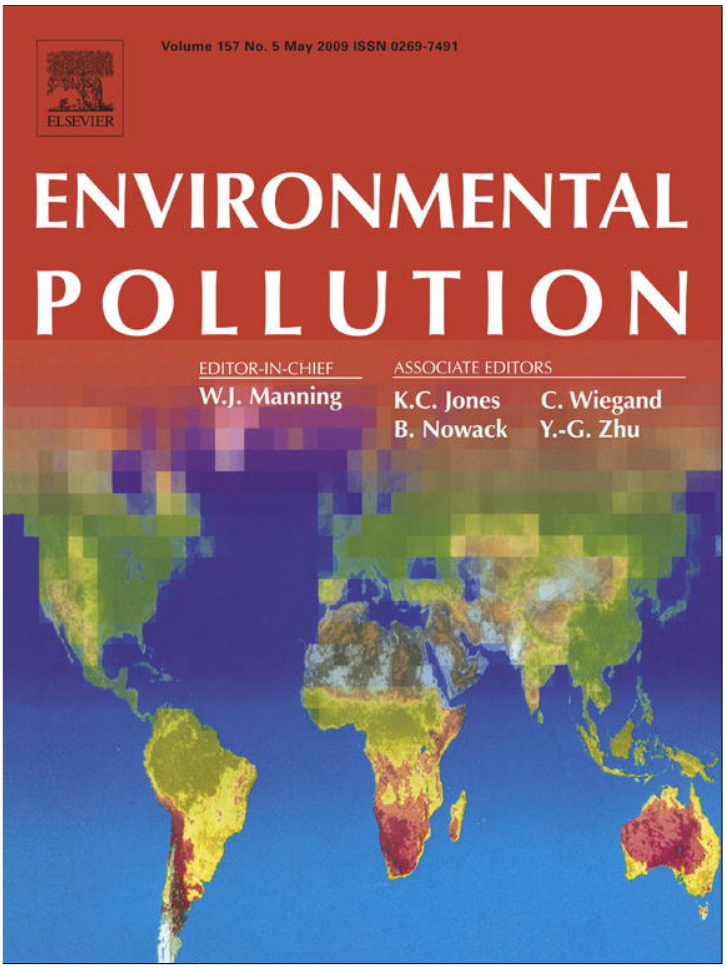

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Effect of 3 years' free-air exposure to elevated ozone on mature Norway spruce (*Picea abies* (L.) Karst.) needle epicuticular wax physicochemical characteristics

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Free-air ozone exposure induced changes in needle wax characteristics of mature Picea abies.

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ABSTRACT

We examined the effect of ozone (O_3) on Norway spruce (*Picea abies*) needle epicuticular wax over three seasons at the Kranzberg Ozone Fumigation Experiment. Exposure to 2× ambient O_3 ranged from 64.5 to 74.2 μl O_3 l^{-1} h AOT40, and 117.1 to 123.2 nl O_3 l^{-1} 4th highest daily maximum 8-h average O_3 concentration. The proportion of current-year needle surface covered by wax tubes, tube aggregates, and plates decreased ($P = 0.011$) under 2× O_3. Epistomatal chambers had increased deposits of amorphous wax. Proportion of secondary alcohols varied due to year ($P = 0.004$) and O_3 treatment ($P = 0.029$). Secondary alcohols were reduced by 9.1% under 2× O_3. Exposure to 2× O_3 increased ($P = 0.037$) proportions of fatty acids by 29%. Opposing trends in secondary alcohols and fatty acids indicate a direct action of O_3 on wax biosynthesis. These results demonstrate O_3-induced changes in biologically important needle surface characteristics of 50-year-old field-grown trees.

1. Introduction

Tropospheric ozone (O_3) is a secondary pollutant generated from primary air emissions of nitrogen oxides (NO_x) and volatile organic compounds (VOC) reacting under warm temperatures and in the presence of sunlight. The concentration of O_3 has more than doubled during the last century and is currently increasing at an annual rate of 0.5–2%, mostly due to human activity (Vingarzan, 2004). Ozone is currently an important stressor of >25% of the world's forests, and some 50% of forests (17 M km^2) may be exposed to O_3 concentrations exceeding 60 nl l^{-1} by 2100 (Fowler et al., 1999). With the continued growth in energy demand and the rise in gross domestic productivity in rapidly industrializing countries, and the increase in vehicle miles traveled, it is likely that O_3 will continue to threaten the world's forests well into this current century (Percy et al., 2003; Ashmore, 2005).

A large scientific literature has documented O_3 impacts on forest trees (cf. Karnosky et al., 2007a; Matyssek et al., 2007). Ozone effects are known to cascade through tree gene expression, biochemistry and physiology, ultimately feeding back to productivity, predisposing trees to pest attack and causing changes in water-use efficiency (Percy et al., 2002; Karnosky et al., 2003, 2005; Matyssek and Sandermann, 2003). Ozone can be phytotoxic at certain concentrations, affecting plant growth, development and condition. Ozone enters plant tissues through the stomata, generating reactive oxygen species (ROS), and triggering an oxidative burst (Kangasjarvi et al., 2005). Perception of ROS (and O_3) can induce several signal transduction pathways involved in plant response to oxidative stress. Reactive oxygen species can inhibit photosynthesis, reduce growth and result in premature foliar senescence (Matyssek and Sandermann, 2003). It can cause foliar necrosis similar to wounding reaction or pathogen attack (Heath and Taylor, 1997). However, until the advent of Free-Air or FACE (Free-Air Carbon Dioxide Enrichment) technology in the mid 1990s (McLeod et al., 1985; Hendrey et al., 1999), our mechanistic understanding of O_3 impacts on forest tree biochemistry was based largely on short-term research in chambered environments. These data on tree response to O_3 are often from controlled exposures of young trees, constrained in pots or by limited rooting zone in chambers (Kolb and Matyssek, 2001). Manning (2005) has stated that much of the data derived from chambered environments appear to have limited utility for extrapolation to risk analysis

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owing to differences in climatic and growth environment between chambered and ambient air situations. Therefore, establishing cause–effect relationships for ambient O_3 exposure and tree growth has been difficult (Karnosky et al., 2005).

Foliar surfaces of all higher plants carry a partial or continuous coverage of amorphous epicuticular wax (EW). These thin films are often surmounted by embedded crystalline wax structures, the shape (plates, tubes, ribbons, filaments, etc.) of which is predetermined by the chemical composition of the wax precursors (Jeffree et al., 1975). Deposits of EW comprise the outermost portion of the leaf cuticle and are extremely important for plant vigor and survival, especially under the current changing growth environment (Jenks and Ashworth, 1999). Epicuticular wax is sensitive to changes in plant physical (temperature, relative humidity, soil moisture) and chemical (inorganic and organic air pollutants) environments (Baker, 1982; Percy et al., 1994).

It has long been known that the structure of EW crystallites is conferred by the chemical composition of the dominant constituents (Jeffree et al., 1975). Chemical composition of EW is under very tight genetic regulation and strong enzyme inhibition (Mikkelsen, 1978); severe treatment or stress (Baker, 1982; Jenks and Ashworth, 1999) are usually required to modify the inherent rate of *de novo* synthesis and subsequent partitioning of carbon between competing biosynthetic pathways (von Wettstein-Knowles, 1995). The production, chemical composition and structure of EWs have been found to be sensitive to O_3. Elevated O_3 has been shown to increase wax production, modify wax ultrastructure and alter wax chemical composition, leading to stomatal occlusion by wax deposits in several forest tree species (Barnes et al., 1988; Percy et al., 1994, 2002; Karnosky et al., 2002; Mankovská et al., 2005). Exposure to O_3 results in effects on EW biosynthetic pathways, rather than from oxidative transformation of wax deposits in situ (Jetter et al., 1996). These changes have been connected to changes in plant quality and to phylloplane resistance to insect and disease populations (Karnosky et al., 2002; Percy et al., 2002).

