

VIEWPOINT: PART OF A SPECIAL ISSUE ON PLANTS AND CLIMATE CHANGE

## From observations to experiments in phenology research: investigating climate change impacts on trees and shrubs using dormant twigs

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● **Background and Aims** Climate change is advancing the leaf-out times of many plant species and mostly extending the growing season in temperate ecosystems. Laboratory experiments using twig cuttings from woody plant species present an affordable, easily replicated approach to investigate the relative importance of factors such as winter chilling, photoperiod, spring warming and frost tolerance on the leafing-out times of plant communities. This Viewpoint article demonstrates how the results of these experiments deepen our understanding beyond what is possible via analyses of remote sensing and field observation data, and can be used to improve climate change forecasts of shifts in phenology, ecosystem processes and ecological interactions.

● **Scope** The twig method involves cutting dormant twigs from trees, shrubs and vines on a single date or at intervals over the course of the winter and early spring, placing them in containers of water in controlled environments, and regularly recording leaf-out, flowering or other phenomena. Prior to or following leaf-out or flowering, twigs may be assigned to treatment groups for experiments involving temperature, photoperiod, frost, humidity and more. Recent studies using these methods have shown that winter chilling requirements and spring warming strongly affect leaf-out and flowering times of temperate trees and shrubs, whereas photoperiod requirements are less important than previously thought for most species. Invasive plant species have weaker winter chilling requirements than native species in temperate ecosystems, and species that leaf-out early in the season have greater frost tolerance than later leafing species.

● **Conclusions** This methodology could be extended to investigate additional drivers of leaf-out phenology, leaf senescence in the autumn, and other phenomena, and could be a useful tool for education and outreach. Additional ecosystems, such as boreal, southern hemisphere and sub-tropical forests, could also be investigated using dormant twigs to determine the drivers of leaf-out times and how these ecosystems will be affected by climate change.

**Key words:** Dormant twigs, woody plants, phenology, leaf-out, flowering time, phenology, climate change, winter chilling, photoperiod, humidity, frost tolerance, trees, shrubs, invasive species.

### INTRODUCTION

Climate change is already shifting the spring leaf-out and flowering times of trees, shrubs and vines in temperate ecosystems, with major implications for ecosystem processes and interactions among species (Rosenzweig *et al.*, 2008; Richardson *et al.*, 2010; Walther, 2010). Spring warming is one of the major drivers of leaf-out in temperate plants (Polgar and Primack 2011), and warming conditions and earlier leaf-out are lengthening the temperate growing season, shifting patterns of carbon dioxide absorption and biomass accumulation (Richardson *et al.*, 2010, 2013; Migliavacca *et al.*, 2012), and altering the competitive interactions of tree and shrub species (Willis *et al.*, 2008; Cleland *et al.*, 2012; Fridley, 2012). Phenological shifts and associated ecosystem alterations will progress in coming decades as climate conditions continue to change (Kirtmann *et al.*, 2013).

Currently, observed changes in leaf-out and their associated drivers are primarily studied via remote sensing and field observations, whereas field experiments have aimed at process

understanding. These methods have provided many insights into the phenology and ecology of woody plants (Fitter and Fitter, 2002; Menzel *et al.*, 2006; Ibáñez *et al.*, 2010; Richardson *et al.*, 2010), but they also have several key limitations. Remote sensing data often fail to parse out variation at the species level. Field observations of leaf-out times can provide species-level information, but they lack predictive power for no-analogue climates. More importantly, using only observational methods of leaf-out times, it is difficult to quantify independently the influence of key environmental drivers – such as winter chilling requirements, spring warming and photoperiod (Chuine *et al.*, 2010; Körner and Basler, 2010) – and to investigate systematically the role of rare and unpredictable events, such as late spring frosts, droughts and insect outbreaks. Field experiments allow researchers to disentangle and systematically investigate drivers of leaf phenology, but experiments on communities of woody plant species growing in the wild can be methodologically challenging and expensive.

Increasingly, scientists are conducting experiments on dormant twigs of woody plants as a proxy for wild plants in the

field. Experiments using dormant twigs in the lab provide an alternative method for quantifying the environmental factors that affect leaf-out times, flowering times and other spring phenomena. This method is providing new insights into many of the processes that affect plant responses to climate change, such as winter chilling requirements, photoperiod requirements, spring warming requirements, humidity requirements and frost tolerance (Miller-Rushing and Primack, 2008; Basler and Körner, 2012; Lenz *et al.*, 2013; Basler and Körner, 2014; Dantec *et al.*, 2014; Laube *et al.*, 2014a, b; Polgar *et al.*, 2014; Vitasse and Basler, 2014). The use of dormant twigs provides unique opportunities for climate change and ecological research, and will probably expand in coming years. In this Viewpoint article, we review the ways in which twig experiments are presently being used, examine some benefits and challenges of this approach, and suggest some additional approaches that could provide new insights.

