

Phenology and Physiology of White Ash in Relation to Climate Extremes

By

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Phenology and Physiology of White Ash in Relation to Climate Extremes

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## Abstract

The industrial revolution began approximately 200 years ago, and since then atmospheric [CO<sub>2</sub>] has increased from 270 to 402 ppm, and global temperatures have warmed by an average of 0.85 °C. Anthropogenic climate change is expected to alter both climate mean conditions and variability, leading to the more frequent occurrence of intense climate extremes such as heat waves and drought. This increase in climatic variability is likely to profoundly affect trees, as phenological and physiological processes of these species have been shown to be more sensitive to climate extremes than changes in the mean state. Yet, investigations of physiology and phenology of trees in relation to climate extremes remains limited, especially studies that address effects of climate extremes among- and within-populations of tree species.

Investigations of the effects of weather extremes on phenology and physiology among- and within-populations of trees can provide important information on future phenological and physiological patterns that may affect ecosystem-level processes such as carbon cycling.

To assess phenological and physiological responses of a tree to weather extremes, I measured phenology and physiology among 43 populations of *Fraxinus americana* (white ash) growing in a common garden during the extreme warm year of 2012, which was also one of the driest years on record at this site, and relatively non-extreme years. Intraspecific phenological responses across extreme and non-extreme years were assessed by observing the timing of leaf emergence whereas intraspecific physiological responses across extreme and non-extreme years were assessed by measuring leaf-level carbon isotopic signatures ( $\delta^{13}\text{C}$ ). Additionally, we tested the performance of a commonly used phenology model in extreme and non-extreme years.

I also identified three major ploidy levels of white ash at this site (diploids, tetraploids, and hexaploids), and this allowed me to investigate the consequences of polyploidy (within-population variation) on plant water relations by measuring leaf-level mid-day water potentials, gas exchange,  $\delta^{13}\text{C}$ , xylem-specific hydraulic conductance ( $K_{smax}$ ), xylem fatigue, and xylem density across three populations within which all cytotypes were represented.

Leaf emergence among white ash populations was accelerated by 22 d during the extreme warm year of 2012 relative to non-extreme years. Additionally, thermal requirements shifted such that greater amounts of warming were required for leaf emergence during the extreme year relative to non-extreme years and this constrained the potential for even earlier leaf emergence by 7 d. I also found rank order for leaf emergence was maintained among populations across extreme and non-extreme years, suggesting at least the relative timing of leaf emergence may be predictable during future extreme years. Most concerning was the finding that responses of white ash to an extreme warm year altered the reliability of conventional models in predicting leaf emergence.

In relation to physiology, we found that the rank order in leaf-level  $\delta^{13}\text{C}$  among populations was maintained across extreme and non-extreme years. This result suggest that the origin of white ash populations may play a role in controlling physiological functioning, and that the relative physiological relationships among populations will be maintained across extreme and non-extreme years. While population had a strong effect on  $\delta^{13}\text{C}$ , we did not find that average  $\delta^{13}\text{C}$  among populations varied significantly across years. Thus, populations of white ash differ constitutively in leaf-level  $\delta^{13}\text{C}$  with little environmentally induced change. We did find a significant linear relationship

between average leaf-level  $\delta^{13}\text{C}$  and growing season (March-Aug.) vapor pressure deficit (VPD); however, this relationship was significantly weakened during 2013. This suggests that the effects of extreme years on white ash physiology may persist into non-extreme years.

As intense droughts are projected to become more frequent over the next century, we investigated the effect of polyploidy on white ash water relations. Different degrees of ploidy within plants can produce morphological and physiological differences in response to local environments, including larger xylem vessels and larger stomata due to increased cell size, and these traits can affect drought tolerance of individuals. I found significant effects of cytotype on mid-day leaf-level water potentials whereby water potentials increased with increasing ploidy level. I also found significant effects on leaf-level gas exchange between cytotypes where diploids showed higher rates of photosynthesis ( $A_{max}$ ), stomatal conductance to water vapor ( $g_s$ ), and transpiration ( $E$ ). However, I did not find any differences across populations or between cytotypes nested within populations in leaf-level  $\delta^{13}\text{C}$ ,  $K_{smax}$ , xylem fatigue, or xylem density. These results suggest polyploidy may affect leaf-level responses in white ash across the growing season.

This research advances our understanding of phenological and physiological responses among- and within-populations of white ash to weather extremes. I report on one of the first studies to document intraspecific variation in phenology during extreme and non-extreme years, and show extreme years will fundamentally alter phenology and its cues. I also find strong population level effects on  $\delta^{13}\text{C}$ , but marginal shifts in these responses across extreme and non-extreme years, suggesting populations have similar leaf-level  $\delta^{13}\text{C}$  with little environmentally induced change. The study on the influence of

polyploidy on water relations of white ash indicates genome duplication may affect physiological responses of white ash during extremely dry periods across the growing season. My documentation of phenology and physiology among- and within-populations of white ash during extreme and non-extreme years provide novel insights into the potential of white ash to respond to rapid environmental changes that are expected under future climate change scenarios with more commonly occurring weather extremes.

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## Table of Contents

Title Page	i
Acceptance Page	ii
Abstract	iii
Acknowledgements	vii
Chapter 1: Warmest Extreme Year in U.S. History Alters Thermal Requirements for Tree Phenology; Carter <i>et al.</i> in review, <i>PNAS</i>	1
Chapter 2: Intraspecific Responses in Leaf-Level $\delta^{13}\text{C}$ of <i>Fraxinus americana</i> are Constant across Extreme and Non-Extreme Years	41
Chapter 3: Ploidy Level of White Ash Trees Influences Water Relations in a Common Garden	69
Chapter 4: Preventing the Decline of Pollinators in the United States: An Overview of Executive-Level Policy	101
Conclusions	137

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**Title:** Warmest Extreme Year in U.S. History Alters Thermal Requirements for Tree Phenology

**Short Title:** Extreme Warm Years Alters Tree Phenology

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## **Abstract**

The frequency of extreme heat events has increased across the majority of the planet. Shifts in plant phenology in response to heat extremes can impact plant survival, productivity, and synchrony with pollinators. Despite extensive study of plant phenology in response to climate change, little is known about the influence of extreme warm years, particularly at the intraspecific level. Here we compare the timing of leaf emergence among 43 different populations of white ash trees (*Fraxinus americana*) during the warmest extreme year in U.S. history (2012) with relatively non-extreme years. This is among the earliest reports to document intraspecific variation in phenology during an extreme warm year, incorporating population-level variation from across the species range in a common garden. We show that (a) leaf emergence among white ash populations was accelerated by 22 d during the extreme warm year of 2012 relative to non-extreme years, (b) rank order for leaf emergence was maintained among populations across extreme and non-extreme years, (c) thermal requirements shifted such that greater amounts of warming were required for leaf emergence during the extreme year relative to non-extreme years and this constrained the potential for even earlier leaf emergence by 7 d on average, and (d) responses of white ash to an extreme warm year fundamentally altered the reliability of conventional models in predicting leaf emergence. Moreover, these results demonstrate a critical need to better understand how extreme warm years will affect tree phenology, since these years are predicted to become the climate norms of the future.

## **Significance Statement**

Phenology responses to heat extremes can impact plant survival, productivity, and interactions with pollinators. We studied leaf emergence among white ash populations during the warmest extreme year in U.S. history (2012). We show that (a) leaf emergence was accelerated by 22 d during the extreme year, (b) rank order for leaf emergence was maintained among populations across years, (c) greater amounts of warming were required for leaf emergence during the extreme year that buffered further accelerations in phenology, and (d) extreme year responses reduced our ability to predict leaf emergence using conventional modeling approaches. These results demonstrate a need to better understand how extreme years will impact phenology, being that these conditions will become the climate norms of the future.

## Introduction

Since the industrial revolution began ( $\approx 200$  years ago), atmospheric [CO<sub>2</sub>] has increased from 270 to 402 ppm, and global temperatures have warmed by an average of 0.85 °C (IPCC, 2012). In addition, recent analysis of temperature anomalies indicates that extreme warm events at the seasonal level are occurring much more frequently over the last 30 years relative to the past (Weaver, Kumar, & Chen, 2014). The IPCC reports that increases in daily temperature extremes and reductions in cold extremes are “virtually certain” to occur in the future (IPCC, 2012). Furthermore, Hansen *et al.* (2012) showed that extremely hot summers ( $>3 \sigma$  warmer than average) that were nearly absent during 1951-1980 (0.2% of land area) are now common across 10% of global land area (Hansen, Sato, & Ruedy, 2012).

Plant phenology (timing of major life cycle events) is an important driver of ecosystem processes and may impact plant survival (Augspurger, 2013), particularly saplings, productivity (Chang, Wang, & Huang, 2013), and synchrony with pollinators and/or herbivores (Forrest, 2015). Little is known about phenological responses to extreme warm years, and the few studies that have been conducted report unprecedented accelerations in flowering times and/or leaf emergence. For example, Rutishauser *et al.* (Rutishauser, Luterbacher, Defila, Frank, & Wanner, 2008) found that the flowering times of cherry trees in Switzerland advanced by 28 d during the extreme year of 2007 compared with the long-term mean (1702-2007). Friedl *et al.* (Friedl *et al.*, 2014) reported that leaf emergence was accelerated by as much as 14 d relative to long-term averages among tree species at Harvard Forest and Hubbard Brook Experimental Forest during the extreme years of 2010 and 2012. In addition, Ellwood *et al.* (Ellwood, Temple, Primack, Bradley, & Davis, 2013) observed the earliest flowering

times in recorded history among 32 plant species in Massachusetts, USA during the extreme years of 2010 and 2012 relative to long-term records dating back to the 19<sup>th</sup> century. In this case, some species advanced flowering by as much as six weeks during 2012. Furthermore, Ellwood *et al.* (Ellwood *et al.*, 2013) showed that the timing of flowering in extreme warm years can be estimated across species by extrapolating historical relationships between flowering times and mean spring temperatures. Unfortunately, little is known about the *intraspecific* phenological responses of plants to extreme warm seasons/years and whether population-level responses can be extrapolated to extreme events from thermal requirements that occur during non-extreme events. Such knowledge is critical for fully determining how species will respond as extreme years become the climate norms of the future (Hansen *et al.*, 2012; IPCC, 2012).

Leaf emergence in temperate trees is a major phenological event because it marks the period when the majority of photosynthetic carbon assimilation begins. In addition, shifts in the timing of leaf emergence in response to temperature extremes can have long-term implications for ecosystem functioning and carbon sequestration. For example, warmer temperatures in early spring have been linked to accelerated phenology that has made trees more susceptible to frost damage, which can ultimately reduce long-term carbon gain and tree survival (Augspurger, 2013). The environmental sensing mechanisms that control the timing of leaf emergence in trees are poorly understood, although it is clear that they can involve both the influence of temperature and photoperiod (Polgar & Primack, 2011). Temperature is considered to be the most dominant factor in influencing leaf emergence in woody species of the temperate zone (Polgar & Primack, 2011), and this factor is generally separated into two main



components. First, temperate trees require a specific number of chilling degree units (CDUs) in winter to break dormancy. Once dormancy is broken, trees begin tracking warming temperatures (referred to as growing degree units, GDUs) until thermal requirements are met. At this point a given phenological event can occur, assuming that other requirements such as photoperiod are met (Polgar & Primack, 2011). In addition, there is thought to be an interplay between chilling and warming requirements such that relatively more GDUs may be required for leaf emergence if CDUs are not met during winter months (Vihera-Aarnio, Sutinen, Partanen, & Hakkinen, 2014). Thus, extreme seasonal temperatures can alter the interplay between CDU or GDU requirements, with major implications for tree phenology.

Although this theoretical framework with thermal units has been applied since the 1950s (Weinberger, 1950), Clark *et al.* (Clark, Salk, Melillo, Mohan, & Anten, 2014) recently questioned this approach, in part because temperature sensing by trees may respond to continuous changes in temperature throughout the year rather than discrete time intervals. Clark *et al.* (Clark *et al.*, 2014) also pointed out that uneven warming during tree development may produce low predictive performance for traditional degree-day models, as these conditions can lead to interactions between developmental state and seasonality of warming. In addition, it has been commonly assumed that CDU and GDU requirements for leaf emergence remain **constant** within species and across years (Luedeling, Zhang, McGranahan, & Leslie, 2009). Despite this assumption, it has not been determined if thermal requirements will be similar in extreme years relative to non-extreme years, and if these responses vary across populations at the intraspecific level.

To address this latter issue, we compared the timing of leaf emergence among 43 populations of *Fraxinus americana* (white ash) during the warmest extreme year in U.S. history (2012) with relatively non-extreme years (2005, 2010, 2011, 2013, and 2014) in a common garden in the Midwestern U.S. This is among the earliest reports to document intraspecific variation in phenology during an extreme warm event, and it incorporates population variation across the majority of the species range. The phenology of ash species is known to be highly affected by temperature, making it an ideal species to investigate the effects of extreme years on leaf emergence (Vitasse, Porte, Kremer, Michalet, & Delzon, 2009). During the extreme year of 2012, average winter (November-February of 2011-2012) and spring (March-May of 2012) temperatures at the common garden (Lawrence, KS) were 4.5 and 17.6 °C (Fig. S1), respectively, which were 2.0 ° and 4.6 °C warmer than average non-extreme year conditions. Along with this being the warmest year on U.S. record, the common garden is located at the western-most edge of the species range, and therefore is warmer and drier than the majority of white ash habitats (Fig.1, S2). Thus, many of the experimental populations were operating outside of the thermal environments to which they were adapted, much like is expected to occur under a warmer climate of the future. Thus, the anomalously warm year of 2012 allowed us to test whether phenological predictions based on the interplay between GDUs and CDUs are maintained in an extreme year using both empirical and modeling approaches (see materials and methods). Moreover, our study provides insights into how extreme warm years that are expected to increase in the future will affect our ability to forecast shifts in phenology at the intraspecific level.

We addressed the following questions: 1) how does an extreme year affect leaf emergence of white ash relative to non-extreme years, 2) how do populations

originating from a wide range of locations vary in their timing of leaf emergence and thermal requirements across extreme and non-extreme years, and 3) how does the interplay between CDUs and GDUs affect the timing of leaf emergence during extreme versus non-extreme years?

## **Results and Discussion**

We conducted a comprehensive intraspecific analysis of 43 white ash populations by comparing phenology responses during extreme and non-extreme years in a common garden. We show that (a) average leaf emergence among white ash populations was accelerated by 22 d during the extreme warm year of 2012 relative to non-extreme years, (b) rank order for leaf emergence was generally maintained among 43 populations of white ash across extreme and non-extreme years, (c) thermal requirements shifted such that greater amounts of warming were required for leaf emergence during the extreme year relative to non-extreme years and this constrained the potential for even earlier leaf emergence by an average of 7 d among populations, and (d) responses of white ash to an extreme warm year fundamentally altered the reliability of conventional models in predicting leaf emergence.

### **Timing of leaf emergence of white ash during the warmest year in U.S. history:**

Average leaf emergence occurred 22 d earlier in 2012 compared with non-extreme years ( $p < 0.0001$ ), with population means ranging from 17 d (Onondaga, NY) to 26 d earlier (Jackson, IN) (Fig. 2). These accelerations are consistent with other reported phenological responses during extreme years (Ellwood et al., 2013; Friedl et al., 2014; Rutishauser et al., 2008), although in this case we documented this at the intraspecific level. Recent evidence suggests that species that are able to accelerate

leaf emergence in response to warming may have a competitive advantage over other species, mainly because this can extend the growing season, providing a longer period for carbon accumulation (Cleland et al., 2012). It is important to note, however, that the potential for white ash to utilize an extended growing season will also depend on leaf damage and lower survival due to early frost (Augspurger, 2013), the influence of warming on leaf senescence (Way, 2011), the effects of drought that are predicted to increase over much of the species range (IPCC, 2013), and mortality following introduction of the emerald ash borer beetle (Poland & McCullough, 2006).

### **Population rank order for leaf emergence across extreme and non-extreme years:**

Interestingly, we found that the 43 white ash populations maintained rank order for leaf emergence across extreme and non-extreme years in the common garden (Fig. 2; cronbach's alpha >0.9). This finding indicates that the *relative* timing of leaf emergence among populations may at least be predictable during extreme warm years. In addition, white ash populations that originated from more northern latitudes consistently showed later leaf emergence than populations originating from southern latitudes (Fig. 2). This suggests that populations may have different thermal requirements depending on the climate norms from which they were adapted and these requirements vary systematically across a latitudinal gradient. From a large interspecific meta-analysis, it was determined that species from more northern latitudes generally exhibit earlier leaf emergence than southern species in common garden studies, likely because northern-derived species have lower warming requirements (Zohner & Renner, 2014). However, in our white ash common garden, leaves generally emerged earlier in southern populations followed later by northern populations. This alternative trend is commonly seen among populations of ring-porous species (such as white ash) relative

to diffuse-porous trees (Salk, 2011). Furthermore, Salk (Salk, 2011) found that among 20 common garden studies investigating intraspecific variation in phenology of ring porous species, leaves *always* emerged in order of warm-adapted to cool-adapted genotypes (*i.e.*, south to north, or low to high altitude), likely due to higher thermal requirements with decreasing temperature of origin or a chilling requirement that increases with latitude. Ring-porous species likely developed this more conservative strategy because their large diameter xylem vessels are especially vulnerable to frost damage (Wang, Ives, & Lechowicz, 1992).

**Thermal requirements among populations for leaf emergence across extreme and non-extreme years:** Common assumptions made when modeling leaf emergence are that CDU and GDU requirements are met independently and sequentially, and that they remain relatively constant within a species and across years (Chuine, Kramer, & Hanninen, 2003). These assumptions are likely oversimplifications of how environmental factors cue leaf emergence, although models underlain with these assumptions have been used with a high degree of success for over 50 years (see Chuine *et al.* (Chuine et al., 2003) Table 4.1-1 and references therein).

In order to test whether GDU requirements remained relatively the same for white ash populations between extreme and non-extreme years, we compared average accumulated GDUs at leaf emergence between the extreme (2012) and non-extreme years. GDUs were calculated using the common Utah model ((Richardson, Seeley, & Walker, 1974) see materials and methods), and the assumption of fixed GDU requirements is represented by the one-to-one lines in Fig. 3. We find that average GDU accumulation at leaf emergence fell well above the one-to-one line for almost all populations, indicating that more GDUs are required for leaf emergence during the

extreme warm year relative to non-extreme years (Fig. 3). If overall GDU accumulation at leaf emergence had been similar in 2012 as in non-extreme years, average leaf emergence would have occurred 29 d earlier in 2012 compared with the observed acceleration of 22 d. This buffering effect of 7 d may be directly due to greater GDU requirements during the extreme warm year that limited further accelerations in phenology. Alternatively, a different factor such as unfulfilled CDU requirements or photoperiod requirements may have constrained the advancement of leaf emergence during the extreme warm year. Photoperiod is an unlikely candidate, however, since white ash has been shown to be relatively insensitive to this factor (Basler & Körner, 2012). Alternatively, greater GDUs may have accumulated during 2012 because the preceding winter was anomalously warm (Fig. S1), potentially leading to unfulfilled CDU requirements and subsequently a delay in leaf emergence (tested below). While this phenomenon has not been commonly observed, it has been predicted to occur with future winter warming (Yu, Luedeling, & Xu, 2010). In addition, the range of slopes among extreme versus non-extreme years (Fig. 3) is likely the result of variation in winter and spring temperatures among non-extreme years. For example, both the winter and spring of 2011 were relatively warmer than other non-extreme years in the Midwestern U.S. (Fig. S1) and this slope falls closer to the one-to-one line, acting more like an extreme year response (Fig. 3).

To investigate the role that thermal requirements play in initiating leaf emergence among populations, and how this interplay may differ between extreme and non-extreme warm years, we estimated CDU and GDU requirements for leaf emergence using the Luedeling model (Luedeling et al., 2009). This model allows one to estimate both a CDU and GDU requirement for leaf emergence for each population. The model

operates under the common theoretical framework that defines a definitive point in time at which a CDU requirement is fulfilled, marking the end of endodormancy (Fig. S3), and after which time GDUs can accumulate until a given phenological event occurs. Thus, the fulfillment of CDU and GDU requirements are assumed to occur sequentially and during two distinct time periods. We applied this statistical model to observed data for *multiple individual trees and multiple years* across 35 populations (populations with <10 surviving trees were eliminated from the analysis). For each tree during each year, we plotted accumulated chilling (sum of CDUs from Nov. 1<sup>st</sup> to the day when leaf emergence occurred) against remaining heat (GDUs that remain to be accumulated before a phenological event) until leaf emergence occurred (Fig. S4). Because the temperature requirements for reaching leaf emergence are assumed to consist of both a chilling and heating component, simultaneous analysis of both CDUs and GDUs requires projection of the data onto a new coordinate system containing both data types. The new data points are defined by a combined measure of normalized CDUs and GDUs ( $x$ ), and a vector distance of the original data points ( $d$ ). We can then find where the variation in accumulated chilling and remaining heat is minimized across multiple individual trees and across multiple years within a population. The point where the variation in these temperature proxies is minimized (defined as the minimum standard deviation of  $d$ ) estimates the theoretical intersection time point (the  $x,y$  intersection point in Fig. S4a) at which the CDU requirement for a population is fulfilled and GDUs begin to accumulate (see Materials and Methods for more information).

To investigate the impact of individual years on the applicability of the model, we analyzed reduced data sets including 4 of the 5 years (dropping each year in turn). The

minimum standard deviation of  $d$  values for each population ranged from 0.054 to 0.18, depending on what years were included (Table S1). For all but three populations (672809 and 672810, Franklin, TN; 673206, Wayne, OH) out of 35, the overall minimum occurred when data from the extreme warm year of 2012 were dropped. If we restrict analysis to a more conservative range of accumulated CDUs and GDUs, we find that the minimum standard deviation of  $d$  for all populations occurs when data from 2012 are dropped (Table 1). We show an example of this for trees of a randomly chosen population (679204, Hopkins, KY), whereby CDU versus GDU curves are greatly altered during the extreme warm year of 2012 (Fig. S4). These results indicate that CDUs and GDUs drive the timing of leaf emergence for white ash trees very differently during an extreme year relative to non-extreme years, such that a commonly used conventional model is no longer predictive in this framework.

We considered the ability of the model (using data from only the non-extreme years) to accurately predict leaf emergence in the extreme warm year of 2012 by calculating the relative error for the date of leaf emergence  $[(\text{observed} - \text{predicted}) / \text{predicted}]$ . We also considered the relative error in leaf emergence for the non-extreme years whose data were included in the calculations for the predicted date of leaf emergence. The relative error of the model was below 0.5 during non-extreme years, but was especially high (1.25) and more variable for the extreme warm year of 2012 (Fig. S5). Interestingly, the relative error of the model was also high for the non-extreme year of 2013. The model predicted leaf emergence to occur, on average, in early February during spring of 2013, but we observed leaf emergence among all populations to occur, on average, in early May. Model predictions for 2013 were likely imprecise because trees experienced an unusually warm winter in 2012, which comprised the



beginning of the 2012-2013 phenological season (Fig. S1). Temperatures experienced during this period resulted in a high accumulation of CDUs and GDUs under the Utah model, and this was likely the reason the Luedeling model predicted such an early leaf emergence date during 2013.

To further examine our modeling and statistical approaches, we calculated root mean square errors (*RMSE*) for each population in each year, using the predicted leaf emergence date from the combined non-extreme years and the observed leaf emergence date for each tree (see Materials and Methods). We also found average *RMSE* across populations for each year. These errors were generally similar to published *RMSE* for leaf emergence using other phenology models (Fu, Campioli, Deckmyn, & Janssens, 2013). As was the case for the relative error, the root mean square errors were lowest for the non-extreme years of 2010, 2011, and 2014 (17-35), with the years sharing the extremely warm winter temperatures of the 2012-2013 phenological season (2012 and 2013) showing much higher average *RMSE* (49 and 65, respectively).

The deviation of the 2012 data from model predictions indicates that we cannot use the Luedeling model to determine whether CDUs were unfulfilled during the extreme warm year of 2012, mainly because this model predicts much earlier leaf emergence dates than were observed in reality. It should also be noted that this model employs commonly used degree-day approaches that are common to many phenology models. Additionally, we find that the effects of the extreme warm year of 2012 likely persisted into 2013, whereby earlier leaf emergence dates were also predicted for 2013 than were observed. This is likely because there were overlapping warmer winter temperatures between the 2012-2013 phenological season (Fig. S1). These modeling

results could be explained by one or more of the following reasons: **1)** the modeling approach does not take into account other factors that could constrain the acceleration of leaf emergence. For example, Wang *et al.* (Wang *et al.*, 1992) reported a nearly 100% loss in xylem conducting capacity by late winter in white ash trees. To support newly emerging leaves, white ash trees must first form new xylem each spring. Thus, the developmental timing of xylem formation may constrain further advancements of leaf emergence even if GDU and CDU requirements are met prior to this. While the timing of xylem vessel formation has not been studied across extreme and non-extreme years, vessel formation in ring-porous species is generally initiated 2-6 weeks prior to leaf emergence (Suzuki, Yoda, & Suzuki, 1996). Thus, it is unlikely that this factor is constraining further accelerations in leaf emergence in our study. **2)** The extreme year of 2012 altered the temperature sensing mechanisms underlying leaf emergence. Because these sensing mechanisms are not well understood, it is currently not possible to speculate on how an extreme warm year may have altered such responses. **3)** This model only considers how one phenological state (leaf emergence) responds to temperature during a discrete time interval, which may result in imprecise predictions if these aggregated variables (e.g., CDUs and GDUs) are not representative of chilling and warming effects. Future models that incorporate multiple phenological states and that do not aggregate temperature time series into a cumulative sum or average value (as traditional degree-day models do) may be better equipped to deal with phenological responses to extreme warm years (Clark *et al.*, 2014). Clark *et al.* (2014) suggested that uneven warming results in an interaction between phenological state and seasonality of warming, and this effect is likely to be amplified in extreme warm years with major heat pulses, reducing our ability to predict phenological events with traditional approaches.