At the Kranzberg Ozone Fumigation Experiment (KROFEX) in Kranzberg, Germany (Nunn et al., 2002; Werner and Fabian, 2002; Karnosky et al., 2007b), the effect of O_3 on primary metabolites of mature Norway spruce (*Picea abies* (L.) Karst.) was investigated during the growing seasons of 2000 through 2002, the first 3 years of the experiment. Although elevated O_3 had only minor effects on contents of non-structural proteins, and total amino compounds in needles, the defensive capacity of the spruce trees developed under natural environmental stress conditions was found to be generally sufficient to provide protection against twice-ambient O_3 ($2\times$ O_3) concentrations (Alexou et al., 2007). Significant effects noted on sun-exposed foliage during the first 3 years (2000–2002) of fumigation included reduced O_3 uptake in year 1, reduced foliar biomass in year 2, and earlier bud break in years 2 and 3 (Nunn et al., 2005). However, the influence of elevated O_3 on older needles following one or more years of exposure could not be excluded, as found previously by Langbartels et al. (1998). Norway spruce, as is the case with all conifers, differs markedly from most dicotyledenous species in having high proportions of tube-forming secondary alcohols (mainly as nonacosan-10-ol) and diols as dominant constituents of leaf EW deposits. Previous research with recrystallized red spruce (*Picea rubens* Sarg.) EW had demonstrated that the EW deposit was not susceptible to direct oxidative damage when exposed to very high (250 nl l^{-1}) O_3 concentrations (Percy et al., 1994).

In this study, we aimed to characterize EW production and chemical composition on mature Norway spruce trees exposed over 3 years at KROFEX. We were interested to determine if O_3 applied at twice-ambient concentrations could directly affect EW physicochemical characteristics during the period of EW production and whether or not any effects were modulated by interannual change in climate.

2. Materials and methods

2.1. Study site

The KROFEX was initiated in May 2000 at the "Kranzberger Forst" (Freising, Germany, 48°25′N, 11°39′E; 485 m a.s.l.) 35 km NE from Munich. A free-air O_3 exposure system was deployed within a mixed stand of adult beech (*Fagus sylvatica* L.) and Norway spruce trees. Complete details on fumigation methodology and experimental design have been described earlier (Werner and Fabian, 2002; Nunn et al., 2002; Karnosky et al., 2007b; Matyssek et al., 2007). The spruce trees sampled in this study were planted in 1951 and were up to 30 m tall at the time of investigation. Leaf area index was 5.4 (closed canopy) and annual stem production was 6.2 m^3 ha^{-1} yr^{-1} across the stand (cf. Pretzsch et al., 1998). Soil was a luvisol (rooting depth of 1 m) formed from loess above tertiary sediments and provided an adequate supply of nutrients. The loamy texture comprised 17–30% clay, 50–60% silt and 5–33% sand, giving a water capacity of 360 kg m^{-3} (Matyssek et al., 2007).

Long-term averages (1970–2000) of mean annual air temperature and precipitation at the Kranzberger Forst were 7.8 °C and 786 mm, respectively. Air temperature remained >10 °C on average for 155 d each year. The study period reported here extended over three growing seasons (2000–2002) of fumigation, during the first 3 years of the experiment. Meteorological conditions and O_3 exposures recorded during 2000–2002 are listed in Table 1.

2.2. Ozone exposure

The KROFEX O_3 delivery system is fully described in Werner and Fabian (2002) and Karnosky et al. (2007b). Ozone was produced using a commercial O_3 generator supplied with 90% O_2 separated on site from dry, purified air. The design allowed a group of 10 neighboring trees, five European beech (*F. sylvatica* Ehrh.) and five Norway spruce individuals, to be experimentally treated in a controlled manner. Upon generation, ozone was diluted to the target level of experimentally enhanced $2\times$ O_3 treatment, and then released into the respective $2\times$ O_3 stand area (canopy volume approx. 2000 m^3) by means of 117 polytetrafluoroethylene (PTFE) tubes and distributed via approximately 6000 calibrated outlets (0.5 mm inner diameter capillaries) calibrated for equal O_3 efflux. The system was operated from late April or early May through the first week of November. Ozone levels in an adjacent stand area of approximately the same canopy volume were not enhanced and these five Norway spruce were exposed to ambient ($1\times$ O_3) levels.

Ozone concentrations were monitored continuously using eight online monitors and 100 passive samplers (replaced at weekly intervals) positioned at four levels in each of the elevated O_3 ($2\times$ ambient, $2\times$ O_3) and ambient O_3 (control treatment, $1\times$ O_3) sections of the stand (Karnosky et al., 2007b; Matyssek et al., 2007). Measurements carried out near or between the outlets of adjacent PTFE tubes showed horizontal O_3 gradients to stay confined within a maximum distance of about 5 cm around the outlets. Thus, KROFEX achieved a fairly homogeneous O_3 distribution, with hot spots, if occurring at all, restricted to rather short radii around the outlets (Karnosky et al., 2007b). Growing season O_3 exposures measured using active continuous monitors during the study period are reported in Table 1 as calculated using the SUM00 (sum of all hourly average concentrations), AOT40 (Fuhrer et al., 1997), 4th highest daily maximum 8-h O_3 concentration (Canadian Council of Ministers of the Environment (CCME), 2000; Federal Register, 2008).

2.3. Epicuticular wax structure

The same five Norway spruce trees from each O_3 treatment were sampled at the end of each growing season. Five current-year needles were removed from the mid-portions of each of three upper-crown (sun) shoots per tree. Needle sections (5 mm) from the mid-portion of the air-dried needles were mounted onto brass stubs using double-sided tape, and coated with gold palladium (80:20, 45 nm thickness) using a Polaron E5100 (London, UK; year 2000 samples) or with platinum (30 nm) using HR-sputter coater Agar (UK; 2001 and 2002 samples). Needle segments were scored for crystalline EW tube distribution within or adjacent to the stomatal antechamber. Wax tube density (WTD) for three stomata per needle was recorded at 1300× magnification under a field-emission scanning electron microscope (FESEM; JEOL

Table 1
ANOVA results for effect of treatment and year on amount of epicuticular wax recovered from current-year Norway spruce needles.

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Treatment	1	0.13105	0.15236	0.15236	1.98	0.163
Year	2	1.03914	1.03914	0.51957	6.75	0.002
Error	79	6.07677	6.07677	0.07692		
Total	82	7.24696				

JSM-6300F, Tokyo, Japan) operated at 12 kV accelerating voltage. Stomata were selected according to the following protocol: 1) the first stoma assessed did not manifest particulate, fungi or algae that would interfere with observation of the underlying EW structure; 2) the next two stomata were then taken from the same row of stomata. This protocol was evaluated as effectively removing potential for surface deposits and organisms to mask underlying EW structure.

The SEM images ($n = 450$) were initially captured using a Link Isis (Oxford Instruments, London, UK) energy dispersive analyzer (EDS). The images were then transferred with the SemAfore digital image recording system (Insinööritoimisto J. Rimppi Oy, Finland), processed and analyzed using an Image Processing Tool Kit 4.0 Adobe Photoshop program. To enhance the texture of the structural wax area, the BEI-images were processed with a range of Gaussian blur filters; threshold and area fractions were measured with the global filter.

2.4. Wax production and chemical composition

Thirty current-year needles were removed from the same three upper-crown shoots per tree as those examined under the SEM, placed in glass vials, and then immersed in chloroform ($CHCl_3$) for 5 s. The chloroform–wax (recovered from a pooled sample of 30 needles) solution was filtered through a #1 Whatman filter paper. After partial chloroform evaporation to ~500 μl on a rotary evaporator, the solution was transferred to a pre-weighed glass vial. Chloroform in the vials was evaporated using a stream of nitrogen at 40 °C for 5 min, and the pre-weighed (±10 μg) 1.8-ml glass vials weighed again to determine wax quantity. Needles were dried to constant cooled weight in an oven at 65 °C for 3 d. Wax quantity was expressed as the proportion of oven-dried needle mass (mg g^{-1}).

Quantitative analysis of chemical composition of Norway spruce wax was completed according to Gordon et al. (1998) using a Varian 3300 high-temperature gas chromatograph (GC) fitted with a flame ionization detector (FID). Retention times were established on a J&W DB1-HT fused silica capillary column (15 m long, 0.32 mm internal diameter) with methyl silicone liquid phase (0.25 μm film thickness). The carrier gas flow was 4.8 ml helium min^{-1} at 70 °C. Column programming was 70–120 °C at 20 °C min^{-1} and 120–390 °C at 6 °C min^{-1}. The septum-programmable injector was operated for 75–125 °C at 18 °C min^{-1}, 125–395 °C at 12 °C min^{-1}, hold 15 min. The FID was set at 400 °C. Wax samples were derivatized at 55 °C for 30 min with BSA (N,O-bis (trimethysilyl) acetamide) before injection.

