UNIVERSITY OF CALIFORNIA, MERCED

Response of *Pinus flexilis* James seedlings to simulated climate change through gas exchange rates, phenology and morphology

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Environmental Systems

by

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2011

The Thesis of Jennifer Rosemary Wolf is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, Merced

2011

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ACKNOWLEDGEMENTS

This thesis would not have been possible without the vision and support of my adviser, Lara Kueppers. I am also very grateful to my committee members, Stephen Hart and Matthew Germino, for guiding me through this process and for providing insightful feedback. I would like to thank Jeffry Mitton for sharing dearly-collected seeds and information, Akasha Faist for showing me the ropes around the laboratory, Scott Ferrenberg and Andrew Moyes for being statistical and theoretical consultants, and laboratory assistants Ruth Xochihua and Rodney Severson for invaluable help and philosophical laboratory conversations. Thank you to the crew maintaining and openly sharing the Niwot Ridge Ameriflux tower climate data. I am grateful to have shared a laboratory with Kaitlin Lubetkin, Yaqiong Lu, Chelsea Carey and Erin Stacey, all of who made laboratory work joyous. I owe my deepest gratitude to my husband, Alok Singhal, for his mathematical and computer programming prowess, his generosity of time and patience and his unending faith in me. Lastly, I would like to thank the U.S. Department of Energy for grant # DE-FG02-07ER64457, from which I was able to conduct this experiment.

ABSTRACT

Anthropogenic climate change in the western U.S. involves increasing temperatures, decreasing water availability and increasing frequency of extreme weather events. Predicting how subalpine forests will respond to climate change is complicated by variation in intraspecific responses. To address the question of whether subalpine tree populations will be able to tolerate future climatic conditions within their current distribution, I compared the physiological, phenological and morphological response of seedlings of two limber pine (*Pinus flexilis* James) mitochondrial haplotypes (narrow and wide-ranging) to simulated climate change. I conducted a 15-week growth chamber experiment on seedlings under low light conditions and two temperature treatments: ambient (recent climate conditions) and heated (ambient +5°C). I recorded the timing of developmental stages and measured seedling net photosynthetic and dark respiration rates, total biomass, root to shoot mass and length, specific leaf area (SLA) and specific root length (SRL)**.** I found that the ambient chamber had greater average rates of net photosynthesis and dark respiration. Between haplotypes, there was a marginally significant difference in net photosynthesis and a significant difference in dark respiration. There were significant morphological differences between the haplotypes and chambers in root to shoot mass and SLA. Seedlings in the heated chamber germinated, on average, two weeks earlier than in the heated chamber and experienced a five-fold increase in mortality rate. The results of this experiment suggests that limber pine seedlings growing in the shade in a 5°C warmer climate will suffer near zero carbon balance, increased mortality, and will experience a longer growing season.

INTRODUCTION

Conifer Responses to Climate Change

Climate change threatens forest ecosystems in the Western U.S with multiple detrimental impacts. The most recent Intergovernmental Panel on Climate Change report (2007) projected that average global temperatures may increase anywhere between 1.1 and 6.4°C by the year 2100, while in some regions, temperatures will increase more than the global average. In the North American Southwest, summer maximum temperatures and minimum winter temperatures are likely to increase more than the average. Temperature increases in the last century have been the most pronounced and rapid at high altitudes and latitudes (IPCC, 2007). Increasing frequency and duration of drought is also likely to occur with climate change. Areas in the southwestern United States, defined from 95 to 125°W and 25 to 40°N, are expected to experience decade-long droughts by 2100 (Seager et al., 2007). Lastly, the incidence and intensity of extreme weather events is expected to increase (IPCC 2007, Repetto and Easton, 2010).

The effects of anthropogenic climate change on forest ecosystems are numerous and primarily detrimental. Although temperature-limited forests with short growing seasons have been slowly increasing annual growth rates in response to increasing average temperatures (Boisvenue and Running, 2006), semi-arid forests, have been slowing in their growth and suffering higher rates of mortality due to drought stress linked to warming temperatures (McKenzie et al., 2001, Van Mantgem et al. 2009). Drought has been shown to decrease above and belowground productivity, shift phenology, alter species composition and reduce the reproductive success of forests

(Erice et al., 2006, Asseng et al., 1998; Lloret et al., 2009; Llorens and Peñuelas, 2005). Elevated temperatures may combine with drought to cause carbon starvation and hydraulic failure, while multi-decadal oscillations between wet and dry periods may predispose plants to water stress (McDowell et al., 2008). However, extreme climatic events may have the most acute effect on ecosystem structure (Repetto and Easton, 2010). A heat wave is a period of time where temperatures significantly increase above seasonal averages and vary by region in their magnitude, duration, and impact (Gershunov et al., 2009). Heat waves may combine with increasing global average temperatures to drastically alter ecosystem structure and function (Jentsch et al., 2007). Heat waves also exacerbate the effect of drought and cause decreases in radial growth (Pichler and Oberhuber, 2007). Heat waves and other extremes may cause a decrease in mean fitness because they represent an increase in the average deviation from optimum conditions to which populations are adapted (Aitken et al., 2008).

Conifers have various means of surviving climate change. They can tolerate wide climatic variation in the short-term, and in the longer-term, migrate and adapt. Phenotype plasticity may allow adult conifers to tolerate climate change in the short term, from years to decades, and to assist adaptation in the long term (Nicotra et al., 2010). Phenotype plasticity is a physiological, morphological, developmental, or behavioral change in the expression of an organism's genotype in response to environmental triggers (Gienapp et al., 2008). Plant functional traits such as net photosynthetic and dark respiration rates, root to shoot mass ratio, specific leaf area (SLA) and specific root length (SRL) are thought to be plastic (Atkin et al., 2006; Nicotra et al., 2010). Phenotype plasticity combined with longevity of the individual may allow conifers to survive up to a

decade of adverse environmental conditions (Hamrick, 2004). Characterizing the limits of this plasticity is important in determining at what point forests will no longer be able to tolerate conditions and the environment becomes uninhabitable (Walther et al., 2002).

Seed dispersal and seedling establishment may allow conifers to migrate out of areas that are too hot or dry into more favorable areas. Migration occurs through the expansion of a species' range into favorable areas and the contraction of its range in unfavorable areas. While seed dispersal is generally random with respect to how adapted it is to the conditions of where it lands, seedling establishment is selective; those seeds that reach favorable microsites germinate, and those seedlings that have adaptations that match their environment survive and reproduce (Davis and Shaw, 2001). Seedling establishment is an intensely selective process (Campbell, 1979; Stevens and Fox, 1991; Germino et al., 2002; Smith et al., 2003; Maher et al., 2005; and Maher and Germino, 2006). Seedlings are located at the soil surface where temperatures change rapidly and vary greatly, and soil resources are limited. Likely due to their location, seedlings in their first year of life are more vulnerable to heat and drought stress than older individuals (Osmond et al., 1987, McDowell et al., 2008). The majority of high elevation conifer seedlings die in their first year (Smith et al., 2003; Germino et al., 2002; Maher and Germino, 2006). As a climate becomes more unfavorable, years of successful seedling recruitment may become separated by longer intervals (Hamrick et al., 1989). Though increasing temperatures may drive migration into higher altitudes or latitudes, other abiotic factors such as intensity of solar radiation, frost and desiccation may limit establishment (Germino, 2002; Smith et al., 2003). Additionally, the rapid pace of climate change may surpass the migration of slowly establishing conifers (Jump and

Peñuelas, 2005; McDowell, 2008; Davis and Shaw, 2001). If suitable climatic conditions remain within a given mountain or mountain range, species will need to shift 8 km per century as trees migrate upward in altitude (Loarie et al., 2009). However, if suitable climatic conditions disappear from a given mountain or mountain range, latitudinal range shifts of 300 to 700 km per century may be needed for species to track climatic conditions to which they're adapted (Davis and Shaw, 2001; McKenny et al., 2007). However, the most exceptional latitudinal range shifts in the geologic record occurred at 100 to 150 km per century (Davis and Shaw, 2001). Rehfeldt et al. (2002) found that, for 11 field sites across northern Europe and Asia, the genotypes most suitable to survive the climate of the year 2090 were at least 1000 km away. Moreover, for many species, crossing these vast distances has likely become very difficult or impossible due to habitat fragmentation and urbanization (Davis and Shaw, 2001).

In addition to tolerating and moving away from unfavorable areas, to ensure the continuity of their species in the long term, conifers will also need to adapt to climate change. Variation within and among populations enables a species to cope with multiple environmental stresses. If genetic variation within a species is great enough and selection pressure is intense, a species can evolve rapidly (Rehfeldt et al., 2002). The fecundity of a species, interspecific competition and biotic interactions also determine the degree to which a species adapts (Aitken et al., 2008). But again, the rate of climate change is likely to surpass the rate of adaptation (McDowell, 2008; Jump and Peñuelas, 2005). For example, assuming the climate stabilizes in 2090, it could take as many as 13 generations before *Pinus silvestris* would be adapted to the changed climate. That would take as many as 1500 years. If the climate does not stabilize, genetic changes in *P. silvestris* may

perpetually lag behind the changes in climate, causing decreased forest productivity or local extinction (Rehfeldt et al., 2002).

Toleration, migration and adaptation are not mutually exclusive. Phenotype plasticity may play a role in adaptation (Nicotra et al., 2010). For example, during adaptation, individuals with greater phenotype plasticity may be favored at first and later, individuals with phenotypes that match environmental conditions will be favored over plastic phenotypes (Lande, 2009). Recently arrived individuals may be able to tolerate conditions through plasticity of their physiological responses. Adaptation may occur at the same time as migration because the process of seedling establishment is selective and differential growth and reproduction would favor a new combination of physiological traits. For example, individuals migrating from a southern population could recombine with individuals at the more northerly location, resulting in a new combination of temperature response suited to a warmed climate but with a shortened photoperiod to match the northern latitude (Davis and Shaw, 2001). If these tolerance, migration and adaptation strategies fail, without human intervention, a species may be facing drastic range reductions or extinction (Engler et al., 2009).

Factors limiting the expansion of treeline

Models project that treelines will migrate upward in elevation in response to a warming climate (IPCC, 2007; Malcolm, 2002; Holtmeier and Broll, 2007), though some empirical studies have suggested that treelines may be constrained in their movement by a host of factors (Holtmeier and Broll, 2007, Berdanier, 2010). Körner, (1998) hypothesized that cold temperatures may limit shoot and root growth and that this growth

limitation is the cause for treeline formation across the globe. This hypothesis has been supported by multiple observations that treeline conifers have higher amounts of non-structural carbohydrates compared to conifers at lower elevations, suggesting that it is the utilization and not the supply of carbon that limits growth at the treeline (Körner, 1998; Shi et al., 2008). A second hypothesis is that seedling establishment limits the expansion of treeline. That is, microclimate conditions in the alpine, including low temperatures, sky and wind exposure, humidity and soil characteristics, limit seedling establishment by negatively affecting carbon uptake (Smith et al., 2003; Smith et al., 2009; Reinhardt et al., in revision). Seed predators, including small mammals, birds, and, to a lesser degree, insects, also play a role in limiting seedling establishment by decreasing seed availability before and after seed dispersal and by altering the spatial distribution of seeds in the initial seed rain (Benkman, 1995; Castro et al., 1999). These hypotheses all may have some explanatory power in explaining limitations on treeline (Benkman, 1995; Berdanier, 2010). As global average temperatures rise, the temperature-limiting effect on growth may be removed or microclimate conditions may improve and treelines are predicted to migrate upward in altitude. Harsch et al. (2009) demonstrated that already 52% of 166 treelines across the globe have advanced in elevation, while only 1% of them have receded. The results of this study are consistent with both hypotheses; treelines that were diffuse and more likely to be constrained only by temperature, were more likely to advance, while treelines that were abrupt or had krummholz forms and were more likely constrained by stress factors leading to plant damage and mortality were less likely to advance (Harsch et al., 2009).

Conifer Physiological Response to increasing temperatures and drought

Instantaneous net photosynthesis, or net CO_2 assimilation, is the moment-to-moment rate of carbon fixed due to photosynthesis minus carbon losses due to photorespiration and background metabolic processes. Net photosynthesis is measured because it provides a snapshot into a plant's physiology, and an understanding of how it changes provides insight into the environmental and genetic factors that influence plant productivity (Sharkey et al., 2007). Dark respiration is the respiration rate that is measured when the plant is not exposed to light; it includes growth and maintenance respiration. Dark respiration rates that occur during the day and at night differ because light can inhibit mitochondrial respiration (Atkin et al., 2000). Dark respiration generates the energy and carbon skeletons necessary for plant growth (Lambers et al., 2008).

Acclimation is the morphological or physiological adjustment that individuals make to compensate for a decline in a biological process due to an external stressing factor (Teskey and Will, 1999; Lambers et al., 2008). Under acclimation, rates of a physiological process approach pre-stress levels but do not necessarily match them (Tjoelker et al., 1999; Loveys et al., 2003; Atkin et al., 2006; Lambers et al., 2008). The potential to acclimate varies across species, with some showing dramatic short-term abilities and others showing little to no acclimation ability (Tjoelker et al., 1999; Larigauderie and Körner 1995, Way and Sage, 2008). Thermal acclimation is the adjustment of physiological rates in response to temperature (Lambers et al., 2008). Conifers have shown a higher degree of thermal acclimation than broad-leaf species (Tjoelker et al., 1999). Full acclimation occurs in tissues that develop under stress

conditions, while older, pre-existing tissues display acclimation that is partial (Loveys et al., 2003; Atkin et al., 2006; Ow et al., 2008). Both net photosynthesis and dark respiration are physiological processes that can acclimate. When net photosynthesis or dark respiration acclimates, the ratio of net photosynthesis to respiration is often similar between plants that are acclimated to differing temperatures when measured at common temperatures (Atkin et al., 2006). Cold-grown plants display higher rates of net photosynthesis and dark respiration than do warm-grown plants when measured at a common temperature (Arnone and Körner, 1997; Loveys et al., 2002; Atkin et al., 2003; Atkin et al., 2006).

Water availability exerts strong controls over net photosynthesis, total respiration and growth (Ryan, 1991; Chaves, 1991; Flexas et al., 2006). Water stress is correlated with high temperatures, low water availability or high soil solute concentrations (Chaves et al., 2003). Plants experiencing water stress have decreased stomata and mesophyll conductance (Ryan, 1991, Warren et al., 2004). Plants grown under drought conditions can acclimate by down-regulating photosynthetic capacity and slowing metabolic activity, thus decreasing respiration rates (Lambers et al., 2008). While plants can decrease the rate of gross photosynthesis to zero, they cannot completely reduce the rate of respiration (Warren et al., 2004; Flexas et al., 2005). In addition to diffusion of CO_2 limiting photosynthesis, photosynthesis can also be limited by metabolic impairment. Metabolic impairment of the photochemical and biochemical aspects of photosynthesis may occur when stomatal conductance drops below 0.05–0.10 mol H_2O m^2 s^{-1} (Flexas et al., 2004; Flexas et al., 2006). Conifers have many adaptations for drought tolerance (McCune, 1988). Compared to broadleaf deciduous trees, conifers have lower soil-to-leaf

conductance, lower osmotic potential and sunken, lignified guard cells that limit water loss (Gao et al., 2002). Seedlings are particularly susceptible to excessive cavitation, in which air bubbles fill xylem tissues such that plants can no longer transpire, as their limited rooting volume exposes them to more negative soil water potentials (McDowell et al., 2008).

