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Research paper

Fast acclimation of freezing resistance suggests no influence of winter minimum temperature on the range limit of European beech

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Low temperature extremes drive species distribution at a global scale. Here, we assessed the acclimation potential of freezing resistance in European beech (*Fagus sylvatica* L.) during winter. We specifically asked (i) how do beech populations growing in contrasting climates differ in their maximum freezing resistance, (ii) do differences result from genetic differentiation or phenotypic plasticity to preceding temperatures and (iii) is beech at risk of freezing damage in winter across its distribution range. We investigated the genetic and environmental components of freezing resistance in buds of adult beech trees from three different populations along a natural large temperature gradient in north-western Switzerland, including the site holding the cold temperature record in Switzerland. Freezing resistance of leaf primordia in buds varied significantly among populations, with LT_{50} values (lethal temperature for 50% of samples) ranging from −25 to −40 °C, correlating with midwinter temperatures of the site of origin. Cambial meristems and the pith of shoots showed high freezing resistance in all three populations, with only a trend to lower freezing resistance at the warmer site. After hardening samples at −6 °C for 5 days, freezing resistance of leaf primordia increased in all provenances by up to 4.5 K. After additional hardening at −15 °C for 3 days, all leaf primordia were freezing resistant to −40 °C. We demonstrate that freezing resistance of *F. sylvatica* has a high ability to acclimate to temperature changes in winter, whereas the genetic differentiation of freezing resistance among populations seems negligible over this small geographic scale but large climatic gradient. In contrast to the assumption made in most of the species distribution models, we suggest that absolute minimum temperature in winter is unlikely to shape the cold range limit of beech. We conclude that the rapid acclimation of freezing resistance to winter temperatures allows beech to track changing climatic conditions, especially during unusually warm winters interrupted by very cold weather.

Keywords: cold acclimation, *Fagus sylvatica*, frost, hardening, species distribution, sub-zero acclimation.

Introduction

Temperature extremes shape plant distribution on earth (Woodward et al. 1990). In temperate climates, plants need to withstand extremely low temperatures in winter. They adapt to the seasonal change of temperature by restricting active growth to the warmer season, and being dormant in winter with low metabolic activity and a high tolerance to freezing temperatures. The change from the active to the dormant life stage and vice versa is regulated, to minimize the risk of freezing damage (Körner and Basler 2010, Vitasse et al. 2014*b*). In autumn, the dormancy period is environmentally triggered by shorter day length, enhanced by cold, non-freezing temperatures. The increase in freezing resistance upon exposure of plants to cold non-freezing temperatures is referred to as cold acclimation, a process by which trees can reach the maximum level of freezing resistance in midwinter. Cold acclimation is associated with many metabolic changes. The major cause of freezing damage is leakage of biomembranes (Ziegler and Kandler 1980, Steponkus 1984). Thus, many of the metabolic changes during cold acclimation are associated with the stability of membranes, i.e., keeping membranes fluid at freezing temperatures, and tolerating freeze-induced dehydration of the protoplasma, as well as protecting membranes and enzymes within the cell sap (Sung et al. 2003, Larcher 2005, Dauwe et al. 2012). Metabolic adjustments of plant cells to withstand freezing temperatures include increases in certain amino acids, and production of polyamines and glycine betaine, polyols and different carbohydrates such as fructans, the raffinose family of oligosaccharides or mono- and disaccharides (Thomashow 1999, Sung et al. 2003, Kalberer et al. 2006).

Temperate tree species are generally very freezing tolerant, yet with a large variation between species and leaf types (deciduous vs evergreen and broadleaved vs needles). Generally, evergreen broadleaved tree species in the temperate zone only resist freezing temperatures of −5 to −18 °C (Sakai 1978) and, thus, are largely confined to Mediterranean regions. Deciduous broadleaved tree species can resist temperatures of −30 °C to even below −70 °C in some extremely hardy species in the dormant period (Sakai 1978), and conifer species are generally the most freezing-resistant tree types. This large difference in freezing resistance among species correlates well with the distribution of species on large geographic scales (Sakai and Weiser 1973, Daly et al. 2012, Kreyling et al. 2015) and has led people to assume that winter temperatures are generally critical in defining species range limits. Currently, the assumption that absolute minimum temperatures play an important role in the distribution of native temperate tree species prevails, especially in the modelling community. For instance, many studies investigating the relationship between freezing resistance and geographic distribution limits have mainly considered mean minimum temperatures for their analyses (e.g., Prentice et al. 1992, Sykes et al. 1996, Svenning and Skov 2004, Daly et al. 2012). However, the correlation between freezing resistance and mean temperatures is problematic, since for the persistence of a tree species in a given region, it is the actual absolute minimum temperature rather than mean minimum temperatures that are decisive upon life or death.

Large differences in maximum freezing resistance in winter are not only apparent among species but also among populations within the same species. For instance, freezing resistance of beech was reported to range from −13 to −40 °C among different studies (Tranquillini and Plank 1989, Visnjic and Dohrenbusch 2004, Lenz et al. 2013, Kreyling et al. 2014). This large variability in freezing resistance in midwinter can result from (i) genetic differentiation among different populations, (ii) differences in phenology and thus in the timing of hardening and dehardening in autumn and spring or (iii) phenotypic plasticity due to acclimation to actual temperature conditions. In the following, we will explore all three possibilities.

The literature on genetic differentiation of freezing resistance is vast, with many studies from agricultural and horticultural sciences (Larcher 1985, Sakai and Larcher 1987). Genetic differentiation among populations is generally assessed with common garden experiments, where plants from geographically distant populations are grown in a single site (‘common’ garden) and thus experience the same climatic conditions. It has been reported previously that genetic differentiation of freezing resistance can be quite large among different populations. For example, LT_{50} values (lethal temperature for 50% of samples) in winter-dormant buds of *Betula pendula* Roth ranged from −29 to −38 °C among three populations (Li et al. 2003). However, the variation in absolute minimum temperature of the site of origin of populations is usually much larger than the genetic differentiation among populations. For instance, differences in freezing resistance of 6–17 K were observed among several populations of *Quercus rubra* L. grown in a common garden in the period from October to March (Flint 1972), while the minimum temperatures of the sites of origin were much more variable with −46 °C recorded at the coldest site and −23 °C recorded at the warmest site (Flint 1972). Similarly, LT_{50} values of 10 populations of *Fraxinus americana* L. ranged from −34 to −43 °C, while the average annual minimum temperature of the site of origin ranged from −12 to −34 °C (Alexander et al. 1984). In beech trees, reported LT_{50} values varied by >10 K among different provenances from all over Europe (Kreyling et al. 2014, Hofmann et al. 2015).

