Rising air humidity during spring does not trigger leaf-out timing in temperate woody plants

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Introduction

The timing of spring leaf emergence in temperate regions directly influences global biogeochemical cycles and species interactions (Richardson et al. 2013). Understanding the environmental drivers of leaf-out is thus essential to forecasting ecosystem responses to global climate change. These drivers have long been thought to be species-specific combinations of spring temperature, winter chilling, and increasing day length during spring (Heide 1993a,b; Laube et al. 2014a; Polgar et al. 2014; Zohner and Renner 2015; Zohner et al. 2016, 2017). Temperature-related increases in air humidity were suggested as an alternative trigger of the spring bud break recently, based on experimental results from nine Eurasian and American tree species, including two needle-leaved trees (Laube et al. 2014b). The new hypothesis was cast into doubt, however, by experiments on 15 further species, again from both Eurasia and America, with one, Cornus mas, being used in both studies (Zipf and Primack 2017). These contradictory conclusions raise basic questions about (i) the type of experiments in use for studying environmental leaf-out triggers and (ii) the extent of species differences in leaf-out triggers and hence the influence of the identity of species studied on the results.

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In their initial experiment, Laube et al. (2014b) found that high humidity (90% relative air humidity compared to 40% control) advances leaf-out by, on average, 7 days. The experimental set-up relied on twig cuttings (c. 25 cm length) obtained on 3 March 2012 from a forest near Freising (a city near Munich, Germany), cleaned and placed in glass bottles filled with tap water. For each species, 20 twigs (from three individuals) were kept in climate chambers for 30 d, half in each humidity treatment. Temperatures were gradually increased, but daylength was kept constant at 16 h. As a physiological explanation for their results, the authors proposed that plant tissue dehydrates during the chilling period in winter and rehydrates in spring by absorbing water directly through the buds. They concluded that absolute air humidity, not temperature, triggers leaf unfolding in spring. In a follow-up study, Zipf and Primack (2017) again used twig cuttings in glass vials placed in climate chambers, but a gradient of humidity (20 – 94% relative air humidity) and constant temperature (23°C) and light conditions (12 h per day). Leaf-out in three of the 15 species studied was significantly affected by higher humidity: *Carya tomentosa* leafed out significantly earlier, while *Berberis thunbergii* and *Rhamnus frangula*, leafed out significantly later. The remaining 12 species, including *Cornus mas* in which Laube et al. (2014) had found a significant effect of higher humidity, were unaffected. Because both studies relied on the twig-cutting experimental set-up in which the integrity of the hormone and water transport is compromised, Zipf and Primack (2017, p. 2216) concluded that “more work must be done on this topic before researchers can properly interpret the results of Laube et al. (2014b) and determine the effect of humidity on the leafing out process for woody species.”

Outdoor experiments in which twig cuttings were placed in water-filled vials next to the trees from which they had been cut have shown that leaf-out times between donor trees and cut twigs do not differ (Vitasse and Basler 2014), supporting the utility of the twig-cutting experimental approach for studying temperature and day-length effects on leaf unfolding. For addressing potential effects of soil and air moisture on plant phenology, however, twig cuttings may be problematic because the water supply to buds differs from that experienced by plants rooted in soil. Due to more negative water potential in the xylem, twig cuttings exhibit reduced water uptake (Ikeda and Suzaki 1986). Only once roots are developed, does the water potential of dormant cuttings increase (Puri and Thompson 2003). Water uptake in rootless cuttings further depends on the water’s gas and nutrient composition (Erstad and Gislerød 1994), while intact roots allow plants to adjust their water uptake depending on the shoot’s demands. Whether twig cuttings receive sufficient water might thus depend on their ability to take up water from the surrounding air. As far as we are aware, only two studies have used rooted saplings (*Picea glauca*) to study how air humidity affects leaf-out, and they obtained opposite results: Marsden et al. (1996) found delayed bud flush under high air humidity, whereas Roberts and Zwiazek (2001) found advanced bud flush. In a twig-cutting experiment, air humidity had no effect on leaf-out in *Picea abies* (Laube et al., 2014b). Further research is therefore needed to study the role of air humidity *per se* as a regulator of spring leaf-out in temperate trees.

