Impacts of Short Photoperiod, Elevated Temperature, and Elevated CO₂ on Cold Hardening in Eastern White Pine

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Cell and Systems Biology
University of Toronto

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Abstract

Cold hardening in evergreen conifers is induced during autumn by decreasing temperature and photoperiod, and may be delayed or impaired by climate warming. This work aimed to 1) characterize the control of photoperiod versus temperature over the downregulation of photosynthesis, changes in carbohydrate metabolism and development of freezing tolerance that occur during cold hardening in Eastern white pine (Pinus strobus L.) seedlings; and 2) assess the impact of elevated temperature and elevated CO₂ on the timing and extent of cold hardening in P. strobus seedlings under controlled and field conditions.

Under controlled conditions, short photoperiod rapidly induced adjustments of leaf starch and sucrose, while low temperature rapidly induced adjustments of leaf pigments, photosynthesis and accumulation of glucose. Prolonged exposure to short photoperiod and low temperature induced downregulation of photosynthesis, accumulation of cryoprotective carbohydrates, and development of freezing tolerance. A novel 16-kD dehydrin protein was induced by short photoperiod and maximally expressed with the addition of low temperature; expression of this dehydrin strongly correlated with freezing tolerance.
Under controlled conditions, elevated temperature suppressed the downregulation of photosynthesis and accumulation of cryoprotective compounds; freezing tolerance was impaired, but provided sufficient protection against average historical winter temperatures at the seedlings’ native origin. The combination of elevated temperature and elevated CO$_2$ enhanced photosynthesis and, under long photoperiod, enhanced accumulation of starch. Elevated CO$_2$ did not further impair development of freezing tolerance.

Under field conditions, development of freezing tolerance was initiated during early autumn by decreasing photoperiod. Frost exposure in mid-late autumn induced the downregulation of photosynthesis, accumulation of soluble sugars, and strongly enhanced freezing tolerance. However, the projected temperature increase for the year 2050 did not perceptibly delay downregulation of photosynthesis or impair freezing tolerance.

These findings indicate crucial roles for short photoperiod and low temperature during cold hardening. Warmer climates with elevated CO$_2$ levels may allow _P. strobus_ seedlings to benefit from increased carbon uptake and extend photosynthetic activity during the autumn. Elevated temperature and CO$_2$ may impair the development of freezing tolerance, but is unlikely to increase risk of damage incurred by winter exposure for _P. strobus_ seedlings grown in southern Ontario.
Acknowledgements

I am deeply grateful to all of those who have inspired, encouraged and supported me during my pursuit of a Doctoral degree.

Firstly, I wish to express my thanks to my supervisor, Dr. Ingo Ensminger, for his extensive guidance and mentoring, and to the members of my supervisory committee, Drs. Rowan Sage and Timothy Westwood, for their guidance and valuable input over the years. I also wish to thank my collaborators, Drs. Shawn Mansfield and Faride Unda at the University of British Columbia, and Dr. Katharina Bräutigam at the University of Toronto. Working with you has been a wonderful experience and taught me a great deal.

I must also thank the members of my lab, especially Emmanuelle Fréchette and Laura Junker, who have been great colleagues and friends, and with whom I have shared so many unforgettable adventures. I also wish to thank those whose efforts have proved invaluable for facilitating my research: Alex Zubilewich, Daniel Marsden, Dhyani Patel, and Tarek bin Yameen who assisted with data gathering; Christine Tan and the team of UTM engineers who helped to ensure smooth running of the growth chambers; Lisa Cheung and Waldimar Czerwinski who provided assistance with equipment and protocols; and Steph Schneider and the support staff at Koffler Scientific Reserve who helped to keep the T-FACE arrays up and running (and rescued us from a snowy situation or two).

Lastly but not at all least, I am absolutely indebted to my family and friends for all of their support. I would not be who and where I am today without you. Special thanks go to my parents and my sister for being there for me every step of the way; to Grace, for her unparalleled generosity and kindness; and to Gene, Sameer, Fiona, Alix, Stu, Chrissy, Jackie, Ya-Wen, and Janne, for reminding me of all of the other things that matter!

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Table of Contents

Abstract ........................................................................................................................................... ii
Acknowledgements ........................................................................................................................ iv
Table of Contents .............................................................................................................................v
List of Tables ................................................................................................................................... viii
List of Figures ................................................................................................................................... ix
Abbreviations ................................................................................................................................... xii
Statement of Co-authorship ............................................................................................................. xvi

Chapter 1 Literature Review: The Physiology and Molecular Regulation of Cold Acclimation in Evergreen Conifers .................................................................1
  1.1 Abstract ................................................................................................................................ 2
  1.2 Introduction ............................................................................................................................. 2
  1.3 Physiological Acclimation to Decreasing Temperature and Photoperiod .......................3
  1.4 Low Temperature Induction of Cold Response ................................................................. 12
  1.5 Photoperiodic Induction of Cold Response ..................................................................... 15
  1.6 Challenges Imposed by a Future Climate ......................................................................... 17
  1.7 Thesis Framework .............................................................................................................. 18
  1.8 References .......................................................................................................................... 20

Chapter 2 Rapid Adjustment of Nonstructural Carbohydrates and Photosynthesis in Response to Short Photoperiod, Low Temperature or Elevated CO₂ in Eastern White Pine .................26
  2.1 Abstract .............................................................................................................................. 27
  2.2 Introduction ......................................................................................................................... 27
  2.3 Materials and Methods ..................................................................................................... 31
  2.4 Results ............................................................................................................................... 36
  2.5 Discussion ......................................................................................................................... 43
Chapter 3 The Good and the Bad: Elevated Temperature and Elevated CO₂ Stimulate Late Season Photosynthesis But Impair Freezing Resistance in Eastern White Pine Seedlings

3.1 Abstract
3.2 Introduction
3.3 Materials and Methods
3.4 Results
3.5 Discussion
3.6 Conclusions
3.7 Supporting Information
3.8 References

Chapter 4 Sensitivity of Cold Acclimation to Elevated Autumn Temperature in Field-grown Pinus strobus Seedlings

4.1 Abstract
4.2 Introduction
4.3 Materials and Methods
4.4 Results
4.5 Discussion
4.6 Conclusions
4.7 Supporting Information
4.8 References

Chapter 5 Conclusions and Future Directions

5.1 Hypothesis 1: The induction and development of cold hardening, which includes downregulation of photosynthesis, accumulation of cryoprotective carbohydrates and development of freezing tolerance, requires both low temperature and short photoperiod.
5.2 Hypothesis 2: Elevated autumn temperature extends the growing season by delaying the downregulation of photosynthesis and associated changes in carbohydrate metabolism, and impairs the development of freezing tolerance. .................................................144

5.3 Hypothesis 3: The combination of elevated temperature and CO₂ enhances photosynthesis in addition to extending the growing season, but further exacerbates vulnerability to freezing damage. ..................................................................................146

5.4 Conclusions......................................................................................................................148

5.5 References........................................................................................................................148

Appendix Experimental Conditions and Methods........................................................................154

A1 Experimental conditions ..................................................................................................154
A2 Photosynthesis protocols .................................................................................................157
A3 Water status protocols ....................................................................................................160
A4 Photosynthetic pigment protocols ..................................................................................161
A5 Carbohydrate protocols .................................................................................................161
A6 Protein protocols ..............................................................................................................163
A7 Freezing tolerance protocol ............................................................................................165
A8 Statistics methods.............................................................................................................167
A9 References........................................................................................................................169

Copyright Acknowledgements.............................................................................................171
List of Tables

Table 2.1: The effects of treatment and time on photosynthetic gas exchange, chlorophyll fluorescence, photosynthetic pigments, and carbohydrates. ................................................................. 38

Table 2.2: Significant changes in nonstructural carbohydrates, chlorophyll fluorescence, and photosynthetic pigments between night and day. ................................................................. 39

Table 2.3: Significant changes in nonstructural carbohydrates, chlorophyll fluorescence, and photosynthetic pigments between days of the experiment. .......................................................... 39

Table 3.1: The effects of low temperature/ambient CO$_2$ (LTAC), elevated temperature/ambient CO$_2$ (ETAC), and elevated temperature/elevated CO$_2$ (ETEC) on photosynthetic gas exchange, chlorophyll fluorescence, photosynthetic pigments, and carbohydrates. ........................................ 73

Table 3.2: Best predictors of seasonal variation for LT$_{50}$ and 16-kD dehydrin in *P. strobus* needles determined by linear mixed-effects modelling. ...................................................... 82

Table 4.1: Cuvette air temperature .......................................................................................... 106

Table 4.2: Equations for gas exchange and fluorescence parameters. ................................. 107

Table 4.3: Summary of two-way ANCOVA analysis showing the effects of treatment and time (day of year) on gas exchange, chlorophyll fluorescence and photosynthetic pigments. ........... 113

Table 4.4: Curve parameters of 4-parametric sigmoid models presented in Fig. 4.7. ............. 120
List of Figures

Figure 1.1: A timeline of cold acclimation ................................................................. 4

Figure 1.2: Intersections between drought, cold and light response pathways............. 6

Figure 1.3: Modes of nonphotochemical quenching (NPQ) ........................................ 9

Figure 1.4: Initial steps of carbohydrate metabolism following photosynthetic carbon assimilation ................................................................. 11

Figure 2.1: Changes in leaf nonstructural carbohydrates in response to low temperature/ambient CO₂ (LTAC), high temperature/ambient CO₂ (HTAC) and high temperature/elevated CO₂ (HTEC) ................................................................. 37

Figure 2.2: Changes in leaf carbohydrate allocation in response to low temperature/ambient CO₂ (LTAC), high temperature/ambient CO₂ (HTAC) and high temperature/elevated CO₂ (HTEC) 40

Figure 2.3: Response of photosynthetic gas exchange to low temperature/ambient CO₂ (LTAC), high temperature/ambient CO₂ (HTAC) and high temperature/elevated CO₂ (HTEC) .......... 41

Figure 2.4: Response of chlorophyll fluorescence to low temperature/ambient CO₂ (LTAC), high temperature/ambient CO₂ (HTAC) and high temperature/elevated CO₂ (HTEC) ............. 42

Figure 2.5: Changes in photosynthetic and accessory leaf pigment levels in response to low temperature/ambient CO₂ (LTAC), high temperature/ambient CO₂ (HTAC) and high temperature/elevated CO₂ (HTEC) ................................................................................................................................. 44

Figure 2.6: Changes in photoprotective leaf pigments in response to low temperature/ambient CO₂ (LTAC), high temperature/ambient CO₂ (HTAC) and high temperature/elevated CO₂ (HTEC) ................................................................................................................................................................. 45

Figure 3.1: Response of photosynthetic gas exchange to low temperature/ambient CO₂ (LTAC), elevated temperature/ambient CO₂ (ETAC) and elevated temperature/elevated CO₂ (ETEC) ... 70
Figure 3.2: Response of chlorophyll fluorescence to low temperature/ambient CO₂ (LTAC), elevated temperature/ambient CO₂ (ETAC) and elevated temperature/elevated CO₂ (ETEC).... 72

Figure 3.3: Changes in photosynthetic leaf pigments in response to low temperature/ambient CO₂ (LTAC), elevated temperature/ambient CO₂ (ETAC) and elevated temperature/elevated CO₂ (ETEC). ......................................................................................................................................... 75

Figure 3.4: Changes in leaf nonstructural carbohydrate content in response to low temperature/ambient CO₂ (LTAC), elevated temperature/ambient CO₂ (ETAC) and elevated temperature/elevated CO₂ (ETEC). ......................................................................................................................................... 77

Figure 3.5: Changes in leaf protein expression in response to low temperature/ambient CO₂ (LTAC), elevated temperature/ambient CO₂ (ETAC) and elevated temperature/elevated CO₂ (ETEC). ......................................................................................................................................... 79

Figure 3.6: Shoot freezing tolerance at the beginning (day 0) and end (day 36) of the experiment. ....................................................................................................................................................... 80

Figure 3.7: Changes in dehydrin protein expression and development of freezing tolerance during cold hardening in needles of field-grown *P. strobus* seedlings........................................ 81

Figure 3.8. Correlation between relative leaf protein content of 16-kD dehydrin (Dhn) and freezing tolerance (LT₅₀).................................................................................................................................................................................................. 82

Figure 4.1: Seasonal variations in precipitation, day length and temperature from August 1, 2012 to January 31, 2014 at Koffler Scientific Reserve in Ontario, Canada.............................................. 105

Figure 4.2: Effect of elevated temperature on photosynthetic gas exchange in field-grown white pine seedlings during autumn ..................................................................................................... 112

Figure 4.3: Effect of elevated temperature on chlorophyll fluorescence in field-grown white pine seedlings during autumn. ............................................................................................................ 114

Figure 4.4: Effect of elevated temperature on soil water availability and osmotic stress in field-grown white pine seedlings during autumn. .............................................................................................................. 115
Figure 4.5: Effect of elevated temperature on photosynthetic pigments in needles of field-grown white pine seedlings during autumn. ................................................................. 116

Figure 4.6: Effect of elevated temperature on photoprotective metabolites in needles of field-grown white pine seedlings during autumn. ................................................................. 118

Figure 4.7: Relationship of photosynthesis and sustained nonphotochemical quenching with minimum daily temperature and photoperiod................................................................. 119

Figure 4.8: Effect of elevated temperature on nonstructural carbohydrates in needles of field-grown white pine seedlings during autumn. ................................................................. 121

Figure 4.9: Effect of elevated temperature on cold hardening in field-grown white pine seedlings during autumn................................................................. 123
Abbreviations

ΔpH trans-thylakoid pH gradient
ΔF/Fm’, ΦPSII effective quantum yield of photosystem II
Ψw water potential
1-qP excitation pressure at photosystem II
3-PGA 3-phosphoglyceric acid
A, A net rate of photosynthetic carbon assimilation
aa amino acid
ABA abscisic acid
ABRE ABA-responsive element
ANOVA analysis of variance
ANCOVA analysis of covariance
AREB/ABF ABA-responsive binding protein
ATP adenosine triphosphate
Ci intracellular CO2 concentration
CAM calmodulin
Car carotenoids
cas30 cold acclimation-specific gene 30
CBF C-repeat binding factor, also known as DREB1
CCA1 circadian clock-associated 1
cDNA complementary DNA synthesized from RNA
CDPK calcium-dependent protein kinase
Chl chlorophyll
Chl a/b ratio of chlorophyll a to chlorophyll b
CO CONSTANS transcription factor
COP constitutive photomorphogenesis 1
COR cold responsive gene
CRT C-repeat, also known as DRE
Cry cryptochrome
D1 reaction core protein of photosystem II, also known as PsbA
DEPS de-epoxidation state of the xanthophyll cycle
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHAP</td>
<td>dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>Dhn</td>
<td>dehydrin</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOY</td>
<td>day of year</td>
</tr>
<tr>
<td>DRE</td>
<td>dehydration responsive element, also known as CRT</td>
</tr>
<tr>
<td>DREB</td>
<td>dehydration responsive element binding protein</td>
</tr>
<tr>
<td>DW</td>
<td>dry weight</td>
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<tr>
<td>E</td>
<td>evapotranspiration</td>
</tr>
<tr>
<td>EC-PAD</td>
<td>electrochemical pulse amperometric detector</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene-diamine-tetra-acetic acid</td>
</tr>
<tr>
<td>F&lt;sub&gt;m&lt;/sub&gt;</td>
<td>dark-adapted maximum fluorescence of photosystem II</td>
</tr>
<tr>
<td>F&lt;sub&gt;m&lt;/sub&gt;rec</td>
<td>fully recovered dark-adapted maximum fluorescence of photosystem II</td>
</tr>
<tr>
<td>F&lt;sub&gt;m&lt;/sub&gt;'</td>
<td>light-adapted maximum fluorescence of photosystem II</td>
</tr>
<tr>
<td>F&lt;sub&gt;o&lt;/sub&gt;</td>
<td>dark-adapted minimum fluorescence of photosystem II</td>
</tr>
<tr>
<td>F&lt;sub&gt;o&lt;/sub&gt;'</td>
<td>light-adapted minimum fluorescence of photosystem II</td>
</tr>
<tr>
<td>F&lt;sub&gt;t&lt;/sub&gt;</td>
<td>transient fluorescence</td>
</tr>
<tr>
<td>F&lt;sub&gt;v&lt;/sub&gt;/F&lt;sub&gt;m&lt;/sub&gt;</td>
<td>maximum quantum yield of photosystem II</td>
</tr>
<tr>
<td>FACE</td>
<td>free-air CO&lt;sub&gt;2&lt;/sub&gt; enrichment</td>
</tr>
<tr>
<td>FKF1</td>
<td>flavin-binding kelch repeat F-box 1</td>
</tr>
<tr>
<td>FT</td>
<td>flowering locus T</td>
</tr>
<tr>
<td>g&lt;sub&gt;s&lt;/sub&gt;</td>
<td>stomatal conductance</td>
</tr>
<tr>
<td>GAP</td>
<td>GAP – glyceraldehyde 3-phosphate</td>
</tr>
<tr>
<td>GI</td>
<td>GIGANTEA protein</td>
</tr>
<tr>
<td>Glu</td>
<td>glucose</td>
</tr>
<tr>
<td>Hex</td>
<td>hexose</td>
</tr>
<tr>
<td>HOS1</td>
<td>high expression of osmotically responsive gene 1</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HY5</td>
<td>long hypocotyl 5</td>
</tr>
<tr>
<td>ICE1</td>
<td>inducer of CBF expression 1</td>
</tr>
<tr>
<td>IWUE</td>
<td>intrinsic water use efficiency</td>
</tr>
<tr>
<td>LDH</td>
<td>lactose dehydrogenase</td>
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</table>
LEA       late embryogenesis abundant protein
Lhcb1     antenna protein associated with light harvesting complex of photosystem II
LHCII     light harvesting complex of photosystem II
LHY       late elongated hypocotyl
Lut       lutein
LT_{50}   lethal freezing temperature inducing 50% damage in exposed seedlings
LTRE      low temperature response element
MYB, MYC  plant homologs of vertebrate myeloblastosis and myelocytomatosis oncogenes
MYBRS, MYCRS MYB- and MYC-recognition sites
MW        molecular weight
N:P:K      fertilizer mixture of nitrogen, phosphorus and potassium
Neo       neoxanthin
NPQ       nonphotochemical quenching
NPQ_{S}   sustained nonphotochemical quenching, also known as qI
NSC       nonstructural carbohydrate
P_{i}     inorganic phosphate
Pfr       far-red light-responsive isoform of phytochrome
Pr        red light-responsive isoform of phytochrome
PEPC      phosphoenolpyruvate carboxylase
Phy       phytochrome
PIF       phytochrome interacting factor
Phot      phototropin
PPFD      photosynthetic photon flux density
ppm       parts per million
PsbA      reaction core protein of photosystem II, also known as D1
PSI       photosystem I
PSII      photosystem II
PTFE      polytetrafluoroethylene
PUT       putative unique transcript
PVDF      polyvinylidene fluoride
qE        energy-dependent nonphotochemical quenching
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>qI</td>
<td>photoinhibitory nonphotochemical quenching, also known as NPQs</td>
</tr>
<tr>
<td>R&lt;sub&gt;d&lt;/sub&gt;</td>
<td>rate of cellular respiration in the absence of light (dark respiration)</td>
</tr>
<tr>
<td>RbcL</td>
<td>large subunit of Rubisco</td>
</tr>
<tr>
<td>RFO</td>
<td>raffinose family oligosaccharide</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>Rubisco</td>
<td>ribulose-1,5-bisphosphate carboxylase/oxygenase</td>
</tr>
<tr>
<td>RuBP</td>
<td>ribulose-1,5-bisphosphate</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SnRK2</td>
<td>sucrose non-fermenting-1-related kinase 2</td>
</tr>
<tr>
<td>Suc</td>
<td>sucrose</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>T-FACE</td>
<td>temperature free-air controlled enhancement</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine diphosphate</td>
</tr>
<tr>
<td>UDP-glu</td>
<td>uridine diphosphate glucose</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>V&lt;sub&gt;cmax&lt;/sub&gt;</td>
<td>maximum substrate-saturated rate of Rubisco carboxylase activity</td>
</tr>
<tr>
<td>VDE</td>
<td>violaxanthin de-epoxidase</td>
</tr>
<tr>
<td>VPD</td>
<td>vapor pressure deficit</td>
</tr>
<tr>
<td>VWC</td>
<td>volumetric water content</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>ZEP</td>
<td>zeaxanthin epoxidase</td>
</tr>
</tbody>
</table>
Statement of Co-authorship

At the time of the submission of this thesis, Chapter 1, a literature review, was submitted to the *Plant Physiology* in August 2016. Chapter 2, an original research paper, is in preparation for submission to a peer-reviewed journal. Chapter 3 was published as an original research paper in the journal *Plant Physiology* in October 2016. Chapter 4 was published as an original research paper in the journal *Frontiers in Plant Science* in March 2015. For each manuscript included in this thesis, I was the primary author responsible for project design and execution, data analysis, and writing. Guidance and supervision was provided throughout my research endeavors by my thesis advisor, Dr. Ingo Ensminger. Collaborators from the University of British Columbia, Drs. Faride Unda and Shawn D. Mansfield, assisted with carbohydrate sample analysis in Chapters 2-4. Contributions were also made by my colleagues from the University of Toronto Mississauga, fellow Ph.D. student Emmanuelle Fréchette (Chapter 3) and undergraduate research intern Alexandra Zubilewich (Chapter 4). A more detailed description of author contributions for each manuscript is found below.

Chapter 1

I had the lead on writing the manuscript jointly with Dr. Ensminger. Some of the material in this chapter and a modified version of Figure 1.2 were contributed to “Tree Responses to Environmental Cues”, chapter 7 in volume 74 of the book series *Advances in Botanical Research* (Elsevier), which was published in May 2015.

Chapter 2

I designed the study with input from Dr. Ensminger, and performed measurements, samplings, pigment, protein, and data analyses. Dr. Unda analyzed the carbohydrates using HPLC, with input from Dr. Mansfield. I had the lead on writing the manuscript with input and guidance on data interpretation from Dr. Ensminger.
Chapter 3

Dr. Ensminger and I designed the study. Emmanuelle Fréchette and I performed measurements and samplings. I performed pigment and protein analyses, freezing tests, and data analyses. Dr. Unda analyzed the carbohydrates using HPLC, with input from Dr. Mansfield. I had the lead on writing the manuscript jointly with Dr. Ensminger. All authors provided editorial input and approved the final manuscript.

Chapter 4

Dr. Ensminger and I designed the study. I performed field measurements and samplings. I developed the freezing test protocol together with Alexandra Zubilewich, who performed the freezing tests with my guidance and input. Dr. Unda and Dr. Mansfield developed the carbohydrate HPLC protocol and Dr. Unda analyzed the carbohydrates. I performed pigment and data analyses. I had the lead on writing the manuscript with input and guidance on data interpretation from Dr. Ensminger. All authors provided editorial input and approved the final manuscript.
Chapter 1
Literature Review: The Physiology and Molecular Regulation of Cold Acclimation in Evergreen Conifers


Status: Under revision.

(Section 1.7: Thesis Framework was not included in the submitted manuscript.)
1.1 Abstract

During autumn, exposure to decreasing temperature, photoperiod and changes in light quality activate a network of signaling pathways that control cold acclimation in cold-hardy plants from high latitudes. In evergreen conifers, cold acclimation involves the development of freezing tolerance, cessation of growth, bud dormancy, changes in carbon metabolism, and the downregulation of photosynthesis. Here we review recent developments in understanding the responses of evergreen conifers to temperature and light cues during cold acclimation and discuss the implications of these findings in the context of climate warming. In the first section, we examine how evergreen conifers adjust metabolism during cold acclimation to defend overwintering tissues against winter stresses. In sections two and three, we explore how perception of low temperature and photoperiod regulate the induction of cold acclimation in plants. Finally, we identify challenges imposed by climate change on overwintering conifers and pinpoint open avenues of research in the field.

1.2 Introduction

In overwintering plants, decreasing temperature and photoperiod during autumn act as environmental signals for the initiation of cold acclimation (Li et al. 2004) and eventually the development of cold hardiness (Ensminger et al. 2006, 2012). For these plants, cold hardiness is critical for surviving long periods of low temperature and osmotic stress and avoiding freezing damage during winter months. Cold hardening initially involves the development of tolerance to chilling, low above-freezing temperatures, followed by the development of tolerance against below-freezing temperatures (Allen and Ort 2001, Ensminger et al. 2012). In evergreen conifers, a complex suite of mechanisms contributes to the ability to survive winter stress, including enhanced protection of the chloroplast (Ensminger et al. 2006, Demmig-Adams et al. 2012, Crosatti et al. 2013), accumulation of cryoprotective compounds such as soluble sugars (Schrader and Sauter 2002, Knaupp et al. 2011) and dehydrins (Close 1997, Kjellsen et al. 2013), and modifications of the cell membrane (Moellering et al. 2010, Vaultier et al. 2006).
1.3 Physiological Acclimation to Decreasing Temperature and Photoperiod

Development of Cold Hardiness

Low temperature and short photoperiods signal the development of cold hardiness (Fig. 1.1), which is achieved upon full cold acclimation and can provide extensive chilling and freezing tolerance. Although cold hardiness is a trait observed in many plant species, it is absent in many tropical plants such as banana or avocado; ergo, exposure to low temperature does not necessarily induce cold acclimation (Lyons, 1973). Low-temperature inhibition of enzymatic activity disrupts metabolic processes, such as CO₂ fixation, and also inhibits synthesis of proteins that normally undergo rapid turnover, such as the PSII reaction center core protein D1 (Crosatti et al. 2013). Plants exposed to freezing temperatures are additionally challenged by mechanical stress, due to the formation of intracellular or extracellular ice crystals and increased brittleness of tissues, as well as osmotic stress since water is trapped in the form of ice (Steponkus 1984). The loss of membrane integrity incurred by freezing and thawing results in plasmolysis and solute leakage from cells and organelles that are insufficiently cryoprotected, as demonstrated for example in cold-sensitive *Coffea* plants (Campos et al. 2003). Consequently, water loss and collapsed cell structures are often observed in plants upon exposure to temperatures below their tolerance level (Steponkus 1984).

Cold-hardy plants use a suite of inducible cryoprotective mechanisms including protein stabilization, increased solute concentrations, and adjustment of the composition of membrane lipids to improve membrane fluidity (Crosatti et al. 2013). These cryoprotective mechanisms are triggered by hundreds of cold-responsive (COR) genes that are activated in response to decreasing temperature and photoperiod (Fig. 1.2; Welling and Palva 2006). In *Picea sitchensis*, these genes include members of the late embryogenesis abundant (LEA) protein family, antifreeze proteins, lipid desaturases, and activators of sucrose, raffinose and proline biosynthesis (Holliday et al. 2008). LEAs are a large, diverse family of proteins that are upregulated in response to chilling, freezing, drought and salt stress (Shih et al. 2008), and typically accumulate in trees during late autumn (e.g. Holliday et al. 2008, Ueno et al. 2013). LEAs perform diverse functions associated with osmo- and cryoprotection (Shih et al. 2008). In *Arabidopsis*, a key cold-induced LEA protein, Cor15a, mitigates freezing damage by forming oligomers that bind to
Figure 1.1: A timeline of cold acclimation.
The downregulation of photosynthesis occurs in response to combined short photoperiod and temperature signals during late autumn. As photosynthesis is downregulated, changes in chloroplast redox state increase excitation pressure at PSII, causing an enhancement of photoprotection in response. While de-epoxidation of the xanthophyll cycle (DEPS) and sustained nonphotochemical quenching (NPQ) increase during late autumn, the transition from energy-dependent to sustained NPQ is not completed until winter. Soluble carbohydrates including sucrose and raffinose accumulate in response to low temperature. Freezing tolerance initially develops in response to short photoperiod and is enhanced in response to low temperature. GC – growth cessation; BD – bud dormancy; CM – carbohydrate metabolism; CH – cold hardiness.
enzymes such as Rubisco and lactate dehydrogenase (LDH) and protect them against freeze-induced inactivation (Nakayama et al. 2007).

