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# Age-dependent xylogenesis in timberline conifers

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## Summary

• Neither anatomical change nor physiological abnormalities have been observed in the cambia of older trees. However, different sensitivity and period of significant responses to climate suggest the existence of some age-related change in the patterns of cambial activity and/or wood cell formation.

• Here, weekly cambial activity and timing and duration of xylem cell enlargement and wall thickening were compared in adult (50–80 yr) and old (200–350 yr) trees of *Larix decidua*, *Pinus cembra* and *Picea abies* at the Alpine timberline during 2004 and 2005.

• Timings and durations of xylogenesis differed between adult and old trees, with 2–3 wk shorter cambial activity found in the latter. The delayed onset of cambium division and lower cell production in old trees, with respect to adult trees, led to reductions of 15–20% in the overall duration of xylem differentiation.

• These results demonstrate that cambial dynamics change during the tree lifespan and that the time window of tree-ring production shortens with age. Variations in the period of xylem growth may be the cause of age-dependent responses to climate. The observed shorter xylogenesis in older plants at the Alpine timberline could be related to a size effect and not just to age *per se*.

**Key words:** Alps, cambial activity, cell differentiation, timberline, tree age, tree ring, wood formation, uniformitarian principle.

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## Introduction

Throughout the lifespan of the plants, cambial cells maintain their ability to divide and, even in 4700-yr-old *Pinus longaeva*, no evidence has been found of an age-related change in the cambial meristems (Lanner & Connor, 2001; Lanner, 2002). However, older trees, or older parts of the stem, exhibit thinner tree rings than younger trees or younger parts of the stem, resulting in a declining trend of ring-width series across the diameter from pith to bark (Cook *et al.*, 1990; Panyushkina *et al.*, 2003). This long-term growth pattern, typical in all trees, is related to advancing age and, as a result, increasing size (Fritts, 1976). Trees with larger stem diameters show higher numbers of cambial cells around the circumference, and, as a consequence, need fewer cells on the radial files to maintain the required water supply for the crown. Is there a specific age-related timing associated with this changing tree ring width? Compared with younger trees, the thin tree rings of old trees could result from either slower growth rates or shorter growing periods. The question whether the dynamics of intra-annual wood growth, such as timing, duration and rate, changes over a tree's lifespan remains unanswered.

The period when xylem is developing corresponds to the time window during which trees and their wood cells are open to directly receive environmental signals (Frankenstein *et al.*, 2005), resulting in tree rings being an archive of long-term meteorological proxy data (Alley, 2001; Bräker, 2002). Dendrochronology uses these proxies, which originate from the

bridging technique of shorter time-series of tree-ring widths. The technique requires rings produced by young plants to be connected and alternate in the chronology with rings produced by old trees (Kaennel & Schweingruber, 1995). These procedures are based on the adoption of James Hutton's uniformitarian principle, which states that the mechanisms linking biological activities in tree rings to environmental conditions remain unaltered over time (Hutton, 1788 in Bräker, 2002; Hughes, 2002). One assumption behind the adoption of Hutton's principle in biology is that no difference in timing and duration of tree-ring formation should occur during the lifespan of a tree, but this has never been proved.

In climate–growth relationships based on tree-ring chronology, tree age is never considered except to remove low-frequency variations from the time series (Cook *et al.*, 1990; Kaennel & Schweingruber, 1995). Growth responses are supposed to be age-independent on standardized series even if Bräker (2002) asserts that the elimination of ageing could result in considerable differences in the remaining residual signal. In *Pinus cembra* and *Larix decidua*, young trees (< 100 yr) were weakly influenced by climate variability, whereas growth responses to maximum temperatures were higher in older trees (Carrer & Urbinati, 2004). The responses of radial growth to temperatures in *Picea glauca* showed different values between trees under and over 200 yr old, although there were unclear site-specific components in the results (Szeicz & MacDonald, 1994). Shifts in temperature responses were also observed: the period with significant positive responses to growing season temperatures became shorter with tree age (Szeicz & MacDonald, 1995). At an anatomical level, Deslauriers *et al.* (2003) described xylem cell formation in *Abies balsamea*, observing early onsets of radial enlargement in stems of younger trees. It is reasonable to suppose from these results that there are age-related changes in patterns or timings of cambial activity and/or xylem cell formation.

This paper tests the hypothesis that timing and duration of xylogenesis, one of the most important features of stem radial growth, changes with tree age. Xylem cell production and differentiation were analysed at weekly scale for 2 yr on 15 adult (50–80 yr) and 15 old (200–350 yr) trees of the three main conifers in the Alps in order to compare age-related intra-annual dynamics of tree-ring formation.