We here report on experiments addressing the effect of air humidity on leaf-out in potted saplings of broad-leaved species, including one also studied by Laube et al. (2014b). The first experiment compared leaf-out timing between a control and a high-humidity treatment under natural spring light and temperature fluctuations. The second experiment compared saplings kept under fully-controlled environmental conditions under 20%, 50%, and 80% air humidity. These experiments allow us to detect effects of air humidity on leaf-out timing without possible artefacts introduced by the modified water uptake in twig cuttings.
Materials and Methods

Experiment 1

Our first experiment tested the effect of elevated air humidity under natural spring light and temperature fluctuations in greenhouse chambers. It was conducted in the Munich Botanical Garden (48°09’ N, 11°30’ E; 501 m a.s.l.) between 1 March and May 2018 on two-year old saplings of 10 temperate tree species (Table 1). From Autumn until the start of the experiment, plants were kept outdoors under natural conditions. Between 12 to 28 individuals per species were exposed to two treatments in climate-controlled glasshouse chambers with an openable top, and the effects on phenology then followed until the end of May. In the humid chamber, relative air humidity was elevated to 96.0 ± 0.6% (mean ± SE) using a humidifier placed at plant height (Luftbefeuchter CEZIO, C. Unger, Scheidt, Germany), resulting in an average absolute air humidity of 11.5 ± 0.5 g/m³ (Figure 1a). Absolute air humidity was calculated as a function of temperature and relative air humidity, using the Clausius-Clapeyron equation implemented in the humidity R-package. In the control chamber, plants were watered if needed, but air humidity was otherwise not modified, resulting in an average relative air humidity of 70.0 ± 1.4% and an average absolute air humidity of 8.3 ± 0.3 g/m³. All other factors (temperature, light intensity, day-length) were identical between chambers, exposing plants to natural fluctuations in temperature and sunlight. Temperatures were ~3°C warmer relative to outdoor temperatures and followed the natural outdoor fluctuations (mean temperature humid chamber = 13.4 ± 0.6°C; mean temperature control chamber = 13.5 ± 0.8°C control). We observed bud phenology twice a week from 15 March until 22 May 2018. Individual leaf-out was defined as the date when the first leaf had fully unfolded. We used climate loggers (HOBO Pro v2, Onset Computer Corporation, Bourne, MA, USA) to monitor temperature and humidity in both chambers.

Experiment 2

Our second experiment tested the effects of air humidity on spring leaf-out under fully-controlled environmental conditions in climate chambers. This experiment was conducted in Zurich (47°37’ N, 8°55’ E; 452 m a.s.l.) between 22 March and May 2019 on two-year old saplings of Carpinus betulus, Fagus sylvatica, and Quercus robur. From December 2018 until the start of the experiment, plants were constantly kept at 4°C, 50% relative air humidity, and 8 h days. Between 18 to 32 individuals per species were exposed to three treatments. Relative air humidity in the chambers was set to 20%, 50%, and 80% (Table 1). Actual air humidities were within ±10% of the prescribed values (see Fig. 1b). All plants were regularly watered to ensure that soil moisture did not affect leaf-out timing. In all chambers, temperature was set to 20°C during the day and 8°C during the night. Actual temperatures were within ±0.8°C of the prescribed values. Daily mean temperatures in the 20%, 50%, and 80% chambers were 14.4 ± 0.9°C, 14.3 ± 0.9°C, and 14.4 ± 0.9°C, respectively (see Fig. 1b). Daily mean absolute air humidities were 2.6 ± 0.2 g/m³, 6.4 ± 0.3 g/m³, and 10.4 ± 0.5 g/m³, respectively. Day length was set to 12 h at the start of the experiment and extended in weekly intervals following the natural day length progression. We observed bud phenology every three days. Individual leaf-out was defined as the date when at least three unfolded leaves, pushed out all the way to the petiole, were visible on the respective individual.

Statistical analysis

For both experiments, linear mixed effects models including species as random effect using the nlme R-package were performed to test for differences in leaf-out dates between the treatments across all species. Additionally, we performed multivariate ANOVAs including an interaction term between species and treatment to test whether the effect of air humidity on
leaf-out differs among species. To test for significant differences in leaf-out dates between the two treatments within each species in experiment 1, a Kruskal-Wallis H Test was performed. To test for differences in leaf-out dates between the three treatments in experiment 2, the KruskalMc post-hoc test (multiple comparison after Kruskal–Wallis) was used (Siegel & Castellan 1988). All statistical analyses were performed with the R software (R 3.4.1, R Core Team 2019).