Dehydrins are an important subgroup of the LEA proteins contributing to cold hardening. Accumulation of some dehydrins during the cold season is associated with the development of freezing tolerance in trees, as recently described in *Picea obovata* (Kjellsen et al. 2013). Dehydrins contain one or more repeats of several distinctive peptide motifs. A highly conserved motif in higher plants is a lysine-rich region known as the K-segment (sequence: EKKGIMDKIKEKLPG; Close 1997). The K-segment has been strongly implicated in membrane binding (Eriksson et al. 2011). This is consistent with the observation that several dehydrins associate with membrane proteins in order to prevent coagulation, suggesting that dehydrins, like other LEAs, are involved in osmo- and cryoprotective functions (Close 1997). In *Citrus unshiu*, the dehydrin CuCOR19 was shown to scavenge oxygen radicals produced during cold or drought stress (Hara et al. 2004).

Cold hardening can result in extreme freezing resistance. Deciduous trees adapted to survive freezing in higher latitudes, including *Betula* and *Populus*, and conifers including *Abies*, *Picea*, *Pinus*, *Larix* and *Pseudotsuga*, have evolved extensive freezing tolerance (Sakai and Weiser 1973, Sakai 1983). Proper development of cold hardiness allows these species to survive temperatures as low as -70°C (Sakai 1983); in *Pinus sylvestris*, freezing resistance to even -196°C was observed (Sakai and Weiser 1973). Evidently, cold hardiness confers a huge advantage for overwintering species, particularly in higher latitudes.

**Cessation of Growth and Bud Dormancy**

In trees, growth cessation and the development of cold hardiness are synchronized with bud dormancy. These processes are controlled by both endogenous and exogenous factors (Welling and Palva 2006). Bud dormancy is regulated by hormones including ABA, auxin, ethylene and gibberellin, and is controlled by a network of pathways integrating photoperiod response, ABA response, and the circadian clock (Fig. 1.2; Cooke et al. 2012). The initial stage in the development of bud dormancy is called endodormancy. In this stage, bud development is inhibited by endogenous factors within the meristem, and buds that have entered endodormancy have a chilling requirement to release dormancy (Welling and Palva 2006, Cooke et al. 2012).
Figure 1.2: Intersections between drought, cold and light response pathways.
Drought, cold, and light signals induce a network of pathways that control drought and temperature responses, growth and bud phenology. Italics indicate pathway activators; ovals indicate key signalling mechanisms; rectangles indicate target cis-acting elements. Figure represents a composite pathway based on: Imaizumi et al. (2003), Chinnusamy et al. (2007), Jiao et al. (2007), Sawa et al. (2007), Umezawa et al. (2010), Catalá et al. (2011), Andrés and Coupland (2012), Cooke et al. (2012), Petterle et al. (2013). Figure modified from Ensminger et al. (2015).
Endodormancy occurs during early autumn and coincides with the initial development of chilling tolerance. In *Quercus petraea*, endormant tissue exhibited upregulation of genes involved in ABA response, cell wall modification, drought regulation and chlorophyll degradation (Ueno et al. 2013). Ecodormancy, in which bud development is no longer endogenously inhibited but is suppressed by environmental conditions, occurs during late autumn as temperatures drop below zero. During ecodormancy, a substantial increase in freezing tolerance is observed (Welling and Palva 2006, Kjellsen et al. 2013) together with the induction of genes involved in response to cold – e.g. dehydrins and other LEAs – as well as oxidative stress, ABA and gibberellins (Ueno et al. 2013).

In many tree species, growth cessation, the termination of shoot elongation and stem diameter growth, is initiated during late summer and early autumn (Repo et al. 2000). In many tree species, the signal that triggers growth cessation is the decrease in photoperiod (Downs and Borthwick 1956, Li et al. 2003). The induction of bud set by photoperiod has been described in many deciduous trees, including *Populus* (Böhlenius et al. 2006), *Salix* (Cooke et al. 2012), and *Betula* (Li et al. 2003), as well as in the evergreen conifer *Picea* (Gyllenstrand et al. 2007). A study of North American and European conifers, including *Picea, Pinus, Abies*, and *Larix*, revealed a strong correlation between the cessation of secondary growth and photoperiod; the rate of growth decreased sharply following the summer solstice, although air temperature remained high for another month (Rossi et al. 2006). However, growth cessation is not triggered in all tree species by a decrease in photoperiod. For example, *Malus* and *Pyrus* respond solely to low temperature and not photoperiod (Heide and Prestrud 2005). Meta-analyses of bud phenology studies have revealed that sensitivity to temperature and photoperiod is highly species-specific (Körner and Basler 2010, Cooke et al. 2012) and may be an adaptation that confers competitive advantage in early vs. late successional communities (Körner and Basler 2010).

**Downregulation of Photosynthesis**

Evergreen conifers face a unique challenge when retaining their leaves with much of their pigments intact throughout winter (Ensminger et al. 2004, Ensminger et al. 2006). Low temperatures impose significant limitations on photosynthesis by decreasing fluidity of the thylakoid membrane (Crosatti et al. 2013). Low temperatures also impact the photochemical
reactions, e.g. by inhibiting regeneration of the PSII reaction center core protein D1 (Aro et al. 1993, Ottander et al. 1995, Ensminger et al. 2004), and the Calvin cycle, by inhibiting regeneration of RuBP and decreasing efficiency of Rubisco carboxylation (Ensminger et al. 2012, Crosatti et al. 2013). However, chlorophyll retained in conifer needles captures light throughout winter, when most if not all of the light energy absorbed is in excess of what can be used for photosynthesis (Ensminger et al. 2006). The excess light energy resulting from the combination of light and low temperature can induce photoinhibition (Fig. 1.1; Huner et al. 1998, Ensminger et al. 2006) and produce excited triplet chlorophylls ($^3$Chl*), singlet oxygens ($^1$O$_2$*), and other reactive oxygen species (ROS), which can incur significant photo-oxidative damage (Adams et al. 2004).

While deciduous trees and cold-sensitive annuals shed their leaves and avoid high light stress during winter, evergreen conifers have evolved mechanisms for mitigating the negative consequences of excess light energy in overwintering leaves. During the downregulation of photosynthesis, conifers can downregulate light absorption by decreasing levels of leaf chlorophylls and integral PSII thylakoid membrane proteins and upregulation of photoprotective carotenoids (see below) (Ottander et al. 1995). As a consequence, photochemical efficiency of PSII decreases and nonphotochemical quenching (NPQ) increases to enhance the capacity for safe dissipation of excess light energy (Fig. 1.3). Under these conditions, PSI activity is enhanced relative to PSII, and can contribute to increased cyclic electron transport to supplement linear electron transport (Fréchette et al. 2015).

Low temperature induced NPQ is facilitated through the accumulation of lutein and xanthophyll cycle pigments (Fig. 1.3). In higher plants, NPQ consists of two components that relax at different rates following light exposure: the fast-relaxing component, also called energy-dependent quenching (qE), is dominant under warm temperatures; the slow component, also called photoinhibitory quenching (ql) or sustained NPQ (NPQs), is dominant during the winter (Demmig-Adams et al. 2012). qE is induced within seconds of light exposure, as photosynthetic electron transport causes protons to accumulate in the thylakoid lumen, generating a trans-thylakoid pH gradient (ΔpH). The acidification of the thylakoid lumen activates violaxanthin deepoxidase (VDE, pH optimum 5.2), which converts violaxanthin via antheraxanthin to zeaxanthin. Zeaxanthin enables dissipation of excess energy as heat via an unknown mechanism (Fig. 1.3; Demmig-Adams et al. 2012, Brooks et al. 2014). Two proposed roles for zeaxanthin
Figure 1.3: Modes of nonphotochemical quenching (NPQ).
A) During energy-dependent quenching (qE), light-induced water splitting and photosynthetic electron transport generate a trans-thylakoid pH gradient (ΔpH). Low pH activates the xanthophyll cycle enzyme violaxanthin de-epoxidase (VDE). VDE converts violaxanthin via antheraxanthin to zeaxanthin. Zeaxanthin facilitates dissipation of excess energy via heat. In the dark, the pH of the thylakoid lumen is neutralized and zeaxanthin is re-converted back to violaxanthin by zeaxanthin epoxidase (ZEP). B) Photoinhibitory quenching (qI) can occur during extended exposure to low temperatures. During qI, the pH of the thylakoid lumen fails to neutralize in the dark. Consequently, ZEP activity is suppressed and zeaxanthin is accumulated, enabling constant dissipation of excess light energy.
are direct quenching of energy from excited chlorophyll molecules (Brooks et al. 2014), and/or allosteric regulation of PSII (Brooks et al. 2014) by binding to the light harvesting complexes, which induces conformational changes (Demmig-Adams et al. 2012, Papageorgiou and Govindjee 2014). In the dark, the thylakoid lumen rapidly relaxes to a neutral pH and zeaxanthin is reconverted to violaxanthin by zeaxanthin epoxidase (ZEP; pH optimum 7.5).

$qI$ is induced by prolonged periods of high excitation pressure, e.g. under high light or low temperature (Demmig-Adams et al. 2012). During $qI$, thylakoid lumen pH can remain low for days following light exposure, thus inhibiting the re-conversion of zeaxanthin to violaxanthin and enabling excess light energy to be constitutively quenched without enzyme mediation (Fig. 1.3; Demmig-Adams et al. 2012, Papageorgiou and Govindjee 2014).

**Changes in Carbohydrate Metabolism**

Plants coordinate carbohydrate metabolism in order to balance carbon assimilation, storage and growth (Smith and Stitt 2007). Carbon balance within the leaf is achieved through regulation of photosynthesis and carbon export (Ensminger et al. 2006, Ainsworth and Bush 2011). Photosynthates in leaves are largely allocated among distinct carbohydrate pools that serve different functions: hexose (immediate use), sucrose (export to sink tissues), and starch (transient storage in the leaf) (Fig. 1.4; Smith and Stitt 2007). Decreasing photoperiod and temperature induce major changes in carbohydrate metabolism. The cessation of growth decreases demand for photoassimilates, resulting in loss of leaf starch and enhanced export of carbohydrates from leaves to stem and root sink tissues (Oleksyn et al. 2000, Hoch et al. 2003). Low temperature can also inhibit enzyme-mediated carbohydrate metabolism, resulting in accumulation of triose phosphates in the cytosol and a depletion of inorganic phosphate within the chloroplast. As a result, the regeneration of ribulose-1,5-bisphosphate (RuBP), and consequently photosynthesis, is inhibited (Stitt and Hurry 2002).

Low temperature also induces accumulation of soluble carbohydrates in conifers, including sucrose, glucose, fructose, maltose, trehalose, raffinose, and raffinose family oligosaccharides (RFOs) (Strimbeck et al. 2008, Dauwe et al. 2012). Of these carbohydrates, elevated levels of glucose, sucrose and raffinose are known to confer freezing tolerance and are associated with cold hardening (Fig. 1.1; Uemura and Steponkus 2003, Strimbeck et al. 2009, Knaupp et al.
While the accumulation of glucose occurs as a result of decreased glycolysis (Dauwe et al. 2012), sucrose and raffinose production is stimulated under low temperature by upregulation of biosynthetic enzymes, which enhances enzyme activity in spite of temperature-limited kinetics (Guy et al. 1992, Schrader and Sauter 2002, Dauwe et al. 2012).

The precise roles of soluble carbohydrates in chilling and freezing tolerance are not fully understood, but it is hypothesized that they contribute to freezing tolerance by increasing solute concentrations and lowering the freezing point of the cytoplasm (Schrader and Sauter 2002). Soluble carbohydrates also may perform specific functions in abiotic stress tolerance, particularly in mitigating oxidative stress. Studies in *Arabidopsis* indicate a role for maltose in protecting photochemical efficiency of PSII against cold shock (Kaplan and Guy 2005) and for

**Figure 1.4: Initial steps of carbohydrate metabolism following photosynthetic carbon assimilation.** In the chloroplast, the Calvin cycle produces triose phosphates glyceraldehyde 3-phosphate (GAP) and its isomer dihydroxyacetone phosphate (DHAP), which are exported to the cytosol. Triose phosphates are converted into sucrose, which can either be exported from the leaf or enter the pentose phosphate pathway. Black boxes indicate major metabolic processes. 3-PGA – 3-phosphoglyceric acid; DHAP – dihydroxyacetone phosphate; GAP – glyceraldehyde 3-phosphate; RuBP – ribulose-1,5-bisphosphate; UDP – uridine diphosphate; UDP-glu – uridine diphosphate glucose. Figure adapted from Rolland et al. (2006).
raffinose in the stabilization of photosystem II (PSII) under low temperature conditions (Knaupp et al. 2011). Accumulation of sucrose and glucose may activate sugar signaling pathways to upregulate cold and oxidative stress response (Rolland et al. 2006, Van den Ende and El-Esawe 2014), or mitigate production of ROS by suppressing oxidative metabolism (Keunen et al. 2013). Furthermore, RFOs have been implicated in radical scavenging (Van den Ende and El-Esawe 2014). These findings indicate that overwintering plants have the ability to adjust and modify carbohydrate metabolism to improve photoprotection and cold hardening during cold acclimation.

1.4 Low Temperature Induction of Cold Response

Sensing Low Temperature

In the following sections, we provide an overview of the molecular regulation of the cold response, which is largely based upon extensive work done using Arabidopsis and other herbaceous model species. We highlight relevant studies performed in trees where available; however, most of the pathways detailed below have not been investigated in conifers.

As we have previously discussed, physiological consequences of the exposure to cold and freezing are well characterized. However, the molecular mechanisms behind cold sensing are not fully elucidated. Known components involved in low temperature sensing include the plasma membrane, redox status of the chloroplast, and calcium as a second messenger.

The composition and physical properties of the plasma membrane are highly responsive to changes in temperature; thus, cell and organelle membranes are considered to be involved in temperature-sensing (Uemura et al. 2006), where the temperature signal is likely transduced via the interaction of membrane-bound proteins and the low temperature signaling pathway (Los and Murata 2004). Enhanced membrane fluidity was correlated with increased freezing tolerance and was also implicated in the regulation of gene expression in a study using Medicago sativa (Örvar et al. 2000). In this study, membrane rigidification was chemically induced at 25°C, which resulted in increased expression of a cold acclimation-specific gene (cas30) and increased freezing tolerance. In contrast, chemical enhancement of membrane fluidity at 4°C inhibited cas30 expression and impaired freezing tolerance (Örvar et al. 2000). In Arabidopsis, low
temperature-induced membrane rigidification activates a signal transduction pathway mediated by diacylglycerol kinase (Vaultier et al. 2006). Transcriptome studies have shown that several cold-induced genes code for fatty acid desaturases which increase the number of desaturated phospholipids, which in turn increases membrane fluidity (Los and Murata 2004).

The redox state of the plastoquinone pool in the thylakoid membrane may also act as an environmental sensor due to the sensitivity of photosynthesis to changes in environmental conditions such as temperature, light, and availability of water and nutrients (Huner et al. 1996, Foyer and Noctor 2003). Bräutigam and colleagues (2009) demonstrated in Arabidopsis that changes in the redox status of the plastoquinone pool dynamically altered metabolite pools and expression of genes involved in photosynthesis, carbohydrate metabolism and amino acid metabolism. This indicates a prominent role of the chloroplast redox state for the long-term regulation of photosynthesis and metabolism in addition to rapid response to changing light conditions (Bräutigam et al. 2009). Under low temperature and high light conditions, the plastoquinone pool of the photosynthetic electron transport chain in the thylakoid membrane becomes increasingly reduced, which impedes energy transfer through the photosystems and results in increased excitation pressure (Huner et al. 1996). As discussed in section 1.3, the photosynthetic apparatus can acclimate to low temperatures, relieving excitation pressure and improving tolerance to high light stress (Huner et al. 1996, Ensminger et al. 2006, Demmig-Adams et al. 2012).

Calcium also acts as a messenger to transmit the low temperature signal by activating the signal transducer calmodulin (CAM), which mediates several pathways including development, defense against pathogens, and abiotic stresses, e.g. temperature, drought, salinity, and light (Fig. 1.4; Conde et al. 2011). Ca$^{2+}$ strongly accumulates during initial exposure to cold and is required for cold hardening in Arabidopsis (Knight et al. 1996). The early influx of extracellular calcium ions into protoplasts was found to be correlated with the expression of cold acclimation-specific genes in Medicago sativa protoplasts (Monroy and Dhindsa 1995). Diverse roles of calcium-dependent protein kinases (CDPKs) in cold signalling have also been described. Mutants overexpressing CDPKs exhibited enhanced drought and freezing tolerance in Populus euphratica (Chen et al. 2013) and Oryza sativa (Saijo et al. 2000); conversely, Zea mays mutants overexpressing the CDPK ZmCPK1 exhibited repression of cold stress markers and impaired freezing tolerance (Weckwerth et al. 2015).
Temperature Induction of Cold Response is Mediated by the CBF Pathway

The primary regulators of cold response genes are the CBF/DREB1 (C-repeat Binding Factors, also known as Dehydration Responsive Element Binding proteins) family of transcription factors that were originally described in *Arabidopsis* (Shinozaki et al. 2003). DREBs are a highly conserved family of ABA-independent cold and drought-induced transcription factors that are rapidly activated following 15 minutes of exposure to 4°C (Thomashow 2010). They fall into two subfamilies, DREB1a-c (or CBF1-3), which respond to low temperature, and DREB2, which responds to osmotic stress (Fig. 1.2; Shinozaki et al. 2003). DREB1 and DREB2 both bind to the cis-acting DREs (Dehydration Responsive Elements), also known as rd29A in *Arabidopsis*, that subsequently induce expression of stress responsive genes (Fig. 1.2; Shinozaki et al. 2003). In *Arabidopsis*, the CBF family is regulated upstream by ICE1 (Inducer of CBF Expression 1) which induces CBF3 and inhibits the CBF-repressing transcription factor MYB15 under low temperature conditions (Fig. 1.2; Chinnusamy et al. 2007, Thomashow 2010).

CBF1-3 function upstream of a series of cold-regulated (COR) genes (Fig. 1.2), including those that promote the synthesis of dehydrins (Puhakainen et al. 2004), raffinose (Cook et al. 2004) and proline (Dörffling et al. 2009), metabolites known to enhance freezing tolerance via membrane or protein stabilization. In *Arabidopsis*, overexpression of DREB1a/CBF3 enhances overall freezing tolerance as well as proline and soluble carbohydrate levels (Gilmour et al. 2000). Transgenic *Oryza sativa* overexpressing DREB1 and grown at 28°C were shown to accumulate glucose, fructose, sucrose and raffinose to similar or higher levels than wild-type plants exposed to 4°C cold stress (Ito et al. 2006), illustrating that the CBF pathway controls cold-induced soluble carbohydrate synthesis.

In addition to the CBF pathway, an ABA-dependent signalling pathway mediates development and abiotic stress response (Fig. 1.2; Umezawa et al. 2010). ABA biosynthesis is upregulated under low temperature and drought conditions (Li et al. 2003, Dörffling et al. 2009). Increased ABA levels activate protein kinase pathways, including a pathway controlled by sucrose non-fermenting-1-related protein kinase 2 (SnRK2) (Umezawa et al. 2010). SnRK2 regulates activity of the AREB/ABF (ABA-responsive Element Binding Factor) transcription factors which mediate drought response via rd29B (Fig. 1.2; Umezawa et al. 2010). ABA also activates MYC/MYB transcription factors, which regulate drought-responsive genes such as rd22.
(Umezawa et al. 2010). Interestingly, the Arabidopsis gene CBF4 is a homolog of DREB1 but is unresponsive to low temperature alone; instead, CBF4 is activated by drought and ABA (Fig. 1.2; Haake et al. 2002). Like other CBFs, CBF4 binds to the promoter of rd29A; overexpression of CBF4 suppresses growth and activates COR genes (Haake et al. 2002). Thus, although the ABA-independent DREB1 family is recognized as the major cold response regulator, ABA still plays a significant role in cold response.

1.5 Photoperiodic Induction of Cold Response

Phytochromes Can Mediate Low Temperature Responses

Plants sense light with photoreceptors that are predominantly sensitive to red, far-red, and blue wavelengths and include phytochromes (Phy), cryptochromes (Cry) and phototropins (Phot) (Fig. 1.2). These photoreceptors are integral for sensing light signals and mediate photomorphogenesis, flowering induction and dormancy (Jiao et al. 2007). Exposure of phytochromes to red or far-red wavelengths causes conversion to active (Pfr) or inactive (Pr) isoforms, respectively; Pfr activates kinase-mediated pathways in the cytosol or nucleus (Jiao et al. 2007). In Arabidopsis, Pfr represses CBF activity under long day conditions (Lee and Thomashow 2012). Pfr also represses the constitutive repressor of photomorphogenesis COP1, which induces degradation of the transcription factor elongated hypocotyl 5 (HY5) (Saijo et al. 2003). The accumulation of HY5 activates cold response genes via the Z-box/low temperature responsive element (LTRE) pathway, which functions independently of the CBF-mediated pathway (Fig. 2; Catalá et al. 2011, Lee and Thomashow 2012).

Photoperiodic Induction of Cold Response and Growth Cessation is Gated by the Circadian Clock

The circadian clock is a network of negative feedback loops that is synchronized to changes in photoperiod by phytochromes and cryptochromes, which induce the expression of clock-associated transcription factors CCA1 and LHY (Fig. 2; Jiao et al. 2007). Experiments using Arabidopsis have shown that the circadian clock controls the cold response via the CBF pathway. Fowler and colleagues (2005) demonstrated increased accumulation of CBF1-3 transcripts in plants exposed to cold at 4 hours after dawn compared to plants exposed to cold at 16 hours after dawn. Dong and colleagues (2011) confirmed that cold-induced expression of
CBF1-3 is controlled by CCA1 and LHY, with peaks of CBF expression occurring 8 hours after
dawn, followed by induction of key cold response genes including the cryoprotectant Cor15a,
dehydrin Cor47 and low temperature-induced protein Cor78; double mutants lacking CCA1 and
LHY exhibited impaired freezing tolerance.

Recently, phytochrome interacting factors (PIFs) have been identified as key integrators of
photoperiod and temperature cues mediating a cold response (Lee and Thomashow 2012).
Transcription of PIFs are controlled by the circadian clock component CCA1 (Niwa et al. 2009,
Lee and Thomashow 2012), and are differently expressed in response to short photoperiod
(Ruttink et al. 2007, Niwa et al. 2009, Lee and Thomashow 2012). In Arabidopsis, PIFs were
shown to suppress CBF expression under long photoperiods (Lee and Thomashow 2012) and to
promote growth under short photoperiods (Niwa et al. 2009). However, in Populus, PIFs were
expressed under short photoperiods during the cessation of growth (Ruttink et al. 2007),
indicating a different role of PIFs in the growth regulation of Populus from that characterized in
Arabidopsis.

Another key mechanism involved in sensing photoperiod and controlling growth cessation and
bud set is the highly conserved CONSTANS (CO)/FLOWERING LOCUS T (FT) module,
which is located downstream of the circadian clock (Fig. 1.2; Böhlenius et al. 2006, Andrés and
Coupland 2012, Cooke et al. 2012). CO protein is constitutively synthesized, but is degraded
during the night (Andrés and Coupland 2012). Long photoperiods allow for sufficient CO
accumulation to activate FT transcription in leaves (Andrés and Coupland 2012). When
translocated to shoot apical meristems, FT activates downstream transcriptional pathways that
result in flowering and vegetative growth (Andrés and Coupland 2012). Under short photoperiod,
Degradation of CO inhibits FT transcription, which initiates cessation of growth and bud set (Fig.
2; Böhlenius et al. 2006, Cooke et al. 2012). In photoperiod-sensitive trees, such as Populus,
Betula and Salix, interaction of red-light-sensitive phytochromes with the CO/FT module
determines the critical day length required to induce cessation of growth and bud set (Böhlenius
et al. 2006, Cooke et al. 2012, Petterle et al. 2013). The putative blue-light photoreceptor FKF1
has also been implicated in photoperiod sensing upstream of CO/FT (Fig. 1.2; Sawa et al. 2007).

Changes in photoperiod alone are sufficient to induce cold hardening. Lee and Thomashow
(2012) established that photoperiod regulates CBF expression: Arabidopsis plants grown under
warm temperature and short-day conditions periodically enhanced transcription of CBFs 8 hours after dawn and exhibited increased freezing tolerance compared to plants grown under warm temperature and long-day conditions. Rostad and colleagues (2006) demonstrated that *Picea abies* seedlings grown under 8-hour day length also exhibited greater freezing tolerance than counterparts grown under 16- to 18-hour day length. These observations underline the importance of photoperiod as a signal for the induction of cold acclimation.

### 1.6 Challenges Imposed by a Future Climate

The accumulation of greenhouse gases continues to contribute to increases in global air temperature and this warming is exacerbated at higher latitudes (IPCC 2014), where evergreen conifers dominate the boreal forest biome. Elevated temperature creates a mismatch in the phasing of temperature and photoperiod during spring and autumn, which can impact plant phenology. Over the past two decades, an increase in the length of the growing season has been observed in the northern hemisphere, with an earlier onset of greening during spring and a delay of senescence of vegetation during autumn (Piao et al. 2007). Rising temperatures during the 20th century have already induced migration of trees in Canada and the northern United States to higher latitudes (e.g. Gamache and Payette 2005) and altitudes (e.g. Beckage et al. 2008). The rapid rate of climate change makes long-lived species such as trees particularly vulnerable (Loarie et al. 2009). Even species that can rapidly migrate may be restricted in range by their dependence upon photoperiod and/or temperature cues (Körner and Basler 2010). In order to comprehend the complexity of tree responses to climate and improve risk assessment for future forests, it is necessary to integrate findings from genetic, molecular, physiological and ecological studies (Aitken et al. 2008).

The delay of phenological events during autumn may negatively impact cold acclimation in overwintering species. As we have discussed, temperature and light cues are integral to the induction of cold responses. Open questions remain as to whether warmer autumns may delay or impair the development of cold acclimation, and whether shorter photoperiod alone can sufficiently induce cold acclimation during autumn in the absence of the low temperature signal. Furthermore, much of our current understanding of the molecular basis of cold acclimation relies on studies using herbaceous annuals, such as spinach, soybean and *Arabidopsis*, or deciduous trees, such as *Populus*. Until recently, conifers have been notoriously difficult to sequence due to
their large genomes (~100 times the size of the *Arabidopsis* genome); consequently, conifers have been largely excluded from a number of genetic and molecular approaches available to model plant species (Neale and Kremer 2011). Technological advances have enabled the sequencing of *Picea abies* (Nystedt et al. 2013), *Picea glauca* (Birol et al. 2013) and *Pinus taeda* (Neale et al. 2014) genomes. The advent of these resources opens exciting avenues of investigation that will facilitate a deeper understanding of the genetic and molecular bases underlying tree responses to climate change.