#### THE TWIG METHOD: HISTORY AND PRACTICE

The twig method is not new – people have long cut twigs from woody plants and exposed them to warm temperatures to force leaf-out or flowering. For example, it was a folk Roman Catholic custom in Central Europe to cut dormant cherry twigs on 4 December (Saint Barbara's day), take them home and watch them flower at Christmas (Gulevich, 2003). Today, the same principle of using twigs or cuttings is employed in horticulture (Balandier *et al.*, 1993; Ruiz *et al.*, 2007; Campoy *et al.*, 2012; Sønsteby and Heide, 2014) and ecology (Heide, 1993a; Miller-Rushing and Primack, 2008; Basler and Körner, 2012, 2014; Laube *et al.*, 2014a; Polgar *et al.*, 2014) to determine winter chilling requirements, obtain predictions for budburst and flowering dates or otherwise investigate twig and bud physiology or behaviour.

Methods can vary depending on the species, purpose of the study and facilities available. Twig cuttings of variable length (usually 10–30 cm) are brought indoors and put into tap water (Basler and Körner, 2012; Laube *et al.*, 2014a; Polgar *et al.*, 2014) or distilled water (Heide, 1993a), at times with the addition of nutrients or sucrose (Ruiz *et al.*, 2007; Campoy *et al.*, 2012; Sønsteby and Heide, 2014). Often disinfectants are used to clean the cut ends of the twigs (Basler and Körner, 2012, 2014; Laube *et al.*, 2014a). These twigs are placed in controlled conditions, such as laboratory conditions or growth chambers with artificial lights, and monitored until they leaf-out or reach the stage of interest (Fig. 1).

Recent studies have found that cut twigs develop similarly to those remaining on donor trees in the wild when exposed to similar conditions (Laube *et al.*, 2014a; Polgar *et al.*, 2014; Sønsteby and Heide, 2014; Vitasse and Basler, 2014), supporting the use of cut twigs as proxies for the behaviour of woody plants in the field. It remains unclear how differences in twig preparation and treatments influence experimental results. However, one-node cuttings develop differently from longer twigs and whole plants, and their use is discouraged (Sønsteby and Heide, 2014). Methods that result in fungal growth, embolisms or otherwise alter water movement in the plants can also affect results. However, when done carefully, the results from twig experiments seem broadly robust; they reflect observations

in the wild and can aid predictions of phenological and other behaviours in future climate conditions (Laube *et al.*, 2014a; Polgar *et al.*, 2014). For instance, models used to estimate the fulfilment of chilling requirements of fruit and nut trees are based in part on data from controlled twig experiments, and are used to predict future impacts of climate change (Luedeling, 2012).

#### PRIMARY ENVIRONMENTAL DRIVERS OF LEAF-OUT PHENOLOGY

Twig experiments are well suited to investigate environmental factors that influence the leaf-out times of woody plants. Leaf-out times are widely considered to be controlled by a combination of winter chilling, photoperiod and spring warming (Murray *et al.*, 1989; Heide, 1993a; Chuine *et al.*, 2010; Körner and Basler, 2010; Hänninen and Tanino, 2011; Polgar and Primack, 2011; Way and Montgomery, 2014). However, so far the evidence for this is based on a limited number of experiments and species (e.g. Murray *et al.*, 1989; Heide, 1993a, b; Caffarra and Donnelly, 2011; Taeger *et al.*, 2015), combined with analyses of long-term observations (e.g. Menzel *et al.*, 2006; Ibáñez *et al.*, 2010; Yu *et al.*, 2010) and phenological models describing leaf-out (Chuine, 2000; Morin *et al.*, 2009; Blümel and Chmielewski, 2012; Olsson and Jönsson, 2014). Additionally, the magnitude and nature of phenological responses to environmental conditions, such as warming spring temperatures and reduced winter chilling, are known to vary substantially from species to species, and we currently lack information on the responses of most species.

Three recent experiments (Basler and Körner, 2012; Laube *et al.*, 2014a; Polgar *et al.*, 2014) highlight the potential value of twig experiments for leaf-out studies; they used dormant twigs, clipped from wild temperate woody plants during the winter and warmed in laboratory conditions, to investigate species-specific leaf-out requirements. These studies investigated dozens of tree, shrub and vine species, and found that the extent of spring warming strongly affected leaf-out phenology in all species studied. In contrast, the influence of winter chilling was highly variable among species, and photoperiod affected only a limited number of species. These three experiments were successful at isolating the roles of various factors and comparing large numbers of species in a manner previous studies had not accomplished.