Previous studies have demonstrated that seedlings and adults differ in their physiological and morphological traits. Seedlings have different rates of carbon uptake and patterns of carbon allocation (Norgren, 1996), and differ from adults in that they have much less photosynthetic tissue to provide materials for growth (Bansal and Germino, 2010). Seedlings also have a greater SLA, leaf area ratio (the ratio of leaf area to whole plant dry mass), and leaf nitrogen concentrations than adults (Reinhardt et al., in revision). Achieving a positive carbon balance is difficult for seedlings and can be a cause of their demise. Despite the differences between adults and seedlings, adults have been the primary subjects of ecophysiological investigations (Germino, 2002; Bansal and Germino, 2010).

Phenological shifts in response to climate change

Phenology is the timing of seasonal life stages of a given species in relation to seasonal cues (Linderholm, 2006). Shifts in phenology are some of the first well-documented effects of ongoing climate change, with data sets going back at least 30-40 years (Walther et al., 2002). Earlier budding, leafing, and flowering are some of the documented changes in response to increasing temperature (Linderholm, 2006). Spring activities have shifted by 2.3 days per decade (Parmesan and Yohe, 2003), and, in some

places, fall events are occurring later (Menzel and Fabian, 1999). The result is that from 1959 to 1996, the global growing season lengthened by about 11 days (Linderholm, 2006). Lengthened growing season may mean increased forest productivity, though recent studies suggested negative or conflicting growth responses at treeline (Linderholm and Linderholm, 2004**;** Wilmking et al., 2004). Species competitive interactions, species composition of forests and species ranges may all be affected as a consequence of differing species responses to lengthened growing season (Kramer et al., 2000).

Intraspecific Variation

The interaction of genetic inheritance and environment may be critical to predicting how forests will respond to climate change. For example, most models that treat forests as homogenous entities, predict that a warmer climate will have positive effects on forest productivity, while models that incorporate genetic variation of forest species predict that climate change will have a negative effect on productivity (O'Neill et al., 2008). Intraspecific variation is the genetic variation within a species that results within or between different populations of the species becoming locally adapted to differing evolutionary forces. Provenance studies demonstrate that moderate to strong local adaptation is frequently observed in conifers despite high levels of gene flow (Aitken et al., 2008; Wang et al., 2010). Traits such as germination rates, growth rate, phenology of bud development and burst, growth form, disease resistance and susceptibility to frost damage vary intraspecifically within conifers (Mitton, 1995). In general, high elevation and low elevation populations often differ in that high elevation populations have higher growth potential, greater leaf nitrogen, and increased root to

shoot ratio (Oleksyn et al., 1998; Rehfeldt et al., 2002). Populations from northern and southern parts of their range also display differences in biomass accumulation, height, cold tolerance, and at what times growth begins and stops within a day and across a growing season (Savolainen et al., 2007; Bower and Aitken, 2008). Many locally adapted traits are controlled by multiple genes and therefore, even with strong selection, these traits may be slow to change (Lynch, 1996). Also, due to their complex nature and the size of the conifer genome, identifying the genes responsible for locally adapted traits is difficult. There are several approaches currently being developed to achieve this goal including identifying candidate genes, quantitative trail locus mapping and genome-wide scans using single nucleotide polymorphisms (Neale and Savolainen, 2004; Hurme et al., 2000; Namroud et al., 2008). Under a changing climate of increasing temperatures and decreasing water availability, populations originating from hot and dry conditions may already be pre-adapted to climate change, though gene flow from cold and wet areas may slow adaptation (Savolainen et al., 2007).

A haplotype is a group of genes that are inherited together and from one parent. In the context of this experiment, I will use haplotype to refer to groups of individuals that have one or more sequences of DNA in common. Haplotypes are created when populations are separated from each other for a long enough period of time for mutations to occur and become fixed within the population. Other traits along with the mutation could have been under local selection pressure so that the haplotypes reflect local adaptation. These haplotype groups would then differ in the way that they function and the functional differences may still be detectable even when the ranges of the two haplotypes began to overlap again.

Characteristics of Limber Pine

Limber pine (*Pinus flexilis* James) is a long-lived, shade-intolerant, primarily bird-dispersed species that has wide morphological variance. Its ability to colonize extreme habitats and to withstand climatic variability makes it a suitable subject for stress physiology research in the context of climate change (Letts et al., 2009). Meanwhile, extensive common garden experiments have not been performed to evaluate local adaptation (Schoettle, 2001). It ranges discontinuously across wide latitudinal and elevation gradients, from the southwest United States to southwest Canada and from 870 to 3810 m. With up to kilometers-wide dispersal through the Clark's nutcracker, *Nucifraga columbiana,* limber pine is thought to be a genetic generalist, characterized to have wide physiological tolerances, tolerating average air temperatures in July varying from 13°C to 22°C (Schoettle and Rochelle, 2000). Limber pine's temperature optimum in terms of net photosynthesis is 15°C but it has a wide temperature response curve; the maximum photosynthetic rate only deviates 12% away from the optimum across temperatures of 10-35°C (Lepper, 1980). It reaches reproductive maturity at 50 years and can live up to 600 years (Letts et al., 2009). High needle longevity, low SLA, low leaf nitrogen concentration and low stomatal density are thought to be traits related drought and stress tolerance (Schoettle and Rochelle, 2000; Letts et al., 2009). Limber pine rates of net photosynthesis have been shown to decline when there is a high leaf-to-air vapor pressure deficit, demonstrating a high degree of stomatal control (Letts et al., 2009).

Limber pine, like most other species in the genus *Pinus*, has different genetic modes of dispersal and inheritance via its pollen and seeds. Mitochondrial DNA (mtDNA), responsible for controlling cellular respiration, is maternally inherited, while

chloroplast DNA (cpDNA), responsible for controlling photosynthesis, is paternally inherited (Mitton et al., 2000a). MtDNA travels with the seed: as little as 100 m with the wind or 12 to 22 km by the Clark's Nutcracker (Tomback and Linhart, 1990; Schoettle and Rochelle, 2000; Mitton et al., 2000b). First dispersed with pollen and later in seeds, the potential for gene flow is much greater for cpDNA. As a consequence, cpDNA inheritance is homogenous and mtDNA inheritance shows a spatial pattern (Mitton et al., 2000a; Mitton et al., 2000b). Few studies have tested the physiological or demographic consequences of variations in mtDNA and cpDNA for any species, though regional differences in genetic adaptation to climate zones can be very pronounced. For example, moving outside a zone even of 300 m decreased survival of Douglas-fir (*Pseudotsuga menziesii*) seedlings by half (Campbell, 1979).

A study by Jørgensen et al. (2002) found that, when compared to genus means, limber pine is 18% more genetically variable within populations and 50% more variable among populations at polymorphic loci. The among-population differentiation may be a result of local adaptation, as it occurs over a wide range of elevations. The greater than average within-population variability is surprising as most species with fragmented distributions like limber pine have little within-population variation (Jørgensen et al., 2002). Populations separated by over 400 m in elevation can have different pollination phenologies, which would limit genetic exchange (Schuster et al., 1989). Limber pine populations in the Colorado Front Ranges have high among and within population genetic diversity relative to more northern and western populations (Jørgensen et al., 2002).

There are at least seven distinct mitochondrial haplotypes of limber pine within its range resulting from divergence during the last glacial maximum (Mitton et al., 2000a). These haplotypes differ at the NAD1 intron, a region of non-coding DNA. Two of the haplotypes co-occur on Black Mountain in the Pike National Forest, Colorado. One haplotype is wide-ranging, found above and below 3000 m, while the other is narrow-ranging, so far found only above 3000 m. It is unknown whether the narrow-ranging haplotype is restricted to high elevations because of biotic or abiotic factors (J.B. Mitton, University of Colorado Boulder, personal communication) and whether the haplotype differences correspond to differences in phenotype.

Goals and Research Questions

The goals of my study were to help address the question of whether underlying genetic differences have different functional responses to climate change and to help establish the limits of physiological tolerance to climate change in limber pine seedlings. The main questions, related to these goals, that I designed my experiment to answer are:

(1) How does simulated climate change affect gas exchange, phenology and morphology?

(2) Do haplotypes respond differently to climate change?

(3) Do haplotypes differ in their gas exchange, phenology or morphology?

(4) How does gas exchange change during a heat wave?

I compared seedlings of two mitochondrial haplotypes of limber pine in their physiological, phenological and morphological response to simulated climate change, including a heat wave event. I conducted growth chamber experiments mimicking recent

climate conditions (ambient) and simulated warmer conditions (heated), and measured seedling net photosynthesis and dark respiration, as well as numerous phenological and morphological traits. Since the two haplotypes differed in a marker in their mitochondrial DNA, and the mitochondria regulate cellular respiration, I expected that the marker might correspond to other functional differences in the DNA and that therefore haplotypes would respond physiologically, phenologically and morphologically different to climate change. Additionally, I expected that I might find differences in physiology, phenology or morphology between haplotypes. I expected that seedlings grown under heated conditions would have differences in their net photosynthetic and dark respiration rates. In response to the 3°C heat wave, I expected that seedling gas exchange would decrease during the heat wave compared to the weeks before and after the heat wave. In accordance with previous findings (Kueppers et al., unpublished data), I expected heated chamber seedlings to germinate earlier and to reach the development stage in which they shed their seed coats faster. Due to temperature differences across the chambers, I expected that there would be more germinants in the heated chamber. While I expected there would be more mortality in the heated chamber as a consequence of greater average temperature, I expected that there would be more mortality in the heated chamber among the narrow-ranging haplotype seedlings because I expected them to be less adapted to warm and dry conditions.

MATERIALS AND METHODS

Experimental Design Overview

To test seedling physiological and developmental responses to climate warming, I designed a growth chamber experiment in which I took gas exchange measurements on two mitochondrial haplotypes grown under two different temperatures. I implemented a watering treatment that mimicked field conditions in all trays and in both chambers. I programmed a heat wave into both of the growth chambers to test for differing short-term responses between the haplotypes to increased air temperatures. I measured instantaneous net photosynthesis and dark respiration rates using a portable gas exchange analyzer. I recorded dates of emergence and development, morphological characters, mortality and biomass to test for differences between the two haplotypes and between the chambers.

Preparing and planting the seeds

Seeds from five limber pine trees were collected from one location at 3295 m on Black Mountain in the Pike National Forest, CO (38°43'409" N, 105°41'574" W). In fall 2010, leaf tissue samples from each of the trees were sequenced at the NAD B/C intron on the mitochondrial genome at University of Colorado, Boulder (CU). Seeds collected from four of the five trees were of the wide-ranging haplotype, while the fifth was of the narrow-ranging haplotype (J.B. Mitton, personal communication). I randomly selected seeds from two of the four wide-ranging haplotype trees (trees 1 and 5) to assess maternal effects and to compare against the seeds from the one narrow-ranging haplotype tree (tree 3).

Beginning on February 20, 2010, I stratified 450 seeds from each tree for 30 days at 4°C following naked stratification procedures outlined in Schopmeyer (1974). Specifically, I kept the seeds moist in permeable bags and rinsed them briefly in 1% hydrogen peroxide solution once a week to prevent mold growth, while discarding individual seeds that showed visible signs of mold. Prior to stratification, I weighed the seed mass of 312 randomly selected seeds (103 tree 1, 105 tree 3, 104 tree 5).

Native soil was collected from the Mountain Research Station (MRS), Nederland, CO, approximately 300 m north of 40° 02' 09" N, 105° 32' 09" W. Soil at this site has been classified as loamy-skeletal, mixed, superactive Typic Dystrocryepts (Soil Survey Staff, USDA). Due to limited amounts native soil available, I filled 66 ml Pine Cell conetainers (Stuwe and Sons, Inc., Tangent, OR) with layers of soil and autoclaved sand; I filled the top ~2/3 of each conetainer with sifted soil (mesh size #10, retaining particles <2 mm) while I filled the remaining bottom 1/3 with sand. I placed the conetainers into 6 trays, each containing 200 conetainers. On March 19, 2010, I planted one seed per conetainer, 2.5 cm in depth. I planted seeds in equal proportions across the three trays in each chamber for a total of 200 seeds from each tree per chamber and their location within the tray was selected randomly. I shifted trays among shelves and rotated their position within a shelf each time I watered them on Monday, Wednesday and Friday. I observed and recorded signs of germination before each time that I watered them for the duration of the experiment: 15 weeks. At harvest, I emptied the contents of all conetainers. To measure dry biomass, I separated roots and shoots and dried them in envelopes in a convection oven at 105°C for three days.

Growth chamber programming and heat wave

I programmed two growth chambers (Percival Scientific, Perry, IA, model # E-36VL, with external dehumidifiers model # IAT-50) based on recent climate data from the CU/Ameriflux Tower (Burns and Monson 2010. I used a program that estimated recent climatic conditions from hourly averages of 7 years of air temperature and relative humidity data at 2 m off the ground during the frost-free growing season, from 1999 through 2005. I programmed the first growth chamber (Ambient) using hourly averages, while I programmed the second (Heated) by adding 5°C to every time-point of the hourly average temperature from the field data. Figure 1 shows the results of the programming. Both light banks in the growth chambers were turned on following a diurnal cycle and light conditions in the two chambers were the same throughout the experiment; they received 14 hours of light (at approximately 300 μmol m^{-2} s^{-1}) and 10 hours of darkness. This light level approximates the mid-summer light level under the forest canopy at midday, around 350 μmol m^{-2} s^{-1} (J.R. Wolf, unpublished data).

In order to test for physiological responses to an extreme climatic event, on week 11 of the experiment, I programmed both growth chambers to simulate a heat wave. I defined a heat wave to be a period of 6 consecutive days in which the maximum daily temperature is above the 90th percentile of the data during the frost-free days (Klein Tank 2009, Definition #14) and also marked by daily minimums peaking above the 90th percentile (Gershunov et al. 2009). After computing the 90th percentile for the maximum and minimum temperatures for 1999 to 2005, I computed the difference between the 90th percentile maximum temperature and the average temperature for the same time period in each year of the record with temperatures below the 90th percentile. From 1999 to 2005,

during time periods that my definition identified as heat waves, temperatures increased an estimated 3°C above average. I added a heat wave to the program during week 11, resulting in ambient and heated chamber temperatures increasing 3°C above average for 7 days (Figure 1). Figure 2 shows the results of the relative humidity programming.