### 1.7 Thesis Framework

The research presented in this thesis aims to elucidate the impact of photoperiod, temperature and elevated CO$_2$ on the induction and development of autumn cold acclimation in the evergreen conifer *Pinus strobus* L. We combined physiological measurements (photosynthetic gas exchange, chlorophyll fluorescence, water potential, and freezing tolerance) and analyses of key molecules (photosynthetic pigments, nonstructural carbohydrates, and proteins) to assess three major processes involved in cold acclimation: downregulation of photosynthesis, changes in carbohydrate metabolism and the development of freezing tolerance. Experiments were designed to test the following hypotheses:

1. The induction and development of cold hardening, which includes downregulation of photosynthesis, accumulation of cryoprotective carbohydrates and development of freezing tolerance, requires both low temperature and short photoperiod.

2. Elevated autumn temperature, e.g. only exposure to a short photoperiod, extends the growing season by delaying the downregulation of photosynthesis and associated changes in carbohydrate metabolism, and impairs the development of freezing tolerance.

3. The combination of elevated temperature and elevated CO$_2$ enhances photosynthesis in addition to extending the growing season, but at the expense of further impairing freezing tolerance.

Three sets of experiments were performed as follows:

The experiment presented in Chapter 2 was designed to determine how quickly leaf carbohydrates and photosynthesis are adjusted in response to a shift from long to short
photoperiod under warm conditions, and how these adjustments are affected by the combination of either short photoperiod and low temperature, or short photoperiod and elevated CO₂. Diurnal adjustments of photosynthesis and carbohydrate metabolism were assessed during the first 3 days to investigate how short photoperiod, low temperature and elevated CO₂ affect energy and carbon balance in evergreen conifer needles. Leaf nonstructural carbohydrates, photosynthesis and photosynthetic pigments were analyzed at pre-dawn and after four hours of light exposure.

The experiment presented in Chapter 3 was designed to determine how induction and development of cold acclimation processes are affected when seedlings are shifted from long to short photoperiod and low temperature, warm temperature, or the combination of warm temperature and elevated CO₂. Photosynthesis, photosynthetic pigments, nonstructural carbohydrates, freezing tolerance and proteins associated with photosynthesis, carbohydrate metabolism and freezing tolerance were analyzed over the course of 36 days to examine the impact of elevated temperature and elevated CO₂ on timing and extent of cold acclimation. In this experiment, I also identified a novel dehydrin protein whose expression is strongly correlated with freezing tolerance.

The experiment presented in Chapter 4 was designed to assess the development of cold acclimation under the projected temperature for 2050 in southern Ontario. *P. strobus* seedlings grown in a field environment were exposed to either ambient or elevated air temperature using temperature free-air-controlled enhancement (T-FACE) infrared warming arrays. Photosynthesis, water potential, photosynthetic pigments, nonstructural carbohydrates and freezing tolerance were assessed over the course of autumn.

In Chapter 5, I discuss the implications of my findings. The results of the experiments performed in my thesis project reveal that during autumn, photoperiod controls carbohydrate metabolism and contributes to the development of freezing tolerance, while temperature controls photosynthesis and also contributes to the development of freezing tolerance. The results also demonstrate that under warmer climate, *P. strobus* can exhibit impairment of freezing tolerance, but the extent of impairment does not increase risk of winter freezing damage for seedlings growing in southern Ontario. Instead, elevated temperature and CO₂ may benefit *P. strobus* by extending the period of photosynthetic activity and enhancing carbon uptake during autumn.
1.8 References

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Chapter 2
Rapid Adjustment of Nonstructural Carbohydrates and Photosynthesis in Response to Short Photoperiod, Low Temperature or Elevated CO$_2$ in Eastern White Pine


Status: In preparation.
2.1 Abstract

During transition from long to short photoperiod, plants decrease daily carbon uptake, resulting in a transient source-sink imbalance that is restored by adjusting carbohydrate metabolism and photosynthesis. These adjustments are likely altered by low temperature, which inhibits both photosynthesis and carbohydrate metabolism, and elevated atmospheric CO2, which enhances photosynthesis and increases carbohydrate supply. We measured leaf nonstructural carbohydrates and photosynthesis to assess how Eastern white pine (Pinus strobus) seedlings diurnally regulate leaf carbon pools and energy use when shifted to short photoperiod, or in combination with either low temperature or elevated CO2. Control seedlings were acclimated to long day (14 h photoperiod), high temperature (22°C /15°C day/night), and either ambient (400ppm) or elevated (800ppm) CO2. All seedlings were then shifted to growth conditions with short day (8 h photoperiod) combined with either low temperature with ambient CO2 (12°C/5°C, 400ppm), high temperature with ambient CO2 (22°C/15°C, 400ppm), or high temperature with elevated CO2 (22°C/15°C, 800ppm). After three days of exposure to short photoperiod, seedlings exhibited increased nighttime depletion of leaf starch. Short photoperiod alone also did not induce any change in photosynthesis. The combination of short photoperiod and low temperature induced accumulation of leaf hexose and downregulation of photosynthetic gas exchange within 24 hours. Downregulation of light reactions and enhancement of photoprotection only occurred after several days of low temperature exposure. Elevated CO2 caused carbon uptake to exceed sink capacity under long photoperiod, but carbon demand was restored within 24 hours of shift to short photoperiod. Our findings suggest that P. strobus is able to rapidly adjust leaf carbohydrate pools to maintain carbon balance under short photoperiod at either ambient or elevated CO2. In contrast, the combination of low temperature and short photoperiod inhibits adjustment of carbohydrate pools and photosynthesis, and requires rapid induction of photoprotection to dissipate increased excess energy.

2.2 Introduction

Decreasing photoperiod and temperature trigger cold acclimation in evergreen conifers, which includes changes such as the downregulation of photosynthesis (Oleksyn et al. 2000, Ensminger et al. 2004, Busch et al. 2007, Busch et al. 2008, Frêchette et al. 2016), cessation of growth (Tanino et al. 2010, Petterle et al. 2013), bud dormancy (Tanino et al. 2010, Cooke et al. 2012),
and changes in carbon partitioning among tissues (Hoch et al. 2003, Strimbeck et al. 2008). Rising temperature and atmospheric CO₂ levels may influence the timing and extent of cold hardening. Several studies have revealed that the downregulation of photosynthesis in conifers can be delayed by warming (Wang 1996, Busch et al. 2007, Stinziano et al. 2015, Fréchette et al. 2016), whereas cessation of growth and bud dormancy can be accelerated by elevated temperature in trees that are photoperiod-sensitive, e.g. *Populus* (Kalcsits et al. 2009), or delayed in trees that are more temperature-sensitive, e.g. *Malus* and *Pyrus* (Heide and Prestrud 2005). In *Picea mariana* seedlings, exposure to elevated CO₂ decreased photosynthetic quantum yield and dark respiration but increased maximum photosynthetic carbon assimilation rate and accelerated bud dormancy (Bigras and Bertrand 2006). These seasonal-scale studies reveal that elevated temperature and CO₂ may have long-term impacts on photosynthesis, metabolism and dormancy during cold hardening. However, a key point is still unknown: how rapidly is cold hardening initiated by downregulation of photosynthesis and adjustment of carbohydrate metabolism in response to changes in photoperiod, temperature and CO₂?

In plants, the complex interactions between photosynthesis, carbohydrate metabolism and carbon partitioning are commonly described using a source-sink model (Kozlowski 1992, Krapp and Stitt 1995, Dietze et al. 2014). Using this model, photosynthesis is regulated to balance energy absorption (energy source) and photochemistry (energy sink) (Huner et al. 1998, Ensminger et al. 2006), while carbohydrate metabolism is regulated to balance photoassimilate production (carbon source) and utilization (carbon sink) (Ainsworth and Bush 2011). Autumn, characterized by decreasing photoperiod and temperature, presents a recurring challenge to energy and carbon balances in evergreen conifers. Shorter day length limits carbon uptake, while continuous metabolic activity during an extended length of the night further depletes shrinking carbon supplies. In late autumn, low temperature impairs metabolic activity and limits energy sink capacity while leaves continue to absorb light energy. As a result, conifers absorb excess amounts of light, resulting in an energy imbalance (Huner et al. 1998, Ensminger et al. 2006).

Allocation of nonstructural carbohydrates (NSCs) between sucrose and starch pools allows plants to regulate carbon balance between photosynthesis and carbohydrate metabolism (Smith and Stitt 2007). Newly synthesized photoassimilates typically face one of three fates within the leaf: 1) Immediate use in metabolic processes, e.g. glycolysis; 2) storage in the form of transitory starch, to fuel metabolic processes over night; or 3) export to sink tissues in the form of sucrose,
for growth or to accumulate reserves for future use (Smith and Stitt 2007, Dietze et al. 2014). In leaves, starch and sucrose are hydrolyzed into reducing sugars, e.g. glucose and fructose, to fuel metabolism. When carbon sink capacity is limited, leaf sucrose export decreases, resulting in accumulation of leaf sucrose, decreased photosynthetic activity and enhanced starch synthesis (Krapp and Stitt 1995, McCormick et al. 2008, Ainsworth and Bush 2011). Short photoperiod can also induce downregulation of carbohydrate metabolism. In Arabidopsis, exposure to short photoperiod initially induces nighttime carbon starvation (Gibon et al. 2004, 2009). Subsequent adjustments in leaf starch and sucrose synthesis rapidly restore the carbon balance within 2-3 days (Gibon et al. 2004, 2009). However, extended exposure to short photoperiod inhibits growth due to the decrease in net daily carbon gain (Gibon et al. 2009, Sulpice et al. 2014). Plants grown under short photoperiod exhibit lower overall protein content and activity of enzymes related to carbohydrate metabolism, glycolysis and respiration, but not photosynthesis (Gibon et al. 2009).

When energy sink capacity is limited, such as during low temperature exposure, an energy bottleneck is created at the photosystems as light energy is continuously absorbed but cannot enter the photochemical pathway (Ensminger et al. 2006). The accumulation of excess light energy encourages formation of reactive oxygen species (ROS) which may damage tissues or even induce programmed cell death (Adams et al. 2004). To avoid such oxidative damage and restore the energy balance, plants adjust the pigment composition of the thylakoids, which includes loss of leaf chlorophylls to minimize light absorption and the accumulation of photoprotective carotenoids to dissipate excess energy via nonphotochemical quenching (NPQ) (Ottander et al. 1995, Busch et al. 2007). The xanthophyll cycle mediates NPQ by reversibly de-epoxidizing violaxanthin, via the intermediate antheraxanthin, to zeaxanthin in order to dissipate excess light energy as heat (Demmig-Adams et al. 2012). At warm temperatures, the xanthophyll cycle can rapidly adjust to changing light conditions and performs energy-dependent quenching (qE); extended exposure to low temperature suppresses the conversion of zeaxanthin to violaxanthin, resulting in the constitutive quenching of absorbed light energy as heat known as sustained nonphotochemical quenching (NPQs) (Demmig-Adams et al. 2012). The violaxanthin-derived xanthophyll, neoxanthin, contributes to stability of the photosynthetic apparatus by preventing oxygen from entering the light harvesting complexes (LHCs) (Mozzo et al. 2008).
Lutein, a xanthophyll derived from α-carotene, performs quenching of triplet chlorophyll in the LHCs of photosystem II (PSII) together with violaxanthin (Dall’Osto et al. 2006).

Elevated atmospheric CO₂ levels facilitate photosynthesis in C3 plants by favoring the carboxylation reaction over the oxygenation reaction of Rubisco, thereby suppressing photorespiration (Leakey et al. 2009). CO₂ enrichment studies indicate that trees exposed to elevated CO₂ tend to increase carbon assimilation, photosynthetic quantum yield, growth, and aboveground dry matter production (Ceulemans and Mousseau 1994, Ainsworth and Long 2005, Leakey et al. 2009). However, trees do not necessarily enhance dark respiration in response to elevated CO₂ (Ceulemans and Mousseau 1994, Amthor 2000, Leakey et al. 2009). Trees that were artificially exposed to elevated CO₂ also accumulated significantly higher leaf nonstructural carbohydrates than trees growing at ambient CO₂ (Ainsworth and Long 2005). Similarly, *Quercus pubescens* growing near a geothermal vent, where natural CO₂ exposure ranged from 500-1000ppm, exhibited greater accumulation of NSCs in leaves and branch wood in comparison with trees growing at a control plot (Körner and Miglietta 1994). These findings suggest that trees constantly maintain reserves of assimilated carbon. Long-term CO₂ enrichment studies have also shown that extended exposure to elevated CO₂ often results in downward acclimation of photosynthesis (Ainsworth and Long 2005, Leakey et al. 2009). These findings indicate that excessive carbon uptake can limit carbon sink capacity, causing downregulation of photosynthesis to match the decreased carbon sink.

Our study aimed to characterize how leaf carbohydrate content and photosynthesis are rapidly adjusted in response to a shift from long photoperiod and warm conditions to 1) short photoperiod and warm temperature, 2) short photoperiod and low temperature, or 3) short photoperiod and warm temperature under elevated CO₂ in Eastern white pine (*Pinus strobus* L.) seedlings. Changes in leaf carbohydrates, photosynthetic rates, energy quenching, and photosynthetic leaf pigments were recorded over three days. We hypothesized that 1) seedlings strongly deplete leaf NSCs when initially shifted from long to short photoperiod and upregulate photosynthesis to restore carbon balance; 2) low temperature inhibits carbon utilization and mitigates carbon depletion under short photoperiod, but induces downregulation of photosynthesis; and 3) elevated CO₂ enhances carbon uptake and mitigates carbon depletion under short photoperiod, and increases photosynthesis.
2.3 Materials and Methods

Plant Material and Growth Conditions

Two replicate experiments were conducted during summer of 2012 using three-year-old (3+0) bare-rooted Eastern white pine (*Pinus strobus* L.) seedlings from a local seed orchard (Ontario seed zone 37, Somerville Seedlings, Everett, Canada). The seedlings were approximately 30-40 cm in height at the start of the experiment. In April, seedlings were planted in deep conifer pots with a volume of 3 L, filled with a mixture of sand and sphagnum peat moss (1:2 v/v) and fertilized with 28:10:10 (N:P:K) Water Soluble Evergreen & Acid Loving Plant Food (Miracle-Gro, Scotts, Maryville, OH, USA). Potted seedlings were then kept outside in an experimental garden and transferred to three growth chambers (two model GC20-BDAFLT and one model GC20-BDAF, Biochambers, Winnipeg, Canada) in early June.

Seedlings were acclimated to long day (LD, 14 h day length), high temperature (22°C/15°C day/night) conditions at either ambient (400 ppm) or elevated (800 ppm) CO₂. After 6 weeks, seedlings grown under ambient CO₂ were shifted to short day (SD, 8 h day length) conditions with either low temperature/ambient CO₂ (LTAC: 12°C/5°C day/night, 400 ppm CO₂) or high temperature/ambient CO₂ (HTAC: 22°C/15°C day/night, 400 ppm CO₂), while seedlings grown under elevated CO₂ were shifted to SD conditions with high temperature/elevated CO₂ (HTEC: 22°C/15°C day/night, 800 ppm CO₂). Photosynthetic active radiation at the top of the canopy was maintained at 1400 µmol quanta m⁻² s⁻¹ during the day and at 400 µmol quanta m⁻² s⁻¹ during the first and last half hour of each day. Light was provided using an equal distribution of metal halide and high pressure sodium lamps (for more details, see Appendix A1). Humidity was set to 60% RH.

Initial measurements and samples were taken after 6 weeks of acclimation to long day conditions before transfer to short day conditions (day 0). Chlorophyll fluorescence measurements and samples were taken from unique individuals on days 1 and 3 following shift to short photoperiod, while gas exchange measurements were taken on days 1 and 4. On each measuring day, measurements and samples were taken 1 h before growth lights turned on (night), and again after 4 hours of exposure to growth light (day). Seedlings were watered every 2-3 days and rotated...
within each chamber once per week. The experiment was replicated two times, and treatments were rotated among chambers between treatments.

**Nonstructural Carbohydrates**

Nonstructural leaf carbohydrates (NSCs) were assessed as indicators for changes in leaf carbohydrate metabolism. One sample of mature, current-year needle tissue (approximately 50 needles) was collected per seedling from five unique seedlings per time point, immediately flash-frozen in liquid nitrogen and stored at -80°C. Each sample was individually homogenized using a mortar and pestle in liquid nitrogen. An aliquot of 100 mg of the ground needle sample was then lyophilized. Leaf soluble carbohydrates were subsequently extracted according to Park et al. (2009). A total of 250 µg galactitol internal standard was added to 30-40 mg of the homogenized, lyophilized needle tissue. The mixture was incubated in 4 ml of extraction buffer (methanol:chloroform:water, 12:5:3 v/v/v), overnight at 4°C. The mixture was centrifuged at 6,000 rpm for 10 min and the supernatant collected. The pellet was washed twice in extraction buffer followed by centrifugation. A total of 5 ml of water was added to the total supernatant, mixed, and centrifuged at 4000 rpm for 4 min. Lastly, the upper aqueous phase was collected. Two ml of the soluble carbohydrate extract were dried using a Vacufuge 5301 (Eppendorf, Hamburg, Germany) and re-suspended in 1 ml of nanopure water. The re-suspended extract was filtered through a 0.45 µm pore nylon syringe filter (Chromatographic Specialties Inc., Brockville, ON, Canada) and analyzed using an ICS-5000 anion-exchange HPIC (Dionex, Sunnyvale, CA, USA) equipped with a Hi-Plex Ca column (Agilent Technologies, Santa Clara, CA, USA) and an electrochemical pulse amperometric detector (EC-PAD). Soluble sugars were eluted with water at a flow rate of 0.170 ml min⁻¹ with a column temperature of 70°C. Post-column detection was performed using NaOH at a rate of 100 mM min⁻¹.

Leaf starch was determined using the residual tissue pellet from the soluble sugar extraction according to Park et al. (2009). The pellet was dried overnight at 55°C. Roughly 20-30 mg of pellet was re-suspended in 5 ml of 2% H₂SO₄ and autoclaved for 5 min at 120°C. Once cooled, the extract was spun at 500 rpm for 5 min and the supernatant was collected, filtered using a 0.45 µm nylon filter and analyzed using a DX-600 anion-exchange IC/HPLC (Dionex, Sunnyvale, CA, USA) equipped with a Carbo-Pac PA1 column (Dionex, Sunnyvale, CA, USA) and EC-PAD. The extract was eluted with water at a flow rate of 1 ml min⁻¹ with a column temperature
of 30°C. Post-column detection was performed using NaOH at a rate of 100 mM min⁻¹. Peak detection and quantification of all nonstructural carbohydrates were performed using ChemStation software (Agilent Technologies).

All nonstructural carbohydrates were expressed as weight per dry weight. Soluble sugars were calculated as the sum of glucose, fructose, and sucrose. Hexose was calculated as the sum of glucose and fructose. To assess leaf carbohydrate usage, the ratio of sucrose to starch (Suc/Starch) was calculated as an indicator of carbohydrate allocation, where an increase in Suc/Starch represents an increase in the availability of carbon immediately usable for growth or export versus carbon transiently stored in the leaf (Muñoz et al. 2005, Smith and Stitt 2007). The ratio of hexose to sucrose (Hex/Suc) was also calculated as an indicator of sucrose hydrolysis (Zrenner et al. 1996), where an increase in Hex/Suc indicates an increase in carbon usable for metabolic processes versus export.

**Photosynthetic Gas Exchange**

Gas exchange was measured to assess changes in leaf carbon uptake and respiration. Gas exchange was measured at days 0, 1 and 4 inside the growth chambers on attached current-year needles of 5 seedlings after ≥2 h of exposure to growth light using a GFS-3000 (Walz, Effeltrich, Germany). The measuring cuvette was set to the following conditions: 400 or 800 ppm CO₂, 12 or 22°C, and 60% RH. Dark respiration (R₉) was measured after 40 minutes of dark adaptation. Net photosynthetic carbon assimilation (Aₙ₉) was subsequently measured at growth light intensity (1400 µmol quanta m⁻² s⁻¹) once steady state assimilation was achieved, after approximately 5-7 minutes. On each measuring day, the infrared gas analyzer was first warmed up for 30-60 minutes. Prior to each measurement, analyzer readings were assessed with a closed, empty cuvette to ensure that the baseline was zero. During initial measurement, needle area was arbitrarily set to 1 cm². All measurements were performed on attached needles that were arranged into a flat surface and secured using breathable tape prior to insertion in the cuvette. After the cuvette was closed, a puff of air was applied by mouth on the cuvette seal to test for leaks, the presence of which would be indicated by a spike in CO₂.

All measurements were performed on attached needles from the topmost branch. The needles were arranged in a flat, single-needle layer and secured with breathable tape before being
inserted into the cuvette. Following measurement, the measured region of needles within the cuvette was harvested and scanned to estimate the light-exposed needle surface area, using WinSeedle software (Regent Instruments Inc., Québec, QC, Canada). The correct surface area was then used to recalculate the original measurements using the GFS-Win software (Walz, Effeltrich, Germany).

**Chlorophyll Fluorescence**

Chlorophyll fluorescence was measured to assess changes in photochemical efficiency and dissipation of excess energy. Chlorophyll fluorescence was measured in the growth chamber on attached current-year needles of 5 different seedlings at each time point, using a Dual-PAM-100 (Walz, Effeltrich, Germany). Minimum PSII fluorescence ($F_o$) and maximum PSII fluorescence ($F_m$) were measured one hour before lights switched on, and again after four hours of light exposure. During daytime, needles were dark-adapted in the leaf clip prior to measurement of $F_o$ and $F_m$. This was followed by exposure to 1400 µmol quanta m$^{-2}$ s$^{-1}$ of actinic light for 3-5 minutes, after which light-adapted minimum PSII fluorescence ($F_o'$), light-adapted maximum PSII fluorescence ($F_m'$), and transient fluorescence ($F_t$) were measured. Maximum quantum yield of PSII was calculated as $F_v/F_m = (F_m - F_o)/F_m$, and effective quantum yield of PSII was calculated as $\Delta F/F_m' = (F_m' - F_t)/F_m'$ (Genty et al. 1989). The excitation pressure at PSII was calculated as $1 - qP = 1 - (F_m' - F_t)/(F_m' - F_o')$ (Maxwell and Johnson 2000). Nonphotochemical quenching was calculated as $NPQ = (F_{mrec}/F_m') - 1$ (Bilger and Björkman 1990, Ensminger et al. 2004). Sustained nonphotochemical quenching was calculated as $NPQ_S = (F_{mrec}/F_m) - 1$ (Maxwell and Johnson 2000, Ensminger et al. 2004, Porcar-Castell 2011, Chang et al. 2015). A good estimation of NPQ requires $F_m$ to be measured when the photosynthetic apparatus is in a fully relaxed state. Under low temperature, $F_m$ is depressed and does not relax rapidly in the dark, and consequently NPQ will be underestimated (Demmig-Adams et al. 2012). Thus, we estimated the fully recovered maximum fluorescence ($F_{mrec}$) as $F_o * 5$, according to Schreiber et al. (1995) and Ensminger et al. (2004). This estimation is based on two assumptions: firstly, the ratio of fully recovered $F_m/F_o$ is approximately equal to 5, which has been demonstrated in multiple plant species (Björkman and Demmig 1987), including conifers (Adams and Demmig-Adams 1994); and secondly, unlike $F_m$, $F_o$ shows little seasonal variation (Ottander et al. 1995).
Photosynthetic Pigments

Photosynthetic pigments were measured to assess changes in thylakoid membrane composition according to Junker and Ensminger (2016). Needle samples were collected from mature current-year needles following each measurement, flash-frozen in liquid nitrogen and stored at -80°C. The needle tissue from each individual tree was homogenized using a mortar and pestle in liquid nitrogen. About 50-60 mg of the homogenized needle powder was extracted at 4°C in 700 µl of 98% methanol buffered with 2% 0.5 M ammonium acetate for 2 h in the dark. The extract was centrifuged at 4°C at 14,000 rpm for 5 min and the supernatant collected. The pellet then was washed twice with 700 µl of 100% methanol at 4°C, followed by centrifugation to fully recover all pigments from the sample. Finally, the total supernatant was filtered using PTFE syringe filters with a pore size of 0.2 µm (Thermo Scientific, Rockwood, TN, USA).

Photosynthetic pigments were separated using a reverse-phase C30 column (model YMC Carotenoid, 5 µm, 250×4.6mm; YMC America Inc., Allentown, PA, US) and analyzed with an Infinity 1260 series high performance liquid chromatography (HPLC) system equipped with a UV-diode array detector (Agilent Technologies, Santa Clara, USA). Pigments were eluted using a gradient of methanol, water buffered with 0.2% ammonium acetate, and tert-butyl methyl ether at a flow rate of 1 ml min⁻¹ at a column temperature of 25°C. Wavelengths were scanned from 250 nm to 680 nm. Chromatograms at 290 nm, 450 nm and 656 nm were analyzed using Chemstation software (Agilent Technologies). Peak quantification was performed via Chemstation software, using standards for chlorophyll a and chlorophyll b from Sigma Aldrich (St. Louis, MO, USA), and antheraxanthin, α-carotene, β-carotene, lutein, neoxanthin, violaxanthin and zeaxanthin from DHI Lab products (Hørsholm, Denmark).

Total chlorophylls were expressed as the sum of chlorophylls a and b per fresh weight. The ratio of chlorophyll a to chlorophyll b, was expressed as mol mol⁻¹. All carotenoid concentrations were normalized to chlorophyll content to reflect the changing capacity for energy dissipation in relation to light absorption in conifer needles under the treatment conditions. Total carotenoids (Car) included violaxanthin (V), antheraxanthin (A), zeaxanthin (Z), neoxanthin, lutein, α-carotene and β-carotene, and were normalized to chlorophyll content. Total xanthophyll cycle pigments were calculated as the sum of V, A, and Z. The de-epoxidation state of the xanthophylls...
cycle pigments (DEPS) was calculated as \((0.5A+Z)/(V+A+Z)\), according to Thayer and Björkman (1990).

Statistical Analyses

The effects of treatment and time on photosynthetic pigments, carbohydrates, gas exchange and chlorophyll fluorescence were assessed using two-way ANOVA. Statistics were performed in R v3.1.1 (www.r-project.org/) using the lmer function of the lme4 package (Bates et al. 2014). The model used was: Variable ~ Treatment * Time + (1|Trial) + (1|Individual), where treatment and time represented categorical fixed factors, and trial (2 experiment replicates) and individual (5 biological replicates) represented random factors (Table 2.1). Tukey’s HSD post-hoc tests were used to contrast 1) between treatments at each time point (letters on the relevant figures), 2) between night and day for each experiment day (Table 2.2), and 3) between days of the experiment (Table 2.3). The Tukey contrasts were performed in R using the glht function of the multcomp package (Hothorn et al. 2008).

2.4 Results

Nonstructural Carbohydrates

On days 0 and 1, seedlings grown at high temperature and elevated CO₂ (HTEC) contained higher nonstructural carbohydrates (NSCs), including higher starch, soluble sugars and sucrose than seedlings grown at high temperature and ambient CO₂ (HTAC) or low temperature and ambient CO₂ (LTAC) (Fig. 2.1A-D). All measured carbohydrates significantly responded to treatment; total NSCs, soluble sugars and sucrose significantly responded to time (Table 2.1). Under long day length (LD: 14 h day), needles grown under elevated CO₂ contained significantly higher NSC and starch content during the day (Fig. 2.1A,B) than needles grown under ambient CO₂. By day 3 after shift to short day length (SD: 8 h day), total NSC and starch content of HTEC needles no longer differed from HTAC or LTAC needles.