Specifically, these three studies demonstrated that 34 out of the 36 species tested in Germany had winter chilling requirements (Laube *et al.*, 2014a), as did the majority of species tested in North America (Polgar *et al.*, 2014). Photoperiod influenced leaf-out times for five of 14 native species in Europe (Basler and Körner, 2012) and one of 17 species in North America (Polgar *et al.*, 2014) (Fig. 1A). In Germany, an experiment of both winter chilling and photoperiod revealed that 12 of 36 species responded to photoperiod; however, even in those 12 species photoperiod had only a minor effect on leaf-out time, and was not significant once winter chilling requirements had been met (Laube *et al.*, 2014a) (Fig. 1B). Although the twig method has only been used in a handful of studies investigating the ecological drivers of leaf-out times, it has already

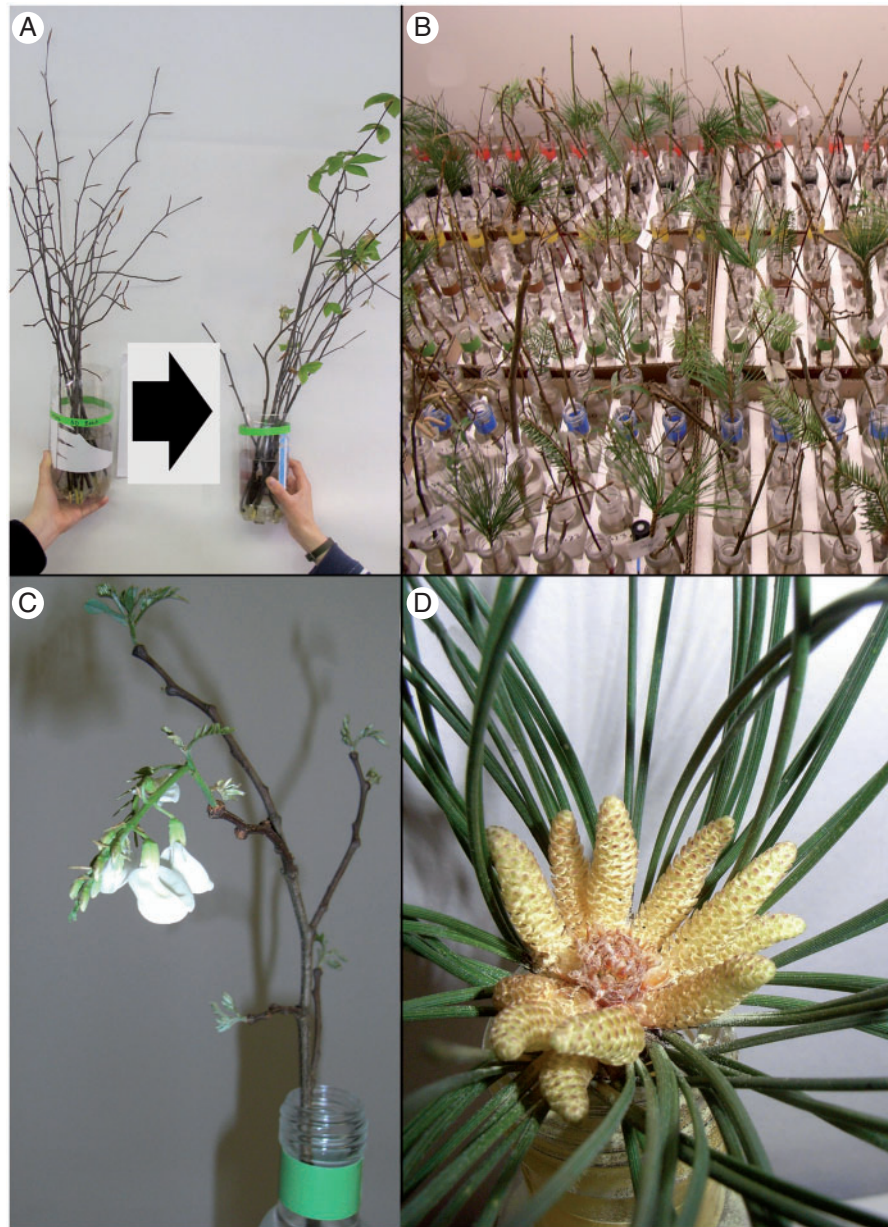


FIG. 1. Examples of research projects using twig experiments; all photographs are of twigs sampled during winter and then monitored for leaf-out and flowering. (A) Beech (*Fagus grandifolia*) twigs kept in ambient (left) and extended (right) daylength (demonstrating that twigs can be used to study the influence of photoperiod on leaf-out). (B) A diversity of twigs exposed to a common set of conditions to investigate species differences in winter chilling requirements. (C) Flowering twig of locust (*Robinia pseudoacacia*) (flowering in the lab during a leaf-out study, demonstrating that twigs can be used to study flowering). (D) Pollen release in a twig of an evergreen mountain pine (*Pinus nigra*) (demonstrating the ability to study reproductive phenology and pollen amount or allergen content with twigs). (Photographs by J. Laube and R. Primack.)

proven to be a simple and effective approach for disentangling multiple drivers and studying many species simultaneously.