The drawback to these future models is that measurements at multiple states of leaf development are required, and the full sensing mechanisms to establish such parameters are still unknown. Additionally, using traditional degree day models may not work during extreme years if thermal sensing mechanisms of trees are altered. Along this line, Luedeling (Luedeling & Brown, 2011) pointed out that chilling models (e.g., Utah model used in this study) are not as accurate at estimating CDUs in warmer regions (e.g., sub-tropical or tropical regions) as compared to cooler temperate regions. It is possible that even temperate regions, for which these models were designed, are now becoming too warm for accurate predictions from these models, at least during extreme years. Moreover, regardless of *why* the Luedeling model fails to accurately predict leaf emergence in the extreme warm year of 2012, our data suggest leaf emergence is fundamentally altered in an extreme warm year relative to non-extreme years as evidenced by the ability of these methods to only be able to estimate a common combination of chilling and heating for individual populations across years when extreme year data are excluded from the analysis.

In contrast to extreme years, the Luedeling model performed well for non-extreme years (Fig. S5). From responses occurring during non-extreme years, we find that predictions of GDU requirements increase with increasing latitude of origin of populations ( $p < 0.01$ , Fig. S6), whereas there is no common trend for CDU requirements. These results suggest that populations from more northern locations have higher thermal requirements for leaf emergence than southern locations. This may explain why we find that white ash populations originating from more northern latitudes consistently show later leaf emergence than populations originating from more southern latitudes in the common garden. The freeze-free period is shorter at northern latitudes,

and therefore these results may point to a more conservative strategy by northern populations to avoid damage due to early spring frosts.

In conclusion, we found that leaf emergence of white ash trees advanced by 22 d on average across 43 populations during the extreme warm year of 2012 relative to non-extreme years. In addition, leaf emergence was buffered from an additional advancement of 7 d on average as a result of greater thermal accumulation prior to leaf emergence during the extreme year relative to non-extreme years. Recent evidence has shown that species that are able to accelerate leaf emergence in response to future warming may have a competitive advantage over other species (Cleland et al., 2012). However, earlier leaf emergence may lead white ash to be more vulnerable to early frost damage and other environmental stressors. We also found that rank order for leaf emergence was generally maintained among 43 populations across extreme and non-extreme years. This finding suggests that the *relative* timing of leaf emergence among white ash populations may at least be predictable during extreme warm years. Most concerning, however, is the finding that a commonly used phenology model that incorporates assumptions that have been applied for over 50 years cannot accurately estimate thermal requirements for leaf emergence during an extreme year. While we cannot say for certain *why* this model performed poorly during the extreme warm year, we show multiple lines of evidence that the relationship between thermal requirements and leaf emergence are fundamentally altered during an extreme year. Thus, we need to rethink the use of traditional phenological models to project future shifts in phenology under climate change scenarios where extreme warm years are becoming more common. Moreover, these results demonstrate a critical need to better understand how

extreme warm years will affect the phenology of plants, mainly because these years are predicted to become the climate norms of the next century.

## **Materials and Methods**

### **Common Garden Location and Climate**

White ash seeds originating from 43 populations throughout the species range were collected by the U.S. Forest Service from open-pollinated native parent trees. Seeds were planted in 1976 at the University of Kansas Field Station in Lawrence, KS (35.0°N, 95.1°W, 299 m a.s.l.; Fig.1, S1). 25 replicate trees from each population were planted, although long-term mortality reduced the number of living trees in the present study (2005, n=763; 2010, n=758; 2011, n=729; 2012, n=733; 2013, n=710; 2014, n=700).

The field station receives an average annual precipitation of ~900 mm per year with more than 70% of this falling in the growing season from April-September. Temperature fluctuates greatly both diurnally and annually with monthly average temperatures ranging from below -7 °C in January to slightly above 32 °C in July. Year 2012 was the warmest year on record for the United States since record keeping began in 1895. At the white ash common garden site during the extreme warm year of 2012, average winter (November-February of 2011-2012) and spring (March-May of 2012) temperatures at the common garden were 4.5 and 17.6 °C (Fig. S1), respectively, which were 2.0 ° and 4.6 °C warmer than average non-extreme years. Total annual precipitation was lowest during the extreme year of 2012 (622 mm) relative to non-extreme years; however, we did not find a significant correlation between precipitation and leaf emergence, and therefore we did not consider this factor further.

White ash leaves are pinnately compound with leaflets oppositely arranged. Each leaf has 7-12 leaflets. We considered a tree to have undergone leaf emergence when clear spacing could be seen between the first five leaflets for one leaf. Each individual tree was observed every 48 hr until leaf emergence occurred.

## **Modeling Leaf Emergence**

### ***Chilling and Forcing Model***

We used the Utah Model proposed by Richardson *et al.* (1974) to calculate chilling degree units (CDU) and growing degree units (GDU) (Richardson *et al.*, 1974). The Utah model has been used extensively to calculate CDUs and GDUs since its development in the 1970's (Luedeling, 2012). According to this model, CDUs decline if temperatures become too cold or warm. Growing degree units are calculated based on a linear forcing model with a fixed threshold,

$$GDU(k) = \sum_{i=r}^k \sum_{h=1}^{24} \max[0, T_h(i) - T_b] \quad (\text{Equation 1})$$

for a given day  $k$ , where  $r$  is the day when the chilling requirement is fulfilled ( $k > r$ ),  $T_h(i)$  is the hourly mean temperature at hour  $h$  and day  $i$ , and  $T_b$  is the threshold (or base) temperature. The Utah Model uses a base temperature of  $T_b = 4.4^\circ\text{C}$ . Accumulating CDUs and GDUs were calculated for each hour of the temperature record, with the beginning of the season set to November 1<sup>st</sup>. Under the Utah Model, the accumulation of chilling degree units occurs with 1 CDU equaling 1 hour of exposure to  $6^\circ\text{C}$ . This chilling contribution drops below 1 CDU as the temperature either drops below or rises above a window around this optimum value (below  $2.5^\circ\text{C}$  or above  $9.1^\circ\text{C}$ ). Finally, there is a negative contribution to the CDU accumulation for temperatures above  $16^\circ\text{C}$ . Therefore, under the Utah Model, CDU accumulation for a given day  $k$  is given by

$$CDU(k) = \sum_{i=1}^{24} T(t_i) \quad (\text{Equation 2a})$$

$$T(t_i) = \begin{cases} 1 & 2.5 \leq t_i \leq 9.1 \\ 0.5 & 1.5 \leq t_i \leq 2.4 \quad \text{or} \quad 9.2 \leq t_i \leq 12.4 \\ 0 & t_i \leq 1.4 \quad \text{or} \quad 12.5 \leq t_i \leq 15.9 \\ -0.5 & 16 \leq t_i \leq 18 \\ -1 & t_i > 18 \end{cases} \quad (\text{Equation 2b})$$

where  $t_i$  gives the temperature in °C at hour  $i$ .

To determine if accumulated GDUs were similar between the extreme year of 2012 and non-extreme years, we plotted accumulated GDUs at leaf emergence for each population. We compared these points to a hypothetical one-to-one line representing a hypothesis of fixed GDU requirements between years. In addition, we also regressed these points between years using a general linear model in SAS 9.2 (Cary, North Carolina, USA).

### ***Luedeling Model***

To determine the intersection point between plotted GDUs versus CDUs for a given population, we use a statistical approach developed by Luedeling *et al.* (2009) (Luedeling *et al.*, 2009). For long-term phenology datasets (>15 years), an alternative method utilizing partial least squares (PLS) regression can be used; however, this method is not recommended for shorter term data sets such as the one utilized here (Luedeling, pers. comm.). Since no chilling or forcing model is absolutely accurate, not all curves will intersect through one well-defined point; therefore, it was necessary to develop a statistical technique to determine the most well-defined point between generated CDU and GDU curves. The goal of the model generated by Luedeling *et al.*

(2009) was to determine which of many chilling models was able to determine the most well-defined intersection point between CDU and GDU curves. Therefore, in this model all data are normalized by minimum remaining heat on the first day of the winter season (November 1<sup>st</sup>), and the minimum accumulated CDU. The normalization of data allows comparisons across different chilling models. The temperature requirements for reaching leaf emergence consist of both a chilling and a heating requirement, requiring a projection of the data onto a new coordinate system consisting of both types of data. Following the methods described in Luedeling et al. (2009), data points are projected into a new coordinate system by tilting the  $y$ -axis by  $45^\circ$  (see Fig. 3 of Luedeling et al. (2009)). In this coordinate system, the new data points are then defined by an  $x'$  coordinate (where  $x' = \text{normalized chilling} - \text{normalized heat}$ ) and a distance  $d$  of the original unprojected data point from the unprojected  $x$ -axis at a  $45^\circ$  angle ( $d = \text{normalized heat} / \sin 45^\circ$ ). Finding the  $x'$  value where the standard deviation of  $d$  is minimized gives the best approximation of the theoretical intersection point between multiple GDU versus CDU curves (given by specific  $x$  and  $y$  values in the original data plot) and allows one to determine which combination of chilling and heating most accurately predicts reality.

Although Luedeling et al. (2009) used this technique to determine which chilling models were able to best predict the intersection point between CDU and GDU curves, we used this model to determine the most well-defined intersection point for white ash populations across sets of years. We analyzed the standard deviation of  $d$  and determined the minimum standard deviation of  $d$  by dropping only *one* year of data at a time in which leaf emergence was observed. We then determined which set of years predict the most well-defined intersection point between the multiple GDU vs. CDU



curves. All  $d$  values correspond to an  $x'$  value for this analysis;  $x'$  values were rounded to three significant figures. We restricted analysis of the minimum standard deviation of  $d$  to  $x'$  values between  $-0.5$  and  $+1.0$ ; a similar range was defined by Luedeling *et al.* (2009). We define this limit because the minimum standard deviation of  $d$  often occurred towards the outer  $x'$  values when no chilling had accumulated. A *Mathematica* notebook (*Mathematica* vers. 8.0, Wolfram™) was written to carry out the analysis steps described above (available upon request).

### Prediction Errors and Statistical Analyses

To consider the ability of the model to accurately predict leaf emergence in the extreme warm year of 2012, we calculated the relative error for the date of leaf emergence [(observed – predicted)/predicted]. We calculated root mean square errors (*RMSE*) for each population in each year, using

$$RMSE_{j,k} = \sqrt{\frac{\sum_i (y_{i,j,k} - \hat{y}_{j,k})^2}{n_{j,k}}}, \quad (\text{Equation 3})$$

where the summation  $i$  is over all individual trees,  $j$  indicates the population and  $k$  the year, using the predicted leaf emergence date ( $\hat{y}_{j,k}$ ) from the combined non-extreme years (2010, 2011, 2013, 2014), the observed leaf emergence date for each tree ( $y_{i,j,k}$ ), and the number of trees for each population in each year ( $n_{j,k}$ ). Average *RMSE* across all 35 populations for each year were also calculated, using

$$RMSE_k = \frac{1}{35} \sum_j RMSE_{j,k}. \quad (\text{Equation 4})$$

To assess the effects of both population and the extreme year of 2012 on the timing of leaf emergence, we used a two-way ANOVA. To test the internal reliability of rank among populations across years, a cronbach's alpha internal reliability measure was calculated. We used a simple regression calculation to compare accumulated growing degree units at average leaf emergence for each population between extreme (2012), and non-extreme years. All statistical analyses were carried out in SAS 9.2 (Cary, North Carolina, USA).

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## Figure Legends

**Fig. 1** The range of *Fraxinus americana* (white ash) is shown in the shaded grey area. Locations from which white ash populations originated are shown by black circles. Trees from each of these populations were grown in the University of Kansas Field Station common garden, Lawrence, KS (red star). Trees from all populations grew side-by-side in this common garden for 36 years to date. Adapted from Marchin et al., 2008 and USDA Forest Service ([www.na.fs.fed.us](http://www.na.fs.fed.us)).

**Fig. 2** Timing of leaf emergence (in Julian dates) among 43 populations grown in a common garden at the University of Kansas Field Station. Symbols are mean leaf emergence times  $\pm 1$  SE. The total number of trees observed each year is as follows: 2005, n=763; 2010, n=758; 2011, n=729; 2012, n=733; 2013, n=710; 2014, n=700. Sample sizes within populations range from 2-25 depending upon long-time survivorship. The figure legend shows original source locations for each population with latitude of origin in parentheses. Populations are color coded with more southern latitude populations in warmer colors (red-yellow) and more northern populations in cooler colors (cyan-dark blue).

**Fig. 3** Average growing degree units (GDU), in thousands, accumulated at leaf emergence for 43 white ash populations regressed between the extreme year of 2012 and non-extreme years of (a) 2010, (b) 2011, (c) 2013, and (d) 2014. The one-to-one line (dashed line) is a hypothetical line showing the response if there was no difference in required growing degree units between 2012 and the non-extreme year. The solid line shows a linear regression in the data between 2012 and the non-extreme year with the corresponding regression equation and  $R^2$

value. Red lines represent 95% confidence intervals (CIs) calculated for the observed slope. Populations are represented by the same symbols and colors as those used in the legend of Fig. 2.





Fig. 1

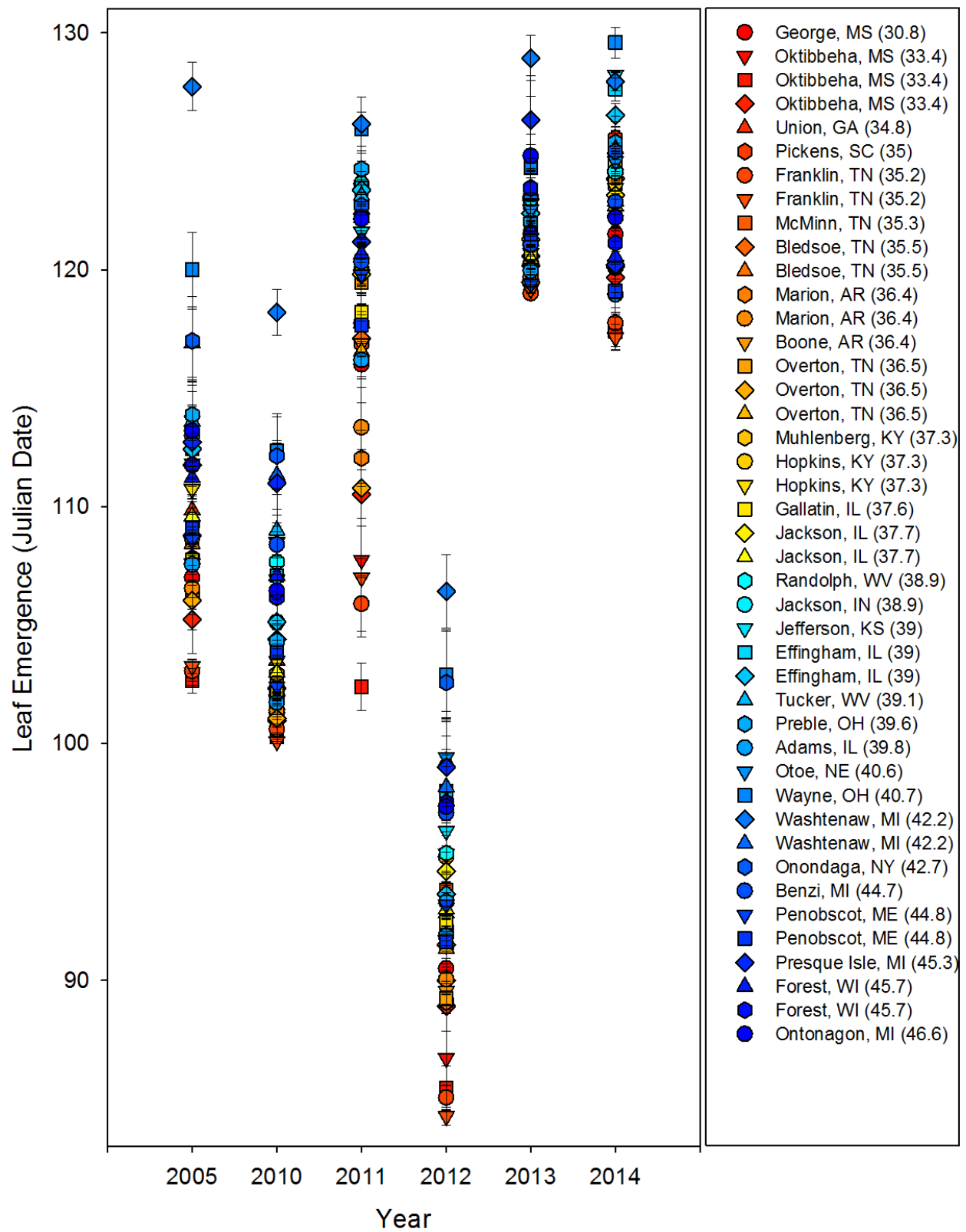
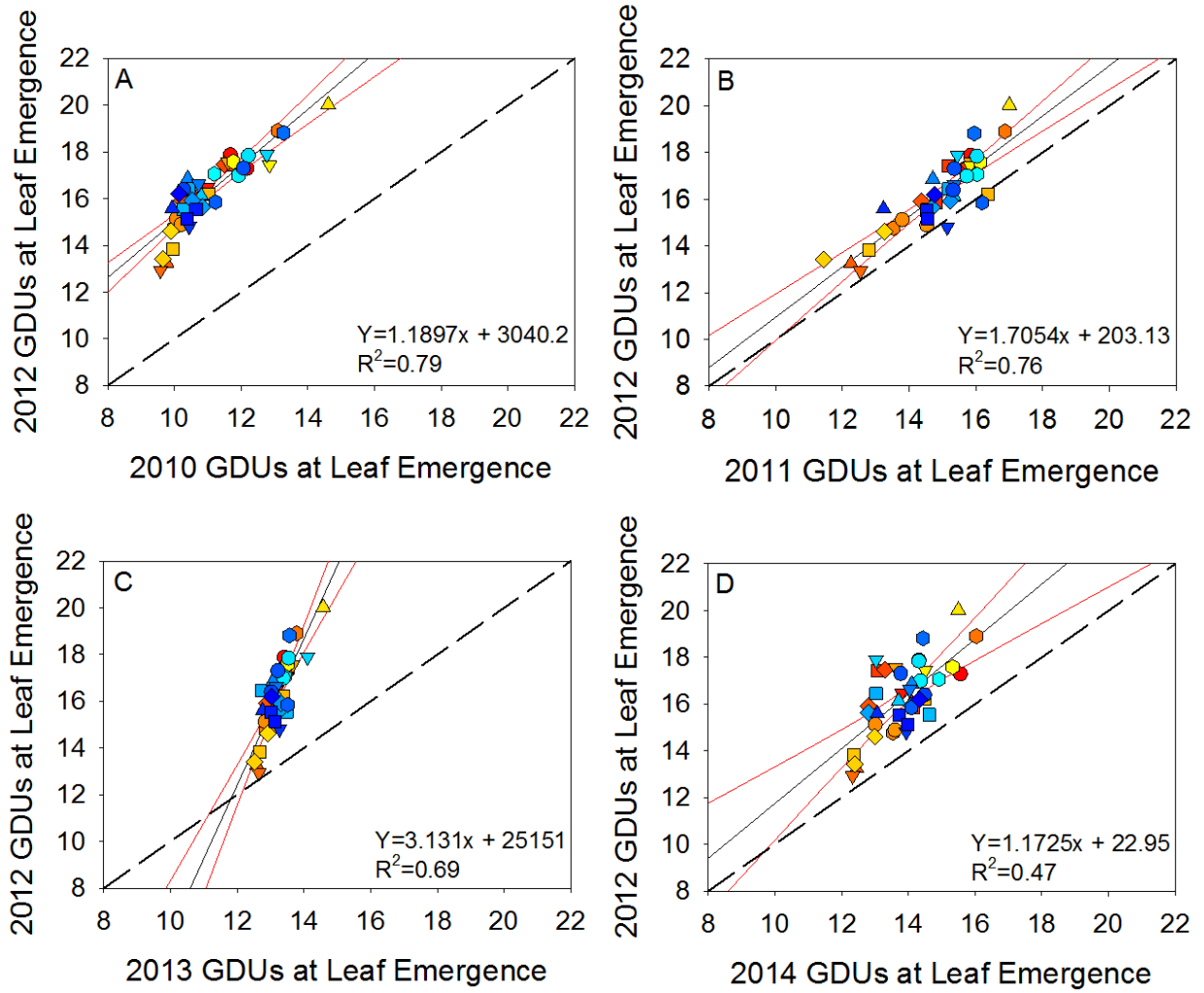


Fig. 2



**Fig. 3**

### Supplementary Tables and Figures

**Table S1** The minimum standard deviation (st. dev.) of  $d$  (normalized heat/sin 45°) as a function of  $x'$  (normalized chilling – normalized heat) (see Figure 3e of Luedeling et al. 2009) for 35 white ash populations sourced from different locations across the US and grown in a common garden in Lawrence, KS. The st. dev of  $d$  is calculated dropping one year in turn for all 5 years (where the year dropped is shown at the top of the column). For all but three populations, the minimum standard deviation of  $d$  (bolded

Family						X	Y
	2010	2011	2012	2013	2014	(CDU)	(GDU)
Jefferson, KS	0.090	0.139	<b>0.062</b>	0.122	0.141	949.703	7071.98
Otoe, NE	0.138	0.118	<b>0.072</b>	0.133	0.149	962.37	6603.6
Jackson, IL	0.133	0.116	<b>0.077</b>	0.176	0.173	955.246	6337.6
Jackson, IL	0.099	0.126	<b>0.064</b>	0.155	0.146	963.33	6254.93
Gallatin, IL	0.094	0.110	<b>0.078</b>	0.178	0.156	957.043	6091.83
Adams, IL	0.102	0.105	<b>0.054</b>	0.150	0.164	982.937	5671.4
Franklin, TN	<b>0.068</b>	0.090	0.076	0.115	0.110	906.811	5700.69
Franklin, TN	<b>0.054</b>	0.086	0.074	0.106	0.103	965.945	5366.68
Wayne, OH	0.118	0.125	0.092	<b>0.088</b>	0.130	1037.79	5715.99
Marion, AR	0.094	0.115	<b>0.081</b>	0.140	0.142	984.25	5558.85
Marion, AR	0.096	0.113	<b>0.068</b>	0.139	0.150	984.995	5566.66
Boone, AR	0.088	0.119	<b>0.075</b>	0.163	0.136	957.161	6103.81
Preble, OH	0.102	0.122	<b>0.073</b>	0.141	0.141	937.421	6721.39
Oktibbeha, MS	0.089	0.098	<b>0.082</b>	0.130	0.116	902.842	5659.02
Oktibbeha, MS	0.089	0.116	<b>0.076</b>	0.128	0.141	980.411	5518.54
Washtenaw, MI	0.122	0.120	<b>0.060</b>	0.125	0.151	962.541	8485.39
Washtenaw, MI	0.133	0.115	<b>0.079</b>	0.133	0.147	986.529	6515.67
Effingham, IL	0.112	0.117	<b>0.076</b>	0.113	0.129	958.912	6946.16
Effingham, IL	0.102	0.115	<b>0.080</b>	0.117	0.129	944.133	6781.55
Randolph, WV	0.091	0.106	<b>0.063</b>	0.107	0.126	946.105	6802.15
Tucker, WV	0.117	0.118	<b>0.074</b>	0.111	0.133	951.493	6858.45
Union, GA	0.091	0.122	<b>0.060</b>	0.133	0.135	928.642	6556
Pickens, SC	0.104	0.119	<b>0.091</b>	0.185	0.152	1005.25	5779.31
Penobscot, ME	0.094	0.120	<b>0.067</b>	0.161	0.149	955.537	6335.04
Muhlenberg, KY	0.091	0.113	<b>0.076</b>	0.147	0.143	937.112	6514.71
Hopkins, KY	0.105	0.106	<b>0.075</b>	0.145	0.151	956.275	6083.87
Onondaga, NY	0.116	0.109	<b>0.078</b>	0.098	0.130	960.265	7079.25
Jackson, IN	0.101	0.129	<b>0.063</b>	0.140	0.130	944.266	6718.16
Benzi, MI	0.121	0.113	<b>0.066</b>	0.126	0.136	985.282	6316.44
McMinn, TN	0.104	0.107	<b>0.077</b>	0.144	0.150	970.828	5897.35
Overton, TN	0.086	0.120	<b>0.076</b>	0.148	0.128	932.968	6463.46
Overton, TN	0.117	0.112	<b>0.078</b>	0.167	0.170	975.838	5470.53
Overton, TN	0.108	0.115	<b>0.076</b>	0.179	0.154	949.648	6150.41
Bledsoe, TN	0.080	0.112	<b>0.075</b>	0.159	0.133	925.425	6431.85
Bledsoe, TN	0.104	0.109	<b>0.086</b>	0.161	0.162	950.614	6035.93

text) occurs when 2012 data are dropped. Applying the Luedeling model to only non-extreme years, the predicted X (chilling requirements, or CDUs) and Y (thermal requirements, or GDUs) are given.