## Materials and Methods

### Study site and tree selection

The study site is located at 2080 m above sea-level (asl) close to the Cinque Torri mountain group (Cortina d'Ampezzo, BL) in the eastern Italian Alps (46°27′ N, 12°08′ E), on a south-facing and shallow calcareous soil. Mean annual temperature is 2.4°C with mean annual precipitation of 1150 mm and June–August precipitation of *c.* 500 mm (Carraro *et al.*, 2001). The forest is composed of mixed and open clumps of trees of *Larix decidua* Mill., *Pinus cembra* L. and *Picea abies* (L.) Karst., corresponding to the timberline ecotone (Fig. 1a,b). Two forest stands located 250 m from one another, at the same altitude, with similar aspect and stand density but with trees of different age classes (adult 40–70 yr-old trees and old 200–350 yr-old trees) were selected. Fifteen dominant trees of *L. decidua*, *P. cembra* and *P. abies* (five trees per species) with upright stems and homogeneous diameters were chosen in each stand (Table 1). Trees with polycormic stems, partially dead crowns, reaction wood or evident damage were avoided.

### Xylem sampling and preparation

Tree-ring formation was studied from April to October 2004 and 2005. Wood microcores (15 mm long, 2 mm diameter) were collected weekly on the stem from 30 cm below to 30 cm above breast height (1.3 m) using Trephor (Rossi *et al.*, 2006a). The very small wound inflicted by the thin Trephor piercing tube and the consequently narrow traumatized tissues around the sampling points allows repetitive samplings during the year without any tree reactions (Forster *et al.*, 2000). Wood samples were always taken at least 5 cm apart to avoid getting resin ducts on adjacent cores, which is a common disturbance reaction in conifers (Deslauriers *et al.*, 2003). About 1560 microcores were collected from the 30 trees sampled over the two years. However, only some tens of sections showed tangentially oriented clusters of resin ducts in the developing tree ring, suggesting that the disturbance reaction of the xylem to the wound only occasionally spread to adjacent samples. Samples usually contained the previous four or five tree rings and the developing annual layer with the cambial zone and

**Table 1** Age and size of the sampled trees

| Species | Age | | Diameter (cm) | | Height (m) | |
|---|---|---|---|---|---|---|
| | Adult | Old* | Adult | Old | Adult | Old |
| *Larix decidua* | 60.6 ± 18.7 | 288.0 ± 43.3 | 26.8 ± 4.8 | 59.5 ± 8.9 | 11.6 ± 1.0 | 19.2 ± 3.1 |
| *Pinus cembra* | 60.4 ± 7.9 | 244.0 ± 25.2 | 29.2 ± 8.6 | 79.6 ± 10.6 | 10.1 ± 0.7 | 17.5 ± 1.6 |
| *Pinus abies* | 45.0 ± 8.7 | 297.5 ± 32.0 | 22.4 ± 6.2 | 81.2 ± 13.4 | 10.4 ± 0.6 | 22.7 ± 2.9 |

Values are means ± SD.
*Ages in old trees could be underestimated because of decayed inner stems.

 