Areas of individual peaks integrated above a zeroed chromatogram baseline and percentage (±0.001%) homologue composition were calculated using Varian Workstar® version 4.0 software. Relative retention times (RRT) were determined from a dominant alkyl ester homologue (C_{40}, octadecyl docosanoate TMS ester). Gas chromatography assignments were based on pre-injected pure reference homologues and an extensive RRT library for six *Picea* species. Identified peaks represented some 99% of GC peaks recorded from a 0.2 μg (2/1 w/v) injection of analite. Gas chromatography assignments were subsequently confirmed using a Hewlett–Packard 5989 GC–MS. A DB1-HT column was programmed from 70 °C to 345 °C at 12 °C min^{-1} (hold 30 min). Helium carrier gas flow rate was 1.0 ml min^{-1} constant flow. Injection was on-column at 250 °C. Electron impact (EI) mass spectra were searched against Wiley, NIST, and CFS reference libraries. Ion source and quadrupole temperatures were 275 °C and 100 °C.

2.5. Statistical analysis

The KROFEX experimental design resulted in exposure of two stand areas to either ambient (1× O_3), or elevated (2× O_3) concentrations of ozone. The two treatments were not replicated within the stand. Accordingly, individual spruce trees sampled were treated as sampling units and treatment means calculated for each area of the stand. Initial statistical analysis using nested ANOVA indicated that differences due to canopy position (three full sun-exposed shoots per tree) did not significantly ($P < 0.05$) affect variable response.

Data on WTD, needle dry weight, EW production and chemical composition (wax class level) were analyzed using a two-way general linear model (GLM) ANOVA. Differences between O_3 treatments (1× O_3, 2× O_3) and years (2000–2002) were tested with treatment ($n = 2$) fixed, and year ($n = 3$) random (Table 1). Data were tested for normality of distribution (Kolmogorov–Smirnov) and equality of variance (Levene's test) before analysis. Percentage data were arcsin transformed. Needle weight and wax quantity data were log transformed before analysis.

3. Results

3.1. Meteorology and ozone exposure

Growing season (1 May–30 September) temperature varied to a small degree during the 2000–2002 study period. Mean growing season temperature decreased from 2000 (16.0 °C) to 2001 (15.6 °C), then increased from 2001 to 2002 (16.4 °C) (Table 2). Precipitation amount varied to a larger degree. Most precipitation was received in the final year of this study (538.7 mm), a 13.7% and 11.4% increase over 2001 and 2000, respectively. Soil moisture content increased only slightly during the 3 years and ranged from 30.6% in 2000 to 32% in 2002 (Table 2).

Table 2
Growing season meteorology and O_3 exposure during 2000–2002.

	2000	2001	2002
AOT40 1× O_3 (μl O_3 l^{-1} h)[a]	15.7	16.5	16.4
AOT40 2× O_3 (μl O_3 l^{-1} h)	64.5	74.2	71.9
4th highest 1× O_3 (nl O_3 l^{-1})[b]	88.3	86.2	81.4
4th highest 2× O_3 (nl O_3 l^{-1})	123.2	117.1	123.2
SUM0 1× O_3 (μl O_3 l^{-1} h)	132.7	129.8	125.5
SUM0 2× O_3 (μl O_3 l^{-1} h)	212.9	244.7	226.5
Mean air temperature (°C)[c]	16.0	15.6	16.4
Precipitation (mm)[d]	483.4	473.8	538.7
Soil moisture (vol.%)	30.6	31.1	32.0

[a] Accumulated Over Threshold (AOT)-based sum of all growing season daytime (0700–2059 h; >50 W m^{-2}) ozone concentrations >40 ppb (Fuhrer et al., 1997).
[b] Growing season 4th highest daily max. 8-h average O_3 concentration (US EPA NAAQS and Canadian CWS metric and from CCME, 2000; Federal Register, 2008).
[c] Measured at canopy height 32 m.
[d] 1 May–30 September.

Ozone exposure was calculated from continuous canopy-level hourly average data using commonly reported air quality indices (Table 2). Norway spruce exposure calculated using the AOT40 index ranged from 15.7 to 16.5 μl O_3 l^{-1} h under 1× O_3 and 64.5 to 74.2 μl O_3 l^{-1} h under 2× O_3. Ambient growing season 4th highest daily maximum 8-h O_3 concentrations ranged from 81.4 nl O_3 l^{-1} to 88.3 nl O_3 l^{-1} under 1× O_3 and 117 nl O_3 l^{-1} to 123 nl O_3 l^{-1} under 2× O_3. Cumulative SUM00 exposure ranged from 125.5 to 132.7 μl O_3 l^{-1} h under 1× O_3 and 212.9 to 244.7 μl O_3 l^{-1} h under 2× O_3 (Table 2).

3.2. Needle weight and epicuticular wax production

Averaged across treatments, mean sample weight (30 pooled needles) varied significantly ($P = 0.001$) between years and was largest in 2000 (0.107 g), decreased by 21.9% in 2001 (0.083 g) and increased by 26.2% in 2002 (0.105 g) (Fig. 1a). Needle weight was significantly ($P < 0.05$) smaller in year 2 (2001) than in 2000 and 2002. Current-year needle dry weight was not affected by O_3 treatment ($P = 0.17$) and, there was no year × treatment interaction (Fig. 1a).

Averaged across treatments, the amount of EW recovered from current-year needles increased from 140.8 mg g^{-1} in 2000 to 181.9 mg g^{-1} (2001), and then decreased to 147.1 mg g^{-1} in 2002 (Fig. 1b). Current-year needle wax amount differed significantly between years ($P < 0.001$) and was significantly greater ($P < 0.05$) in year 2 of the study (2001) than in 2000 or 2002. Amount of EW recovered from current-year needles did not ($P = 0.69$) differ between O_3 treatments. There was a statistically significant ($P = 0.007$) year × treatment interaction. Interestingly, on an absolute weight basis, EW per needle (EW amount × needle weight) averaged over three years was significantly ($P = 0.029$) larger by 8.9% after exposure to 2× O_3.

3.3. Epicuticular wax structure

Epicuticular wax structure within/adjacent to epistomatal chambers on current-year needles is shown in Fig. 2. Wax structure under 1× O_3 was similar at the beginning (Fig. 2a) and end of the experiment (Fig. 2b), although density of the crystallite structures differed. Epistomatal chambers were partially occluded with tubular waxes. Within stomatal rows, wax crystals were aggregated (Fig. 2b) and occasional upright plates were noted (Fig. 2a).

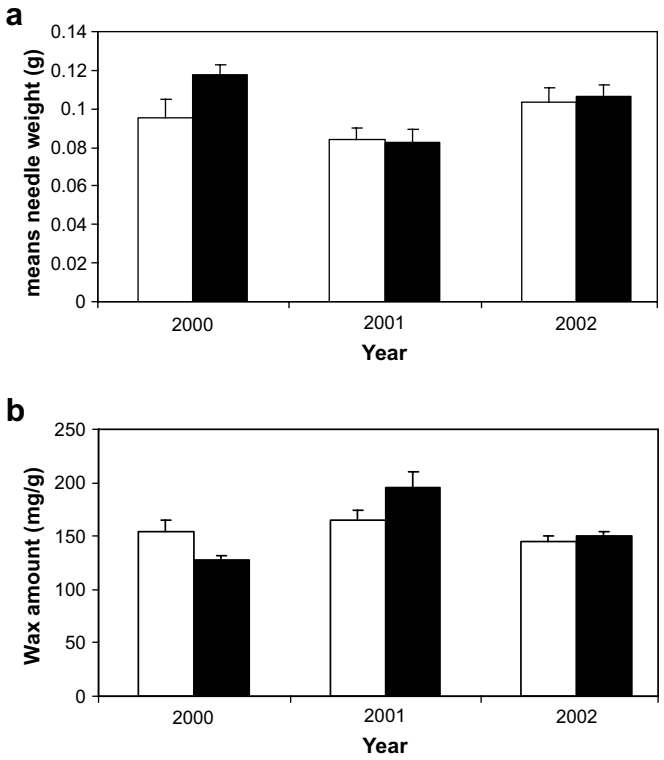

Fig. 1. Effects of O_3 treatment and year of exposure on Norway spruce current-year needles. (a) Effect of O_3 on needle dry weight from pooled sample of 30 needles; (b) Effect of O_3 on epicuticular wax amount. Data are means ± 1 SE ($n = 82$).