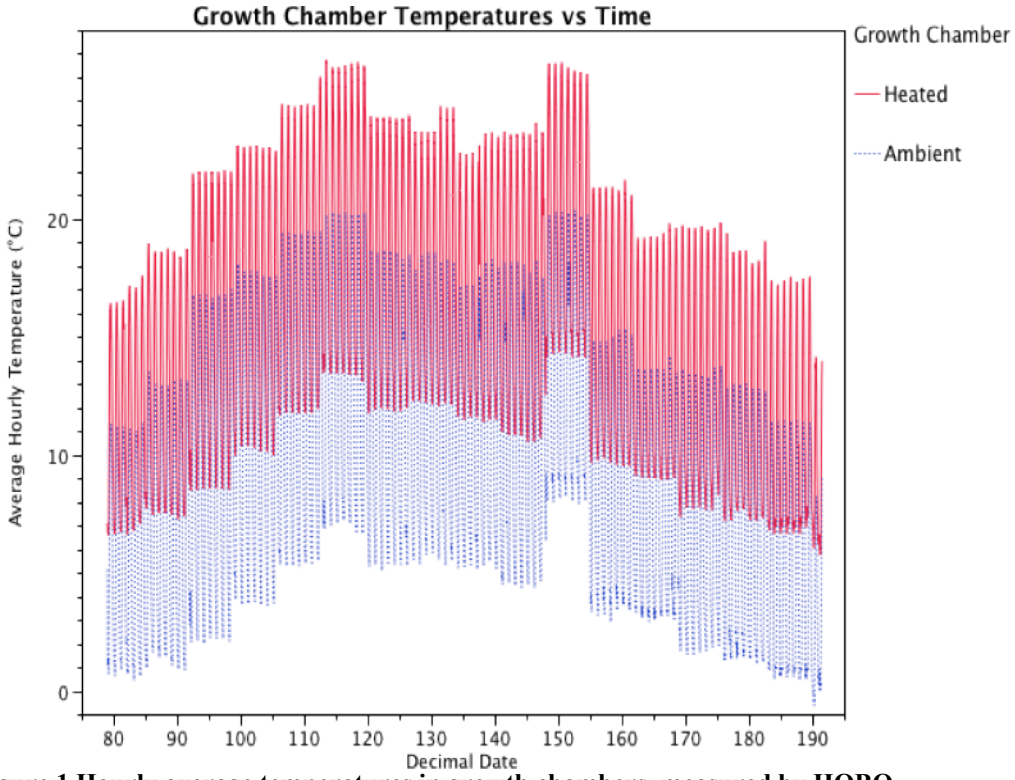

Figure 1 Hourly average temperatures in growth chambers, measured by HOBO temperature probes. Decimal date is continuous time format that incorporates day of year with hour, minute and second. The heated chamber was programmed to be similar to a climate at the end of the 21st century, with a 5°C increase at every time point. The ambient chamber was programmed to be similar to recent climatic conditions in the Colorado Rocky Mountains. Shown here are the results of the programming. The ambient chamber high temperatures overlap with low temperatures in the heated chamber. The second peak in temperatures around decimal day 145 shows the heat wave.

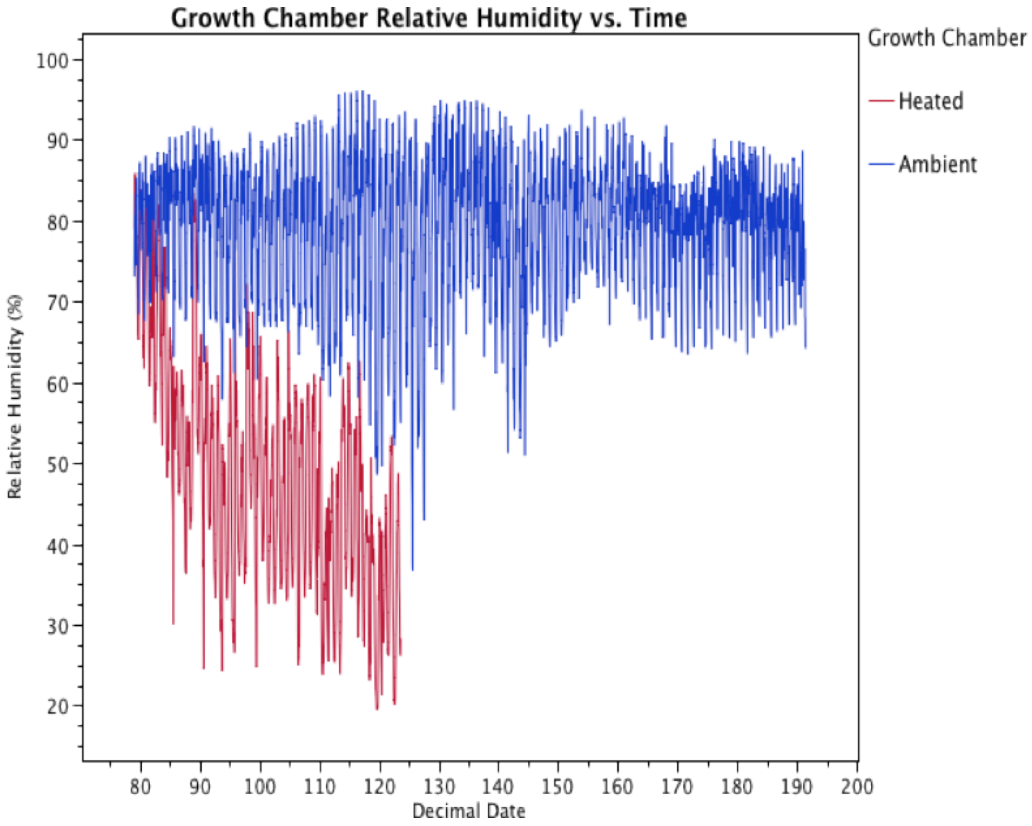

Figure 2 Relative humidity in growth chambers, measured by HOBO relative humidity probes. Relative humidity was programmed to be at the same level in both chambers, in the range of the ambient chamber. However, due to increased temperatures in the heated chamber, relative humidity was less than the programming target in the heated chamber. A period of heated chamber data, from week 7 to week 15, was not recorded due a malfunctioning probe.

Watering

I designed the soil moisture regime to mimic average field conditions at the Niwot Ridge Ameriflux tower. However, to prevent massive seedling mortality, I set target-watering conditions to be slightly wetter than field conditions. I used three years of data (2006-2008) to determine average field water content at 5 cm depth from the ground surface, which is approximately within the rooting zone of conifer seedlings (Figure 3).

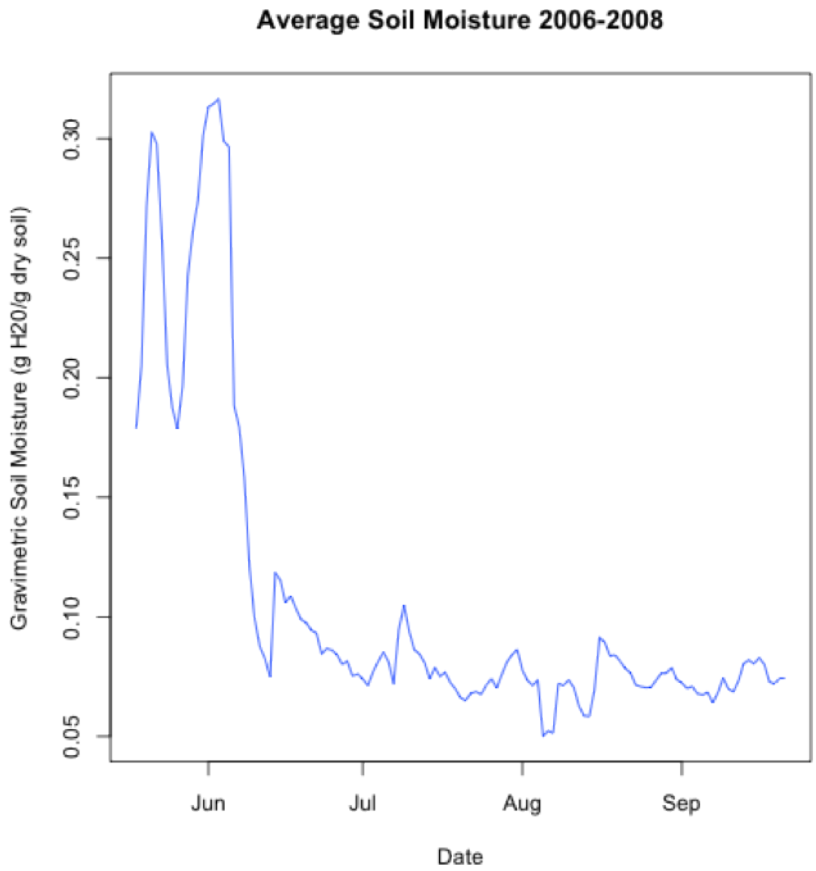

Figure 3 Niwot Ridge Ameriflux Tower gravimetric soil moisture data at 5 cm from ground surface, 2006-2008 This graph shows the average soil gravimetric water content (m^3/m^3) for 2006-2008 at the Ameriflux Tower, MRS, CO.

In the field, as snow melts, the soil has a high gravimetric water content (GWC) for a period of time and then it later dries down, with periodic rewetting periods due to summer storms. For the lab experiment, I set two watering targets for simplicity. The first

target, around 0.3 g H_2O/g dry soil, which is at or near field capacity (Figure 3), was maintained for two weeks. By decreasing watering gradually over the course of two weeks, the trays approached the second watering target, around 0.2 g H_2O/g dry soil, which is 0.1 g H_2O/g soil greater than average mid-summer conditions in the field (Figure 3).

I watered the trays using a spray nozzle attached via PVC tube to a faucet supplying reverse osmosis water, pH=6.6, Nitrate (NO_3)=1.2 mg/L (sampled August 2008). The trays were watered for three minutes initially, with a gradual decrease in time down to one minute. Trays in both the heated and ambient chambers were watered for approximately the same amount of time, while the spray nozzle maintained the same amount of flow, thus trays in both chambers received the same amount of water. In order to account for increased evaporation rates in the heated chamber, heated chamber trays were watered up to 30 seconds more than the ambient chamber trays. However, the increase in time was not sufficient to make up for the increased evaporation in the heated chamber; average GWC was 0.06 g H_2O/g soil lower in the heated chamber across the entire experiment ($p = 0.0001$, Figure 4).

To keep track of soil water content in trays, I weighed a subset of conetainers after watering each Monday, Wednesday and Friday. I selected a randomly chosen subset of 16 conetainers per tray; I weighed the same set by location across all trays. At the end of the experiment, I destructively sampled soil from this set of cells and I determined what the GWC was for the experiment duration.

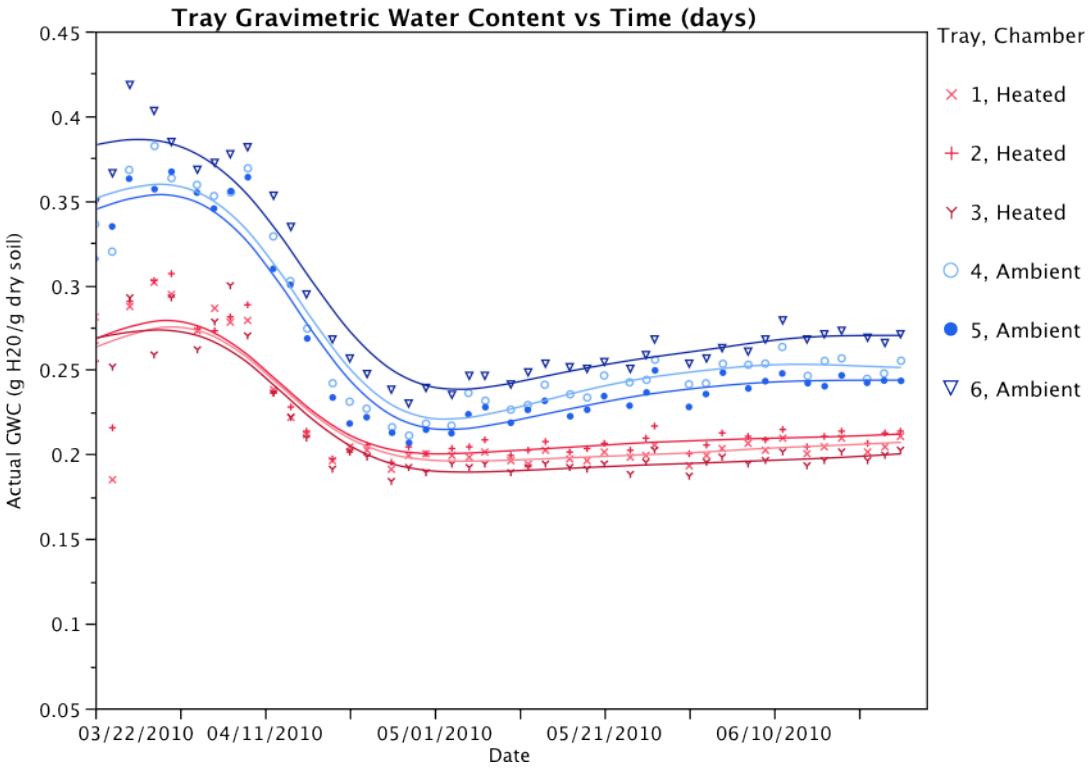

Figure 4 Actual gravimetric water content by tray. The lines are averages of each tray. Trays 1-3 were in the heated chamber while trays 4-6 were in the ambient chamber. Differences in rates of evaporation led to different gravimetric water content outcomes despite relatively similar watering times.

Development and Morphology Measurements

I tracked seedling development in terms of the number of days from germination to the first date when they shed their seed coat. Before watering, I scanned all cells in a tray visually for signs of germination and/or development. After shedding their seed coat, I tracked seedlings for mortality, which I defined as being desiccated to the point of having brittle tissue. I recorded when these developmental steps occurred in order to calculate the timing of each event and to examine whether there were differences between haplotypes and chambers.

On three separate occasions, spaced approximately one month apart, I measured all seedlings for morphological characteristics including plant height, from soil to cotyledon node, and for cotyledon length (of a randomly selected cotyledon) and number. At harvest, I measured seedlings for root and shoot length, as well as cotyledon length and number. I defined the shoot as the vertical length of the plant (stem plus leaf) that was above the soil, and the root as the remaining vertical length. From these data, I calculated the ratios of the root and shoot masses and lengths, SLA (at the whole shoot level), and SRL. I expected these characteristics to display differences among haplotypes and plastic responses to growth chamber conditions.

Many cells initially showed signs of soil breakthrough (i.e., the soil cracked or was pushed up) but a seedling never broke through the soil. At harvest, I discovered many of these cells to have seedlings that had germinated but had failed to break the soil surface and died. I measured these germinants for root and shoot length and cotyledon number and length when possible but categorized them separately from seedlings that had emerged fully from the soil.

