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Original Article

Tree-ring stable isotopes record the impact of a foliar fungal pathogen on CO_2 assimilation and growth in Douglas-fir

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ABSTRACT

Swiss needle cast (SNC) is a fungal disease of Douglas-fir (*Pseudotsuga menziesii*) that has recently become prevalent in coastal areas of the Pacific Northwest. We used growth measurements and stable isotopes of carbon and oxygen in tree-rings of Douglas-fir and a non-susceptible reference species (western hemlock, *Tsuga heterophylla*) to evaluate their use as proxies for variation in past SNC infection, particularly in relation to potential explanatory climate factors. We sampled trees from an Oregon site where a fungicide trial took place from 1996 to 2000, which enabled the comparison of stable isotope values between trees with and without disease. Carbon stable isotope discrimination ($\Delta^{13}C$) of treated Douglas-fir tree-rings was greater than that of untreated Douglas-fir tree-rings during the fungicide treatment period. Both annual growth and tree-ring $\Delta^{13}C$ increased with treatment such that treated Douglas-fir had values similar to co-occurring western hemlock during the treatment period. There was no difference in the tree-ring oxygen stable isotope ratio between treated and untreated Douglas-fir. Tree-ring $\Delta^{13}C$ of diseased Douglas-fir was negatively correlated with relative humidity during the two previous summers, consistent with increased leaf colonization by SNC under high humidity conditions that leads to greater disease severity in following years.

Key-words: *Phaeocryptopus gaeumannii*; *Pseudotsuga menziesii*; stable isotopes; Swiss needle cast; tree-rings.

INTRODUCTION

Swiss needle cast (SNC) disease of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] is caused by stomatal blockages by the fruiting bodies (pseudothecia) of the fungus *Phaeocryptopus gaeumannii* (T. Rohde) Petr., which results in premature needle abscission (Manter *et al*. 2000). SNC was present in the Pacific Northwest before it was considered an economically important disease. The first record of the fungus and SNC appeared in the mid-1920s after a severe SNC epidemic of U.S.-imported Douglas-fir in Switzerland (Boyce 1940). It was not until the late 1980s that the fungus began causing serious needle loss and growth reductions to Douglas-fir in the United States. Attempts to understand the potential influence of climate on the recent rise in disease severity have suggested that warm winter and spring temperatures and spring/summer leaf wetness may facilitate fungal growth and reproduction (Rosso & Hansen 2003; Manter *et al*. 2005; Coop & Stone 2007; Latta *et al*. 2009; Black *et al*. 2010; Zhao *et al*. 2011).

Previous research using Douglas-fir tree-ring widths attempted to identify historic SNC epidemics and the cause of the sudden prevalence of the disease (Black *et al*. 2010). Although Black *et al*. (2010) provided one potential avenue for understanding the history and onset of SNC, tree-ring widths can be sensitive to numerous environmental variables that could confound a SNC signal or yield false positives for infection when there was no disease (e.g. climate signals, or disturbances associated with defoliating insect outbreaks, fires, windstorms, etc.; McCarroll & Loader 2004; Barnard *et al*. 2012). To more accurately quantify past SNC symptoms in tree-rings, a method is needed that can distinguish physiological responses to SNC from environmental noise. With this information, it would not only be possible to improve our knowledge of the epidemiology of SNC, but also to define clearer relationships between SNC symptoms and the climate factors that exacerbate them. As explained later, stable tree-ring isotope signatures should provide such information.

SNC restricts gas exchange of Douglas-fir needles. In spring, spores land and germinate on current-year foliage, and hyphae begin colonizing the needle surface and interior (Hansen *et al*. 2000). In the most severe cases, the fungus produces pseudothecia that occlude stomata and reduce stomatal conductance (g_s) by about 83% and carbon dioxide assimilation rate (A) by about 72% by the following late spring or early summer (Manter *et al*. 2000). The ratio of intercellular $[CO_2]$ (c_i) to that in the surrounding atmosphere (c_i/c_a) is modified by g_s and A, such that a decrease in g_s will

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cause c_i to decrease, and a decrease in A will cause c_i to increase, but not enough to compensate for the impact of the decline in g_s (Farquhar *et al.* 1989). Biochemical discrimination against the heavier stable carbon isotope ^{13}C ($\Delta^{13}C$) during CO_2 assimilation is directly related to c_i/c_a, and thus, $\Delta^{13}C$ is also influenced by g_s and A (Farquhar *et al.* 1982; Evans *et al.* 1986). The physical blockage of stomata in trees with SNC causes a reduction in g_s and a weaker decline in A, such that c_i/c_a should decrease, causing the $\Delta^{13}C$ of fixed carbohydrates to decrease. In addition, reduced g_s in trees with SNC could potentially cause changes in tree-ring oxygen isotope fractionation ($\Delta^{18}O$) (Barbour 2007). According to the original model of Farquhar & Lloyd (1993), a reduction in g_s and therefore, transpiration (E) would result in decreased advection of unenriched xylem water to the sites of carboxylation. The resulting increase in the relative impact of back diffusion of ^{18}O-enriched water from the sites of evaporation to the sites of carboxylation would cause a potential increase in ^{18}O fixed into carbohydrates. Alternatively, sufficiently low levels of E, comparable with those reported for Douglas-fir (e.g. Cermak *et al.* 2007), have been reported to cause the effective path length (L) for water movement through the leaf to increase exponentially with decreasing g_s and E (Song *et al.* 2013). Taken together, because changes in g_s and A likely affect the $\Delta^{13}C$ and $\Delta^{18}O$ of carbohydrates fixed by diseased trees, tree-rings could record the signal imparted by the effects of SNC on g_s and A in a given year. If SNC indeed has an effect on tree-ring $\Delta^{13}C$ and/or $\Delta^{18}O$, then we need to understand how $\Delta^{13}C$ and $\Delta^{18}O$ change in response to disease symptom severity to be able to reconstruct past disease severity using these stable isotopes in tree-rings, and to better understand the response of the disease to meteorological variables.

The objectives of the present study were to determine: (1) whether analysis of tree-ring $\Delta^{13}C$ and $\Delta^{18}O$ can serve as a diagnostic tool for the detection of past SNC infection in Douglas-fir and (2) which climate factors may modify the strength of the signal. We sampled trees at the site of a fungicide trial where there are known years of severe SNC and years of low SNC. We hypothesized that $\Delta^{13}C$ would be higher in tree-rings during years of low SNC than during years of severe SNC. In contrast, we hypothesized that tree-ring $\Delta^{18}O$ during years of low SNC would be lower than that of years of severe SNC, based on the model of Farquhar & Lloyd (1993). For both carbon and oxygen analyses, we used co-occurring western hemlock [*Tsuga heterophylla* (Raf.) Sarg.] as a non-host reference species. Previous research suggests that severity of SNC infection increases under warmer winters and wetter springs and summers [e.g. greater fog, precipitation, relative humidity (RH)], thus we expected to find lower $\Delta^{13}C$ and higher $\Delta^{18}O$ following winters with above average temperatures or wet conditions during spring and summer of the previous year.