Under LD, soluble sugar (Fig. 2.1C) and especially sucrose (Fig. 2.1D) levels were lower during the night than during the day in needles grown under ambient CO₂, but remained significantly higher in needles grown under elevated CO₂. After shift to SD, HTAC needles continued to exhibit low nighttime and high daytime soluble sugar and sucrose content. However, on day 3,
Figure 2.1: Changes in leaf nonstructural carbohydrates in response to low temperature/ambient CO$_2$ (LTAC), high temperature/ambient CO$_2$ (HTAC) and high temperature/elevated CO$_2$ (HTEC). 

A) Total nonstructural carbohydrates; B) starch content; C) soluble sugar content; D) sucrose content; E) hexose content. Vertical line separates long day (LD, 14 h day) and short day (SD, 8 h day) measurements; grey background indicates night period, white background indicates day period. Points represent the average of $n = 8-10 \pm$ SE, from two replicate experiments with $n = 5$ samples. Letters where present indicate significantly different treatment groups at each time point, determined by Tukey’s HSD ($\alpha = 0.05$).
Table 2.1: The effects of treatment and time on photosynthetic gas exchange, chlorophyll fluorescence, photosynthetic pigments, and carbohydrates.

Two-way ANOVA was applied using the following formula: \( \text{Variable} \sim \text{Treatment} \times \text{Time} + (1|\text{Trial}) + (1|\text{Individual}) \). Treatment and Time were included as categorical fixed factors. Trial, representing experiment replicates, and Individual, representing biological replicates, were included as random factors.

P-values in bold indicate statistical significance (\( \alpha = 0.05 \)).

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<tr>
<th>Parameter</th>
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<th>Time</th>
<th>Treat × Time</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
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<tr>
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<td>Starch</td>
<td>14.443</td>
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<td>17.276</td>
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<td>57.910</td>
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<td>0.233</td>
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<tr>
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<td>38.169</td>
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<td>18.109</td>
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Table 2.2: Significant changes in nonstructural carbohydrates, chlorophyll fluorescence, and photosynthetic pigments between night and day.
Tukey contrasts between night and day values on each experiment day. Only parameters with significant effects are shown. Bolded P-values indicate statistical significance (α = 0.05).

<table>
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<th>Parameter</th>
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<th>HTEC</th>
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<td>Night vs Day 1</td>
<td>Night vs Day 3</td>
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</tr>
<tr>
<td>DEPS</td>
<td>0.979</td>
<td>0.259</td>
<td>0.005</td>
</tr>
<tr>
<td>Vio/Chl</td>
<td>0.413</td>
<td>0.005</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ant/Chl</td>
<td>0.999</td>
<td>0.992</td>
<td>0.012</td>
</tr>
<tr>
<td>Zea/Chl</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Table 2.3: Significant changes in nonstructural carbohydrates, chlorophyll fluorescence, and photosynthetic pigments between days of the experiment.
Tukey contrasts between days of experiment. Only parameters with significant effects are shown. Bolded P-values indicate statistical significance (α = 0.05).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HTAC</th>
<th>LTAC</th>
<th>HTEC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0 vs 1</td>
<td>Day 1 vs 3</td>
<td>Day 0 vs 3</td>
</tr>
<tr>
<td>Hexose</td>
<td>1.000</td>
<td>0.125</td>
<td>0.044</td>
</tr>
<tr>
<td>A_{net}</td>
<td>1.000</td>
<td>1.000</td>
<td>0.999</td>
</tr>
<tr>
<td>R_d</td>
<td>0.996</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>F_{v}/F_{m}</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>NPQ_{S}</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>V+A+Z/Chl</td>
<td>1.000</td>
<td>0.992</td>
<td>1.000</td>
</tr>
<tr>
<td>DEPS</td>
<td>1.000</td>
<td>1.000</td>
<td>0.999</td>
</tr>
<tr>
<td>Zea/Chl</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>
LTAC needles retained soluble sugar and sucrose at night, while HTEC needles conversely began to exhibit nighttime depletion of soluble sugars and sucrose similar to HTAC needles.

Under LD, hexose content was significantly lower during daytime in needles grown under elevated CO₂ than needles grown under ambient CO₂ (Fig. 2.1E). After 3 days of SD treatment, hexose content significantly decreased in HTAC needles (Table 2.3), resulting in similar hexose levels as HTEC needles (Fig. 2.1E). In contrast, hexose levels in LTAC needles remained significantly elevated in comparison with HTAC and HTEC needles.

**Nonstructural Carbohydrate Partitioning**

Under LD, the ratio of sucrose to starch (Suc/Starch) increased from night to daytime in HTAC needles on day 1, but remained unchanged in needles grown under elevated CO₂ (Fig. 2.2A). The ratio of hexose to sucrose (Hex/Suc, Fig. 2.2B) responded strongly to treatment and time (Table 2.1). Under LD, Hex/Suc was significantly higher in needles grown under ambient CO₂ than needles grown under elevated CO₂. Hex/Suc also significantly decreased from nighttime to daytime in LTAC needles on day 1 (Table 2.2), but did not change in needles grown under elevated CO₂. After 3 days, LTAC needles retained higher Hex/Suc than both HTAC and HTEC needles.

![Figure 2.2: Changes in leaf carbohydrate allocation in response to low temperature/ambient CO₂ (LTAC), high temperature/ambient CO₂ (HTAC) and high temperature/elevated CO₂ (HTEC). A) Suc/Starch, ratio of sucrose to starch; B) Hex/Suc, ratio of hexose to sucrose. Vertical line separates long day (LD) and short day (SD) measurements; grey background indicates night period, white background indicates day period. Points represent the average of n = 8-10 ± SE, from two replicate experiments with n = 5 samples. Letters where present indicate significantly different treatment groups at each time point, determined by Tukey’s HSD (α = 0.05).]
Gas Exchange and Chlorophyll Fluorescence

Photosynthetic carbon assimilation ($A_{net}$), dark respiration ($R_d$), maximum quantum yield of PSII ($F_v/F_m$), and sustained nonphotochemical quenching (NPQs) responded strongly to both treatment and time (Table 2.1). Under LD, needles grown at ambient and elevated CO$_2$ exhibited similar $A_{net}$ (Fig. 2.3A) or $R_d$ (Fig. 2.3B). After shift to SD, HTAC needles retained high $A_{net}$ and exhibited a significant increase in $R_d$ by day 4 (Fig. 2.3B; Table 2.3). In contrast, both LTAC and HTEC needles exhibited decreased $A_{net}$ by day 1 and no change in $R_d$.

Under LD, seedlings exhibited similar photosynthetic quantum yield ($F_v/F_m$, $\Delta F/F_m'$), excitation pressure at PSII (1-$qP$), and nonphotochemical quenching (NPQ) irrespective of CO$_2$ level. No significant changes were observed until three days after shift to SD. On day 3, LTAC needles exhibited a significant decrease in both maximum quantum yield of PSII ($F_v/F_m$; Fig. 2.4A) and effective quantum yield of PSII ($\Delta F/F_m'$; Fig. 2.4A) (Table 2.3). Sustained NPQ (NPQs; Fig. 2.4D) was also significantly increased in LTAC seedlings on day 3 but did not develop in either HTAC or HTEC seedlings (Table 2.3).

![Figure 2.3: Response of photosynthetic gas exchange to low temperature/ambient CO$_2$ (LTAC), high temperature/ambient CO$_2$ (HTAC) and high temperature/elevated CO$_2$ (HTEC).](image-url)
A) $A_{net}$, photosynthetic carbon assimilation; B) $R_d$, dark respiration. Measurements were taken under growth conditions. Vertical line separates long day (LD) and short day (SD) measurements. Points represent the average of $n = 10 \pm SE$, from two replicate experiments with $n = 5$ samples. Letters where present indicate significantly different treatment groups at each time point, determined by Tukey’s HSD ($\alpha = 0.05$).
Figure 2.4: Response of chlorophyll fluorescence to low temperature/ambient CO₂ (LTAC), high temperature/ambient CO₂ (HTAC) and high temperature/elevated CO₂ (HTEC).

A) $F_{v}/F_{m}$, maximum quantum efficiency of PSII and $\Delta F/F_{m}'$, effective quantum efficiency of PSII; B) $1-qP$, excitation pressure at PSII; C) NPQ, nonphotochemical quenching; D) NPQ₅, sustained nonphotochemical quenching. Measurements were taken under growth conditions. Vertical line separates long day (LD) and short day (SD) measurements; grey background indicates night period, white background indicates day period. Points represent the average of $n = 8-10 \pm SE$, from two replicate experiments with $n = 5$ samples. Letters where present indicate significantly different treatment groups at each time point, determined by Tukey’s HSD ($\alpha = 0.05$).
Photosynthetic Pigments

Leaf chlorophylls (Fig. 2.5A) had a significant overall response to both treatment and time; chlorophyll a/b (Fig. 2.5B), β-carotene (Fig. 2.5D) and the ratio of carotenoids to chlorophylls (Car/Chl; Fig. 2.5E) responded significantly to treatment, while α-carotene (Fig. 2.5C) responded significantly to time (Table 2.1). However, none of these pigments significantly differed between treatments at each time point, or over the course of the experiment within each treatment. Under LD, chlorophyll a/b, β-carotene and Car/Chl were both slightly higher during the day in needles grown under elevated CO₂ in comparison with ambient CO₂; this minor effect disappeared upon shift to SD.

The de-epoxidation state of the xanthophyll cycle (DEPS; Fig. 2.6B), violaxanthin (Fig. 2.6C), neoxanthin (Fig. 2.6D) and zeaxanthin (Fig. 2.6G) significantly responded to both treatment and time; antheraxanthin (Fig. 2.6E) responded only to time, while lutein (Fig. 2.6F) responded only to treatment (Table 2.1). DEPS, violaxanthin, antheraxanthin and zeaxanthin exhibited significant diurnal responses (Table 2.2). While DEPS and violaxanthin levels exhibited significant diurnal changes in all three treatments, antheraxanthin was only diurnally induced in HTAC and LTAC needles, and zeaxanthin was only diurnally induced in LTAC needles (Table 2.2). The xanthophyll cycle responded most strongly to the LTAC treatment. Daytime levels of the xanthophyll cycle pigment pool (V+A+Z/Chl), DEPS and zeaxanthin were strongly increased within 24 hours of shift to LTAC conditions (Table 2.3). Under LD, needles grown under elevated CO₂ exhibited slightly higher DEPS, antheraxanthin, and zeaxanthin during the day than needles grown under ambient CO₂; this minor effect also disappeared upon shift to SD.

2.5 Discussion

Short photoperiod limits net daily carbon uptake and may temporarily upset leaf carbon balance, requiring adjustments in carbohydrate metabolism and photosynthesis to restore homeostasis. In this study, we assessed leaf nonstructural carbohydrates, photosynthesis and leaf pigments in order to better understand how conifer seedlings diurnally adjust carbohydrate metabolism and photosynthesis when shifted to short photoperiod. We also aimed to determine how response to short photoperiod is affected by low temperature or elevated CO₂.
Figure 2.5: Changes in photosynthetic and accessory leaf pigment levels in response to low temperature/ambient CO$_2$ (LTAC), high temperature/ambient CO$_2$ (HTAC) and high temperature/elevated CO$_2$ (HTEC).

A) Total chlorophylls; B) chlorophyll a/b, ratio of chlorophyll a to b; C) α-carotene; D) β-carotene; E) total carotenoids per chlorophyll. Vertical line separates long day (LD) and short day (SD) measurements; grey background indicates night period, white background indicates day period. Points represent the average of $n = 8-10 \pm SE$, from two replicate experiments with $n = 5$ samples.
Figure 2.6: Changes in photoprotective leaf pigments in response to low temperature/ambient CO$_2$ (LTAC), high temperature/ambient CO$_2$ (HTAC) and high temperature/elevated CO$_2$ (HTEC).

A) Xanthophyll cycle pigments including V, violaxanthin, A, antheraxanthin, and Z, zeaxanthin; B) DEPS, de-epoxidation state of the xanthophyll cycle; C) violaxanthin; D) neoxanthin; E) antheraxanthin; F) lutein; G) zeaxanthin. Vertical line separates long day (LD) and short day (SD) measurements; grey background indicates night period, white background indicates day period. Points represent the average of n = 8-10 ± SE, from two replicate experiments with n = 5 samples. Letters where present indicate significantly different treatment groups at each time point, determined by Tukey’s HSD (α = 0.05).
Short photoperiod rapidly induces an adjustment of leaf carbohydrates but does not impact photosynthesis

The impact of short photoperiod on the diurnal regulation of leaf NSCs has been extensively studied in herbaceous plants such as *Arabidopsis*, but is poorly understood in conifers. Plants growing under short photoperiod may deplete carbon reserves due to the constraints to carbon uptake during the decreased daylight hours. Under a 12 h photoperiod, *Arabidopsis* maintains constant levels of sucrose over day and night (Gibon et al. 2004), strongly accumulates leaf starch during the day (Gibon et al. 2004, Sulpice et al. 2014), and exhausts leaf starch reserves by the end of the night (Gibon et al. 2004, 2009). After shift to short photoperiod, *Arabidopsis* exhibits premature exhaustion of leaf starch during the first night, adjusts starch and sucrose levels during the following day, and no longer prematurely exhausts leaf starch by the second night (Gibon et al. 2004). In plants grown for several weeks under short photoperiods, enhanced photosynthesis increases daily net carbon uptake (Sulpice et al. 2014), while the rate of nighttime starch degradation is decreased to ensure sufficient starch reserves throughout the night period (Gibon et al. 2009, Graf et al. 2010, Sulpice et al. 2014).

We hypothesized that *Arabidopsis* and conifers would similarly regulate leaf carbon in response to short photoperiod. However, it appears that *P. strobus* regulates leaf carbohydrates differently from *Arabidopsis* under both long and short day conditions. When shifted from long to short photoperiod, *P. strobus* seedlings did not fully exhaust leaf carbohydrates at the end of the first night. Instead, seedlings in all treatments exhibited only a 15-30% loss of leaf starch and sucrose at the end of the third night (Fig. 2.1B,D). Under long photoperiod, increased daytime sucrose levels contributed to a higher sucrose-to-starch ratio and a lower hexose-to-sucrose ratio (Fig. 2.2), reflecting that a greater proportion of carbohydrates was available during the day for leaf carbon export than was used for metabolism or transiently stored as starch. The reverse relationship was observed at night, as lower sucrose levels contributed to lower Suc/Starch and higher Hex/Suc. After seedlings were transferred from long to short photoperiod, seedlings also retained a high proportion of soluble leaf carbohydrates, with daytime Suc/Starch ranging from approximately 0.4 to 0.75, in comparison with *Arabidopsis* in which daytime Suc/Starch ranged from approximately 0.1 to 0.3 (Gibon et al. 2004).
Unlike herbaceous annuals such as *Arabidopsis*, trees typically reserve a proportion of assimilated carbon in stem and root tissues, and to a lesser extent in leaves, which is not invested in growth (Hoch et al. 2003, Körner 2003, Wiley and Helliker 2012). This carbon reserve can be remobilized years after initial storage in response to carbon limitation, e.g. from defoliation (Vargas et al. 2009, Barry et al. 2012, Dietze et al. 2014). Remobilization of carbohydrates from stem and root tissues to leaves has also been suggested in trees exposed to carbon limitation via drought stress (Adams et al. 2013). In our experiment, short photoperiod only had a minor impact on leaf starch levels, while a large pool of soluble leaf carbohydrates was consistently maintained. These findings suggest that *P. strobus* seedlings may utilize externally stored carbohydrate reserves to buffer against carbon limiting conditions such as short photoperiod. However, further studies are needed to characterize how carbon is transported among conifer leaf, root and stem tissues in response to short photoperiod.

Daytime respiration increased after four days of short photoperiod exposure in HTAC seedlings; however, respiration did not change in seedlings that were exposed to a combination of short photoperiod and low temperature, or short photoperiod and elevated CO2 (Fig 2.3B). In addition, photosynthesis was not upregulated in *P. strobus* seedlings in response to short photoperiod. Photosynthetic carbon assimilation, quantum yield of photosynthesis, and nonphotochemical quenching responded only to low temperature (Fig. 2.3A, 2.4). Likewise, photosynthetic pigment pools did not change in seedlings that were exposed to only short photoperiod (Fig. 2.5, 2.6). Evidently, short photoperiod did not upset leaf carbon balance enough to affect photosynthesis in *P. strobus* seedlings.

Components of the downregulation of photosynthesis, accumulation of carbohydrates and enhanced photoprotection are induced at different rates by low temperature

Seedlings that were exposed to a combination of short photoperiod and low temperature contained significantly higher hexose levels in comparison with seedlings exposed solely to short photoperiod (Fig. 2.1E). However, sucrose and starch were not significantly affected by low temperature exposure during our experiment. These findings differ from previous studies on *Arabidopsis*, which reported simultaneous hexose accumulation, increased starch degradation and increased sucrose synthesis in response to low temperature (Klotke et al. 2004, Kaplan et al.
In these studies, the accumulation of hexose was alternately interpreted as a chilling response which stimulated glycolysis and the pentose phosphate pathway (Klotke et al. 2004) or stimulated sucrose synthesis (Kaplan et al. 2007). However, LTAC seedlings did not exhibit a significant increase in either dark respiration or leaf sucrose levels which would support such conclusions. Instead, I suggest that the accumulation of hexose reflects low temperature inhibition of the activity of enzymes involved in hexose metabolism (Graham and Patterson 1982).

The downregulation of photosynthesis in evergreen conifers that is induced by low temperature and short photoperiod has previously been characterized using long-term studies spanning weeks or months (e.g. Savitch et al. 2002, Busch et al. 2007, Chang et al. 2015, Fréchette et al. 2016). Our findings indicate that the downregulation of photosynthesis can be rapidly initiated within three days of low temperature and short photoperiod exposure. The Calvin cycle is particularly sensitive to low temperature, which inhibits activity of enzymes involved in CO₂ assimilation including FBPase and SBPase (Sassenrath et al. 1990, Allen and Ort 2001, Ensminger et al. 2012). Indeed, seedlings exhibited decreased photosynthetic carbon assimilation within the first 24 h of exposure to low temperature and short photoperiod (Fig. 2.3A, Table 2.3). Interestingly, both effective and maximum quantum yield of PSII did not significantly decrease in LTAC seedlings until the third day of treatment (Fig. 2.4A, Table 2.3), indicating that the downregulation of photosynthetic gas exchange precedes the downregulation of the light reactions.

The xanthophyll cycle and lutein are two distinct groups of photoprotective pigments involved in the dissipation of excess energy. Within the first 24 hours of LTAC treatment, seedlings exhibited increases in leaf xanthophyll cycle pigment content (Fig. 2.6A) and the de-epoxidation state of the xanthophyll cycle (Fig. 2.6B) due to the daytime accumulation of large amounts of zeaxanthin (Fig. 2.6G). However, NPQ₅ did not develop until day 3, when increased overnight retention of antheraxanthin (Fig. 2.6E) and zeaxanthin (Fig. 2.6G) contributed to elevated nighttime DEPS (Fig. 2.6B). These results reflect the increased activation of the xanthophyll cycle to minimize risk of cold-induced photo-oxidative damage, as well as inhibited relaxation of the xanthophyll cycle in the dark. The role of the xanthophyll cycle as a rapid defense mechanism against photo-oxidative stress during energy-dependent quenching (qE) is well established (Demmig-Adams et al. 2012). Likewise, accumulated zeaxanthin is strongly...
associated with the development of NPQs under low temperatures during photoinhibitory quenching (Demmig-Adams et al. 2012).

Lutein functions in triplet chlorophyll quenching in vitro and can partially restore NPQ in the absence of the xanthophyll cycle (Dall’Osto et al. 2007). In *P. strobus*, lutein typically increases by 1.5- to twofold during mid to late autumn, when temperature has decreased below the freezing point and photosynthesis is being downregulated (Verhoeven et al. 2009, Chang et al. 2015). In our study, lutein slightly increased by day 3 in LTAC needles, but the change in pigment content was not significant between treatments or over time (Fig. 2.6F; Table 2.1). Stable amounts of lutein precursors α-carotene (Fig. 2.5C), β-carotene (Fig. 2.5D), as well as neoxanthin (Fig. 2.6D), were also observed throughout the experiment. A seasonal study of *P. strobus* and *Abies balsamea* reported stable neoxanthin and β-carotene levels, but a strong depletion of α-carotene that corresponded with a strong increase in lutein during autumn as temperature and photoperiod decreased (Verhoeven et al. 2009). Our findings indicate that low temperature can rapidly induce the xanthophyll cycle, but does not cause lutein to accumulate during short-term exposure.

**Under long photoperiod, elevated CO₂ causes carbon uptake to exceed leaf sink capacity**

*P. strobus* seedlings grown under elevated CO₂ exhibited higher accumulation of NSCs (Fig. 2.1A), particularly starch (Fig. 2.1B) and nighttime sucrose pools (Fig. 2.1D). Similarly, elevated CO₂ induced increased leaf starch content in *Pinus sylvestris* and *Picea abies* grown under a 22-hour photoperiod (Sallas et al. 2003). Accumulation of leaf NSCs was also observed in *Quercus pubescens* and *Q. ilex* growing near a geothermic vent, where CO₂ levels naturally ranged from 500 ppm to 1000 ppm (Körner and Miglietta 1994). Both *Quercus* species appeared to assimilate carbon without visibly increasing investment in structural growth, which the authors interpreted as a sign of CO₂ saturation (Körner and Miglietta 1994).

In our experiment, seedlings grown under elevated CO₂ and long photoperiod did not exhibit nighttime depletion of either starch or sucrose. Under long photoperiod, seedlings grown under elevated CO₂ contained significantly higher daytime starch (Fig. 2.1B), as well as significantly higher nighttime levels of soluble sugars (Fig. 2.1C) and sucrose (Fig. 2.1D), than seedlings grown under ambient CO₂. Furthermore, HTEC seedlings exhibited less diurnal variation in leaf
starch and sucrose levels, and maintained strikingly low Suc/Starch in comparison with seedlings grown under ambient CO₂ throughout the experiment (Fig. 2.2A). HTEC seedlings also exhibited both lower hexose content (Fig. 2.1E) and lower Hex/Suc than seedlings grown under ambient CO₂ (Fig. 2.2B). Unlike HTAC seedlings, HTEC seedlings did not increase dark respiration after shift to short photoperiod (Fig. 2.3B). Together, these findings indicate that *P. strobus* seedlings tend to increase carbon storage and export, rather than carbon usage, when exposed to elevated CO₂.

We also observed evidence that elevated CO₂ may cause *P. strobus* seedlings to exceed carbon sink capacity under long photoperiod. On day 0, seedlings grown under elevated CO₂ exhibited slightly lower total chlorophyll content during the day (Fig. 2.5A) and particularly chlorophyll b, the form of chlorophyll primarily associated with light harvesting antenna proteins (Murphy 1986), resulting in an increased chlorophyll a/b ratio (Fig. 2.5B). Seedlings grown under elevated CO₂ also exhibited lower Fₐ/Fₘ than seedlings grown under ambient CO₂ (Fig. 2.4A), reflecting their diminished capacity for photochemistry. Decreased leaf chlorophylls and decreased Fₐ/Fₘ were also observed in current-year needles of *Pinus sylvestris* after 7 weeks of acclimation to elevated CO₂ exposure under simulated summer conditions in a growth chamber experiment (Sallas et al. 2003). Similarly, decreased chlorophylls and decreased maximum rates of photosynthetic electron transport have been reported for grasses, crops and woody species exposed to elevated CO₂ using FACE experiments (Ainsworth and Long 2005).

Under long photoperiod, seedlings grown under elevated CO₂ contained larger pools of total carotenoids (Fig. 2.5E) and xanthophyll cycle pigments (Fig. 2.6A). These seedlings also exhibited mild increases of photoprotective pigments during the day, including increased antheraxanthin (Fig. 2.6E), zeaxanthin (Fig. 2.6G) and higher DEPS (Fig. 2.6B). The accumulation of these photoprotective pigments enabled HTEC seedlings to quench excess light energy away from the photosystem reaction centers (Demmig-Adams et al. 2012) and resulted in slightly higher NPQ on day 0 (Fig. 2.4C). Under long day conditions, NPQ was higher in *Picea mariana* seedlings (Bigras and Bertrand 2006) and shoots from mature *Pinus sylvestris* (Wang and Kellomäki 1997) exposed to elevated CO₂ in comparison with ambient CO₂. Elevated CO₂ also exacerbated light stress in two species of *Eucalyptus* (Roden and Ball 1996) exposed to high light, resulting in impaired photosynthesis, increased NPQ, and lower thresholds for photoinhibition.
Carbon sink demand was quickly restored after shift to short photoperiod. The slightly elevated daytime levels of antheraxanthin (Fig. 2.6E), zeaxanthin (Fig. 2.6G) and DEPS (Fig. 2.6B) present in HTEC seedlings on day 0 were no longer observed on days 1 or 3. Following shift to short photoperiod, HTEC seedlings also no longer contained significantly higher sucrose or starch than HTAC seedlings (Fig. 2.1B,C). Together, these responses suggest that the limitation on daily net carbon assimilation induced by short photoperiod allowed HTEC seedlings to utilize enough carbohydrates to restore local sink demand. As a consequence, increased sink capacity alleviated the energy imbalance and removed the necessity for enhanced photoprotection under elevated CO$_2$ conditions.

2.6 Conclusions

Our findings indicate that short-term exposure to short photoperiod induces moderate adjustments of leaf sucrose and starch pools within three days, but has a negligible effect on photosynthesis in *P. strobus* seedlings. The combination of short photoperiod and low temperature affects seedlings rapidly. Within 24 hours, leaf hexose accumulates and the downregulation of photosynthetic gas exchange occurs. After three days, downregulation of the light reactions and development of sustained nonphotochemical quenching occurs. Though seedlings exposed to long photoperiod and elevated CO$_2$ accumulate enough carbohydrates to exceed carbon sink capacity, shift to short photoperiod rapidly restores carbon sink demand within 24 hours. Our findings suggest that *P. strobus* is able to quickly re-equilibrate carbon balance under warm temperatures. This is achieved by adjusting carbohydrate pools in response to short photoperiod to avoid carbon limitation under ambient CO$_2$ conditions or carbon excess under elevated CO$_2$ conditions. However, low temperature inhibits both photosynthesis and carbohydrate metabolism, requiring additional photoprotection to restore energy balance.