Despite these successful experiments, investigators should be concerned about how the results of twig experiments are affected by variations in the experimental treatments. Each of four recent experimental studies that investigated the primary drivers of leaf-out phenology (Basler and Körner, 2012; Dantec *et al.*, 2014; Laube *et al.*, 2014a; Polgar *et al.*, 2014) used different combinations of daylength and temperature, different observation frequency and different methods of calculating

growing degree days (Table 1). Direct comparison of the results is not possible for most pairs of studies due to the use of different species. However, the studies by Basler and Körner (2012) and Laube *et al.* (2014a) had 11 species in common and a highly significant positive correlation in the growing degree days of spring warming required for leaf-out among the species (Spearman rank correlation  $\rho = 0.72$ ). At the species level, both studies found that *Larix decidua* had the lowest requirement for growing degree days, and that *Fraxinus excelsior* had the highest requirement. This comparison of two studies in



TABLE 1. Experimental procedures of four recent studies using dormant twigs from woody plants to investigate leaf-out requirements (Basler and Körner, 2012; Dantec et al., 2014; Laube et al., 2014a; Polgar et al., 2014)

Study	Light treatments (daylength)	Temperature treatments (night/day)	Observation frequency	Development stage	Base temperature used for GDD calculation	Chilling days prior to experiment	Species groups used
Polgar	Constant: 14 h	Constant: 22/22 °C	1 per week	Leaf-out	5 °C	114	Shrubs, trees and vines
Dantec	Constant: 16 h	Constant: 25/25 °C	3 per week	Budburst	5 and 10 °C	100	Trees
Basler	Increasing: 9-5 to 12 h, and 11 to 14 h	Increasing: 5 to 10 °C; night/day difference 5 °C	3 per week	Budburst	0 °C	92–119	Trees
Laube	Constant: 8, 12 and 16 h	Increasing: 7 to 27.5 °C; night/day difference 5 °C	3 per week	Budburst	0 °C	33–110	Shrubs and trees

The studies differed in the number of chilling days experienced by twigs prior to collection, exposed twigs to different light and temperature treatments in the laboratory, had different frequencies of observations, monitored different stages of leaf-out and used different formulas to calculate the growing degrees (GDD) required in order for twigs to leaf out.

which twigs were exposed to different temperature and photoperiod conditions suggests that the use of twigs is fairly robust to variations in the experimental treatment.

### INVESTIGATING FLOWERING TIMES

Apart from leaf-out studies, twig experiments have also been used to investigate the environmental drivers of flowering times (and pollen release in the case of gymnosperms) in temperate trees. For example, studies have used twigs to investigate flowering times in birches (Miller-Rushing and Primack, 2008) and the early stages of flower bud development of apricots (Ruiz et al., 2007; Campoy et al., 2012). The same factors that affect the breaking of dormancy of leaf-out similarly affect the breaking of dormancy of flower buds (Campoy et al., 2011). Therefore, twig experiments can be used to study the role of temperature, light, frost and other factors in influencing flowering times of woody plants (Fig. 1C, D). This has special economic and cultural significance for ornamental plants, such as the blossoming of Japanese cherry trees, and for fruit trees in which earlier flowering results in earlier fruit set.

For species that are major contributors to pollen allergies, such as pines, oaks, poplars, hazels and birches, twig experiments can be used to investigate the climatic factors that contribute to the date of pollen release. For example, a study using dormant birch twigs showed that species release pollen earlier in warmer temperatures (Miller-Rushing and Primack, 2008). The study also showed that species vary in their responses – species with higher water content in the catkins release pollen later in the spring. Such studies on pollen release from catkins of spring-flowering, wind-pollinated tree species have practical value in predicting the allergy season, with significant public health consequences. Seasonal timing, population differences or other environmental factors, such as ozone, that influence major pollen allergens (Beck et al., 2013), could be readily studied with twig experiments.

### HUMIDITY AND OTHER ENVIRONMENTAL DRIVERS

Winter chilling, photoperiod and spring warming are not the only factors that control leaf-out phenology. In a recent study (Laube et al., 2014b), twigs were used to investigate the

importance of air humidity in the leaf-out process. The study showed that high air humidity accelerates leaf-out phenology, suggesting that twigs and buds may take up moisture from the air during early spring development.

The study highlights the lack of physiological understanding of the spring leaf-out process of trees. While much of the current knowledge is based on correlative studies, the findings of the twig humidity experiment conducted by Laube et al. (2014b) illustrate a weakness in resolving confounding environmental triggers, since absolute humidity is often positively correlated with temperature, i.e. warm air holds more moisture than cold air. Thus, earlier leaf-out times of woody plants in warmer conditions may actually be due, at least in part, to higher humidity rather than higher temperatures. Twig experiments present the opportunity to explore the role of other complex factors on leaf-out phenology, such as nutrient availability, temperature variability and the role of daytime vs. night-time temperatures.