MATERIALS AND METHODS

Carbon stable isotope conceptual approach

We investigated $\Delta^{13}C$ patterns of plant cellulose ($\Delta^{13}C_{cell}$) as influenced by SNC because this signal should integrate, at the canopy level, the physical and biochemical processes known to be affected by SNC at the leaf level. Because the carbon isotope ratio of the atmospheric CO_2 ($\delta^{13}C_{atm}$) changes over time and influences the carbon isotope ratio of plant cellulose ($\delta^{13}C_{cell}$), this effect can then be accounted for by calculating $\Delta^{13}C_{cell}$ (‰), following Farquhar *et al.* (1982):

$$\Delta^{13}C_{cell} = \frac{\delta^{13}C_{atm} - \delta^{13}C_{cell}}{1 + \delta^{13}C_{cell}}, \quad (1)$$

where annual values of past $\delta^{13}C_{atm}$ have been estimated by McCarroll & Loader (2004; data from 1989 to 2003) and for more recent years from monitoring at Mauna Loa, Hawaii (http://cdiac.ornl.gov/; data from 2004 to 2011). C_3 photosynthesis causes $\Delta^{13}C_{cell}$ to be related directly to c_i/c_a, as described by Farquhar *et al.* (1989):

$$\Delta^{13}C_{cell} = a + (b - a)\left(\frac{c_i}{c_a}\right), \quad (2)$$

where a is fractionation resulting from diffusion of CO_2 from the ambient air through the stomata to the intercellular spaces (4.4‰), and b is fractionation associated with carboxylation by rubisco (~27‰). Equation 2 is an approximation that does not account for other variables that influence $\Delta^{13}C$ such as differences between c_i and $[CO_2]$ at the sites of carboxylation (c_c) related to variation in mesophyll conductance. Nevertheless, Eqn 2 has proven to be adequate for relating variation in plant tissue $\Delta^{13}C$ to variation in c_i/c_a driven by changes in A/g_s in a broad range of species (Cernusak *et al.* 2013).

SNC infection starts in newly expanded leaves, but in the most severe cases, the fungus does not affect gas exchange until the pseudothecia emerge during spring in the year following infection (Manter *et al.* 2000; Stone *et al.* 2008). The physical blockage of stomata causes a decline in A, but a stronger relative reduction in g_s, such that c_i/c_a should decrease, causing the $\Delta^{13}C$ of fixed carbohydrates to decrease (Manter *et al.* 2000). However, there are potential limitations on the utility of tree-ring $\Delta^{13}C_{cell}$ values as integrators of temporal variation in the severity of SNC symptoms. SNC-induced biochemical limitations on assimilation rates (Manter *et al.* 2000) may eventually cause c_i/c_a to increase resulting in higher $\Delta^{13}C_{cell}$ values. Additionally, hyphae in the leaf may continue to increase in mass through the summer following initial infection, causing further decreases in photosynthesis (Manter 2000), which could cause the needles experiencing the most severe symptoms to contribute less to tree-ring $\Delta^{13}C_{cell}$. Another consideration is that in the most extreme cases of infection, nearly all needles on the tree eventually develop a negative carbon balance (i.e. consuming more carbon than they fix) and abscise during the second year of infection (Manter *et al.* 2003), thus reducing the impact of photosynthate from these needles on $\Delta^{13}C_{cell}$. Despite these potential caveats, the effect of the fungus on $\Delta^{13}C_{cell}$ should be strong enough to be detected in the wood due to the overwhelming presence and abundance of pseudothecia on diseased trees. As a precaution, we focused on the central portion of the tree-ring, hereafter referred to

as *middlewood* (*sensu* Roden *et al*. 2011), which contains cellulose synthesized from sugars produced in spring and early summer before abscission of the most severely infected needles.

A final consideration is the effect of the relationship between leaf mesophyll conductance (g_m) and $\Delta^{13}C_{cell}$. Flexas *et al*. (2008) restrict the definition of g_m to the diffusion of CO_2 through leaf mesophyll, including intercellular air spaces, the cell wall, and the intracellular liquid pathway. Several studies have addressed how changes in g_m due to a variety of influences, such as water and nutrient stress, can lower c_c without a concurrent change in g_s (Flexas *et al*. 2008, 2012; Seibt *et al*. 2008). Reduced c_c results in less discrimination against ^{13}C and a lower $\Delta^{13}C_{cell}$, assuming there are no concurrent changes in the biochemical capacity of photosynthesis to offset the changes due to a reduction in g_m. Capitano (1999) demonstrated that there is significant hyphal growth inside the needles of Douglas-fir with SNC, which could potentially cause g_m to decrease, and thus $\Delta^{13}C_{cell}$ to decrease. The potential impact of the fungus on g_m should amplify the $\Delta^{13}C_{cell}$ signal in tree-rings by reducing c_c, although previous research with Douglas-fir has suggested that $\Delta^{13}C_{cell}$ may remain rather constant under changing g_m (Warren *et al*. 2003). In either case, changes in g_m should not negatively affect the ability to use the analysis of $\Delta^{13}C_{cell}$ as a diagnostic tool for the detection of past SNC infection in Douglas-fir tree-rings, but it does limit the extent to which changes in $\Delta^{13}C_{cell}$ could be directly attributed to changes in g_s.

Oxygen stable isotope conceptual approach

We used stable isotope values of ^{18}O from the tree-ring cellulose of Douglas-fir as another potential indicator of variation in SNC symptom severity. The carbohydrates that become tree-ring cellulose carry a signal imparted by the oxygen stable isotope ratio ($\delta^{18}O$) of leaf water, which is primarily influenced by the isotopic composition of source water entering the leaf from the xylem and ^{18}O-enrichment at the sites of evaporation within the leaf (Craig & Gordon 1965; Dongmann *et al*. 1974). According to the Craig–Gordon model (Craig & Gordon 1965), the steady-state isotopic enrichment of oxygen over plant source water at the sites of evaporation in the leaf ($\Delta^{18}O_{es}$) is:

$$\Delta^{18}O_{es} = \varepsilon^{+} + \varepsilon_k + (\Delta^{18}O_v - \varepsilon_k)\left(\frac{e_a}{e_i}\right), \quad (3)$$

where ε^{+} is the equilibrium fractionation factor between liquid water and gaseous water (i.e. vapour); ε_k is the kinetic fractionation factor as vapour diffuses from leaf intercellular spaces to the atmosphere; $\Delta^{18}O_v$ is the isotopic difference between vapour and plant source water; and e_a/e_i is the ratio of atmospheric to intercellular vapour pressure.

Evaporative enrichment is complicated by several factors, such as the mass flow of unenriched water from the xylem that opposes the diffusion of enriched water from the sites of evaporation back towards the xylem (i.e. the Péclet effect; Farquhar & Lloyd 1993), which results in spatial heterogeneity in the isotopic composition of leaf water from the xylem to the site of evaporation (Wang & Yakir 1995). Evaporative enrichment is also affected by non–steady-state conditions (Farquhar & Cernusak 2005). Based on previous research that has shown little difference between steady- and non–steady-state models of leaf water enrichment in conifers (Lai *et al*. 2006; Seibt *et al*. 2006; Barnard *et al*. 2007; Snyder *et al*. 2010), we used the steady-state model for isotopic enrichment of mean lamina mesophyll water ($\Delta^{18}O_L$) proposed by Farquhar & Lloyd (1993) for this study. $\Delta^{18}O_L$ is estimated using the equation (Farquhar & Lloyd 1993):

$$\Delta^{18}O_L = \frac{\Delta^{18}O_{es}(1 - e^{-\wp})}{\wp}, \quad (4)$$

where $\wp$ is the Péclet number, a dimensionless ratio of the advection of unenriched source water to the evaporative sites to the back diffusion of enriched water from evaporative sites, which is calculated as follows:

$$\wp = \frac{EL}{CD}, \quad (5)$$

where E is the leaf transpiration rate (mol m^{-2} s^{-1}), L is the effective path length (m) for water movement from the veins to the site of evaporation, C is the density of water (mol m^{-3}), and D is the diffusivity (m^2 s^{-1}) of $H_2{}^{18}O$ in water. The parameter L is a product of the distance of the pathway from the xylem to the evaporative surface and a scaling factor that adjusts for the tortuosity of the path (Farquhar & Lloyd 1993).