2.7 References


Chapter 3
The Good and the Bad: Elevated Temperature and Elevated CO$_2$
Stimulate Late Season Photosynthesis But Impair Freezing Resistance in Eastern White Pine Seedlings


Status: Published.
3.1 Abstract

Rising temperature and CO₂ levels may sustain late season net photosynthesis of evergreen conifers, but may also impair the development of cold hardiness. Our study investigated how elevated temperature, and the combination of elevated temperature with elevated CO₂, affected photosynthetic rates, leaf carbohydrates, freezing tolerance, and proteins involved in photosynthesis and cold hardening in Eastern white pine (*Pinus strobus* L.). We designed an experiment where control seedlings were acclimated to long photoperiod (day/night 14/10h), warm temperature (22°C/15°C), and either ambient (400ppm) or elevated (800ppm) CO₂, and then shifted seedlings to growth conditions with short photoperiod (8/16h) and low temperature/ambient CO₂ (LTAC; 12°C/5°C), elevated temperature/ambient CO₂ (ETAC), or elevated temperature/elevated CO₂ (ETEC). Exposure to LTAC induced downregulation of photosynthesis, development of sustained non-photochemical quenching, accumulation of soluble carbohydrates, expression of a 16-kD dehydrin absent under long photoperiod, and increased freezing tolerance. In ETAC seedlings, photosynthesis was not downregulated, while accumulation of soluble carbohydrates, dehydrin expression, and freezing tolerance were impaired. ETEC seedlings revealed increased photosynthesis and improved water use efficiency, but impaired dehydrin expression and freezing tolerance similar to ETAC seedlings. 16-kD dehydrin expression strongly correlated with increases in freezing tolerance, suggesting its involvement in the development of cold hardiness in *P. strobus*. Our findings indicate that exposure to elevated temperature and elevated CO₂ during autumn can delay downregulation of photosynthesis and stimulate late season net photosynthesis in *P. strobus* seedlings. However, this comes at the cost of impaired freezing tolerance.

3.2 Introduction

Land surface temperature is increasing, particularly in the northern hemisphere (IPCC 2014) which is dominated by boreal and temperate forests. In higher latitudes, trees rely on temperature and photoperiod cues to detect changing seasons and to trigger cessation of growth and cold hardening during the autumn (Ensminger et al. 2015). For boreal and temperate evergreen conifers, cold hardening involves changes in carbohydrate metabolism, downregulation of photosynthesis, accumulation of cryoprotective metabolites, and development of freezing tolerance (Crosatti et al. 2013, Ensminger et al. 2015). These processes minimize freezing
damage and enable conifers to endure winter stresses. However, rising temperatures result in asynchronous phasing of temperature and photoperiod characterized by delayed arrival of first frosts (McMahon et al. 2010), which may impact the onset and development of cold hardening during autumn.

Short photoperiod induces the cessation of growth in many tree species (Downs and Borthwick 1956, Heide et al. 1974, Repo et al. 2000, Böhlenius et al. 2006). As a consequence, carbon demand in sink tissue decreases towards the end of the growing season, and the bulk of photosynthetic assimilates are translocated from source tissues to storage tissues (Hansen and Beck 1994, Oleksyn et al. 2000). In addition, cryoprotective soluble sugars including sucrose, raffinose, and pinitol accumulate in leaf tissues to enhance freezing tolerance (Strimbeck et al. 2008, Angelcheva et al. 2014). Thus, by winter, leaf nonstructural carbohydrates are mainly comprised of mono- and oligosaccharides, and only minimal levels of starch remain (Hansen and Beck 1994, Strimbeck et al. 2008). The concurrent decrease of photosynthetic assimilates and demand for metabolites that occur during the cessation of growth also impacts the citric acid cycle, which mediates between photosynthesis, respiration, and protein synthesis. The citric acid cycle generates NADH to fuel ATP synthesis via mitochondrial electron transport, as well as amino acid precursors (Shi et al. 2015). In C3 plants, the enzyme phosphoenolpyruvate carboxylase (PEPC) converts phosphoenolpyruvate (PEP) to oxaloacetic acid in order to supplement the flow of metabolites to the citric acid cycle, and thus controls the regulation of respiration and photosynthate partitioning (O’Leary et al. 2011).

Cessation of growth, low temperature, and presumably short photoperiod decrease the metabolic sink for photoassimilates, resulting in harmful excess light energy (Öquist and Huner 2003, Ensminger et al. 2006) and increased generation of reactive oxygen species (ROS) (Adams et al. 2004). During autumn and the development of cold hardiness, conifers reconfigure the photosynthetic apparatus, in order to avoid absorption of excess light and formation of ROS. This involves a decrease in chlorophylls and PSII reaction center core protein D1 (Ottander et al. 1995, Ensminger et al. 2004, Verhoeven et al. 2009), as well as aggregation of light harvesting complex (LHC) proteins (Ottander et al. 1995, Busch et al. 2007). Additionally, photoprotective carotenoid pigments accumulate in leaves, especially the xanthophylls zeaxanthin and lutein, which contribute to non-photochemical quenching (NPQ) via thermal dissipation of excess light energy (Busch et al. 2007, Verhoeven et al. 2009, Demmig-Adams et al. 2012). Prolonged

In conifers, freezing tolerance is initiated during early autumn in response to decreasing photoperiod (Rostad et al. 2006, Chang et al. 2015), and continues to develop through late autumn in response to the combination of short photoperiod and low temperature (Strimbeck and Schaberg 2009, Chang et al. 2015). In addition to changes in carbohydrate content, freezing tolerance also involves the expression of specific dehydrins (Close 1997, Kjellsen et al. 2013). Members of the dehydrin protein family are involved in responses to osmotic, salt and freezing stress (Close 1996). Correlations between dehydrins and improved freezing tolerance have been reported in many species including spinach (Kaye et al. 1998), strawberry (Houde et al. 2004), cucumber (Yin et al. 2006), peach (Wisniewski et al. 1999), birch (Puhakainen et al. 2004), and spruce (Kjellsen et al. 2013). In angiosperms, a characteristic lysine-rich dehydrin motif known as the K-segment interacts with lipids to facilitate membrane binding (Koag et al. 2009, Eriksson et al. 2011). Several in vitro studies have demonstrated dehydrin functions including prevention of aggregation and unfolding of enzymes (using Vitis riparia; Hughes and Graether 2011), radical scavenging (using Citrus unshiu; Hara et al. 2004), and suppression of ice crystal formation (using Prunus persica; Wisniewski et al. 1999). To date, dehydrin functions have not been demonstrated in planta.

Rising temperatures since the mid-twentieth century have delayed the onset of autumn dormancy and increased length of the growing season in forests across the northern hemisphere (Boisvenue and Running 2006, Piao et al. 2007, McMahon et al. 2010). Studies have shown that elevated temperatures ranging from +4 to +20°C above ambient can delay downregulation of photosynthesis in several evergreen conifers. These findings were consistent among climate-controlled chamber studies exposing Pinus strobus seedlings to a sudden shift in temperature and/or photoperiod (Fréchette et al. 2016), as well as chamber studies exposing Picea abies seedlings to simulated autumn conditions using a gradient of decreasing temperature and photoperiod (Stinziano et al. 2015). Similar findings were also demonstrated in open-top chamber experiments exposing mature Pinus sylvestris to a gradient of decreasing temperature and natural photoperiod (Wang 1996). Elevated temperature (+4°C above ambient) also impaired cold hardening in Pseudotsuga menziesii seedlings (Guak et al. 1998) and mature P. sylvestris...
Repo et al. 1996) exposed to a decreasing gradient of temperature and natural photoperiod using open-top chambers. In contrast, a recent study showed that smaller temperature increments (±1.5 to +3°C) applied using infrared heaters did not delay downregulation of photosynthesis or impair freezing tolerance in field-grown *P. strobus* seedlings which were acclimated to larger diurnal and seasonal temperature variations (Chang et al. 2015). For many tree species, photoperiod determines cessation of growth (Tanino et al. 2010, Petterle et al. 2013), length of the growing season (Bauerle et al. 2012), and development of cold hardiness (Welling et al. 1997, Li et al. 2003, Rostad et al. 2006). However, effects of warming climate on tree phenology are complex and can be unpredictable due to species- and provenance-specific differences in sensitivity to photoperiod and temperature cues (Körner and Basler 2010, Basler and Körner 2012, Basler and Körner 2014).

The effect of elevated CO₂ further increases uncertainties in the response of trees to warmer climate. Similar to warmer temperature, elevated CO₂ may also delay the downregulation of photosynthesis in evergreens and extend the length of the growing season, as demonstrated in mature *P. sylvestris* (Wang 1996). Elevated CO₂ increases carbon assimilation (Curtis and Wang 1998, Ainsworth and Long 2005) and biomass production (Ainsworth and Long 2005) during the growing season. This may continue during the autumn if dormancy or growth cessation is delayed, which suggests that elevated CO₂ may increase annual carbon uptake. However, long-term exposure to elevated CO₂ can also downregulate photosynthesis during the growing season (Ainsworth and Long 2005). Prior studies which have aimed to determine the impact of the combination of elevated CO₂ and/or temperature on cold hardening in evergreens have largely focused on freezing tolerance, with contrasting results. Open-top chamber experiments showed that the combination of elevated temperature and CO₂ both delayed and impaired freezing tolerance of *P. menziesii* seedlings (Guak et al. 1998) and evergreen broadleaf *Eucalyptus pauciflora* seedlings (Loveys et al. 2006), but did not affect freezing tolerance of mature *P. sylvestris* (Repo et al. 1996). A recent field experiment on mature trees revealed that *Larix decidua*, but not *Pinus mugo*, exhibited enhanced freezing damage following six years of exposure to combined soil warming and elevated CO₂ (Rixen et al. 2012). In contrast, a climate-controlled study showed that exposure to elevated CO₂ advanced the date of bud set and improved freezing tolerance in *P. mariana* seedlings (Bigras and Bertrand 2006). In a second study on similar seedlings conducted by the same authors, exposure to elevated CO₂ also
enhanced freezing tolerance but impaired the accumulation of sucrose and raffinose (Bertrand and Bigras 2006). The abovementioned experiments were tested using conditions involving gradual decreases in temperature and natural photoperiod. Their contrasting findings clearly imply that more information is needed to understand the sensitivity of processes involved in cold hardening to photoperiod, temperature and CO₂.

Our study aimed to determine 1) how induction and development of the cold hardening process is affected by a shift from long to short photoperiod under warm conditions, and 2) how the combination of warm air temperature and elevated CO₂ affects photoperiod-induced cold hardening processes in Eastern white pine (*Pinus strobus* L.). To assess the development of cold hardening, we measured photosynthetic rates, changes in leaf carbohydrates, freezing tolerance, and proteins involved in photosynthesis and cold hardening over 36 days. We hypothesized that warm temperature, and the combination of warm temperature and elevated CO₂, will prevent seedlings growing under autumn photoperiod from downregulating photosynthesis. We further hypothesized that warm temperature, and the combination of warm temperature and elevated CO₂ will impair the development of freezing tolerance, due to a lack of adequate phasing of the low temperature and short photoperiod signals.

### 3.3 Materials and Methods

**Plant Material and Growth Conditions**

Two replicate experiments were conducted in 2012 and 2013 using three-year-old (3+0) Eastern white pine (*Pinus strobus* L.) seedlings from a local seed orchard (Ontario seed zone 37, Somerville Seedlings, Everett, Ontario, Canada). In April, seedlings were planted in 3-liter pots filled with a mixture of sand and sphagnum peat moss (1:2 v/v) and fertilized with 28:10:10 (N:P:K) Water Soluble Evergreen & Acid Loving Plant Food (Miracle-Gro, Scotts, Maryville, OH, USA). Potted seedlings were then kept outside in an experimental garden at the University of Toronto Mississauga campus and transferred to growth chambers in early July (Biochambers, Winnipeg, Canada).

Seedlings were acclimated for 6 weeks to long photoperiod at either ambient CO₂ (AC: 22°C day/15°C night, 14 h day length, 400 ppm CO₂) or elevated CO₂ (EC: 22°C day/15°C night, 14 h day length, 800 ppm CO₂). Following acclimation to long photoperiod treatment, seedlings
grown under ambient CO2 were shifted to a short photoperiod treatment of either low
temperature/ambient CO2 (LTAC: 12°C day/5°C night, 8 h day length, 400 ppm CO2) or
elevated temperature/ambient CO2 (ETAC: 22°C day/15°C night, 8 h day length, 400 ppm CO2),
while seedlings grown under elevated CO2 were shifted to a short photoperiod treatment with
elevated temperature/elevated CO2 (ETEC: 22°C day/15°C night, 8 h day length, 800 ppm CO2).
Diurnal temperature variations in southern Ontario, where the seedlings originated, often exceed
10°C (Environment Canada 2016). We thus chose treatment ranges that differed by 10°C that
were physiologically relevant, but higher than the small temperature ranges (+1.5 to +3°C) that
we have used previously in long-term monitoring field experiments (Chang et al. 2015). The
12°C/5°C temperature range for the LTAC treatment represents typical chilling temperatures
experienced during mid-autumn (Fig. 3.7A), while the 22°C/15°C range for the ETAC treatment
represents conditions in which only the short photoperiod, but not low temperature, signal is
perceived. Photosynthetic active radiation at the top of the canopy was maintained at 1400 µmol
quanta m⁻² s⁻¹ during the day and at 400 µmol quanta m⁻² s⁻¹ during the first and last half hour of
each day. Light was provided using metal halide and high pressure sodium lamps. Humidity was
set to 60% RH.

Measurements and samples were taken after 6 weeks of long photoperiod acclimation (day 0)
prior to transferring seedlings to short photoperiod conditions, and repeated on days 1, 4, 8, 16,
26 and 36 following transfer. The duration of the experiment and measuring points were selected
to assess early, mid and late-stage responses to a change in photoperiod and temperature, given
constant exposure to 400 or 800 ppm CO2. We did not perform control measurements with
unchanged photoperiod and temperature, since no change in photosynthesis, pigments, and
proteins occur when photoperiod and temperature remain constant (Busch et al. 2007). All
measurements were taken at growth temperature. Throughout the experiment, seedlings were
watered every 2-3 days. Seedlings were rotated within each chamber once per week. During the
second replicate experiment, treatments were rotated among chambers.

Mature current-year needle samples were obtained from field-grown seedlings during August 17,
September 26, October 26, and November 16 of 2013 for the analysis of leaf dehydrins and
freezing tolerance. Three-year-old (3+0) seedlings obtained from the same seed orchard used in
the growth chamber experiment were established in five replicate field plots during May 2012 at
the Koffler Scientific Reserve of the University of Toronto, located in southern Ontario.
(44°050'N, 79°483’W). Plots were excavated to 30 cm deep, filled with a mixture composed of one-third peat, one-third sand and one-third local soil, and tilled prior to planting. Ambient canopy temperature was recorded using infrared sensors (Model IRT-P5, Apogee Instruments, Logan, UT, USA) and a CR1000 datalogger (Campbell Scientific Inc., Edmonton, AB, Canada). 5-day running averages for mean ambient temperature and minimum ambient temperature were calculated using Graphpad Prism v6.04 (GraphPad Software, Inc., La Jolla, CA, USA).

Photosynthetic Gas Exchange

Gas exchange was measured at growth conditions on current-year needles attached to the seedlings after ≥2 h of exposure to growth light using a GFS-3000 (Walz, Effeltrich, Germany). The measuring cuvette was set to the following growth conditions: 400 or 800 ppm CO₂, 12 or 22°C, and 60% RH. Dark respiration (Rd) was measured after 40 minutes of dark adaptation. Net photosynthetic carbon assimilation (ANet), stomatal conductance (gs), and evapotranspiration (E) were subsequently measured at growth light intensity (1400 µmol quanta m⁻² s⁻¹) once steady state assimilation was achieved. Intrinsic water use efficiency (IWUE) was calculated as ANet/gs, according to Silva and Horwath (2013).

A/Ci curves were measured on days 0 and 36. Assimilation was assessed after 2-3 minutes of exposure to CO₂ levels of 400, 300, 250, 200, 150, 100, 50; 400, 550, 650, 800 and 1000 ppm CO₂, based on a protocol by Long and Bernacchi (2003). Measurements were taken at 25°C and 1400 µmol quanta m⁻² s⁻¹. The initial slope of the A/Ci curve, which represents the Rubisco-limited rate of carboxylation \( W_c \), was used to calculate the maximum substrate-saturated rate of Rubisco carboxylation \( V_{cmax} \) at 25°C using the following equation:

\[
W_c = \frac{V_{cmax}(C_i)}{C_i + K_c(1 + \frac{O}{K_o})}
\]

where \( C_i \) represents the intracellular partial pressure of CO₂, \( O \) represents the partial pressure of oxygen at 25°C, and \( K_c \) and \( K_o \) represent the Michaelis-Menten constants of Rubisco for the competing carboxylation and oxygenation reactions, respectively (Farquhar et al. 1980, Sage 1990).
All measurements were performed on attached needles from the topmost branch. The needles were arranged in a flat, single-needle layer and secured with breathable tape before being inserted into the cuvette. Following measurement, the measured region of needles within the cuvette was harvested and scanned to estimate the light-exposed needle surface area, using WinSeedle software (Regent Instruments Inc., Québec, QC, Canada).

**Chlorophyll Fluorescence**

Chlorophyll fluorescence was measured at growth conditions on attached current-year needles using a Dual-PAM-100 (Walz, Effeltrich, Germany) after ≥2 h of exposure to growth light. Dark-adapted minimum PSII fluorescence ($F_o$) and maximum PSII fluorescence ($F_m$) were obtained after 40 min of dark adaptation in the leaf clip, followed by assessment of light-adapted minimum PSII fluorescence ($F_o'$), light-adapted maximum PSII fluorescence ($F_m'$), and transient fluorescence ($F_t$) after exposure to 1400 µmol quanta m$^{-2}$ s$^{-1}$ of actinic light for 3-5 minutes. Maximum quantum yield of PSII was calculated as $F_v/F_m = (F_m - F_o)/F_m$, and effective quantum yield of PSII was calculated as $\Delta F/F'_m = (F_m' - F_t)/F_m'$ (Genty et al. 1989). The excitation pressure at PSII was calculated as $1 - qP = 1 - (F_m' - F_t)/(F_m' - F_o')$ (Maxwell and Johnson 2000). Nonphotochemical quenching was calculated as $NPQ = (F_{m\text{rec}}/F_m') - 1$ (Bilger and Björkman 1990) with fully recovered $F_m$ ($F_{m\text{rec}}$) estimated as $F_o \times 5$, according to Chang et al. (2015). Sustained nonphotochemical quenching was calculated as $NPQ_S = (F_{m\text{rec}}/F_m) - 1$ (Ensminger et al. 2004, Maxwell and Johnson 2000, Porcar-Castell 2011, Chang et al. 2015).

**Photosynthetic Pigments**

Photosynthetic pigments were analyzed according to Junker and Ensminger (2016). Needle samples, collected from chamber-grown seedlings after ≥2 h of exposure to growth light, were flash-frozen in liquid nitrogen and stored at -80°C. Samples were individually homogenized to a fine powder in liquid nitrogen using a mortar and pestle. Photosynthetic pigments were extracted from 50-60 mg homogenized frozen needle tissue at 4°C in 700 µl of 98% methanol buffered with 2% 0.5 M ammonium acetate for 2 h in the dark. The extract was centrifuged at 4°C at 14,000 rpm for 5 min and the supernatant collected. The pellet then was washed twice with 700 µl of methanol at 4°C followed by centrifugation, and the supernatant was again collected.
Finally, the total supernatant was filtered using 0.2 µm pore polytetrafluoroethylene (PTFE) syringe filters (Thermo Scientific, Rockwood, TN, USA).

Photosynthetic pigments were separated using a reverse-phase C30 column (5 µm, 250×4.6mm; YMC Co., Ltd., Kyoto, Japan) and analyzed with an Infinity 1260 series high performance liquid chromatography (HPLC) system equipped with a UV-diode array detector (Agilent Technologies, Santa Clara, USA). Pigments were eluted using a gradient of methanol, water buffered with 0.2% ammonium acetate, and tert-butyl methyl ether at a flow rate of 1 ml min⁻¹ at a column temperature of 25°C. Pigments were calibrated using standards from Sigma Aldrich (St. Louis, MO, USA) and DHI Lab products (Hørsholm, Denmark). Peak detection and pigment quantification were performed using ChemStation software (Agilent Technologies).

Total chlorophylls were expressed as the sum of chlorophylls a and b in µmol g⁻¹ fresh weight. The ratio of chlorophyll a to chlorophyll b was expressed as mol mol⁻¹. All carotenoid concentrations were normalized to chlorophyll content and expressed as mmol mol⁻¹. Total carotenoids (Car) were expressed as the sum of violaxanthin (V), antheraxanthin (A), zeaxanthin (Z), neoxanthin, lutein, α-carotene and β-carotene. Total xanthophyll cycle pigments were calculated as the sum of V, A, and Z. The de-epoxidation state of the xanthophyll cycle pigments (DEPS) was calculated as \((0.5A+Z)/(V+A+Z)\), according to Thayer and Björkman (1990).

**Nonstructural Carbohydrates**

Needle samples, collected shortly after midday from chamber-grown seedlings, were flash-frozen in liquid nitrogen and stored at -80°C. Each sample was individually homogenized using a mortar and pestle in liquid nitrogen. The ground needle sample was then lyophilized. Leaf soluble carbohydrates were extracted according to Park et al. (2009). A total of 250 µg galactitol internal standard was added to 30-40 mg homogenized, lyophilized needle tissue. The mixture was incubated in 4 ml of extraction buffer (methanol:chloroform:water, 12:5:3 v/v), overnight at 4°C. The mixture was centrifuged at 6,000 rpm for 10 min and the supernatant collected. The pellet was washed twice in extraction buffer followed by centrifugation. A total of 5 ml of water was added to the total supernatant, mixed, and centrifuged at 4000 rpm for 4 min. Lastly, the upper aqueous phase was collected.
An aliquot of 2 ml of the soluble carbohydrate extract were dried using a vacuum centrifuge and re-suspended in 1 ml of nanopure water. The re-suspended extract was filtered through a 0.45 µm pore nylon syringe filter (Chromatographic Specialties Inc., Brockville, ON, Canada) and analyzed using an ICS-5000 anion-exchange HPIC system (Dionex, Sunnyvale, CA, USA) equipped with an electrochemical pulse amperometric detector (EC-PAD). Glucose, sucrose, fructose and pinitol were eluted using a Hi-Plex Ca column (Agilent Technologies, Santa Clara, CA, USA) with water at a flow rate of 0.170 ml min⁻¹ with a column temperature of 70°C. Raffinose was eluted using a Carbo-Pac PA1 column (Dionex, Sunnyvale, CA, USA) with 150 mM NaOH (isocratic) at a flow rate of 1 ml min⁻¹. For all soluble carbohydrates, post-column detection was performed using NaOH at a rate of 100 mM min⁻¹.

Leaf starch was determined using the residual tissue pellet from the soluble sugar extraction according to Park et al. (2009). The pellet was dried overnight at 55°C. Roughly 20-30 mg of pellet was re-suspended in 5 ml of 2% H₂SO₄ and autoclaved for 5 min at 120°C. Once cooled, the extract was spun at 500 rpm for 5 min and the supernatant was collected, filtered using a 0.45 µm nylon filter and analyzed using a DX-600 anion-exchange IC/HPLC system (Dionex, Sunnyvale, CA, USA) and EC-PAD. The extract was eluted with water at a flow rate of 1 ml min⁻¹ with a column temperature of 30°C. Post-column detection was performed using NaOH at a rate of 100 mM min⁻¹. Peak detection and quantification of all nonstructural carbohydrates were performed using PeakNet software (Dionex).

Leaf starch content was expressed as percent dry weight. Soluble sugars were expressed as mg g⁻¹ dry weight. Total soluble sugars were calculated as the sum of glucose, fructose, sucrose, raffinose and pinitol.

**Protein Extraction, SDS-PAGE and Immunoblotting**

Proteins were extracted according to Busch et al. (2007) from 50-60 mg homogenized frozen needle tissue obtained from chamber-grown and field-grown seedlings. The extraction buffer consisted of 60 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 15% (w/v) sucrose, 20 mM dithiothreitol, and Complete EDTA-free proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentration was assessed using the RC/DC protein assay kit (Bio-Rad)
Laboratories, Hercules, CA, USA). Precisely 5 µg total protein per lane was loaded on 10% NuPage Novex Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and separated at 120V for 90 min at room temperature using the XCell Mini gel system. Following separation, proteins were transferred to a PVDF membrane (0.2 µm pore size, Bio-Rad) using the Xcell II Blot Module (Invitrogen) for 1 h at 30V on ice. The membrane was blocked using 5% nonfat milk in TBS for 45 minutes at room temperature (RT), then probed with antibodies against PsbA (1:5000, AS05084), Lhcb1 (1:5000, AS09522), RbcL (1:5000, AS01017), PEPC (1:1000, A209458), or Dhn (1:1000, AS07206). The Dhn antibody was raised against the K-segment peptide sequence from the dehydrin C-terminal, EKKGIMDKIKEKLPG. Goat anti-rabbit (AS09602) or chicken anti-rabbit (AS10833) IgG conjugated with horseradish peroxidase (1:75,000) were used as secondary antibodies for chemiluminescent protein detection using the Amersham ECL Prime kit (GE Healthcare, Buckinghamshire, UK) and a ChemiDoc MP (Bio-Rad) for visualization. All antibodies were obtained from Agrisera (Vännäs, Sweden). Optical band density was quantified using Image Lab software (Bio-Rad).

Mass Spectrometry

Precisely 40 µg of total protein from a sample containing high levels of the 16-kD dehydrin, as previously determined by Western blot, was loaded on a 12% Bolt Bis-Tris gel (Invitrogen) and separated at 100 V for 2 h 45 min at room temperature using the Bolt Mini Gel Tank (Invitrogen). Presence of dehydrins in the 16-kD band was confirmed on a replicate gel using Western blot, as described above. Following separation, the gel was stained using Coomassie Blue for 1 h, then destained overnight. The 16-kD band was subsequently excised and immersed in 1% acetic acid prior to analysis. Trypsin digest of the sample, followed by LC-MS/MS analysis, was performed at the SPARC BioCentre mass spectrometry facility of the Hospital for Sick Children (Toronto, ON, Canada). Peptide sequences were predicted by protein BLAST against nr and Uniprot databases, using PEAKS Studio v7.5 (Bioinformatics Solutions Inc., Ma et al. 2003). Peptides containing fragments of the dehydrin K-segment were aligned using MUSCLE (www.ebi.ac.uk/Tools/msa/muscle/, Edgar 2004) and manually reviewed. The aligned peptides were then used to generate a consensus K-segment peptide using Jalview v2.9.0b2 (www.jalview.org/, Waterhouse et al. 2009) (Fig. S3.2). Similarity among the consensus K-

Freezing Tolerance

Chlorophyll fluorescence was used to assess freezing tolerance using a modified protocol after Sutinen et al. (1992). Samples were taken from chamber-grown seedlings after 36 days of LTAC, ETAC or ETEC treatment, and from field-grown seedlings on August 17, September 26, October 26, and November 16 of 2013. Sections of current-year shoots from five seedlings per treatment were cut, dark-adapted for 40 minutes, and Fv/Fm measured. Shoots were then individually wrapped in moist paper towel and aluminum foil and sealed in plastic bags.

Shoots were exposed to a range of freezing temperatures at 5°C intervals from 0°C to -40°C using a Thermotron SM-16-8200 environmental test chamber (Thermotron Industries, Holland, MI, USA). The initial decrease from 0°C to -1°C was achieved over 1 hour, followed by a maximum cooling rate of 5°C h⁻¹ to reach target temperature. Each target temperature was held for 8-12 h. At the end of each interval, shoot sections were transferred to a 4°C refrigerator for 24 h, followed by transfer to RT, where the shoots were allowed to recover for 24 h. Following the 24 h recovery period, shoots were unwrapped and exposed to 1 h light exposure at 800 µmol quanta m⁻² s⁻¹ in order to stimulate PSII, then dark-adapted for 40 minutes (Burr et al. 2001). Fv/Fm was then recorded. The temperature at which 50% of seedlings were damaged by freezing (LT₅₀) was estimated as a 50% reduction in the post-recovery Fv/Fm, which was used as a proxy to indicate damage to the photosynthetic apparatus. LT₅₀ was calculated using the midpoint of a sigmoidal curve fit to the data, using the method described previously (Chang et al. 2015). Fitted sigmoidal model curves are presented in Fig. S3.4.