### DIFFERENCES AMONG SPECIES ACCORDING TO ECOLOGICAL STRATEGY

Researchers using twig experiments have found that different groups of woody species, such as trees and shrubs, pioneer and climax species, and native and non-native invasive species can differ in their phenological responses to changes in climate (Basler and Körner, 2012; Laube et al., 2014a; Polgar et al., 2014). Polgar et al. (2014) found that across 43 species from a single community in North America, non-native invasive shrub species had lower winter chilling requirements and faster leaf-out than did native shrub and tree species (Fig. 2). In a study of 36 species from a single site in Germany, both pioneer and non-native invasive shrub and tree species showed lower chilling requirements and faster leaf-out than did native climax tree species (Laube et al., 2014a). A study in Switzerland (Basler and Körner, 2012) suggests that photoperiod effects, although minor, affect plants differently based on their ecological strategies; for 14 tree species in Switzerland in one chilling treatment, climax species responded more strongly to photoperiod than did pioneer species. The fact that these studies differed in methodology (Table 1) and species, but were each able to detect an ecological pattern, suggests that it would be worthwhile

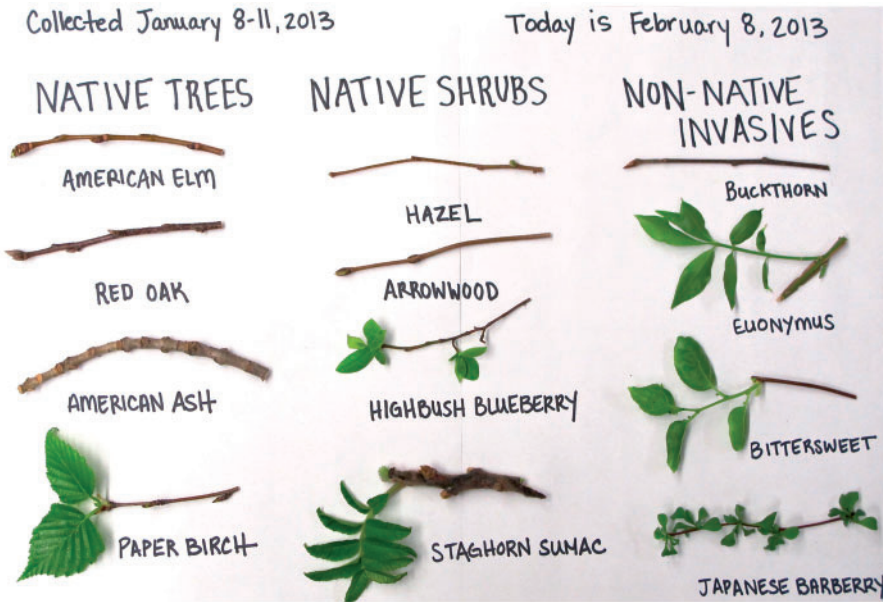


FIG. 2. Twig experiments allow species differences in spring development to be easily communicated. In this case, twig experiments revealed that non-native invasive shrubs (right) on average leaf out earlier and have a lower winter chilling requirement than native trees (left), with native shrubs (middle) intermediate. (Photograph by A. Gallinat and R. Primack.)

to expand on these studies using a wider range of species and ecosystems.

#### SEPARATING GENETIC AND ENVIRONMENTAL FACTORS

Twigs of different populations or provenances, as well as from across a species' range, have been examined under controlled conditions, with individuals from different elevations showing different photoperiod requirements (Basler and Körner, 2012) and different thermal requirements (Dantec *et al.*, 2014) for leaf-out. Thus, twig experiments can also be used to determine genetic differences in response to environmental drivers, i.e. genetic variation within and between populations or provenances. Twigs can also be used in reciprocal transplant experiments. Traditional reciprocal transplant experiments are used to investigate genetic and environmental contributions to observed population differences. These experiments are conducted by transplanting individual plants among different sites or gardens, which can limit the number of species and replicates in each study. However, the twig method could be used to test the same hypotheses as traditional reciprocal transplant experiments, by collecting twigs of the same species from multiple locations and exposing them to reciprocal field sites or to environmental treatments in the laboratory. For instance, twigs from different populations could be sampled and brought to different sites along an elevation gradient to study leaf-out times and other phenological characteristics. This would be both faster and less expensive than establishing long-term experimental gardens along altitudinal gradients. In such reciprocal transplant experiments, researchers should consider the clonal nature of the species under investigation, especially for shrub species, and the possibility of residual environmental effects of the source population that take more than one season to subside.

#### INVESTIGATING FROST TOLERANCE

Twig experiments, field experiments and observational studies show that leaf-out and flowering are advancing with warmer spring temperatures, and that this phenomenon can increase the risk of frost damage to woody plants if a late frost occurs (Inouye, 2008; Augspurger, 2013; Vitasse *et al.*, 2014a). Frost damage to young leaves and flowers can affect both wild plants in natural habitats and economically important plants, such as fruit trees (Palonen and Buszard, 1997). Twig experiments offer a method for examining potential drivers of frost tolerance, and variation in frost tolerance among multiple species in the laboratory.