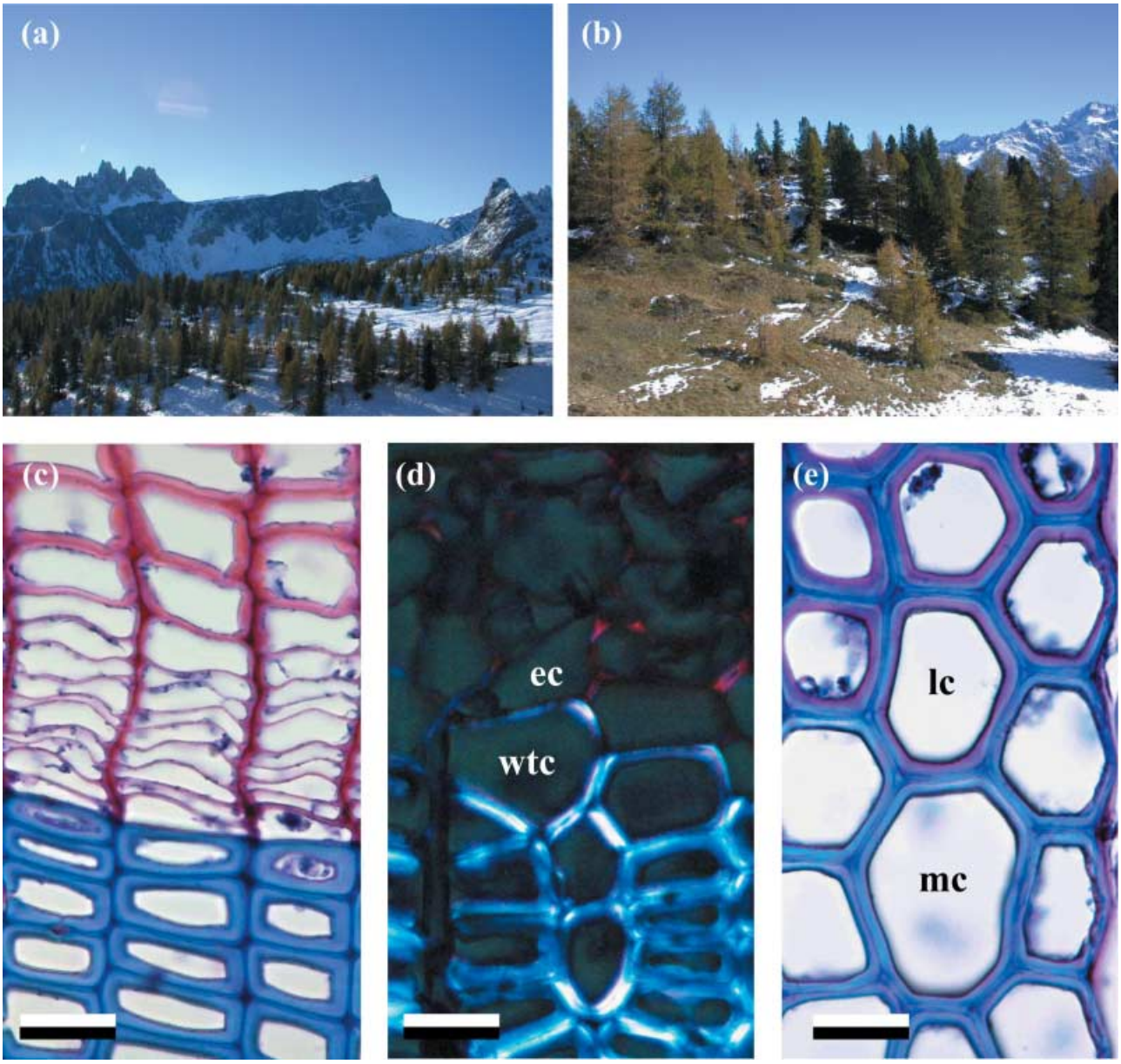


**Fig. 1** (a,b) Mixed open forests of *Larix decidua*, *Pinus cembra* and *Picea abies* in the Cinque Torri area (Dolomites, Italian Alps) corresponding to the timberline ecotone. (c) Dormant cambial cells of *P. cembra* at the end of summer. (d) Enlarging (ec) and wall thickening (wtc) cells of *P. cembra* under polarized light. (e) Cells completing lignification (lc) and mature cells (mc) of *L. decidua* stained with cresyl violet acetate. Bars, 20 μm. Photographs by S. Rossi.

adjacent phloem. Dead outer bark was removed before sampling. The collected microcores were placed in Eppendorf microtubes with an ethanol solution (50% in water) and stored at 5°C to avoid tissue deterioration. Each sample was oriented by marking the transverse side with a pencil under a stereo-microscope at ×10–20 magnification. The microcores were dehydrated with successive immersions in ethanol and D-limonene, embedded in paraffin and transverse sections of 6–10 μm thickness were cut with a rotary microtome (Rossi *et al.*, 2006a).

## Analysis of xylem development

Sections were stained with cresyl violet acetate (0.16% in water) and examined within 10–25 min with visible and polarized light at magnifications of ×400–500 magnifications to distinguish the developing xylem cells. For each sample, the radial number of cells in the cambial zone, radial enlargement phase, cell wall thickening phase and mature cells were counted along three radial files according to Rossi *et al.* (2006b). In cross-section, cambial cells were characterized by thin cell walls and

small radial diameters (Timell, 1980; Fig. 1c). During cell enlargement, the tracheids contained a protoplast that was still enclosed in the thin primary wall but with radial diameter at least twice that of a cambial cell. In this phase, deformed files of tracheids were frequently observed, representing the enlargement process occurring despite strong compression between xylem tissues and bark. Observations under polarized light discriminated between enlarging and cell wall thickening tracheids. Because of the arrangement of the cellulose microfibrils, the developing secondary walls glistened when observed under polarized light (Fig. 1d), whereas no glistening was observed in enlargement zones where the cells were still just composed of primary wall (Abe *et al.*, 1997). The progress of cell wall lignification was detected with cresyl violet acetate reacting with the lignin (Antonova & Shebeko, 1981). Lignification appeared as a colour change from violet to blue (Fig. 1e). This colour change over the whole cell wall revealed the end of lignification and the reaching of tracheid maturity (Gričar *et al.*, 2005).

The cell numbers in the three radial files per tree were averaged and used to assess onset, ending and duration of xylem growth. In spring, when at least one row of cells was observed in the enlarging phase, xylem formation was considered to have begun. In late summer, when no cells were observed in wall thickening and lignification, xylem formation was considered accomplished.