**Fig. S1** The average winter (November-December of the previous year, and January-February of the current year) and spring (March-May of the current year) temperatures for the years 2010, 2011, 2012, 2013, and 2014 at a common garden located in Lawrence, KS, USA.

**Fig. S2** A satellite image of the white ash common garden (outlined by the yellow box).

**Fig. S3** Graphical representation of a commonly used theoretical framework used to explain how leaf emergence is controlled by temperature. Under this framework, chilling is ineffective during the time in which heating accumulates and vice versa. Thus, the accumulation of both heating and chilling occur in two distinct periods, and requirements are assumed to be fulfilled sequentially.

**Fig. S4** An example of the theoretical intersection point between chilling ( $x$ ) and thermal requirements ( $y$ ) as predicted by the Luedeling model for a randomly chosen population (679204) that originated from Hopkins, KY. The top panel (a) shows data for all years except 2012, as well as the predicted heating ( $y = 6083.87$ , horizontal line) and chilling ( $x = 956.275$ , vertical line) requirements. The bottom panel (b) shows the 2012 data for this population plotted against the same predicted  $y$  and  $x$  values. The curves clearly lie far outside of the predicted intersection point, and similar results were seen for all other populations, except for three (Table 1).

**Fig. S5** The relative error ( $[(\text{observed} - \text{predicted})/\text{predicted}]$ ) for average leaf emergence across all populations during the extreme year of 2012 relative to non-extreme years (2010, 2011, 2013, and 2014). The relative error was below 0.5 for all non-extreme years except 2013. The higher relative error in 2013 is likely due to the anomalously warm temperatures experienced in 2012, which were included in the calculated CDUs and GDUs for 2013.

**Fig. S6** The average growing degree unit (GDU) requirement for leaf emergence for 35 white ash populations (black dots) as predicted from the Luedeling model regressed against populations' latitude of origin. There is a significant trend that suggests populations originating from more northern latitudes have higher GDU requirements for leaf emergence than populations originating from more southern latitudes.

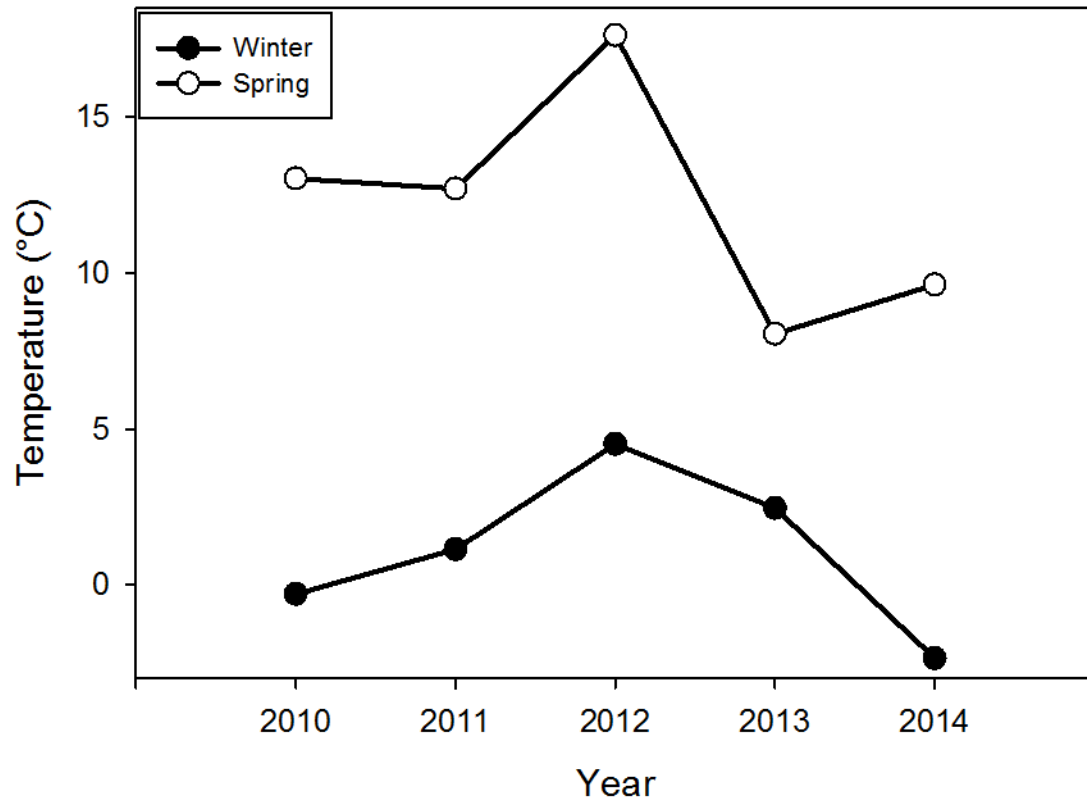


Figure S1



**Figure S2**

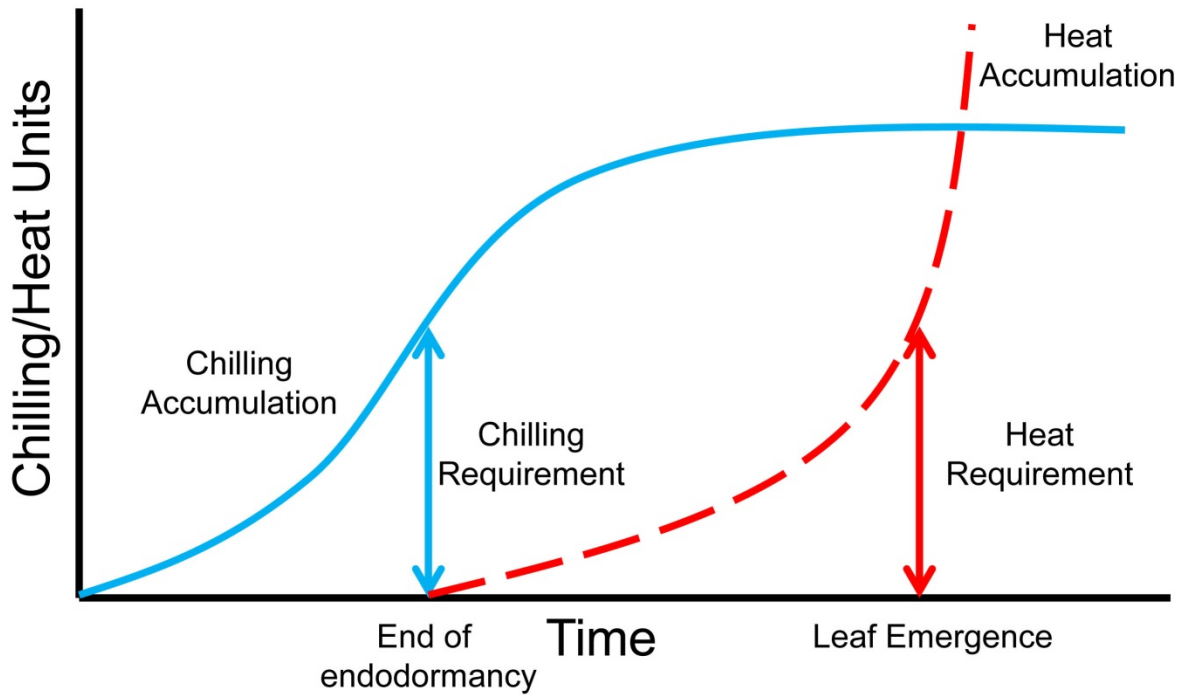


Figure S3



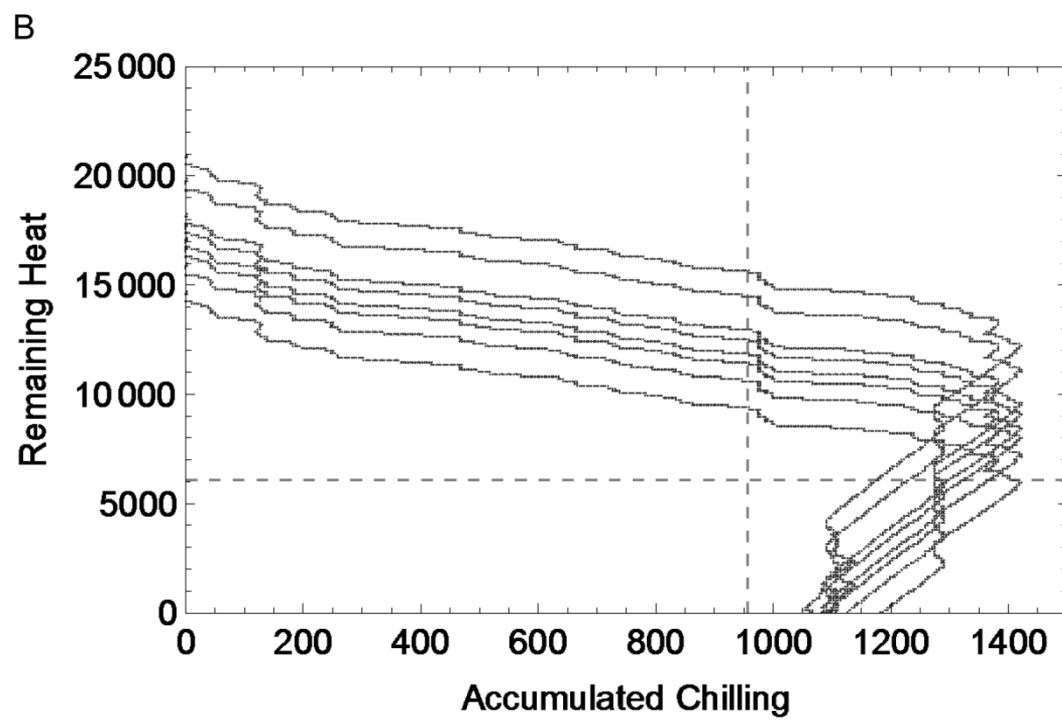
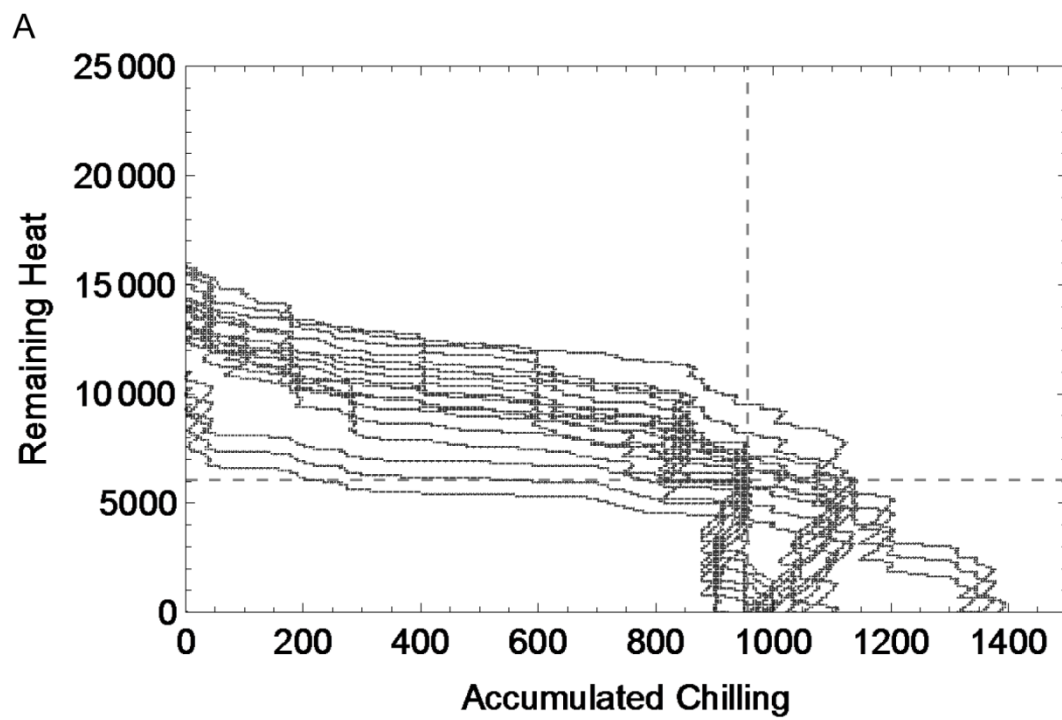
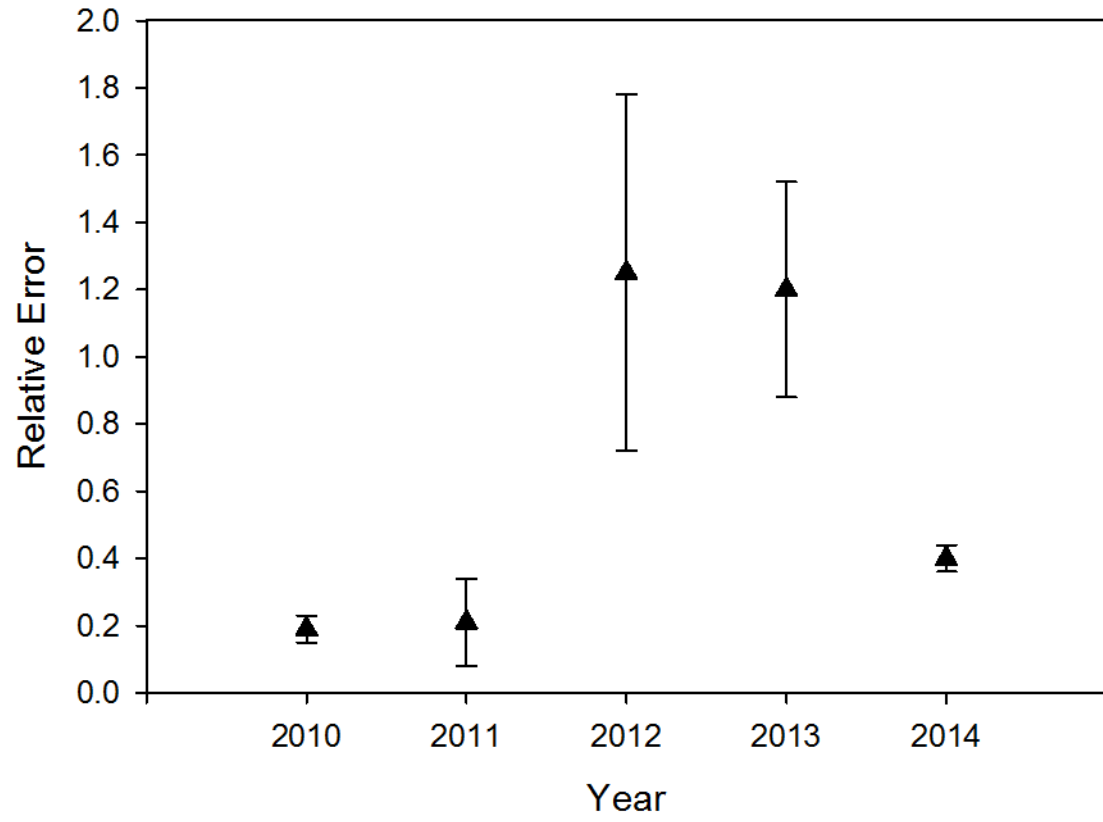


Figure S4



**Figure S5**

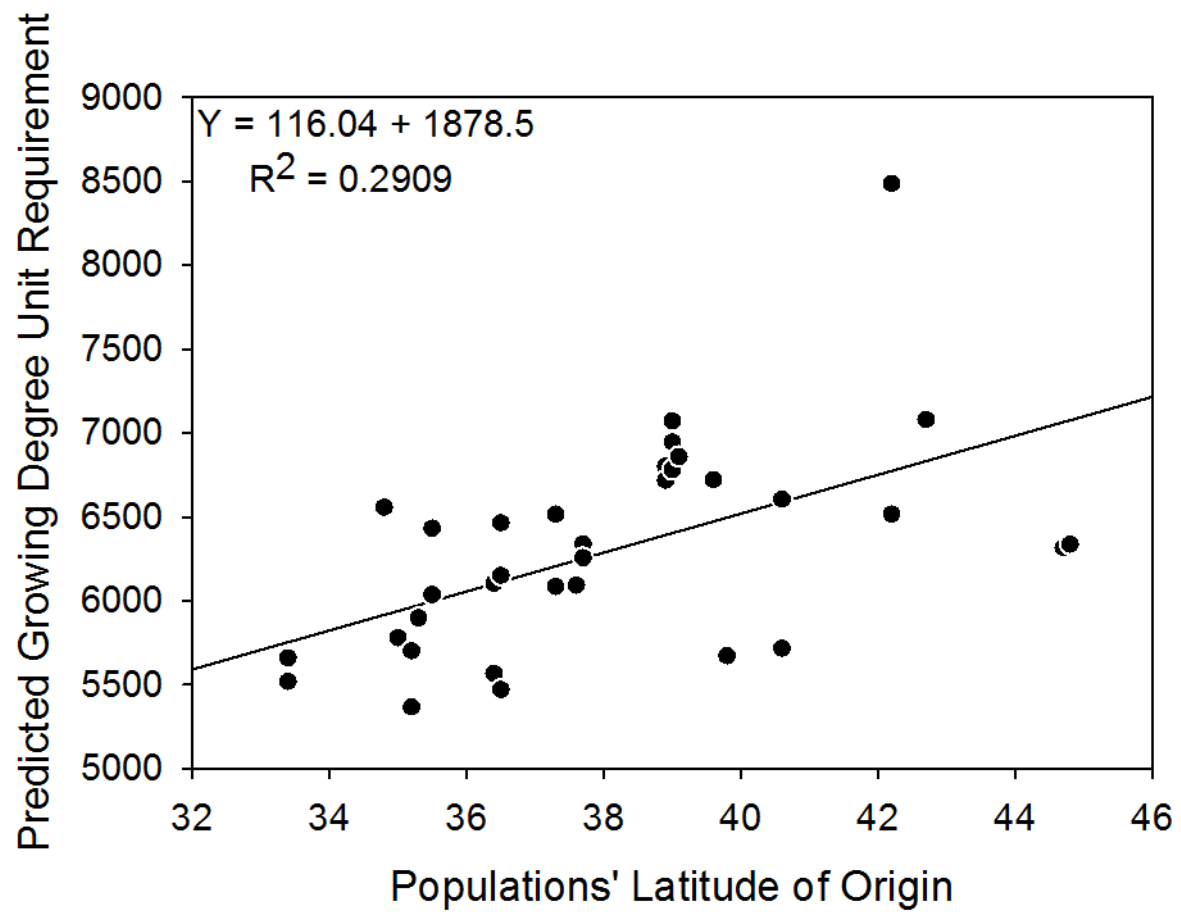


Figure S6

## Chapter 2: Intraspecific Responses in Leaf-Level $\delta^{13}\text{C}$ of *Fraxinus americana* are Constant across Extreme and Non-Extreme Years

### Abstract

Extreme years (i.e., years in which temperature or precipitation dramatically deviate from long-term mean conditions) are expected to become more common as climate change progresses. Trees are expected to be especially vulnerable to weather extremes since these conditions may push them past their physiological limits. However, the effect of extreme versus non-extreme years on physiological traits of trees is still poorly understood, especially at the intraspecific level. A high level of population-level variation in physiological traits may be one way trees can adapt to future climate changes. Investigations of intraspecific responses in important and potentially adaptive physiological traits using common garden experiments can provide information on the potential of populations to respond to changing environmental conditions across space and/or time. We conducted a comprehensive *intraspecific* analysis of leaf-level  $\delta^{13}\text{C}$  responses in a common garden of white ash located in Lawrence, KS across 7 years, including the warmest year in U.S. history, which was also one of the driest years on record at this site. Average leaf-level  $\delta^{13}\text{C}$  did not respond differently across extreme and non-extreme years at the population level suggesting little environmentally induced change in leaf-level  $\delta^{13}\text{C}$ . However, we found a strong effect of population on leaf-level  $\delta^{13}\text{C}$ , and the rank order in leaf-level  $\delta^{13}\text{C}$  among populations remained relatively uniform across extreme and non-extreme years. This result suggests that the origin of white ash populations may play a role in dictating physiological functioning, and that the relative physiological relationships among populations will be maintained across

extreme and non-extreme years. We did not observe a population x year interaction for leaf-level  $\delta^{13}\text{C}$  suggesting that leaf-level  $\delta^{13}\text{C}$  responses of populations are similar across extreme and non-extreme years.

## Introduction

Over the next century, climate change will occur 10 times faster than it has at any time in at least the past 65 million years (Diffenbaugh & Field, 2013). As a result, average global temperatures are predicted to increase at a rate of 0.25 °C per decade by 2020 in the northern hemisphere (Smith, Edmonds, Harlin, Mundra, & Calvin, 2015). In addition to these increases in mean temperature, high seasonal extremes are expected to become more common over this time period (Hansen et al., 2012; IPCC, 2012). A 1-in-20 year hottest day in the current climate is expected to become a 1-in-2 year event by the end of this century across the majority of the planet (Diffenbaugh & Field, 2013). The impacts of weather extremes on plant physiology are largely unknown, even though it is understood that weather extremes can affect the link between plant physiology and ecosystem-level processes, such as carbon cycling (W.R.L Anderegg et al., 2015). As many plant species are adapted in their physiology to the current climate, climate change may increase species' vulnerability to decline or extinction in the future (Urban, 2015).

The  $\delta^{13}\text{C}$  of plant tissues is an integrative measure of the ratio of the concentration of intercellular to atmospheric  $\text{CO}_2$  ( $c_i/c_a$ ) (Farquhar, Oleary, & Berry, 1982). This ratio is the result of a balance between stomatal conductance and photosynthetic rates over the growing season in which carbon was fixed (Farquhar, Ehleringer, & Hubick, 1989). Because  $c_i/c_a$  also reflects a trade-off between photosynthetic carbon gains to water loss at the leaf level, intrinsic water-use efficiency (WUE) can be inferred from  $\delta^{13}\text{C}$  values. While  $\delta^{13}\text{C}$  does not provide a *direct* measure of WUE, the two measures are highly correlated. Higher WUE has been shown to be favored among species growing in dry areas with lower rainfall (Ehleringer, 1993a;

Heschel & Riginos, 2005; Knight et al., 2006), therefore this trait may be predictive of the capacity of populations to adapt under future drier conditions (Nicotra & Davidson, 2010). Furthermore, because leaf-level  $\delta^{13}\text{C}$  is an integrative measure of physiological functioning across the entire growing season, it is likely to be related to plant fitness (Casper *et al.*, 2005).

It has been suggested that a high level of variation in physiological traits among populations (or provenances) may aid adaptation of species to rapid environmental changes expected under future climate change scenarios (Aitken, Yeaman, Holliday, Wang, & Curtis-McLane, 2008). Over 250 years of common garden studies (also referred to as provenance trials) have provided a wealth of information on the population differentiation of trees for many physiological traits (Alberto et al., 2013). Many of these studies have documented that differences in physiological traits among populations reflect differences in a populations' climate of origin. This is likely because tree populations are often (although not always) adapted to local environmental conditions, having the highest fitness in their home environments (e.g., local adaptation) (Alberto et al., 2013; Kawecki & Ebert, 2004; Savolainen, Pyhajarvi, & Knurr, 2007). Among 112 provenance trial studies, involving 59 tree species and 19 adaptation-related traits, genetic differentiation among populations and clinal variation along environmental gradients was commonly observed (90% and 78% of cases, respectively (Alberto et al., 2013). Variation in  $\delta^{13}\text{C}$  at the population-level is often consistent over time and space. For example, Ehleringer (1993) found that the relative ranking of leaf-level  $\Delta$  values of *Encelia farinosa* shrubs at the population level was relatively uniform through time (Ehleringer, 1993b). Additionally, Voltas *et al.* (2008) observed that the ranking of  $\delta^{13}\text{C}$  among 25 *Pinus halepensis* provenances was relatively uniform across

two sites with different environmental conditions (Voltas, Chambel, Prada, & Ferrio, 2008). These observations suggest that evaluations of leaf-level  $\delta^{13}\text{C}$  responses at a particular point in time or location may be useful in extrapolating to long-term patterns in leaf-level  $\delta^{13}\text{C}$  responses (Ehleringer, 1993a). However, as environmental conditions shift with climate change, locally adapted populations may no longer be physiologically adapted to their home environment, and this is likely to have fitness consequences (see Fig. 1 of (Anderson, Panetta, & Mitchell-Olds, 2012).

Here, we report on variation in the leaf-level  $\delta^{13}\text{C}$  among 43 populations of *Fraxinus americana* (white ash) planted in a common garden in Lawrence, KS (Fig. 1). This study was conducted across 7 years, including the most extreme warm year in U.S. history, which was also one of the driest on record in Lawrence, KS (Fig. 2). Because increased temporal variation in temperature and precipitation is expected to occur over the next century, with an increased occurrence of extreme years, it is critically important to understand how physiological responses will vary under strong climatic variation. Additionally, assessing variation in physiological responses at the *intraspecific* level across multiple years with high variation in temperature and precipitation, as is done in this study, can provide insight into how population-level responses may vary with rapid environmental changes that are more likely to occur with future climate change.