Studies have indicated that frost tolerance is genetically conserved and is frequently the limiting factor in the species ranges of fruit trees (Palonen and Buszard, 1997). Still, relatively little is known about the relative frost tolerance of young leaves in wild woody species. In a recent study, Lenz *et al.* (2013) used twigs to test the hypothesis that the earliest tree species to leaf-out in the spring are more frost tolerant than species that leaf-out later. They demonstrated that for eight deciduous tree species, the earliest species to leaf-out are the most frost tolerant (Lenz *et al.*, 2013) (Fig. 3A). They also demonstrated that dormant twigs were far more frost tolerant than twigs undergoing leaf-out (Fig. 3B), and that variation in the age of the plants did not have significant effects on the degree of frost tolerance (Vitasse *et al.*, 2014b). The study by Lenz *et al.* (2013) also suggests that the visual inspection of frost damage is as effective as more elaborate biochemical and anatomical tests (Fig. 3C), a result which greatly simplifies the experimental procedures needed to conduct frost studies (Lenz *et al.*, 2013).

Twig experiments can be used to advance our understanding of the drivers of frost tolerance in wild plants and to identify vulnerable species. For example, lower winter chilling requirements of invasive species, demonstrated by previous twig experiments

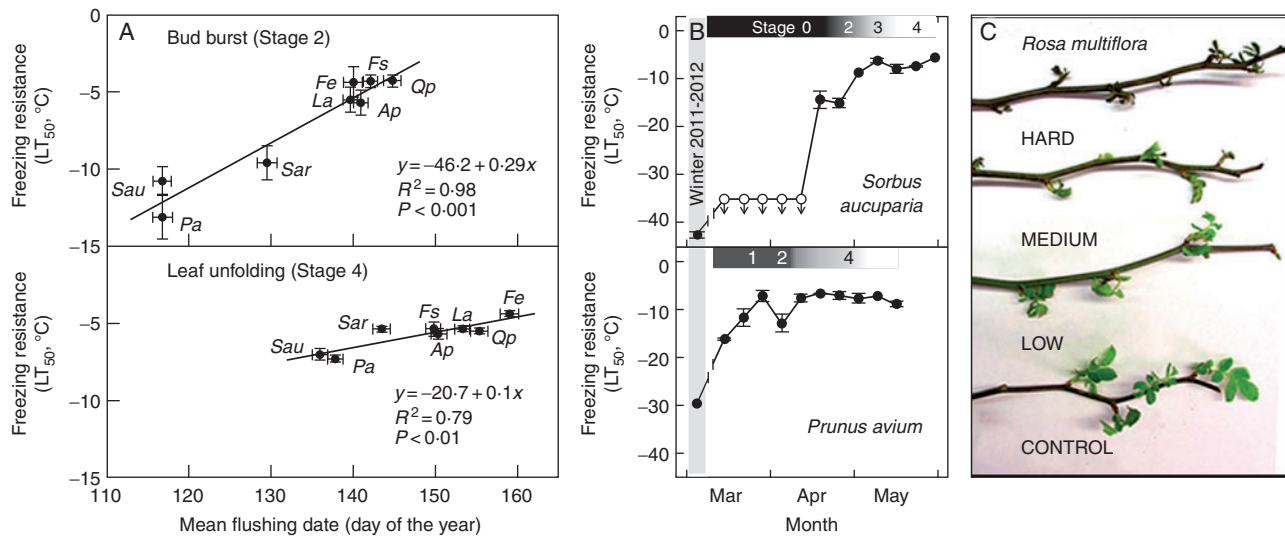


Fig. 3. Freezing resistance during spring development using twigs. (A) Twig experiments show that tree species with later dates of budburst and leaf-out dates have lower frost tolerance (adapted from Lenz *et al.*, 2013). Ap, *Acer pseudoplatanus*; Fe, *Fraxinus excelsior*; Fs, *Fagus sylvatica*; La, *Laburnum alpinum*; Pa, *Prunus avium*; Qp, *Quercus petraea*; Sar, *Sorbus aria*; Sau, *Sorbus aucuparia*; y-axis: lethal temperatures (at which 50 % of the samples died). (B) Twig experiments are used to show a decrease in frost tolerance with successive stages of phenological development (adapted from Lenz *et al.*, 2013). Stage: phenological development from 0 (no development) to 4 (leaf unfolding); y-axis: lethal temperatures (at which 50 % of the samples died). (C) Use of twigs to assess frost tolerance in multiflora rose (*Rosa multiflora*) using exposure to different frost temperatures: hard (−5 °C), medium (−3 °C), light (−1 °C) and control (5 °C). The amount of frost damage increases with lower temperatures. (Photograph by A. Gallinat.)

(Laube *et al.* 2014a; Polgar *et al.*, 2014), suggest that invasive species are at greater risk of encountering late frosts. Thus, information on the frost tolerance of invasive species is important to predict whether frost may limit their ability to invade an area or whether their frost tolerance could facilitate spread. Researchers can also use twig experiments to explore the molecular and anatomical mechanisms of frost tolerance, identify how frost tolerance is contributing to current range shifts, and examine interactions with other factors, such as exposure to sun and wind.