### Rate of cell production

The total number of cells produced during 2004 and 2005 was calculated as the sum of all producing (cells in the cambial zone), developing (enlarging and secondary wall thickening and lignification cells) and mature tracheids observed during the season. For each tree, growth curves were fitted with Gompertz functions using the NLIN procedure (NonLINear regression in SAS (1999)) with the Marquardt iterative method (Motulsky & Ransnas, 1987). The Gompertz function was defined as:

$$y = A \exp(-e^{\beta - \kappa t}) \qquad \text{Eqn 1}$$

where $y$ is the weekly cumulative sum of cells; $t$ is the time computed in day of year; $A$ is the upper asymptote (maximum growth expressed as cell number); $\beta$ is the $x$-axis placement parameter; and $\kappa$ the rate of shape change (Rossi *et al.*, 2006c). The residuals were regressed onto the model partial derivatives with respect to the Gompertz parameters until the estimates converged. Several possible starting values were specified for each parameter, so that the NLIN procedure evaluated each combination of initial values using the interactions producing the smallest residual sums of squares. To evaluate the general goodness-of-fit of each regression, the proportion of variation accounted for ($R^2$), standard errors and linearity of each parameter and distribution of the residuals were calculated (Motulsky & Ransnas, 1987; Ratkowsky, 1990; Draper & Smith, 1998). From the estimated constants, the weighted mean absolute rate of cell production ($r$) was calculated according to Richards (1959):

$$r = \frac{A\kappa}{2(\nu + 2)} \qquad \text{Eqn 2}$$

where the parameter $\nu$ was set at 0.0001, since the Gompertz function is a special case of the Richards function when $\nu = 0$ (Deslauriers *et al.*, 2003).

### Statistical analyses

For each tree, onset, ending and duration of cell differentiation were computed in days of the year. Comparisons between groups in timing of differentiation, number of tracheids in the tree ring and rate of cell production were performed using analysis of variance (ANOVA). Tests of the normal distribution and homogeneity of variances were performed using the Shapiro–Wilk and Levene tests, respectively (Scherrer, 1984). Both tests produced nonsignificant results ($P > 0.05$) thus assuring the achievement of the required conditions for the ANOVA test.

## Results

### Cambial activity

In the cambial zones, similar but delayed annual dynamics were observed between age classes and species (Figs 2 and 3). In spring and autumn, when no cells were produced, the dormant cambium was composed of five to eight closely spaced cells in all observed trees. In May–June, the cambial zone began to widen rapidly (within a week) as the number of cells increased revealing the onset of cell division. The earliest start of cambial activity was observed in adult *P. cembra* at the beginning of May in 2004 and at the end of April in 2005, and 2 wk later in adult *L. decidua* and *P. abies*. In old trees, cambial activity started 2–3 wk later than in adult trees of the same species.

Once annual activity had ended and the cambium stopped dividing, the number of cells in the cambial zone gradually decreased to the minimum value, corresponding to quiescence conditions of the meristems. Cambial activity terminated from the end of July in *P. cembra* to the beginning of August in *P. abies* and *L. decidua*. Cambial activity ended at about the same time in adult and old trees because of the contemporary reaching of the minimum cell number in the cambial zone. Different durations of cell production were observed between age classes. In 2004, cambial activity lasted from 65 to 72 d in adult trees and from 48 to 55 d in old trees (Fig. 2), corresponding to a 15–30% reduction in the overall period for cell production in the older trees.

 