Differences in freezing resistance within the same species might not be caused by genetic differentiation, but can also result from differences in phenology. In spring, freezing resistance of species is strongly dependent on the phenological stage of the tree (Taschler et al. 2004, Lenz et al. 2013). A similar effect can be expected in autumn. Hardening of tissues in buds can only be achieved once bud set has occurred and trees have stopped meristematic activity. Autumnal leaf senescence and bud set generally occur earlier in tree populations originating from high elevation or northern latitudes when grown in common gardens (Alberto et al. 2013, Vitasse et al. 2013). An earlier bud set allows tree species to enter tissue hardening earlier in autumn, and reach greater maximum freezing resistance in midwinter. Consistently, northern populations of *B. pendula* achieved an earlier hardening and a higher freezing resistance under a short day treatment combined with a cold temperature treatment compared with southern populations (Li et al. 2003), suggesting that both phenology and the extent of hardening have a genetic component.

Freezing resistance might also depend on the prevailing air temperature at a given site. Hardening is initiated by short photoperiod and cold, non-freezing temperatures in autumn. Maximum hardiness of plants is only reached after they experience freezing temperatures (Weiser 1970). Temperature can even overrule the effect of photoperiod on hardening. For instance, low temperature induced a strong increase in freezing resistance in buds of *Picea abies* (L.) H. Karst and *Pinus sylvestris* L. even under a constant long photoperiod, while a short photoperiod with cold temperatures was more effective to harden saplings (Christersson 1978). The first temperatures below freezing induced a strong increase in freezing resistance in apple bark in autumn, even under a constant long photoperiod (Howell and Weiser 1970). In order to achieve survival of twigs at ultra-low temperature (−196 °C), a pre-freezing at −30 °C was found to be necessary in several woody species (Sakai 1960). Interestingly, cold hardiness of the same individual tree was found to differ among winters. For instance, LT_{50} values of −22 and −29 °C were measured in *F. sylvatica* in two consecutive years in the vicinity of Göttingen, Germany, in midwinter (Till 1956).

In the present study, we investigated freezing resistance of three beech populations growing along a strong temperature gradient in the Swiss Jura Mountains, from one of the coldest regions in Switzerland to a significantly warmer region. We measured the midwinter freezing resistance of leaf primordia in buds and of cambial meristems and pith tissue in twigs of European beech (i) directly after sampling, (ii) after a moderate artificial hardening treatment and (iii) after a maximum hardening treatment. These treatments allowed us to disentangle the genetic and environmental components involved in the development of winter freezing resistance. The plasticity of freezing resistance to prevailing temperature conditions was assessed by comparing the actual freezing resistance among the three populations with the freezing resistance after the moderate and the maximum hardening treatment. Genetic differentiation of freezing resistance among the populations was assessed after fully hardening the samples, since any difference in freezing resistance after maximum hardiness was reached was hypothesized to reflect genetic differentiation among the populations. In detail, we addressed the following questions: (i) Are beech populations growing in a colder climate more freezing resistant than beech populations from warmer climates? (ii) To what extent do beech trees acclimate their freezing resistance in winter to variations of actual temperature? (iii) Do possible differences in freezing resistance result from genetic differentiation or phenotypic plasticity? (iv) Does beech encounter a risk of being damaged by freezing temperatures in winter at the coldest site in Switzerland?

Materials and methods

Study sites

Beech trees were sampled at three sites along a 90-km natural temperature gradient in the Swiss Jura Mountains, from the absolute coldest site in Switzerland at low elevation (La Brévine, 1100 m above sea level (a.s.l.)), hereafter called the cold site, to a warmer site near Montfaucon at ~1000 m a.s.l., hereafter called the intermediate site, and the warmest site in this study near Hofstetten at ~550 m a.s.l., hereafter called the warm site (Table 1). La Brévine is a special site where cold air drainage frequently occurs resulting in the formation of 'cold air lakes'. As a consequence, air temperature can drop quite fast to values significantly below −20 °C in the valley (see Figure 1), and the coldest temperature in Switzerland (−41.8 °C) was recorded at this site in January 1987, when the trees we sampled grew already in that area. The other two sites are considerably warmer, with an absolute minimum temperature of −29.9 °C recorded in Montfaucon and −23.3 °C in Hofstetten (Table 1).

Definitions

Here, we use the general ecological definition of the term 'acclimation' for processes that occur rapidly (within days to weeks) during the lifetime of an organism in response to environmental changes, which is in contrast to adaptation. Acclimation can, therefore, easily be observed in controlled conditions. In the cold hardiness field of research, the term sub-zero acclimation is used to refer to an acclimation of freezing resistance in response to a sub-zero artificial hardening treatment that occurs within hours to days. This use of 'acclimation' should not be confused with the term 'cold acclimation', which generally refers to the increase of freezing resistance from autumn to winter over

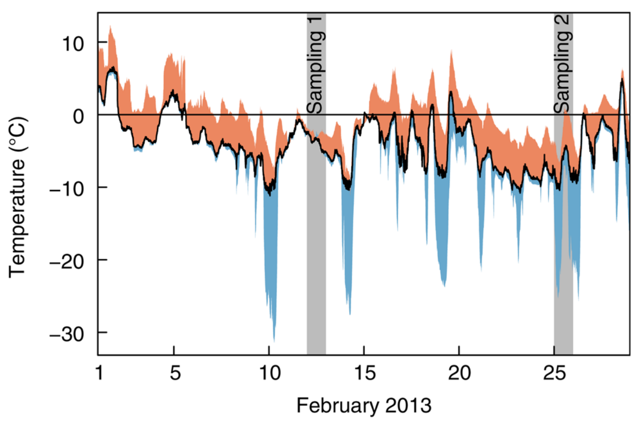

Figure 1. Air temperature recorded by climate stations (10-min intervals) at all three sites during February 2013. The line indicates temperature at the intermediate site, with the temperature difference to the warm and cold site shown as the grey (brown and blue online) area. Vertical grey bars indicate the two sampling occasions.

