87
		Year	1.02	0.32
		Treatment × Year	4.25	0.02 *
	Percentage of total vessel area	Treatment	0.02	0.98
		Year	0.03	0.87
		Treatment × Year	4.37	0.02 *
	Grouped KH	Treatment	0.40	0.68
		Year	1.10	0.30
		Treatment × Year	4.03	0.02 *
	Percentage of grouped KH	Treatment	0.92	0.42
		Year	4.92	0.03 *
		Treatment × Year	4.25	0.02 *

"*" indicates $p < 0.05$.

Table 2. ANOVA results showing effects of nitrogen (N) addition treatments, year and their interaction effects on xylem-related traits in *Quercus variabilis* Blume during 2013–2018 in Central China.

Xylem Related Traits	Source of Variation	*F*-Value	*p*-Value
Tree-ring width	Treatment	4.27	0.03 *
	Year	3.18	0.08
	Treatment × Year	0.26	0.77
Annual mean vessel area	Treatment	1.27	0.31
	Year	0.01	0.91
	Treatment × Year	0.86	0.43

Table 2. *Cont.*

Xylem Related Traits	Source of Variation	*F*-Value	*p*-Value
Annual KH	Treatment	1.78	0.20
	Year	2.59	0.11
	Treatment × Year	0.90	0.41
Percentage of annual total vessel area	Treatment	2.01	0.16
	Year	1.26	0.26
	Treatment × Year	2.23	0.11

"*" indicates $p < 0.05$.

4. Discussion

Increasing N deposition has affected the availability of active N for tree growth. Tree-ring growth and vessel-related traits are key indicators of tree growth response to environmental changes. Therefore, understanding effect of increased N deposition on these traits is critical for improving predictions of tree growth response to future global change. In this study, we analyzed effect of canopy (CN) and understory (UN) N addition treatments on tree-ring width and vessel-related traits of *Q. variabilis* over six years. According to the distribution of vessels along tree-ring, these vessels along tree-ring were divided into three groups (i.e., group I, II and III). Results showed a negative impact of N addition on tree-ring growth, but only the effect of UN was significant. Meanwhile, no significant effect was detected for either CN or UN on any of the vessel-related traits (mean vessel area, theoretical xylem hydraulic conductivity (KH), percentage of total vessel area and relative ratio of KH of each group). However, our results did indicate some diverging influencing trends between CN and UN on some vessel-related traits, which are specifically discussed in the following discussions. These results suggest that future N deposition might have a negative impact on tree-ring growth and have different effects on different-sized vessels along tree-ring during growing season.

4.1. Vessel Distributions along Tree-Ring in Different Treatments

Different-sized vessels along tree-ring had the same distribution pattern between control, CN and UN treatment. Vessels formed at the beginning of tree-ring were larger and their sizes then rapidly decreased between 0th and 30th% of tree-ring, followed by a slow decrease in the rest of tree-ring (Figure 2). This kind of vessel distribution was similar with vessel distribution of other oak species, such as *Q. canariensis* Willd. and *Q. robur* L. [26,34].

In this study, CN and UN differed in their influence on large vessels located in the first 0%–15% along tree-ring, as these vessels were large in CN-trees and smaller in UN-trees, indicating these vessels might be increased by CN but decreased by UN treatment (Figure 2). One possible reason for this diverging effect might be the different amount of carbon (e.g., nonstructural carbohydrate, NSC) stored from the previous year. It was reported that these large vessels at the start of tree-ring are formed before initiation of photosynthesis of the current year and mainly use the NSC stored from the previous year [35,36]. Moreover, previous work at the same experimental site has shown that CN increased leaf N content and fine root biomass [16], which might improve photosynthesis and, consequently, the amount of carbon stored in previous year. By contrast, trees in UN treatment showed a decrease in fine root biomass and soil pH [27,28], which might inhibit carbon production for the initiation of the large vessels in the next year. These findings indicated that effect of atmospheric N deposition on these large vessels formed at the beginning of tree-ring might be underestimated in studies of using understory N addition method. N processes in the canopy of trees during natural N deposition should be taken into account in the future.

4.2. Effect of N Addition Treatments on Different-Sized Vessels along Tree-Ring

Statistically, no significant effects were observed between CN and UN treatments on vessel-related traits (mean vessel area, percentage of total vessel area, grouped KH

and percentage of KH) in group I (Table 1). However, mean vessel area of group I was generally smaller in UN treatment than in control, while the percentage of vessel area was slightly higher in UN treatment (Figures 3 and 4). This might represent an adaptation to maintain a balance between maximizing water conductivity efficiency and minimizing risk of embolism. The large vessels formed at the beginning of the growing season play a vital role in water transport in ring-porous tree species [32,37]. These large vessels could ensure efficient water transport, but they also increase the risk of embolism and cavitation [38–40]. Therefore, to maximize tree growth and reduce the risk of vessel cavitation, trees need to reduce the hydraulic diameter (i.e., vessel size) and increase vessel density to adapt to stressful conditions [7,26,41]. In our study, UN treatment, which directly applied soluble N to the soil, could reduce soil water potential and create conditions that might be unfavorable for water absorption from soil, thereby inducing a drought-like condition at the roots [28]. To ensure water transport efficiency while reducing the risk of embolism, trees need to make adjustments, such as reduce vessel size and increase vessel density [42], which might also explain the slightly higher KH observed in group II in the UN-trees (Figure 3, Supplementary Figure S3).