The integration of $\Delta^{18}O_L$ into plant cellulose ($\Delta^{18}O_{cell}$) is described by the following equation (Barbour & Farquhar 2000):

$$\Delta^{18}O_{cell} = \Delta^{18}O_L(1 - p_{ex}p_x) + \varepsilon_o, \quad (6)$$

where p_{ex} is the proportion of oxygen atoms that exchange with xylem water during cellulose construction at the meristem, p_x is the proportion of unenriched xylem water at the site of cellulose construction (value of 1 when the cellulose was collected from mature, suberized stems), and ε_o is a fractionation factor of +27‰ to adjust for water/carbonyl exchange (Sternberg 1989; Yakir & Deniro 1990).

In the case of SNC, reduced g_s and E in diseased Douglas-fir trees could cause increases in $\Delta^{18}O_{cell}$ due to a reduction in the Péclet number provided the relative impact of the fungus on stomatal blockage and suppression of E is greater than its potential to increase L through internal proliferation of hyphae (Farquhar & Lloyd 1993). For this study, we utilize $\delta^{18}O_{cell}$, without calculating the difference from source water ($\Delta^{18}O_{cell} = \delta^{18}O_{cell} - \delta^{18}O_{sw}$) because we do not have information on the year-to-year variation in source water isotopes. However, because all sampled trees were the same age and located on the same site with the same primary source water, we assumed that differences in $\delta^{18}O_{cell}$ between trees and treatments in any year reflected differences in $\Delta^{18}O_L$ rather than $\delta^{18}O_{sw}$.

Treatment group	Age (years)	DBH (cm)	Height (m)	Crown width (m)
Treated Douglas-fir	29 (2)	31.7 (1.2)	22.2 (1.0)	6.7 (0.7)
Untreated Douglas-fir	28 (1)	28.0 (4.2)	19.7 (1.4)	5.0 (1.0)
Western hemlock	23 (4)	37.0 (9.3)	21.4 (2.5)	5.3 (1.5)

Table 1. Characteristics of each treatment group ($n = 6$ trees per treatment group), including age at breast height, diameter at breast height (DBH), height, and crown width (diameter) with one standard deviation from the mean in parentheses

Field site and sampling

We sampled trees in two Douglas-fir stands and one western hemlock stand at a coastal site near Beaver, Oregon (45.3°N, 123.8°W) where Douglas-fir trees exhibit chronic, severe SNC symptoms (Stone *et al.* 2007). From 1996 to 2000, the Oregon Department of Forestry applied Bravo® fungicide (chlorothalonil, Syngenta Crop Protection, Greensboro, NC, USA) twice annually to the trees in one stand (treated Douglas-fir), which dramatically reduced SNC disease severity, while the trees in the other stand remained unsprayed (untreated Douglas-fir; Johnson *et al.* 2003; Stone *et al.* 2007). This experimental site thus provided a unique opportunity to quantify the impact of variation in SNC disease severity on tree-ring $\Delta^{13}C_{cell}$ and $\delta^{18}O_{cell}$ through comparative analyses of stable isotope chronologies in treated and untreated Douglas-fir prior to, during, and after fungicide application. We determined the site severity index (site average of approximately 1.5 to 2 years of needle retention) using the standard SNC sampling method (e.g. Maguire *et al.* 2002). In January 2012, we collected four increment cores (12 mm diameter) from each of six trees of similar age, diameter at breast height, height and crown width in both the fungicide-treated stand and the untreated stand (Table 1). We also collected four cores from each of six trees in a nearby western hemlock stand to use as a baseline of climate impacts on stable isotope chronologies in a co-occurring species that does not become infected by SNC.

Additionally, we collected twigs and foliage from the terminal branches of six Douglas-fir trees at another coastal site with severe SNC near Tillamook, Oregon, called Prairie Hill (45.5°N, 123.8°W) to characterize the $\Delta^{13}C$ signal closer to the source of carbohydrate synthesis in the crown. Sunlit upper canopy branches were not readily accessible at the site where fungicide had been applied. The branches were separated into segments representing growth increments from the years 2012, 2011, 2010 and 2009 (cohort years), and then separated into twigs and foliage. Bark and phloem were removed from the twigs to isolate the twig wood for isotopic analysis.

Annual growth analysis

We measured the ring widths of increment cores with a stereo microscope interfaced with a Velmex measuring table and Measure J2X® measuring software (http://www.voortech.com/projectj2x/). The cross-dating program COFECHA (Holmes 1983; Grissino-Mayer 2001) was used to detect either missing or false rings. The ring widths for the four cores from each tree were averaged for each year and basal area increment (BAI, the transverse area of a given growth ring, cm^2) was calculated assuming ring circularity to characterize annual tree growth from 1989 to 2011.

Stable isotope analyses

The middlewood was sliced from each tree-ring from 1989 to 2011 with a scalpel and ground into a fine powder using a ball-mill. Middlewood was defined as the portion of the ring that included the latter 75% of earlywood and the initial 25% of attached latewood from the same year. The ring material from all four cores was pooled for each tree to minimize the effect of differences in isotope signatures around the circumference of the tree. The α-cellulose of each tree-ring was isolated following Leavitt & Danzer (1993). The tree-ring $\delta^{13}C_{cell}$ and $\delta^{18}O_{cell}$ were measured by stable isotope ratio mass spectrometry (IRMS). All $\delta^{13}C_{cell}$ measurements were carried out at the Oregon State University (OSU) in the College of Earth, Ocean and Atmospheric Sciences (CEOAS) Stable Isotope Laboratory using an elemental analyser (NA 1500, Carlo Erba, Milan, Italy) connected to an IRMS (DeltaPlus, Thermo Finnigan, Bremen, Germany). All $\delta^{18}O_{cell}$ measurements were carried out at the University of California, Davis, Stable Isotope Facility in the Department of Plant Sciences using a pyrolysis system (PyroCube, Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to an IRMS (PDZ Europa 20-20, Sercon Ltd., Cheshire, UK). Measurement precision (1 SD) was better than 0.06‰ for $\delta^{13}C_{cell}$ and 0.24‰ for $\delta^{18}O_{cell}$ of tree-rings as determined from sample replicates.