Table 1. Elevation and location of study sites with the absolute minimum temperature and the mean number of freeze days.

Site	Location	Elevation (m a.s.l.)	Latitude	Longitude	Absolute minimum temperature (°C)[1]	Mean number of freeze days[2] per year
Cold	La-Brévine	1080	46°59′14″N	06°36′40″E	−41.8	39
Intermediate	Montfaucon	1000	47°16′25″N	07°02′04″E	−29.9	28
Warm	Hofstetten	550	47°28′08″N	07°30′16″E	−23.3	13

[1]For the period from 1959 to 2013.
[2]A freeze day is a day with temperatures below freezing during the whole day.

weeks, also called the hardening period, and which is strongly associated with dormancy in trees.

Assessment of freezing resistance

At each site, branches of six dominant mature beech trees were sampled on 12 and 25 February 2013. For each tree, a well-exposed larger branch in the upper crown was collected with a pole-pruner. We were able to reach up to 6 m and cut branches with a maximum diameter of 5 cm. Immediately after cutting, the branches were packed into plastic bags and kept at 0–4 °C in a cooler for transport to the laboratory. All samples were processed immediately after returning to the laboratory and placed into the freezers within <10 h after collection. From each tree, several small branches comprising in total at least eight buds per target freezing temperature were equally distributed among six computer-controlled freezers, one control chamber at 4 °C and a negative control freezer at −80 °C.

To run the freezing treatments, we used customized commercial freezers (Liebherr GN 1056 Premium No Frost, with an integrated heating system; Liebherr, Ochsenhausen, Germany), modified to allow computer control of the freezing and thawing process (see Lenz et al. 2013 for technical details). The freezing system allowed for an independent freeze–thaw cycle for each target temperature. We employed a freezing and re-thawing rate of 3 K h^{-1} and kept samples for 4 h at the target freezing temperature (see Table S1 available as Supplementary Data at *Tree Physiology* Online). The duration of freezing may have a considerable effect on the damage observed, with prolonged exposures at sub-zero leading to more damage. At the three study sites, the mean duration of the minimum temperature in single freezing events is 4 h (see Figure S1 available as Supplementary Data at *Tree Physiology* Online). Similar to the duration of a freeze event, the cooling and rewarming rate has a strong influence on damage. Air temperature usually does not drop faster than 5 K h^{-1} in nature, and the rate of 3 K h^{-1} has been successfully employed in several studies (Lenz et al. 2013, Rehm et al. 2014, Palacio et al. 2015). Freezing ramps were programmed so that all samples reached 4 °C at the same time after thawing. The temperature within the freezers was recorded using Pt-100 temperature sensors placed within the bags containing the samples (see Table S1 available as Supplementary Data at *Tree Physiology* Online). After the freezing programme, samples were kept at 4 °C for 4–10 h, so freezing damage had time to develop visual symptoms. Buds were visually observed for survival following the protocol of Lenz et al. (2013). After cutting buds with a razor blade, we observed damage in leaf primordia in buds, the cambial meristem and pith tissue in twigs. Freezing damage is visible as browning caused by the oxidation of polyphenols, and as a characteristic odour due to de-compartmentalization and autolysis of the protoplast. For the maximum hardening treatment (see below), we additionally employed electrolyte leakage of the treated plant material to complement the visual rating following the protocol of Lenz et al. (2013).

Artificial hardening treatments

To assess the adaptive potential of beech trees to colder temperatures, freezing resistance of buds was assessed after three different treatments. First, LT_{10} and LT_{50} values (temperature that is lethal for 10 and 50%, respectively, of samples) were assessed directly after sample collection on both sampling occasions (12 February and 25 February 2013), hereafter referred to as actual freezing resistance. In addition, two artificial hardening treatments were applied, but only with samples from the second sampling occasion on 25 February. For the moderate hardening treatment, a third of the samples was placed in a freezer set up at −6 °C for 5 days in complete darkness, with a cooling rate from 4 to −6 °C at 3 K h^{-1}. For the maximum hardening treatment, another third of the samples from the second sampling date was first exposed to −6 °C for 5 days, and thereafter to −15 °C for another 3 days, again, with a cooling rate of 3 K h^{-1} between the treatment temperatures.

In situ temperature

We recorded air temperature at all three sites at 2 m height in full shade using fully sealed data loggers (TidbiT v2; Onset Computer Corp., Cape Cod, MA, USA), at the two warmer sites from 12 February 2013 to 17 October 2013, and at the coldest site from 12 February 2013 to 12 February 2015. We extrapolated daily mean temperatures to these in situ temperatures with temperatures from the nearest climate stations for the last few days before 12 February 2013. We used climate stations from La Brévine (1050 m a.s.l., 46°59′N 6°37′E), La Chaux-de-Fonds (1018 m a.s.l., 47°05′N 6°48′E) and Binningen (316 m a.s.l., 47°32′N 7°35′E) for the cold, intermediate and warm sites, respectively. Because temperature extremes are very hard to extrapolate, we used temperatures from climate stations to assess the long-term risk of freezing damage during winter in beech at the three sites during the period of 1959–2014 (see below). The use of climate station data in this assessment of risk is unproblematic, since it is based on the relationship between mean and extreme temperatures measured at exactly the same site. Climate station data offer longer time series, and are thus useful for this risk assessment. We use °C for absolute temperature values, and K for temperature differences throughout the manuscript, to avoid confusion between the two parameters, as suggested by McVicar and Körner (2013).

Data analysis

The values of LT_{10} and LT_{50} were calculated using logistic regressions for visually observed damage and with nonlinear Gompertz models for electrolyte leakage data (see Lenz et al. 2013 for details). Both LT values were calculated separately for each sampled tree and tissue ($n = 6$ per treatment). Lethal temperature for 50% of bud samples assessed by electrolyte leakage correlates well with LT_{50} values of leaf primordia from visual observation (see Lenz et al. 2013), allowing us to verify LT_{50} values of leaf primordia after the maximum hardiness treatment. We calculated analyses of variance to assess (i) potential differences in LT_{10} and LT_{50} values among different tissues and sites and (ii) potential differences in LT_{10} and LT_{50} values between treatments and sites. When interactions between treatments and sites were significant, we calculated Tukey honest significant differences (Tukey-HSD) post hoc tests. Further, we used a linear model to test the relationship between LT_{10} and LT_{50} values in leaf primordia and in situ temperatures before sampling. To assess whether beech trees can sufficiently acclimate their freezing resistance in nature to tolerate freezing temperatures, we (i) correlated the mean temperature of the last 3 days (including the day of sampling) before each strong freezing event (absolute minimum temperatures below −20 °C) with the absolute minimum temperature reached during these freezing events. (ii) Based on the correlation of LT_{10} or LT_{50} values with in situ temperature, we extrapolated LT_{10} and LT_{50} values of leaf primordia with the mean temperature of the last 3 days before each strong freezing event, and correlated these extrapolated LT_{10} and LT_{50} values with absolute minimum temperatures. All analyses were performed using R 2.15.3 (R Development Core Team 2013) using the R-package nlme to calculate Gompertz models (Pinheiro et al. 2013) and the R-package multcomp to calculate post hoc tests (Hothorn et al. 2008).