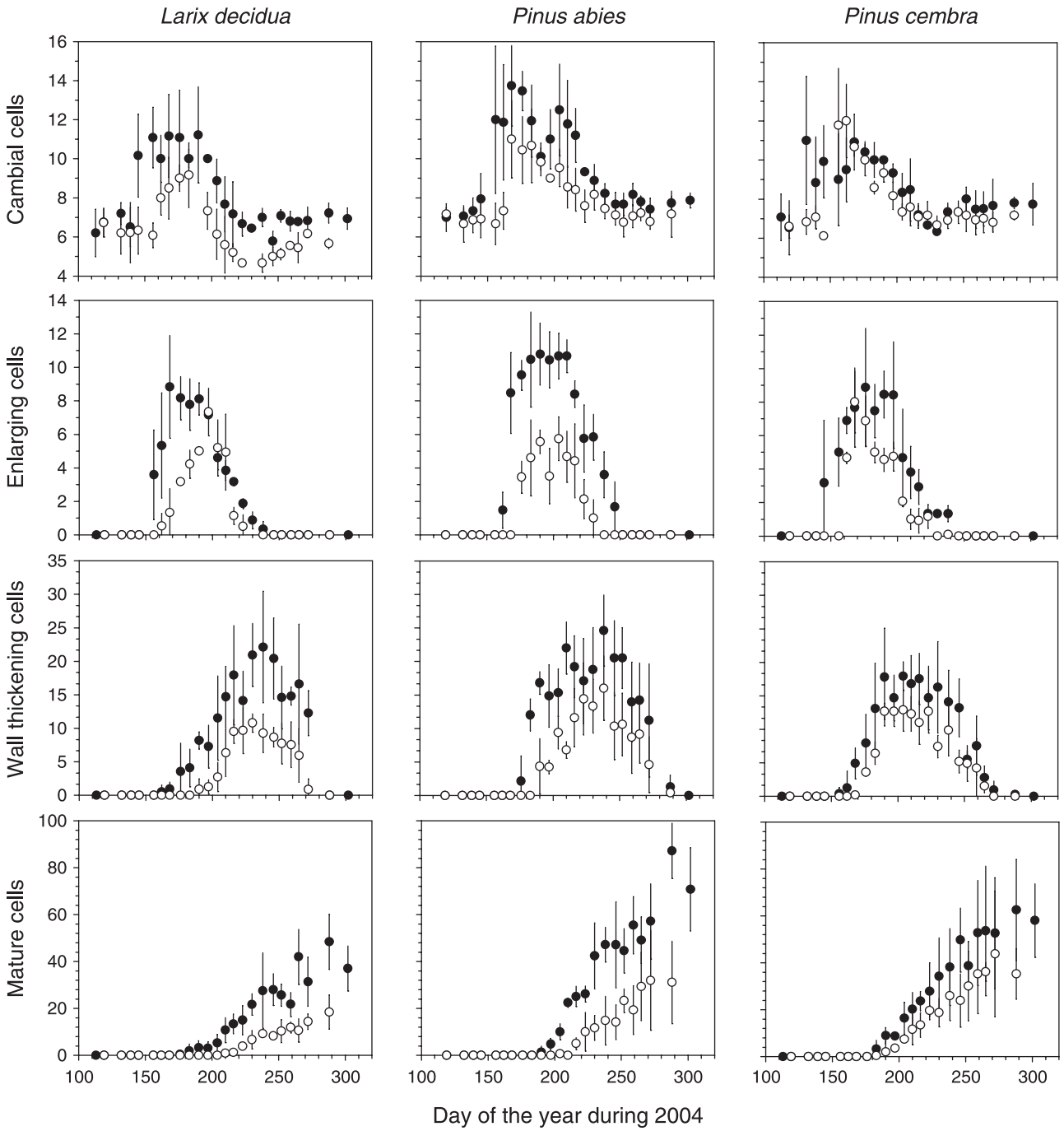


**Fig. 2** Numbers of cells in the cambial zone, in radial enlargement, in wall thickening and lignification and mature cells in adult (closed circles) and old (open circles) trees during 2004. Error bars indicate ± SD for five trees per sampling date.

## Pattern of xylem differentiation

Adult and old trees showed the same trend of xylem formation but with different timings of cell differentiation. The radial files of differentiating cells had a clear pattern of variation during the year in all species (Figs 2 and 3), related to the number of tracheids in the different development phases and resulting in two delayed bell-shaped curves (radial enlargement and wall thickening) and a growing curve (mature cells). In *L. decidua* in 2004, for example, at the beginning of cell differentiation (days of the year 119–132, Fig. 2), the number of tracheids undergoing radial enlargement increased. Once wall thickening and lignification began (day of the year 132, Fig. 2), the number of cells in radial enlargement gradually