Gas Exchange Measurements

I used a LI-COR 6400 XT with a 6400-02B LED Light Source leaf chamber (LI-COR Biosciences, Lincoln, Nebraska, USA) to measure net photosynthesis and dark respiration on seedlings that had shed their seed coat before, during and after the heat wave (weeks 10 to 13). I created an automated program to minimize experimenter influence while measuring both photosynthesis and dark respiration. The program for photosynthesis took a total of 45 data points over the course of up to 8 minutes. It set

chamber conditions to flow rate = 200 μmol s^{-1}, CO_2 = 400 μmol mol^{-1}, and photosynthetic active radiation input (PARi) = 300 μmol m^{-2} s^{-1}. These settings were chosen according to LI-COR recommendations for small fluxes, ambient CO_2 levels in the laboratory, and the maximum light level available in the growth chambers, which was measured using the LI-COR External Quantum Sensor. After I started the program, the program waited 30 seconds and then matched the sample and reference infrared gas analyzers (IRGAs), and waited another 30 to 120 seconds, depending upon stability of the sample CO_2 and water vapor concentrations, the flow rate, and the calculated values for transpiration and conductance. After waiting for stability, the LI-COR logged 5 data points consecutively, all within 1-2 seconds. 15 seconds later, the LI-COR logged a second round of data points, and after 15 more seconds, it took a third round of data points.

The program for dark respiration took a total of 39 data points over the course of up to 4.25 minutes. I ran the dark respiration program after the photosynthesis program on the same seedling without opening the chamber. It used the same conditions as the photosynthesis program except that PARi was set to 0 μmol m^{-2} s^{-1}. The program waited 30 seconds after the light turned off before it matched the IRGAs. Then another 60-180 seconds elapsed depending upon the same stability conditions used for the photosynthesis program before 4 rounds of logging 5 points, again within 1-2 seconds, and after waiting 15 seconds between measurement periods.

Data Verification

Potential sources of error related to the use of an automated program include insufficient time for the LI-COR and/or seedling to acclimate to conditions, changes of pressure within the lab leading to changes of flow within the LI-COR, leaks in the leaf chamber, and excessive matching of IRGAs. Therefore, rather than computing the average of the 45 and 20 data points generated by the programs, I examined my data for measures of quality including whether they had a trend over time and how variable they were (Figure 5).

Internal concentration of CO_2 (C_i), the mole fraction of CO_2 in the sample air, and the net assimilation rate, served as the first indicators of data quality. C_i is a value calculated by the LI-COR based on stomatal and boundary layer conductance (which are themselves calculated from flow rates and the water mole fractions). Data points with negative values for C_i are impossible and I excluded them. Additionally, C_i should be less than the ambient concentration of CO_2 (C_a) if net photosynthesis measurements are positive due to the drawdown of CO_2. Therefore, I excluded positive net photosynthesis data points that had C_i values greater than C_a and negative net photosynthesis data points that had C_i values less than C_a. Respiration values should be negative, indicating a net efflux of carbon, and so I excluded respiration data points that were positive.

Second, in order to minimize the effects of leaks and drift, I examined the data for variability and whether there was a trend in the data over time. I did this by categorizing the data using the ratio of standard deviation to mean and the correlation coefficient of the net photosynthesis and dark respiration data versus the time over the period that they were recorded (Figure 5). The plots of all seedlings were then visually inspected for a

trend over time. The last 15 data points for photosynthesis and the last 14 for respiration were averaged if the standard deviation/absolute value of the mean ratio was less than 1, and the magnitude of the correlation coefficient of the time series was less than 0.2 and if the last 15 or 14 data points were not visual outliers in the data. Individuals with visual outliers were excluded because a sudden change in the last few data points would suggest that the seedling had not stabilized to LI-COR conditions. The selection process yielded 92 net photosynthesis and 96 dark respiration data points for further analysis (33 net photosynthesis and 30 dark respiration data points were excluded). Given the stringent criteria applied, the number of eliminated data points may have been larger than necessary, but my automated programs for net photosynthesis and dark respiration had recorded more data than was necessary and I wanted to be sure that my data were of a high quality.

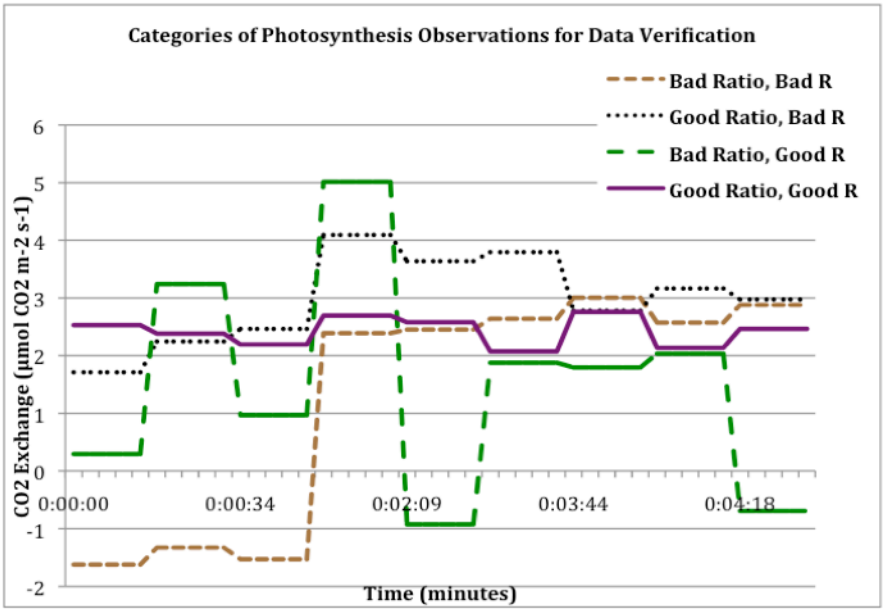

Figure 5. Categorization of net photosynthesis and dark respiration observations based on (1) the ratio of the standard deviation to mean (Ratio) and (2) the linear correlation coefficient versus time elapsed during measurement (R). Definitions: good ratios <1, good R<0.2. Depicted are four representative observations of the categories. The bad ratio, bad R example is 1.56, 0.88, respectively (cell 759, 5/25/10). The good ratio, bad R is 0.25 and 0.48, respectively (cell 959, 5/25/10). The bad ratio, good R is 1.19 and -0.16, respectively (cell 317, 6/3/10). The good ratio and good R, is 0.10 and 0.04, respectively (cell 003, 5/24/10).

Data Analysis

I carried out all the statistical tests in JMP version 8.0.1 (SAS Institute Inc., Cary, NC). A priori, all statistical tests used $p=0.050$ as a significance threshold and factors or co-variables that had $0.050<p<0.120$ were classified as marginally significant. The cutoff for marginal significance was greater than 0.1 so that I could include covariates that were slightly greater than 0.1. Prior to analysis, I tested for independence among trays and seedlings within trays and maternal effects, using tree as a factor, in the wide-ranging haplotype. I transformed data that were not normally distributed using the best fitting distribution according to the smallest value of Akaike's Information Criterion (AICc) to meet the assumptions for ANOVA (Akaike, 1987). Next, I tested the resulting transformations for normality using the Chi Squared test and for equality among variances of different groups using the Unequal Variance Test. The data that were normally distributed data also had equal variances and so I used ANOVA or ANCOVA models to test for significance. For those data that could not be transformed to a normal distribution, I used the non-parametric Median Test.

The net photosynthesis data were transformed using Generalized Log function, the stomatal conductance and dark respiration data sets were transformed using the Johnson SI distribution, and the ratio of net photosynthesis to dark respiration was transformed using the Johnson SU distribution (SAS Institute Inc., 2008). These distributions were the best fit according to the AICc values of each distribution. For each response variable, I formed a model based on my hypotheses (the hypothesized model) and then used a mixed stepwise regression to determine which factors best explained the variation in net photosynthesis, stomatal conductance and dark respiration (the best

model). To evaluate whether water availability had an indirect role on net photosynthesis, I used a mixed stepwise regression model to determine the relative importance of chamber, weekly average GWC and at-hour average temperature (i.e., the temperature of the growth chamber that a seedling was experiencing when selected for gas exchange measurement). I transformed the ratio of net photosynthesis to dark respiration using the Johnson SU distribution. I used the non-parametric Median Test to determine whether there was a significant difference in the number of seedlings with mean negative net photosynthesis values. I measured the gas exchange of 11 seedlings twice. For these seedlings, I alternated selecting the first and then the second measurement of a pair of repeated measurements and included them in the model, while excluding the remaining repeated measurements from the data set.

For the phenology data, I calculated the percent of seedlings that germinated, reached the seed coat shed stage and died. I calculated percent germination as the percentage of seedlings that successfully emerged through the soil. I used the non-parametric Median Test to determine whether the percentages differed by tree, haplotype or chamber. The seed mass data set was cube root-transformed and analyzed to test whether seed mass differed between tree seed sources. I evaluated phenology by analyzing number of days until emergence, seed coat shed or mortality using the nonparametric Median test.

For each morphological trait, I tested whether haplotype, chamber, seedling age and the interaction of haplotype and chamber was significant. I calculated total biomass from the dry mass of roots plus the dry mass of the shoots and transformed the data using the Johnson SI distribution. I calculated the ratio of root and shoot mass and transformed

it using the Johnson SU distribution. I calculated the ratio of root to shoot length and I transformed the data using the Johnson SI distribution. I took the maximum cotyledon length and plant height out of the three measuring dates. I transformed maximum cotyledon length using the Johnson SI distribution; plant height was normally distributed. I calculated SLA using the entire area and mass of the photosynthetic shoot, including the cotyledons and the green stem. I calculated leaf area assuming 0.1 cm for cotyledon width and for stem radius, based on previous seedling measurements (J.R. Wolf, unpublished data). I multiplied the product of the width, the lengths and the number of cotyledons and added the cylindrical area of the shoot. I calculated SRL by dividing the root length by the dry mass of the root and transformed SRL using the Johnson SI distribution.

RESULTS

Examining Confounding Factors

As the first step of data analysis, I identified potential confounding factors and tested their influence on the gas exchange data. I planned for the unit of replication to be at the level of cells within a tray. In order to test this, I determined whether tray, shelf or position within the tray significantly explained variation in the data using ANOVA tests. Within chambers, tray was not a significant factor in explaining either net photosynthesis or dark respiration. Location within the tray (i.e., whether a seedling was in the exterior or interior half of the tray) was a marginally significant factor and I included it in the hypothesized and main models. Shelf was not a significant factor. Experiment wide, the heated chamber average GWC was 22.7% smaller than the ambient chamber ($p<0.0001$), and so I included weekly average GWC as a covariate in the hypothesized model, although it is confounded with chamber temperature. At-hour average temperature is the average temperature recorded in the growth chamber during the hour from which a seedling was taken. Including at-hour average temperature in the model would indicate that the effect of chamber on seedling physiology was not due to temperature alone and so it was included in the hypothesized models for net photosynthesis and dark respiration. The two wide-ranging elevation seed sources, trees 1 and 5, were not significantly different from each other in terms of gas exchange (Table 1). Seedling age at time of measurement was of concern, since seedlings in the ambient growth chamber were on average 32 days younger at time of measurement than seedlings in the heated chamber

and gas exchange rates may vary inherently with seedling age. I included it as a covariate in the hypothesized model.

Table 1 Analysis of confounding factors. Gas exchange data were analyzed using single factor ANOVA tests. Significant factors ($p \leq 0.05$) are in bold, marginally significant factors ($0.05 < p < 0.12$) are italicized. Ps=net photosynthesis, R=dark respiration.

Dependent Variable	Factor	DF	SS	F Ratio	Prob>F
Ps	Trays 1-3	2	1.556	1.064	0.3543
	Trays 4-6	2	1.744	1.023	0.3675
	Shelf	2	0.112	0.054	0.9474
	Interior/Exterior Location	1	3.652	3.719	*0.0569*
	Tree Seed Source 1 vs 5	1	0.254	0.297	0.5879
R	Trays 1-3	2	1.846	1.845	0.1700
	Trays 4-6	2	0.452	0.481	0.6211
	Shelf	2	2.845	1.420	0.2469
	Interior/Exterior Location	1	0.320	0.314	0.5766
	Tree Seed Source 1 vs 5	1	0.224	0.206	0.6520

Factors Affecting Net Photosynthesis

The average photosynthetic rate in seedlings grown in the heated chamber (0.72 μmol CO_2 m^{-2} s^{-1}) was 68% less than the photosynthetic rate of seedlings grown in the ambient chamber (2.27 μmol CO_2 m^{-2} s^{-1}). The narrow-ranging haplotype had an average net photosynthetic rate (1.16 μmol CO_2 m^{-2} s^{-1}) that was 33% smaller than the wide-ranging haplotype (1.72 μmol CO_2 m^{-2} s^{-1}).

The hypothesized models were based on a list of factors, in order of importance, which I thought might affect net photosynthesis, stomatal conductance and dark respiration (Table 2). Despite the heat wave treatment the hypothesized model shows that net photosynthesis is not significantly different between weeks (before, during, and after the heat wave). Additionally, though water availability differed significantly between the chambers, weekly average GWC was not a significant factor in the hypothesized model. There is also no interaction between haplotype and chamber, indicating that haplotypes responded similarly to the temperature treatment.

The best model was created using a mixed stepwise regression model out of the hypothesized model and explains 28% of the variation in net photosynthesis (Table 3). Chamber temperature was significant ($p<0.0054$). Meanwhile, haplotype was marginally significant ($p=0.0511$). Two covariates were also significant: the number of days since the seedling lost its seed coat ($p=0.0441$) and whether the seedling was located in the interior half or the exterior half of the tray ($p=0.0329$). Additionally, at-hour average temperature was a marginally significant factor in the best model ($p=0.0895$), indicating that the temperature seedlings were being exposed to within the chambers may explain some variation in net photosynthesis (Figure 6).

Factors Affecting Stomatal Conductance

Heat chamber seedlings had an average stomatal conductance (0.01 mol H_2O m^{-2} s^{-1}) that was 72% smaller than ambient chamber seedlings (0.05 mol H_2O m^{-2} s^{-1}), likely mainly due to decreased water availability in the heated chamber. However, the factor that explains more of the variation in stomatal conductance is chamber and not GWC. In a mixed model stepwise regression, weekly average GWC is significant in explaining the variability in stomatal conductance only when chamber is locked out of the model ($p<0.0001$, adjusted $R^2=0.467$). When chamber is entered into the model it is significant ($p=0.0008$) and weekly average GWC is no longer significant ($p=0.9704$). In my hypothesized model, I found that stomatal conductance did not differ between haplotype, the interaction of haplotype and chamber, and GWC (Table 2). After running the hypothesized model through mixed stepwise regression, I found that chamber ($p<0.0001$) and week ($p=0.0296$) were significant. Figures 7 and 8 graphically depict the effects of chamber and weekly GWC on stomatal conductance and net photosynthesis using one linear regression model grouped across chambers (black line) and a second linear regression grouped within chambers (red and blue dashed lines). Regressions across chambers are significant ($p<0.0001$) but regressions within chambers are not. Thus, seedlings within chambers had similar stomatal conductance and net photosynthetic rate across a range of GWC values.