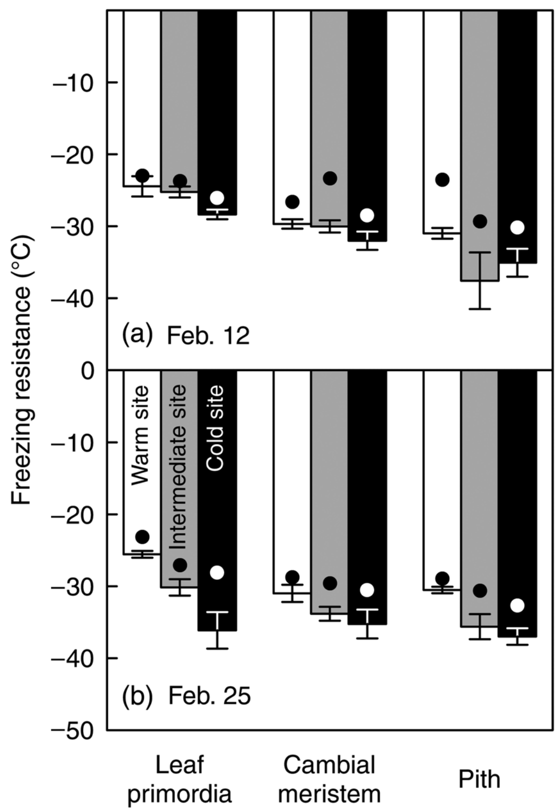

Figure 2. Lethal temperature for 50% of leaf primordia samples in buds, and cambial meristems and pith tissue in twigs of European beech at the cold, intermediate and warm sites at the first sampling occasion (a) and the second sampling occasion (b). Lethal temperature for 10% of samples is indicated as points. Mean ± SE are shown.

Results

Air temperature at the study sites

Temperatures in the week before sampling were below freezing for most of the time at the two colder sites for both sampling occasions (Figure 1). The mean air temperature ranged from −8.4 °C at the coldest site to −5.2 °C at the intermediate site and −1.4 °C at the warmest site in the last 5 days before the first sampling occasion (7–11 February). Mean air temperatures were slightly colder before the second sampling, ranging from −9.2 to −1.7 °C between the coldest and the warmest site. Interestingly, the absolute minimum temperature differed significantly more than the mean temperature between the two sampling occasions. Temperature dropped once and approximately four times below −20 °C at the coldest site in the 5 days preceding the first or second sampling occasion, respectively. At the two warmer sites, the absolute minimum temperatures differed only slightly between the two sampling occasions, ranging from −11.2 to −5 °C. Remarkably, the temperature course among the three different sites was quite similar, except for the strong temperature drops during nights at the coldest site, most likely resulting from cold air pooling into the valley of La Brévine (Figure 1).

Actual freezing resistance

Actual freezing resistance, directly after sampling, differed significantly among the three tissue types and the three sites at both sampling occasions (Figure 2, Table 2). Independent of site, leaf primordia were the most sensitive tissues, with LT_{10} values ranging from −23 to −28 °C and LT_{50} values ranging from −25 to −36 °C. Cambial meristems had 5–6 K lower LT_{50} values (more freezing resistant) than leaf primordia, and pith tissue had the lowest LT_{10} and LT_{50} values (Figure 2). Irrespective of tissue, both LT_{10} and LT_{50} values were more negative at the colder sites. Interestingly, LT_{50} values at the two colder sites

Table 2. Summary of the analysis of variance on LT_{10} or LT_{50} values dependent on tissue (leaf primordia, cambial meristem and pith tissue), the site of origin (cold, intermediate or warm) and the interaction between tissue and site for both sampling dates. Significant results ($P < 0.05$) are given in bold.

Factor	Degrees of freedom	First sampling		Second sampling	
		F-value	*P*-value	*F*-value	*P*-value
LT_{10} values					
Site of origin	2	**5.8**	**<0.01**	**9.2**	**<0.001**
Tissue	2	**3.7**	**<0.05**	**18.9**	**<0.001**
Site of origin × tissue	4	2.4	0.07	1.0	0.43
LT_{50} values					
Site of origin	2	**5.8**	**<0.01**	**20.5**	**<0.0001**
Tissue	2	**27.7**	**<0.0001**	**7.1**	**<0.01**
Site of origin × tissue	4	1.7	0.18	1.6	0.20

decreased (became more negative) markedly from the first to the second sampling occasion (Figure 2). Thus, the most negative LT_{50} values (around −36 °C) were observed at the coldest site after the second sampling.

Effect of temperature on freezing resistance

Both LT_{10} and LT_{50} values directly after sampling (actual freezing resistance) strongly correlated with the mean temperatures at the three sites shortly before sampling, although the correlation with LT_{50} values was stronger. The strongest correlation (highest R^2 value) was observed with the mean temperature of the last 2 days before the assessment of freezing resistance, including the day when samples were collected (Figure 3). Most interestingly, the correlation became statistically even stronger when two LT_{50} values collected at another site ~90 km from the transect, sampled in winter 2012 (Morcle, 1350 m a.s.l., 46°12′53″N 7°2′15″E), were included (Figure 3b).