*Fraxinus spp.* (ash trees) are important hardwood resources in the U.S. It is estimated that over 7.5 billion ash trees grow in the U.S. (Gould & Bauer, 2009) and their value is estimated at \$282.3 billion (Federal Register, 2003). However, populations of ash trees are rapidly declining in the U.S. because of the introduced non-native emerald ash borer (EAB) beetle (MacFarlane & Meyer, 2005; Poland & McCullough,



2006). The loss of ash trees is expected to have significant economic and ecological impacts (Kovacs et al., 2010; Poland & McCullough, 2006). White ash (*Fraxinus americana*) is one of the most widely distributed (Fig. 1) and commercially important ash species in the Eastern U.S. (MacFarlane & Meyer, 2005). Across this range, populations experience average annual temperatures between 4-22°C and average total annual precipitation between 700-1600 mm (see Table 1 of (Marchin, Sage, & Ward, 2008)). Models predicting suitable habitat for white ash trees under future climate change scenarios indicate that its range will decrease as a result of a shift northward in optimum growing conditions (Iverson, Prasad, Matthews, & Peters, 2008). Additionally, mean total annual precipitation is expected to decrease over much of the species range and severe droughts are expected to become more common over this same area during the next century (IPCC, 2013). Thus, the ability of white ash to persist under future climate change will depend on the potential of the species to respond to both shifts in the *mean*, as well as interannual variation, in total annual precipitation.

Our overall goal was to assess the potential for white ash to respond physiologically to strong temporal variation at the *intraspecific* level across extreme and non-extreme years using  $\delta^{13}\text{C}$ . Our specific objectives were to: 1) determine how variable physiological functioning is across extreme and non-extreme years on average, 2) test whether physiological functioning of a population is consistently maintained across extreme and non-extreme years, 3) and test if populations are responding physiologically similar across extreme and non-extreme years (population level x interannual variation).

## Materials and Methods

### *Common Garden Location and Climate*

White ash seeds originating from 43 populations throughout the species range were collected by the U.S. Forest Service from open-pollinated native parent trees. Seeds were planted in 1976 at the University of Kansas Field Station in Lawrence, KS (35.0°N, 95.1°W, 299 m a.s.l.; Fig. 1). Twenty five replicate trees from each population were planted at this site.

The field station receives an average annual precipitation of ~900 mm per year with more than 70% of this falling in the growing season from March-August. Temperature fluctuates greatly both diurnally and annually with monthly average temperatures ranging from below -7 °C in January to slightly above 32 °C in July. Year 2012 was the warmest year on record for the United States since record keeping began in 1895. At the white ash common garden site during the extreme warm year of 2012, the average annual temperature was 16.5 °C, which was 4.5 °C warmer than average non-extreme years (2005, 2007, 2009, 2010, 2011, and 2013). Total annual precipitation was lower during the extreme year of 2012 (482 mm) relative to the average total annual precipitation during non-extreme years (892 mm; Fig. 2). The total precipitation that fell during the growing season (March-August) also was lower during the extreme year of 2012 (290 mm) relative to the average total growing season precipitation during non-extreme years (576 mm). Growing season vapor pressure deficit was calculated using the saturated vapor pressure at the mean daily temperature subtracted from the actual vapor pressure (Murray, 1967).

### *Stable Isotope Measurement of $^{13}\text{C}$ in Leaf Tissue*

We measured stable carbon isotopic ratios ( $^{12}\text{C}:^{13}\text{C}$ ) in leaf tissue in 43 populations of white ash originating throughout the species range and grown in a common garden in Lawrence, KS (Fig. 1, red star). Isotope measurements were performed at the Keck Paleoenvironmental and Environmental Stable Isotope Laboratory (KPESIL) at the University of Kansas.  $\delta^{13}\text{C}$  were calculated using the following formula:

$$\delta = R_{\text{sample}} / R_{\text{standard}} - 1 \text{ (equation 1)}$$

where  $R$  is the ratio of  $^{13}\text{C}/^{12}\text{C}$ , using belemnite carbonate from the Pee Dee Formation, Hemingway, SC (PDB) as the standard. Data were converted to “per mil” (‰) notation by multiplying  $\delta$  values by 1000. Leaves used in the analysis were collected during August (2005, 2007, 2009, 2010, 2011, 2012, and 2013) from fully mature sunlit leaves on the south side of trees from one block at the site (block 2). Trees are interspersed equally throughout the common garden, and populations are clumped in rows (5 trees each) in each of 5 blocks. We found no difference in relative water content or soil nutrients between blocks and, therefore, no longer considered the effect of block in our analyses. The number of trees that were sampled varied by year (2005,  $n=126$ ; 2007,  $n=149$ ; 2009,  $n=169$ ; 2010  $n=159$ ; 2011  $n=125$ ; 2012,  $n=166$ ; 2013,  $n=149$ ). Two leaves were collected from each tree and dried at 60 °C for 48 hours, and then ground together in liquid nitrogen to a fine powder using a mortar and pestle. Tissues were then weighed (3-3.5 mg) and loaded into tin capsules (3.5 x 5.5 mm). A ThermoFinnigan MAT 253 Dual Inlet System was used for  $\delta^{13}\text{C}$  analysis. We assumed near constancy in the

difference between chloroplastic and intercellular CO<sub>2</sub> concentration at the population level, to assess population-level variation in  $\delta^{13}\text{C}$ .

### *Statistical Analysis*

To assess the effects of both population and year on leaf-level  $\delta^{13}\text{C}$ , we used a two-way ANOVA. To test the internal reliability of rank among populations across years, a cronbach's alpha internal reliability measure was calculated. We used a simple regression calculation to test for a relationship between growing season atmospheric vapor pressure deficit (VPD) and average leaf-level  $\delta^{13}\text{C}$  across populations within a year. All statistical analyses were carried out in SAS 9.2 (Cary, North Carolina, USA).

### **Results and Discussion**

We conducted a comprehensive *intraspecific* analysis of 41 white ash populations growing in a common garden comparing leaf-level  $\delta^{13}\text{C}$  responses during extreme and non-extreme years. In this study we show that **A)** average leaf-level  $\delta^{13}\text{C}$  across all populations during the extreme year of 2012 (-28.7‰ on average across populations) was not significantly different from non-extreme years (-29.1‰ on average across populations). However, we found a significant linear relationship between leaf-level  $\delta^{13}\text{C}$  and growing season (March – August) VPD across 2005, 2007, 2009, 2010, 2011, and 2012 ( $R^2 = 0.64$ ), but this relationship was significantly weakened when data from 2013 were included ( $R^2 = 0.26$ ). **B)** The relative ranking of leaf-level  $\delta^{13}\text{C}$  responses among populations remained relatively consistent across extreme and non-

extreme years. **C)** There was no significant population x year interaction, suggesting that there is a physiologically set aspect of water use efficiency among populations and across extreme and non-extreme years.

*Leaf-level  $\delta^{13}\text{C}$  response during the extreme year of 2012 relative to non-extreme years*

Average leaf-level  $\delta^{13}\text{C}$  values among 41 white ash populations did not significantly vary across years. Average leaf-level  $\delta^{13}\text{C}$  responses were not significantly different between the extreme year of 2012 (-28.7‰) and non-extreme years (-29.1‰; Fig. 3). Studies on grassland species have reported marginal shifts in  $\delta^{13}\text{C}$  between simulated extreme drought and wet conditions (Kahmen, Perner, & Buchmann, 2005; Zavalloni et al., 2009). Investigating the leaf-level  $\delta^{13}\text{C}$  response of grass species under simulated extreme drought and wet conditions, Zavalloni *et al.* (2009) found only marginal differences between the two environmental conditions. Authors suggested that lack of larger differences in leaf-level  $\delta^{13}\text{C}$  between the two treatments were likely due to significant decreases in photosynthesis and growth rates under the extreme drought conditions. These decreased rates limited the amount of carbon incorporated in leaf tissues under the extreme drought so that isotopic signatures were not fully reflective of the extreme drought conditions. In our study, leaf-level gas exchange rates were similar throughout May and June during the extreme year of 2012 (data not shown), but leaf-level gas exchange ceased during July, 2012. Unfortunately, we did not assess whether this affected leaf-level biomass and/or growth. Therefore, it may be possible that the *full* effects of decreased precipitation during 2012 were not reflected by  $\delta^{13}\text{C}$  if carbon containing sugars were not made and subsequently incorporated in leaf tissues across the entirety of the drought. The contribution of older carbon (i.e., stored from previous years) to newly developing leaves is variable in deciduous species, but on average is

around 30% (Keel, Siegwolf, & Korner, 2006). Therefore, a majority of leaf structural tissues in fully grown and mature leaves are composed of carbon assimilated during their current season of growth (Bruggemann et al., 2011).

While the leaf-level  $\delta^{13}\text{C}$  responses among 41 populations of white ash did not vary significantly across years, there was a significant linear relationship between average leaf-level  $\delta^{13}\text{C}$  and growing season (March-August) vapor pressure deficits (VPD) across years ( $p < 0.001$ ; Fig. 4). This suggests that the small variation in average leaf-level  $\delta^{13}\text{C}$  across years is driven by differences in growing season VPD. Leaf-level  $\delta^{13}\text{C}$  is primarily modulated by stomatal conductance, often through responses to VPD (Comstock & Ehleringer, 1992). Interestingly, the relationship between growing season VPD and average leaf-level  $\delta^{13}\text{C}$  is non-significant when data from 2013 are included ( $p > 0.05$ ). While growing season VPD was lower in 2013 (.484 kPa) relative to the extreme year of 2012 (1.028 kPa), average leaf-level  $\delta^{13}\text{C}$  responses among all populations in 2012 and 2013 were virtually the same (-28.6841‰ and -28.6807‰, respectively). Additionally, total growing season precipitation was only slightly higher in 2013 than in 2012 (Fig. 2). Therefore, while atmospheric conditions (VPD) during 2013 were suitable for high rates of stomatal conductance, the rate of stomatal conductance may have been limited by soil water availability leading to increased  $\delta^{13}\text{C}$ . These results may also point to a legacy effect whereby the impacts of the extreme year of 2012 persisted into future years. Using a modeling analysis to predict the timing of leaf emergence at this site, we found evidence that the strong effects of the extreme year of 2012 on phenology persisted into 2013 (Carter *et al.*, 2015 – *in review*). Legacy effects of extreme years have been shown in other species. A severe drought that occurred from 2000-2003 across western North America was observed to lead to widespread die-

off of *Populus tremuloides* that commenced in 2004-2005 and continued until 2011 (W. R. L. Anderegg et al., 2013). This lag effect was the result of damage to xylem vessels during the drought that lead to increases in xylem fatigue, and thus less resistance to xylem vessel cavitation, in subsequent years (W. R. L. Anderegg et al., 2013).

#### *Population rank order in leaf-level $\delta^{13}\text{C}$ across extreme and non-extreme years*

Leaf-level  $\delta^{13}\text{C}$  significantly varied across populations ( $p < 0.0001$ ). We also observed that the relative rank order of leaf-level  $\delta^{13}\text{C}$  responses among 41 white ash populations was maintained across extreme and non-extreme years (Fig. 5; Cronbach' alpha  $> 0.8$ ). This finding indicates that the *relative* responses of leaf-level  $\delta^{13}\text{C}$  among populations may at least be predictable during future extreme years. In order to test if the relationship between leaf-level  $\delta^{13}\text{C}$  and VPD varied across a precipitation gradient, we selected a subset of 10 populations originating within a narrow latitudinal range along an east-west precipitation gradient (VPD also varies across the source location of these populations; see Table 1 of Marchin *et al.*, (2008)). Average leaf-level  $\delta^{13}\text{C}$  among these 10 populations did not significantly vary with either annual precipitation ( $p = 0.2$ ) or growing season VPD ( $p = 0.07$ ) from their source locations. These results are in contrast to previous findings at this site. Marchin *et al.* (2008) reported a strong longitudinal trend in leaf-level  $\delta^{13}\text{C}$  for these same 10 populations growing at the white ash common garden site during 2005. Variation in leaf-level  $\delta^{13}\text{C}$  among these 10 populations during 2005 was much higher (std. dev. = 0.92) compared to other years used in this study (std. dev = 0.38-0.52). We are uncertain why leaf-level  $\delta^{13}\text{C}$  was more variable in 2005 among these 10 populations spanning a precipitation gradient, but such variation likely allowed a significant trend to be elucidated during 2005. While we do not find a significant relationship among these 10 populations between population source

precipitation and leaf-level  $\delta^{13}\text{C}$ , on average, populations sourced from eastern locations have decreased  $\delta^{13}\text{C}$  relative to populations sourced from western locations (Fig. 6), which are similar to trends reported by Marchin *et al.* (2008).

#### *Testing Population Level $\delta^{13}\text{C}$ Responses to Environmental Change*

In order to determine if there were population specific differences in leaf-level  $\delta^{13}\text{C}$  responses among 41 white ash populations across extreme and non-extreme years, we tested for a significant population x year interaction for this trait. We did not find a significant interaction between population and year ( $p>0.9$ ) providing evidence that leaf-level  $\delta^{13}\text{C}$  responses among populations are similar across extreme and non-extreme years.

#### **Conclusions**

Predicted shifts in the *mean* temperature and precipitation over the next century will be accompanied by increased interannual variation in these variables, with extreme years becoming more common (Hansen *et al.*, 2012; IPCC, 2013). Because trees have long generation times, and their migration rates are likely too slow to track rapid shifts in climate zone boundaries, population-level variation may allow species to adapt under future climate change scenarios (Aitken *et al.*, 2008; Alberto *et al.*, 2013). However, the impacts of weather extremes on tree physiology are largely unknown (W.R.L Anderegg *et al.*, 2015). Common garden studies, or provenance trial experiments, can be useful for investigating intraspecific variation in physiological traits that may be relevant to the adaptive capacity of a population as climate changes.

Across 41 populations of white ash growing in a common garden in Lawrence, KS, we found little potential for leaf-level  $\delta^{13}\text{C}$  to respond to variation in temperature and



precipitation across extreme and non-extreme years. While we observed a significant effect of population on leaf-level  $\delta^{13}\text{C}$ , we did not observe a significant population x year interaction. Thus, populations of white ash differ constitutively in leaf-level  $\delta^{13}\text{C}$  with little environmentally induced change. Additionally, the rank order in leaf-level  $\delta^{13}\text{C}$  response among populations remained relatively consistent across extreme and non-extreme years. Thus, the *relative* response in leaf-level  $\delta^{13}\text{C}$  of a population may be predictable even during extreme years in the future. Intraspecific variation in leaf-level  $\delta^{13}\text{C}$ , as is seen here, may be beneficial for white ash to adapt to future climate change, unless populations are unable to migrate to new areas with environmental conditions that they have been historically adapted to. Lastly, we found that a relationship between average leaf-level  $\delta^{13}\text{C}$  and growing season VPD was significantly weakened when data from 2013 were included. While this observation may have been due to soil water availability, it may also suggest that the effects of 2012 persisted into 2013.

Leaf-level  $\delta^{13}\text{C}$  is only one trait that may be relevant to the adaptive capacity of white ash under future climate change, since it is likely to be related to plant fitness. The impact of extreme years on physiological traits of trees under future climate change scenarios is still poorly understood. Forests ecosystems are ecologically and economically important, but will be especially vulnerable to decline or extinction under future climate change, and especially during extreme years (Allen et al., 2010; W. R. L. Anderegg, Anderegg, Sherman, & Karp, 2012; W. R. L. Anderegg et al., 2013; W.R.L. Anderegg et al., 2015). Because extreme years are expected to become much more common over the next century, it is critical that we understand how ecophysiological traits that will likely be relevant under future climate change will respond to strong variation in climate.

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## Figure Legends

**Fig. 1** The range of *Fraxinus americana* (white ash) is shown in the shaded grey area. Locations from which white ash populations originated are shown by black circles. Trees from each of these populations were grown in the University of Kansas Field Station common garden, Lawrence, KS (red star). Trees from all populations grew side-by-side in this common garden for 36 years to date. Adapted from Marchin et al., 2008 and USDA Forest Service ([www.na.fs.fed.us](http://www.na.fs.fed.us)).

**Fig. 2** Total annual precipitation (solid black line) and total growing season precipitation (March-August, dashed black line) across seven years (2005, 2007, 2009, 2010, 2011, 2012, 2013) at a white ash common garden in Lawrence, KS.

**Fig. 3** Average leaf-level  $\delta^{13}\text{C}$  values across non-extreme years (black bar) was only marginally different (0.4‰) the average leaf-level  $\delta^{13}\text{C}$  of 2012 (grey bar).

**Fig. 4** Average leaf-level  $\delta^{13}\text{C}$  values (open circles) among 41 populations of white ash trees closely tracked growing season (March-August) vapor pressure deficit (VPD) across seven years of study (2005, 2007, 2009, 2010, 2011, 2012, and 2013).

**Fig. 5** Timing of leaf emergence (in Julian dates) among 41 white ash populations grown in a common garden at the University of Kansas Field Station. Symbols are mean leaf-level  $\delta^{13}\text{C}$  values. The total number of trees observed each year is as follows: 2005, n=126; 2007, n=149; 2009, n=168; 2010, n=159; 2011, n=125; 2012, n=166; 2013, n=149. Sample sizes within populations range from 1-10 depending upon long-time survivorship. Numbers in parentheses are shown if multiple families were sourced from that population. Populations were color coded based on their rank during a randomly selected year (2009) – populations with less negative leaf-level  $\delta^{13}\text{C}$  values



are coded darker relative to populations with more negative leaf-level  $\delta^{13}\text{C}$  values, which are coded in lighter colors.

**Fig. 6** The relationship between the average leaf-level  $\delta^{13}\text{C}$  across 7 years (2005, 2007, 2009, 2010, 2011, 2012, and 2013) for each of 10 populations sourced across a precipitation gradient (east-west longitudinal gradient) and growing in a common garden in Lawrence KS with a) total annual precipitation of the populations source location, and b) growing season (April-September) VPD from the populations source location. A simple linear regression line is shown with its corresponding equation.



Fig. 1

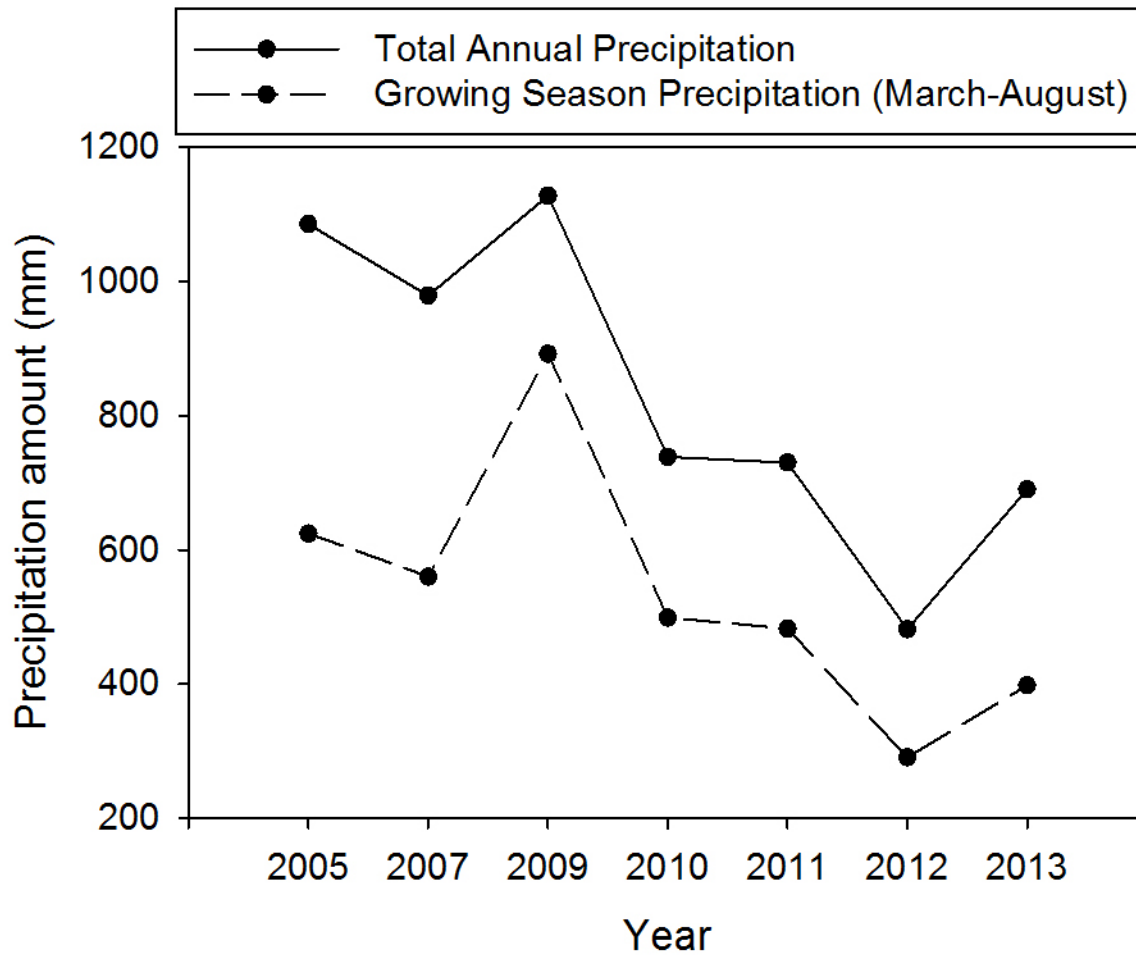
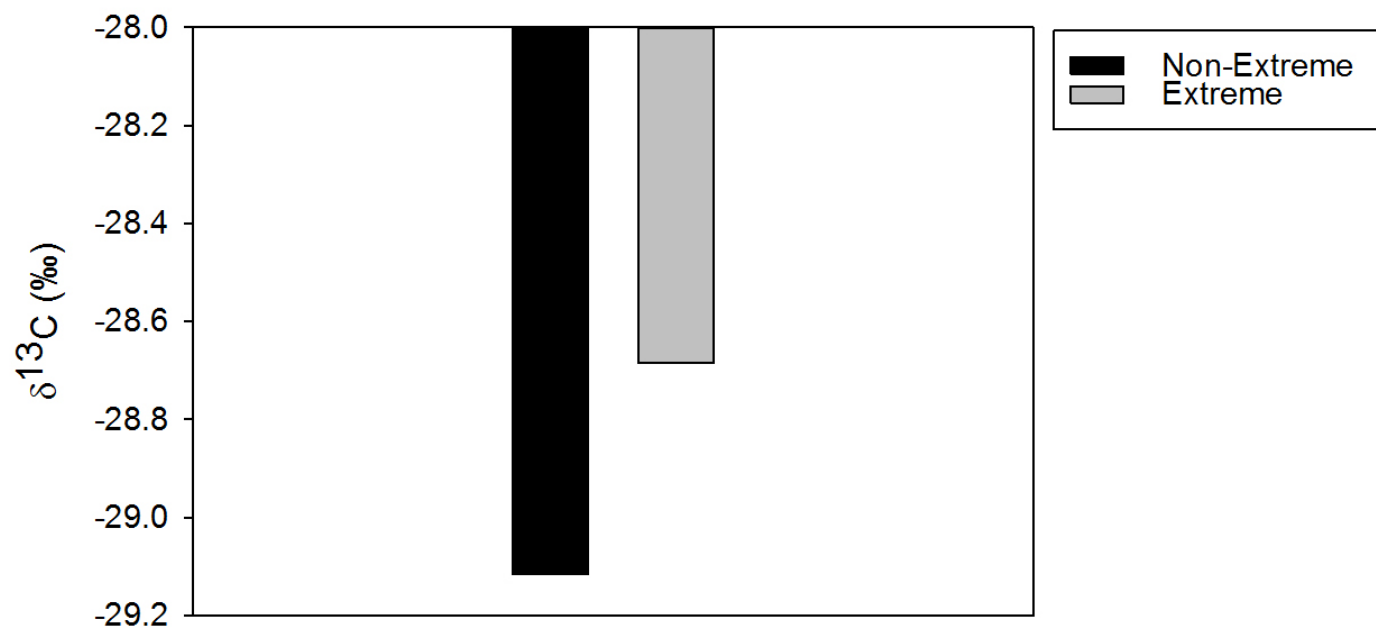