Factors Affecting Dark Respiration

The mean dark respiration rate in the heated chamber (-1.60 μmol CO_2 m^{-2} s^{-1}, where a negative value indicates net production of CO_2 by the leaf) was 65% less than the mean dark respiration rate in the ambient chamber (-4.53 μmol CO_2 m^{-2} s^{-1}, $p<0.0001$). The narrow-ranging haplotype had an average respiration rate (-2.88 μmol CO_2 m^{-2} s^{-1}) that was 11% smaller than the wide-ranging haplotype (-3.23 μmol CO_2 m^{-2} s^{-1}, $p=0.0480$).

Three factors were not significant in the hypothesized model for predicting dark respiration (Table 2). The interaction between haplotype and chamber was not significant, indicating that haplotypes responded similarly to the temperature treatments. Second, weekly average GWC was not significant, indicating that soil water availability had no effect in explaining dark respiration variation, even when chamber was locked out of the model. And last, at-hour average temperature was not significant, indicating that the most recent growth chamber temperature that seedlings experienced did not affect dark respiration measurements.

The best model explained 53% of the variation in dark respiration (Table 3). Chamber temperature was a highly significant factor in explaining the dark respiration rate ($p<0.0001$). The respiration rates of the two haplotypes were significantly different ($p=0.0480$). Unlike the results for net photosynthetic rate, week was a significant factor in explaining respiration rate ($p=0.0002$). A linear fit to the data shows that respiration rates increased over time by 0.45 μmol CO_2 m^{-2} s^{-1} per week (Figure 9). Seedling age (in days from date of soil breakthrough) was also a significant covariate in explaining dark

respiration and Figure 10 shows how dark respiration values decreased with increasing seedling age (p=0.0088).

Net Photosynthesis to Dark Respiration Ratio

The ratio of net photosynthesis to dark respiration was not significantly different between chambers (p=0.1795), haplotypes (p=0.8084) or the interaction of haplotype and chamber (p=0.5647).

Table 2 Hypothesized factors to explain variation in net photosynthesis and dark respiration. Certain factors were hypothesized to explain the observed variation in net photosynthesis and dark respiration of limber pine seedlings and included these in full models. Significant factors (p<0.050) are in bold, marginally significant factors (p<0.120) are italicized.

Dependent Variable	Factor	DF	SS	F Ratio	Prob > F	n	Model Adjusted R^2
Net Photosynthesis	**Chamber**	1	4.51	6.06	**0.0159**	92	0.273
	Haplotype	1	3.53	4.74	**0.0323**		
	Haplotype*Chamber	1	0.18	0.24	0.6249		
	Week	1	0.94	1.26	0.2653		
	Weekly Avg GWC	1	0.65	0.87	0.3527		
	# Days without Seed Coat	1	4.11	5.52	**0.0212**		
	Location Within Tray	1	3.69	4.96	**0.0286**		
	At-hour Avg Temperature	1	3.61	4.85	**0.0304**		
Stomatal Conductance	**Chamber**	1	5.03	12.04	**0.0008**	93	0.549
	Haplotype	1	0.53	1.26	0.2647		
	Haplotype*Chamber	1	0.24	0.58	0.4469		
	Week	1	2.17	5.20	**0.0251**		
	Weekly Avg GWC per tray	1	0.13	0.32	0.5722		
	Location Within Tray	1	1.10	2.62	*0.1091*		
Dark Respiration	**Chamber**	1	4.16	1.82	**0.1814**	96	0.522
	Haplotype	1	8.12	3.55	*0.063*		
	Haplotype*Chamber	1	1.68	0.73	0.3942		
	Week	1	22.10	9.65	**0.0026**		
	Weekly Avg GWC per tray	1	0.76	0.33	0.5671		
	# Days since Emergence	1	16.19	7.07	*0.0093*		
	At-hour Avg Temperature	1	0.38	0.17	0.6856		

Table 3 Best model to explain variation in net photosynthesis and dark respiration. I used a mixed model stepwise regression to generate the Best Model from the full Model to explain net photosynthesis and dark respiration of limber pine seedlings. Significant factors ($p<0.050$) are in bold, marginally significant factors ($p<0.120$) are italicized.

Dependent Variable	Factor	DF	SS	F Ratio	Prob > F	n	Model Adjusted R^2
Net Photosynthesis	**Chamber**	1	6.01	8.14	**0.0054**	92	0.279
	Haplotype	1	2.89	3.91	*0.0511*		
	# Days without Seed Coat	1	3.08	4.18	**0.0441**		
	Location Within Tray	1	3.47	4.70	**0.0329**		
	At-hour Avg Temperature	1	2.18	2.95	*0.0895*		
Stomatal Conductance	**Chamber**	1	46.03	110.12	**<.0001**	93	0.549
	Week	1	2.04	4.89	**0.0296**		
Dark Respiration	**Chamber**	1	57.58	25.58	**<.0001**	96	0.530
	Haplotype	1	9.04	4.02	**0.048**		
	Week	1	34.19	15.19	**0.0002**		
	# Days since Emergence	1	16.12	7.16	**0.0088**		

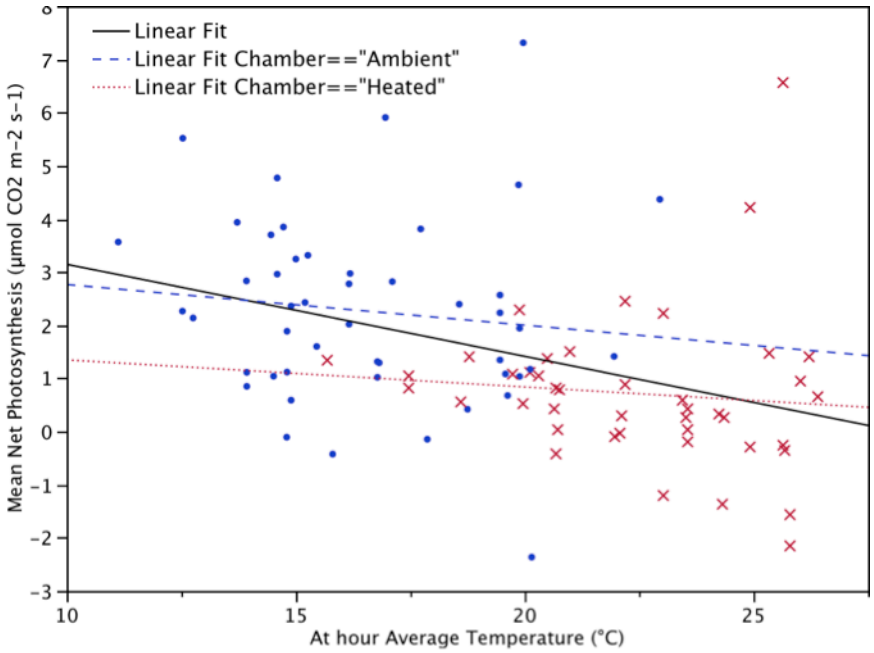

Figure 6. Average net photosynthesis versus at-hour average temperature. The solid line shows a linear regression across chambers. The patterned lines show linear regression lines for each chamber (see inset key). Heated chamber data are X's, while ambient chamber data are dots. Regression line equations are:
Ps (across chambers) = 4.8814654 - 0.1732845*Temperature, $p<0.0001$, $R^2_{adj}=0.14$
Ps (ambient chamber) = 3.5293905 - 0.0763769*Temperature, $p=0.4269$, $R^2_{adj}=0.00$
Ps (heated chamber) = 1.856531 - 0.0508669*Temperature, $p=0.5350$, $R^2_{adj}=0.00$

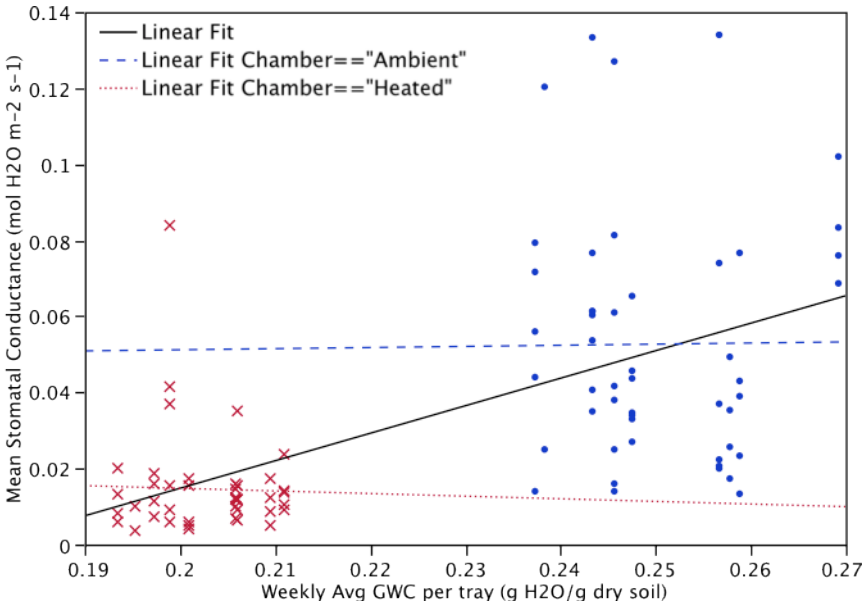

Figure 7. Mean stomatal conductance versus weekly average GWC per tray. The solid line shows a linear regression across chambers. The patterned lines show linear regression lines for each chamber (see inset key). Heated chamber data are X's, while ambient chamber data are dots. Regression line equations are:
Conductance (across chambers) = -0.126355 + 0.7089689*GWC, p<0.0001, R^2_{adj}=0.33
Conductance (ambient chamber) = 0.0452786 + 0.0295791*GWC, p=0.9536, R^2_{adj}=0.00
Conductance (heated chamber) = 0.0285592 - 0.0689102*GWC, p=0.8470, R^2_{adj}=0.00

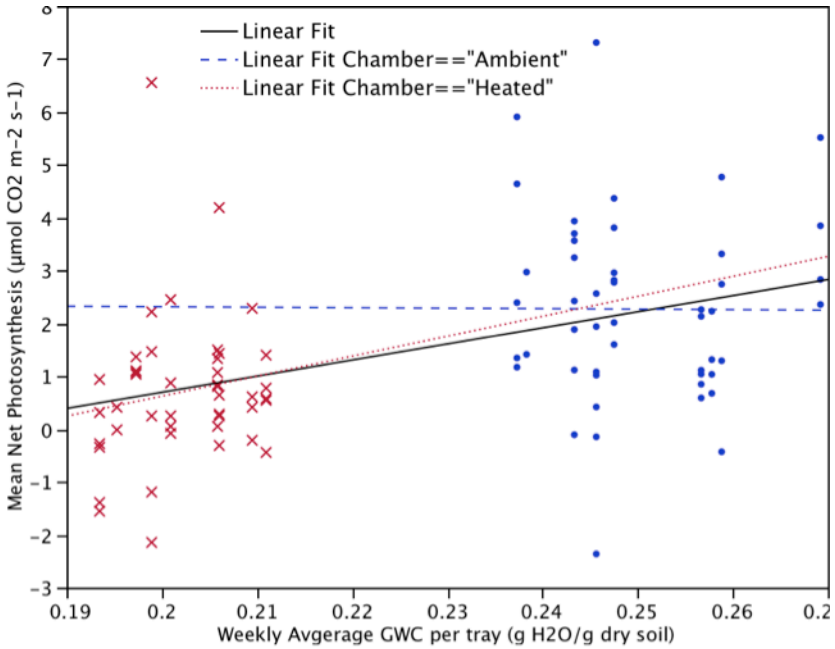

Figure 8. Average net photosynthesis versus weekly average GWC per tray. The solid line shows a linear regression across chambers. The patterned lines show linear regression lines for each chamber (see inset key). Heated chamber data are X's, while ambient chamber data are dots. Regression line equations are:
Ps (across chambers) = -5.17258 + 29.535763*GWC, p<0.0001, R^2_{adj}= 0.17
Ps (ambient chamber) = 2.5008013 - 0.9154952*GWC, p=0.9739, R^2_{adj}=0.00
Ps (heated chamber) = -6.925994 + 37.759839*GWC, p=0.3142, R^2_{adj}=0.00

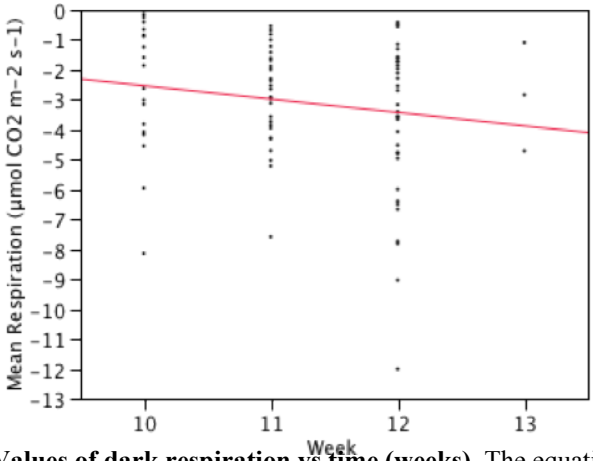

Figure 9 Values of dark respiration vs time (weeks). The equation of the linear fit is Mean Respiration = 1.96 - 0.45*Week, p=0.0908, R^2_{adj}=0.02.

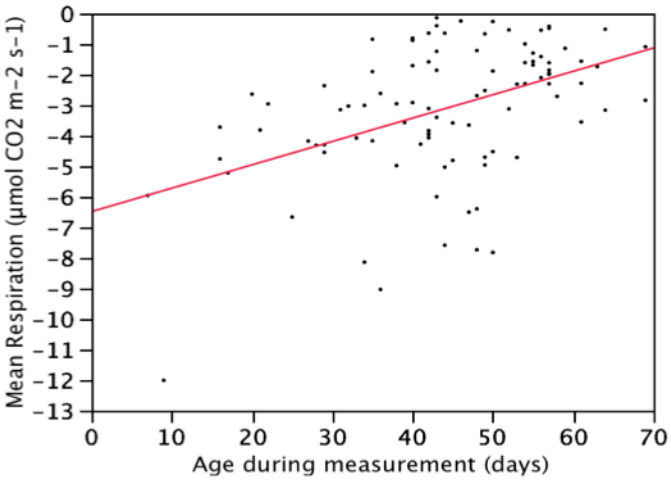

Figure 10 Values of dark respiration vs seedling age at time of measurement. The equation of the linear fit is Mean Respiration = -6.48 + 0.08*Days since emergence, p<0.0001, R^2_{adj}=0.20.

Seedling Germination, Development and Mortality

Thirty-three percent of seedlings died in the heated chamber, while 5% died in the ambient chamber. There was no significant difference in germination rate between the chambers (p=0.6469), however significantly more seedlings reached the seed coat shed stage and died in the heated chamber compared to the ambient chamber (p=0.0013 and p<0.0001, respectively, Figure 11).