Freezing resistance after artificial hardening treatments

After the moderate hardening treatment, LT_{10} and LT_{50} values were more negative in populations from all three sites and tissues, although not to the same extent (Figure 4, Table 3). In leaf primordia, LT_{50} values decreased significantly by ~4.5 K in beech populations from the warm and the intermediate site in response to the moderate hardening treatment, but not in populations from the cold site (Figure 4a), leading to a significant interaction between site of origin and treatment (Table 3). Interestingly, LT_{10} values decreased significantly by 3–5 K in all three sites in response to the moderate hardening treatment. After the maximum hardening

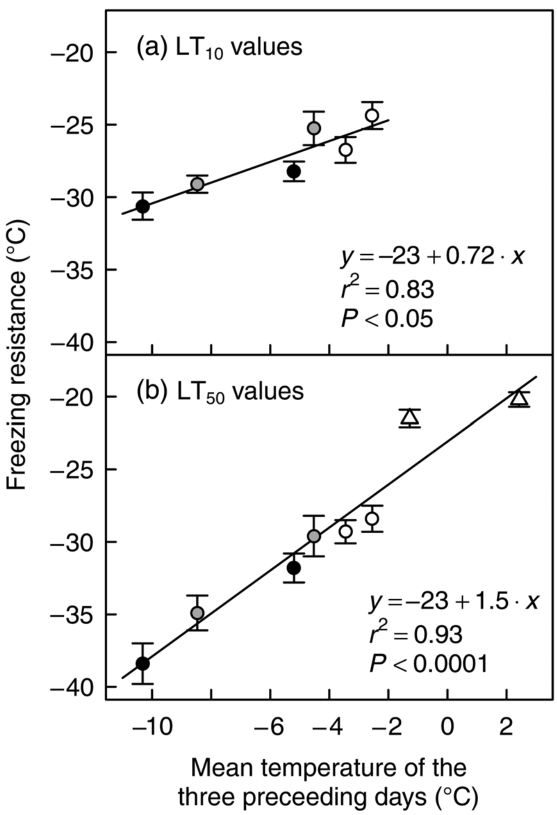

Figure 3. Correlation of LT_{10} (a) or LT_{50} (b) values of leaf primordia with the mean temperature of the 3 days before we assessed freezing resistance, including the day of sampling. Circles indicate the mean LT_{10} or LT_{50} value (±SE) of six individual beech trees at the warm (white), intermediate (grey) and cold (black) site for the two sampling occasions. Triangles indicate mean LT_{50} values (±SE) for leaf primordia of beech buds of five individual trees measured twice in winter, extracted from Lenz et al. (2013).

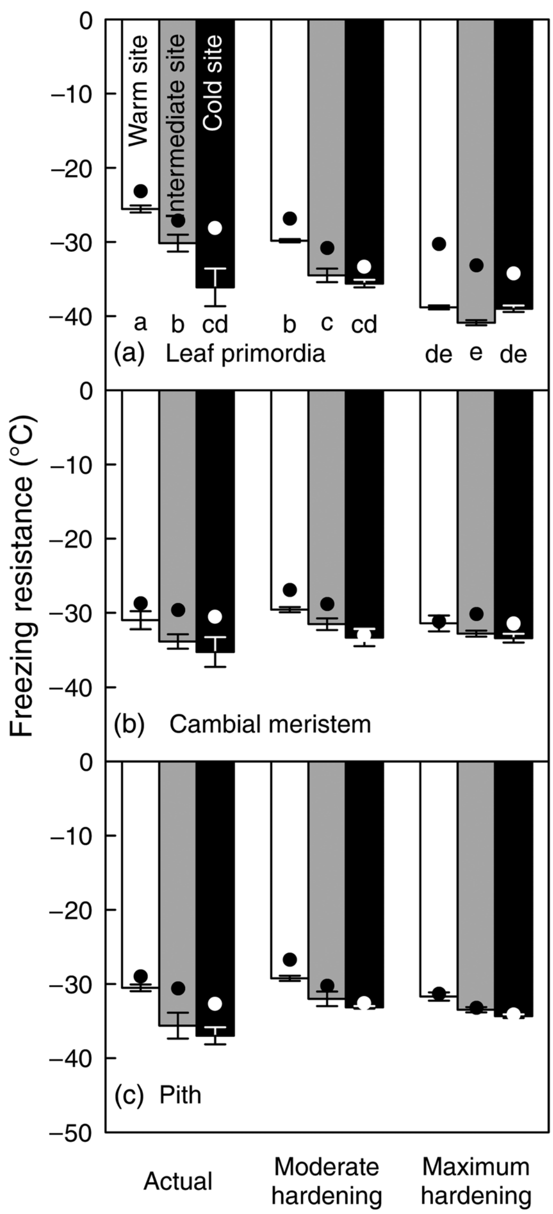

Figure 4. The actual freezing resistance, freezing resistance after the moderate hardening treatment and freezing resistance after the maximum hardening expressed as LT_{50} values of (a) leaf primordia in buds, (b) cambial meristems and (c) pith tissue in twigs of beech among the three sites. Lethal temperature for 10% of samples is shown as points. Letters indicate significant differences calculated by a Tukey-HSD post hoc test, if the interaction between site of origin and treatment was significant (see Table 3). Hardening treatments were only performed after the second sampling occasion.

Table 3. Summary of the analysis of variance between LT_{10} and LT_{50} values of leaf primordia, cambial meristem and pith tissue dependent on the site of origin (cold, intermediate or warm), the treatment (actual freezing resistance[1], moderate hardening[2] or maximum hardening[3]) and the interaction between site and treatment. Significant results ($P < 0.05$) are given in bold.

Factor	Degrees of freedom	Leaf primordia		Cambial meristem		Pith	
		F-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
LT_{10} values							
Site of origin	2	**38.4**	**<0.0001**	**5.6**	**<0.01**	**24.1**	**<0.0001**
Treatment	2	**54.1**	**<0.0001**	1.5	0.25	**14.3**	**<0.0001**
Site of origin × treatment	4	0.8	0.5	2.1	0.1	1.3	0.29
LT_{50} values							
Site of origin	2	**28.4**	**<0.0001**	**8.3**	**<0.001**	**21.9**	**<0.0001**
Treatment	2	**77.0**	**<0.0001**	2.7	0.08	**9.3**	**<0.001**
Site of origin × treatment	4	**8.4**	**<0.0001**	0.4	0.79	1.7	0.17

[1]Actual freezing resistance: freezing resistance measured directly after sampling.
[2]Moderate hardening: 5 days at −6 °C.
[3]Maximum hardening: 5 days at −6 °C and 3 days at −15 °C.

treatment, LT_{50} values of leaf primordia reached ca −40 °C in populations from all three sites, thus increased by 5–9 K compared with the moderate hardening treatment (Figure 4a). Lethal temperature for 10% of leaf primordia samples was less responsive to the maximum hardening treatment, and ranged from −30 to −34 °C among the tree populations. Overall, freezing resistance increased significantly with both hardening treatments, with a difference of >15 K between the actual LT_{50} values and the LT_{50} values after the maximum hardening treatment in the warmest site (Figure 4a). In contrast to leaf primordia, the hardening treatments had no effect on LT_{50} values of cambial meristems, and only a small and insignificant effect on LT_{50} values of pith tissue below buds at all three sites (Figure 4b and c, Table 3).