Twig experiments to determine the frost tolerance of flowers are particularly appropriate for investigations of early-flowering species grown for commercial fruit production, such as apples, pears, peaches, cherries and blueberries, and ornamental species grown for early flowering, such as forsythias, magnolias and redbuds. Commercial fruiting species are often selected for early flowering times in order to have earlier fruits for selling. However, it is currently unknown whether the selection for earlier flowering makes species more or less vulnerable to late frosts. While early-flowering species are more likely to be exposed to frost, Reig *et al.* (2013) used the twig method on flowering peach stems to demonstrate that the earliest flowering cultivars are the most frost tolerant. The flowering times and frost tolerance of other species can be studied by monitoring flowering times of twigs in the lab and subjecting these flowering twigs to frost tolerance experiments similar to those which have been carried out on young leaves.

#### ADVANTAGES AND DISADVANTAGES OF TWIG EXPERIMENTS

Twig experiments have the advantages of being simple and highly repeatable relative to experiments carried out under field

conditions. Twigs can be brought into laboratory conditions where light, temperature and other environmental variables can be controlled, and unpredictable field conditions can be avoided. Several lines of evidence suggest that dormant twigs used in laboratory and field experiments are effective model systems for investigating the responses of adult plants (Laube *et al.*, 2014a; Polgar *et al.*, 2014; Sønsteby and Heide, 2014; Vitasse and Basler, 2014). In addition, while seedlings and young plants may be easier to manipulate than adult plants, they often exhibit different phenological patterns (Augspurger, 2008; Vitasse, 2013; Vitasse *et al.*, 2014b), and so twigs taken from adult plants offer an easily manipulated alternative that is more reflective of adult plant phenology.

Because twigs can be gathered in large numbers, and their maintenance requires limited space, it is easy to have many samples or replicates and carry out frequent observations. It can be difficult and expensive to set up experiments with large numbers of rooted tree seedlings, and, as previously stated, they can show unwanted ontogenetic effects. Daily observations of large numbers of plants in the wild are often restricted by logistics.

Moreover, the development of standardized protocols using twigs is relatively easy, such as using a fixed set of temperatures, frost, light and humidity conditions, which could facilitate extensive international collaborations. As mentioned above, even when twigs are exposed to different sets of laboratory conditions, the key differences among species in their winter chilling requirements are still apparent. Because of their relative ease, twig experiments could promote phenological research in understudied ecosystems, such as southern hemisphere temperate forests, tropical deciduous and semi-deciduous forests, boreal forests and dry shrublands.



There are also disadvantages to using twigs in the laboratory. For instance, a certain small percentage of plant species do not leaf-out well from twigs, perhaps due to damage to the xylem or phloem systems, especially when having long vessels, or increased chance of fungal infections and other disease affecting the developing tissues. A frequent problem in these experiments is fungal moulds growing on the stems in water; these fungi can be reduced and removed by weekly scrubbing, re-cutting the bottom of each stem, changing the water in the containers and through the use of chemical solutions. Insect outbreaks, from occasional dormant or developing insects brought in from the field on the twigs, can damage the tender young leaves and bud tissues. Also, sometimes the twigs and buds simply dry out and fail to leaf-out. However, our experience is that only a small number of species (<10 %), (e.g. *Buddleja davidii*, *Lonicera subsessilis* and *Euonymus sieboldianus*) are not appropriate for twig experiments. Further work is needed to determine if the success of using twigs can be increased by techniques to prevent embolisms (air bubbles) and blockages in the xylem, such as re-cutting twigs under water. Further research could also investigate whether problematic species share certain wood anatomical traits (such as ring porous stem anatomy or twig diameter), phylogenetic affinities or growth habits (such as the shrubs mentioned above).

Additionally, using twigs in laboratory conditions does not replicate factors affecting the root system, including soil conditions and snow cover (e.g. Inouye, 2008; Cornelius *et al.*, 2013); nor does it always replicate the fluctuating levels of temperature and light, and other aspects of microsite conditions in the field, depending on the sophistication of the laboratory conditions. Frost tolerance experiments in particular might be affected by environmental conditions, particularly if light levels are too low for adequate photosynthesis and sugar production prior to freezing (Gusta and Wisniewski, 2013). High quality growth chambers can more accurately mimic field conditions of spring temperature, frost, humidity and the precise qualities of light, but on the other hand they can be costly to buy and maintain. However, simpler alternatives exist or can be built, and may be adequate for many studies. For example, various published winter chilling experiments have used both growth chambers and ordinary laboratory conditions with no substantial difference in the results (Laube *et al.* 2014a; Polgar *et al.*, 2014), or have used a wide range of growing conditions already available for other purposes (Miller-Rushing and Primack, 2008). For frost tolerance experiments, one approach is to use fairly expensive scientific, programmable freezers or growth chambers, but it is also possible to achieve a suitable range of temperatures by modifying and re-programming commercial refrigerators and freezers (Lenz *et al.*, 2013). In many temperate and boreal systems, snow cover often protects low-lying shrubs from frost damage and desiccation since it affects soil temperature and the depth of soil freezing. Combining frost tolerance experiments in the lab with field experiments that manipulate snow cover and depth for wild shrubs could be an effective approach in future climate change research.