Fig. 2



**Fig. 3**

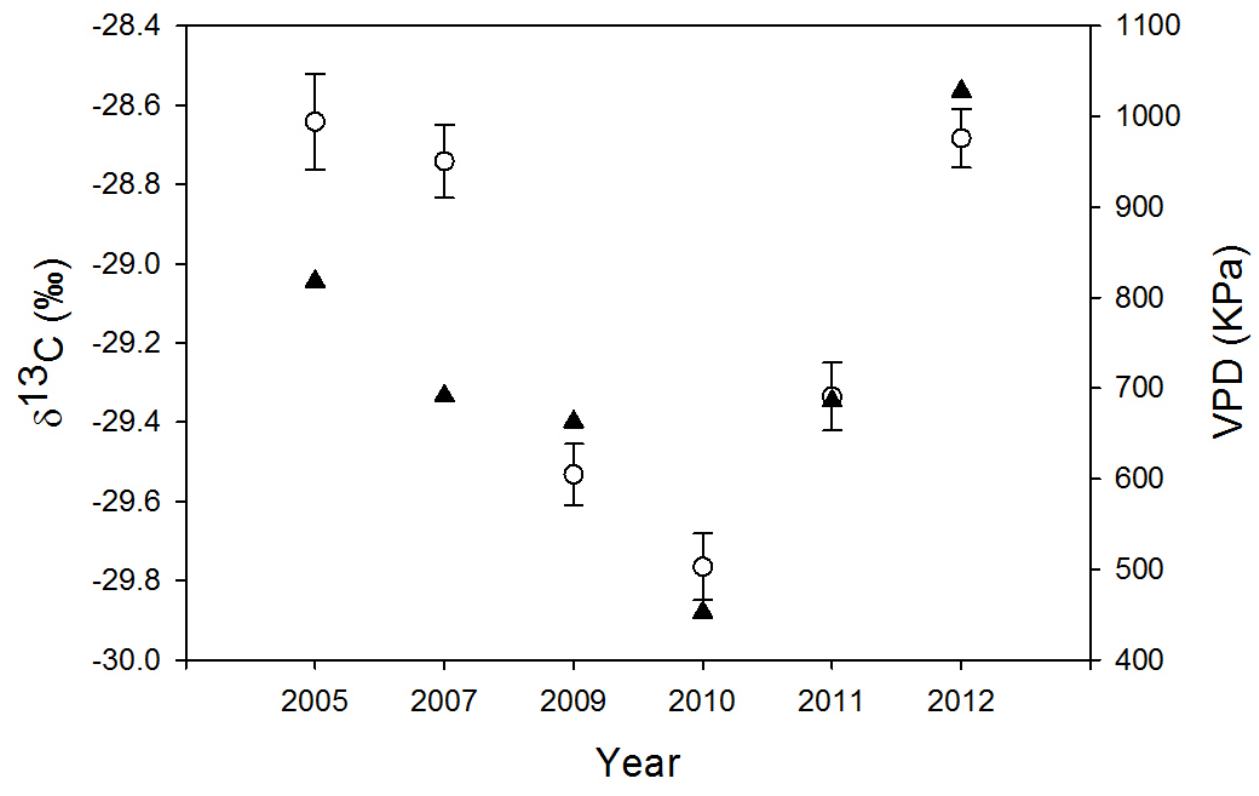


Fig. 4

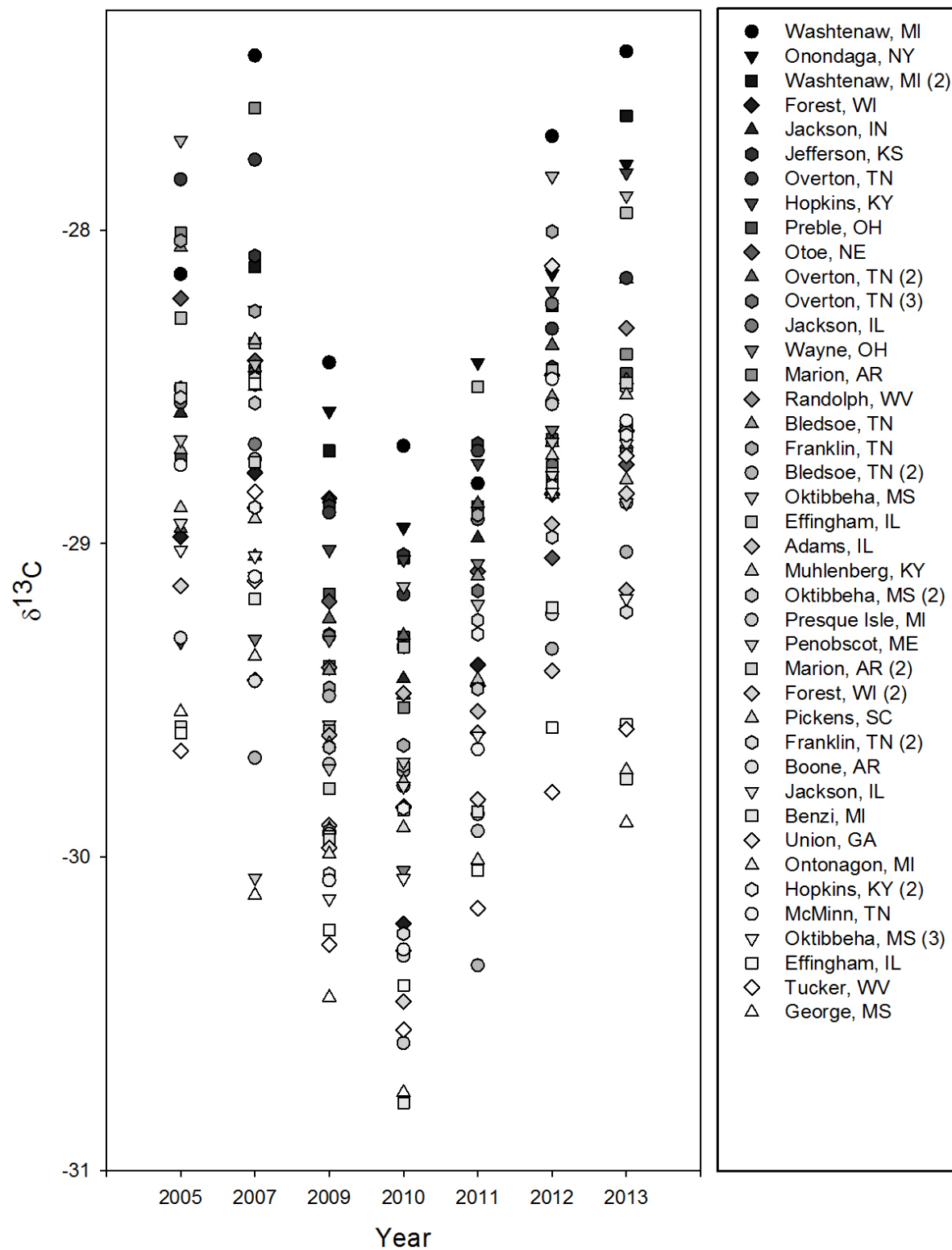
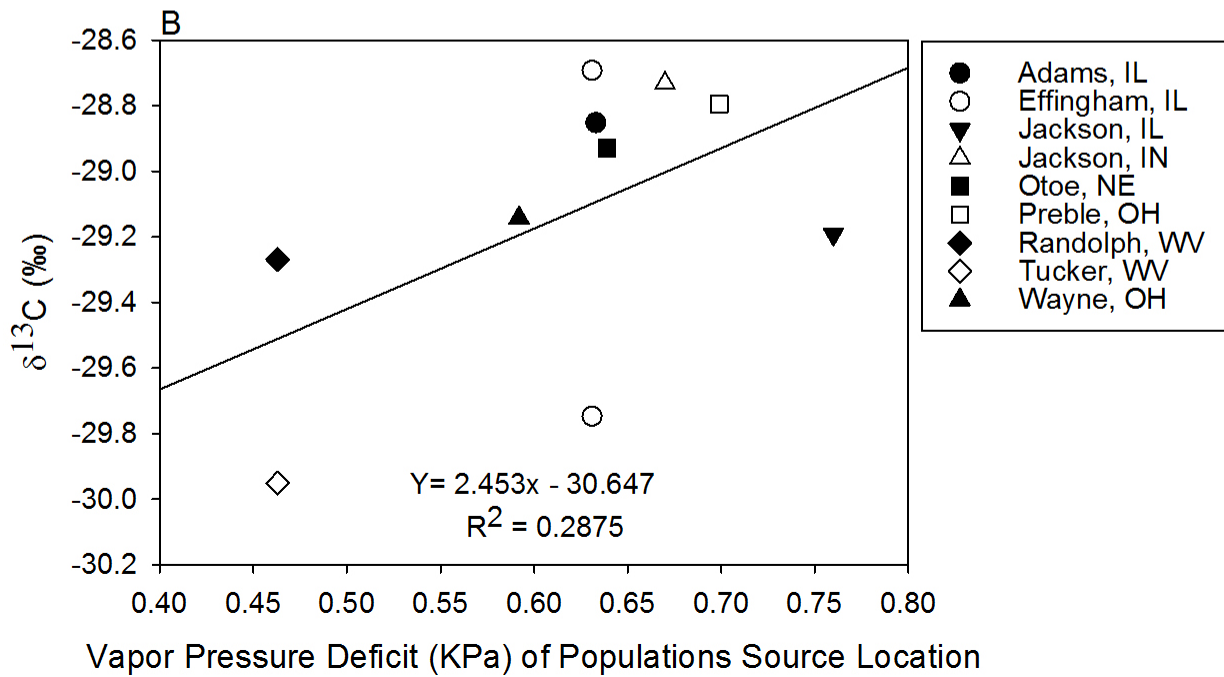
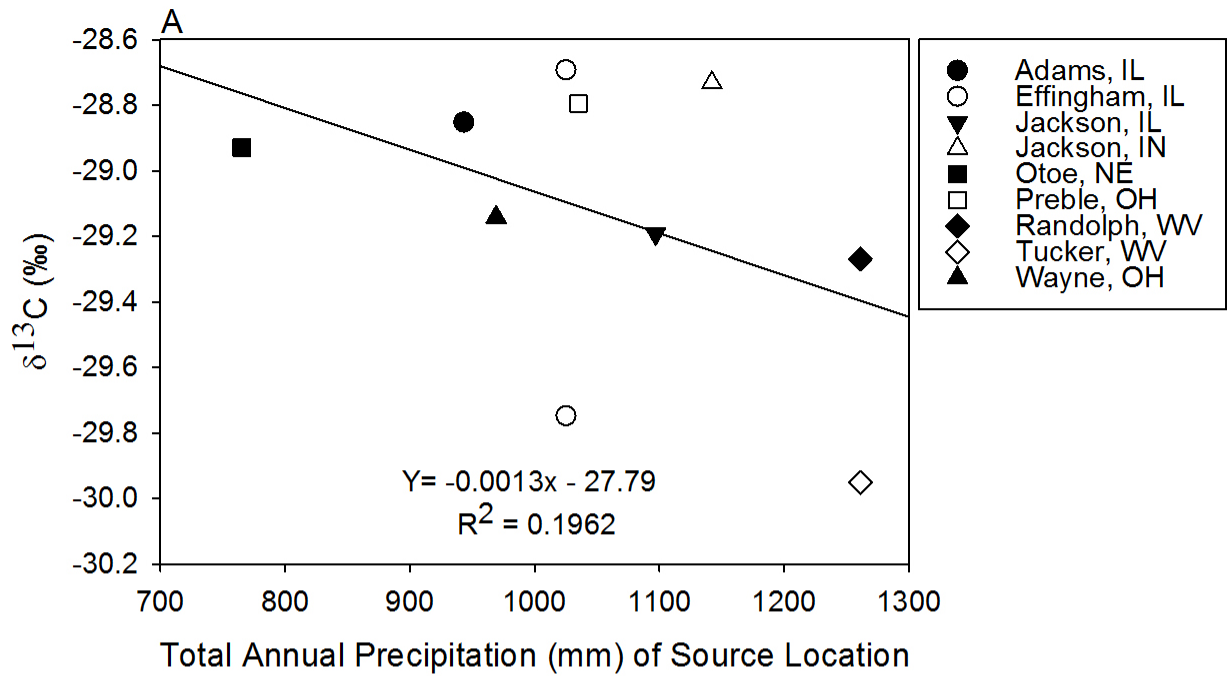


Fig. 5



**Fig. 6**

## Chapter 3: Ploidy Level of White Ash Trees Influences Water Relations in a Common Garden

### Abstract

Different degrees of ploidy within plants can produce morphological and physiological differences in response to local environments including larger xylem vessels and larger stomata due to increased cell size. It is predicted that larger xylem vessels among polyploids may allow for higher hydraulic conductance relative to diploid progenitors under wet conditions, but this could lead to enhanced embolism with subsequent blockage of water transport under dry conditions. However, polyploids often occupy drier habitats relative to their diploid congeners, which may suggest that polyploids possess xylem properties that confer increased drought tolerance relative to diploids. If water-use strategies differ among cytotypes within a species, then this also should be reflected in leaf-level physiological responses under common conditions. Unfortunately, the physiological characteristics of different cytotypes are not well understood at the leaf level. Moreover, because ploidy is often closely correlated with geographical distribution, the effects of genome duplication versus population origin on physiological functioning can be confounding, and few studies have been able to tease apart these variables. Here, we report on the physiological characteristics of diploid, tetraploid, and hexaploid white ash trees, whereby all cytotypes are represented within populations originating from diverse locations. Additionally, all measurements are made within a common garden in Lawrence, KS. Using this unique approach we were able to control for variation between populations and isolate the effects of polyploidy level on key physiological traits. We observed a significant effect of cytotype nested within population on mid-day leaf-level water potentials ( $\Psi_w$ ) whereby average  $\Psi_w$  increased with increasing ploidy within all populations. Additionally, we found leaf-level saturated



photosynthesis ( $A_{max}$ ), stomatal conductance to water vapor ( $g_s$ ), and transpiration ( $E$ ) were significantly different between cytotypes nested within populations. We found diploids had higher leaf-level gas exchange rates relative to polyploids. Taken together, leaf-level mid-day water potentials and rates of gas exchange may reflect differences in sensitivity to drought among cytotypes, and this may explain the different distributions of cytotypes across the range of white ash. However, we also found that differences in leaf-level mid-day water potentials and gas exchange were not reflective of physiological functioning across the whole growing season as evidenced by the lack of difference in leaf-level  $\delta^{13}\text{C}$  among cytotypes nested within populations. We also observed no differences in xylem-area specific hydraulic conductance, xylem fatigue, or xylem density across populations and cytotypes nested within populations. Thus, the effects of polyploidy on physiological functioning may only have implications for the responses of white ash to future droughts at shorter temporal scales. Further studies that incorporate measurements across more populations and the entire growing season are needed to provide a more comprehensive understanding of the consequences of ploidy for white ash water relations, especially as extreme droughts are expected to become more common across the species range over the next century.

## Introduction

Polyploidy, the condition of an organism possessing more than two paired sets of chromosomes in their somatic cells (Stebbins, 1971), has arisen recurrently throughout the evolutionary history of most extant angiosperms (Cui et al., 2006; Soltis & Soltis, 1999). In addition, polyploidy is regarded as a key mechanism of sympatric speciation (Wood et al., 2009), but the factors that contribute to the establishment and persistence of polyploids remain unresolved (Soltis, Buggs, Doyle, & Soltis, 2010). Because polyploids often form in sympatry with their diploid parents, competition is likely to reduce the establishment probability of polyploids (Glennon, Ritchie, & Segraves, 2014). The establishment probability of polyploids may be enhanced if they possess distinct physiological tolerances that allow them to exploit new niches and escape competition. Habitat differentiation between cytotypes (i.e., different levels of ploidy) is commonly reported with observations showing that the frequency of polyploids increase at the edge of species ranges (Fowler & Levin, 1984), and polyploids often occupy some of the most ecologically extreme environments relative to their diploid congeners (Brochmann et al., 2004; Hijmans et al., 2007). Physiological differences can contribute to habitat differentiation between cytotypes (Fowler & Levin, 1984; Maherali, Walden, & Husband, 2009; Manzaneda et al., 2012; Ramsey, 2011; Segraves & Thompson, 1999; Senock, Barrow, Gibbens, & Herbel, 1991; te Beest et al., 2012). However, it is often unknown if these physiological differences between cytotypes occupying different areas are a direct consequence of genome size or geographic distribution (O'Keefe, Tomeo, Nippert, & Springer, 2013). Moreover, the consequences of polyploidy for many physiological traits are still unknown (Soltis et al., 2010).

Polyploidy can increase xylem conduit size (Hao, Lucero, Sanderson, Zacharias, & Holbrook, 2013), xylem lignin content (Nassar, Graciano-Ribeiro, Fernandes, & Araujo, 2008), and cell wall thickness of xylem vessels (Maherali et al., 2009). These differences in xylem properties between cytotypes may explain the more frequent occurrence of polyploids in dry versus wet habitats (Hao et al., 2013; Maherali et al., 2009; Manzaneda et al., 2012). This is because such differences in xylem properties are likely to affect the hydraulic function of xylem conduits, which could in turn impact leaf-level physiology including the assimilation of CO<sub>2</sub> (Brodribb & Holbrook, 2004; Santiago et al., 2004), and survivorship in drought-stressed environments (McDowell et al., 2008). There is currently a paucity of studies investigating the relationship between ploidy, xylem properties, and hydraulic function (but see (Hao et al., 2013; Maherali et al., 2009).

Extreme droughts are predicted to become more common across the U.S. over the next century (IPCC, 2013). As evapotranspiration increases during droughts, this will impose constraints on plant water uptake and transport (Adams et al., 2009). Lower soil water potentials during drought will likely heighten tension on the water column in xylem conduits causing cavitation, the rupture of the water column in xylem conduits, that will subsequently lead to embolism of xylem conduits and blockage of water transport (J. S. Sperry, Hacke, Oren, & Comstock, 2002). An inverse relationship between hydraulic transport efficiency and safety is often reported in stems, whereby stems with larger xylem conduits can transport higher amounts of water to leaves under wet conditions, but are more prone to cavitation and embolism under dry conditions relative to stems with smaller xylem conduits (Fan et al., 2011; Martinez-Vilalta, Prat, Oliveras, & Pinol, 2002). This trade-off is thought to be related to the vulnerability of pit-

membrane pores to air-seeding, the process by which a small bubble of air (water vapor) enters a xylem vessel through the pore of a pit membrane, which occurs at progressively higher (or less negative) xylem tensions as pit membrane pores increase in size (Tyree, 1997; Wheeler, Sperry, Hacke, & Hoang, 2005). There is a positive relationship between the maximum size of pit membrane pores and xylem conduit size (Christman, Sperry, & Adler, 2009); therefore, it is hypothesized that larger xylem conduits are more prone to air-seeding (Wheeler et al., 2005). Thus, plants face a “compromise” between the ability to maintain a high flux of water to leaves, which can increase CO<sub>2</sub> assimilation under wet conditions, and the ability to cope with water stress under dry conditions. This compromise is likely a reason why drought tolerant species are often displaced from more mesic areas (Engelbrecht et al., 2007). The fact that polyploids occupy drier sites more often than diploids may be explained by this trade-off in hydraulic efficiency and safety.

Hydraulic transport in xylem conduits is inextricably linked to leaf-level gas exchange, and stomatal control plays a significant role in regulating water loss from leaves, which can impact growth and survival (Mansfield & Davies, 1985; McDowell et al., 2008). To maintain tissue hydration and photosynthesis, water lost via stomata through transpiration ( $E$ ) must be replaced. Transpiration ( $E$ ) creates tension that pulls water from the soil through the plant to the crown, where it is eventually lost to the atmosphere through diffusion from open stomata. The rate of  $E$  can be described based on the soil-plant-atmosphere hydraulic continuum,

$$E = K_l (\Psi_s - \Psi_l - h_{p_{wg}}) \text{ (Eqn. 1 from (McDowell et al., 2008))}$$

where  $K_l$  is leaf-specific hydraulic conductance,  $\Psi_s$  and  $\Psi_l$  are soil and leaf water potentials, and  $h\rho_w g$  is the gravitational pull on a water column of height  $h$  and density  $\rho_w$ . If no cavitation occurs, then  $K_l$  remains constant and the tension difference ( $\Psi_s - \Psi_l$ ) across the plant increases in proportion to  $E$ . When tension ( $\Psi_s - \Psi_l$ ) does increase, such as during drought,  $K_l$  decreases due to air-seeding in pit pore membranes, and this decrease in  $K_l$  places an upper limit on  $E$  ( $E_{crit}$ ). If  $E$  exceeds  $E_{crit}$ ,  $K_l$  approaches 0, and water movement through the plant ceases (McDowell et al., 2008). The tension that determines  $E_{crit}$  varies widely among species (Pockman & Sperry, 2000; Pockman, Sperry, & O'Leary, 1995). Plants can maintain  $E$  below  $E_{crit}$  through regulating leaf-level stomatal closure ( $g_s$ ). The degree of closure is often linked to leaf-level water potentials ( $\Psi_w$ ) that result in embolism ( $\Psi_{crit}$ ) (J. S. Sperry et al., 2002), which is linked to xylem properties such as the presence of large pit pores (Wheeler et al., 2005). Thus, these physiological traits are inextricably linked, and can provide valuable information regarding an individual's sensitivity to drought (McDowell et al., 2008).

Investigations of plant hydraulics and water relations between cytotypes have produced mixed results. Hao *et al.* (2013) found that tetraploid and hexaploid populations of *Atriplex canescens* growing in more water-limited soils than diploids in the Chihuahuan Desert of southern New Mexico, US, had lower leaf-area specific hydraulic conductances and were more resistant to embolism relative to diploid populations. However, Hao *et al.* (2013) also found that xylem vessel diameters were larger in polyploids relative to diploids. Because larger xylem conduits are generally associated with a higher number of large pit membrane pores that are more susceptible to air seeding relative to smaller pores (Wheeler et al., 2005), this result suggest that the size of pit membrane pores may not scale with xylem conduit size across cytotypes

(Hao et al., 2013). Thus, larger xylem vessel diameters of polyploid *A. canescens* may not be related to higher hydraulic requirements, but solely to the effect of genome duplication. In contrast to this finding, Maherali *et al.* (2009) reported hexaploid *Chamerion angustifolium* individuals had higher hydraulic conductances relative to diploids, but vulnerability to embolism was not different between cytotypes. Further studies are thus warranted to investigate the consequences of ploidy on plant hydraulics and water relations.

White ash (*Fraxinus americana* L.) is one of the most widely distributed (Fig. 1) and commercially important ash species in the Eastern U.S. (MacFarlane & Meyer, 2005). There are three naturally occurring cytotypes distributed throughout the range of white ash, a diploid (46 chromosomes), a tetraploid (92 chromosomes), and a hexaploid (138 chromosomes) (Black & Beckmann, 1983). The geographic range of the diploids completely encompasses that of the tetraploids and hexaploids; tetraploids have a more westward distribution and are restricted to regions south of latitude 35°N, and hexaploids have a more eastern distribution and are found between latitudes 35°N and 40°N (Black & Beckmann, 1983; Nesom, 2010). In addition to differences in geographic distribution, Armstrong and Funk (1982) reported xylem vessel elements were 27% and 43% longer in tetraploid and hexaploid trees, respectively (Armstrong, 1982). The distribution of tetraploids further south and west in the U.S. where water is more limiting suggest that tetraploids may have increased drought tolerance relative to diploids and hexaploids. However, longer and larger xylem conduits may make both tetraploids and hexaploids more prone to cavitation and embolism, since these xylem conduits may have larger pit membrane pores relative to diploids. In the present study, we investigated leaf-level mid-day water potential, gas exchange, the isotopic signature of

$^{13}\text{C}$ , xylem hydraulic conductivity, xylem fatigue, and xylem density in three major identified ploidy levels of white ash (diploid, tetraploid, and hexaploid trees) in a common garden. These physiological traits often explain variation in drought resistance among plant species (W. R. L. Anderegg et al., 2013; Donovan & Ehleringer, 1994; Jacobsen et al., 2007; McDowell et al., 2008). We identified three populations (Jackson, IL; Hopkins, KY; Overton, TN) sourced from a narrow precipitation gradient (~300 mm total annual precipitation difference between populations) in which all cytotypes are represented. Investigating variation in physiological traits across cytotypes nested within populations allowed us to assess the influence of geographic origin versus genome duplication on plant water relations. We tested the following hypotheses: 1) the effect of population source location is greater than the effect of genome duplication on plant water relations, 2) the effect of genome duplication, due to increases in xylem conduit size, is greater than the effect of population source location, and 3) tetraploids show physiological traits that are more sensitive to drought stress relative to diploids and hexaploids, due to their distribution across more water limited areas.

## **Materials and Methods**

### *Common Garden Location and Climate*

White ash seeds originating from 43 populations throughout the species range were collected by the U.S. Forest Service from open-pollinated native parent trees. Seeds were planted in 1976 at the University of Kansas Field Station in Lawrence, KS (35.0°N, 95.1°W, 299 m a.s.l.; Fig. 1). Twenty five replicate trees from each population were planted at this site. We collected data on three populations at this site (Jackson, IL; Hopkins, KY; Overton, TN) that are located along a narrow precipitation gradient

where total annual precipitation at Jackson, IL (1097 mm) is lower than that of Hopkins, KY (1220 mm) and Overton, TN (1408 mm). All three identified white ash cytotypes (diploid, tetraploids, and hexaploids) were represented within these three populations. Cytotypes of white ash have been shown to differ in discrete morphological traits including leaf scar shape and leaf pubescence (Nesom, 2010). Furthermore, polyploids are separated and relatively isolated from each other geographically suggesting that there truly are three different cytotypes (Nesom, 2010).

The field station receives an average annual precipitation of ~900 mm per year with more than 70% of this falling in the growing season from March-August. Temperature fluctuates greatly both diurnally and annually with monthly average temperatures ranging from below -7 °C in January to slightly above 32 °C in July. In the present study, data were collected during 2013 and 2015. The total amount of precipitation that fell during the growing season at this site in 2013 (397.8 mm) was slightly less than 2015 (421.4 mm) to date, and temperatures were cooler during the growing season of 2013 at this site (14.8 °C) than in 2015 to date (18 °C).

#### *Xylem-Specific Hydraulic Conductance, Xylem Density, and Xylem Fatigue*

Xylem-specific hydraulic conductivity ( $K_s$ ) was calculated as the maximum rate of water flow (with all embolisms removed) through a stem segment per xylem cross-sectional area, following the methods of Sperry *et al.* (1988), Kolb and Davis (1994), and Martinez-Vilalta *et al.* (2002). One branch from 2-4 individual trees within each cytotype per population was collected from the white ash common garden site. Branches were either cut directly from the tree with a branch submerged under water, or were cut longer than the maximum vessel length recorded for white ash (>3m) to



prevent air from artificially being introduced into the cut branches. Branches were submerged under water and trimmed from alternate ends until a final stem segment, 14 cm in length and between 6-8 mm in diameter, was obtained. Stems were connected to a tubing system and flushed with a 20 mM KCl solution that was degassed (membrane contactor, Liqui-Cel Minimodule 1.7 X 5.5, Charlotte, NC, USA) and filtered (0.1  $\mu\text{m}$  inline filter, GE Water and Process Technologies, Trevose, PA, USA). Stems were flushed at a pressure of 100 kPa for one hour to remove gas emboli from xylem vessels. Maximum hydraulic conductivity ( $K_{hmax}$ ) of stems was then measured gravimetrically using a conductivity apparatus (John S. Sperry & Tyree, 1988).