Results and Discussion
Our results are not in agreement with the study of Laube et al. (2014b) who inferred that, instead of temperature fluctuations, seasonal fluctuations in absolute air humidity are the main regulator of spring phenology in temperate woody plants. In our first experiment, increased air humidity led to slightly (by 1.5 days) delayed leaf-out when including all 10 species in a linear mixed effects model \((p = 0.02, N = 213)\). There was a significant interaction between treatment effect and species (ANCOVA: \(p < 0.01\)), i.e., the effect of air humidity on leaf-out dates differed across species. Separate analysis for each species revealed significantly earlier leaf-out under elevated air humidity in one of the ten study species: *Aesculus hippocastanum* advanced leaf unfolding in the humid chamber by, on average, 3 days (Table 1; Figure 2). High air humidity significantly delayed leaf unfolding in *Acer campestre*, *Sorbus torminalis*, and *Carpinus betulus* by, on average, 6, 5, and 3 days, respectively. In the remaining six species, no significant differences were detected (Figure 2, Table 1). In our second experiment, no significant difference in leaf-out dates across treatments could be detected across the three species used in the experiment (linear mixed effects model: \(p = 0.62, N = 69\)). Separate analysis for each species also showed no significant differences among treatments (Figure 3).

The effects of air humidity detected for *Acer campestre*, *Aesculus hippocastanum*, *Carpinus betulus*, and *Sorbus torminalis* in experiment 1 show that air humidity can affect leaf-out to some degree, but the effect is of inconsistent direction (Figure 2). The delays in leaf-out under high humidity found in two species by Zipf and Primack (2017 using cut twigs) and in three species in our study (using rooted saplings) may be the result of a stress reaction. Although physiological responses to elevated air humidity before leaf unfolding have never been tested, there is evidence that high humidity affects plant behaviour once the leaves are out. For instance, thermoregulation in plants exposed to high air humidity is impaired because of lower transpiration rates (van de Sanden and Veen 1992; Fordham et al. 2001), leading to reduced photosynthesis (Mortensen 1986; Rezaei Nejad and van Meeteren 2008). Water-stress hormone concentrations, such as Abscisic Acid 8’-Hydroxylase (ABA), are lower when exposed to high humidity, leading to changes in stomatal behaviour (Rezaei Nejad and van Meeteren 2008; Okamoto 2009). As a result, leaves kept under high humidity fail to close their stomates when needed (Fordham et al. 2001), resulting in shoot and leaf elongation (Mortensen 1986, Roberts and Zwiazek 2001) and possibly a longer time to full leaf unfolding of the newly developed shoots. Slightly delayed leaf-out under elevated air humidity might thus not result from physiologically delayed dormancy release, but instead be due to a slowed leaf development during the initial stages of bud opening.

Conclusion
Here, we tested the effects of air humidity on leaf-out in potted saplings, not twig cuttings, to keep the integrity of the hydraulic and hormonal systems. We exposed saplings of 10 woody species to elevated or ambient air humidity in glasshouse chambers under natural light, and subsequently exposed three of the species to 20%, 50%, and 80% air humidity in light- (and temperature) controlled chambers. Our results, in agreement with those of Zipf and Primack (2017), using a different experimental set-up, refute the hypothesis that air humidity is a main
trigger of spring leaf unfolding in temperate woody plants. By using rooted plants instead of cuttings in water-filled vials to study the effects of air humidity on leaf-out phenology, we rule out artefacts associated with water supply and hormonal responses in plants disconnected from their root system. As such, our results uphold the overriding role of temperature (chilling and forcing) and day length in triggering spring leaf-out, increasing our confidence in projections of future ecosystem responses based on the existing phenological models.

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Author contributions
The study was conceived and developed by CMZ. The experiments were performed by CMZ, AFTS, and FB. Analyses were performed by CMZ and AFTS. The manuscript was written by CMZ with assistance from SSR. YV and FB reviewed and provided input on the manuscript.

References


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Table 1: Species list and sample sizes (number of individuals).

<table>
<thead>
<tr>
<th>Species</th>
<th>Experiment 1</th>
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<th>Experiment 2</th>
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<tr>
<td></td>
<td>Total</td>
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<td>50%</td>
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</table>

Figure 1. Temperature and humidity profiles of the treatments applied in experiments 1 (a) and 2 (b). a, Mean daily relative humidity (upper panel), absolute humidity (middle panel), and temperature (lower panel) from March 14 until May 1 for the control and elevated humidity treatment in experiment 1. b, Daily temperature and humidity profiles for the 20%, 50%, and 80% humidity treatments of experiment 2.

Figure 2. Results of experiment 1 in which saplings of 10 woody species were exposed to ambient and elevated air humidity. Boxplots show the quartiles and medians of the days to leaf-out (from 1 Jan) for each species and treatment. Points show individual leaf-out dates. Significance levels from Kruskal-Wallis H test: ns, P ≥0.05; *, P <0.05; **, P <0.01; ***, P <0.001.

Figure 3. Results of experiment 2 in which saplings of three woody species were exposed to 20%, 50%, and 80% air humidity. Boxplots show the quartiles and medians of the days to leaf-out (from 1 Jan) for each species and treatment. Points show individual leaf-out dates. No significant differences were found across treatments (Kruskal-Wallis H test: P ≥0.05).