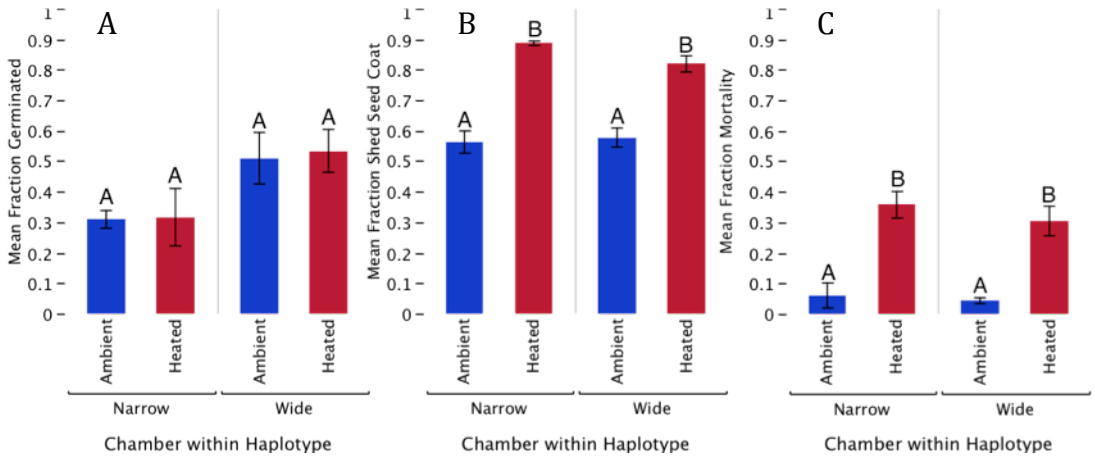

Figure 11 Developmental stages by haplotype and chamber. (A) Percent germination is the percent of seeds planted that germinated. (B) Percent Shed Seed Coat is the percent of germinated seedlings that developed sufficiently to shed their seed coat. (C) Percent mortality is the percent of germinated seedlings that developed a brown, shriveled appearance.

Nine percent of seeds germinated but failed to break through the soil, but there were no significant differences in the rate of soil breakthrough failure between haplotypes or chambers. Median tests revealed that tree 5, a wide-ranging haplotype, had significantly higher germination rates than trees 1 and 3 (p=0.0122) and that it also had greater rates of soil breakthrough failures (p=0.0084). There were no significant differences between the trees in terms of shedding seed coats or in mortality. Tree 5 had a

23% and a 24% smaller average seed mass than trees 1 and 3, respectively (Tukey's HSD test, $p<0.0001$). Haplotypes were marginally significantly different in germination rates ($p=0.0519$), but not different in rates of shedding seed coats or in mortality, likely due to the greater germination rates in tree 5.

Phenology

Seedlings in the heated chamber germinated a median of 14 days earlier ($p<0.0001$) and reached the seed coat shed stage a median 25 days earlier ($p<0.0001$). For those seedlings that died, seedlings in the heated chamber had a median lifespan, from emergence to mortality, which was 16 days longer than seedlings in the ambient chamber ($p=0.0428$). There was no significant difference in phenology between haplotypes or trees.

In order to capture development trends with time, I calculated percent weekly mortality and percent cumulative germination for seedlings within each chamber (Figure 12). Percent weekly mortality is the number of number of seedlings that died in a given week out of the cumulative number of seedlings that had germinated. Percent cumulative germination is the number of seedlings that germinated out of the 200 total seeds of each tree type planted in a growth chamber. In the heated chamber, the number of new seedlings categorized as dead peaked at week ten and then declined dramatically by week 12. In the ambient chamber, percent weekly mortality for tree 3 reached a much smaller peak (3.5%) at week 9 and dropped back to zero by week 11. For the wide-ranging haplotypes (trees 1 and 5) in the ambient chamber, percent weekly mortality peaked 1

week later than in the heated chamber. Percent cumulative germination reached its peak in the heated chamber in week 6 and in week 12 in the ambient chamber.

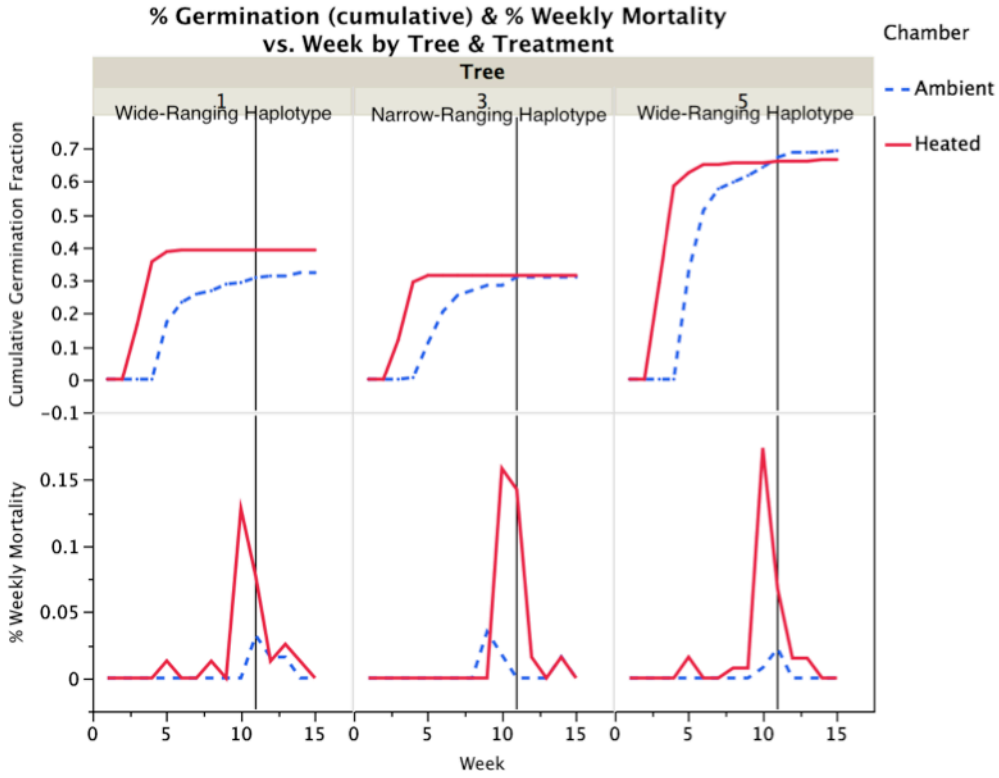

Figure 12 Cumulative germination and weekly mortality rate by chamber temperature and tree. % Weekly Mortality is the number of seedlings that died in a given week out of the cumulative number of seedlings that had germinated. % Cumulative Germination is the % of seedlings that germinated out of the total number of seeds of a given type planted in a growth chamber. Trees 1 and 5 are the wide-ranging haplotype, tree 3 is the narrow-ranging haplotype. The vertical line shows the heat wave week during

Morphological characters

Average total biomass increased with seedling age ($p<0.0001$). Since seedlings in the heated chamber were older, they on average had 0.007 g greater total biomass, but the difference between chambers was not significant when seedling age was included as a covariate in the model. Additionally, for total biomass, there was no significant difference between haplotypes and there was no interaction of haplotype and chamber (Table 4). Total biomass is positively correlated with net photosynthesis ($p<0.0001$, Adjusted $R^2=0.17$, Figure 13).

The root to shoot mass ratio was significantly different between haplotypes ($p=0.0371$) and chambers ($p=0.0035$); the root to shoot mass ratio for the narrow-ranging haplotype (0.94:1) was 7.9% smaller than the wide-ranging haplotype (1.02:1). Seedlings in the ambient chamber had a root to shoot mass ratio (0.97:1) that was 6.5% less than the mass ratio in the heated chamber (1.04:1). There was no interaction of haplotype and chamber and seedling age was not significant in explaining root to shoot mass.

The interaction between haplotype and chamber was significant for root to shoot length ($p=0.0048$, Table 4): the wide-ranging haplotype had approximately the same root to shoot length in the ambient chamber as the narrow-ranging haplotype but it decreased its root to shoot length in the heated chamber. Seedling age was not a significant factor in explaining root to shoot length.

Week of germination was a significant factor for maximum needle length ($p<0.0001$, Table 4); seedlings that germinated early had longer maximum needle lengths. The interaction of haplotype and chamber was marginally significant ($p=0.0766$); the trend was that narrow-ranging seedlings had longer needle lengths in the ambient

chamber than in the heated chamber, while the wide-ranging had shorter needle lengths in the ambient chamber compared to the heated chamber.

Two factors, the interaction of haplotype and chamber and week of germination, were significant for maximum plant height ($p=0.0174$ and $p<0.0001$, respectively, Table 4). The wide-ranging haplotype overall had a greater maximum plant height than the narrow-ranging haplotype but its maximum plant height increased in the heated chamber compared to the ambient chamber. Plant height increased as seedling age increased.

SLA, calculated at the whole shoot level (photosynthetic area of stem+cotyledons/shoot mass), was significantly different between haplotypes ($p=0.0391$) and chambers ($p=0.0060$, Table 4). The narrow-ranging haplotype had a SLA (9.82 m^2/kg) that was 8.2% smaller than the wide-ranging haplotype (10.70 m^2/kg). Seedlings in the ambient chamber had an average SLA (9.98 m^2/kg) that was 8.9% smaller than the heated chamber (10.95 m^2/kg). The interaction of haplotype and chamber and week of germination were not significant factors.

The interaction of haplotype and chamber was the only factor that was significant for SRL ($p=0.0403$, Table 4). The interaction was such that the wide-ranging haplotype had a greater SRL in the ambient chamber than in the heated chamber, and the pattern was reversed for the narrow-ranging haplotype.

Table 4 Tests for whether various functional characters can be explained by haplotype or chamber. Factors were tested for significance using ANCOVA. Week of germination is a proxy for seedling age.

Dependent Variable	Factor	DF	SS	F Ratio	Prob > F	n	Model Ajusted R^2
Total Biomass	Haplotype	1	0.051	0.062	0.8033	493	0.044
	Chamber	1	1.131	1.380	0.2407		
	Week of Germination	1	12.925	15.764	**<.0001**		
	*Haplotype*Chamber*	1	2.693	3.285	*0.0705*		
Root to Shoot Mass	**Haplotype**	1	3.925	4.371	**0.0371**	493	0.071
	Chamber	1	7.709	8.585	**0.0035**		
	Week of Germination	1	2.577	2.870	*0.0909*		
	Haplotype*Chamber	1	0.167	0.186	0.6667		
Root to Shoot Length	**Haplotype**	1	5.301	5.675	**0.0176**	489	0.072
	Chamber	1	6.380	6.830	**0.0092**		
	Week of Germination	1	0.283	0.303	0.582		
	Haplotype*Chamber	1	7.514	8.044	**0.0048**		
Max Needle Length	*Haplotype*	1	2.299	2.432	*0.1195*	491	0.096
	Chamber	1	0.421	0.445	0.5049		
	Week of Germination	1	27.415	29.001	**<.0001**		
	*Haplotype*Chamber*	1	2.976	3.149	*0.0766*		
Max Plant Height	**Haplotype**	1	1.717	11.172	**0.0009**	491	0.230
	Chamber	1	1.887	12.282	**0.0005**		
	Week of Germination	1	3.577	23.277	**<.0001**		
	Haplotype*Chamber	1	0.875	5.694	**0.0174**		
Specific Leaf Area	**Haplotype**	1	67.786	4.282	**0.0391**	483	0.020
	Chamber	1	120.394	7.605	**0.006**		
	Week of Germination	1	3.691	0.233	0.6294		
	Haplotype*Chamber	1	15.688	0.991	0.32		
Specific Root Length	Haplotype	1	0.008	0.009	0.9261	489	0.019
	Chamber	1	1.181	1.197	0.2745		
	Week of Germination	1	0.013	0.014	0.9074		
	Haplotype*Chamber	1	4.174	4.229	**0.0403**		

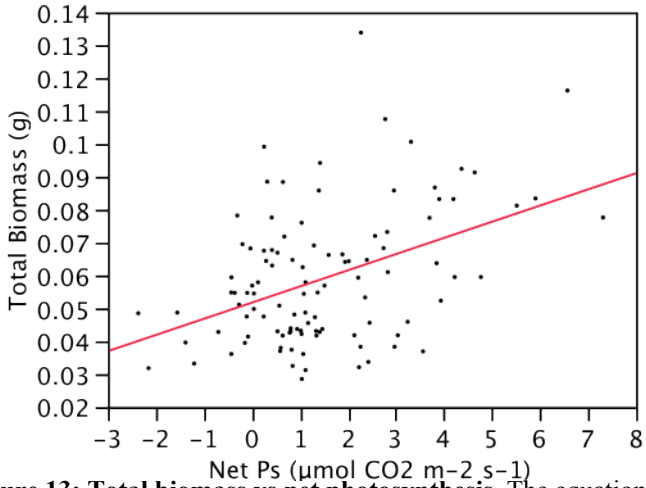

Figure 13: Total biomass vs net photosynthesis. The equation of the linear fit is Total Biomass = 0.052 + 0.005*Mean Ps, $p<0.0001$ $R^2_{adj}=0.17$.

DISCUSSION

Overview

The simulated climate change of the heated growth chamber (increased temperatures and decreased water availability (unintentional)) resulted in seedlings with decreased net photosynthesis and dark respiration, decreased the time until germination, increased mortality, and altered carbon allocation to roots and shoots and SLA. The general lack of interaction of haplotype and chamber for gas exchange and morphology data (except for root to shoot length, plant height and SRL) indicated that the haplotypes responded similarly to the simulated climate change. However, haplotypes did have differences in their gas exchange rates, root to shoot mass and SLA. There was no detectable effect of the 3°C heat wave on seedling gas exchange. Unexpected findings were an increasing trend in dark respiration rates over time and that haplotype had a marginal effect on net photosynthesis in the best model generated by mixed stepwise regression. Contrary to my hypothesis, germination rates were not different between chambers and haplotypes did not have different rates of mortality. Given the 5-fold increase in mortality in the heated chamber (33% of the seedlings died in the heated chamber vs 5% in the ambient chamber), heated chamber conditions were notably harsher than in the ambient chamber. However, surviving heated chamber seedlings, despite lower rates of gas exchange, accumulated more biomass and had greater plant heights and needle lengths than the ambient chamber, suggesting that their growth was stimulated by heated conditions.

Why did chamber temperature have a large effect on gas exchange?

Chamber was a significant factor for net photosynthesis, stomatal conductance and dark respiration. It is likely that differences in gas exchange rates measured at a common temperature were large due to the seedlings acclimating to chamber conditions. Acclimation is the morphological or physiological adjustment that individuals make to compensate for a decline in a biological process due to an external factor (Teskey and Will, 1999; Lambers et al., 2008). Temperature, water and light availability all affect the acclimation of gas exchange rates in plants and likely all played a role in this experiment.