Following determination of  $K_{hmax}$ , the level of xylem fatigue in stems was assessed using the simple centrifuge technique (Alder, Pockman, Sperry, & Nuismer, 1997). Stems were spun at a prescribed speed of rotation in order to generate a known negative pressure (-0.25 MPa) on the water column in xylem vessels (Alder et al., 1997). Stems were then reconnected to a conductivity apparatus, and their hydraulic conductance was measured gravimetrically using a low pressure-head (1.5-2.0 kPa) to avoid displacing air from embolized vessels. The xylem cross-sectional area was estimated by calculating the area of a circle ( $\pi r^2$ ) using the diameter of the middle of the stem. The xylem specific  $K_s$  value ( $\text{kg m}^{-1} \text{MPa}^{-1} \text{s}^{-1}$ ) was then calculated as  $K_h$  divided by cross-sectional xylem area.

Xylem density was determined by measuring the mean dry mass per fresh volume of stems from 6-8 individuals per cytotype within population. The pith was removed from stems by cutting the stems longitudinally with a razor blade. The stems were previously saturated with degassed and filtered KCl solution until they reached their maximum wet mass. Water-saturated volume was determined by water

displacement in a graduated cylinder. The stems were dried at 60 °C for 72 hours and then dry mass was measured. Xylem density was then determined by dividing mass by volume (Hacke, Sperry, Pockman, Davis, & McCulloch, 2001).

### *Stable Isotope Measurement of <sup>13</sup>C in Leaf Tissue*

We measured stable carbon isotopic ratios (<sup>12</sup>C:<sup>13</sup>C) in leaf tissue of 2-15 trees of each of three cytotypes within three different populations of white ash. Isotope measurements were performed at the Keck Paleoenvironmental and Environmental Stable Isotope Laboratory (KPESIL) at the University of Kansas.  $\delta^{13}\text{C}$  were calculated using the following formula:

$$\delta = R_{\text{sample}} / R_{\text{standard}} - 1 \text{ (equation 1)}$$

where  $R$  is the ratio of <sup>13</sup>C/<sup>12</sup>C, using belemnite carbonate from the Pee Dee Formation, Hemingway, SC (PDB) as the standard. Data were converted to “per mil” (‰) notation by multiplying  $\delta$  values by 1000. Leaves used in the analysis were collected during August of 2013 from fully mature sunlit leaves on the south side of trees from one block at the site (block 2). Trees are interspersed equally throughout the common garden, and populations are clumped in rows (5 trees each) in each of 5 blocks. We found no difference in relative water content or soil nutrients between blocks and, therefore, no longer considered the effect of block in our analyses. Leaves were dried at 60 °C for 48 hours and then ground in liquid nitrogen to a fine powder using a mortar and pestle. Tissues were then weighed (3-3.5 mg) and loaded into tin capsules (3.5 x 5.5 mm). A ThermoFinnigan MAT 253 Dual Inlet System was used for  $\delta^{13}\text{C}$  analysis.

### *Leaf-Level Gas Exchange and Mid-Day Water Potentials*

We measured gas exchange using a LiCor-6400 infra-red gas analyzer with a red/blue light source and a CO<sub>2</sub> injector (LiCor, Lincoln, Nebraska, USA) on two leaves for 2-15 trees per cytotype within each of the three populations used in this study. Gas exchange was measured seven times during July, 2013 and once during August, 2013 (8 sample dates). Irradiance inside the cuvette was 2,000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , CO<sub>2</sub> concentration was 400 ppm and relative humidity was maintained at ambient. Gas exchange measurements were made on new, mature leaves growing in full sunlight on the south side of trees between 0900 and 1300 hours Central Daylight Time (CDT). Measurements included photosynthetic rate at 2000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  ( $A_{\text{sat}}$ ), stomatal conductance to water ( $g_s$ ), and transpiration ( $E$ ). Water potential measurements were conducted at midday (1300-1500 hours CDT) using a Scholander pressure bomb (PMS instruments, Albany, Oregon, USA) on two leaf samples per ploidy within populations (n= 2-15 trees).

### *Statistical Analyses*

Data were analyzed using a mixed effects model ANOVA in SAS 9.1 (Cary, North Carolina, USA), where population and cytotype nested within population were fixed effects, whereas sampling date was a random effect to account for repeated measures in the design. Gas exchange measurements were not recorded during 2015 at this site.

### **Results and Discussion**

In the present study, we show that **A**) average mid-day leaf-level water potentials ( $\Psi_w$ ) significantly varied across cytotypes nested within three white ash populations

(Overton, TN; Jackson, IL; Hopkins, KY) whereby  $\Psi_w$  became more positive as ploidy level increased. For example, for cytotypes sourced from Hopkins, KY, average  $\Psi_w$  was -2.8, -2.4, and -1.9 MPa for diploids, tetraploids, and hexaploids, respectively. **B)** leaf-level gas exchange significantly varied by cytotype nested within populations. While trends were different among populations, diploids showed the highest  $A_{max}$ ,  $g_s$ , and  $E$  relative to polyploids, whereas these values were lowest in tetraploids relative to diploids and hexaploids. However, we observed no significant differences across populations or cytotypes within populations in a more integrative measure of leaf-level physiology ( $\delta^{13}C$ ), which may suggest cytotypes respond differently as abiotic conditions change across the growing season. **C)**  $K_{smax}$ , xylem fatigue, and xylem density did not significantly vary across populations or between cytotypes within a population.

#### *Leaf-level Water Potentials and Gas Exchange Vary by Cytotype*

We observed significant differences in midday leaf-level water potentials ( $\Psi_w$ ) across cytotypes nested within three white ash populations (Overton, TN; Jackson, IL; Hopkins, KY; Fig. 2A). Average  $\Psi_w$  became more positive as ploidy level increased in each population (Fig. 2A). For example, average  $\Psi_w$  for individual trees sourced from Hopkins, KY was -2.8, -2.4, and -1.9 MPa for diploids, tetraploids, and hexaploids, respectively (Fig. 2A). These results suggest that there may be differences in water stress among cytotypes. Leaf-level gas exchange that included measurements of  $A_{max}$ ,  $g_s$ , and  $E$ , were not different across populations, but did differ among cytotypes nested within populations ( $p < 0.01$ , Fig. 2 B-D). However, there are no obvious trends in leaf-level gas exchange across cytotypes nested within populations (Fig. 2 B-D).

Because population did not explain variation in leaf-level gas exchange, we investigated trends in the averaged leaf-level gas exchange data as well as leaf-level mid-day water potentials across populations to assess the effects by cytotype. In doing so, we found that leaf-level gas exchange was consistently higher for diploids relative to polyploids, and consistently lower for tetraploids relative to diploids and hexaploids (Table 1). Leaf-level midday water potentials *marginally* increased with ploidy level from -2.7 to -2.2 MPa (diploids and hexaploids, respectively; Table 1). Taken together, these results may reflect that diploids function closer to their hydraulic margin of safety (i.e., the difference between  $E$  and  $E_{crit}$ ) than polyploids, since they maintain higher levels of gas exchange at lower (more negative) water potentials relative to polyploids. We cannot say this for certain as we are unable to estimate an  $E_{crit}$  value in this study since variation in  $\Psi_w$  across individuals within cytotypes was low (Table 1), and estimation of  $E_{crit}$  requires observations of  $E$  across a range of  $\Psi_w$ . If diploids do function physiologically closer to their hydraulic margin of safety, we expect that diploids would be more vulnerable to drought relative to polyploids, unless they have xylem traits that make them more resistant to drought-induced cavitation. Diploids may have higher resistance to drought-induced cavitation if their xylem conduits and pit membrane pores are smaller than those of polyploids (Wheeler et al., 2005). Differences in leaf-level gas exchange and mid-day water potentials between cytotypes also may be explained by the ratio of leaf area to sapwood area ( $A_l:A_s$ ) (McDowell et al., 2002). According to the “pipe model,” the leaf area of a stem should scale in proportion (scale isometrically with) to the cross-sectional area of xylem (sapwood) that supports it (Shinozaki, Yoda, Hozumi, & Kira, 1964). Under dry conditions, high vapor pressure deficits generally imply that a larger  $E$  must be maintained in order to achieve a given rate of  $A$ , which

decreases the optimal  $E:A$ . In lieu of decreasing  $A$  under dry conditions, plants can maintain a smaller  $A_l:A_s$  so that photosynthesis remains at similar rates under dry and wet conditions (Togashi et al., 2015). In our study, diploids showed higher rates of  $A_{max}$  relative to polyploids (Table 1), and this may suggest that there are differences in  $A_l:A_s$  among cyotypes that are likely driven by specific leaf area. Hao *et al.* (2013) found higher  $A_l:A_s$  in polyploid *A. canescens* individuals relative to diploids because polyploids had larger leaves and less stem thickening (i.e., smaller sapwood area). Additionally, other leaf-level factors such as stomatal size and chlorophyll content, which are known to differ between cyotypes (Masterson, 1994; Warner & Edwards, 1989), may explain the differences we see here in our study. Regardless of the mechanism that explains the observed differences in leaf-level gas exchange between cyotypes in our study, we find that diploids have higher  $A_{max}$  relative to polyploids. If  $A_{max}$  translates into higher growth rates for diploids relative to polyploids across the range of white ash, and if higher growth rates make diploids more competitive than polyploids, then these results may reflect why there is a wider distribution of diploids across the species range.

We found no significant differences in leaf-level  $\delta^{13}\text{C}$  across populations or between cyotypes nested within populations (Fig. 2E). Because leaf-level  $\delta^{13}\text{C}$  is an integrative measure of the ratio of  $c_i/c_a$  across the growing season, and reflects a balance between  $A_{max}$  and  $g_s$ , we would expect leaf-level  $\delta^{13}\text{C}$  to reflect, partially, instantaneous leaf-level gas exchange measurements. Precipitation greatly fluctuated throughout the growing season of 2013 (Fig. 3). Leaf-level gas exchange was measured nearly exclusively during the month of July, and a lower amount of precipitation fell during this month of the growing season (Fig. 3). Therefore, these instantaneous measures of leaf-level gas exchange are likely more reflective of physiological

functioning during a drier time of the growing season. Thus, the  $\delta^{13}\text{C}$  presented here suggest similar rates of leaf-level gas exchange between cytotypes are likely maintained across a majority of the growing season.

*Xylem-area specific hydraulic conductance, xylem fatigue, and xylem density*

As genome duplication can increase xylem conduit size, we expected higher maximum hydraulic conductance in polyploids relative to diploids. Additionally, leaf-level gas exchange and mid-day water potential data suggested that diploids may be most resistant to xylem fatigue. Furthermore, if genome duplication alters xylem conduit size, we would expect to see higher xylem density in diploids relative to polyploids since diploids are likely to have smaller xylem conduits that can be more tightly compacted in sapwood. However, we found no significant differences among populations or cytotypes nested within populations for xylem-area specific hydraulic conductance, xylem vessel fatigue, or xylem density (Fig. 4 A-C). These results may suggest that these xylem properties are strongly controlled by environmental conditions; however, xylem conduit size and hydraulic conductance between cytotypes are generally affected by ploidy (Hao et al., 2013; Maherali et al., 2009), and therefore this is unlikely. Data presented here were measured when xylem conduits were not fully lignified (Fig. 5). The time in which stems are collected can significantly affect hydraulic conductance and resistance to drought-induced cavitation and embolism (Jacobsen, Pratt, Davis, & Tobin, 2014). Because xylem were not fully formed at the time when measurements were taken, a majority of xylem conduits imploded (i.e., lost the totality of their hydraulic functioning) when stems were placed and spun in a centrifuge. Thus, we were not able to construct vulnerability curves. Additionally, because xylem were not fully formed this may explain the high variability in hydraulic conductance data observed in this study (Fig. 4 A-B). We

plan to follow up on this project by repeating these measures in the future with more developed xylem.

## **Conclusions**

Habitat differentiation is often reported among cytotypes with a more frequent occurrence of polyploids occupying drier sites relative to diploid congeners. Physiological differences often contribute to habitat differentiation between cytotypes. Differences in plant water relations among cytotypes may explain the more frequent occurrence of polyploids on drier sites relative to diploids, since these traits are often linked to drought tolerance. However, few studies have investigated the effects of genome duplication on plant water relations to date.

In the present study, we found that mid-day leaf-level water potentials significantly increased with level of ploidy in three major identified cytotypes of white ash (diploids, tetraploids, and hexaploids). This suggested that water stress may differ among cytotypes. We saw a significant effect of cytotype nested within populations on leaf-level gas exchange. Averaging leaf-level gas exchange by cytotype across populations, we observed diploids had higher leaf-level gas exchange than polyploids. Taken together, this data may reflect different drought sensitivities among cytotypes, which may explain the geographic distribution of cytotypes across the range of white ash. However, we also found no difference in leaf-level  $\delta^{13}\text{C}$  across populations or cytotypes nested within populations. This suggested that leaf-level gas exchange measurements were reflective of physiological responses to drier conditions during the month (July, 2013) in which measurements were taken. Additionally, a lack of difference in leaf-level  $\delta^{13}\text{C}$  suggests that these populations and cytotypes have similar



physiological functioning across a majority of the growing season. We also found no differences across populations or cytotypes nested within populations for  $K_{smax}$ , xylem fatigue, or xylem density. Thus, differences observed at the leaf-level are likely not underlain by differences in xylem properties. However, leaf-specific hydraulic conductance in this study may be imprecise due to the lack of lignin in xylem conduits when measurements were taken.

In conclusion, while we find evidence that physiological differences between cytotypes exist at the leaf-level during a drier month, these differences did not explain physiological functioning of cytotypes over the whole growing season. Furthermore, we did not find evidence that such leaf-level differences are underlain by corresponding differences in xylem properties. Thus, the effects of polyploidy on physiological functioning may only have implications for the responses of white ash to future drought at short temporal scales. Future studies should incorporate more populations, measure leaf-level gas exchange during both wet and dry conditions throughout the growing season, and investigate stem hydraulics at a time when xylem are fully lignified. Extreme droughts are expected to become more common across the range of white ash such that a more comprehensive understanding of the effects of polyploidy on drought tolerance in the species is warranted.

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**Table 1** The average maximum photosynthetic rate ( $A_{max}$ ), stomatal conductance to water vapor ( $g_s$ ), transpiration ( $E$ ), and mid-day water potential ( $\Psi_w$ ) across three ploidy levels of white ash. Here, data were averaged by cytotype across populations since leaf-level gas exchange and mid-day water potentials did not significantly vary by population.

<b>Cytotype</b>	<b><math>A_{max}</math></b>	<b><math>g_s</math></b>	<b><math>E</math></b>	<b><math>\Psi_w</math></b>
<b>Diploid</b>	11.25 ± 0.32	0.18 ± 0.009	4.6 ± 0.16	-2.7 ± 0.07
<b>Tetraploid</b>	9.10 ± 0.54	0.12 ± 0.011	3.3 ± 0.21	-2.4 ± 0.10
<b>Hexaploid</b>	10.36 ± 0.41	0.14 ± 0.010	3.7 ± 0.19	-2.2 ± 0.11

## Figure Legends

**Fig. 1** The range of *Fraxinus americana* (white ash) is shown in the shaded grey area. Trees from populations across the species range (black circles) have been growing in a common garden at the University of Kansas Field Station (red star) since 1976. Populations used in this study are shown with red dots.

**Fig. 2** Leaf-level mid-day water potential (A), photosynthetic rate (B), rate of transpiration (C), and  $^{13}\text{C}$  isotopic signatures among diploid, tetraploid, and hexaploid white ash trees (n= 2-15 trees) . Cytotypes were nested within three populations – Hopkins, KY (closed circles), Jackson, IL (open circles), and Overton, TN (closed triangles).

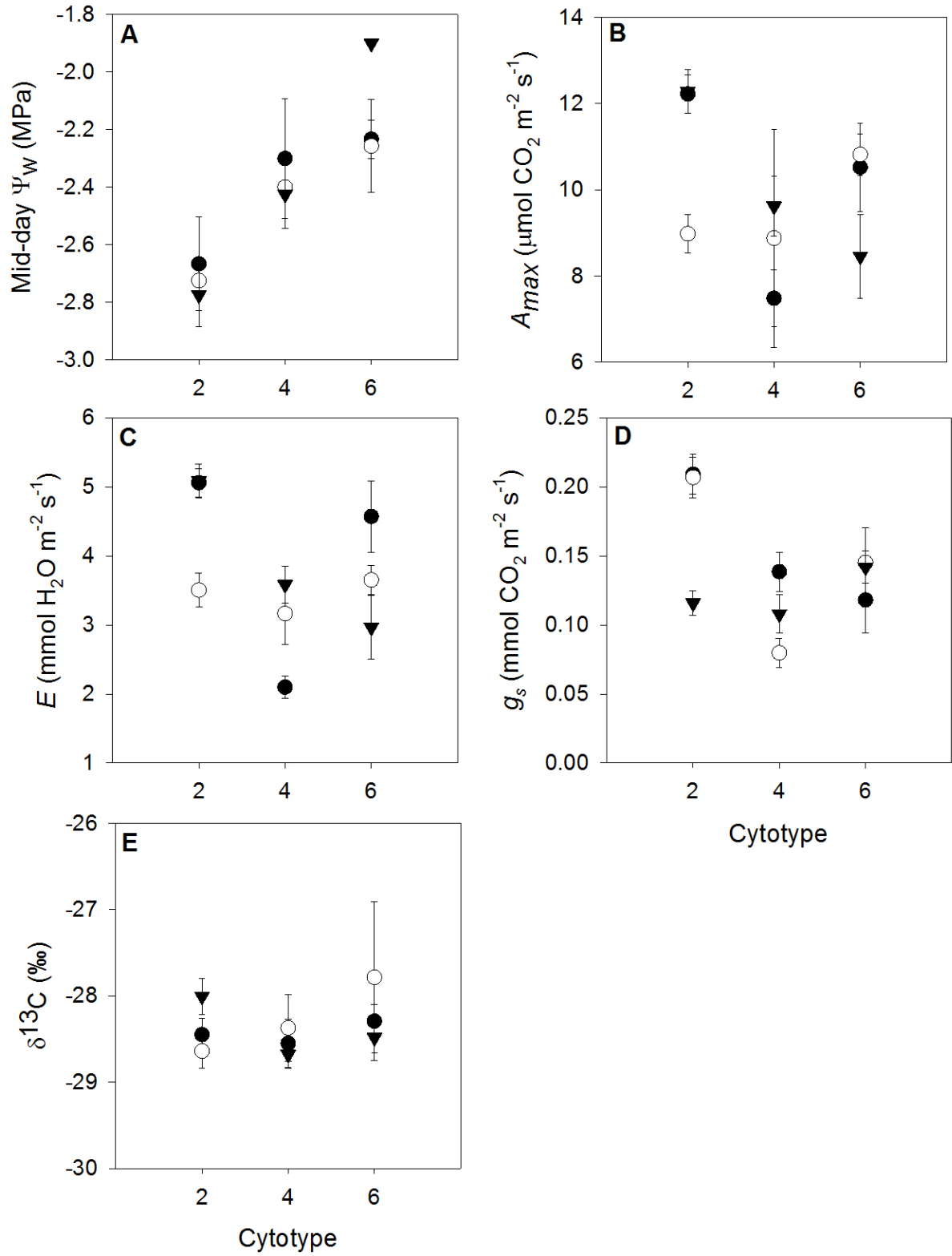
**Fig. 3** Total precipitation (mm) across the growing season (March-August) during 2013 at the white ash common garden site located in Jefferson, KS.

**Fig. 4** Xylem area-specific conductivity at xylem tensions of 0 MPa (max; A) and -0.25 MPa (B), and xylem density (C). Sample sizes for  $K_s$  were between 2-4 trees, whereas samples sizes for xylem density were between 6-8 trees. Populations are coded the same as in Fig. 2.

**Fig. 5** A cross section of a white ash stem where the arrow shows vessels beginning to form for the current year of growth (2015; photo taken by A. Jacobsen).



Fig. 1



**Fig. 2**

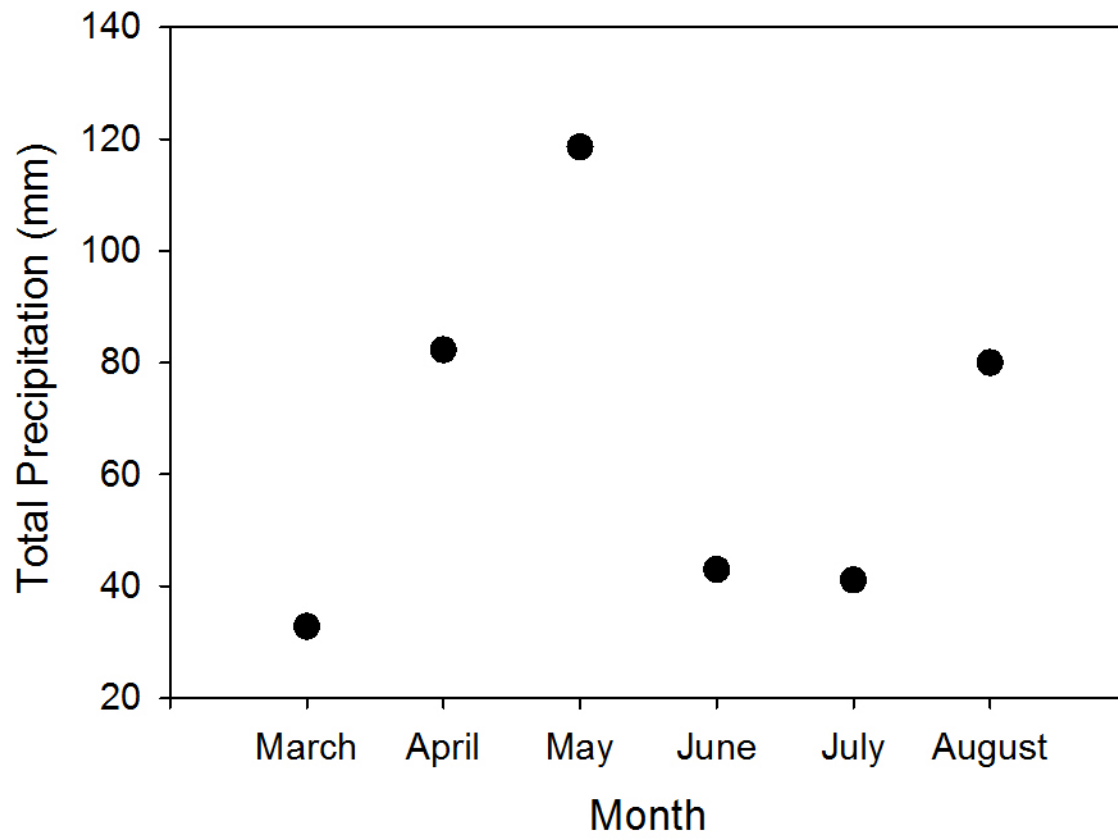
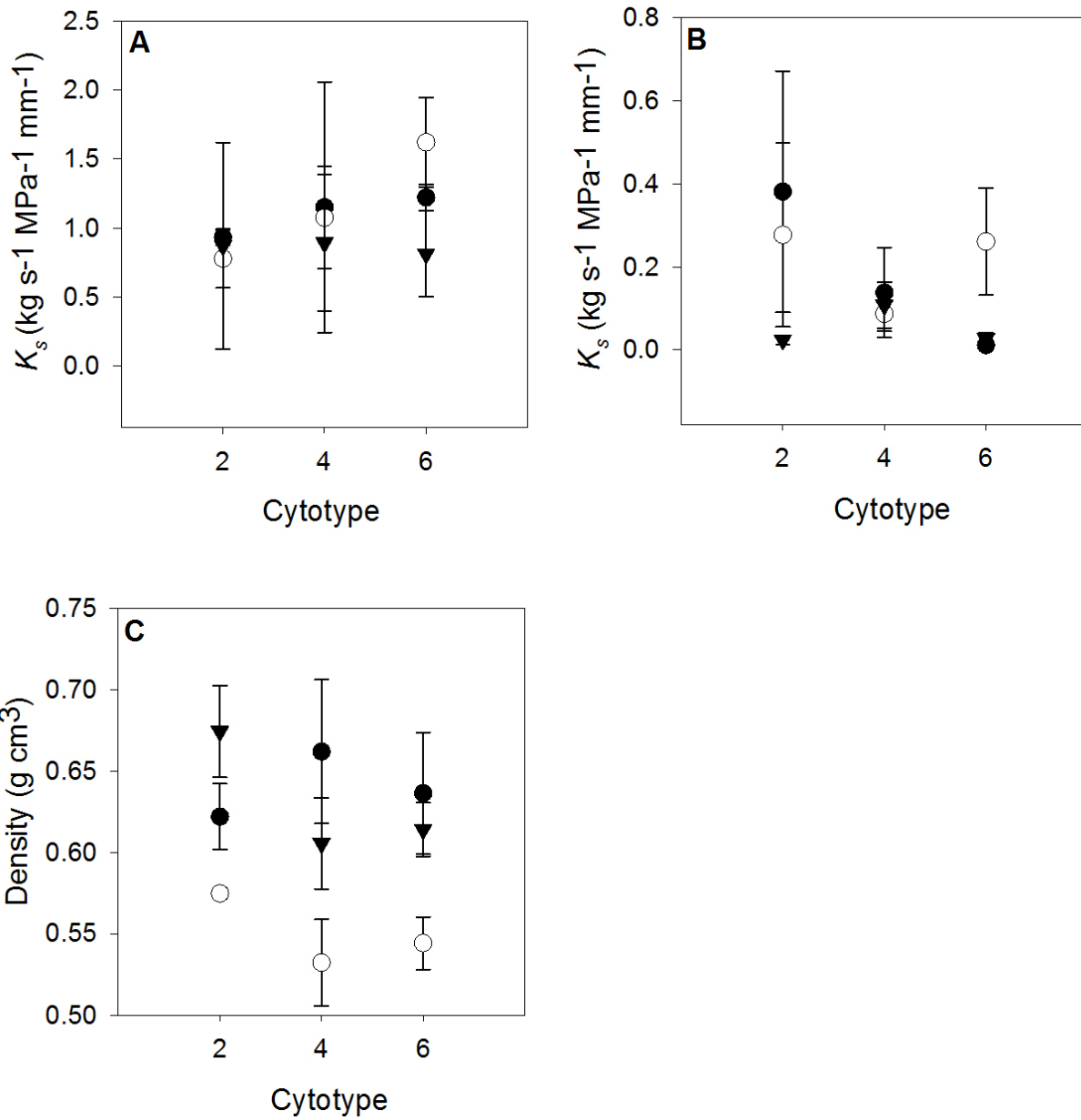
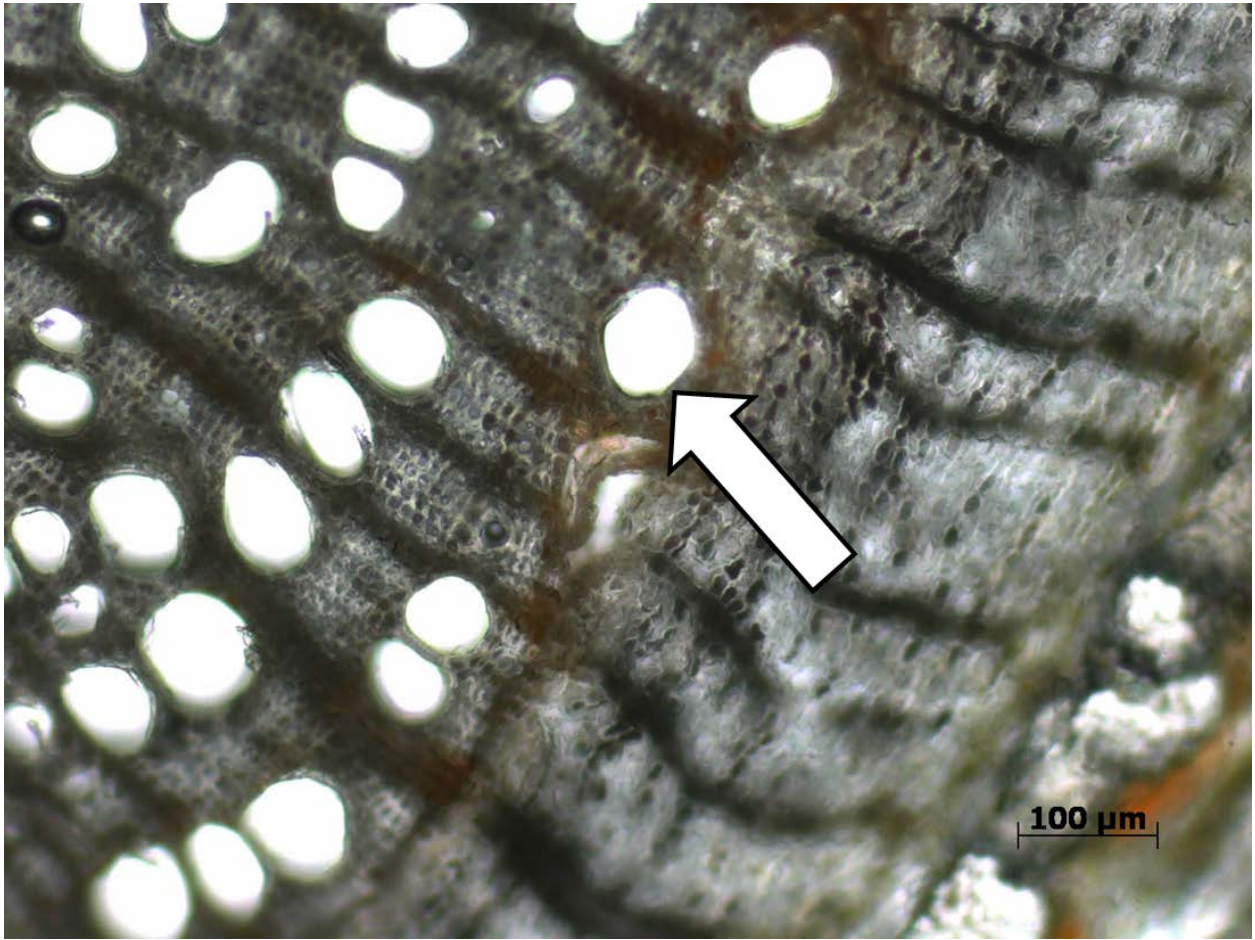