Short-term acclimation of freezing resistance in nature

Using long-term climate data from all three sites, we correlated mean air temperatures of the last 3 days before each strong freezing event (absolute minimum temperature below −20 °C) with the absolute minimum temperature reached during these freezing events. Whenever absolute minimum temperatures fell below −20 °C, the mean temperature of the last 3 days including the day with the extreme low freezing temperature was always below 0 °C (Figure 5a). Whenever absolute minimum temperatures were below −30 °C, the mean temperature of the preceding 3 days was always below −7 °C (i.e., at a temperature range where hardening of beech does occur). According to our temperature models, beech was always safe from severe freezing damage with temperatures below LT_{50} values in winter over the time period from 1959 to 2014 at all three study sites (Figure 5c), except for one occasion in La Brévine on 3 February 1977, when temperatures suddenly dropped from −3 °C (mean temperature of the last 3 days) to −29.3 °C. Thus, extrapolated LT_{50} values (by using the mean temperature of the last 3 days) are always lower than the minimum temperature reached at each freezing event. However, extrapolated LT_{50} values below −40 °C should be regarded with caution (Figure 5c), since the extrapolation goes beyond the data range of our correlation analysis (Figure 3b). In a more conservative model with LT_{10} values, damage would occur more frequently, however, still in only 1.7% of all days included in the analysis, and only in La Brévine, where the weather station is situated at the valley bottom and trees grow on the slopes and most likely do not experience the same temperatures as the weather station due to cold air pooling (Figure 5b). Again, many of the extrapolated LT_{10} values should be regarded with caution, since the extrapolation goes beyond the data range of the correlation (Figure 3a).

Discussion

We subjected cuttings of mature European beech trees originating from three populations to the same controlled sub-zero acclimation regimes, allowing them to reach maximum hardiness, and compared the maximum hardiness with in situ acclimated freezing resistance. This approach allowed us to disentangle the effects of genetic differentiation (maximum hardiness among the different populations) and phenotypic plasticity (acclimation potential of a given population) of freezing resistance. Our results demonstrated a substantial and relatively fast acclimation potential of freezing resistance to actual temperatures in dormant buds, but overall no genetic differentiation in freezing resistance among populations along the steep temperature gradient. Interestingly, the large acclimation potential enables beech trees to become more freezing resistant while extreme cold temperatures prevailed. We thus suggest that beech trees can easily survive temperatures in winter throughout the distribution range, and potentially even beyond.

Acclimation potential of freezing resistance

Freezing resistance of species is very responsive to temperature in midwinter, even though trees are in dormancy. Freezing

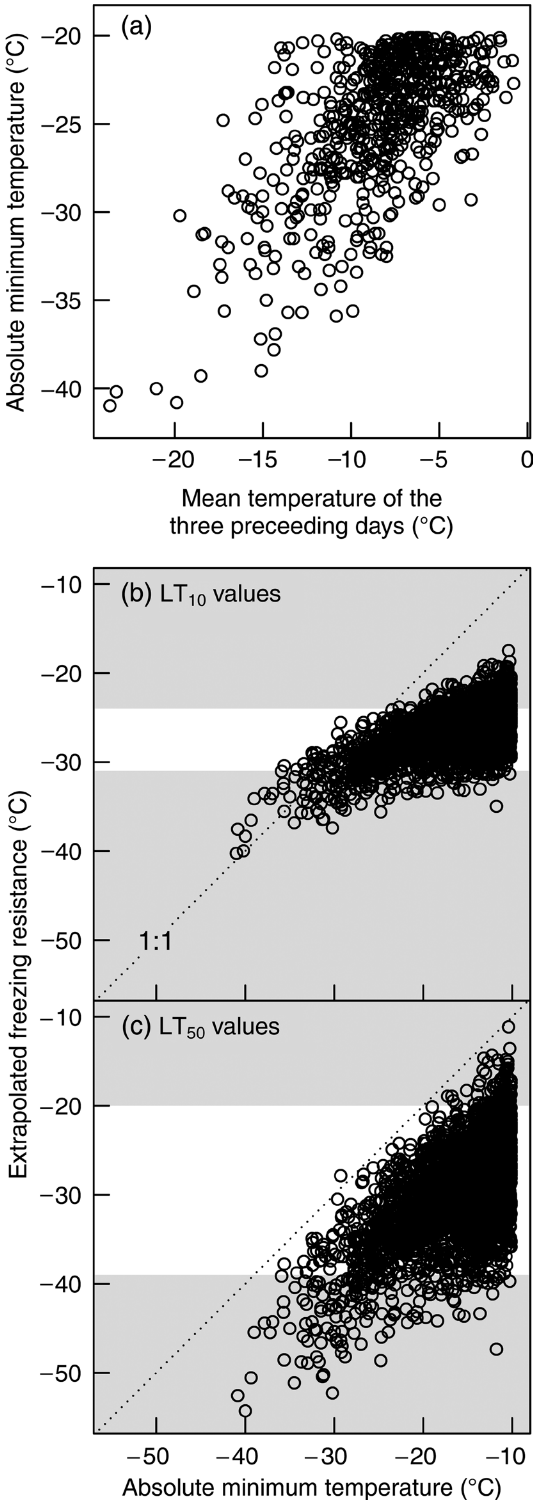

Figure 5. Relationship between the absolute minimum temperature with the mean temperature of the three previous days, including the day when the absolute minimum temperature was reached (a), with extrapolated LT_{10} values of leaf primordia (b) and with LT_{50} values of leaf primordia extrapolated by the mean temperature of the two previous days (c). Temperature was recorded by climate stations at all three sites from 1959 to 2014. The grey shade in (b) and (c) denotes the range of LT values extrapolated beyond the range of available data (i.e., above −24 or below −31 °C for LT_{10} values, and above −20 or below −39 °C for LT_{50} values) and should be regarded with caution.