Researchers using twig experiments need to be vigilant in detecting artefacts caused by the experimental process itself, and to be aware that the rates of response in the lab might be different from those in the field. In recent warming studies, for example, plants have been shown to have lower responses to

experimental temperature variation than are indicated by long-term observations of wild plant populations (Wolkovich *et al.*, 2012).

## CONCLUSIONS

Because of their numerous advantages, twig experiments can continue to advance our understanding of phenology in important ways.

### *Additional factors that may influence phenology*

Many factors, such as nutrient status (Jochner *et al.*, 2013), precipitation (Fu *et al.*, 2014) and light quality (Linkosalo and Lechowicz, 2006), could influence spring leaf-out phenology, but their effects are understudied. Chief among these factors may be the influence of phylogeny, i.e. do closely related species tend to behave similarly? A few recent studies involving numerous species have found phylogenetic patterns in leaf-out and flowering times (Fridley, 2012; Davies *et al.*, 2013; Panchen *et al.*, 2014). Twig experiments could be used to extend these studies to determine if closely related species tend to share similar characteristics, such as photoperiod and winter chilling requirements.

### *Interactions*

The flexibility and large sample sizes possible in twig experiments make them particularly attractive for studies investigating interactions among factors that influence phenology and among plant traits. For example, twig experiments that couple measurements of moisture content during dormancy and spring leaf-out with experimental frost treatments could deepen our knowledge of how tissue moisture, phenological development and frost tolerance interact. As another example, ecological chilling experiments in which twigs were collected from the wild on different dates have not separated out temperature and photoperiod factors; twigs cut earlier in winter had experienced both less chilling and shorter daylength than twigs cut in late winter with both more chilling and longer daylength. However, further improvements are easily possible with a combination of controlled dormancy treatments, as already used in the horticultural field, and controlled forcing and photoperiod conditions.

### *Test model assumptions, parameters and thresholds*

Experiments in which twigs are exposed to a range of environmental conditions, especially factorial experiments, could significantly improve current models of leaf-out responses to climate change. Specifically, the somewhat arbitrary temperature thresholds for chilling or forcing and photoperiod requirements derived from model optimization routines (Kramer 1994; Hänninen, 1995; Richardson *et al.*, 2006; Linkosalo *et al.*, 2008; Blümel and Chmielewski, 2012; Migliavacca *et al.*, 2012; Olsson and Jönsson, 2014) need thorough experimental testing for individual species. It is quite likely that many individual species have winter chilling requirements that are

somewhat different from the standard models developed for a small number of fruit species, or show differing temperature thresholds and photoperiod requirements for the start of physiological processes.

#### Other spring phenomena

The use of twig experiments could provide a method for examining other spring phenomena, such as the increasing photosynthetic activity of young leaves as they expand in size and mature, the effects of plant nutrient status and light levels on the physiological ecology of the leaves, the timing of production of secondary plant compounds within the leaf tissue, the related timing of herbivore damage on leaf tissue, and the shifting balance of plant hormones during development. For investigations of herbivore damage, leaves of different ages could be exposed to herbivores for short intervals of time to determine the effects of plant secondary compounds and leaf toughness on insect growth and feeding preference.

#### Further possibilities

In addition, the use of twigs might have applications for investigating the effects of climate change on plants in other seasons of the year. In autumn especially, twig cuttings with a reduced number of leaves could potentially be used to investigate the effects of frost, temperature and photoperiod on leaf senescence and dormancy induction. Twigs collected in the winter could be exposed to brief warm periods, simulating the effects of unseasonable warm periods, then returned to the cold temperatures of winter, to investigate how more variable climatic conditions, or extreme events (Jentsch *et al.*, 2009), will affect the phenological development. However, it remains to be seen if such experiments will work in practice.

Lastly, twig experiments could be incorporated into environmental education, school science lessons and science fair projects related to climate change and plant science. Such experiments are valuable because twigs will leaf-out earlier or later in the range of temperatures and light environments found in school buildings and homes during the winter and early spring, demonstrating principles of species variability and the effects of environmental drivers on seasonal plant processes.

Dormant twigs provide a valuable and underutilized approach for investigating a wide range of topics relevant to climate change biology, ecosystem ecology, phenology research and plant physiological ecology. The most obvious next step is to expand the use of twig experiments to investigate how different facets of climate change impact the ecophysiology and leaf dynamics for a wide diversity of trees, shrubs and vine species, and across a range of ecosystems. In addition, experiments on frost tolerance and humidity requirements can be used to predict which species may benefit from a changing climate and which species may decline and become locally extinct. Information from these experiments can then be used to improve climate change forecasts of shifts in phenology, ecosystem processes and ecological interactions.

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