Fig. 3



**Fig. 4**



**Fig. 5**

*The following chapter stems from the University of Kansas and National Science Foundation Integrative Graduate Education and Research Traineeship (IGERT) program titled C-CHANGE: Climate Change, Humans, and Nature in the Global Environment. This program provided interdisciplinary training in climate change science. C-CHANGE trainees were afforded the opportunity to undertake a policy internship to better understand the relationship between climate change science and public policy. This chapter provides an overview of the major project undertaken during the authors' internship at the Office of Science and Technology Policy in the Executive Office of the President of the U.S. during the spring of 2014.*

## **Chapter 4: Preventing the Decline of Pollinators in the United States: An Overview of Executive-Level Policy**

### **Introduction**

Pollinating bees, birds, butterflies, bats, and other animals ensure that flowering plants continue to exist, providing a vital but often unnoticed service. But many pollinators' populations are declining rapidly across the globe. Pollinators play a critical role in maintaining diverse ecological systems and support the production of many important agricultural species. It is estimated that pollinators are responsible for providing one in every three bites of food. Pollination from honey bees alone was estimated to provide \$15 billion in crop revenue during 2009 in the United States (Calderone, 2012). All pollinators were estimated to provide \$29 billion to the U.S. economy in 2009 (Calderone, 2012). Pollinators also pollinate an estimated three-fourths of the world's flowering plants (National-Research-Council, 2007). The loss of pollinating services could significantly affect agricultural production, the U.S. agricultural



economy, the maintenance of diverse natural systems and the services that they provide (e.g., carbon sequestration, water filtering).

On June 20<sup>th</sup>, 2014, President of the U.S. (POTUS), Barack Obama, released a Presidential Memorandum calling for strategies to be undertaken by the federal government to reverse pollinator losses (C.F.R. Doc. 2014-14946; White House 2014). The memorandum established the “pollinator health task force,” a group of representatives from Federal agencies and departments, and chaired by the Secretary of Agriculture and Administrator of the Environmental Protection Agency (EPA). The group was tasked by POTUS with creating a national pollinator health strategy that included specific goals, milestones, and metrics within 180 days of the release of the presidential memorandum. On May 19<sup>th</sup>, 2015, the pollinator health task force released the, “National Strategy to Promote the Health of Honey Bees and other Pollinators,” as well as the, “Pollinator Research Action Plan” (White-House, 2015a, 2015b).

These two documents together form strategies that expand or add to actions already undertaken across many Federal departments and agencies to increase pollinator health. It addresses many factors that affect the health of pollinators, including declining habitat for foraging, pesticides, diseases, and parasitism. These strategies also call for an “all hands on deck” approach asking for Federal departments and agencies, private citizens, and corporate businesses to *all* play a part to increase the health of pollinators in the U.S.

The White House National Strategy to Promote the Health of Honey Bees and other Pollinators (2015a) outlines three overarching goals:

1. Reduce honey bee colony collapse disorder during winter (overwintering mortality) to no more than 15% within 10 years.
2. Increase the Eastern population of the monarch butterfly to 225 million butterflies occupying an area of approximately 15 acres (6 hectares) in the overwintering grounds in Mexico, through domestic/international actions and public-private partnerships, by 2020.
3. Restore or enhance 7 million acres of land for pollinators over the next 5 years through Federal actions and public-private partnerships.

This paper will address the following interconnected issues surrounding the recent efforts of the U.S. federal government to restore declining pollinator populations and increase pollinator health: 1) the history of pollinator decline in the U.S., 2) the recent decline of managed honey bee colonies, 3) pesticide use and the politics surrounding this issue, and 4) an analysis of the strategies, and their potential effectiveness, proposed by the U.S. Pollinator Health Task Force.

### **History of Pollinator Decline in the United States**

While all native pollinators are considered to be important for plant life to flourish in the U.S., the non-native honey bee (*Apis mellifera* L.) is considered to be the *most* important pollinator due to its importance in agriculture. Honey bees are a commercialized species in the U.S. that are managed throughout the U.S. by beekeepers. Beekeepers transport honey bees to various farms and orchards in need of pollinating services. The hives are literally loaded and transported to their destination/s on large trucks (Fig. 1). Honey bees may be transported as far as 2,800 miles for their pollination services (Ahn, Xie, Riddle, Pettis, & Huang, 2012). Pollinating services from

honey bees in the U.S. are estimated to provide \$15 billion in agricultural crop sales per year (Calderone, 2012). But where and when did honey bees come from? And when did honey bee populations begin to decline in the U.S.?

The native range of honey bees extends from the southern regions of Scandinavia to Central Asia and throughout Africa (Seeley, 1985; Sheppard & Meixner, 2003). But the honey bee can now be found in nearly every habitable part of the world. Most of the honey bees' range expansion is due to deliberate human transport (Crane, 1999). Honey bees' most important contribution presently is the pollination service that they provide, but this has not always been considered their most important contribution to society. The impetus to transport honey bees began as a need for honey (Ransome, 1937). Honey was the only form of sweetener readily available to most peoples until methods were developed to refine sugar. While we do not consider honey the *most* important contribution of honey bees to society today, honey remains an important global commodity. An estimated 1.07 metric tons of honey was produced globally during 2007 alone (FAO, 2009).

The descendants of medieval Europe were primarily responsible for spreading the art of keeping bees, as well as bees themselves, to the new world (Ransome, 1937). In North America, honey bees were unknown to Native Americans. Early American writers reported that honey bees were known as, "white man's flies" (Crane, 1999; Engel, Hinojosa-Diaz, & Rasnitsyn, 2009). When honey bee colonies were spotted, Native Americans would note that white settlers were not far behind. The first bee hive was introduced to the United States in 1622 in Virginia, and there are reports of bee colonies found throughout Massachusetts by 1639 (Turpin, 1999). Once honey bees were introduced, they migrated and established colonies throughout the U.S.

Honey bees establish new colonies by swarming. Swarms are able to migrate several miles (Crane, 2004). Honey bees reportedly swarmed to Kansas by 1843 (Turpin, 1999). The rest of their natural westward migration was slow causing humans to aid their full colonization in the U.S. Mormons brought honey bees to Utah in 1848, which allowed swarms to reach Nevada by 1852 (Turpin, 1999). A botanist, C.A. Shelton, aided colonization of bees into the Pacific Coast by using a sea route along the East Coast and crossing Panama before using the Pacific Ocean for the final part of the journey (Oertel, 1980). It should be noted that transportation of bees would have not been easy in the past, and this should highlight the importance of honey bees to early U.S. settlers. The sea voyage from England to the east coast of the U.S. lasted six to eight weeks, a time frame that would have not been easy to keep confined bees alive. In fact, many of the attempts to transport bees to the new world were not successful as earlier writings indicate (Turpin, 1999).

Honey-producing bee colonies in the U.S. are declining (USDA-NASS, 1976, 2015). The number of honey-producing colonies is counted by the United States Department of Agriculture (USDA) National Agricultural Statistics Service (NASS). NASS has been tabulating the number of honey-producing colonies since 1943. But NASS's honey bee census has been scrutinized. NASS did not count the number of honey-producing colonies in the U.S. during 1982-1985 (vanEngelsdorp & Meixner, 2010). During these years, the number of honey-producing colonies in the U.S. was estimated by the Agricultural Stabilization and Conservation Service (Rodenberg, 1992). The annual census developed by NASS was developed to count colonies that produce honey per state (USDA-NASS, 1976). This means that if a colony is transported and produces honey in another state, it is counted multiple times. This could inflate the

number of honey-producing colonies counted in NASS's annual census (NRC, 2007). Additionally, in 1985 NASS changed the methods of its census to no longer count beekeepers hives if they managed five or fewer hives (Rodenberg, 1992). Because of this switch in the census method, there is a steep decline in the number of honey-producing colonies between 1985 and 1986 (Fig. 2). Even considering the issues of NASS's bee colony census, it is still remarkable that the number of honey-producing colonies in the U.S. has dropped 61% from 5.9 million managed hives in 1947 to 2.3 million in 2008 (Fig. 2).

Honey bee colonies have been declining since the 1940's in the U.S., but recent declines have been severe (vanEngelsdorp & Meixner, 2010). A survey conducted by the Apiary Inspectors of America estimated that between 651,000 and 875,000 of the nation's 2.4 million honey bee colonies were lost during the winter of 2006-2007 (vanEngelsdorp, Underwood, CaronD, & Hayes Jr., 2007). Some beekeepers reported up to 90% loss of their honey-producing colonies (Underwood & vanEngelsdorp, 2007). Many of these colony losses were caused by known bee threats (e.g., pesticides, mites, diseases), but 25% were categorized as an *unexplained* rapid loss of the colony's adult bee population (vanEngelsdorp et al., 2007). The unexplained loss of adult bees of a hive has been given the name, "colony collapse disorder," or CCD (Underwood & vanEngelsdorp, 2007). Collapsed colonies have only the queen left with a small number of young worker bees (Stokstad, 2007). There are plenty of food stores left in the hives of collapsed colonies, and few dead bees are found. It is as if the colony vanished into thin air.

Many different factors likely provide the key to explain the decline of honey-producing colonies in the U.S. No *one* factor can explain the loss of colonies. Many

factors that are likely responsible for the decline of honey-producing colonies in the U.S. can occur simultaneously and probably influence each other. The next section of this chapter aims to discuss the most likely factors responsible for the decline of honey-producing honey bee colonies in the U.S.

### **Honey Bee Colony Collapse Disorder and Decline**

Honey bee colonies often die over winter in the U.S. But recent overwintering losses since 2006 have averaged 31% (Fig. 3), which far exceeds the economically sustainable overwintering loss rate of 15-17% (Steinhauser et al., 2015). While the loss of honey bee colonies is typically counted over winter months because this is when most colonies die, losses can be higher when these numbers are coupled with losses during other times of the year. For example, an estimated 27.4% of bee colonies were lost during the summer of 2014 and 14.7% during the 2014-2015 winter for a total annual loss of 42.1% (Steinhauser et al., 2015). Because of these steep annual losses, meeting the demand for pollination services in agriculture is difficult. Many beekeepers split their hives into two by providing a queen to the “new” hive as well as supplemental food. This is largely done to meet demand for pollination services to almond orchards in California. Approximately 60-75% of all U.S. commercial honey bee colonies are required to fulfill pollination contracts with almond orchards during spring (Bond, Plattner, & Hunt, 2014). It is imperative that we understand *why* honey bee colonies are collapsing to maintain many of our important agricultural crops as well as diverse ecosystems.

It is nearly impossible to pinpoint a reason for the death of a honey bee colony. Colonies must be consistently monitored yearlong to provide clues as to why they are

lost. A considerable amount of time may pass before a beekeeper notices that a colony has died, and by this time it is difficult to discern the cause of death. There are some longitudinal studies that monitor honey bee hives consistently over several years. The most well-known of these studies is likely the German Honey Bee Monitoring Program (Genersch et al., 2010). Researchers monitor approximately 1200 honey bee colonies in this program. The program has provided valuable data that have allowed researchers to determine some relationships between risk factors and the death of honey bee colonies. There are multiple factors including diseases, parasites, predators, pests, and climate change that can adversely affect honey bee colonies. This overview will focus on the most significant factors known to adversely affect honey bee colonies, specifically those factors thought to play some role in CCD.

### ***Varroa destructor***

*Varroa destructor* (varroa mite) is a mite that has contributed to the loss of honey bee colonies in the U.S. (Fig. 3). The introduction of varroa is recent. A 1980-1982 survey of samples from 4,400 apiaries in the U.S. found no evidence of mites in the U.S. or Canada (Shimanuki, Knox, Delfinado, & Lima, 1983). The mite was first reported in the U.S. in 1987 (NRC, 2007) and has since spread across the nation. The parasite is not native to the U.S. and it is unknown how it arrived in the country (NRC, 2007).

The female parasites feed and reproduce on workers and male pupae (NRC, 2007). Varroa mites feed on the hemolymph (similar to blood in humans) of honey bees. Pupae that have been parasitized exhibit a number of adverse conditions as adults. These adverse conditions include weight loss (De Jong, De Jong, & Goncalves, 1982),

wing deformations (Aratanakul & Burgett, 1975), and reduced longevity (De Jong & De Jong, 1983). The colony, if left untreated, can die in as fast as 6 months after a varroa mite infestation (De Jong, 1990). An infestation of varroa mites can also indirectly affect the queen of the hive. The queen has lower reproductive output during a varroa mite infestation because males that have been parasitized show smaller seminal vesicles, lower sperm production, and decreased flight performance during mating (Bubalo, Pechhacker, Licek, Kezic, & Sulimanovic, 2005; Duay, De Jong, & Engels, 2002).

Colonies that have been infected with varroa mites also show less resistance to diseases. Varroa mites serve as vectors for many diseases, and bees are less resistant to diseases when parasitized by varroa mites because the parasitism results in a weakened immune system (Bailey & Ball, 1991). Some of the diseases spread by varroa mites can be extremely contagious and deadly. For example, in 2012, varroa mite infestations of honey bee colonies led to a global spread of a very deadly strain of a disease called, “deformed wing virus” (Martin et al., 2012).

The negative impact of *V. destructor* on individual colonies is quite clear (Rosenkranz, Aumeier, & Ziegelmann, 2010). But the *overall* impact of the varroa mite to honey bee colonies is difficult to discern. As mentioned previously, many beekeepers do not closely or consistently monitor their hives, and this lack of observation makes it difficult to determine why a hive is lost. In the U.S., following the introduction of the varroa mite in 1987, 26% of managed bee colonies were lost (USDA-NASS, 1988). This loss is often associated with the introduction of the varroa mite (NRC, 2007). But in years following 1987 the rate of honey bee colony decline (1.09% per year) is barely different from the rate of decline during 1947-1987 (1.11% per year) before the mite was introduced (vanEngelsdorp & Meixner, 2010). It has been suggested by some



researchers that the steep rate of decline following the introduction of the varroa mite into the U.S. was likely due to the fallout of smaller apiaries. Forty percent of apiaries went out of business during 1987-2002 (Daberkow, Korb, & Hoff, 2009). These apiaries only managed approximately 10% of honey bee colonies in the U.S. Because of their smaller operations, these apiaries probably had difficulty in managing varroa mite infestations. At the same time that many of these smaller apiaries went out of business, there is a 66% increase in the number of managed bee colonies by the remaining apiaries (Daberkow et al., 2009). There are some examples where stronger evidence exists to support the loss of colonies due to the introduction of varroa mites. For example, in the winter of 1995-1996, Pennsylvania beekeepers reported an average honey bee colony loss of 53% (Finley, Camazine, & Frazier, 1996). However, Pennsylvania beekeepers that treated their hives with Apistan (varroa mite control) during the fall of 1995 reported an average honey bee colony loss of 26% (Finley et al., 1996). This data suggest the varroa mite was largely responsible for the increase in the rate of decline of honey bee colonies in Pennsylvania during 1995-1996.

Varroa mites also have affected the honey bee pollinating services market. Controlling varroa mite infestation is costly and these costs are often passed on to producers renting bees for their pollination services (Burgett, 2004). Additionally, beekeepers may have to buy new hives to replace those lost to varroa mites. These hives are often smaller and less productive (NRC, 2007). Varroa mites are considered to be one of the most significant causes of the loss of honey bee colonies over winter (vanEngelsdorp, Hayes Jr., Underwood, & Pettis, 2008).

## ***Nosema spp.* Infections**

The *Nosema* genus contains microsporidians that have been recently reclassified as fungi that adversely affect honey bees. Some regard *Nosema* as the most economically important disease for beekeepers, as well as the most widespread and common honey bee disease (Fries, 2010). *Nosema* reproduces through the release of small spores, which are resistant to extreme desiccation and freezing (Fenoy, Rueda, Higes, Martin-Hernandez, & del Aguila, 2009). Spores can remain viable in a hive for over a year. A honey bee is infected with *Nosema* when it accidentally eats a spore (Traver & Fell). The spore then settles in the bees' mid-gut where the *Nosema* will live as a parasite in epithelial cells (Fries, 1993). *Nosema* utilizes the contents of these epithelial cells as food. These epithelial cells aid in the digestion of pollen for the bee; therefore, during an infection of *Nosema*, a bee is unable to properly digest and absorb its' food weakening the bee. The *Nosema* parasite multiplies in these cells producing more spores. When enough spores have been made, the cell burst releasing more spores into the bees' mid-gut and starting the cycle over. A heavily infected bee can contain 30-50 million spores (Fries, 1993). These spores also pass into the rectum of the bee where they are eventually voided in the bees' feces (Fries, 1993). Worker bees that remove feces from the hive are exposed to *Nosema* spores and become infected. When a bee is heavily infected with *Nosema*, it becomes too weak to fly back to the hive while foraging for pollen and nectar. When enough worker bees do not return to the hive, the colony will collapse.

## **American Foulbrood Disease**

American foulbrood disease (AFB) is caused by the species, *Paenibacillus larvae* (a bacteria), and is considered one of the most deleterious bee diseases (Genersch, 2010). The reason that AFB is so dangerous to honey bee colonies, particularly managed honey bee colonies, is that it is highly virulent and spreads easily (Genersch, 2010). In the 1940's, AFB caused the loss of hundreds of thousands of colonies globally (Barrett, 1955). In the latter half of the 20<sup>th</sup> century, the incidence of AFB was reduced due to the formation of antibiotics and state policies requiring the burning of hives that showed symptoms of AFB. American foulbrood disease has had the largest impact on the beekeeping industry. Even with effective management of AFB, the pathogen has caused annual economic losses up to \$5 million (Eischen, Graham, & Cox, 2005).

American foulbrood disease will likely remain a threat to honey bee colonies. While many countries allow for the prophylactic use of antibiotics, like the U.S., many countries still do not. This is because antibiotics are costly and do not kill spores of the pathogen. Additionally, antibiotic resistance of *P. larvae* was observed in the U.S. in 1994 and is now becoming widespread (Cox, Eischen, & Graham, 2005; Shimanuki & Knox, 1994).

## **Pesticides**

While diseases have certainly played a role in the decline of managed honey bee colonies in the U.S., pesticides have as well. The use of pesticides as a form of pest management in agriculture has made the regulation of these chemicals to protect colonies of honey bees a contentious issue. Pesticides often spread outside of the area that they are intended to target, and because honey bees forage nearly anywhere

pollen and nectar sources are available, they are often exposed to these toxic chemicals. Policies of the past primarily focus on the direct exposure of honey bees to pesticides.

Many countries have national policies that protect pollinators from direct exposure of pesticides. In the U.S., this policy is the Federal Insecticide Fungicide and Rodenticide Act ("Federal Insecticide, Fungicide, and Rodenticide Act," 1972). This legal framework gives the EPA authority to regulate the sale and use of pesticides in the U.S. The EPA generally defers to the state to enforce rules and regulations of FIFRA, but will override state authority if the state is not meeting its obligations. Under current regulations by FIFRA, pesticide manufacturers are required to label chemicals if they could adversely affect pollinators, including honey bees. The labels are mandated to read such statements as, "do not apply this product while bees are foraging" (Bradbury, 2013). Beekeepers have found this type of language to be unenforceable and unrealistic (Beyond-Pesticides). It has been suggested that people who use pesticides are often noncompliant with pesticide labels (Beyond-Pesticides). If true, this noncompliance places responsibility on beekeepers to report violations of FIFRA.

The majority of the concern of direct poisoning is with a group of pesticides known as neonicotinoids. Many seeds that farmers sow are dressed with neonicotinoids. The plant absorbs the pesticide as it germinates which kills insect pests (Sur & Stork, 2003). Plants that are sold commercially at retail stores like Home Depot and Wal-Mart also contain neonicotinoids (Friends-Of-Earth, 2013). Neonicotinoids also may be present in the pollen or nectar of the plant (Schmuck, Schoning, Stork, & Schramel, 2001). Honeybees can be exposed to neonicotinoids through ingesting pollen or nectar of treated plants. Honey bees that are exposed to neonicotinoids can

experience problems with flying and navigation, have reduced taste sensitivity, and they also learn tasks slower (Blacquiere, Smagghe, van Gestel, & Mommaerts, 2012). These effects of neonicotinoids all affect the success of honey bees' ability to forage. Exposure to neonicotinoids also may make honey bees more susceptible to parasites and pathogens (Alaux et al., 2010), which are known to lead to the loss of colonies. In some cases, the neonicotinoid dressing on seeds has become loosened. This creates a toxic dust that can be blown into non-targeted areas. In the spring of 2008 in southern Germany, more than 11,000 honey bees were damaged and/or killed by neonicotinoid pesticides that had become loosened from corn seeds (vanEngelsdorp & Meixner, 2010). This particular neonicotinoid was Clothianidin. Because of the incident in 2008, Clothianidin can no longer be sold or used in Germany.