Rates of net photosynthesis and dark respiration across chambers show evidence for thermal acclimation. The presence of thermal acclimation in plants can be determined in several ways (Loveys et al., 2002). Plants that developed under differing growth temperatures are acclimated if (1) when measured at their respective growth temperatures, net photosynthesis and dark respiration rates are similar between cold-grown and warm-grown plants, (2) when measured at a common temperature, the ratio between net photosynthesis and dark respiration rates is similar between cold-grown and warm-grown plants, or (3) when measured at a common temperature, the rates of net photosynthesis and dark respiration are lower in plants developed under a warmer growth temperature, and vice versa (Loveys et al., 2002; Loveys et al., 2003; Atkin and Tjoelker, 2003; Atkin et al., 2006). The seedlings in this experiment were grown at two temperature regimes that fluctuated diurnally but were always 5°C apart, allowing them the potential to fully acclimate to chamber conditions (Loveys et al., 2003; Atkin and Tjoelker, 2003, Atkin et al., 2006, Ow et al., 2008). I did not measure the gas exchange of seedlings at their respective growth temperatures and cannot determine whether criteria

(1) applied. Instead, I measured seedlings at a common temperature (26°C) and found support for criteria (2) and (3). The ratio of net photosynthesis to dark respiration did not differ between chambers when measured at 26°C ($p=0.1795$), similar to the findings of Gifford (1995), Dewar et al. (1999**),** Loveys et al. (2002), Campbell et al. (2007), and Ow et al. (2010). Additionally, ambient chamber seedlings had higher rates of net photosynthesis and dark respiration at 26°C than did heated chamber seedlings. This finding is in line with trends in the literature that show that cold-grown plants have greater photosynthetic and respiratory capacities than warm-grown plants when measured at common temperatures (Arnone and Körner, 1997; Teskey and Will, 1999; Loveys et al., 2003; Luomala et al., 2003; Atkin et al., 2006).

In this experiment, there is also evidence for acclimation to water availability. However, the effect of water availability is confounded with the temperature effect of the growth chambers and so it is difficult to make direct linkages. Low stomatal conductance suggests that seedlings experienced water stress (Letts et al., 2009). The average stomatal conductance in the heated and ambient chambers was 0.015 mol H_2O m^{-2} s^{-1} and 0.05 mol H_2O m^{-2} s^{-1}, respectively. However, Figure 7 depicts how, within chambers, stomatal conductance did not change with increasing water availability, suggesting that seedlings had acclimated by limiting their stomatal conductance to match the water availability in each chamber.

Limited water availability likely reduced photosynthetic rates. Under water stress, decreased stomatal and mesophyll conductance limits the diffusion of CO_2, which in turn limits photosynthesis and respiration (Dewar et al., 1999; Warren et al., 2004; Atkin et al., 2006; Lambers et al. 2008). For example, Wertin et al. (2010) found that *P. taeda* L.

seedlings with restricted water availability had significantly lower rates of net photosynthesis compared to seedlings with high water availability. Under extreme water stress, (i.e., when stomatal conductance falls below 0.15-0.20 mol H_2O $m^{-2}s^{-1}$), metabolic failure of photochemistry/biochemistry is thought to occur, generally across plant species (Grassi and Magnani, 2005; Flexas et al., 2006; Galmés et al., 2007). This is a non-stomatal limitation on photosynthesis, in which photosynthetic capacity is down-regulated (Lambers et al., 2008). Because stomatal conductance in both chambers approximate the range of extreme water stress, it is likely that seedlings in both chambers down-regulated their photosynthetic capabilities.

Water availability also interacts with thermal acclimation (Rodríguez et al., 2009). Though not a significant factor on its own and demonstrated to be secondary to chamber through mixed stepwise regression, water availability could have had indirect effects in exacerbating the effect of temperature and low light availability. Furthermore, limited water availability in the chambers could have been the primary cause of seedling mortality. Due to limited rooting volume, seedlings are particularly vulnerable to hydraulic failure through excessive cavitation (McDowell et al., 2008).

Why was haplotype a weak factor in explaining gas exchange?

In contrast to the large effects that chamber temperature had on net photosynthetic rate and dark respiration, haplotype had a smaller effect on gas exchange. The wide-ranging haplotype had higher rates of dark respiration and marginally higher rates of net photosynthesis. The small difference between haplotypes may reflect a common ability to acclimate among different populations (Teskey and Will, 1999). Other studies have

demonstrated that genetic background has had similar weak effects in explaining physiological differences. Tjoelker et al. (2008) found no correlation between the climate-origin of 20 populations of *P. banksiana* individuals growing together in common gardens and acclimated respiration rate, suggesting that the respiration rate was not differentiated among climatic or geographic clines. Cantin et al. (1997) similarly found that there was no difference in the net photosynthesis or growth of seedlings in 15 maternal families of *P. banksiana* L. in response to elevated temperature and CO_2. In *P. taeda* L. seedlings, Teskey and Will (1999) also found that there were no differences in net photosynthesis between provenances and families. However, there were significant differences in respiration rates between regions, with higher rates of respiration occurring in seedlings coming from warmer regions, when measured under a common temperature.

Why did the heat wave have no effects on net photosynthesis or dark respiration?

Net photosynthesis did not change significantly in either chamber over time as was expected. The hypothesis was that net photosynthetic rates would decrease during the heat wave and rebound back to normal levels during the recovery week. The lack of effect could be due to the small magnitude of the temperature increase (3°C) relative to the temperature variability experienced by the seedlings even over their short lifetimes. Seedlings likely can handle such temperature fluctuations because they are exposed to a wide range of temperatures on a diurnal basis. At 2 m above the ground, temperatures ranged from -12 to 27°C at the Ameriflux Tower during the 2008 growing season. Temperatures in the growth chambers had a smaller range: in the ambient chamber, temperatures fluctuated from 6 to 20°C diurnally and, experiment-wide, from 0 to 20°C.

Limber pine has been shown to have a wide temperature response curve, with its optimum at 15°C, it can tolerate temperatures across 10-35°C with only a 12% deviation from its maximum photosynthetic rate (Lepper, 1980). Further, the net photosynthesis of seedlings of another species, *Pinus taeda* L., was insensitive to a season-wide 2°C increase in temperature, possibly due to a broad temperature optimum (Wertin et al., 2010).

I also did not observe a decrease in respiration rates during the heat wave week as I had hypothesized. Respiration rates can acclimate within 1-2 days (Atkin and Tjoelker, 2003), so it is possible that early on during the heat wave week, seedlings acclimated to small temperature increase of 3°C, resulting in no significant change in respiration rates. While at-hour average temperature was a marginally significant factor for net photosynthesis, it was not significant for dark respiration. This lack of effect suggests that the dark respiration of the seedlings was similar to that of other species in being quick to adjust to temperature changes. However, there was an overall significant increasing trend in dark respiration across the measurement weeks. Dark respiration values increased in magnitude by 0.45 μmol CO_2 m^{-2} s^{-1} per week (Figure 9). I observed this effect while at the same time, as seedling age increased, the magnitude of dark respiration decreased (Figure 10). The measurement weeks took place towards the end of the simulated growing season, and after the heat wave, average temperatures decreased by 1°C per week. Seedlings may have been acclimating to decreasing temperature by increasing respiration rates (Atkin and Tjoelker, 2003, Tjoelker et al., 2008; Ow et al., 2010). For example, Tjoelker et al. (2008) found that the foliar respiration of *Pinus banksiana* increased when air temperatures decreased across the season.

Why did tree 5, a wide-ranging haplotype, have higher rates of germination?

Tree 5 had a significantly smaller seed mass than trees 1 and 3 and it also had greater germination rates. The observed differences in the seed mass and germination rate of tree 5 could be due to maternal effects, though replication for the wide-ranging haplotype was 2 and replication for the narrow-ranging haplotype was 1. Climatic conditions during seed development could have effects on seed mass and epigenetic effects on seedling growth and phenology (Aitken et al., 2008). Originating from the same site likely decreased most of the variation among seeds but microclimatic conditions could have favored tree 5. Reinhardt et al. (in revision) found that low provenance seedlings had a seed mass that was approximately 10% smaller but had greater rates of germination and survival. Norgren (1996) found that though *Pinus contorta* seedlings had a smaller seed mass, they accumulated more biomass at the end of a 4-year growing period than did *Pinus sylvestris* L., seedlings. In contrast, Oleksyn et al. (1998) found that *Picea abies* seed mass decreased as elevation of origin increased. In this study, despite higher germination rates, there were no differences in the percent of seedlings reaching the seed coat shed stage or in mortality, suggesting that the differences between maternal trees did not correlate with seedling development or gas exchange.

What are the impacts of a shifting phenology?

I designed this experiment to mimic a growing season from snowmelt to first frost. In this time, approximately the same number of seedlings germinated in both chambers but more seedlings in the heated chamber shed their seed coats and died. Additionally, seedlings in the heated chamber germinated a median 2 weeks earlier and

shed their seed coats 25 days earlier. Unexpectedly, heated chamber seedlings also survived longer; they had a median age at death that was greater than in the ambient chamber.

If the shifts in phenology observed in this experiment held true for field conditions under climate change, earlier emergence in the growing season might mean more seedling mortality due to early or late season frost occurrences or to greater environmental exposure such as droughts (Holtmeier and Broll, 2007). Intervals between precipitation events may lengthen and the longer duration from snowmelt to first autumn frost would mean that the soil would have more time to desiccate (Boisvenue and Running, 2006). It is unclear whether growth cessation also shifts in limber pine. For plants in general, growth onset is cued by temperature, as temperatures rise, growth begins earlier and growth cessation is cued by shortening photoperiod and in some species by high or low temperatures (Hänninen and Tanino, 2011). If increasing temperatures does not shift growth cessation in limber pine, the species may experience a longer growing season. In the absence of drastic weather events, this experiment suggests that a longer growing season can benefit conifer seedlings. At the end of the experiment, the surviving heated chamber seedlings, despite having lower average rates of gas exchange, had greater total biomass, longer needle lengths and a greater plant height than seedlings in the ambient chamber (Table 4). This suggests that seedlings grew at a slower pace than ambient chamber seedlings (their carbon balance was near zero) but they grew for longer, resulting in larger seedlings at the end of the simulated growing season.

Effects of chamber and haplotype on morphological plasticity

Various morphological attributes differed between chambers, suggesting that both haplotypes are plastic in their phenotypes. I had expected haplotypes to vary morphologically but had not anticipated the effect of chamber temperature on morphology. Chamber had significant effects on the mass ratio of roots to shoots and SLA. The shifted biomass allocation to favor roots in the heated chamber seedlings is similar to the findings of Cantin et al. (1997). The authors found that *P. banksiana* L. seedlings grown in at 4°C elevated temperatures invested more biomass in their roots than shoots. In contrast, Teskey and Will (1999) found that foliage biomass of *P. taeda* seedlings increased with a 5°C increase in temperature. These differing findings are likely due to differing species responses since carbon allocation is genetically controlled (Retzlaff et al., 2001). SLA of seedlings was greater in the heated chamber. Similarly, other studies have found that the SLA increased as growth temperature increased (Woodward 1979; Loveys et al., 2002). Because cell division and growth is temperature dependent, warmer temperatures likely stimulated shoots to grow, resulting in a greater leaf area per unit mass (Woodward, 1979; Hänninen and Tanino, 2011).

Haplotypes had fewer morphological differences between them than did chambers, but the haplotype differences suggest that some of their functional characters may be locally adapted to different regions. Despite factors minimizing phenotypic differences such as their seeds originating from the same site and being grown under the same growth chamber conditions, seedlings from the narrow-ranging haplotype were less photosynthetically and metabolically active, had more massive roots compared to their shoots and had a smaller SLA. The narrow-ranging haplotype has been found only above

3000 m (J.B. Mitton, personal communication) and the differences in traits may be a result of the narrow-ranging haplotype being adapted to high elevations. For example, low rates of gas exchange and a small SLA might reflect that they are adapted to a high light environment. Since light is not a limiting resource at high elevations near treeline, these seedlings may be predisposed to invest less mass in shoot tissue and to invest more in root tissue.

If these differences were due to the haplotypes being adapted to differing elevations, I would expect them to have shown similar morphology patterns as seedlings from high and low elevation populations of a common haplotype. Similar to the findings of this experiment where the narrow-ranging haplotype had a smaller SLA, New Zealand tree species growing at high elevations have a smaller SLA than trees growing at lower elevations (Körner, 1986). A smaller SLA may be adaptive to stressful conditions, while a larger SLA fosters a greater short-term relative growth rate and greater carbon uptake (Shipley and Vu, 2002; Bansal and Germino, 2010). The wide-ranging haplotype having greater rates of net photosynthesis and dark respiration and a larger SLA is similar to the finding of Rehfeldt et al. (2002), who found that populations originating from warmer climates had greater growth potentials than did populations originating from colder ones. Thus, the wide-ranging haplotype may be faster growing than the narrow-ranging haplotype, which may be more stress adapted. More support comes from two common garden experiments, in which SLA remained constant or decreased with increasing elevation of seed origin in *P. menziesii*, *P. ponderosa* and *P. abies* (Zhang and Marshall, 1995; Oleksyn et al., 1998). However, contrary to the results of this experiment, another common garden experiment found that a high elevation provenance of the wide-ranging

haplotype of limber pine had a greater SLA and a greater root to shoot mass than did a low elevation provenance of the wide-ranging haplotype (Reinhardt et al., in revision). Similarly, Schoettle and Rochelle (2000) found that SLA increased in natural populations of limber pine with increasing elevation; however the authors infer that this trend may be due to less stress experienced at the higher elevation. Given contradictory results in the literature, the narrow-ranging haplotype is like some high elevation ecotypes and unlike others.

The phenotypic differences between haplotypes are small in magnitude. These small differences are statistically significant but may not be biologically relevant. Assuming that they are maintained over time, small percentage changes in the height of young trees are magnified exponentially when the differences are extrapolated to volumes of wood per unit area (Rehfeldt et al., 2002). It may be the case that these small differences in root to shoot mass and length and total biomass be indicative of large future differences between trees (e.g., Strauss and Ledig, 1985). Additionally, the observed differences between growth chambers shows that limber pine is fairly phenotypically plastic and that the environment (temperature) had a larger effect on phenotype expression than inherent genetic variation (haplotype). However, genetic variation might have played a larger role if this experiment had incorporated seeds from more haplotypes and a higher replication of each haplotype.