The xylem of group I in CN-trees showed a slight increase in all the detected vessel-related traits compared to C-trees (Figures 3 and 4). One reason might be more carbon storage from the previous year, induced by possible enhancement of photosynthesis by CN treatment [27]. Previous work at the same experimental site showed that CN treatment increased leaf N content and leaf area, suggesting an enhanced leaf photosynthesis rate [27], which in turn would increase carbon storage in the previous year. The stored carbon from the previous year could be served as the primary energy and carbon source for vessel formation and tree-ring growth at the beginning of the growing season before leaf emergence [43]. For this reason, more carbon and energy might be available for the growth of group I vessels in CN-trees, leading to a slight increase in all the vessel-related traits of group I.

In group II, vessel-related traits showed no obvious difference between CN and control, indicating that CN treatment barely impacted the small and abundant vessels formed during the middle of growing season. The reasons might be complicated, because during the time when these vessels were formed, new leaves were formed, growing and maturing, and thus could generate carbon for tree growth, including formation of vessels, growth of new buds and leaves and elongation of new shoots and trunk, etc. In this case, carbon allocation strategy would be changed according to the external environment to maintain balance between tree growth and risk. Therefore, how these vessels respond to N deposition during their formation still requires further exploration in the future studies.

For vessels in group III, N treatments had no significant effect on any of the vessel-related traits, whereas the interaction of treatment and year was significant for all the detected vessel traits. This result indicates that, on average, N treatment did not affect vessel-related traits, while the effect varied in different years probably due to the different climatic and environmental condition between different years, especially the different precipitation conditions among years showed in Figure 1. These small vessels formed at the end of the growing season have been less studied due to their small size and low sensitivity to environmental changes compared to the large vessels formed at the beginning of tree-ring growth [44,45]. As reported in some other oak tree studies, these small and abundant vessels could still be functional in several adjacent older tree-rings to maintain xylem hydraulic conductivity [46,47], while the large vessels formed at the beginning of growing season would irreversibly cavitate and lose their ability of hydraulic conductivity in the year after formation [48,49]. The lower variation in the small vessels in group III under N addition treatments in our results, to some extent, confirmed the resistance of small vessels to the changing environment or stressful conditions. However, based on the results in this study, these small vessels of group III formed at the end of tree-ring were possibly influenced by a co-effect of N addition and other environmental factors in different years (Table 1). Thus, our results suggested that the small-sized vessels formed at the end

of the growing season could also be a meaningful indicator for environmental changes, especially for the multi-influencing environmental factors, and should be furtherly studied to provide more climatic information for future studies.

4.3. Effect of N Addition Treatments on Annual Tree-Ring and Annual Vessel-Related Traits

4.3.1. Annual Tree-Ring Growth

Tree-ring width was decreased in both CN and UN treatments, while only the effect of UN was statistically significant (Table 2 and Table S2). This was consistent with previous studies showing that N addition had a negative impact on radial growth [50,51]. The negative effect found in these studies might reflect influenced of environmental stresses (e.g., soil acidification, nutrient imbalances) caused by N addition on tree growth. For instance, at our study site, soil pH was significantly decreased after CN and UN treatments, with lower pH value showed in UN treatment [27], and this could partially explain the smaller tree-rings seen in UN-trees than that in CN-trees. In addition, a previous study at the same experimental site found that N addition treatment decreased base ion concentration and increased aluminum accumulation in soil [28] (Shi et al., 2016), and these changes might negatively affect plant cell division and elongation [52–54], and consequently interfere with tree-ring growth. Moreover, changes in carbon allocation strategy during tree growth could also explain our results. At the same experimental site, researchers found that both CN and UN treatment increased fine root turnover and promoted the release of carbon and nutrients into soil [27]. These changes might result in a reduction in carbon allocation to tree-ring formation. Furthermore, increase in foliar N content was also greater in CN-trees than that in UN-trees [16], and this might enhance leaf photosynthesis [55,56], thereby promoting a greater allocation of photosynthates to tree-ring growth in CN-trees [57]. All these above changes might result in less tree-ring growth under UN than CN treatment.