Twig and foliage tissues were oven-dried and ground to a fine powder, packaged as whole tissue into tin capsules, and analysed for $\delta^{13}C$ using an IRMS at the OSU CEOAS Stable Isotopes Laboratory. Measurement precision was better than 0.10‰ for $\delta^{13}C$ for twig and foliage tissues as determined from sample replicates. Current-year needles were not expected to show significant fungus-induced alteration in their gas exchange, and thus, current-year wood attached to current-year foliage should not show a strong isotopic signal from the fungus unless a substantial fraction of current-year wood was derived from carbohydrates produced by older foliage. Because the effect of the fungus on needle physiology increases over time, we sought to detect the $\delta^{13}C$ signal in the newer wood on older shoot extension increments that was built with carbohydrate produced by older needles, and therefore was expected to contain a stronger carbon isotope signal associated with fungal disruption of gas exchange. Since older tissues of twig wood are composed of different layers of wood made in different years, twig wood $\delta^{13}C$ values were adjusted with a calculation that estimated the $\delta^{13}C$ of wood produced in the most recent year for each growth increment cohort year. This calculation assumes that the amount of wood from each year was the same for growth increments older than year 0, such that the calculated value of $\delta^{13}C$ for growth increment x ($\delta^{13}C_{calcx}$) based on the

observed value for growth increment x ($\delta^{13}C_{obsx}$) where x equals the number of years beyond the sampling year (i.e. 0, 1, 2, 3 years) was:

$$\delta^{13}C_{calc1} = (\delta^{13}C_{obs1} \times 2) - \delta^{13}C_{obs0}, \quad (7)$$

$$\delta^{13}C_{calc2} = (\delta^{13}C_{obs2} \times 3) - \delta^{13}C_{obs0} - \delta^{13}C_{calc1}, \quad (8)$$

$$\delta^{13}C_{calc3} = (\delta^{13}C_{obs3} \times 4) - \delta^{13}C_{obs0} - \delta^{13}C_{calc1} - \delta^{13}C_{calc2}. \quad (9)$$

The preceding equations do not take into account the potential contribution of non-structural carbohydrates formed in prior years to the current-year twig wood increment. Incorporation of photosynthate produced by younger, less heavily infected needles would tend to dampen the expected twig age-related trend in the $\delta^{13}C$ signal. Values of $\delta^{13}C_{calc}$ were transformed to $\Delta^{13}C$ using the procedure described above for tree-ring $\delta^{13}C_{cell}$.

Climate data

Estimates of climate data, including monthly and annual average precipitation, temperature, and dew point, were obtained from the PRISM Climate Group at the OSU (http://prism.oregonstate.edu). Average monthly and annual RH values were calculated from temperature and dew point temperature values. Fog occurrence data were acquired from J. Johnstone, using the methods employed by Johnstone & Dawson (2010). The average fog frequencies of the Southwest Oregon Regional Airport in North Bend, Oregon, and of the Astoria Regional Airport in Warrenton, Oregon, were calculated and averaged to estimate the monthly and annual average fog frequency for the Oregon coast. Growing season averages included data from May through July, and summer averages included data from June through September.

Statistical analyses

Three separate repeated measures analysis of variance tests with an AR1 covariance structure were used to determine the difference in mean BAI, as well as mean tree-ring $\Delta^{13}C_{cell}$ and $\delta^{18}O_{cell}$, between treated and untreated Douglas-fir, and western hemlock, at the Beaver site for each year from 1989 to 2011. A linear model using generalized least squares with an AR1 covariance structure was used to compare the relationship between mean tree-ring $\Delta^{13}C$ and mean BAI for the treated Douglas-fir trees. Another linear model using generalized least squares with an AR1 covariance structure was used to describe and compare mean $\Delta^{13}C$ of twig wood, and foliage, from the Prairie Hill site with increasing tissue age.

To detect the impact of climate on disease severity, we analysed climate influences (mean monthly temperature, precipitation, RH and fog occurrence) on the two tree-ring stable isotope series of each species (mean tree-ring $\Delta^{13}C_{cell}$ and $\delta^{18}O_{cell}$ of untreated Douglas-fir and western hemlock) using Pearson's correlation coefficients (r). Correlations with a magnitude of at least 0.42 corresponds approximately with the 95% confidence level for a Gaussian white noise process (n = 23 years; 1989–2011), so we considered any correlation exceeding ± 0.42 as significant and an indicator of an important relationship to inform our interpretation of the data.

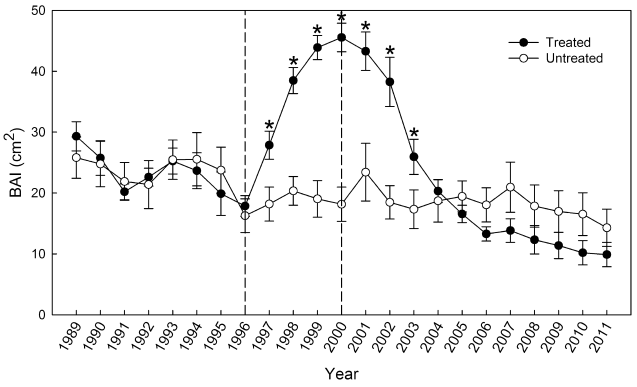

Figure 1. Mean basal area increment (BAI) of fungicide-treated and untreated Douglas-firs at the Beaver, Oregon site during the years of observation (1989–2011; n = 6 trees per treatment group). The treatment period is indicated by vertical dashed lines (1996–2000, inclusive). Bars represent one standard error. Asterisks indicate years of significant difference between treated and untreated Douglas-fir (results of repeated measures analysis of variance with an AR1 covariance structure).

RESULTS

Annual growth

Because of the significant interaction between the effect of treatment and year on mean annual BAI ($F_{22, 230}$ = 4.79, P = 0.0001), the rest of the analyses discussed later will describe a difference in mean annual BAI between treatments for each year of observation separately. The annual growth trajectories of the treated and untreated Douglas-fir were essentially indistinguishable before imposing the fungicide treatment in 1996 (Fig. 1). There was a 1 year lag before BAI differed between treatments after treatment began. Mean annual BAI of the fungicide-treated Douglas-fir was significantly greater than that of untreated Douglas-fir beginning in the second year of treatment (1997) and increased each year until a peak average difference of about 27.4 cm^2 between treatment groups [95% confidence interval (CI): (19.4, 35.5)] in the final year of treatment (2000). Thereafter, growth of the treated trees declined over three years until 2004 when there was no longer a statistically significant difference between treated and untreated trees.

Branch carbon stable isotopes

At the Prairie Hill site where we collected branches from Douglas-fir trees with severe SNC, there was a significant interaction between tissue type (twig wood or foliage) and growth increment age on mean $\Delta^{13}C$ ($F_{1, 41}$ = 12.84, P = 0.001), indicating that the rates of change in $\Delta^{13}C$ per increase in

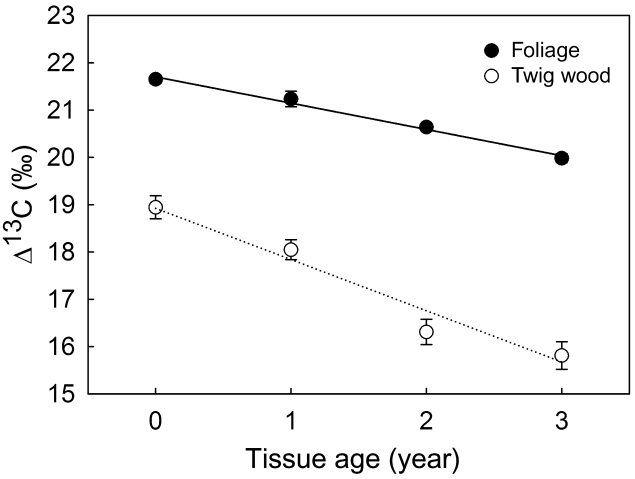

Figure 2. $\Delta^{13}C$ of foliage and twig wood samples from branch cohorts aged 0–3 years old from heavily infected Douglas-fir at the Prairie Hill site ($n = 6$ trees). Lines represent the regression results of a general least squares model with an AR1 covariance structure fit to foliage (solid) and twig wood (dotted; $P = 0.001$). Twig wood values were adjusted as described in *Materials and Methods*. Bars represent 1 SE.

growth increment age for twig wood and foliage were significantly different. The mean $\Delta^{13}C$ of twig wood decreased with tissue age by about 1.08‰ per cohort year [95% CI: (–1.27, –0.89)], and the mean $\Delta^{13}C$ of foliage decreased by about 0.56‰ per cohort year [95% CI: (–0.77, –0.34)] (Fig. 2).