Exposure to 2× O_3 caused a marked change to wax structure. Epistomatal chambers were more occluded in 2000 (Fig. 2c) than under 1× O_3 (Fig. 2a). Crystalline structure was less evident and most wax had an amorphous structure, with flat plates often with crystalline structures protruding from them (Fig. 2c). In 2002, the epistomatal chamber was less evident and was almost fully occluded with amorphous deposits. The stomata were surrounded by large and more extensive amorphous deposits having long (~3 μm) tubes protruding through them, and additional upright, irregular plates (Fig. 2d).

3.4. Crystalline wax tube density

The proportion of current-year needle surface area covered by tubular epicuticular wax crystallites (mainly nonacosan-10-ol) was assessed using SEM. Over 3 years, there was a significant ($P < 0.001$) increase by year (more pronounced in control) in wax tube density in both treatments (Fig. 3). Averaged over treatments, crystalline tube density increased significantly ($P < 0.05$) from 50.9% of needle surface area in 2000 to 59.7% in 2001 and to 70.6% in 2002 (Fig. 3).

Crystalline wax tube density also differed significantly ($P = 0.011$) due to treatment. Tube density in 2002 was considerably lower under 2× O_3 (63.4%) than under 1× O_3 (77%). There was no ($P = 0.13$) year × treatment interaction.

3.5. Epicuticular wax chemical composition

Epicuticular wax recovered from current-year needles comprised eight wax classes and two minor constituents (Table 3). Major wax classes (1× O_3 treatment; mean ± 1SE) in order of decreasing proportion averaged over the 3-year study period were: secondary alcohols (66.3 ± 2.7%), fatty acids (19.3 ± 2.0%), diols (6.6 ± 1.2%), alkyl esters (3.1 ± 0.6%), methyl esters (2 ± 0.3%), primary alcohols (0.6 ± 0.1%), alkanes (0.6 ± 0.2%) and alkenes (0.5 ± 0.2%). Secondary alcohols comprised two C_{29} and one C_{31} homologues (Table 3). Twelve fatty acid homologues were confirmed, ranging from C_{12} to C_{30}, with even-numbered chains dominating. All five diol homologues resolved were C_{29} isomers, with 5,10 nonacosane diol most prominent. Alkyl esters comprised 12 homologues, with even-numbered C chains dominating. Octadecyl-tetracosanoate (C_{42}) was most prominent. Only small proportions of primary alcohols in two homologues were resolved, mostly hexacosanol (C_{26}). Both even- and odd-numbered saturated alkane homologues were confirmed, odd-numbered C chains predominating. Two alkene homologues were confirmed in current-year Norway spruce needle wax. Minor constituents (<1%) resolved and confirmed by GC–MS included abietic acid and 16-hydroxyhexadecanoic acid (Table 3).

There were significant differences due to year ($P = 0.004$) and O_3 treatment ($P = 0.029$) in proportions of secondary alcohols resolved in EW recovered from current-year Norway spruce needles. Averaged across treatments, secondary alcohols formed a greater proportion (66.1%) of the wax deposit in the final year (2002) than in 2000 (61.3%) or 2001 (55.89%) (Fig. 4). Averaged across years, secondary alcohols in Norway spruce wax were reduced under 2× O_3 (59.5%) compared with ambient O_3 (65.4%). Within years, the amount of secondary alcohols resolved in needle wax was significantly ($P < 0.05$) reduced by 2× O_3 in 2002 only (Fig. 4). ANOVA determined a year × treatment interaction ($P = 0.014$) for secondary alcohols.

In contrast to secondary alcohols, exposure to 2× O_3 significantly ($P = 0.037$) increased proportions of fatty acids recovered in Norway spruce needle wax. Averaged across years, fatty acid proportions were higher under 2× O_3 (18.6%) than under 1× O_3 (14.3%) (Fig. 5). Fatty acid proportions were also significantly ($P = 0.004$) different between years. Fatty acids contributed less to the wax deposit in 2002 (13.1%) than in either 2000 (19.3%) or 2001 (20.1%) (Fig. 5). ANOVA determined a significant ($P = 0.05$) interaction between treatment and year.

Proportions of the third most important constituent wax class diols differed ($P < 0.001$) due to year, but were not ($P = 0.43$) affected by O_3 treatment (Fig. 6). Averaged across treatments, diol proportions were significantly different by year and there was a decreasing trend from 2000 (9.4%) to 2001 (6.8%) to 2002 (4.1%). Diol proportions were more variable than secondary alcohols and fatty acids. Across years, diol proportions were similar under 1× O_3 (7.1%) and 2× O_3 (6.5%). ANOVA determined a statistically significant ($P = 0.002$) interaction between treatment and year.

Alkane proportions differed significantly ($P = 0.017$) between years (Fig. 7). Alkanes in needle wax comprised a much smaller proportion in 2000 (0.8%) than in 2001 (2%) or 2002 (2.4%). Like diols, there was a trend of increasing proportions over time. Alkanes on needles exposed to 2× O_3 (1.9%) did not differ significantly from those under 1× O_3 (1.5%).

There were no statistically significant differences due to O_3 treatment or year detected in proportions of alkyl esters, methyl esters, primary alcohols, alkenes or the two minor constituents.

4. Discussion

In this study, we co-measured seasonal meteorology with O_3 concentrations (Karnosky et al., 2007b), and recovered Norway spruce needle EW at the end of each growing season. This study revealed that free-air exposure to elevated O_3 may induce significant changes in Norway spruce needle epicuticular biosynthesis

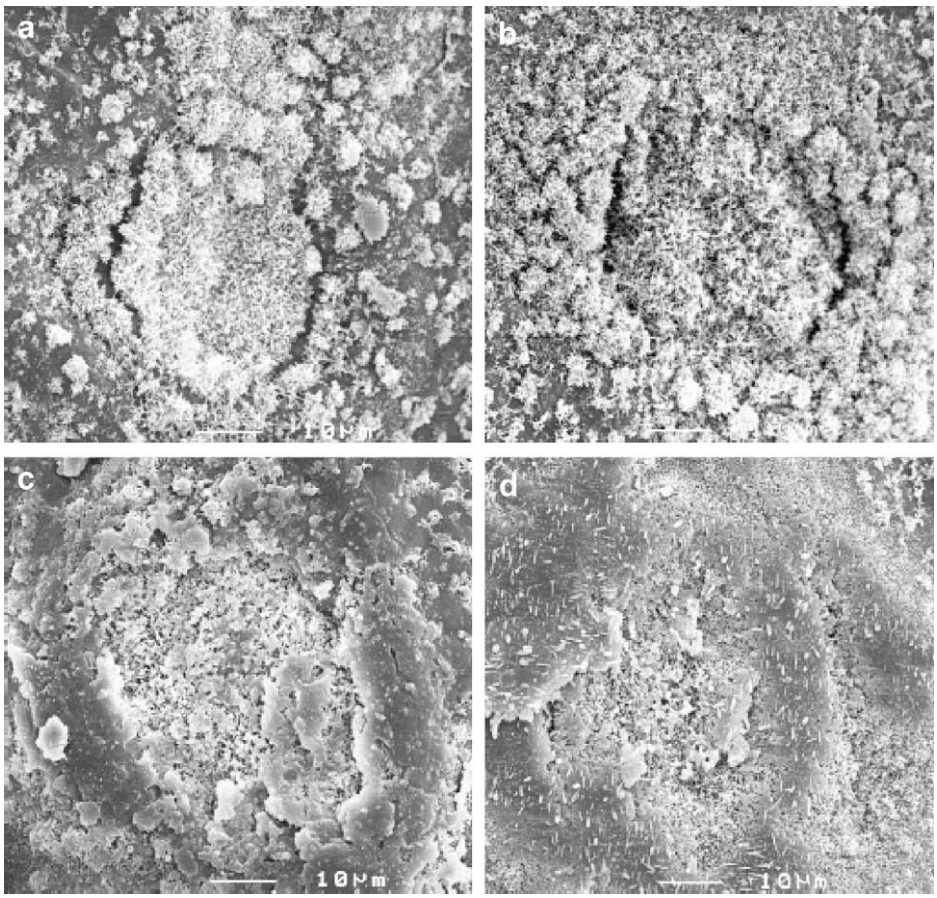

Fig. 2. Scanning electron micrographs of epicuticular wax structure within and adjacent to epistomatal chambers of current-year Norway spruce needles. Shown are images of needle surfaces exposed to ambient O_3 in 2000 (a) and 2002 (b) and to 2× ambient O_3 in 2000 (c) and 2002 (d).

under comparatively little variation in year-to-year climate, and, importantly, without measurable changes in needle weight and *de novo* synthesis.