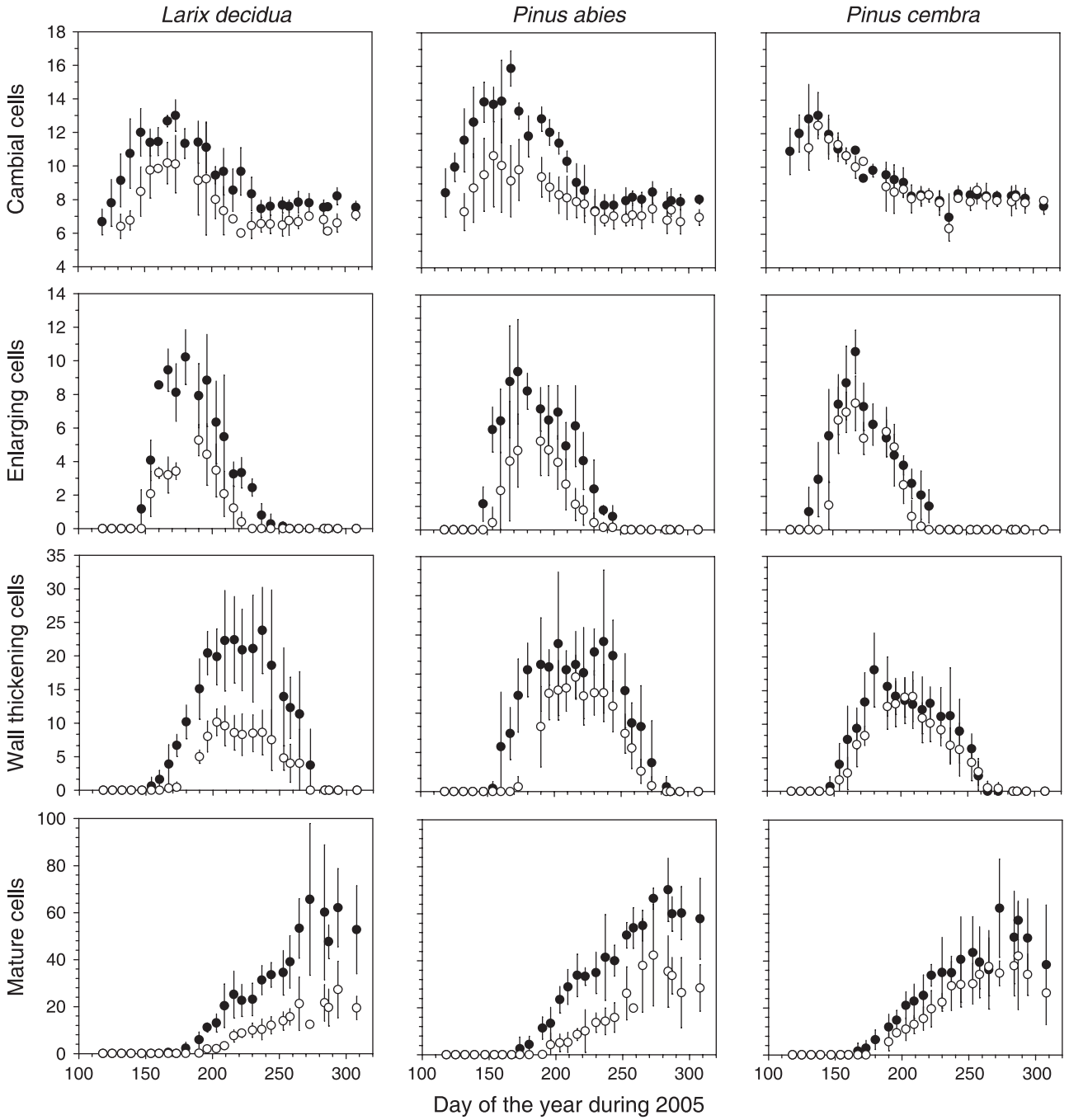


**Fig. 3** Numbers of cells in the cambial zone, in radial enlargement, in wall thickening and lignification and mature cells in adult (closed circles) and old (open circles) trees during 2005. Error bars indicate ± SD for five trees per sampling date.

reduced while the number of cells in secondary wall formation increased. The curves of mature cells were associated with the gradual accumulation of mature tracheids in the tree ring.

Compared with adult trees, old ones had between 30 and 60% fewer cells (Fig. 4) in each development phase and for mature tracheids in both *L. decidua* ($F = 45.06$, $P < 0.0001$) and *P. abies* ($F = 91.01$, $P < 0.0001$). However, no difference was observed in *P. cembra* ($F = 4.45$, $P > 0.05$). The three species showed different numbers of tracheids in the tree ring, with higher cell production observed in *P. abies*, but no difference was detected between the two years (ANOVA, $P > 0.05$).

## Timing and duration of xylem differentiation

The onset of radial enlargement lasted from mid-May to mid-June, occurring 7–17 d earlier in adult trees than in old

 