Tree-ring carbon stable isotopes

Because the treatments were applied during 5 years of our 23 year time series, treatment effects (treated Douglas-fir, untreated Douglas-fir, western hemlock) on mean tree-ring $\Delta^{13}C_{cell}$ varied over time causing a significant interaction between time and treatment (1989–2011; $F_{44, 325} = 1.54$, $P = 0.02$). The mean tree-ring $\Delta^{13}C_{cell}$ of treated Douglas-fir became steadily greater after treatment began in 1996, and was significantly greater than untreated Douglas-fir from 1999 to 2002 (Fig. 3). The mean difference in tree-ring $\Delta^{13}C_{cell}$ between treated and untreated Douglas-fir reached a maximum in 2001 [about 1.59‰; 95% CI: (0.66, 2.52)], and thereafter declined until there was no statistically significant difference between treated and untreated Douglas-fir in 2003.

Before the fungicide treatment was applied (1989 to 1995), mean tree-ring $\Delta^{13}C_{cell}$ differed significantly between treated Douglas-fir and western hemlock by about 1.81‰, but from the year that treatment began through 3 years following the end of treatment (1996–2003), there was no statistically significant difference between these two groups (Fig. 3). Following 2003 and to the end of the observation period in 2011, there was a significant difference in mean tree-ring $\Delta^{13}C_{cell}$ between treated Douglas-fir and western hemlock. Mean tree-ring $\Delta^{13}C_{cell}$ values between untreated Douglas-fir and western hemlock were significantly different in every year of the observation period except for two years (1989 and 2000). The mean difference in $\Delta^{13}C_{cell}$ between untreated Douglas-fir and western hemlock was about 1.39‰. The mean difference in $\Delta^{13}C_{cell}$ between treated Douglas-fir and western hemlock was 0.06‰ for the years 1999–2002.

The relationship between mean BAI and mean tree-ring $\Delta^{13}C$ of treated Douglas-fir over the observation period (1989–2011) was such that BAI increased by 9.57 cm^2 for every 1‰ increase in $\Delta^{13}C_{cell}$ [95% CI: (5.36, 13.78), $P = 0.0002$; Fig. 4].

Tree-ring oxygen stable isotopes

There was not a significant interaction between treatment (treated Douglas-fir, untreated Douglas-fir, western hemlock) and year of observation on mean tree-ring $\delta^{18}O_{cell}$ between 1989 and 2011 ($F_{22, 367} = 1.40$, $P = 0.06$; Fig. 5). After accounting for year of treatment in the model, there was no

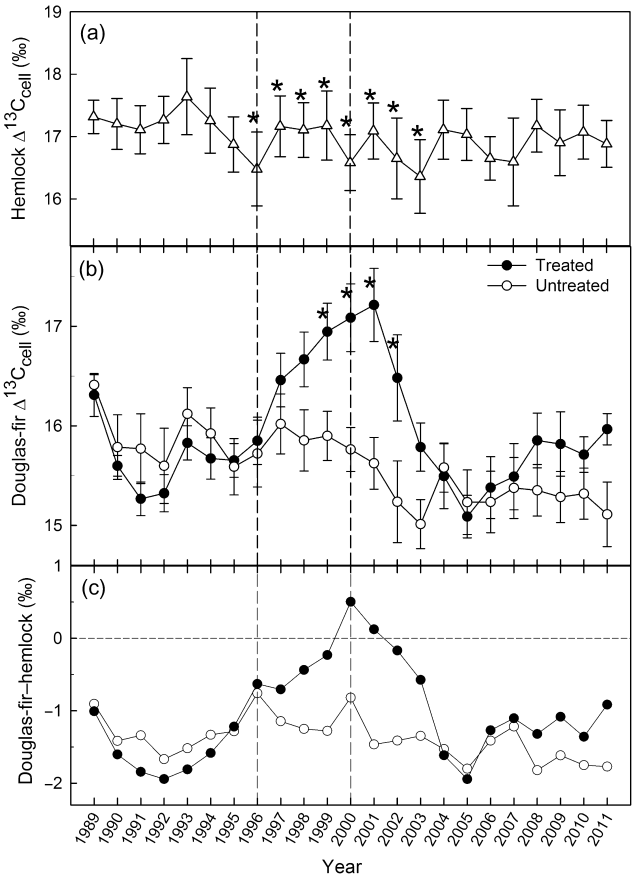

Figure 3. Mean $\Delta^{13}C_{cell}$ of western hemlock (a), fungicide-treated and untreated Douglas-fir (b) and the difference in $\Delta^{13}C_{cell}$ between treated and untreated Douglas-fir and hemlock (c) at the Beaver, Oregon site during the years of observation (1989–2011; $n = 6$ trees per treatment group). The treatment period is indicated by vertical dashed lines (1996–2000, inclusive). Bars represent 1 SE. Asterisks in (a) indicate years of no difference between western hemlock and treated Douglas-fir, and in (b) indicate years of significant difference between treated and untreated Douglas-fir (results of repeated measures analysis of variance with an AR1 covariance structure).

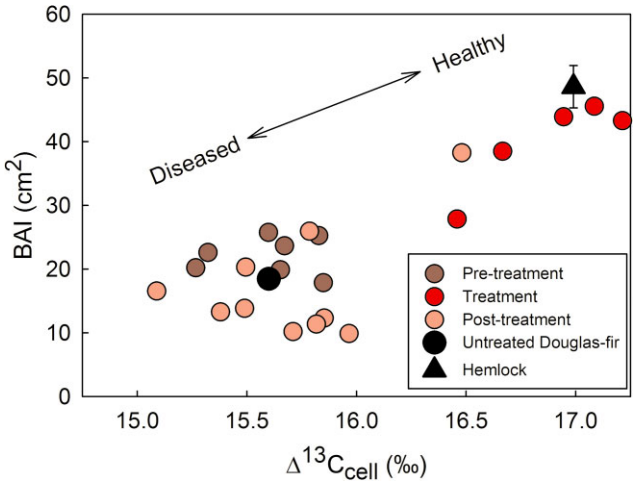

Figure 4. Scatterplot of mean tree-ring $\Delta^{13}C_{cell}$ and mean basal area increment (BAI) of fungicide-treated Douglas-fir post-, during and pre-treatment. Values for untreated Douglas-fir and western hemlock were averaged across all trees over the entire observation period (1989–2011) within each group. Bars represent 1 SE.

significant difference in mean tree-ring $\delta^{18}O_{cell}$ between treated and untreated Douglas-fir (Fig. 5). The mean tree-ring $\delta^{18}O_{cell}$ of western hemlock was about 0.61‰ greater than that of treated Douglas-fir [95% CI: (0.38, 0.84)], and about 0.60‰ greater than that of untreated Douglas-fir [95% CI: (0.37, 0.82); Fig. 5].