In ambient air (1× O_3) oven-dry current-year needle weight was maximized in the year (2002) when average temperature, precipitation amount and soil moisture content were greatest. Coincidentally, AOT40 and 4th highest daily maximum 8-h O_3 concentration were lowest during 2002 (Table 1). Mean needle weight was maximized under 2× O_3 when average temperature (16 °C) and precipitation (483.4 mm) were mid-range, and soil moisture content (30.6%) lowest (Table 2). However, wax amount was greatest when temperature and precipitation were lowest, soil moisture content was mid-range and AOT40 (74.2 µl O_3 l^{-1} h) and 4th highest daily maximum 8-h O_3 concentrations were greatest. Under lower precipitation amounts, one might intuitively expect a stimulation of EW *de novo* synthesis leading to increased wax amounts (Baker, 1982).

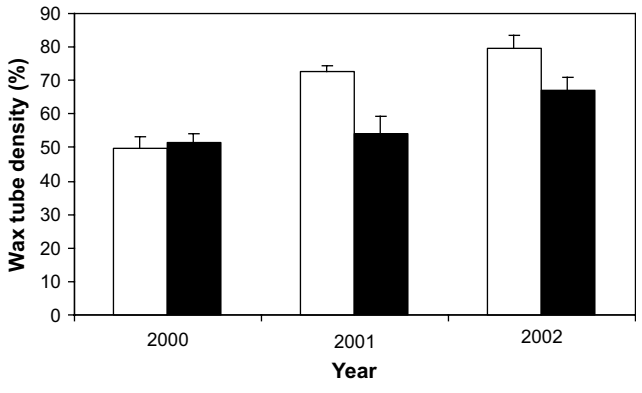

Fig. 3. Effect of O_3 treatment on Norway spruce current-year needle epicuticular wax crystalline tube density. Data are means ± 1 SE ($n = 450$).

Previously, Dixon et al. (1998) using open-top chambers reported an increase in Norway spruce seedling wax quantity at four different O_3 concentrations, but no change due to O_3 alone nor any O_3 drought interaction on wax quantity or quality. In contrast, Barnes and Brown (1990) reported no significant decrease due to O_3 fumigation in wax quantity recovered from Norway spruce seedlings grown in solar domes. Günthardt-Goerg and Keller (1987) reported that wax quantity was considerably reduced after fumigation with 150 nl l^{-1} O_3, although SEM examination revealed a new "smooth" layer underneath the soluble wax layer and within epistomatal chambers.

Our data could point to a cumulative impact (i.e., "memory effect") of O_3 over the 3-year period as advanced for conifers by Langbartels et al. (1998). Nunn et al. (2005) also reported significant ($P < 0.05$) reductions in Norway spruce sun-exposed foliar biomass after 2 years (in 2001) of fumigation at Kranzberger Forst. Epicuticular wax production in *Picea* spp. is known to be responsive to changes in physical growth environment (Baker, 1982; Cape and Percy, 1993) as well as to plant exposure to wet- and dry-deposited air pollutants such as acid rain (Percy and Baker, 1990, 1991), O_3 (Percy et al., 1990) and UV-B (Gordon et al., 1998). Although no statistically significant interactions between year and treatment were determined in this 3-year study, the data on needle weight

Table 3
GC–MS confirmed epicuticular wax chemical composition of wax recovered from current-year needles of adult trees in the Kranzberg Ozone Fumigation Experiment.

Wax class	Molecular ion (m/z)	Major MS fragments (m/z)
Secondary alcohols[a]		73, 103, 147
Nonacosan-10-ol	496	[M – 15, 481], 369, 229
Nonacosan-6-ol	496	[M – 15, 481], 425, 173
Hentriacontan-10-ol	524	[M – 15, 509], 369, 267
Fatty acids[b]		73, 117, 129, 132, 145
Dodecanoic acid	272	[M – 15, 257]
Tetradecanoic acid	300	[M – 15, 285]
Pentadecanoic acid	314	[M – 15, 299]
Hexadecanoic acid	328	[M – 15, 313]
Heptadecanoic acid	342	[M – 15, 327]
Octadecanoic acid	356	[M – 15, 341]
Docosanoic acid	384	[M – 15, 369]
Dodocosanoic acid	412	[M – 15, 397]
Tetracosanoic acid	440	[M – 15, 425]
Hexacosanoic acid	468	[M – 15, 453]
Octacosanoic acid	496	[M – 15, 481]
Tricosanoic acid	524	[M – 15, 509]
Diols[a]		73, 103, 147
10,13 nonacosane diol	584	457, 359, 327, 229
7,10 nonacosane diol	584	499, 317, 369, 187
5,10 nonacosane diol	584	527, 369, 317, 159
4,10 nonacosane diol	584	541, 369, 317, 145
3,10 nonacosane diol	585	555, 369, 317, 171
Alkyl esters		74, 87, 143, [$M^+ - 43$]
C32	480	
C34	508	
C36	536	
C38	564	
C40	592	
C42	620	
C43	634	
C44	648	
C45	662	
C46	676	
C47	690	
C48	704	
Methyl esters		74, 143 [$M^+ - 43$]
C30	466	
C32	494	
C34	522	
Primary alcohols[a]		75, 103, 147
Octadecanol	342	[M – 15, 327]
Hexacosanol	454	[M – 15, 439]
Alkanes		57, 71, 85, 99, 114
Tricosane	324	
Tetracosane	338	
Pentacosane	352	
Hexacosane	366	
Heptacosane	380	
Nonacosane	408	
Triacontane	436	
Tritriacontane	464	
Hexatriacontane	506	
Alkenes		55, 71, 85, 99, 114
1-Docosene	308	
1-Tetracosene	336	
Minor constituents[b]		
Abietic acid	374	73, 185, 241, 256, 359, 374
16-hydroxyhexadecanoic acid	416	75, 311, 385, 401, 416

73: Underline denotes the base peak.
[a] Resolved as TMSi ethers.
[b] Resolved as TMSi esters.

and EW amount do indicate the role each variable may play in a long-lived species.

Exposure of Norway spruce seedlings over three consecutive growing seasons to elevated O_3 in open-top chambers resulted in significant decreases in net photosynthesis and chlorophyll fluorescence, indicating interaction between effects of O_3 and photo-inhibition (Mikkelsen and Ro-Poulsen, 1994). Earlier, Barnes and Brown (1990) exposed Norway spruce seedlings to 156 $\mu g\, m^{-3}$ O_3 and reported a 16% increase in daily transpiration associated with slower stomatal closure in response to increasing water deficit in O_3-exposed needles. Although they did not note a significant effect of O_3 on amount of wax recovered, they did report a marked increase in needle wettability that they predicted to have potentially important physiological consequences. Epicuticular wax characteristics are a major factor affecting leaf surface wetting (leaf-droplet contact angle). Key characteristics include crystalline chemical composition and arrangement over the surface (Holloway, 1970). The chemistry of the EW determines its crystalline form (Jeffree et al., 1975). Pollutant-induced changes to leaf surface wettability across a range of plant forms have been linked with underlying changes in chemical composition of the EW deposit (Percy and Baker, 1988, 1991; Percy et al., 1990, 1992).