Statistical Analysis

The effects of treatment, time and the interaction of treatment and time on photosynthesis, pigment content, nonstructural carbohydrates and freezing tolerance of chamber-grown seedlings were assessed by two-way ANOVA, using the lmer function of the lme4 package (Bates et al. 2014) in R v3.1.1 (www.r-project.org/). The ANOVA model used treatment and day as categorical fixed factors, and individual and replicate as random factors. Tukey’s HSD post-hoc tests were used to contrast between treatments at each time point, and were performed using the
glht function of the *multcomp* package (Hothorn et al. 2008) in R v3.1.1. The effects of treatment on protein content were assessed by one-way ANOVA, using GraphPad Prism v6.04. LT$_{50}$ values for the chamber-grown seedlings were compared among treatments with an extra sum-of-squares F test (p < 0.05), using GraphPad Prism v6.04.

Linear mixed models were used to estimate the best predictor for seasonal changes in 16-kD levels and freezing tolerance assessed in field-grown seedlings using the lmer function of the *lme4* package (Bates et al. 2014) in R v3.1.1. Plot was included as a random factor in all models. Models were scored using the Akaike information criterion (AIC) to evaluate goodness-of-fit. ΔAIC was calculated by subtracting the AIC score of each predictor from that of the null model. Significance of predictors was calculated by comparing the null model with the predictor using ANOVA.

In order to evaluate the strength of the relationship between levels of the 16-kD dehydrin and freezing tolerance, as well as to correlate dehydrin levels and freezing tolerance to photoperiod and air temperature, R$^2$ values were obtained from nonlinear regression of the data using the exponential growth equation $Y = Y_0 e^{kX}$, where $k$ is the rate constant. Nonlinear regressions were performed using GraphPad Prism v6.04.

### 3.4 Results

#### Photosynthetic Gas Exchange

Under long photoperiod, net photosynthetic CO$_2$ assimilation ($A_{\text{net}}$) was approximately 20% higher in seedlings grown under elevated CO$_2$ compared to seedlings grown under ambient CO$_2$ (Fig. 3.1A, day 0). After transfer to short photoperiod treatments, $A_{\text{net}}$ decreased in the low temperature/ambient CO$_2$ (LTAC) seedlings by 50% from day 0 to day 1 and remained significantly lower for the entire experiment than in seedlings transferred to elevated temperature/ambient CO$_2$ (ETAC) or elevated temperature/elevated CO$_2$ (ETEC) conditions (Table 3.1). In comparison, seedlings transferred to ETAC maintained stable $A_{\text{net}}$ throughout the experiment. Seedlings transferred to ETEC performed significantly higher $A_{\text{net}}$ (20-30%) than seedlings grown under ETAC throughout the experiment, except for a brief decrease in $A_{\text{net}}$ that occurred on days 1 and 4 of treatment.
Figure 3.1: Response of photosynthetic gas exchange to low temperature/ambient CO$_2$ (LTAC), elevated temperature/ambient CO$_2$ (ETAC) and elevated temperature/elevated CO$_2$ (ETEC).
A) $A_{net}$, net photosynthetic CO$_2$ assimilation; B) $g_s$, stomatal conductance; C) $R_d$, dark respiration; D) IWUE, intrinsic water use efficiency; E) $V_{cmax}$, maximum rate of Rubisco carboxylation. Grey background indicates long photoperiod controls; white background indicates short photoperiod treatments. Measurements for $A_{net}$, $g_s$, $R_d$ and IWUE were taken at 1400 µmol quanta m$^{-2}$ s$^{-1}$ and growth temperature; measurements for $V_{cmax}$ were taken at 1400 µmol quanta m$^{-2}$ s$^{-1}$ and 25°C. Points represent the average of $n = 8-10 \pm$ SE, from two replicate experiments with $n = 5$ samples. Letters in panel E indicate significantly different treatment groups determined by one-way ANOVA ($p < 0.05$).
At day 0, stomatal conductance (gs, Fig. 3.1B) and respiration (Rd, Fig. 3.1C) did not differ between treatments, while intrinsic water use efficiency (IWUE, Fig. 3.1D) was higher in seedlings grown under elevated CO2. In LTAC seedlings, IWUE decreased rapidly at day 1 and stabilized, while gs revealed some fluctuations throughout the experiment. In contrast, ETAC seedlings retained similar gs and IWUE at the beginning and end of the experiment. Seedlings transferred to ETEC exhibited significantly lower gs and significantly higher IWUE, in comparison with seedlings grown under ETAC (Table 1).

At day 0, there was no difference in dark respiration (Rd) between seedlings grown under ambient and elevated CO2 (Fig. 3.1C). However, after transfer to short photoperiod conditions, ETAC seedlings retained similar gs, E, and IWUE at the beginning and end of the experiment. Seedlings transferred to ETEC exhibited significantly lower gs and significantly higher IWUE, in comparison with seedlings grown under ETAC (Table 3.1).

Rd increased approximately threefold until day 8 of the experiment in all treatments. This was followed by a decrease in Rd until day 26 and a subsequent increase in all treatments by day 36. Over the course of the experiment, Rd was significantly lower in LTAC seedlings than in ETAC seedlings (Table 3.1). Nonetheless, by day 36, Rd was similar in seedlings of all three treatments.

At day 0, seedlings grown under elevated CO2 exhibited slightly lower maximum substrate-saturated rate of Rubisco carboxylation (Vcmax) in comparison with seedlings grown under ambient CO2 (Fig. 3.1E). After 36 days, seedlings grown under LTAC exhibited significantly lower Vcmax, whereas Vcmax in ETAC and ETEC seedlings was not significantly affected.

**Chlorophyll Fluorescence**

In contrast to photosynthetic gas exchange, chlorophyll fluorescence was only responsive to low temperature and was unaffected by either photoperiod or elevated CO2. On day 0, seedlings grown under elevated CO2 levels exhibited slightly lower effective quantum yield of PSII (ΦPSII), and slightly higher NPQ and PSII excitation pressure (1-qP) compared with seedlings grown under ambient CO2. Maximum quantum yield of PSII (Fv/Fm) and ΦPSII decreased in seedlings within the first four days of exposure to LTAC conditions, and remained significantly lower for the duration of the experiment when compared to seedlings exposed to ETAC or ETEC treatments (Fig. 3.2A,B, Table 3.1). In contrast, NPQ (Fig. 3.2C), sustained non-photochemical
Figure 3.2: Response of chlorophyll fluorescence to low temperature/ambient CO₂ (LTAC), elevated temperature/ambient CO₂ (ETAC) and elevated temperature/elevated CO₂ (ETEC). A) F_v/F_m, maximum quantum efficiency of photosystem II; B) Φ_{PSII}, effective quantum yield of photosystem II; C) NPQ, total non-photochemical quenching; D) NPQ_s, sustained non-photochemical quenching; E) 1-qP, excitation pressure at photosystem II. Grey background indicates long photoperiod controls; white background indicates short photoperiod treatments. Measurements were taken at growth conditions; light-adapted measurements were taken at 1400 µmol quanta m⁻² s⁻¹. Points represent the average of n = 8-10 ± SE, from two replicate experiments with n = 5 samples.
Table 3.1: The effects of low temperature/ambient CO₂ (LTAC), elevated temperature/ambient CO₂ (ETAC), and elevated temperature/elevated CO₂ (ETEC) on photosynthetic gas exchange, chlorophyll fluorescence, photosynthetic pigments, and carbohydrates.

Tukey’s HSD multiple comparisons of means indicate significant differences (Sig.) between treatment pairs following two-way ANOVA (Table S3.1). (*** P < 0.001, ** P < 0.01, * P < 0.05)

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<th>Parameter</th>
<th>LTAC vs. ETAC</th>
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<th>ETAC vs. ETEC</th>
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<th>LTAC vs. ETEC</th>
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<td>***</td>
<td>&lt;0.001</td>
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<td>&lt;0.001</td>
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<td>0.697</td>
<td>n.s.</td>
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<td>***</td>
</tr>
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</table>
quenching (NPQs; Fig. 3.2D), and 1-qP (Fig. 3.2E) significantly increased in LTAC seedlings in comparison to seedlings grown under ETAC and ETEC (Table 3.1). Growth under ETAC conditions did not affect $F_v/F_m$, $\Phi_{PSII}$, NPQ, NPQs or 1-qP compared to day 0.

Photosynthetic Pigments

On day 0, needles from seedlings grown under elevated CO$_2$ levels contained 50% less chlorophyll (Fig. 3.3A) and 40% higher carotenoids per chlorophyll (Fig. 3.3B) compared with seedlings grown under ambient CO$_2$. Following the first day of transfer to short photoperiod, total chlorophylls in LTAC and ETAC seedlings were decreased by 50%, while total carotenoids increased by 50% in LTAC seedlings and ETAC seedlings were unaffected. ETEC seedlings did not exhibit changes in chlorophylls, but exhibited a transient increase in carotenoids during the first two weeks of treatment that subsided by day 16. By day 36, seedlings from all three treatments contained similar chlorophyll levels, and LTAC seedlings retained higher carotenoids than both elevated temperature treatments.

On day 0, the ratio of chlorophyll a to chlorophyll b (Chl a/b) was slightly, but not significantly, lower in seedlings grown under elevated CO$_2$ compared to seedlings grown under ambient CO$_2$ (Fig. 3.3C). After the first day of treatment, both LTAC and ETAC seedlings exhibited decreased Chl a/b, with a larger initial decrease in ETAC seedlings. By day 16, Chl a/b in LTAC seedlings continued to decline while ETAC seedlings recovered to values similar to day 0. In contrast, ETEC seedlings did not experience an initial decrease in Chl a/b and maintained relatively constant Chl a/b throughout the experiment.

On day 0, seedlings grown under elevated CO$_2$ exhibited higher leaf xanthophyll cycle pigment content (Fig. 3.3D) and de-epoxidation state of the xanthophyll cycle (DEPS, Fig. 3.3E) than seedlings grown under elevated CO$_2$. After the first day of transfer to LTAC treatment, xanthophyll cycle pigment content and DEPS increased considerably and remained significantly higher in LTAC seedlings in comparison with ETAC and ETEC seedlings (Table 3.1). Xanthophyll pool size did not respond to the ETAC treatment, but DEPS increased threefold from day 0 to day 1 and remained elevated until the end of the experiment. In ETEC seedlings, xanthophyll cycle pigments transiently increased during the first 16 days, but DEPS did not
Figure 3.3: Changes in photosynthetic leaf pigments in response to low temperature/ambient CO₂ (LTAC), elevated temperature/ambient CO₂ (ETAC) and elevated temperature/elevated CO₂ (ETEC).

A) Tot Chl, total chlorophylls expressed per gram fresh weight; B) Tot Car, total carotenoids expressed per mol chlorophyll; C) Chl a/b, ratio of chlorophyll a to chlorophyll b; D) V+A+Z, total xanthophyll cycle pool, comprised of violaxanthin, antheraxanthin and zeaxanthin; E) DEPS, de-epoxidation state of the xanthophyll cycle. Grey background indicates long photoperiod controls; white background indicates short photoperiod treatments. Points represent the average of n = 8-10 ± SE, from two replicate experiments with n = 5 samples.
change. By day 36, ETEC and ETAC seedlings exhibited similar xanthophyll cycle pigment content and DEPS.

**Nonstructural Carbohydrates**

Seedlings grown under elevated CO₂ contained almost double the leaf starch present in seedlings grown under ambient CO₂ on days 0 and 1. Leaf starch content continuously decreased in seedlings from all three short photoperiod treatments over the course of the experiment (Fig 3.4A). By day 36, all three treatments exhibited similar starch content.

Total and individual soluble carbohydrates (Fig. 3.4B-F) increased in seedlings from all three treatments during the first four days before stabilizing. Total leaf soluble carbohydrate content did not differ among seedlings of all three treatments (Fig. 3.4B, Table 3.1). Seedlings grown under LTAC exhibited significantly higher leaf sucrose, hexose and raffinose content compared to seedlings grown under ETAC, but this was offset by significantly lower pinitol (Fig. 3.4C-F, Table 1). Notably, hexose (Fig. 3.4D) and raffinose (Fig. 3.4F) levels increased during the first week of treatment in LTAC seedlings and remained elevated for the duration of the experiment. In contrast, hexose and raffinose concentrations were increased in seedlings grown under ETAC and ETEC during the first week of treatment, but by day 16, levels of both soluble carbohydrates returned to levels similar to values recorded on day 0. Neither hexose nor raffinose content differed significantly between ETAC and ETEC seedlings (Table 3.1).

All seedlings exhibited similar amounts of pinitol on day 0, and pinitol levels increased following transfer to all three short photoperiod treatments (Fig. 3.4F). Pinitol levels stabilized on day 16 in seedlings grown under LTAC, while pinitol continued to accumulate in seedlings grown under ETAC and ETEC throughout the experiment. Pinitol content was significantly lower in LTAC seedlings compared to ETAC seedlings, but did not differ between ETAC and ETEC seedlings (Table 3.1).
Figure 3.4: Changes in leaf nonstructural carbohydrate content in response to low temperature/ambient CO$_2$ (LTAC), elevated temperature/ambient CO$_2$ (ETAC) and elevated temperature/elevated CO$_2$ (ETEC).

A) Starch content, expressed as percent dry weight; B) total soluble sugars, composed of the sum of C-F; C) sucrose content; D) hexose content (glucose + fructose); E) pinitol content; F) raffinose content, expressed per unit dry weight. Grey background indicates long photoperiod controls; white background indicates short photoperiod treatments. Points represent the average of $n = 8-10 \pm SE$, from two replicate experiments with $n = 5$ samples. Letters indicated on the insets for D and F indicate significantly different treatments determined by two-way ANOVA ($p < 0.05$).
Proteins

On day 0, protein content for photosynthetic proteins RbcL, Lhcb1 and D1 did not differ between seedlings grown under ambient or elevated CO2. RbcL did not reveal a significant response to temperature, photoperiod or CO2 (Fig. 3.5A). In contrast, Lhcb1 and D1 decreased by 50% in LTAC seedlings after 36 days (Fig. 3.5B,C). ETAC seedlings also exhibited significantly decreased D1 and slightly decreased Lhcb1 concentrations, although this difference was statistically not significant. ETEC seedlings did not exhibit changes in RbcL, Lhcb1 or D1.

Phosphoenolpyruvate carboxylase (PEPC) was significantly decreased in seedlings grown under elevated CO2 in comparison with seedlings grown under ambient CO2 at the beginning of the experiment (Fig. 3.5D). When seedlings were transferred from long to short photoperiod treatments, PEPC decreased in the treatments with ambient CO2 and remained low in seedlings growing at elevated CO2, and there was no difference in PEPC between treatments at the end of the experiment (Fig. 3.5D).

We detected two dehydrins of size 52 and 16 kD. The 52-kD dehydrin was constitutively expressed and revealed only minor non-significant differences among treatments and over the course of the experiment (Figs. 3.5E,F). In contrast, the 16-kD dehydrin was not detected in samples collected from seedlings grown under long photoperiod (day 0), but was expressed in all three short photoperiod treatments on day 36 (Fig. 3.5G). Furthermore, the 16-kD protein showed increased expression in all three treatments starting on day 16 and then continued to increase until day 36 (Fig. 3.5H). Expression of the 16-kD dehydrin was more than two-fold higher in LTAC seedlings than in ETAC or ETEC seedlings. Total leaf protein content did not vary significantly during the course of the experiment or between treatments (Fig. S3.1).

We further investigated the 16-kD dehydrin in order to elucidate the amino acid sequence of this protein using mass spectrometry (MS). A tentative consensus sequence for a K-segment of the dehydrin was generated, which shared 73% identity with the angiosperm K-segment, EKKGIMDKIKEKLPG (Close 1996) and 60% identity with the gymnosperm K-segment, [Q,E]K[P,A]G[M,L]DKIK[A,Q][K,M][I,L]PG (Jarvis et al. 1996) (Fig. S3.2).
Figure 3.5: Changes in leaf protein expression in response to low temperature/ambient CO$_2$ (LTAC), elevated temperature/ambient CO$_2$ (ETAC) and elevated temperature/elevated CO$_2$ (ETEC).

A) RbcL, rubisco large subunit; B) Lhcb1, light harvesting complex protein of photosystem II; C) D1, reaction center core protein of photosystem II; D) PEPC, phosphoenolpyruvate carboxylase; E-H) Dhn, dehydrin. The average optical density of the day 0 AC control was arbitrarily scaled to 1 for RbcL, Lhcb1, D1, PEPC, and 52-kD Dhn (panels A-F); the average optical density of day 36 LTAC treatment was scaled to 1 for 16-kD Dhn (panels G-H). Bars represent the average of $n = 8$-10 ± SE, from two replicate experiments with $n = 5$ samples. Letters, where present, indicate significantly different treatment groups determined by one-way ANOVA ($p < 0.05$). Representative blots shown were loaded with 5 µg total protein per lane.
Freezing Tolerance

Freezing tolerance in seedlings acclimated to long photoperiod and warm temperature ranged from -7 to -10°C on day 0, with no significant difference between seedlings grown under ambient or elevated CO₂ (Fig. 3.6; Fig. S3.4A). After 36 days, freezing tolerance was significantly increased in all treatments (Fig. 3.6; Fig. S3.4B). Freezing tolerance was -30.4°C in seedlings grown under LTAC, which was significantly lower than freezing tolerance of seedlings grown under ETAC (-26°C) and ETEC (-23.9°C) (Fig. 3.6; Fig. S3.4B).

Figure 3.6: Shoot freezing tolerance at the beginning (day 0) and end (day 36) of the experiment. Low temperature/ambient CO₂ (LTAC), elevated temperature/ambient CO₂ (ETAC), and elevated temperature/elevated CO₂ (ETEC). Grey background indicates long photoperiod controls; white background indicates short photoperiod treatments. Bars represent average LT₅₀ of n = 10, from two replicate experiments with n = 5 samples, estimated using sigmoidal curves fit to the data (Fig. S3.3); error bars represent 95% confidence intervals. Letters, where present, indicate significantly different treatment groups determined by extra sum-of-squares F test.

Correlation between Dehydrins and Freezing Tolerance

The relationship between the cold and photoperiod-responsive 16-kD dehydrin and freezing tolerance was further investigated using field grown seedlings. Levels of the 16-kD dehydrin increased during autumn (Fig. 3.7C), particularly as minimum temperatures decreased below freezing (Fig. 3.7A), while levels of the 52-kD dehydrin remained unchanged (Fig. 3.7B). Seedlings also exhibited greater freezing tolerance after exposure to freezing temperatures (Fig. 3.7D). Using a mixed linear model approach, we revealed that both expression of the 16-kD dehydrin and freezing tolerance were best represented by photoperiod, followed by minimum air temperature (Tₘᵢₙ) and the interaction of photoperiod and Tₘᵢₙ (Table 3.2). Freezing tolerance correlated strongly with levels of the 16-kD dehydrin (Fig. 3.8).
Figure 3.7: Changes in dehydrin protein expression and development of freezing tolerance during cold hardening in needles of field-grown *P. strobus* seedlings.

A) Photoperiod (Phot), mean (T\text{mean}) and minimum (T\text{min}) ambient air temperature measured at field site; dotted vertical lines indicate sampling dates. B) Constitutively expressed 52-kD and C) autumn-induced 16-kD dehydrin levels. The average optical density of day 36 LTAC, used as a reference, was arbitrarily scaled to 1. Representative blots shown were loaded on an equal protein basis. Bars represent the average of n = 5 ± SE plot replicates. D) Shoot freezing tolerance. Bars represent the average of n = 5 ± 95% confidence interval. Letters, where present, indicate statistically different groups determined by one-way ANOVA (p < 0.05).
Table 3.2: Best predictors of seasonal variation for LT<sub>50</sub> and 16-kD dehydrin in <i>P. strobus</i> needles determined by linear mixed-effects modelling.

T<sub>min</sub>, minimum daily temperature; T<sub>mean</sub>, mean daily temperature. Akaike information criterion (AIC) scores are provided to indicate goodness-of-fit for each predictor model; higher ΔAIC scores indicate higher goodness-of-fit.

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Figure 3.8. Correlation between relative leaf protein content of 16-kD dehydrin (Dhn) and freezing tolerance (LT<sub>50</sub>). Each point indicates plot average of three individuals. R<sup>2</sup> value indicates goodness-of-fit for an exponential relationship. P-value indicates whether log-transformed slope differs significantly from zero.
3.5 Discussion

Under field conditions, simultaneous decreases in photoperiod and temperature induce cold hardening in conifer seedlings. To investigate the impact of elevated temperature and atmospheric CO₂ levels, we aimed to assess 1) how the onset and development of cold hardening are affected by a shift from long photoperiod to short photoperiod when air temperature remains warm, and 2) how the combination of warmer air temperature with elevated CO₂ affects the progress of the short photoperiod-induced cold hardening process.

Warm temperature suppresses the downregulation of photosynthesis in seedlings growing under short photoperiod

Shifting *P. strobus* seedlings from long photoperiod and warm temperature to short photoperiod and low temperature (LTAC) induced cold hardening and resulted in the expected downregulation of photosynthesis (Fig. 3.1A), modifications in thylakoid membrane protein composition (Fig. 3.5B,C) and changes in photosynthetic pigments (Fig. 3; Öquist and Huner 2003, Ensminger et al. 2006, Busch et al. 2007, Chang et al. 2015). This reorganization of the chloroplast further led to the development of sustained non-photochemical quenching (Fig. 3.2D), an important mechanism for the safe dissipation of excess light energy required by cold-hardy evergreen plants during winter (Ensminger et al. 2004, Busch et al. 2007, Verhoeven et al. 2009).

Short photoperiod alone was insufficient to induce the downregulation of photosynthesis. Under short photoperiod combined with warm temperature (ETAC), photosynthetic carbon assimilation (Fig. 3.1A), Fᵥ/Fₘ (Fig. 3.2A) and Φₚₛᵢ₂ (Fig. 3.2B) remained unaffected, although some components of the light reactions were downregulated, indicated by decreases in total chlorophyll content (Fig. 3.3A) and minor losses of Lhcb1 (Fig. 3.5B) and D1 (Fig. 3.5C). Warm temperature also affected the ability of ETAC seedlings to quench excess energy in comparison with LTAC seedlings: ETAC seedlings did not increase photoprotective pigments (Fig. 3.3B,D), showed smaller increases in DEPS (Fig. 3.3E), and suppressed the development of NPQs (Fig. 3.2D). Together, this indicates that the combination of short photoperiod and low temperature is required to downregulate photosynthesis and upregulate photoprotective processes in *P. strobus* seedlings. In contrast, field-grown *P. strobus* seedlings did not exhibit a delay in the downregulation of photosynthesis over the autumn when canopy-level air temperature was
elevated by +1.5°C during the day and +3°C during the night (Chang et al. 2015). Evidently, field-grown plants acclimated to larger day-to-day temperature variations and exposed to a much smaller temperature increment of +1.5/+3°C exhibit a much weaker response of photosynthesis to short photoperiod and warmer temperature than seedlings grown under the controlled conditions of this experiment.

The combination of warm temperature and elevated CO2 stimulates photosynthesis in seedlings growing under short photoperiod

Seedlings shifted to the combination of short photoperiod, elevated temperature and elevated CO2 (ETEC) increased rates of photosynthetic carbon uptake by 33% compared to seedlings grown under ETAC (Fig. 3.1A). Elevated CO2 stimulates carbon assimilation in woody species during the growing season, as shown from both growth chamber (Curtis and Wang 1998) and free-air CO2 enrichment (FACE) studies (Ainsworth and Long 2005). Few studies, however, have investigated the impact of elevated temperature or elevated CO2 on photosynthesis during autumn when cold hardiness is induced in the field. During mid and late autumn, Bigras and Bertrand (2006) observed increased light-saturated photosynthesis in *Picea mariana* seedlings exposed to elevated CO2. In our experiment, seedlings grown under short photoperiod, warm temperature and elevated CO2 also greatly improved water use efficiency (Fig. 3.1D), which was a consequence of increased A (Fig. 3.1A) and decreased stomatal conductance (Fig. 3.1B). This is attributable to the effect of CO2 enrichment on photosynthesis, a phenomenon that has been well-described for plants actively growing during the vegetative season (Ceulemans and Mousseau 1994, Curtis and Wang 1998, Ainsworth and Long 2005, Leakey et al. 2009), but not for plants exposed to conditions triggering the development of cold hardiness.

The stimulation of photosynthetic carbon uptake under elevated CO2 also caused an excess of leaf carbohydrates in *P. strobus* seedlings, indicated by the greater accumulation of starch (Fig. 3.4A), and by the lower $V_{\text{cmax}}$ (Fig. 3.1E) observed in EC seedlings compared with AC seedlings at the beginning of the experiment. Under short photoperiod, i.e. extended night, respiration is increased relative to carbon assimilation and may decrease daily net carbon uptake. As a consequence, depletion of carbohydrates may at least partially restore carbon sink capacity in seedlings during cold hardening. Carbon depletion resulting from an extended night period has been demonstrated in *Arabidopsis* leaves under warm growth conditions (e.g. Gibon et al. 2004),
but not under cold or elevated CO₂ conditions in conifers. Indeed, transfer to ETEC also decreased xanthophyll cycle pool size (Fig. 3.3D) and DEPS (Fig. 3.3E), suggesting that enhanced photoprotection was no longer required under short photoperiod. Consistent with this hypothesis, other studies using conifer seedlings, e.g. *Picea mariana* (Bigras and Bertrand 2006) and *Pinus sylvestris* (Wang and Kellomäki 1997), have also reported high levels of NPQ in plants growing under long photoperiod and elevated CO₂ conditions, and relaxation of NPQ following a decrease in photoperiod.

**A major shift in carbohydrate metabolism is induced by short photoperiod**

Seedlings from all three treatments exhibited degradation of leaf starch and accumulation of sucrose, which formed the bulk of soluble carbohydrates in the leaf (Fig. 3.4A-C). During autumn, these changes in leaf carbohydrate composition in field-grown conifers such as *Pinus sylvestris* reflect the seasonal translocation of carbohydrates from leaves to roots (Oleksyn et al. 2000). This seems to be largely triggered by photoperiod and was observed in our experiment within the first four days following transfer to short photoperiod, indicated by increases in leaf sucrose (Fig. 3.4C) and dark respiration (Fig. 3.1C). In contrast, a transient decrease in leaf starch content was accomplished at a much slower rate and over the first two weeks (Fig. 3.4A).