Climate Analyses

Because of the lack of difference in mean tree-ring $\delta^{18}O_{cell}$ between treatments during the treatment period, and thus the absence of evidence in support of using tree-ring $\delta^{18}O_{cell}$ as an indicator of variability in SNC disease severity, we only discuss tree-ring $\Delta^{13}C_{cell}$ in our climate analyses. The average previous December temperature was negatively correlated with $\Delta^{13}C_{cell}$ of western hemlock ($r = -0.47$; Fig. 6). Previous July precipitation displayed a positive correlation with $\Delta^{13}C_{cell}$ of untreated Douglas-fir ($r = 0.43$). There were also significant positive relationships between previous December fog occurrence and $\Delta^{13}C_{cell}$ of untreated Douglas-fir ($r = 0.51$). The strongest significant correlations were between RH in the previous June, July, August and September, growing season, summer and summer two years previous, and $\Delta^{13}C_{cell}$ of untreated Douglas-fir ($r = -0.51$, -0.49, -0.54, -0.57, -0.51, -0.62, -0.60, respectively), indicating that $\Delta^{13}C_{cell}$ decreased with increasing humidity of the previous years. There were no significant correlations between RH and $\Delta^{13}C_{cell}$ of western hemlock.

DISCUSSION

Annual BAI of treated Douglas-fir increased soon after the fungicide treatment began and was more than twice that of untreated trees by the fifth year of treatment (Fig. 1). Tree-ring $\Delta^{13}C_{cell}$ of treated Douglas-fir (low SNC) was greater than that of untreated Douglas-fir (severe SNC) from the fourth year of treatment through two years after the treatment ended, reaching a peak difference of about 1.5‰ in 2001 (Fig. 3). The increase in $\Delta^{13}C_{cell}$ during the fungicide treatment and the increase in radial growth of treated trees were consistent with decreased diffusional limitation on needle gas exchange. We also found evidence of a cumulative effect of *P. gaeumannii* on $\Delta^{13}C$ of tissues in the canopy, where older twig wood and foliage had lower $\Delta^{13}C$ than newer tissues (Fig. 2). The relationship between BAI and tree-ring $\Delta^{13}C_{cell}$ (Fig. 4) demonstrated that treated Douglas-fir had values similar to western hemlock during the treatment period (Fig. 3), which suggests that the use of $\Delta^{13}C_{cell}$ in Douglas-fir tree-rings may be a practical technique for tracking SNC disease history, provided a suitable, co-occurring, non-infected reference species like western hemlock is also used to develop a reference $\Delta^{13}C_{cell}$ chronology. When we used the climate response difference between Hemlock and untreated Douglas-fir $\Delta^{13}C_{cell}$ as a proxy for disease symptom severity in Douglas-fir, disease severity was most strongly correlated with high RH in previous summer and growing season months (Fig. 6). In contrast to our tree-ring $\Delta^{13}C_{cell}$ results, there were no distinguishable differences in tree-ring $\delta^{18}O_{cell}$ between treated and untreated Douglas-fir during the years of the fungicide treatment and both are offset by ~0.6‰ from western hemlock (Fig. 5).

Annual growth and carbon stable isotopes

In agreement with previous research at the Beaver site, annual growth of treated Douglas-fir increased in response to the fungicide treatment (Johnson *et al.* 2003). We identified a 1 year lag in the annual growth response after the first

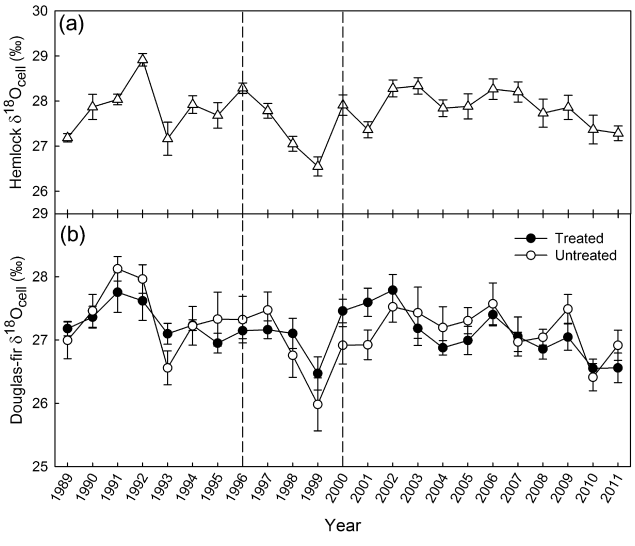

Figure 5. Mean tree-ring $\delta^{18}O_{cell}$ of western hemlock (a), and fungicide-treated and untreated Douglas-firs (b) at the Beaver, Oregon site during the years of observation (1989–2011; $n = 6$ trees per treatment group). The treatment period is indicated by vertical dashed lines (1996–2000, inclusive). Bars represent 1 SE.