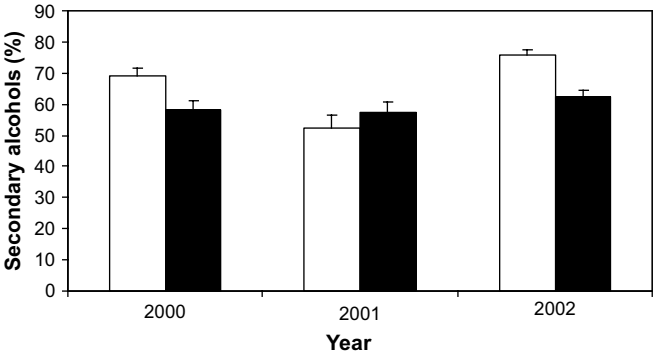

Fig. 4. Effect of O_3 treatment on proportion of secondary alcohols in Norway spruce needle epicuticular wax. Data are means ± 1 SE ($n = 82$).

There are numerous reports in the literature of accelerated EW structural degradation caused by O_3 fumigation. Usually, SEM studies with Norway spruce seedlings and O_3 have reported a conversion from a crystalline array of wax consisting of "fine tubes" to an amorphous layer with no definable structure. Typical of this phenomenon was the higher frequency of occlusion of epistomatal chambers often linked to accelerated structural degradation (Barnes et al., 1988). In this study we used the SEM to observe marked within-year structural changes in EW deposits on current-year needles sampled at the end of each growing season. Exposure to 2× O_3 resulted in a shift from an amorphous film deposit surmounted by crystalline tube aggregates and erect plates to a structure dominated by large flat amorphous plates with

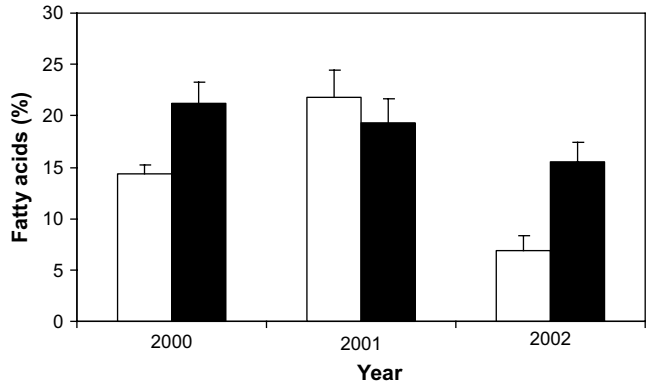

Fig. 5. Effect of O_3 treatment on proportion of fatty acids in Norway spruce needle epicuticular wax. Data are means ± 1 SE ($n = 82$).

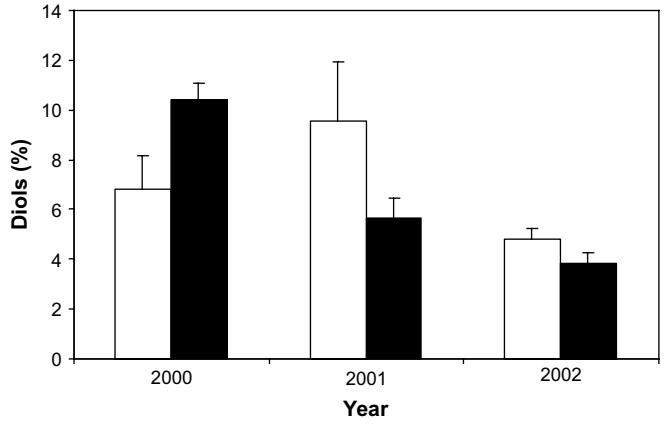

Fig. 6. Effect of O_3 treatment on proportion of nonacosane diols in Norway spruce needle epicuticular wax. Data are means ± 1 SE ($n = 82$).

some upright plates and isolated tube crystallites, particularly surrounding and within the epistomatal chamber (compare Fig. 2a with c). It also appeared that the degree of change was greater in 2002 (Fig. 2c) than in 2000 (Fig. 2d). To quantify this observed modification caused by elevated O_3, we used an SEM procedure modified from Percy and Baker (1988) to measure proportion of needle surfaces covered by crystalline tubes, aggregates and plates in the interstomatal regions. The increased density of crystalline structures in 2001 relative to that in 2000 is in agreement with the increase in wax quantity and may reflect the aforementioned change in growing season climate, particularly as the change was greatest under 1× O_3. On the other hand, the fact that the change was proportionally much less under 2× O_3 may imply change mediated more by altered C allocation to biosynthetic pathways and less of a response to climate. This is supported by the significant reduction in density under 2× O_3 in years 2 and 3. Given our tree age and the relatively short time span over which this study occurred, it is unclear why wax tube density increased consistently over the 3 years. There are reports of significant differences in wax characteristics in conifers (Frannich et al., 1978), but only among widely separated stages of plant development.

The chemical composition of EW recovered from current-year needles of mature Norway spruce at Kranzberger Forst was similar in overall class composition and homologue distribution to that reported for other areas in Europe (Günthardt, 1986; Prügel, 1994; Cape and Percy, 1998) but different from that reported by Lütz et al. (1990) who reported a less complex composition from seedling

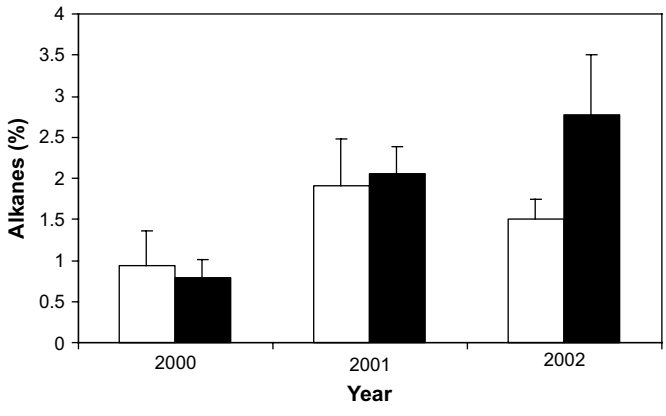

Fig. 7. Effect of O_3 treatment on proportion of alkanes in Norway spruce needle epicuticular wax. Data are means ± 1 SE ($n = 82$).

stage Norway spruce clones. As in most conifers, the dominant wax homologue was the secondary alcohol nonacosan-10-ol that comprised some 61% of the wax deposit. Although proportions of several wax classes varied between years, only secondary alcohols and the secondmost-prominent class fatty acids (17%) differed with O_3 treatment. This study, the first with mature Norway spruce exposed using free-air technology over multiple growing seasons, provides additional support to that previously offered (Percy et al., 1994; Jetter et al., 1996) for direct O_3 action on wax biosynthesis as opposed to oxidative action in situ. Here, the two dominant (88% of wax deposit) constituent classes produced by two competing biosynthetic pathways showed opposite direction of effect under 2× O_3. Averaged across years, secondary alcohols produced via the decarboxylative pathway were significantly reduced under 2× O_3 (59.5%) compared with 1× O_3 (65.4%). Across years, fatty acids produced from straight-chain elongation were significantly higher under 2× O_3 (18.6%) than under 1× O_3 (14.3%). Barnes and Brown (1990) had reported that Norway spruce needle-droplet contact angle was decreased following exposure to O_3 alone or to acid mist. With red spruce, Percy et al. (1990, 1992) found that O_3 was more damaging to EW than was acid fog. Needles exposed to O_3 had significantly less secondary alcohols, diols, alkyl esters, fatty acids and hydroxy-fatty acids than those exposed to charcoal-filtered air. These changes to EW chemical composition resulted in significant decreases in contact angles. Aggregates of secondary alcohols and diols form a highly hydrophobic surface that repels water, reflects incident radiation (including UV-B) and prevents excessive water loss. Previously, Barnes and Davison (1988) had ascribed part of the influence of elevated O_3 on diminishing winter hardiness to enhanced rates of cuticular transpiration in 1-year-old needles. Earlier work by Cape and Percy (1996) demonstrated the influence of wax development on needle water loss in four spruce species, including Norway spruce.

Taken together, these data provide evidence for a direct action of O_3 in diverting increased proportions of C from reductive to decarboxylative pathways, and allocating more to formation of longer-chain unsaturated fatty acids. As secondary alcohols form tubes and fatty acids form amorphous films, this hypothesis is further substantiated by the tube density data reported above. As wax quantity was not altered under 2× O_3, *de novo* wax synthesis is unlikely to have been directly affected. It is interesting that for both fatty acids and nonacosane diols a significant ($P < 0.05$) O_3 × year interaction was determined, particularly in the case of diols as this class, although not predominant, also forms crystalline structures often aggregated with secondary alcohol tubes between stomatal rows.