Notably, at the end of the experiment, needles from all three short photoperiod treatments exhibited significant decreases in PEPC levels (Fig. 3.5D) and increases in respiration rates (Fig. 3.1C) in comparison with seedlings grown at ambient CO₂ and long photoperiod. The downregulation of PEPC suggests that short photoperiod induced a metabolic shift where mitochondrial respiration was not principally maintained using the endproducts of glycolysis. Instead, pyruvate derived from the conversion of malate by malic enzyme may also have been used to fuel the citric acid cycle, thus bypassing the anaplerotic pathway mediated by PEPC. Indeed, a study of *Picea sitchensis* during autumn cold acclimation revealed a strong downregulation of PEPC transcripts along with key enzymes involved in glycolysis, but increased transcript levels for malic enzyme, citric acid cycle enzymes and concentrations of leaf citrate, malate and succinic acid (Dauwe et al. 2012). Hence, our findings provide strong evidence that the metabolic shift that occurs during the development of cold hardiness is largely triggered by short photoperiod and not low temperature.
Needles of LTAC seedlings also contained higher levels of hexoses (Fig. 3.4D) than needles of ETAC or ETEC seedlings. In general, glucose and fructose are only observed in minor quantities in conifer needles (Hoch et al. 2003, Strimbeck et al. 2008) because they act as transitory intermediates in sucrose synthesis, glycolysis, and regeneration of triose phosphates in the Calvin cycle (Granot et al. 2013). The relatively small but significant increase in glucose and fructose levels in LTAC suggests metabolic adjustments induced by low temperature. This further emphasizes that only certain aspects of the cold hardening process can be triggered by short photoperiod alone.

**Short photoperiod alone increases freezing tolerance and accumulation of some, but not all, cryoprotective carbohydrates**

Low temperature acclimation and the development of freezing tolerance is associated with the accumulation of soluble carbohydrates such as sucrose (Guy et al. 1992, Uemura and Steponkus 2003), raffinose (Knaupp et al. 2011) and pinitol (Angelcheva et al. 2014), which are considered cryoprotectants. Our data suggest that raffinose contributes more effectively than pinitol to cold hardening in *P. strobus*. Accumulation of sucrose and pinitol was induced by short photoperiod in all three treatments (Fig. 3.4C,E). Surprisingly, LTAC seedlings contained 20-30% less pinitol than ETAC and ETEC seedlings by the end of the experiment (Fig. 3.4E). Raffinose synthesis was also induced following shift to short photoperiod, but only accumulated in LTAC seedlings and was depleted in ETAC and ETEC seedlings after the first two weeks (Fig. 3.4F). The higher accumulation of pinitol observed in the two elevated temperature treatments at the end of the experiment did not confer enhanced freezing tolerance in comparison with the LTAC treatment; instead, ETAC and ETEC seedlings exhibited significantly less freezing tolerance (Fig. 3.6). Seedlings from all three treatments exhibited an approximate two- to three-fold increase in freezing tolerance after 36 days of short photoperiod exposure, but maximum freezing tolerance also required a low temperature stimulus (Fig. 3.6). Elevated CO₂ only minimally affected the accumulation of cryoprotective carbohydrates. After transfer to short photoperiod, ETEC seedlings contained slightly more sucrose at the end of the experiment, but similar levels of hexoses, pinitol, and raffinose in comparison with ETAC seedlings (Fig. 3.4C-F).

Field-grown *P. strobus* seedlings also began to develop freezing tolerance in response to decreasing photoperiod prior to low temperature exposure (Fig. 3.7; Chang et al. 2015).
Following frost exposure, the field-grown seedlings also accumulated high levels of soluble carbohydrates, including sucrose, raffinose and pinitol (Chang et al. 2015), and exhibited considerably greater freezing tolerance (Fig. 3.7, Chang et al. 2015).

**Maximum freezing tolerance is achieved by a combination of short photoperiod and low temperature and correlates with the induction of a 16-kD dehydrin**

Several dehydrin proteins are inducible by low temperature in spruce, and their accumulation correlates with the development of freezing tolerance (Kjellsen et al. 2013). We probed total leaf proteins using an antibody specific to the highly conserved K-segment region of dehydrins. We observed a constitutively expressed protein band with a size of approximately 52 kD that was present in all samples (Fig. 3.5E-F). A second protein band, 16 kD in size, was absent under long photoperiod conditions, but was significantly induced in response to short photoperiod and low temperature (Fig. 3.5G-H). Analysis of the 16-kD protein band via mass spectrometry confirmed the presence of a dehydrin and provided a tentative consensus sequence for its K-segment: EKKGILGQVKEKLPG (Fig. S3.2). The structure of the tentative *P. strobus* K-segment was more closely related to the known angiosperm K-segment (73% identity, EKKGIMDKIKEKLPG; Close 1996) than the closest known variant of the gymnosperm K-segment (71% identity, EKAGMDKIKAKLPG; Jarvis et al. 1996) (Fig. S3.3). Intriguingly, this 16-kD dehydrin appeared to be associated with freezing tolerance, and only accumulated after several weeks of treatment (Fig. 3.5H). Although present in all samples exposed to short photoperiod, its accumulation was strongly suppressed by both warm temperature and elevated CO₂. In ETAC seedlings, accumulation of this 16-kD dehydrin was suppressed by 75% (Fig. 3.5G) and freezing tolerance was 14% less than in the LTAC seedlings (Fig. 3.6). In ETEC seedlings, accumulation of the 16-kD dehydrin was suppressed by 90% and freezing tolerance was 21% less than in LTAC seedlings.

We further investigated the relationship between the accumulation of dehydrins and freezing tolerance in needles from field-grown seedlings. We identified the constitutively expressed 52-kD dehydrin band in late summer, autumn and winter (Fig. 3.7A). The 16-kD dehydrin was absent during late summer, but was expressed in early autumn and strongly upregulated in late autumn after temperature decreased below freezing (Fig. 3.7B). In the field-grown seedlings, we
observed that both 16-kD dehydrin accumulation and freezing tolerance were determined by photoperiod and minimum air temperature (Table 3.2). Furthermore, the 16-kD dehydrin correlated strongly with freezing tolerance (Fig. 3.8); therefore, suppression of 16-kD dehydrin synthesis by warm temperature likely contributed to the impaired freezing tolerance observed in ETAC and ETEC seedlings (Fig. 3.6).

Autumn accumulation of dehydrins have previously been reported in *Pinus sylvestris*: Korotaeva et al. (2015) reported constitutive expression of ~70-kD and 45-kD dehydrins, while a 17-kD dehydrin manifested during spring and autumn, but not summer. Similarly, Petrov et al. (2011) reported a 15-kD dehydrin absent during summer, detectable during early autumn, and strongly accumulated by late autumn. However, our study is the first to identify a dehydrin that can be induced by short photoperiod alone, but shows maximum expression when plants are exposed to the combination of low temperature and short photoperiod, and which further shows a strong correlation with the development of maximum freezing tolerance in pine seedlings.

### 3.6 Conclusions

Our findings suggest that warmer temperature has a greater negative impact than elevated CO₂ on the development of cold hardening upon exposure to shorter photoperiod in *Pinus strobus* seedlings. Evidently, elevated temperature, and the combination of elevated temperature and elevated CO₂, can suppress the downregulation of photosynthesis and impair the development of freezing tolerance during autumn. However, all seedlings exposed to short photoperiod, irrespective of temperature or CO₂ level, developed sufficient freezing tolerance to protect against typical winter temperatures at their geographic origin. In southern Ontario, the 30-year historical average minimum daily temperature was -10°C, with a record minimum of -35°C (Environment Canada 2016). We showed that short photoperiod alone induced freezing tolerance to -26°C, while exposure to chilling temperatures of 12°C day/5°C night increased freezing tolerance to -30°C. Under colder temperatures during late November, field-grown seedlings even exhibited freezing tolerance to -60°C. Based on our findings, future increases in temperature and CO₂ may extend the growing season and stimulate late season net carbon uptake, but are unlikely to increase risk of freezing damage in *Pinus strobus* seedlings in Ontario.
3.7 Supporting Information

Table S3.1. The effects of treatment and time on photosynthetic gas exchange, chlorophyll fluorescence, photosynthetic pigments, and carbohydrates.

Two-way ANOVA was calculated as Variable ~ Treatment * Time + (1|Trial) + (1|Indiv). Treatment and Time were included as categorical fixed factors; trial (experimental replicate) and individual (biological replicate) were included as random factors. (** p < 0.01, * p < 0.05)

<table>
<thead>
<tr>
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<th>Treatment</th>
<th>Time</th>
<th>Treat × Time</th>
</tr>
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<tr>
<td></td>
<td>P</td>
<td>Sig.</td>
<td>P</td>
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<tr>
<td>A_{net}</td>
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<tr>
<td>g_{s}</td>
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Figure S3.1. Changes in total leaf protein content A) in response to low temperature/ambient CO$_2$ (LTAC), elevated temperature/ambient CO$_2$ (ETAC), and elevated temperature/elevated CO$_2$ (ETEC) treatments; and B) over autumn under field conditions. Grey background indicates summer treatment. Bars represent the average of A) n = 8-10 ± SE biological replicates and B) n = 5 ± SE plot replicates.
Figure S3.2: A) Predicted sequence of K-segment of the 16-kD dehydrin and B) alignment of peptides used to generate consensus. Consensus K-segment is outlined in red. Accession numbers for homologous proteins matched to each peptide are shown. Amino acids are color coded according to their chemical properties using the Lesk color scheme.
**Fig. S3.3:** Muscle alignment of predicted K-segment sequence for 16-kD protein, angiosperm and gymnosperm K-segments.
Asterisks indicate conserved amino acids; colons indicate shared amino acids with strong similarities (>0.5 score in PAM250 matrix); periods indicate shared amino acids with weakly similar properties (≤0.5 score in PAM250 matrix).

<table>
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</tr>
<tr>
<td>angiosperm</td>
<td>EKKGIMDKIKEKLPG</td>
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<tr>
<td>gymnosperm</td>
<td>EKQG-MDKIKAKLPG</td>
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**Fig. S3.4:** Response of shoot freezing tolerance to A) CO2 level in seedlings grown under long photoperiod; B) low temperature/ambient CO2 (LTAC), elevated temperature/ambient CO2 (ETAC), and elevated temperature/elevated CO2 (ETEC) in seedlings grown under short photoperiod.
Grey background indicates long photoperiod controls; white background indicates short photoperiod treatments after 36 days. Grey vertical lines indicate midpoints representing LT50. Letters, where present, indicate statistically different treatment groups as determined by extra sum-of-squares F test.
3.8 References


Chapter 4
Sensitivity of Cold Acclimation to Elevated Autumn Temperature in Field-grown *Pinus strobus* Seedlings


Status: Published.
4.1 Abstract

Climate change will increase autumn air temperature, while photoperiod decrease will remain unaffected. We assessed the effect of increased autumn air temperature on timing and development of cold acclimation and freezing resistance in Eastern white pine (EWP, Pinus strobus) under field conditions. For this purpose we simulated projected warmer temperatures for southern Ontario in a Temperature Free-Air-Controlled Enhancement (T-FACE) experiment and exposed EWP seedlings to ambient (Control) or elevated temperature (ET, +1.5°C/+3°C during day/night). Photosynthetic gas exchange, chlorophyll fluorescence, photoprotective pigments, leaf nonstructural carbohydrates (NSC), and cold hardiness were assessed over two consecutive autumns. Nighttime temperature below 10°C and photoperiod below 12h initiated downregulation of assimilation in both treatments. When temperature further decreased to 0°C and photoperiod became shorter than 10h, downregulation of the light reactions and upregulation of photoprotective mechanisms occurred in both treatments. While ET seedlings did not delay the timing of the downregulation of assimilation, stomatal conductance in ET seedlings was decreased by 20-30% between August and early October. In both treatments leaf NSC composition changed considerably during autumn but differences between Control and ET seedlings were not significant. Similarly, development of freezing resistance was induced by exposure to low temperature during autumn, but the timing was not delayed in ET seedlings compared to Control seedlings. Our results indicate that EWP is most sensitive to temperature changes during October and November when downregulation of photosynthesis, enhancement of photoprotection, synthesis of cold-associated NSCs and development of freezing resistance occur. However, we also conclude that the timing of the development of freezing resistance in EWP seedlings is not affected by moderate temperature increases used in our field experiments.

4.2 Introduction

Global land-surface temperatures are increasing, particularly in northern latitudes and during winter months (IPCC 2007). Records collected since the mid-twentieth century describe a delay in the onset of dormancy and an increase in growing season length in temperate and boreal forest regions across the northern hemisphere, particularly in North America (Boisvenue and Running 2006, Piao et al. 2007, McMahon et al. 2010). The environmental signals used by trees to sense seasonality and trigger dormancy and development of cold acclimation are the decrease in
temperature and the length of photoperiod during the autumn (Welling et al. 2004). Increasing temperatures, as projected by climate change models, will delay the low temperature signal while photoperiod will remain unaffected. Asynchronous phasing of temperature and photoperiod is expected to impact the onset and development of cold acclimation during autumn. In evergreen conifers from high latitudes, cold acclimation includes the cessation of growth (Rossi et al. 2008), development of bud dormancy (Cooke et al. 2012), changes in chloroplast function and membrane composition (Öquist and Huner 2003, Ensminger et al. 2006, Crosatti et al. 2013), a transition from dynamic to sustained energy quenching (Demmig-Adams and Adams 2006), changes in gene expression (Ruelland et al. 2009), accumulation of intracellular metabolite pools (Stitt and Hurry 2002), and cold hardening (Guy 1990). A critical factor affecting the impact of future elevated autumn temperature is the importance of photoperiod versus temperature for the induction of phenological events. Early conifer studies suggested seasonal variations in photon flux density (Troeng and Linder 1982) and the onset of autumn frosts (Bergh et al. 1998) as regulators of autumn phenology. More recent studies identified differences in the sensitivity of various evergreen conifer species to photoperiod and temperature during autumn bud dormancy (Olsen 2010, Cooke et al. 2012).

Photoperiod induces physiological changes in plants during late summer and early autumn. Decreasing photoperiod results in the depletion of sugars, particularly sucrose, towards the end of the night, as was shown in *Arabidopsis* (Gibon et al. 2009) and *Populus* (Hoffman et al. 2010). The nighttime depletion of sugars acts as a metabolic signal during the following day, inhibiting growth and reducing the rate of starch turnover (Gibon et al. 2009). As temperatures begin to decrease during autumn, low temperature exposure induces the cessation of growth in evergreen conifers by limiting photosynthetic productivity and decreasing the rate of cell differentiation (Rossi et al. 2008). The resulting decrease in carbon sink size affects rates of cellular respiration and induces negative feedback regulation of photosynthetic carbon assimilation (Busch et al. 2007, Bauerle et al. 2012).

Enzymatic reactions of the Calvin-Benson cycle are slowed down under low temperature conditions (Bernacchi et al. 2002). To compensate for the reduced energy sink, evergreen conifers reduce their capacity for harvesting sunlight by adjusting photosynthetic pigment pools, and downregulate the capacity of the light reactions in order to maintain photostasis (Huner et al. 1998, Ensminger et al. 2006, Kurepin et al. 2013). Low temperature also inhibits turnover rates
for the reaction center core protein D1 (Schnettger et al. 1994, Öquist et al. 1995, Zarter et al. 2006), thus decreasing the number of functional PSII reaction centers and limiting photochemical energy conversion (Sveshnikov et al. 2006, Zarter et al. 2006). As a result, the plant’s capacity to quench absorbed light energy via photochemical energy conversion is greatly diminished (Sveshnikov et al. 2006, Zarter et al. 2006, Busch et al. 2007).

As photochemical efficiency decreases under low temperature conditions, light energy absorbed in excess energy can induce the light harvesting complexes (LHCs) to dissociate from photosynthetic reaction centers (Iwai et al. 2010, Johnson et al. 2011), and trigger the formation of thylakoid protein aggregates (Ottander et al. 1995). Excess light energy can also generate highly reactive chlorophyll and oxygen radicals (Ensminger et al. 2006). Plants increase the production of radical scavengers, such as α-tocopherol, β-carotene, neoxanthin and lutein (Havaux and Kloppstech 2001, Busch et al. 2007). Xanthophyll pigments also serve a year-round photoprotective function. High light exposure causes the de-epoxidation of violaxanthin, via antheraxanthin, into zeaxanthin. During the warm seasons, this occurs in a dynamic and reversible process known as the xanthophyll cycle, which is involved in energy-dependent nonphotochemical quenching in response to a trans-thylakoid pH gradient created by photosynthetic electron transport (Öquist and Huner 2003, Sveshnikov et al. 2006, Zarter et al. 2006, Ensminger et al. 2006, Busch et al. 2007). The interaction of zeaxanthin with LHCII, mediated by the PsbS protein (Niyogi et al. 2004), allows excess light energy to be dissipated as heat (Zarter et al. 2006); zeaxanthin also acts as an antioxidant to protect membrane-bound lipids (Johnson et al. 2007). In evergreen conifers, prolonged exposure to cold-induced high light stress arrests the xanthophyll cycle in zeaxanthin form and induces PsbS accumulation at the LHCII aggregates, allowing absorbed energy to be constantly dissipated in a process known as sustained nonphotochemical quenching (Öquist and Huner 2003, Demmig-Adams and Adams 2006, Zarter et al. 2006).

As photosynthesis and growth cease, leaf carbon partitioning is shifted from starch to soluble sugars, enabling mobilization of carbohydrates from leaves to sink tissues (Guy et al. 1992, Strand et al. 1999, Stitt and Hurry 2002, Dauwe et al. 2012). In addition to regulating plant metabolism, decreasing photoperiod causes phytochromes to activate a cold response pathway mediated by the CBF transcription factors (Maibam et al. 2013), resulting in enhanced freezing tolerance (Welling et al. 2002, Li et al. 2003, Welling et al. 2004, Lee and Thomashow 2012).
Low temperature induces a stronger cold response via CBF (Cook et al. 2004) and ABA-mediated (Cuevas et al. 2008) pathways, resulting in strengthened cytoskeleton and cell walls, increased membrane lipid fluidity and synthesis of cryo- and osmoprotectants (reviewed in Crosatti et al. 2012), as well as accumulation of soluble sugars including raffinose and sucrose in leaf tissues (Dauwe et al. 2012). High levels of sucrose, assessed in barley cell cultures (Tabaei-Aghdaei et al. 2003), and raffinose, shown using transgenic petunia (Pennycooke et al. 2003), are correlated with increased freezing tolerance.

Several studies have investigated the effect of elevated temperature on plants and growing season length. Most studies have focused on the effects of spring warming (Hänninnen and Tanino 2011). Studies assessing the response of evergreen conifers to elevated autumn temperature have largely been conducted using climate chambers (e.g. Busch et al. 2007), mesocosms (e.g. Tingey et al. 2007) or open-top chambers (e.g. Murray et al. 1994, Wang et al. 1995, Repo et al. 1996, Guak et al. 1998). However, results obtained from chamber experiments often cannot be directly extrapolated to the field (Aronson and McNulty 2009). Temperature free-air-controlled enhancement (T-FACE) experiments provide an attractive alternative to chamber systems because they do not affect solar radiation, precipitation, soil or wind (Kimball et al. 2008, Aronson and McNulty 2009). Previous T-FACE experiments have focused on herbaceous species, such as wheat (de Boeck et al. 2012), alfalfa (Kimball et al. 2008), rice (Mohammed and Tarpley 2009), and prairie grasses (Luo et al. 2001, Kimball et al. 2008). Studies using T-FACE experiments involving evergreen conifer seedlings have been rare and focused on the effect of elevated temperature on productivity during the growing season (e.g. Zhao and Liu 2009).

The aim of this study was to characterize autumn cold acclimation in the evergreen conifer *Pinus strobus* under field conditions and to assess the effect of elevated autumn temperature at the beginning of the cold hardening process and the subsequent development of cold hardiness. We hypothesized that elevated temperature (i) delays the downregulation of photosynthesis, (ii) delays the transition from dynamic to winter sustained non-photochemical quenching, (iii) delays changes in nonstructural leaf carbohydrates including starch and low temperature-associated soluble sugars, and (iv) impairs the development of freezing tolerance. A T-FACE system was used to increase temperature by 1.5°C during the day and 3°C during the night, in accordance
with 35-year temperature projections for the Canadian provinces of Ontario and Québec (Price et al. 2011).

### 4.3 Materials and Methods

#### Study Site and Plant Material

The experiment was conducted at the Koffler Scientific Reserve of the University of Toronto located near King City, Ontario (44°050’N, 79°483’W). A Temperature Free-Air-Controlled Enhancement (T-FACE) system was set up according to Kimball et al. (2008), consisting of 10 experimental plots, each with a diameter of 3 m. Ambient canopy temperature (AT) was recorded using infrared sensors (Model IRT-P5, Apogee Instruments, Logan, UT, USA) in five unheated control plots. For the elevated temperature (ET) treatment, five plots were arranged with six 1000 W infrared heaters (Mor Electric Heating Association, Comstock Park, MI, USA) per plot in a hexagonal array, where leaf temperature was raised by +1.5°C during the day and +3°C during the night, according to Kimball et al. (2008). Ambient air and canopy temperatures were recorded using a CR1000 datalogger (Campbell Scientific Inc., Edmonton, AB, Canada). Precipitation data were obtained from the Buttonville Airport weather station in Newmarket, ON (Environment Canada), located 25 km from the field site.

Plots were excavated 30 cm deep, filled with a mixture composed of one-third peat, one-third sand and one-third local soil, and tilled prior to planting. Three-year-old (3+0) bare-rooted *Pinus strobus* seedlings were obtained from a local seed orchard (seed zone 37, Somerville Nurseries, Everett, ON, Canada). In early May 2012, ninety seedlings were planted per plot. Gas exchange and fluorescence measurements commenced in mid-August 2012 after seedlings had established, and continued until December 2012. During 2013, measurements were expanded to assess water potential, soil moisture, and freezing tolerance. Measurements in 2013 were taken monthly from August 2013 until November, with final measurements taken in January 2014 (Fig. 4.1). At each time point, three seedlings were randomly selected for measurement from each of five replicate plots per treatment. Soil moisture was measured using a HydroSense™ soil water content sensor (Campbell Scientific Inc., Edmonton, AB, Canada). Soil moisture, measured as percent volumetric water content, was assessed at a depth of 15 cm, 10 cm from the base of each measured seedling, three times per seedling. Deep frozen soil and ice packs prevented
measurements of soil moisture in January 2014. Air humidity and temperature sensors (Hoskin Scientific Limited, Burlington, ON, Canada) were installed in May 2014, in order to assess differences in vapor pressure deficit (VPD), or the difference between actual and saturated air moisture, between heated and unheated plots during July 2014.

Mature current-year needles were collected from measured trees immediately following measurements, flash-frozen in liquid nitrogen, and stored at -80°C until analysis.

**Photosynthetic Gas Exchange and Chlorophyll Fluorescence**

Gas exchange and chlorophyll fluorescence measurements were performed simultaneously using a portable photosynthesis system (LI-6400 XT; Li-Cor Biosciences, Lincoln, NE, USA) with attached leaf chamber fluorometer (6400-40). Topmost, south-facing needles of the primary shoot were arranged in a flat single-needle layer and placed into the cuvette. The cuvette was set to maintain a level of 400 ppm CO₂ and ambient temperature, which was selected based on the predicted daily average (Table 4.1).

Dark-adapted minimum PSII fluorescence (F₀), and dark-adapted maximum PSII fluorescence (Fₘ) were determined after 40 minutes of dark adaptation. Subsequently, plants were exposed to 1200 µmol quanta m⁻² s⁻¹ for 7-12 minutes to obtain measurements of steady-state photosynthesis; this light intensity represents one that is typically observed in boreal environments on clear and sunny days, even during early winter or early spring (Ensminger et al. 2004). Measured parameters included photosynthetic CO₂ assimilation (A), stomatal conductance (gₛ), evapotranspiration (E), light-adapted minimum PSII fluorescence (F₀'), light-adapted maximum fluorescence (Fₘ'), and transient fluorescence (Fᵣ), which were used to calculate gas exchange and fluorescence parameters (Table 4.2).

The seasonal depression of Fₘ due to low temperature does not allow for recovery of the maximum fluorescence signal in the dark, and thus limits its use for the calculation of the fluorescence parameter NPQ (Demmig-Adams and Adams 2006). A good estimation of NPQ requires a dark-adapted control value of Fₘ that is measured when the photosynthetic apparatus is in a fully relaxed state. During winter, when Fₘ is depressed and does not relax rapidly in the dark, NPQ will be underestimated (Demmig-Adams et al. 2012). The non-photochemical
quenching parameter NPQ was therefore calculated as shown in Table 4.2. The fully recovered maximum fluorescence ($F_{m,rec}$) was estimated as $F_o \times 5$, according to Schreiber et al. (1995) and

Figure 4.1: Seasonal variations in precipitation, day length and temperature from August 1, 2012 to January 31, 2014 at Koffler Scientific Reserve in Ontario, Canada. (A) Daily precipitation and (B) 5-day running averages of max (upper dark grey line), mean (light grey line), and minimum ambient air temperature (lower dark grey line), as well as photoperiod (dotted black line). Arrows indicate measuring dates. Black bar above temperature data indicates periods where heaters were operating; grey bar indicates period where heating was on, but data was lost due to a logger malfunction.
Table 4.1: Cuvette air temperature.
Values were measured during each measurement campaign, using the LI-6400 XT gas exchange system, and represent the average of 30 measurements (15 from ambient plots, 15 from elevated temperature plots) ± S.E.

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<tr>
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<td>24.7 ± 0.2</td>
</tr>
<tr>
<td>October</td>
<td>17.2 ± 0.1</td>
<td>6.3 ± 0.4</td>
</tr>
<tr>
<td>November</td>
<td>7.2 ± 0.1</td>
<td>6.5 ± 0.7</td>
</tr>
<tr>
<td>December</td>
<td>0.9 ± 0.1</td>
<td>n/a</td>
</tr>
<tr>
<td>January</td>
<td>n/a</td>
<td>-0.1 ± 0.1</td>
</tr>
</tbody>
</table>

Ensminger et al. (2004). This estimation is based on two assumptions: firstly, the ratio of fully recovered $F_m/F_o$ is equal to 5, which has been demonstrated in multiple plant species (Björkman and Demmig 1987), including conifers (Adams and Demmig-Adams 1994); and secondly, unlike $F_m$, $F_o$ shows little seasonal variation (Ottander et al. 1995). However, this approach might occasionally underestimate NPQ when $F_o$ is strongly decreased.

Each measurement took approximately 15 minutes and were taken from 2 hours after dawn until 2 hours prior to sunset. Measurement campaigns occurred over 2-3 consecutive days. Measurement order was randomized at individual, plot and treatment levels during each campaign in order to minimize confounding diurnal or daily effects. All measurements were performed on attached needles. Following measurement, needles in the cuvette were harvested to estimate the light-exposed needle surface area using a scanner and the WinSeedle software package (Regent Instruments Inc., Québec, QC, Canada).
Table 4.2: Equations for gas exchange and fluorescence parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Equation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IWUE</td>
<td>$\frac{A}{g_s}$</td>
<td>Silva and Horwath 2013</td>
</tr>
<tr>
<td>$F_v/F_m$</td>
<td>$\frac{F_m - F_o}{F_m}$</td>
<td>Genty et al. 1989</td>
</tr>
<tr>
<td>1-qP</td>
<td>$1 - \frac{F_{m'} - F_o}{F_{m'} - F_{o'}}$</td>
<td>Maxwell and Johnson 2000</td>
</tr>
<tr>
<td>$\Phi_{PSII}$</td>
<td>$1 - \frac{F_t}{F_{m'}}$</td>
<td>Genty et al. 1989</td>
</tr>
<tr>
<td>NPQ</td>
<td>$\frac{F_{m't} - F_{o'}}{F_{m'} - F_{o'}} - 1$</td>
<td>Bilger and Björkman 1990, Ensminger et al. 2004, Porcar-Castell 2011</td>
</tr>
<tr>
<td>NPQS</td>
<td>$\frac{F_{m'rec}}{F_m} - 1$</td>
<td>Maxwell and Johnson 2000, Ensminger et al. 2004, Porcar-Castell 2011</td>
</tr>
</tbody>
</table>

Water Potential

Water potential measurements were performed from August to October, 2013. Pre-dawn and midday (noon) water potential ($\Psi_w$) were assessed on individual current-year needles using a Model 1505D Pressure Chamber Instrument (PMS Instrument Company, Albany, OR, USA). During each campaign, measurements were taken from three needles per seedling on three seedlings per plot, five plots per treatment. During November and January water potential was not assessed because the system did not operate at sub-freezing temperatures.