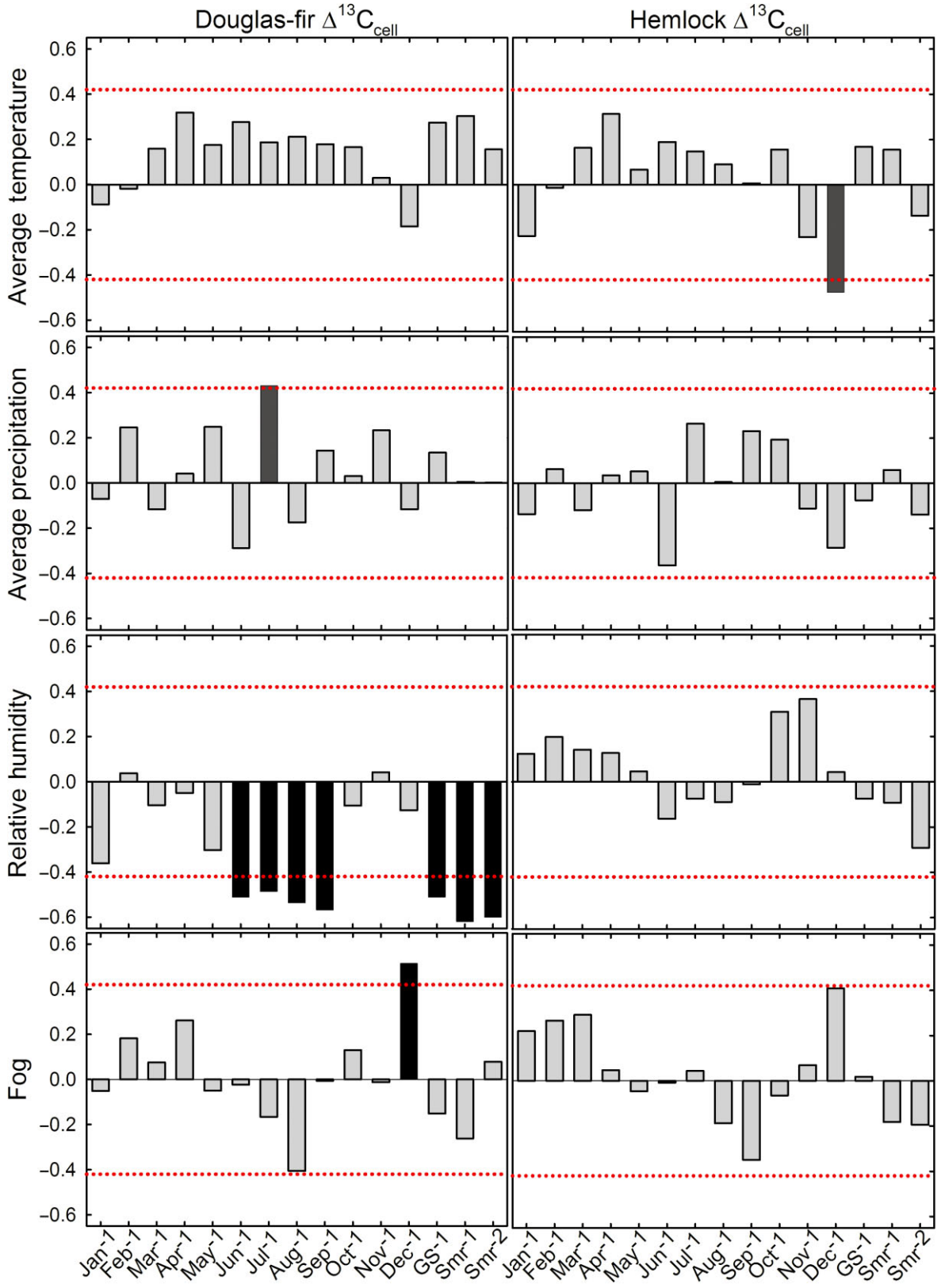

Figure 6. Pearson correlation coefficients (r) of Douglas-fir and western hemlock tree-ring $\Delta^{13}C_{cell}$ and various climate variables at different times of year: monthly values of the previous year (e.g. Jan-1), previous growing season average (May–July; GS-1), previous summer average (June–September; Smr-1), and summer two years previous average (Smr-2). Red, dotted lines represents significance at 95% confidence level (0.42/−0.42).

year of treatment, as well as a 3 year lag in the return to pre-treatment growth levels after the last year of treatment. These lags are likely due to the nature of the life cycle of *P. gaeumannii* and the progression of disease in Douglas-fir foliage (Stone *et al*. 2008). After initial infection of current-year foliage, it takes at least a year for *P. gaeumannii* to produce the pseudothecia that result in disease symptoms that include reduced carbon dioxide assimilation and foliage loss. Thus, it would take a year after the first application of fungicide for there to be a notable difference in foliage retention and annual growth between treated and untreated trees. Furthermore, because the fungus only infects new foliage, each subsequent year of treatment meant that the treated trees had one more cohort of healthy, productive foliage, which explains why annual growth steadily increased over the treatment period and why there was a 1 year lag before annual growth began to decline back to pre-treatment levels after the last year of treatment. Needle retention on the Oregon coast is anywhere between three and four needle cohorts for healthy Douglas-fir (Shaw *et al*. 2011), so the 3 year lag before annual growth of treated trees returned to pre-treatment levels after the final year of treatment represents the steady annual progression of additional diseased needle cohorts in the absence of the fungicide treatment.

The response of tree-ring $\Delta^{13}C_{cell}$ to fungicide treatment over time was similar to the pattern that we observed in annual growth. There was a difference in the number of years that BAI and tree-ring $\Delta^{13}C_{cell}$ between treated and untreated trees were significantly different, such that there was a 3 year lag after treatment began and a 2 year lag after treatment ended for tree-ring $\Delta^{13}C_{cell}$. It is possible that more healthy needle cohorts were required to produce a significant change in tree-ring $\Delta^{13}C_{cell}$ than were required to produce changes in annual growth, which could explain why the difference in $\Delta^{13}C_{cell}$ between Douglas-fir treatment groups required more time to manifest after treatment began and less time to diminish after treatment ended. Incorporation of non-structural carbohydrates produced in prior years into current-year wood may also have contributed to the lag in the appearance of the $\Delta^{13}C$ signal in treated trees. Nevertheless, the overall trajectory of the tree-ring $\Delta^{13}C_{cell}$ response to treatment was essentially consistent with that of annual growth.

We verified the $\Delta^{13}C_{cell}$ signal that was identified in tree-rings was also present in the crown at the source of carbohydrate synthesis. The $\Delta^{13}C$ of both foliage and twig wood decreased with increasing tissue age (Fig. 2), a trend opposite to that reported for branches on healthy Douglas-fir trees in which the $\Delta^{13}C$ of foliage typically decreases with decreasing age from the branch base to tip because of increasing hydraulic constraints on stomatal conductance (Panek & Waring 1995; Panek 1996; Warren & Adams 2000). Thus, the cumulative impact of intensification of SNC symptoms over time on leaf gas exchange dominated over normal axial trends in hydraulic limitation of stomatal conductance. Foliage and twig wood $\Delta^{13}C$ values were clearly different, consistent with previous research showing that non-photosynthetic tissues, such as those found in branch and stem wood, are typically enriched in ^{13}C (lower $\Delta^{13}C$) by between 1 and 3‰ compared with leaves on the same branch or tree (Cernusak *et al*. 2009). Because the secondary growth of twigs is largely derived from carbohydrate exported from needles attached to them, the $\Delta^{13}C$ of both tissues should decrease over time at roughly the same rate. However, twig wood $\Delta^{13}C$ declined at about twice the rate of foliage across the same 4 year sequence. The bulk mass of foliage is cellulose that is laid down in the first year when SNC is still in the early stages of inoculation. Foliar cellulose does not turn over, so changes in foliar $\Delta^{13}C$ with increasing age must be associated with leaf constituents that are produced under increasing SNC-related constraints on stomatal conductance after the needle is developed (e.g. non-structural carbohydrate and other substances that undergo metabolic turnover). Furthermore, the exported carbohydrate from foliage and the secondary growth of the attached twig that it ultimately builds would show progressively lower $\Delta^{13}C$ with each cohort year. Thus, twig wood of diseased trees would be expected to have a greater rate of decrease in $\Delta^{13}C$ with age compared with foliage as the carbohydrate exported from older needles would have a lower $\Delta^{13}C$ signal than the bulk mass of the needle itself.

Mean tree-ring $\Delta^{13}C_{cell}$ of treated Douglas-fir and western hemlock were not significantly different from the year treatment began through three years following treatment, which roughly follows the trend in significant differences between the mean tree-ring $\Delta^{13}C_{cell}$ of treated and untreated Douglas-fir during and after the treatment period. When we examined the positive relationship between mean tree-ring $\Delta^{13}C_{cell}$ and BAI of treated Douglas-fir, $\Delta^{13}C_{cell}$ and BAI during the treatment period were similar to those of western hemlock (Fig. 4). These results suggest that there is clear potential in using tree-ring $\Delta^{13}C_{cell}$ and BAI chronologies as a means to track SNC disease history provided a suitable co-occurring reference species like western hemlock is available. Applying this method, years when disease was low or absent within a Douglas-fir tree-ring $\Delta^{13}C_{cell}$ chronology would not be significantly different from the same years in a tree-ring $\Delta^{13}C_{cell}$ chronology of nearby western hemlock, and the degree of divergence in tree-ring $\Delta^{13}C_{cell}$ of the two species in a given year should be related to SNC disease severity for that year (Fig. 3). It would be beneficial to use BAI in conjunction with tree-ring $\Delta^{13}C_{cell}$ since both variables appear to track disease severity and could potentially identify non-SNC influences on tree growth and physiology when one variable changes while the other remains constant.