Plant species tend to have ecophysiological adaptations to climatic demands of their natural habitats for cuticular water permeability (Schreiber and Riederer, 1996). Cuticular water permeability is not correlated to cuticle thickness or to degree of wax coverage (Reiderer and Schreiber, 2001), although there is also evidence for discrete layers of cuticular wax that have markedly different chemical composition (Jetter et al., 2000). In vitro experiments (Percy et al., 1994; Jetter et al., 1996) simulating in vivo EW structure and chemical compositions have clearly demonstrated that there is no known mechanism for oxidative transformation of in situ waxes at even supra-realistic O_3 concentrations. Epicuticular wax tubes, as well as tube aggregates comprising nonacosan-10-ol and isomers of nonacosane diols always form tubular shapes when crystallized from organic solvents (Jetter and Riederer, 1994). The presence of the (S)-enantiomer of nonacosan-10-ol is necessary for the formation of the highly specific and regular EW tubules formed under the kinetic regime in most conifers (Jetter and Riederer, 1994). Although their in vitro work points toward a spontaneous transition between two crystal modifications of (S)-nonacosan-10-ol being

responsible for EW structural changes reported due to aging and air pollutants, in this study we provide in vivo evidence from 3 years of needle exposure that structural changes seen under the SEM can be explained by altered patterns of C allocation during biosynthesis as determined by GC.

5. Conclusions

Multi-year, free-air, controlled exposure of 50-year-old Norway spruce has demonstrated that current-year needle EW physicochemical characteristics of trees are responsive to elevated O_3 when measured against interannual climate variation. Ozone exposure in the $2\times$ O_3 treatment calculated using the European AOT40 critical level, and the modified (annual averaging time) North American air quality standard 4th highest daily maximum 8-h average O_3 concentration was at the higher end of current measured ambient exposures. Tube-forming secondary alcohols decreased under elevated O_3, whereas amorphous film-forming fatty acids increased. Wax tube density on the needle surface decreased. These changes could affect needle surface properties, and in other species, have been shown to have important biological consequences.

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References

Alexou, M., Hofer, N., Liu, X., Renneberg, H., Haberer, K., 2007. Significance of ozone exposure for inter-annual differences in primary metabolites of old-growth beech (*Fagus sylvatica* L.) and Norway spruce (*Picea abies* L.) trees in a mixed forest stand. Plant Biology 9, 227–241.

Ashmore, M., 2005. Assessing the future global impacts of ozone on vegetation. Plant, Cell and Environment 28, 949–964.

Baker, E.A., 1982. Chemistry and morphology of plant epicuticular waxes. In: Cutler, D.J., Alvin, K.L., Price, C.E. (Eds.), The Plant Cuticle. Academic Press, London, pp. 139–165.

Barnes, J.D., Brown, K.A., 1990. The influence of ozone and acid mist on the amount and wettability of the surface waxes in Norway spruce (*Picea abies* (L.) Karst.). New Phytologist 114, 531–535.

Barnes, J.D., Davison, A.W., 1988. The influence of ozone on the winter hardiness of Norway spruce (*Picea abies* (L.) Karst.). New Phytologist 108, 159–166.

Barnes, J.D., Davison, A.W., Booth, T.A., 1988. Ozone accelerates structural degradation of epicuticular wax on Norway spruce needles. New Phytologist 110, 309–318.

Canadian Council of Ministers of the Environment (CCME), 2000. Canada-wide Standards for Particulate Matter (PM) and Ozone Available from: http://www.ccme.ca.

Cape, J.N., Percy, K.E., 1993. Environmental influences on the development of spruce needle cuticles. New Phytologist 125, 787–799.

Cape, J.N., Percy, K.E., 1996. The interpretation of leaf drying curves. Plant, Cell and Environment 19, 356–361.

Cape, J.N., Percy, K.E., 1998. Use of needle epicuticular wax chemical composition in the early diagnosis of Norway spruce (*Picea abies* (L.) Karst.) decline in Europe. Chemosphere 36, 895–900.

Dixon, M., Le Thiec, D., Garrec, J.P., 1998. Reactions of Norway spruce and beech trees to two years of ozone exposure and episodic drought. Environmental and Experimental Botany 40, 77–91.

Federal Register, March 27, 2008. National Ambient Air Quality Standards for Ozone. Rules and Regulations. US EPA, 40 CFR Parts 50 and 58 Final Rule, vol. 73, No. 60.

Fowler, D., Cape, J.N., Coyle, M., Flechard, C., Kuylenstierna, J., Hicks, K., Derwent, D., Johnson, C., Stevenson, D., 1999. The global exposure of forests to air pollutants. Water, Air and Soil Pollution 116, 5–32.

Frannich, R.A., Wells, L.G., Holland, P.T., 1978. Epicuticular wax of *Pinus radiata* needles. Phytochemistry 17, 1617–1623.

Fuhrer, J., Skärby, L., Ashmore, M.R., 1997. Critical levels for ozone effects on vegetation in Europe. Environmental Pollution 97, 91–106.

Gordon, D.C., Percy, K.E., Riding, R.T., 1998. Effects of UV-B radiation on epicuticular wax production and chemical composition of four *Picea* species. New Phytologist 138, 441–449.

Günthardt, M.S., 1986. Epicuticular waxes of needles of *Pinus cembra*, *Pinus sylvatica*, and *Picea abies*. European Journal of Forest Pathology 16, 400–408.

Günthardt-Goerg, M.S., Keller, T., 1987. Some effects of long-term ozone fumigation on Norway spruce II. Epicuticular wax and stomata. Trees 1, 145–150.

Heath, R.L., Taylor Jr., G.E., 1997. Physiological processes and plant responses to ozone exposure. In: Sandermann, H., Welburn, A.R., Heath, R.L. (Eds.), Forest Decline and Ozone: a Comparison of Controlled Chamber and Field Experiments. Springer-Verlag, Berlin, pp. 317–364.

Hendrey, G.R., Ellsworth, D.S., Lewin, K.F., Nagy, J., 1999. A free-air enrichment system for exposing tall forest vegetation to elevated atmospheric CO_2. Global Change Biology 5, 293–309.

Holloway, P.J., 1970. Surface factors affecting the wetting of leaves. Pesticide Science 1, 156–163.

Jeffree, C.E., Baker, E.A., Holloway, P.J., 1975. Ultrastructure and recrystallization of plant epicuticular waxes. New Phytologist 75, 539–549.

Jenks, M.A., Ashworth, E.N., 1999. Plant epicuticular waxes: function, production and genetics. Horticultural Reviews 23, 1–68.

Jetter, R., Riederer, M., 1994. Epicuticular crystals of noacosan-10-ol: in vitro reconstitution and factors influencing crystal habits. Planta 195, 257–270.

Jetter, R., Riederer, M., Lendzian, K.J., 1996. The effects of dry O_3, SO_2 and NO_2 on reconstituted epicuticular wax tubules. New Phytologist 133, 207–216.

Jetter, R., Schäffer, S., Riederer, M., 2000. Leaf cuticular waxes are arranged in chemically and mechanically distinct layers: evidence from *Prunus laurocerasus* L. Plant, Cell and Environment 23, 619–628.

Kangasjarvi, J., Jaspers, P., Kollist, H., 2005. Signalling and cell death in ozone-exposed plants. Plant, Cell and Environment 28, 1021–1036.

Karnosky, D.F., Percy, K.E., Xiang, B., Callan, B., Noormets, A., Mankovska, B., Hopkin, A., Sober, J., Jones, W., Dickson, R.E., Isebrands, J.G., 2002. Interacting elevated CO_2 and tropospheric O_3 predisposes aspen (*Populus tremuloides* Michx.) to infection by rust (*Melampsora medusae* f. sp. *tremuloidae*). Global Change Biology 8, 329–338.

Karnosky, D.F., Pregitzer, K.S., Zak, D.R., Kubiske, M.E., Hendrey, G.R., Weinstein, D., Nosal, M., Percy, K.E., 2005. Scaling ozone responses of forest trees to the ecosystem level in a changing climate. Plant, Cell and Environment 28, 965–981.