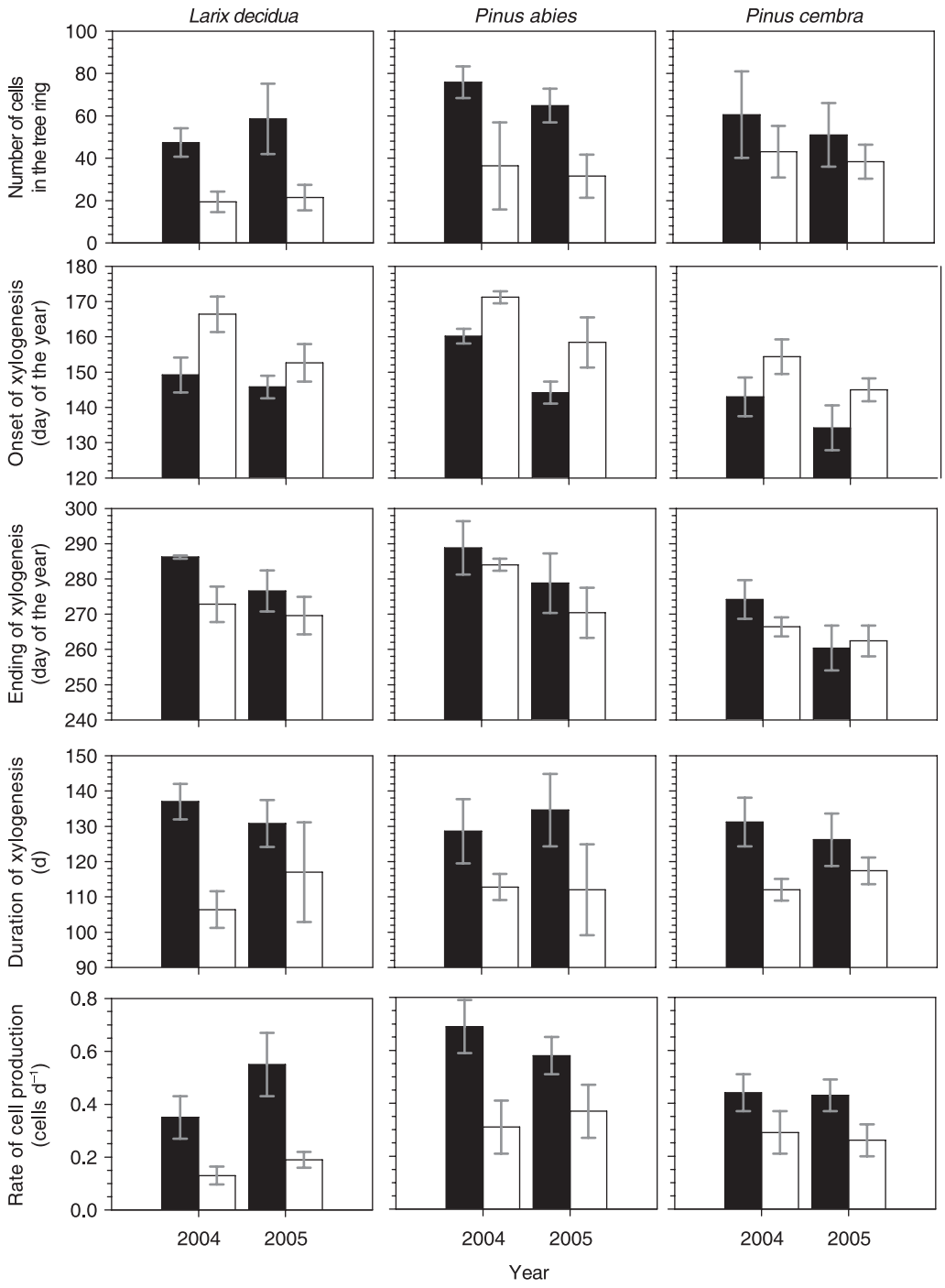


**Fig. 4** Number of cells produced in a radial file of the tree-ring, onset, ending and duration of xylogenesis computed in days of the year and rate of cell production (number of cells produced per day) in adult (closed bars) and old (open bars) trees. Error bars indicate ± SD among five trees.

trees (ANOVA, $F = 68.96$ ($P < 0.0001$) for 2004 and $F = 33.78$ ($P < 0.0001$) for 2005). Significant differences were detected between the three species (ANOVA, $F = 38.19$ ($P < 0.0001$) for 2004 and $F = 1560$ ($P < 0.0001$) for 2005) (Fig. 4), with the earliest start observed in *P. cembra*. Tracheids of *L. decidua* began cell differentiation after *P. cembra* and before *P. abies*.

Xylogenesis was considered terminated when no other cell was observed in radial enlargement, wall thickening or lignification. The end of cell differentiation lasted from mid-September to mid-October (Fig. 4). In 2004, cell differentiation ended earliest in old trees (ANOVA, $F = 17.43$, $P < 0.0001$), while no difference was observed between

the age classes in 2005 (ANOVA, $P > 0.05$). Instead, there were significant differences among the three species (ANOVA, 17.43 ($P < 0.0001$) for 2004 and $F = 11.37$ ($P < 0.0001$) for 2005), with average differences of 10 d between *P. cembra* and *L. decidua* and 14 d between *P. cembra* and *P. abies*. Both adult and old trees of *P. abies* were the last to terminate secondary wall formation in October

The duration of xylogenesis, i.e. the time required to complete cell differentiation for all the tracheids forming the tree ring, varied between 106 d and 137 d (Fig. 4) with significant differences between adult and old trees (ANOVA, $F = 103.45$ ($P < 0.0001$) for 2004 and $F = 17.45$ ($P < 0.001$) for 2005). Longer durations were estimated for adult trees, which required an average of 132.2 d to complete cell differentiation in 2004 and 130.5 d in 2005. For old trees, xylogenesis duration was between 110.4 d and 115.3 d in 2004 and 2005, respectively. No difference was detected between the three species in both years (ANOVA, $P > 0.05$).

Interaction effects between age and species were also tested. Significant results were found only for the duration of xylogenesis in 2004 (ANOVA, $F = 4.33$, $P < 0.05$), indicating that the reduction in the duration of xylem differentiation between adult and old trees was not uniform in the three species for that year. A higher reduction was detected in *L. decidua*, with a difference of 31 d in the overall duration of xylogenesis between adult and old trees.

Along a radial file, different rates of cell production were calculated between the age classes (Fig. 4). Depending on the species, from 0.35 to 0.69 cells $d^{-1}$ were produced in adult trees. Lower rates of cell production were observed in old trees, ranging from 0.13 to 0.38 cells $d^{-1}$ (ANOVA, $F = 33.83$, 74.22 and 25.34 with $P < 0.0001$ for *L. decidua*, *P. abies*, and *P. cembra*, respectively).