4.3.2. Annual Vessel-Related Traits

Annual mean vessel is another morphological anatomical indicator reflecting environmental changes, especially water availability [7,25,44,58]. Although some studies have reported that hydraulic conductivity of xylem could be changed by N addition [16,59], this might not be reflected in the annual mean vessel area in our study, as no obvious or significant responses were seen for either CN or UN treatment. Annual mean vessel area did not show any obvious variation between control and N treatments; however, compare to control, annual KH and percentage of annual total vessel area of CN-trees and UN-trees had a tendency to increase during 2013–2018, although the increase was not statistically significant (Figures 3 and 4, Table 2). Previous studies have shown that N addition could increase plant water conductivity by increasing vessel area or the number of vessels [16,41]. Therefore, we speculated that percentage of total vessel area and KH might be increased by N addition due to increase in the number of vessels rather than increase in vessel size (Figure 4, Supplementary Figure S3).

Overall, no significant effect was observed for either CN or UN for any of the vessel-related traits examined in this study. One explanation might be that formation of the hydraulic architecture of trees is complicated and is related to many different external and internal factors (e.g., the water content in soil, air temperature, precipitation, plant hormone level and dynamics, genetic regulation, etc.) [60–64]. Thus, the most dominant influencing factor for vessel traits might not be the extra added N. Another possible reason is that the amount or the duration of N addition might not be sufficient to reach the threshold or tipping point for a significant change in the vessel traits of mature trees [65–67] (Mo et al., 2008; Pregitzer et al., 2008; Zhang et al., 2017), considering that mature trees are less sensitive than younger trees and non-woody plants to nitrogen uptake [68,69] (Day et al., 2001; Vieira et al., 2009).

5. Conclusions

Given the important role of tree-ring and vessels in tree growth, carbon fixation and water cycle, understanding their responses to increasing N deposition is of great relevance in the context of climate change. In this study, we detected the effect of two different N addition treatments (i.e., CN and UN treatments) on tree-ring growth and vessel-related traits in mature *Q. variabilis* during the years 2013–2018 in a warm-temperate forest of a transitional zone in Central China. Contrary to our hypothesis, tree-ring growth was negatively affected by both N addition treatments, but only the effect of UN treatment was statistically significant. No significant response was seen over the six-year-long N addition treatment (either CN or UN) in vessel-related traits, indicating that six years of N addition was still unable to significantly change the hydraulic conductance unit and features of *Q. variabilis*. Our hypotheses were therefore rejected.

However, we have to admit that, although the effects of N addition treatments on vessel-related traits were not statistically significant, N addition to canopy and understory did have a tendency to pose diverging influences on some of the vessel-related traits. For instance, the size of large vessels developing at the beginning of tree-ring formation increased with canopy N addition and decreased with understory N addition treatment. Similarly, although the mean vessel area of small vessels developed during the middle to end of tree-ring formation did not respond to N addition treatments, their KH increased, probably due to an increased vessel density. Therefore, it could be presumed that the ongoing increasing N deposition might negatively or positively influence tree-ring growth and some vessel-related traits, thereby influencing the performance of trees in warm-temperate transitional zones and consequently changing forest structure and carbon dynamics if this influence continues.

Based on our study, we therefore concluded that the traditional N simulation approach of applying N to the understory of trees might provide inappropriate evaluations of the effect of natural N deposition on tree growth, especially with respect to wood formation and xylem hydraulic architecture. This study would help to accurately evaluate the impacts of N deposition on forest productivity and carbon-water dynamics at the transitional zone from warm temperate to subtropical zones under the climate change scenario.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13071075/s1, Figure S1: Experimental design of this study. Note: canopy N addition treatment with the amount of 50 kg N $ha^{-1}yr^{-1}$ (CN), understory N addition treatment (UN) with the amount of 50 kg N $ha^{-1}yr^{-1}$; Figure S2: A cross-sectional sample for vessel measurements (under magnification of 40×). a and b represent the diameters along long and short axis of vessel, respectively. Figure S3: Vessel density of each group (i.e., group I, II, and III) in *Q. variabilis* under Control, canopy N addition (CN) and understory N addition (UN) treatment during 2013–2018. Note: density of vessels (number/mm^2) = number of vessels of groups/the detected xylem area of each year in each tree. Group I is the vessels located between the start, i.e., 0th%, to 30th% of the tree-ring, group II is the 30th%–70th% of the tree-ring and group III is the 70th%–100th% of the tree-ring; Table S1: Tree Height and diameter at breast height (DBH) of the selected *Q. variabilis* for measurements of radial growth and vessel related indexes; Table S2: Multiple Tukey-test comparison ANOVA results showing the different effects on xylem related traits of *Q. variabilis* between Control, CN and UN treatments during 2013–2018 in Central China. "*" indicates $p < 0.05$; Table S3: Supplementary for Table 1 and Table S4: Supplementary for Table 2 were also added to this section to show the degree of freedom from ANOVA results.

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