Karnosky, D.F., Skelly, J.M., Percy, K.E., Chappelka, A.H., 2007a. Perspectives regarding 50 years of research on effects of tropospheric ozone air pollution on U.S. forests. Environmental Pollution 147, 489–506.

Karnosky, D.F., Werner, H., Holopainen, T., Percy, K., Oksanen, T., Oksanen, E., Heerdt, C., Fabian, P., Nagy, J., Heilman, W., Cox, R., Nelson, N., Matyssek, R., 2007b. Free-air exposure systems to scale up ozone research to mature trees. Plant Biology 9, 181–190.

Karnosky, D.F., Zak, D.R., Pregitzer, K.S., Awmack, C.S., Bockheim, J.G., Dickson, R.E., Hendrey, G.R., Host, G.E., King, J.S., Kopper, B.J., Kruger, E.L., Kubiske, M.E., Lindroth, R.L., Mattson, W.J., McDonald, E.P., Noormets, A., Oksanen, E., Parsons, W.F.J., Percy, K.E., Podila, G.K., Riemenschneider, D.E., Sharma, P., Thakur, R.C., Sober, A., Sober, J., Jones, W.S., Anttonen, S., Vapaavuori, E., Mankovska, B., Heilman, W.E., Isebrands, J.G., 2003. Tropospheric O_3 moderates responses of temperate hardwood forests to elevated CO_2: a synthesis of molecular to ecosystem results from the Aspen FACE project. Functional Ecology 17, 289–304.

Kolb, T.E., Matyssek, R., 2001. Limitations and perspectives about scaling ozone impacts in trees. Environmental Pollution 115, 373–393.

Langbartels, C., Heller, W., Fuhrer, G., Lippert, M., Simons, S., Sandermann, H., 1998. Memory effects in the action of ozone on conifers. Ecotoxicology and Environmental Safety 41, 62–72.

Lütz, C., Heinzmann, U., Gülz, P.G., 1990. Surface structures and epicuticular wax composition of spruce needles after long-treatent with ozone and acid mist. Environmental Pollution 64, 313–322.

Mankovská, B., Percy, K.E., Karnosky, D.F., 2005. Impacts of greenhouse gases on epicuticular waxes of *Populus tremuloides* Michx.: results from an open-air exposure and a natural O_3 gradient. Environmental Pollution 137, 580–586.

Manning, W.J., 2005. Establishing a cause and effect relationship for ambient ozone exposure and tree growth in the forest: progress and an experimental approach. Environmental Pollution 137, 443–454.

Matyssek, R., Sandermann, H., 2003. Impact of ozone on trees: an ecophysiological perspective. Progress in Botany 64, 349–404. Springer Verlag, Heidelberg.

Matyssek, R., Bahnweg, G., Ceulmans, R., Fabian, P., Grill, D., Hanke, D.E., Kraigher, H., Oßwald, W., Renneberg, H., Sandermann, H., Tausz, M., Wieser, G., 2007. Synopsis of the CASIROZ case study: carbon sink strength of *Fagus sylvatica* L. in a changing environment—experimental risk assessment of mitigation by chronic ozone impact. Plant Biology 9, 163–180.

McLeod, A.R., Fackrell, J.E., Alexander, K., 1985. Open-air fumigation of field crops: criteria and design for a new experimental system. Atmospheric Environment 19, 1639–1649.

Mikkelsen, J.D., 1978. The effects of inhibitors on the biosynthesis of the long chain lipids with even carbon numbers in barley spike epicuticular wax. Carlsberg Research Communications 43, 15–35.

Mikkelsen, T.N., Ro-Poulsen, H., 1994. Exposure of Norway spruce to ozone increases the sensitivity of current year needles to photoinhibition and desiccation. New Phytologist 128, 153–163.

Nunn, A.J., Reiter, I.M., Häberle, K.-H., Langebartels, C., Bahnweg, G., Pretzsch, H., Sandermann, H., Matyssek, R., 2005. Response patterns in adult forest trees to chronic ozone stress: identification of variations and consistencies. Environmental Pollution 136, 365–369.

Nunn, A.J., Reiter, I.M., Häberle, K.-H., Werner, H., Langbartels, C., Sandermann, H., Heerdt, C., Fabian, P., Matyssek, R., 2002. "Free-air" ozone canopy fumigation in an old-growth mixed forest: concept and observations in beech. Phyton 42, 105–119.

Percy, K.E., Baker, E.A., 1988. Effects of simulated acid rain on leaf wettability, rain retention and uptake of some inorganic ions. New Phytologist 108, 75–82.

Percy, K.E., Baker, E.A., 1990. Effects of simulated acid rain on epicuticular wax production, morphology, chemical composition and on cuticular membrane thickness in two clones of Sitka spruce (*Picea sitchensis* (Bong.) Carr.). New Phytologist 116, 79–87.

Percy, K.E., Baker, E.A., 1991. Effects of simulated acid rain on needle wettability and rain retention by two Sitka spruce (*Picea sitchensis*) clones. Canadian Journal of Forest Research 21, 694–697.

Percy, K.E., Awmack, C.S., Lindroth, R.L., Kubiske, M.E., Kopper, B.J., Isebrands, J.G., Pregitzer, K.S., Hendrey, G.R., Dickson, R.E., Zak, D.R., Oksanen, E., Sober, J., Harrington, R., Karnosky, D.F., 2002. Altered performance of forest pests under CO_2- and O_3-enriched atmospheres. Nature 420, 403–407.

Percy, K.E., Jensen, K.F., McQuattie, C.J., 1992. Effects of ozone and acidic fog on red spruce needle epicuticular wax production, chemical composition, cuticular membrane ultrastructure and needle wettability. New Phytologist 122, 71–80.

Percy, K.E., Krause, C.R., Jensen, K.F., 1990. Effects of ozone and acidic fog on red spruce needle epicuticular wax ultrastructure. Canadian Journal of Forest Research 20, 117–120.

Percy, K.E., Legge, A.H., Krupa, S.V., 2003. Tropospheric ozone: a continuing threat to global forests? In: Karnosky, D.F., Percy, K.E., Chappelka, A.H., Simpson, C., Pikkarainen, J. (Eds.), Air Pollution, Global Change and Forests in the New Millenium. Developments in Environmental Science, vol. 3. Elsevier, Oxford, pp. 85–118.

Percy, K.E., McQuattie, C.J., Rebbeck, J.A., 1994. Effects of air pollutants on epicuticular wax chemical composition. In: Percy, K.E., Cape, J.N., Jagels, R., Simpson, C.J. (Eds.), Air Pollutants and the Leaf Cuticle. NATO ASI Series, vol. G36. Springer-Verlag, Berlin, Heidelberg, pp. 67–79.

Pretzsch, H., Kahn, M., Grote, R., 1998. Die Fichten-Buchen-Mischbestände des Sonderforschungsbereiches, "Wachstum oder Parasitenabwehr?" im Kranzberger Forst. Forstwissenschaftliches Centralblatt 117, 241–257.

Prügel, B., 1994. Contribution à l'étude des modifications chimiques des cires cuticulaires de *Picea abies* (L.) Karst. et de *Picea sitchensis* (Bong.) Carr. en relation avec le dépérissement forestier et l'augmentation du CO_2 dans l'atmosphère. Dissertation. Université de Nancy, Nancy, France.

Reiderer, M., Schreiber, L., 2001. Protecting against water loss: analysis of the barrier properties of plant cuticles. Journal of Experimental Botany 52, 2023–2032.

Schreiber, L., Riederer, M., 1996. Ecophysiology of cuticular transpiration: comparative investigation of cuticular water permeability of plant species from different habitats. Oceologia 107, 426–432.

Vingarzan, R., 2004. A review of surface ozone background levels and trends. Atmospheric Environment 38, 3431–3442.

von Wettstein-Knowles, P., 1995. Biosynthesis and genetics of waxes. In: Hamilton, R.J. (Ed.), Waxes: Chemistry, Molecular Biology and Functions. The Oily Press, Dundee, UK, pp. 91–156.

Werner, H., Fabian, P., 2002. Free-air fumigation of mature trees – a novel system for controlled ozone enrichment in grown-up beech and spruce canopies. Environmental Science and Pollution Research 9, 117–121.