temperature can lead to a strong increase in freezing resistance within a few days, whereas warm temperatures can lead to a decrease in freezing resistance. Consistently, we found a strong correlation of freezing resistance with preceding temperature in beech, and a strong and fast acclimation to our artificial hardening treatments. A similar or even stronger acclimation of freezing resistance to sub-zero temperatures was previously found in other tree species. A single night with freezing temperatures can lead to a significant increase in freezing resistance of *P. abies* (Søgaard et al. 2009), although the first drop of temperatures below freezing in autumn does not necessarily lead to an increase in freezing resistance in temperate and boreal conifers (Strimbeck and Kjellsen 2010). In midwinter, freezing resistance of needles of *Pinus cembra* L. and *P. abies* increases by 9–14 K when kept for 4 days at −6 or −14 °C, and even by 21 K when kept for 1 week at −14 °C (Pisek and Schiessl 1947). More recently, Buchner and Neuner (2011) found a similar increase in freezing resistance of *P. cembra* needles and buds by artificially hardening twigs in situ at −20 °C for 3 weeks. Detached twigs of *Populus nigra* L. could be hardened by 10 K when kept at −3 °C for 10 days in midwinter (Sakai 1966). Even stronger increases in freezing resistance from −15 to −50 °C could be achieved in *Salix* species when acclimated at −3 °C for 14 days in midwinter (Sakai 1970).

Not only is the acclimation potential large in winter, tree species also de-acclimate rapidly in response to warm temperatures. We did not apply a de-acclimation treatment in the current study. However, our sampled beech trees were less freezing resistant at the first sampling date, when in contrast to the second date, temperatures before sampling were frequently above or only slightly below freezing. Similarly, *P. cembra* lost 20 K of freezing tolerance in buds and 10 K in needles when exposed to 10 K warmer temperatures than ambient air temperature in situ (Buchner and Neuner 2011), and LT_{50} values of *P. cembra* and *P. abies* increased (became less freezing resistant) by 10–15 K after 4 days with temperatures of +15 °C (Pisek and Schiessl 1947). Importantly, warm temperatures cannot fully de-acclimate such native trees in winter. Thus, in the study by Pisek and Schiessl (1947), freezing resistance of *P. cembra* was −26 to −28 °C, and ranged from −22 to −24 °C in *P. abies*, irrespective of the level of freezing resistance before the dehardening treatment in midwinter. Even temperatures only slightly above freezing can lead to a strong loss of freezing resistance in midwinter. After 30 days at 0 °C, twigs of *P. nigra* lost freezing resistance from surviving submergence in liquid nitrogen to being damaged at −30 °C (Sakai 1966). In summary, the current and previous studies demonstrated that freezing resistance of temperate tree species shows strong and rapid acclimation and de-acclimation to temperature in winter.

Genetic differentiation of freezing resistance

As in many studies where populations are selected on the basis of the climatic conditions that prevail at the place of origin, we do not know if the selected populations belong to the same metapopulation (i.e., populations away from each other, but still connected by gene flow) as is commonly found along elevational gradients (Alberto et al. 2011), or if they are genetically distinct. However, the temperature gradient is very large among the three selected populations. The coldest site used in this study is the coldest place in Switzerland, with a low temperature record of −41.8 °C, recorded on 12 January 1987 by a weather station from MeteoSwiss installed in the village of La Brévine, whereas the absolute minimum temperature at the warmest site along our investigated transect was >15 K warmer (−24.1 °C on 22 January 1942). Despite this very strong climatic gradient, the geographic distance among populations was relatively small (~90 km), which might also explain why we did not find genetic differentiation in maximum frost hardiness among the different populations investigated. Due to the short distance among the sites, but also to much warmer sites near La Brévine and Montfaucon (<10 km), gene flow is likely large, and genetic differentiation among populations might, therefore, be small. In contrast to this study, genetic differentiation of freezing resistance in beech becomes apparent over much larger geographic areas. For instance, seedlings from different populations of beech originating from the entire north–south axis of its natural distribution range exhibited LT_{50} values from −13 to −19 °C in January, when grown in a common garden in Central Germany (Visnjic and Dohrenbusch 2004). Even larger differences among seedlings of different populations across Europe were observed in two common gardens in Germany, with LT_{50} values of −28 to −40 °C (Kreyling et al. 2014). Only recently, genetic differentiation of freezing resistance had been also documented in adult beech trees (Hofmann et al. 2015). Generally, temperate deciduous trees show no ontogenetic change in freezing resistance with increasing tree age from seedlings to adult size provided that freezing resistance is assessed at the same phenological stage (Vitasse et al. 2014*a*). Interestingly, the phenotypic variation in freezing resistance due to artificial hardening observed in our study was much larger than the genetic differences among beech populations from all over Europe. We therefore suggest that acclimation of freezing resistance should be included in distribution models of tree species.

Mechanism of acclimation at sub-zero temperatures

To date, it is largely unknown how beech trees can achieve increasing levels of freezing resistance by sub-zero hardening temperatures during winter. A purely physics-driven change in freezing resistance at sub-zero temperature is supercooling of water, when water cools below the freezing point. Interestingly, the limit for supercooling is the homogenous ice nucleation point of pure water at approximately −38 °C (Wilson 2012), except for xylem parenchyma cells of certain conifer species, which likely employ deep supercooling, lowering the freezing point of water by certain solutes (Fujikawa et al. 2009). The temperature of homogeneous ice nucleation matches well the lowest freezing resistance we found, suggesting that water in buds of beech does probably supercool. Recent studies show that beech employs freeze avoidance in buds, where water is supercooled (R. Miller and G. Neuner, personal communication). When buds are kept for a longer time at sub-zero temperatures, more water is removed from the primordia, and the low temperature exotherm (the limit for supercooling) occurs at colder temperature (reviewed in Quamme 1995). Thus, the artificial hardening treatments we applied most likely lead to a reduction of water content in leaf primordia and an increase of ice in adjacent tissues, until a new equilibrium between ice and water was reached. Whether this movement of water was actively mediated by aquaporins, as observed in wheat (Herman et al. 2006), or only by diffusion, we cannot answer. The low temperature exotherm, and thus the limit for supercooling, correlates with the minimum air temperature of the last few days (reviewed in Quamme 1995). This possibly explains why we found a strong correlation of temperature and freezing resistance.