Integration of gas exchange with morphological characters

In order to concisely integrate the variables I measured in this experiment, I have constructed a hypothesized diagram showing the cascade of effects (Figure 14). I

hypothesized that abiotic factors, such as water availability, air temperature, light availability and soil nitrogen (a probable factor since I used native soil without fertilizer), began the cascade of effects. I also hypothesized that the factor of most biological importance was survival and that the morphological variables I measured were intermediary between the abiotic factors and seedling survival. Three variables directly impact survival: water availability, net carbon uptake and total biomass. I hypothesized that low water availability negatively impacts seedling survival because it increases the likelihood of excessive cavitation and tissue desiccation (McDowell et al., 2008). A low net carbon uptake puts seedlings at risk of carbon starvation and therefore would also increase mortality. A low net carbon uptake also slows down the accumulation of biomass. I found that heated chamber seedlings had a lower carbon balance than ambient chamber seedlings but that they had accumulated more biomass. This contradiction is resolved when I take seedling age into account; heated chamber seedlings were older and therefore had more time to slowly accumulate biomass. Having a greater total biomass increases the likelihood of survival because it is likely that seedlings with a greater total biomass also have more stored carbon for use in maintenance, growth and during periods of stress when carbon uptake declines (Retzlaff et al., 2001). For example, seedling survival past the first growing season was positively linked to first year biomass accumulation in five species of hardwood seedlings (Walters and Reich, 1996).

Relating short-term gas exchange measurements to long-term growth, or the accumulation of biomass, has been the subject of numerous studies. The majority found a slight positive correlation of net photosynthesis with growth (Greenwood and Volkaert, 1992). I found a positive correlation supporting this literature finding (Figure 13).

However, because gas exchange measurements are momentary and do not represent the long-term carbon balance of the plant, plant growth and gas exchange measurements can become uncoupled (Körner, 2006). However, Poorter and Remkes (1990) found a stronger association between the leaf area ratio (i.e., the ratio of leaf area and total plant mass) and the relative growth rate (grams added of new plant per grams existing plant) than they found between net assimilation rate (the difference between photosynthesis and respiration per unit leaf area) and the relative growth rate. Heated chamber seedlings had more shoot and leaf area than ambient chamber seedlings and so this could have contributed to their greater accumulation of total biomass.

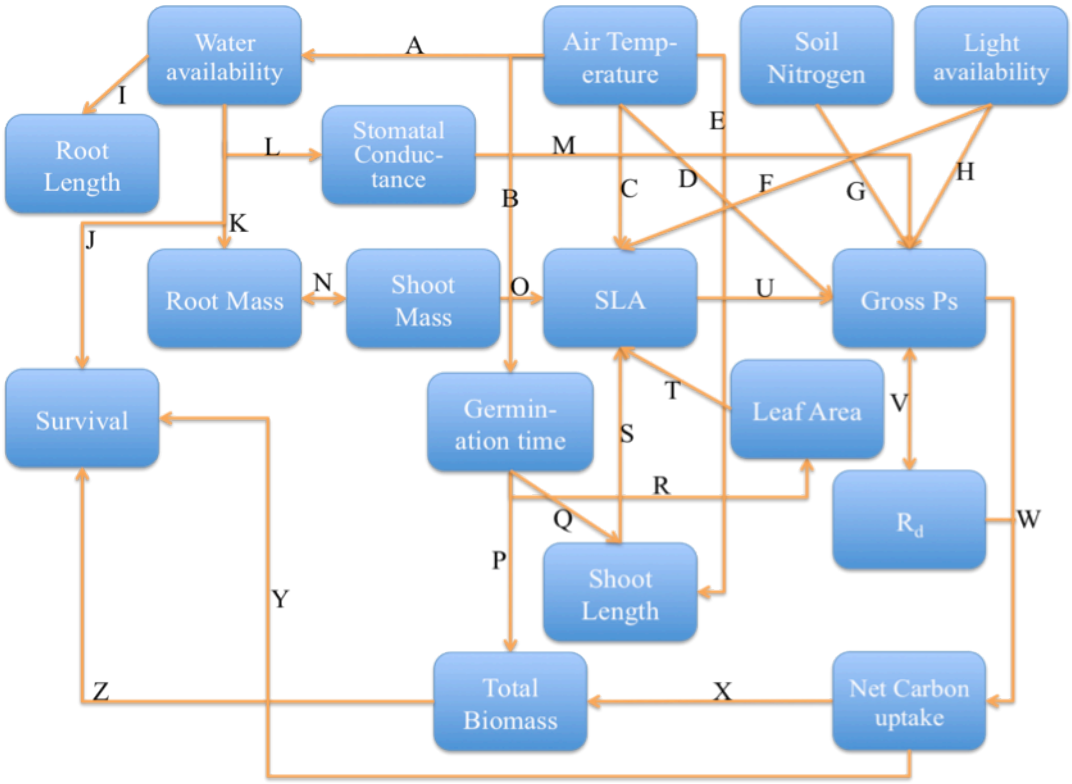

Figure 14: Hypothesized cascade of effects integrating survival with gas exchange and morphological characters. The figure concerns whole seedlings during their first growing season. Letters on the diagram correspond to the following proposed relationships:

A. Warmer air temperatures increased evaporation and decreased RH, decreasing water availability
B. Warmer air temperatures advanced the timing of germination
C. Warmer air temperature increased SLA by decreasing plant cell area and increasing plant cell number
D. Plants acclimated to warmer air temperatures by decreasing gross photosynthesis
E. Warmer air temperatures increased shoot length by stimulating cellular division
F. Plants acclimated to low light availability by increasing their SLA
G. Low soil nitrogen (not measured) limits chlorophyll production and decreases photosynthetic capacity
H. Plants acclimated to low light availability by down-regulating photosynthetic structures, decreasing photosynthetic capacity
I. Low water availability inhibited growth of roots at primary apical root meristem
J. Low water availability decreased survival by increasing occurrence of excessive cavitation
K. Decreased water availability caused plants to allocate more carbon to their roots by increasing fine root tissue
L. Low water availability decreased stomatal conductance
M. Decreased stomatal conductance decreases gross photosynthesis
N. Plant allocation to root mass and shoot mass are inversely related
O. Plant shoot mass is inversely related to SLA
P. Seedlings that germinated earlier accumulated more biomass
Q. Seedlings that germinated earlier had greater plant heights
R. Seedlings that germinated earlier had longer needle lengths, increasing leaf area
S. Increased shoot length increases SLA (SLA was calculated at the whole shoot level including the stem)
T. Increased leaf area increases SLA
U. Increased SLA increases photosynthetic capacity
V. Decreased gross photosynthesis decreases respiration by decreasing the substrate of respiration
W. Decreased photosynthesis and respiration results in a decreased net uptake of carbon
X. A small carbon balance decreased biomass
Y. A small carbon balance decreased likelihood of survival
Z. Increased biomass increases the likelihood of survival by increased stores of carbon assimilates for plant growth and maintenance

Study Limitations

Growth chamber experiments cannot mimic the variability in abiotic factors that are found in the field and thus are best suited to examining in detail the effects of one or two factors on plants. The growth chambers in this experiment were programmed with 7-year averages of temperature and relative humidity and trays were watered following a 3-year average of water availability data. Therefore, the high and low temperatures that the growth chamber seedlings experienced were not as high or as low as what they can potentially experience in the field and the changes in temperature from one week to the next were relatively small, resulting in a relatively stable simulated climate, which is dissimilar to the climate in the field. In the field, temperature extremes may be a very important determinant of survival. Additionally, the effects of temperature and water became confounded in this experiment, thus I cannot make conclusions about the separate effects of temperature and water on physiology, phenology or morphology. Lastly, biotic factors in the field also play an important role in determining whether a given plant lives or dies. This experiment can say nothing about biotic effects since competition nor facilitation among seedlings did not exist.

The air temperature and relative humidity data I used was the data available that was the closest to the ground, at 2 m. However, the maximum plant height of the seedlings in this experiment was 2.7 cm, with an average of 1.1 cm. Leaf temperatures of coniferous seedlings at 2 cm above the ground in the subalpine would be similar to air temperatures at 2 cm near the ground given their small size and shape. Air temperatures at 2 cm differ from air temperatures 2 m from the ground, since heating and cooling of soil surface temperature and cold air drainage influence the air temperature near the

ground. During the day, shading by the forest canopy would prevent soil from heating up and air above the soil would be relatively cool but patches of soil that are exposed to full sunlight would heat up more than surrounding areas and the air above the soil would could become very warm. At night, cold air settles and drains along the ground, exposing seedlings to cold temperatures that would be colder than at 2 m off the ground. Thus, in the ambient chamber, I exposed seedlings to temperatures that were likely warmer at midday and at midnight than what they might experience at the forest floor and in the heated chamber. Therefore, the ambient chamber programming may not have reflected ambient conditions from 1999-2005 and the heated chamber programming may have been even more extreme a scenario than intended.

Measuring gas exchange in small conifer seedlings is prone to error. The operating errors of the LI-COR, such as leaks and edge effects, are magnified under conditions of small fluxes, low CO_2, or when plants are stressed (Warren et al., 2004; Long and Bernacchi, 2003). Light conditions in the growth chamber were low; around 300 μmol m^{-2} s^{-1} photosynthetically active photon flux (PAR) and seedlings were measured at this light level. It is likely that the seedlings were also stressed, in the ambient chamber due to relatively low water availability, and, in the heated chamber due to a combination of high temperatures and low water availability. These factors were likely responsible in creating low fluxes of gas exchange, and measuring these small fluxes could have considerably contributed to the error in this experiment. Lastly, my LI-COR automated programs did not allow enough time for gas exchange processes to stabilize, which caused the rates of net photosynthesis and dark respiration be variable

and to have a trend with time. Like other gas exchange experiments, there is no reason to rule out leaks and edge effects as having not added to the error in this experiment.

The results of this experiment are not a good measurement of the typical net photosynthesis and dark respiration rates for limber pine seedlings in the subalpine forest. The fluxes of photosynthesis and dark respiration were smaller than measurements I took in shaded field conditions, under 300 μmol m^{-2} s^{-1} PAR. This is probably because field seedlings experiencing similar light levels to this experiment typically experience periods of intense solar radiation, or sun flecks. This would allow them to maintain a higher maximum rate of photosynthesis than the seedlings in this experiment. Consistent exposure to low light levels in plants results in low rates of photosynthesis as the photosynthetic machinery is down-regulated. For example, the range of net photosynthesis values for shaded plants in the field was -5 to 23 μmol CO_2 m^{-2} s^{-1} measured at 1200 μmol m^{-2} s^{-1} PAR (J.R. Wolf, unpublished data), while the growth chamber range of values was -2 to 7 μmol CO_2 m^{-2} s^{-1} measured at 300 μmol m^{-2} s^{-1} PAR. However, because all seedlings experienced and were measured under the same conditions, valid comparisons can be made across chambers and haplotypes.

Also, low light levels could have ameliorated the effects of low water availability and high temperatures in the heated chamber. I may have seen no effect of the 3°C heat wave on net photosynthesis and dark respiration because the seedlings could tolerate the combination of high temperatures and low water availability under low light but not if light levels had been higher. Studies show that physiological processes are further impacted when tolerating multiple stressors, such as the combination of high light, low or high temperature and low water availability, than when tolerating only one factor

(Germino and Smith, 1999; Germino and Smith, 2000; Valladares and Pearcy, 1997; Valladares and Pearcy, 2002). However, because the response to multiple stresses cannot be predicted from studying each stressor separately (Mittler, 2006), this experiment provides useful information on what the effect of climate change (increased temperatures, decreased water availability) will be on limber pine seedlings growing in the forest understory. However, limber pine is a shade intolerant species that is typically found in exposed sites where other species cannot establish and so this experiment can only apply to the rare occurrence of seedling establishment within the forest.

To gauge the effect of haplotype more effectively, an ideal experimental setup would have included seeds from many seed sources of the same haplotypes and especially it would include more than one narrow-ranging haplotype individual. Thus, I can only make limited conclusions about the effect the two haplotypes had on my various experimental factors and not about the effect of haplotype in limber pine generally. It may be the case that haplotype differences are much larger than what I observed in this experiment. Also, as a result, maternal effects may have been confounded differences I observed in physiology or morphology. For instance, one observed maternal effect was the higher germination rate in one of the wide-ranging haplotype individuals, tree 5 but otherwise I did not see differences between the two wide-ranging elevation haplotypes.

CONCLUSION

Future Challenges for Forests

Climate change is not the only challenge that faces limber pine and other white pines. Rapid climate change, combined with disease outbreaks as well as fire suppression and forest succession, has been attributed to recent declines in the white pine family (Tomback and Achuff, 2010). White pine blister rust, a non-native wind-borne pathogen, now extends throughout most of the white pine range (Hunt et al., 2010). It causes branch dieback, reproductive failure and mortality (Tomback and Achuff, 2010). Additionally, the mountain pine beetle has also been expanding its range in recent decades into regions previously too cold for it due to increases in temperature and decreases in precipitation (Kurz et al., 2008). Outbreaks of the mountain pine beetle are at their highest in recorded history (Gibson et al., 2008). As forests experience severe mortality, they can change from being a carbon sink to a source, thus fueling further climate change (Kurz et al., 2008). Given its fragmented distribution and the multiple challenges from disease, fire and pests, facilitated migration might be the only strategy to maintaining limber pine and other species like it (Aitken et al., 2008). Planting seeds in areas that have become too dry for other subalpine species may be one solution (Hunt et al., 2010). Small founder populations may live long enough and receive enough genetic variation from far away populations from pollen in order to expand and adapt locally (Schuster and Mitton, 2000; Hamrick, 2004; Aitken et al., 2008). Thus, facilitated migration may involve planting only a few trees in new habitats (McLachlan et al., 2007).

The range of limber pine has contracted and expanded as glaciers expanded and retreated. During contraction of its range, genetic variability may have been lost (Jørgensen et al., 2002) and anthropogenic climate change threatens the loss of more genetic variability. To remain within its current distribution as the climate changes, it is likely that limber pine will need to become increasingly tolerant of warmer temperatures and more frequent and intense droughts. At some threshold, climatic change will exceed its tolerance, causing local extinction. More research is needed to establish the magnitude and combination of abiotic factors that characterize this threshold. The fact that a third of all seedlings in the heated chamber died suggests that the selection pressure on seedling recruitment will be more intense, which might create the potential for the species to rapidly adapt. For example, Kuparinen et al. (2010) found that, though the adaptation of Scots pine (*P. sylvestris*) is expected to lag behind the climatic optimum a century from now, populations experiencing higher rates of mortality are more likely to have the fastest rate of adaptation. On the one hand, small differences between haplotypes in gas exchange and morphology provide evidence for functional genetic variation that will perhaps play a role in the survival of the species. On the other hand, because differences between haplotypes were small, the species in general might be capable of responding to climatic variability. Further research into intra- and inter-species biotic interactions is needed to determine how haplotypes and species will compete in a changing environment.

Overall, environmental factors (temperature and water) played a more central role in determining growth and survival outcomes of limber pine seedlings than did genetic factors (mtDNA haplotypes) in this growth chamber experiment. Decreased rates of net

photosynthesis and dark respiration and increased biomass in heated chamber seedlings demonstrate that limber pine seedlings can tolerate a 5°C overall increase in temperatures and the resulting decreased water availability, but at the cost of a five-fold increase in seedling mortality. Earlier emergence by two weeks in the heated chamber suggests that seedlings will experience a longer growing season in the future and may suffer greater environmental exposure as a result.

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