Oxygen-stable isotopes

Contrary to our hypothesis, tree-ring $\delta^{18}O_{cell}$ did not differ between treated and untreated Douglas-fir in response to treatment. Previous research has demonstrated that high RH (~60–70%) can mask the effects of reduced stomatal conductance on $\delta^{18}O_L$, and thus the signal imparted to tree-ring cellulose, because there is so little evaporative enrichment of leaf water and the isotopic exchange with atmospheric vapour is much higher under high RH (Roden & Ehleringer 1999; Barbour & Farquhar 2000; Brooks & Mitchell 2011). To

explore the effects of variation in g_s and L (effective path length) on $\Delta^{18}O_{cell}$ under the mean growing season RH at our site (78%, May–July), we used Eqns 3–6. A reference value for g_s of 0.14 mol $m^{-2}\,s^{-1}$, typical for healthy, non–water-limited Douglas-fir was applied (Bond & Kavanagh 1999). Stomatal conductance was then varied between 0 and 0.14 mol $m^{-2}\,s^{-1}$ with L set between 33 and 2400 mm, representative of the range of L reported for several conifer species (Wang *et al*. 1998; Song *et al*. 2013). Source water $\delta^{18}O$ was set to the 5 year average for precipitation in Newport, Oregon (–7.05‰; J.R. Brooks, unpublished data). Leaf and air temperature were set for average summer time conditions determined from the PRISM climate data as 15°C. Boundary layer conductance was set to 2 mol $m^{-2}\,s^{-1}$. We assumed that $\Delta^{18}O_V$ was in equilibrium with source water, and therefore was equal to $-\varepsilon^+$ (Barbour 2007). Finally, in Eqn 6 we set p_{ex} to 0.4, and p_x to 1 (Brooks & Mitchell 2011).

The results of this modelling exercise suggested that even under near complete loss of stomatal conductance in diseased trees, the difference in $\Delta^{18}O_{cell}$ between diseased and healthy trees with a typical L (33 mm) would only be about 0.46‰. Moreover, Manter *et al*. (2000) found that Douglas-fir seedlings with SNC that were sampled in May and June had g_s values of around 0.02–0.05 mol $m^{-2}\,s^{-1}$, while control seedlings had values around 0.08–0.12 mol $m^{-2}\,s^{-1}$. Although we sampled mature Douglas-fir and not seedlings, it is possible that the magnitude of difference in g_s between treated and untreated trees was not as great as in this modelling exercise, thus yielding an even smaller difference in $\delta^{18}O_{cell}$ between treatments. Furthermore, the model demonstrates that as L increases the difference in $\Delta^{18}O_{cell}$ from the control value should increase. Although L may be slightly modified by the presence of fungal hyphae in the needle, the lack of difference in our observed tree-ring $\delta^{18}O_{cell}$ between treated and untreated Douglas-fir suggests that any difference in L between treatments was minimal.

In summary, the blockage of stomata by pseudothecia and/or hyphae within the leaf mesophyll may indeed affect $\delta^{18}O_{cell}$ when RH is low, but because RH was rather high at our site, we did not observe any influence of the fungus on $\delta^{18}O_{cell}$. Because SNC is more prevalent in coastal regions where RH is normally high, our results imply that stable isotopes of oxygen in tree-rings would not serve as a reliable indicator of variability in SNC disease severity.

Disease severity and climate

The most prevalent relationships we observed were a negative correlation between previous summer and growing season averages of RH (previous June, July, August, September, growing season, and summer; and two summers previous) and Douglas-fir tree-ring $\Delta^{13}C_{cell}$. The strongest of these correlations were with RH of the previous summer ($r = -0.62$) and two summers previous ($r = -0.60$). A typical physiological response of healthy trees to low RH (and thus, high vapour pressure deficit) is to close stomata, causing $\Delta^{13}C_{cell}$ to decrease during the same year. In the case of Douglas-fir with SNC, we observed the opposite; low RH was related to increased $\Delta^{13}C_{cell}$. Previous research has shown that spring and summer leaf wetness in the year of initial infection is positively correlated with disease severity (Hansen *et al*. 2000; Rosso & Hansen 2003), which is likely due to increased spore germination on leaf surfaces. The negative relationship between previous summer RH and Douglas-fir tree-ring $\Delta^{13}C_{cell}$ likely reflects higher leaf wetness through increased nightly dew frequency and amounts and lower evaporation rates of this dew when the spores are germinating. In turn, greater leaf wetness, particularly from dew that would presumably wash off fewer spores than rainfall, would promote greater spore adhesion to needles and more successful germination on foliage. Subsequent increases in pseudothecia-blocked stomata would result in lower $\Delta^{13}C_{cell}$ in the following year or two when that foliage would be contributing substantial photosynthate. Accordingly, this response would not be observed in a species that is not susceptible to SNC, like western hemlock, which did not demonstrate any relationship between RH and tree-ring $\Delta^{13}C_{cell}$.

Variation in SNC disease severity has been correlated primarily with warm winter temperatures and wet spring and summer conditions, with some studies also reporting a negative relationship between high summer temperatures and disease severity (Rosso & Hansen 2003; Zhao *et al*. 2011). Although our results agreed with the positive relationship between July RH and disease severity reported by Latta *et al*. (2009), we did not find any evidence for a relationship between any of the measures of temperature, precipitation, or fog occurrence and disease severity as measured with tree-ring $\Delta^{13}C_{cell}$. The relationship between climate and SNC severity in past studies has been explored using different measures of disease, such as needle retention (Rosso & Hansen 2003; Coop & Stone 2007; Latta *et al*. 2009; Zhao *et al*. 2011), pseudothecia abundance (Manter *et al*. 2005), and growth (i.e. tree-ring width; Black *et al*. 2010). Although these measures are reasonable assessments of disease signs and symptoms, some have potential confounding factors that could complicate efforts to determine their respective relationships to climate. For example, needle retention can be influenced by stand density, tree age, temperature, water availability, nutritional status, herbivory and functional net photosynthesis (reviewed by Ewers & Schmid 1981; Chabot & Hicks 1982; Pouttu & Dobbertin 2000; Xiao 2003), and tree-ring widths are sensitive to a variety of environmental signals (McCarroll & Loader 2004) independent of disease severity. Although there are certainly factors other than SNC that could potentially affect tree-ring $\Delta^{13}C_{cell}$, differences in carbon stable isotope signatures of diseased Douglas-fir and a healthy reference species may eliminate noise contributed by other factors that affect needle retention and tree-ring width and provide another perspective of the relationship between climate and SNC disease severity.

The results of the present study demonstrated that SNC causes reduced $\Delta^{13}C_{cell}$ values. However, without information regarding disease status, these results could be interpreted very differently with traditional stable isotope theory, particularly when attempting climate reconstruction with tree-ring stable isotopes. Thus, future related studies should

consider the potential impacts of disease when interpreting stable isotope signals in tree-rings.

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