Most likely, the increase of freezing resistance at sub-zero temperature is not exclusively physical, but involves active changes in plant cells. Only a few studies have investigated metabolic changes occurring at freezing temperatures (e.g., Strimbeck et al. 2008, Kjellsen et al. 2010, Angelcheva et al. 2014), with beech being completely uninvestigated in this respect to date. Ultrastructural changes occurring at freezing temperatures are best evidenced, like the alteration of organelle structure, especially in the endoplasmic reticulum and the Golgi apparatus (Herman et al. 2006). Generally, sugar concentration increases in plants kept at freezing temperatures. Starch has been shown to be degraded to free sugars at temperatures between −3 and −10 °C in *Salix sachalinensis* F. Schmidt (Sakai 1966). However, following van't Hoffs law, an increase in sugar concentration can only decrease the freezing point of water by a fraction of a degree (Livingston and Henson 1998), and hence cannot directly prevent freezing at temperatures of below −30 °C as occur at our study sites. All these changes at the cell level are associated with large changes in gene expression at sub-zero temperatures, as evidenced in wheat and *Arabidopsis* (Herman et al. 2006, Le et al. 2015). It remains to be seen whether acclimation at sub-zero temperatures is supported by metabolic and ultrastructural changes in beech as well.

Limitations of the study

The investigated populations are located within the centre of the distribution range of European beech. Even though temperatures in La Brévine are the coldest throughout the range of European beech in winter due to the regular formation of cold air pools in the valley, populations at the northern or eastern range limit might behave differently to a drop in temperature than the investigated populations due to potential genetic adaptation. Nonetheless, the acclimation capacity we found in beech in midwinter is considerable, and occurs in other species as well.

We used cuttings for the acclimation experiments. This has the advantage that we could collect samples from adult trees. Plants are usually more freezing resistant when freezing resistance is directly assessed in situ (Buchner and Neuner 2009, 2011), so our results are rather conservative and adult trees in situ might be able to harden even more in response to a drop in temperature. Further, the use of cuttings could explain why we did not find an increase in freezing resistance in pith tissue and possibly cambial tissue of xylem, since we disrupted the continuity of the xylem. The xylem parenchyma cells and pith cells of beech are known to supercool (Hong et al. 1980). The cut sides of the branches used here most likely lead to ice nucleation at warmer temperatures than in nature, and a subsequently reduced level of supercooling. The presence or absence of intracellular ice nucleators can play an important role in ice formation.

Absolute minimum temperature and the distribution of beech

The absolute minimum temperatures in this study are reached during clear nights with cold air pooling and strong temperature inversion in the valley of La Brévine. During these clear nights, radiative cooling is the most significant driver of the temperature drops. Temperatures measured at climate stations, which are sheltered, are expected to deviate from temperatures measured in the canopy of trees, and of temperatures that buds experience. Interestingly, in a recent study, Kollas et al. (2014*b*) could show that temperatures measured at climate stations are actually cooler than temperatures measured inside forests; however, they match well with temperatures that trees experience in the canopy. Thus, temperatures recorded at weather stations can be used to extrapolate absolute minimum temperatures that trees experience in their crowns.

The temperatures used for the assessment of freezing risk originate from the three climate stations near the study sites. The climate station at the coldest place, in La Brévine, is situated in the bottom of the valley. Beech trees do not occur at the site of the climate station, but only slightly higher on the slopes, where temperatures are warmer during nights with cold air pooling and radiative freezing. Thus, we most likely overestimated the amount of slight damage to beech trees in nights when temperatures fall below LT_{10} values. We only observed temperatures below LT_{10} values of beech trees to occur in La Brévine, which are actually colder than temperatures throughout the entire distribution range of European beech (Vitasse et al. 2014*b*).

The distribution of beech is usually related to a minimum temperature during the coldest month. For instance, pollen records revealed that the distribution of beech correlates well with a mean temperature of −1 and −4 °C in January, both in North America and in Europe (Huntley et al. 1989). Similarly, occurrence data of different beech species correlate well with mean minimum temperature in winter (Fang and Lechowicz 2006). Finally, beech does not occur at sites where the absolute minimum temperature falls below −35 °C in Europe (Bolte et al. 2007). Yet these are statistical correlations, using climatic layers derived from scattered weather stations. The distribution of beech cannot be related to minimum temperature. Thus, in the mentioned studies, other factors like the mean temperature of July, growing season warmth or precipitation and different temperature variables also correlate strongly with the distribution of beech. Because for the freezing survival of a species, absolute temperature extremes, and not means, do matter, the mean minimum temperatures may correlate with the distribution of species, but they likely do not have predictive value in a mechanistic sense. Interestingly, the absolute low temperature extremes (in winter) at the northern range limit of beech are much colder than at the high elevation range limit in the Alps, suggesting that absolute minimum temperatures in winter do not set the physiological range limit of beech (Kollas et al. 2014*a*). Consequently, freezing resistance of different beech populations grown in common gardens and measured in winter does not correlate with the mean minimum temperature of the place of origin in Europe (Kreyling et al. 2014, Hofmann et al. 2015). The LT_{50} values we found are 5–10 K colder than what climate stations at the extreme locations of the distribution of beech recorded in the last 100 years (Vitasse et al. 2014*b*), suggesting that absolute minimum temperature has no predictive value for the distribution limit of beech. Indeed, the distribution limit of beech is rather related to freezing temperatures in spring, and the subsequent mean temperature of the growing season, which ensures shoot maturation and subsequent winter survival (Lenz et al. 2013, 2014, Kollas et al. 2014*a*).

Conclusion

European beech shows a large acclimation potential of freezing resistance and is able to rapidly increase its freezing resistance in response to a drop of temperature in a deeply frozen state in winter. The maximum freezing resistance of beech after artificial hardening is well below absolute minimum temperatures occurring throughout the distribution range of beech. In line with recent studies, our results suggest that winter temperature does not shape the cold range limit of beech. We, thus, conclude that species distribution models should refrain from correlating species distribution with mean minimum temperature in winter, and rather focus on the interaction between low temperature extremes and phenology in spring (Lenz et al. 2013, 2015, Vitasse et al. 2014*b*).

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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