## Discussion

With their long lifespan and their 'eternally youthful' meristem cells, trees present an extraordinary challenge to the general theories of biological ageing, given that the concept of senescence appears not to apply to woody plants (Briand *et al.*, 1993; Lanner & Connor, 2001; Larson, 2001; Thomas, 2002). According to Connor & Lanner (1990), neither anatomical change nor cambial abnormality caused by deleterious mutational phenomena and leading to malfunctions in metabolic processes appear in 4700-yr-old trees of *Pinus longaeva*. However, because of intrinsic or extrinsic factors related to age or size, timing and duration of tree-ring formation change during the tree lifespan. This work has clearly demonstrated that in older trees (> 250 yr) of timberline *L. decidua*, *P. cembra* and *P. abies*, xylogenesis occurs in a shorter period and at lower rates of tracheid production along a radial file than in adult trees (< 80 yr). Cambium division and postcambial growth are strictly connected: delays in cell production obviously lead to delays in all differentiation processes (Rossi *et al.*, 2003, 2006b). So, the 2–3 wk earlier reactivation of cambial activity observed in adult trees corresponded to the 7–17 d earlier start of all other phases of cell differentiation. The higher the number of cells produced along a radial row, the longer the overall period of tree ring formation becomes (Gričar *et al.*, 2005; Rossi *et al.*, 2006b). For this reason, adult trees concluded lignification of latewood tracheids later than older trees despite similar endings of cell division in the cambium.

Our results demonstrate that, in some environments, age is important in tree-ring formation and should be considered when performing growth–climate relationships. Although the same climate variables control tree growth throughout its lifespan, these variables become more limiting with age (Carrer & Urbinati, 2004) because the time window of tree-ring production shortens. As different timings of xylem formation were observed in adult and old timberline conifers, environmental conditions might be experienced in different time-windows during the lifespan, thus explaining the age-related periods and intensities of the observed responses to climate. The declining period, from several months to one, of significant response to growing season temperatures in *Picea glauca* over 100 yr old (Szeicz & MacDonald, 1994, 1995) was the result of the shortening of the time-window available for tree-ring formation in older plants. The longer xylogenetic activity in the younger trees produces a dilution, and then an attenuation, of the climatic signal over a longer period (up to 15–25% longer for cambial activity and cell differentiation in our data) and reduces the response level to climate, as observed by Carrer and Urbinati (2004).

Is the delay in cambial reactivation age or size related? Is the change directly connected to intrinsic physiological features in old trees or induced by the environment? Reactivation of the vascular cambium is promoted by indol-3-acetic acid (IAA). This hormone is produced in the younger shoots of plants and exported basipetally into the subjacent stem to induce the production of xylem and phloem (Larson, 1969; Aloni, 2001) and regulate rate and duration of developmental processes during xylogenesis (Tuominen *et al.*, 1997; Uggla *et al.*, 1998). Larson's hypothesis affirms that, with the IAA basipetal movement, periclinal divisions in the cambium should also begin at the base of the buds and spread downwards toward branches and stem (Larson, 1969; Denne, 1979; Lachaud *et al.*, 1999). As a consequence, cambial activity at the stem base should begin later in taller trees because of the basipetal migration of the cambial growth wave. The 2–3 wk delay in the onset of cell production between adult and old trees compared with the height difference of the two age classes (Table 1) should lead to estimating a cambial growth wave moving at 50–80 cm $d^{-1}$ along the stem of 20 m tall trees – a very high value compared with the 6 cm $d^{-1}$ observed in young *Fagus sylvatica* (Lachaud, 1989). However, there is evidence that the IAA required for cambial resumption is available in dormant conifer tissues in autumn and winter (Little & Wareing, 1981; Sundberg *et al.*, 1991) and Larson's

 

hypothesis still remains to be clearly demonstrated or refuted (Riding & Little, 1986; Sundberg *et al.*, 1991; Uggla *et al.*, 1998; Sundberg *et al.*, 2000; Funada *et al.*, 2002).

In cold climates, cambial activity and tissue production are driven by temperature (Schmitt *et al.*, 2004; Deslauriers & Morin, 2005; Rossi *et al.*, 2007). In spring, the increases in tissue temperature among different stem diameters are partly uncoupled because of the longer-lasting frozen inner parts of the stems and the insulating effect of the thicker bark, thus shifting the reaching of warmer temperature in large old trees (Mayr *et al.*, 2006). Since below a given temperature threshold, assessed as 7–9°C for the stem, cell formation (i.e. cell division and differentiation) could not occur or, if there is any, it will be slowed down (James *et al.*, 1994; Körner, 2003; Rossi *et al.*, 2007), xylogenesis in an old tree could be delayed because of the colder environment induced by the plant size itself. The observed shorter xylogenesis in older plants at the Alpine timberline could then be related to the size effect and not to age *per se*.

In conclusion, this study has demonstrated that, in conifers at the Alpine timberline, timing and duration of xylogenesis are not constant during a tree's lifespan, with older plants showing shorter and delayed periods of cambial activity and xylem cell differentiation. The shorter time-window during which tracheids are open to directly receive environmental signals could explain the age-dependent climate growth responses found previously. These results suggest that the supposed principle linking age-independent processes between environment and tree-ring growth might not be fully applicable in some environments (i.e. in cold climates such as the timberline). As a consequence, the time-independent growth–climate computed models could be partly incorrect. As tree rings are the most diffuse and replicated proxy for reconstructing the past climate, there is now a need to take age-dependent responses into account. The assumption that environment affects tree-ring formation in the same way independently of tree age should be reformulated.

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