To determine the lethal level of pesticide exposure, studies are undertaken to quantify the LD50 (median lethal dose). The LD50 represents the pesticide exposure that kills 50% of a population of honey bees (Blacquiere et al., 2012). Based on these findings, pesticides can then be categorized based on their risk to honey bees. If honey bees are exposed to pesticides under its LD50, it is said that the honey bees are exposed to a sub-lethal dose. Sub-lethal pesticide exposures are not unlawful under FIFRA.

Data are just now emerging to better understand the types of pesticides that hives are exposed to, and how much pesticide exposure is experienced by the hive. In the U.S., a striking number of pollen and beeswax samples were found to contain a considerable number of pesticides (Frazier, Mullin, Frazier, & Ashcraft, 2008). In a survey conducted by the German Honey Bee Monitoring Program, an investigation of

the bee bread of hives revealed only 24% of samples were free of pesticides (vanEngelsdorp & Meixner, 2010).

There are a number of public policies that have been proposed recently due to the growing evidence that pesticides adversely affect honey bees and other pollinators. Most recently, a Federal level policy bill was introduced to congress that would suspend the use of all neonicotinoid pesticides. This suspension would last until a scientific review and field study are undertaken by the EPA to demonstrate that neonicotinoid pesticides cause no harm to pollinators. This bill, the "Saving America's Pollinators Act," was first introduced in 2013 ("Saving America's Pollinators Act," 2013). The bill failed to pass with the 113<sup>th</sup> congress, but was reintroduced to the 114<sup>th</sup> congress. If passed, this would be the first national level policy to limit the use of neonicotinoid pesticides to protect pollinators. There have been similar policies proposed in Eugene, OR ("Save Oregon's Pollinators Act," 2014), Spokane, WA (Geranios, 2014), Seattle, WA ("Resolution," 2014), and Skagway, AK ("Title 8 Health and Safety," 2014).

Policies that place moratoriums on pesticides are controversial. A host of interest groups with powerful D.C. lobbying arms, including farm organizations, seed producers, and pesticide companies such as Monsanto, Bayer, and Syngenta, are fearful of bans places on pesticides. As discussions concerning the harmful of effects of pesticides to honey bees are becoming more prevalent in government, agribusinesses have increased their public relations efforts to show that they are "honey bee friendly." Monsanto hosted a "Honey Bee Health Summit," in St. Louis in 2013, and Bayer has opened "Bee Care Centers" (Barber, 2014). It is likely no coincidence that Bayer opened the first of its' Bee Care Centers in Germany (Barber, 2014) when the European Union considered its ban on neonicotinoids. In 2014, Bayer started a second lobbying

team, Gephardt Group Government, to defend its pesticides (Tau, 2014). Bayer has also released public statements against Maryland's efforts to pass a bill that would require new labeling for neonicotinoid pesticides stating that they are known to kill bees (Lessner, 2015). Becky Langer, a project manager at the Bee Health North America Center for Bayer CropScience, said the bill was "unnecessary." Ms. Langer continued, "Bee numbers are on the rise...locally, bee colonies have risen by 45% since 1960 according to U.N. reports" (Lessner, 2015). While the number of managed honey bee colonies has risen in the U.S., the rate of decline of colonies has not (vanEngelsdorp & Meixner, 2010). But there is disparate evidence linking sub-lethal pesticide exposure (what pollinators are mostly exposed to) to CCD. A study published by Harvard's School of Public Health found a link between CCD and exposure to sub-lethal levels of neonicotinoids (Lu, Warchol, & Callahan, 2014), but the biological mechanisms have not been uncovered. This lack of evidence for an association between the exposure of honey bees to sub-lethal levels of neonicotinoids and CCD will likely make it more difficult to pass federal level policies like the "Saving America's Pollinators Act."

### **Will the National Strategy to Promote the Health of Pollinators Prove Effective?**

The National Strategy to Promote the Health of Honey Bees and other Pollinators, despite its rather bureaucratic title, is pretty straightforward: The government will provide more money for bee habitat and research, and the EPA will re-evaluate the use of neonicotinoids. There is an increase of \$48 million for the Department of Interior (DOI), EPA, and USDA specifically targeted to increase pollinator health (Table 1). In this section, I will detail one of the largest strategies outlined in the White House's plan – the increase or enhancement of pollinator habitat across the Nation.

## **Strategies to Increase and Improve Pollinator Habitat**

The White House's plan to increase pollinator health emphasizes the need to increase and improve pollinator habitat. The strategy calls for Federal agencies and departments to modify existing guidance documents that influence the landscaping and facilities management to encourage the creation of native pollinator habitat. This particular strategy shouldn't modify an agency's mission or alter its' budget. The national strategy also calls for Federal agencies and departments to implement pollinator habitat action on managed lands. This strategy makes sense when considering that the Federal government is the largest land manager in the Nation.

The USDA Farm Service Agency (FSA) will play a large role in increasing and improving pollinator habitat on public and private lands. The USDA FSA currently administers the Conservation Reserve Program (CRP), which creates long-term contracts with farmers across the U.S. to set aside environmentally sensitive land from agricultural production. The creation of CRP lands was created to re-establish valuable land cover to help improve water quality, prevent soil erosion, and reduce loss of wildlife habitat. The CRP has been successful with 24 million acres currently enrolled. The FSA currently has 124,000 acres enrolled in a special CRP category to protect pollinators (CP-42). Under this category, a variety of plant species that provide foraging for native pollinators year-round are planted on CRP lands. As part of the national strategy, FSA has dedicated an additional 76,000 acres under the CP-42 CRP category. The FSA is working with the Natural Resources Conservation Service (NRCS) to develop a more-affordable pollinator-friendly seed mix that can be used on CRP lands. In 2014, FSA



began an \$8 million honey bee incentive program to increase its CP-42 CRP lands in five states (MI, MN, ND, SD, WI). These five states are collectively home to more than 65% of summer honey bee hives. The NRCS also provided \$3.2 million in assistance to CRP participants in these same five states to implement conservation practices that would provide diverse plant forage. The FSA and NRCS are partnering with the U.S. Geological Survey to monitor the effectiveness of these joint efforts in these five key states.

While the FSA and NRCS play a significant role in improving or increasing pollinator habitat under the national strategy, the Fish and Wildlife Service (FWS) will also play a large role. The FWS is working with Canada and Mexico on a Tri-national Monarch Butterfly Conservation Plan. Monarch butterfly population numbers reached a record low in 2013-2014 (Robbins, 2013). The FWS has plans to restore or enhance more than 200,000 acres of monarch habitat on public and private lands. The FWS has allocated \$2 million in priority projects for increasing monarch habitat. One of these priority projects focuses on restoring habitat along the I-35 corridor that runs from Texas to Minnesota that provides breeding habitat in the monarch's key migration corridor. The Federal Highway Administration (FHWA) also has contracted to create materials to support best management practices along on highway right of ways. These materials will provide transportation agencies with the tools necessary to promote increased pollinator habitat along roadways. The FWS and US Department of Transportation (DOT) will work with state DOT's to determine best practices to improve monarch habitat along the I-35 right of way.

## **Will these Strategies be Effective?**

It is difficult to determine if the proposed national strategies will be effective in increasing pollinator health. But these same strategies have been implemented for several years at the state level or in other countries. We can look to these programs that have already been operating to learn about the effectiveness of these strategies as well as the best way to implement them.

While many states have pollination protection plans, most do not include strategies to increase pollinator habitat. The majority of these plans focus on the protection of honey bee hives from pesticides, policies regarding where hives may be located, and notification of where hives are located. Pollinator plans that do include strategies to increase or improve pollinator habitat may be non-regulatory. For example, North Dakota's pollinator plan list strategies for increasing pollinator habitat, but the plan is fully non-regulatory (State-of-North-Dakota, 2014).

The majority of increases in pollinator habitat in the U.S. are due to the FSA's CRP pollinator initiative. But many farmers may not be taking advantage of this initiative. The State of Tennessee recently released a public statement reminding landowners and agricultural producers that assistance was available to create and enhance pollinator habitat (Tennessee-Wildlife-Resources-Agency, 2015). High commodity crop prices have led to a decrease in CRP enrollment, forcing farmers to put even marginal acres back into production (Stubbs, 2014). The incentive to participate in CRP's pollinator initiative practices cannot compete with the revenue of high commodity crops when only covering 50% of the costs of planting the pollinator habitat and paying only a one-time \$150 per acre payment for enrollment (Baylis & Coppess, 2014).

The enhancement of pollinator habitat on farmlands increases bee abundance and diversity. There are a number of studies from around the world that show a positive correlation between the availability of diverse floral resources and bee abundance and diversity on farmlands (Ostler & Harper, 1978; Steffan-Dewenter & Tscharntke, 2001; Wratten, Gillespie, Decourtye, Mader, & Desneux, 2012). Studies have also shown that increasing the diversity of floral resources in peripheral areas around crops (e.g., crop buffer strips, or green lanes) can increase the abundance and diversity of honey bees, bumble bees, and butterflies (Carreck & Williams, 1997; Croxton, Hann, Greatorex-Davies, & Sparks, 2005; Lagerlof, Stark, & Svensson, 1992). The continuing success of pollinator habitat on farmlands depends on their year-to-year management (Haaland, Naisbit, & Bersier, 2011). To my knowledge, there are no studies that have been conducted to investigate the effectiveness of the FSA's CRP pollinator initiative. But it seems likely from other studies that the increase in pollinator habitat that CRP lands will provide as part of the White House's National strategy will increase pollinator abundance and diversity.

Planting and maintaining native wildflowers on roadside right of ways can provide pollinators with valuable habitat, acting as linear refuges or connecting remnant habitat patches. Bees were twice as abundant on roadsides with native flowering plants relative to those dominated by non-native grasses and flowers on roadside in Kansas (Hopwood, 2013). In Iowa, the restoration of native flowering prairie plants increased the abundance of butterflies five-fold as compared to roadsides with non-native grasses (Ries, Debinski, & Wieland, 2001). Hopwood *et al.* (2010) also found sizable populations of bumblebees foraging on restored prairie roadside vegetation in Iowa.

Roadside vegetation has been shown as an important source of food and shelter for pollinators (Quin, Aviron, Dover, & Burel, 2004; Schaffers, Raemakers, & Sykora, 2012).

There is some concern that roadside management (e.g., mowing, herbicides) could kill enough pollinators that it would offset the benefit of the pollinator habitat that is created. This is true if the pollinator habitat created is not managed properly. Mowing can cause the direct mortality of eggs and larval stages of pollinators since they cannot avoid a mower. Mowing has been shown to destroy entire bumble bee colonies (Hatfield, Jepsen, Mader, Black, & Shepherd, 2012). Mowing may also remove flower stalks that pollinators feed on. If mowing is avoided during the growing season, and is done infrequently, it can lead to increases in floral diversity and subsequent increases in pollinator abundance and diversity (Forrester, Leopold, & Hafner, 2005). Other forms of roadside management are of concern as well including the use of herbicides, fire, and grazing. These management techniques can all adversely impact pollinator populations. But if these management techniques are done properly and tailored to a specific site, they can be beneficial to pollinator communities (Ries et al., 2001). In general, since less management is required for roadsides planted with native species that are attractive to pollinators, vegetation management costs decrease. The Florida Department of Transportation estimates that vegetation management costs can be reduced by 30% through the implementation of pollinator friendly roadside practices (Harrison, 2014). Given the evidence that pollinator abundance and diversity increase when states increase or enhance pollinator habitat along roadside right of ways, it seems likely that the White House's strategy to increase habitat along the I-35 corridor would produce similar results.

## Conclusion

The White House's National Strategy to Promote the Health of Honey Bees and other Pollinators seems to have considered the vast amount of scientific evidence in forming its policies. But whether or not these strategies are effective will largely depend on the cooperation between Federal Departments and Agencies, State Departments and Agencies, and private citizens (especially farmers). Furthermore, the pollinator task force should make sure to work with states that have already implemented plans to increase pollinator habitat and health, particularly those with integrated roadside vegetation management plans. The success of this national strategy also will depend on the increase in funding that it seeks for selected agencies (Table 1). Additionally, policies that are being pushed through federal and state-level legislatures will face opposition from well-funded lobbying groups supporting agribusinesses. Pollinators, especially honey bees, are declining worldwide. Because of the valuable services that they provide, it is essential that we protect them. While I have not fully detailed all of the strategies proposed by the Pollinator Health Task Force, the most important strategy (to increase or enhance pollinator habitat) seems to be well supported by scientific evidence.

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**Table 1** Pollinator-specific proposed Fiscal year 2016 budget additions relative to the Enacted Fiscal year 2015 budget for DOI, EPA, and USDA (\$ Million). This table is published as part of the White House’s National Strategy to Promote the Health of Honey Bees and other Pollinators (White-House, 2015a).

Agency	Program	FY 2015 Enacted	FY 2016 Budget	Change from 15 Enacted to 16 Budget
DOI	U.S. Geological Survey (USGS)	0.00	1.56	1.56
	DOI Total	0.00	1.56	1.56
EPA	Office of Pesticide Programs	0.00	1.50	1.50
	State and Tribal Assistance Grants	0.00	0.50	0.50
	EPA Total	0.00	2.00	2.00
USDA	National Agricultural Statistics Service (NASS)	2.40	2.90	0.50
	Agricultural Research Service (ARS)	14.19	21.19	7.00
	National Institute of Food and Agriculture (NIFA)	9.66	31.50	21.84
	Economic Research Service (ERS)	0.28	0.28	0.00
	Land Management Programs			
	Farm Service Agency (FSA) Conservation Reserve Program (CRP)	18.00	18.06	0.06
	Natural Resource Conservation Service (NRCS) Environmental Quality Incentives Program (EQIP)	3.00	4.00	1.00
	Animal and Plant Health Inspection Service (APHIS)	1.00	1.00	0.00
	USDA Total	48.52	78.93	30.41
Agencies Total		48.53	82.49	33.96

## Figure Legends

**Figure 1** Loading a semi-trailer truck with honey bee hives in Garfield County, Colorado. This picture was taken from the Post Independent news, and accessed at [www.postindependent.com](http://www.postindependent.com) on July 3<sup>rd</sup>, 2015.

**Figure 2** The numbers of managed honey-producing honey bee colonies in the United States during 1944-2008. Estimates are provided by the United States Department of Agriculture National Agricultural Statistics Service (USDA-NASS) for every year during this period (solid circles) except for 1982-2005, during which estimates are provided by the USDA Agricultural Stabilization and Conservation Service (open squares). Estimates of the total number of colonies by the AG census (open triangles) are also provided. This figure was published by vanEngelsdorp and Meixner (2010).

**Figure 3** Varroa mites (brown mites) on a honey bee pupa (white pupae). This picture was taken from [agibioresearch.msu.edu](http://agibioresearch.msu.edu) and accessed on July 7<sup>th</sup>, 2015.





Fig. 1

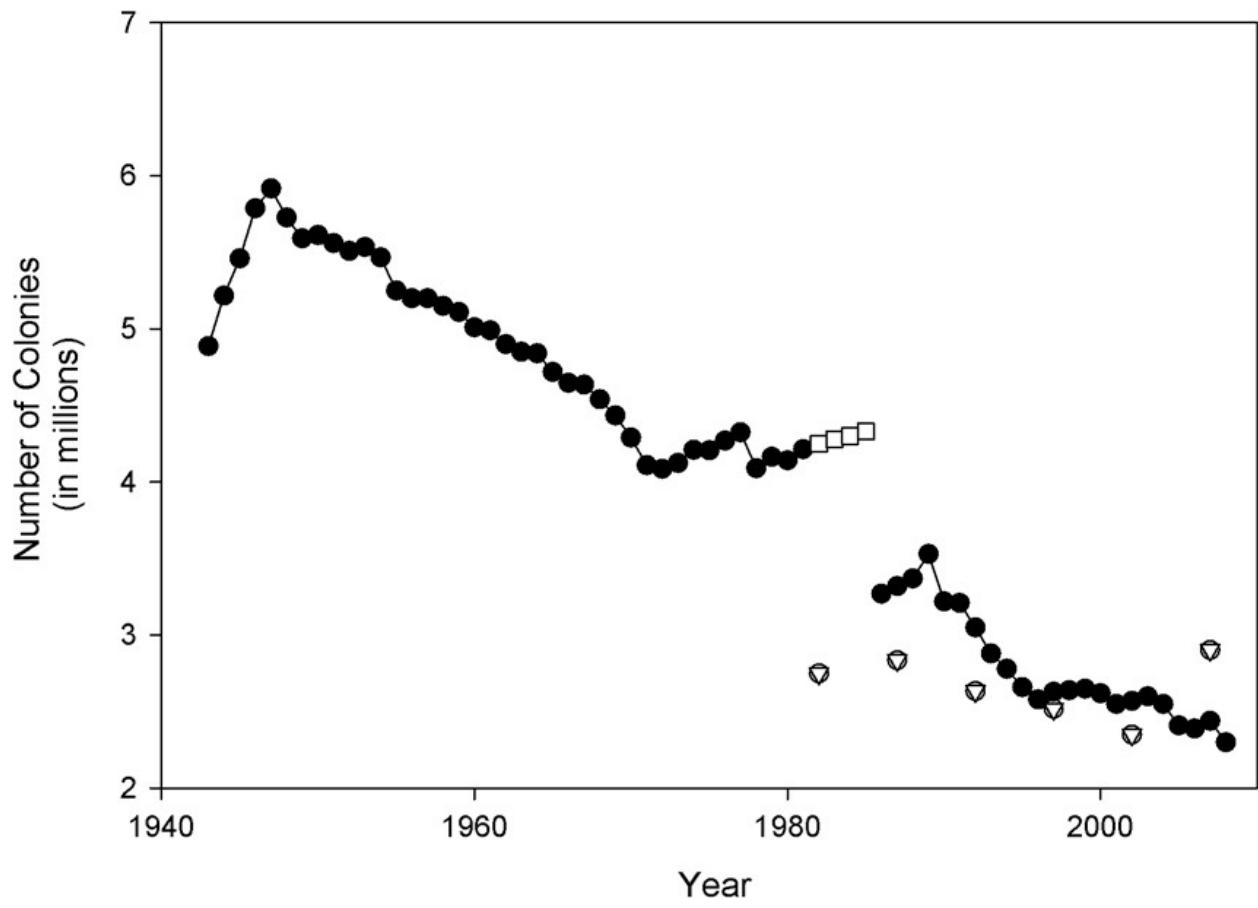


Fig. 2



**Fig. 3**

## Conclusion

Over the next century, climate change will occur 10 times faster than it has at any time in at least the past 65 million years. As a result, average global temperatures are predicted to increase at a rate of 0.25 °C per decade by 2020 in the northern hemisphere. In addition to these increases in mean temperature, the IPCC reports that increases in daily temperature extremes and reductions in cold extremes are “virtually certain” to occur in the future. A 1-in-20 year hottest day in the current climate is expected to become a 1-in-2 year event by the end of this century across the majority of the planet. Furthermore, extremely hot summers ( $>3 \sigma$  warmer than average) that were nearly absent during 1951-1980 (0.2% of land area) are now common across 10% of global land area, and are expected to increase over more global land area in the future. In addition to temperature extremes, droughts are likely to become more intense and frequent across the globe in the future. The effects of weather extremes on tree phenological and physiological processes are largely unknown. Because tree phenology and physiology are linked to ecosystem level processes such as carbon cycling, which can feedback on climate change, it is critically important that we understand how these processes will be impacted by extreme years. Furthermore, it will be important to understand how phenological and physiological responses vary among- and within-populations of trees during extreme years. Trees are expected to be especially vulnerable to weather extremes since these conditions may push them past their physiological limits; however, high amounts of intraspecific variation may aid the capacity of tree species to adapt to future climate change.

In order to investigate intraspecific responses in phenology and physiology, we observed the timing of leaf emergence and measured leaf-level  $\delta^{13}\text{C}$  across 43

populations of white ash that were sampled throughout the species range and planted in the same location in Jefferson, KS. This common garden (or provenance trial) was set-up by the U.S. Forest Service, North Central Forest Experiment Station in 1976 to better understand how genetic variation affected growth, and identify the best seed sources for various geographical locations. Seed was collected from open-pollinated trees when the initial experiment began from 59 locations and planted in multiple common gardens throughout the U.S. Many of these common gardens were not maintained, unfortunately. The U.S. experienced the warmest year in U.S. history during 2012. At the white ash common garden site during the extreme warm year of 2012, the average annual temperature was 16.5 °C, which was 4.5 °C warmer than the average of all non-extreme years (2005, 2007, 2009, 2010, 2011, and 2013) in which we measured phenology or physiology. Total annual precipitation was lower during the extreme year of 2012 (482 mm) relative to the average total annual precipitation during non-extreme years (892 mm; Fig. 2). The total precipitation that fell during the growing season (March-August) also was lower during the extreme year of 2012 (290 mm) relative to the average total growing season precipitation during non-extreme years (576 mm). The occurrence of this year presented a unique opportunity to study the effects of weather extremes among many white ash populations under natural conditions – these observations are very rare because these studies are “opportunistic” in the sense that they cannot be planned. Thus, the conditions at this site afforded me the opportunity to conduct a comprehensive intraspecific study of the effects of extreme years on white ash phenology and physiology. From the results of these studies, I make the following conclusions.

**1)** The effect of extreme years on phenology and physiology of white ash will vary depending on the trait observed.

Leaf emergence accelerated by 22 d on average among 43 populations during the extreme year of 2012 relative to non-extreme years. This was an unprecedented acceleration of phenology during the extreme year of 2012 relative to non-extreme years. In our investigation of leaf-level  $\delta^{13}\text{C}$ , we found that average leaf-level  $\delta^{13}\text{C}$  responses across populations were similar between extreme and non-extreme years (a difference of only 0.4‰). These results suggest that leaf emergence of white ash will be strongly affected by future weather extremes, but leaf level  $\delta^{13}\text{C}$  responses will be similar during extreme and non-extreme years.

**2)** The relative timing of leaf emergence and the relative leaf-level  $\delta^{13}\text{C}$  responses will likely be predictable at the population level for white ash during extreme years.

The rank order in the average timing of leaf emergence among 43 white ash populations was relatively uniform across extreme and non-extreme years. I also found that the rank order in the average leaf-level  $\delta^{13}\text{C}$  responses among white ash populations was relatively uniform across extreme and non-extreme years. Thus, reports of white ash phenology and physiology during the extreme year of 2012 likely provide a snapshot of phenological and physiological responses that may be extrapolated to responses during future extreme years.

**3)** Phenology and physiology of populations of white ash will be affected differently by extreme years.

In our phenology study, white ash populations that originated from more northern latitudes consistently showed later leaf emergence than populations originating from

southern latitudes. This suggests that populations may have different thermal requirements depending on the climate norms from which they were adapted and these requirements vary systematically across a latitudinal gradient. These results also suggest that southern populations of white ash may be more vulnerable to early spring frost under future climate change scenarios. While we did not find a significant relationship between precipitation or growing season VPD from a populations source location and a populations' leaf-level  $\delta^{13}\text{C}$  response, trends suggested that eastern populations have lower (more negative) leaf-level  $\delta^{13}\text{C}$  during extreme years relative to western populations. If droughts become more intense and more common in the future as predicted, these results suggest that eastern populations of white ash may be affected by drought more so than western populations.

**4)** The timing of leaf emergence may be fundamentally altered during extreme years such that the reliability of conventional phenology models is weakened.

In our phenology study, thermal requirements shifted such that greater amounts of warming were required for leaf emergence during the extreme year relative to non-extreme years and this constrained the potential for even earlier leaf emergence by an average of 7 d among populations. Thermal requirements (GDUs) for leaf emergence are expected to remain relatively similar across years, and so the greater GDU requirement we saw during the extremely warm year of 2012 was odd. Thus, we attempted to predict leaf emergence during the extreme warm year of 2012 using a conventional model, and found that the reliability of this model was altered during 2012 such that leaf emergence was unpredictable in this year.

**5)** The effects of polyploidy (within-population variation) on plant water relations may be important to physiological functioning of white ash during extremely dry periods across the growing season.

In the polyploidy analysis, I found that average leaf-level mid-day water potentials and gas exchange were affected by polyploidy during July, 2013, which was one of the driest months during this growing season. Diploids showed higher leaf-level gas exchange, but the lowest (more negative) leaf-level mid-day water potentials relative to polyploids. These results may suggest that diploids are more competitive than polyploids when conditions are drier during the growing season, especially if diploids are more resistant to drought-induced cavitation. However, we found no difference in leaf-level  $\delta^{13}\text{C}$  suggesting physiological responses may be similar between cytotypes during wetter conditions across the growing season.

**6)** Lastly, I find that new policies delivered from the Pollinator Health Task Force of the White House concerning the decline of pollinators are guided by the best science available.

While not directly related to my dissertation, this is an important policy document considering that some bee keeper's colonies in the U.S. have decline by over 90% over the past 20 years and monarch butterfly populations hit their record lowest in 2014. While I have not fully detailed all of the strategies proposed by the Pollinator Health Task Force, the most important strategy and proposed plans (to increase or enhance pollinator habitat) seem to be well supported by scientific evidence.

This research advances our understanding of phenological and physiological responses among- and within-populations of white ash during extreme and non-extreme



years. Collectively, these results have serious implications for understanding how white ash tree populations will respond to future climate change, and the potential for the species to adapt to rapid shifts in environmental conditions.