Analysis of Photosynthetic Pigments

Roughly 50-60 mg homogenized frozen needle tissue was extracted in 2 ml methanol buffered with 2% 0.5 M ammonium acetate according to Junker L. V., Ensminger I., unpublished (see Appendix A4). Samples were filtered using a 0.45µm nylon filter prior to HPLC analysis. Chlorophylls and carotenoids were separated on a reverse-phase C30 column (YMC Carotenoid; Chromatographic Specialties Inc., Brockville, ON, Canada). Pigment extracts were analyzed with an Infinity 1200 series high performance liquid chromatography (HPLC) system equipped with a UV-diode array detector (Agilent Technologies, Santa Clara, CA, USA). De-epoxidation
state (DEPS) was calculated as \((0.5A+Z)/(V+A+Z)\) where \(V\) is violaxanthin, \(A\) is antheraxanthin, and \(Z\) is zeaxanthin. Total chlorophylls and \(\alpha\)-tocopherol were expressed on a per freshweight basis, as the water content of white pine needles fluctuates less than 10% year-round (Verhoeven et al. 2009).

### Analysis of Nonstructural Carbohydrates

Roughly 30-40 mg homogenized and lyophilized needle tissue from samples collected in August, October and December of 2012 were extracted in methanol:chloroform:water (12:5:3) according to Park et al. (2009), with the addition of 250 µg galactitol as an internal standard. An aliquot of 2 ml of the soluble sugar extract was vacuum centrifuged and resuspended in 1 ml of nanopure water. The resuspended extract was filtered using a 0.45 µm nylon filter and analyzed using a DX-600 anion-exchange HPLC (Dionex, Sunnyvale, CA, USA) equipped with a Hi-Plex Ca column (Agilent Technologies, Santa Clara, CA, USA) and electrochemical pulse amperometric detector (EC-PAD). Sucrose, fructose, glucose and pinitol were eluted with water at a flow rate of 0.170 ml/min with a column temperature of 70°C. Post-column detection was performed using NaOH at a rate of 100 mM/min. Raffinose was eluted using a Carbo-Pac PA1 column (Dionex, Sunnyvale, CA, USA) with 150 mM NaOH (isocratic) at a flow rate of 1 ml/min with post-column detection using NaOH at a rate of 100 mM/min.

Starch was determined from the residual tissue pellet from the soluble sugar extraction. The pellet was dried overnight at 55°C. 25-50 mg of the dried pellet were resuspended in 5 ml of 4% \(\text{H}_2\text{SO}_4\), vortexed and autoclaved for 3.5 min. After cooling to room temperature, the extract was spun at 500 rpm for 5 min and the supernatant collected. The supernatant was filtered using a 0.45 µm nylon filter and analyzed using a DX-600 anion-exchange HPLC (Dionex, Sunnyvale, CA, USA) equipped with a Carbo-Pac PA1 column (Dionex, Sunnyvale, CA, USA) and EC-PAD. Glucose was eluted with water at a flow rate of 1 ml/min with a column temperature of 30°C. Post-column detection was performed using NaOH at a rate of 100 mM/min.

### Freezing Tests

Chlorophyll fluorescence was used to assess freezing tolerance in August, September, October, November of 2013 and January 2014, using a modified protocol based on Sutinen et al. (1992). Current-year shoots were dark-adapted for 40 minutes. Each shoot was excised and \(F_v/F_m\) was
measured. The shoots were then individually wrapped in moist paper towel and aluminum foil and sealed in a plastic bag prior to transport on ice back to the laboratory.

Shoots were exposed to freezing temperatures using a Thermotron SM-16-8200 environmental test chamber (Thermotron Industries, Holland, MI, USA). The maximum cooling rate was 2.5°C h⁻¹, with the 0 to -1°C interval achieved over 1 hour. Since freezing resistance varies over the course of the year, preliminary freezing tests were performed throughout the year to identify a range of freezing temperatures suitable to induce freezing damage in white pine seedlings. Target freezing temperatures were then adjusted during each month of the experiment in order to account for the expected change in freezing tolerance, with the aim of selecting a range of freezing temperatures that bracketed the temperature at which 50% of the seedlings were damaged by freezing (LT₅₀). One shoot per tree per freezing temperature was held at the desired temperature for 6-8 h and subsequently thawed in a stepwise manner to room temperature: shoots exposed to ≤ -30°C were kept at -20°C for 24 h, transferred to 4°C for 24 h, and then transferred to room temperature for 24 h recovery. Shoots exposed to ≥ -20°C were transferred directly to 4°C for 24 h and then to room temperature for recovery (Sutinen et al. 1992). Following the 24 h recovery period, shoots were unwrapped and exposed to 1 h light exposure at 800 μmol quanta m⁻² s⁻¹ in order to stimulate PSII, then dark-adapted for 40 minutes (Burr et al. 2001). Fᵥ/Fₘ was then assessed. Since we used chlorophyll fluorescence to evaluate freezing injury at PSII, we defined LT₅₀ as the temperature required to reduce maximum Fᵥ/Fₘ by 50%. Maximum Fᵥ/Fₘ was assessed by subjecting non-frozen shoots to the same protocol of 24 h recovery period, 1h of light exposure, 40 minutes of dark adaptation and measurement. LT₅₀ values were calculated by fitting Fᵥ/Fₘ values measured from freezing-recovered shoots using a modified Richards curve model (von Fircks and Verwijst 1993):

\[ f(x) = \frac{K}{1 + e^{-B(x-M)}} \]

where K represents the upper asymptote, or pre-freezing Fᵥ/Fₘ; B represents the maximum slope at LT₅₀ and M represents LT₅₀. Data was tested for normality using the D’Agostino-Pearson omnibus normality test. The curve for each treatment (elevated vs. ambient temperature) was fitted using the least squares method. LT₅₀ values were compared between treatments using an extra sum-of-squares F test with a P-value cutoff of 0.05. Analysis was performed using Graphpad Prism v6.04 (Graphpad Software, Inc., La Jolla, CA, USA).
Statistical Analyses

Two-way ANCOVA was used to assess the effect of the elevated temperature treatment and time on gas exchange, fluorescence and photosynthetic pigments, while accounting for the effect of seasonal variation introduced by photoperiod and daily temperature. The ANCOVA model used treatment and day of year as categorical fixed factors, photoperiod and minimum daily temperature as continuous numeric covariates, and plot and year as random factors, using the \textit{lme4} package in R v3.1.1 (http://www.r-project.org/). Multiple comparisons were used to contrast treatment within each time point, and were performed using the \textit{multcomp} package in R v3.1.1. \textit{P}-values for multiple comparisons were adjusted using Bonferroni correction.

Starch and soluble sugars were analyzed using two-way ANOVA to identify treatment, time and interaction effects. Tukey’s HSD post-hoc test was used to identify significantly different groups. The statistical analyses for sugars were performed using Graphpad Prism v6.04.1.

Treatment responses of A, Fv/Fm, and NPQs from both years were pooled, independently plotted against minimum daily temperature and photoperiod, and fitted using the least squares method with a 4-parametric sigmoidal curve function:

\[
    f(x) = A + \frac{K - A}{1 + e^{-B(x-M)}}
\]

where K represents the maximal parameter value; A represents the minimal parameter value; B represents the maximum slope and M represents the midpoint of the curve at which estimated values represent 50% of the maximum value of the parameter. \( R^2 \) and 95% confidence intervals were calculated. Midpoints were compared between treatments using a sum-of-squares F test. Modelling and analyses of the sigmoid curves were performed using Graphpad Prism v6.04.1.

4.4 Results

Seasonal Weather Patterns

The field site experienced higher amounts of precipitation during the growing season and lower amounts during winter (Fig. 4.1A). 2012 was characterized by warm early autumn, with daily maximum temperatures remaining above 20°C until the first week of October (Fig. 4.1B). In contrast, during 2013, daily maximum temperatures began to decline below 20°C by the first
week of September. Daily mean temperatures remained above 0°C until November in both years. The first night frost was recorded on October 8 in 2012 and on October 27 in 2013. During October 2012, the temperature dropped rapidly until mid-November and remained between a daily minimum of -5°C and a daily maximum of 10°C until mid-December. In contrast, nighttime temperatures during October and early November 2013 were mild, with minimum temperatures only reaching -2°C and daily maximums above 10°C. Minimum temperatures did not reach -20°C during the winter of 2012 until January 1, while minimum temperature reached -20°C on December 12 in 2013 (Fig. 4.1B).

The variation in weather conditions affected temperature and precipitation during measurement campaigns. Measurements taken during August 2012 occurred after several rainy days, whereas measurements taken in August 2013 were taken after ten days without rainfall (Fig. 4.1A), resulting in decreased soil water content (Fig. 4.4A). In 2012, we recorded a daily mean temperatures of 22°C during our measurements in August, 15°C in September and 15°C in October. In contrast, during 2013 we recorded daily mean temperatures of 20°C during our measurements in August, 7°C in September and 4°C in October (Fig. 4.1B).

**Photosynthetic Gas Exchange**

Photosynthetic carbon assimilation (A) remained unchanged from August to the beginning of October, was downregulated during October and November, and eventually ceased in December and January (Fig. 4.2A). This trend was also observed for stomatal conductance (gs), intrinsic water use efficiency (IWUE) and evapotranspiration (E) (Fig. 4.2B-D). However, we also observed differences between years, e.g. during August and September 2013, when we measured lower rates of A, gs, and IWUE (Fig. 4.2A-C) compared to 2012. In 2012, photosynthetic gas exchange was fully downregulated by mid-November, while photosynthetic activity was still detectable in November 2013 (Fig. 4.2A-C). Treatment had a significant effect on gs, IWUE, and E; the interaction of treatment and time significantly affected gs, IWUE and E (Table 4.3).

From August to early October, seedlings in heated plots that experienced elevated temperature (ET) exhibited lower assimilation in comparison to seedlings in unheated control treatment (Control) that were exposed to ambient temperature (Fig. 4.2A). However, the timing of the
Figure 4.2: Effect of elevated temperature on photosynthetic gas exchange in field-grown white pine seedlings during autumn
(A) Photosynthetic carbon assimilation (A); (B) stomatal conductance (gs); (C) intrinsic water use efficiency (IWUE); (D) evapotranspiration (E). AT and ET, seedlings grown at ambient and elevated temperature, respectively. Each data point represents the average of 5 plots, ± S.E. Asterisks represent significant treatment effect at a single measuring date (** P < 0.01, * P < 0.05).

Autumn downregulation of photosynthesis was not affected by the elevated temperature treatment, as assimilation began to decrease by the end of October during both years, irrespective of treatment (Fig. 4.2A). Between August and October of both years, gs was decreased by 20-30% in ET seedlings compared to Control seedlings. Control seedlings maintained gs between 0.10-0.15 mol H2O m-2 s-1, while the elevated temperature treatment exhibited values between 0.06-0.12 mol H2O m-2 s-1 (Fig. 4.2B). gs was significantly reduced in ET seedlings in heated plots in October 2012 (P = 0.041, Fig. 4.2B). In 2012, IWUE was increased by about 15-20% in seedlings in the heated plots compared to Control seedlings, and was significantly enhanced in October (P = 0.036, Fig. 4.2C). However, in 2013, IWUE was not significantly affected in seedlings in the heated plots (Fig. 4.2C). E was reduced by 20-30% in ET seedlings in heated plots compared to Control seedlings during both years (Fig. 4.2D), particularly during September 2012 (P = 0.009), October 2012 (P = 0.048), and September 2013 (P = 0.003).
Table 4.3: Summary of two-way ANCOVA analysis showing the effects of treatment and time (day of year) on gas exchange, chlorophyll fluorescence and photosynthetic pigments.

Variables were estimated as Variable ~ Treatment * Time + Photoperiod + Temperature + (1|Plot) + (1|Year). Treatment and time were included as categorical fixed factors. Photoperiod and daily temperature were included as continuous numeric covariates. Plot and year were included as random factors. P-values in bold indicate statistical significance ($\alpha = 0.05$).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th></th>
<th></th>
<th>Treatment x Time</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td>Gas exchange</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2.420</td>
<td>0.121</td>
<td>0.765</td>
<td>0.385</td>
<td>0.809</td>
<td>0.369</td>
</tr>
<tr>
<td>gs</td>
<td>7.361</td>
<td><strong>0.007</strong></td>
<td>0.292</td>
<td>0.589</td>
<td>4.911</td>
<td><strong>0.028</strong></td>
</tr>
<tr>
<td>IWUE</td>
<td>4.687</td>
<td><strong>0.032</strong></td>
<td>0.010</td>
<td>0.919</td>
<td>4.684</td>
<td><strong>0.032</strong></td>
</tr>
<tr>
<td>E</td>
<td>12.254</td>
<td><strong>0.001</strong></td>
<td>0.048</td>
<td>0.827</td>
<td>8.302</td>
<td><strong>0.004</strong></td>
</tr>
<tr>
<td>Chlorophyll fluorescence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$F_v/F_m$</td>
<td>1.709</td>
<td>0.192</td>
<td>201.118</td>
<td>&lt;<strong>0.001</strong></td>
<td>1.368</td>
<td>0.243</td>
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<tr>
<td>1-qP</td>
<td>0.403</td>
<td>0.526</td>
<td>9.681</td>
<td><strong>0.002</strong></td>
<td>1.357</td>
<td>0.245</td>
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<tr>
<td>$\Phi_{PSII}$</td>
<td>3.572</td>
<td>0.060</td>
<td>1.669</td>
<td>0.198</td>
<td>1.802</td>
<td>0.181</td>
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<td>NPQ</td>
<td>5.617</td>
<td><strong>0.019</strong></td>
<td>1.343</td>
<td>0.248</td>
<td>2.637</td>
<td>0.106</td>
</tr>
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<td>NPQs</td>
<td>1.476</td>
<td>0.225</td>
<td>200.188</td>
<td>&lt;<strong>0.001</strong></td>
<td>1.073</td>
<td>0.301</td>
</tr>
<tr>
<td>Photosynthetic pigments</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Chl</td>
<td>0.094</td>
<td>0.759</td>
<td>0.532</td>
<td>0.466</td>
<td>0.052</td>
<td>0.821</td>
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<tr>
<td>Chl a/b</td>
<td>4.012</td>
<td><strong>0.046</strong></td>
<td>10.905</td>
<td><strong>0.001</strong></td>
<td>3.123</td>
<td>0.078</td>
</tr>
<tr>
<td>Car/Chl</td>
<td>0.060</td>
<td>0.808</td>
<td>0.795</td>
<td>0.374</td>
<td>0.418</td>
<td>0.518</td>
</tr>
<tr>
<td>$\alpha$-Car/Chl</td>
<td>1.593</td>
<td>0.208</td>
<td>3.954</td>
<td><strong>0.048</strong></td>
<td>3.007</td>
<td>0.084</td>
</tr>
<tr>
<td>$\beta$-Car/Chl</td>
<td>1.137</td>
<td>0.287</td>
<td>28.745</td>
<td>&lt;<strong>0.001</strong></td>
<td>1.866</td>
<td>0.173</td>
</tr>
<tr>
<td>V+A+Z/Chl</td>
<td>0.006</td>
<td>0.938</td>
<td>1.233</td>
<td>0.269</td>
<td>0.131</td>
<td>0.718</td>
</tr>
<tr>
<td>DEPS</td>
<td>1.298</td>
<td>0.256</td>
<td>106.087</td>
<td>&lt;<strong>0.001</strong></td>
<td>1.230</td>
<td>0.268</td>
</tr>
<tr>
<td>Lut/Chl</td>
<td>0.135</td>
<td>0.714</td>
<td>0.031</td>
<td>0.862</td>
<td>0.706</td>
<td>0.402</td>
</tr>
<tr>
<td>Neo/Chl</td>
<td>0.013</td>
<td>0.910</td>
<td>11.269</td>
<td><strong>0.001</strong></td>
<td>0.025</td>
<td>0.874</td>
</tr>
<tr>
<td>$\alpha$-Toc</td>
<td>0.059</td>
<td>0.809</td>
<td>0.908</td>
<td>0.341</td>
<td>0.110</td>
<td>0.741</td>
</tr>
</tbody>
</table>
Chlorophyll Fluorescence

The maximum quantum efficiency of PSII ($F_v/F_m$) was approximately 0.75-0.80 from August to early October, and continuously decreased from late October through January (Fig. 4.3A). The effective quantum yield of PSII ($\Phi_{PSII}$) was downregulated during the autumn transition and reached minimum values towards the end of November (Fig. 4.3B). Non-photochemical quenching (NPQ) was high during both years; sustained NPQ (NPQ$_S$) began to develop during late October and comprised nearly 100% of nonphotochemical processes by January (Fig. 4.3C). Excitation pressure ($1-qP$) increased during October and reached maximum levels in November before relaxing again in December (Fig. 4.3D). In contrast to the substantial interannual variation observed in photosynthetic gas exchange, we did not observe interannual variations in most fluorescence parameters (Fig. 4.3).

Figure 4.3: Effect of elevated temperature on chlorophyll fluorescence in field-grown white pine seedlings during autumn.
(A) Maximum quantum yield of PSII ($F_v/F_m$); (B) effective quantum yield of PSII ($\Phi_{PSII}$); (C) rate constant of total nonphotochemical quenching (NPQ) and sustained nonphotochemical quenching (NPQ$_S$); (D) excitation pressure at PSII ($1-qP$). AT and ET, seedlings grown at ambient and elevated temperature, respectively. Each data point represents the average of 5 plots, ± S.E. Asterisks represent significant treatment effect at a single measuring date (*** $P < 0.001$, * $P < 0.05$).
\( \Phi_{\text{PSII}} \) was significantly lower in August 2013 in the elevated temperature treatment \((P = 0.015,\) Fig. 4.3B). NPQ was significantly higher in August \((P = 0.014)\) and September 2013 \((P < 0.001)\) in the elevated temperature treatment, but was not significantly different during 2012; NPQs was not significantly different between treatments during either year, although ET seedlings in the heated plots exhibited decreased NPQs during November and December (Fig. 4.3C). 1-qP relaxed considerably from November to January under ambient temperature conditions, but did not when exposed to elevated temperature \((P = 0.002,\) Fig. 4.3D). Treatment had a significant effect on NPQ, whereas time had a significant effect on NPQs, \(F_v/F_m\), and 1-qP (Table 4.3).

Figure 4.4: Effect of elevated temperature on soil water availability and osmotic stress in field-grown white pine seedlings during autumn.

(A) Soil moisture content, expressed in percent volumetric water content (% VWC); (B) pre-dawn water potential \((\Psi_w)\); and (C) midday \(\Psi_w\) measured during 2013. AT and ET, seedlings grown at ambient and elevated temperature, respectively. n/a indicates points where water potential was not assessed because the equipment did not operate at sub-freezing temperatures in the field. Each data point represents the average of 5 plots, ± S.E.
Water Potential and Vapor Pressure Deficit

In 2013, soil moisture was lowest during August (Fig. 4.4A). There was a consistent but non-significant reduction in soil moisture in the heated plots during the growing season (Fig. 4.4A). Leaf water potential ($\Psi_w$) was generally high, with values consistently higher than -0.2 MPa (Fig. 4.4B,C). There was no significant difference in $\Psi_w$ during pre-dawn or midday (Fig. 4.4B,C).

The extent of vapor pressure deficit (VPD) imposed by our heating treatment was assessed from July 18 to August 12, 2014 (Fig. S4.1). The difference in VPD between heated and control plots varied depending on air temperature (Fig. S4.1A,B). During the night, an increment of +3°C induced a 20% increase in VPD (0.351 ± 0.089 kPa) in the heated plots, while during the day an increment of +1.5°C induced a 6% increase in VPD (0.179 ± 0.089 kPa) in the heated plots (Fig. S4.1).

Figure 4.5: Effect of elevated temperature on photosynthetic pigments in needles of field-grown white pine seedlings during autumn.
(A) Chlorophyll a + b per fresh weight; (B) ratio of chlorophyll a to chlorophyll b; (C) total carotenoids per total chlorophyll; (D) $\alpha$- and $\beta$-carotene per total chlorophyll. AT and ET, seedlings grown at ambient and elevated temperature, respectively. Each data point represents the average of 5 plots, ± S.E.
Photosynthetic Pigments

Total chlorophylls, measured on a fresh-weight basis, increased from August to September, decreased from October to November, and remained stable in December and January (Fig. 4.5A). Chlorophyll a/b decreased, albeit not significantly, from August to December (Fig. 4.5B), whereas total carotenoids increased from October to November and stabilized in December (Fig. 4.5C). β-carotene showed large variations during the autumn and between the treatments (Fig. 4.5D). β-carotene levels increased over October and November, while α-carotene decreased over the same period (Fig. 4.5D). Inter-annual variations were observed in chlorophyll and carotenoid pools, with chlorophyll a/b decreasing earlier in 2013 than in 2012 (Fig. 4.5B), and slightly higher carotenoid levels during August 2013 compared to August 2012 (Fig. 4.5C), though these differences were not significant.

Photoprotective pigments and metabolites also showed distinct changes during the autumn. Lutein (Fig. 4.6A), neoxanthin (Fig. 4.6B), and xanthophyll cycle pigments (Fig. 4.6C) accumulated through October and November to maximal levels in December. The de-epoxidation status of the xanthophyll-cycle pigments (DEPS) (Fig. 4.6D) transiently increased from October through January. α-tocopherol increased from August to November, followed by stabilization in December (Fig. 4.6E).

Accumulation of lutein (Fig. 4.6A), neoxanthin (Fig. 4.6B), total xanthophylls (Fig. 4.6C) and DEPS (Fig. 4.6D) varied between years. In August 2012, seedlings exhibited low levels of lutein (Fig. 4.6A) and total xanthophylls (Fig. 4.6C) compared to August 2013. DEPS was increased in August 2012 compared to August 2013 (Fig. 4.6D). Increases in lutein (Fig. 4.6A), neoxanthin (Fig. 4.6B), and xanthophylls (Fig. 4.6C) occurred during August and September 2013 which were not observed in 2012. Treatment did not have a significant effect on any of the photosynthetic pigments, whereas time had a significant effect on chlorophyll a/b, α-carotene, β-carotene, DEPS, and neoxanthin (Table 4.3).
Figure 4.6: Effect of elevated temperature on photoprotective metabolites in needles of field-grown white pine seedlings during autumn.

(A) Lutein per total chlorophyll; (B) neoxanthin per total chlorophyll; (C) xanthophyll pool size per total chlorophyll; (D) de-epoxidation state (DEPS); (E) α-tocopherol per fresh weight. AT and ET, seedlings grown at ambient and elevated temperature, respectively. Each data point represents the average of 5 plots, ± S.E. Asterisks represent significant treatment effect at a single measuring date (* $P < 0.05$).
Figure 4.7: Relationship of photosynthesis and sustained nonphotochemical quenching with minimum daily temperature and photoperiod.

(A,B) Photosynthetic carbon assimilation ($A$); (C,D) maximum quantum yield of PSII ($F_v/F_m$); (E,F) sustained nonphotochemical quenching (NPQ$_S$). Each point indicates a single measurement. Lines represent 4-parametric sigmoidal curves fit to the data using the least-squares method. Open circles and dashed lines, elevated temperature (ET); closed circles and solid lines, ambient temperature (AT). Grey lines indicate midpoint of curve at which estimated values represent 50% of the maximum parameter value.
Table 4.4: Curve parameters of 4-parametric sigmoid models presented in Fig. 4.7. Mid indicates the midpoint of the curve at which estimated values represented 50% of the maximum value. For each model curve, mid and slope values were compared between treatments using an extra sum-of-squares F test; bolded values ($P < 0.05$) indicate statistical significance.

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Control of Low Temperature and Photoperiod on the Downregulation of Photosynthesis and Development of Sustained NPQ

In response to the decrease in daily minimum temperature from 10 to -2°C during autumn and winter, we observed a transient decrease in $A$, $F_v/F_m$ and NPQs (Fig. 4.7A,C,E, Table 2.4). $A$ also showed a response to decreasing photoperiod over a range of 11 to 9 h (Fig. 4.7B, Table 4.4). In contrast, rapid downregulation of $F_v/F_m$ and rapid induction of sustained NPQ were observed when photoperiod reached a threshold value of approximately 9.6 h (Fig. 4.7D,F, Table 4.4). Elevated temperature did not significantly affect the response of assimilation to minimum temperature, but did shift the response of $F_v/F_m$ and NPQs (Table 4.4).

Nonstructural Carbohydrates

In both treatments, leaf starch levels remained unchanged from August through December (Fig. 4.8A), while the amount of total soluble sugars decreased slightly from August to October and doubled in December (Fig. 4.8B). Raffinose was absent in August, present in minute quantities in October, and present in large quantities in December (Fig. 4.8C). Sucrose levels were constant during August and October but increased in December (Fig. 4.8D). Fructose levels remained
Figure 4.8: Effect of elevated temperature on nonstructural carbohydrates in needles of field-grown white pine seedlings during autumn.
Samples were collected during August, October and December of 2012. AT and ET, seedlings grown at ambient and elevated temperature, respectively. Each bar represents the average of 5 plots, ± S.E. Letters, where present, indicate significantly different groups ($P < 0.05$).
constant from August through December (Fig. 4.8E), while glucose levels decreased by more
than 50% from August to October, but tripled from October to December (Fig. 4.8F). Pinitol
mirrored the glucose levels, and was reduced by 50% from August to October, but doubled in
December (Fig. 4.8G). Seedlings in the heated plots exhibited significantly higher amounts of
leaf starch ($P = 0.026$, Table S4.1); however, this did not significantly affect the accumulation of
soluble sugars (Table S4.1).

Freezing tolerance

In 2013, seedlings exhibited tolerance to freezing exposure of -10°C during August (Fig. 4.9A), -16°C during September (Fig. 4.9B), -30°C in October (Fig. 4.9C) and were fully cold hardy
below -60°C by November (Fig. 4.9D). The cold hardness of ET seedlings from heated plots
did not differ from that of Control seedlings from unheated plots during August (Fig. 4.9A) or
September (Fig. 4.9B). In October, seedlings from heated plots exhibited significantly greater
freezing tolerance in comparison with freezing tolerance of seedlings from unheated plots ($P = 0.027$, Fig. 4.9C), though this difference may not be biologically relevant.

4.5 Discussion

We explored the effect of a moderate increase of air temperature by +1.5°C during the day and
+3°C during the night on the development of cold acclimation by assessing photosynthesis,
photoprotective NPQ and pigments, carbohydrate metabolism and freezing tolerance in Eastern
white pine in a field experiment. We observed that physiological responses of ET seedlings
exposed from heated plots mainly differed from unheated Control seedlings during August,
September and early October. We also observed that most physiological changes in
photosynthesis (Fig. 4.2, Fig. 4.3) and cold hardiness (Fig. 4.9) occurred during late September
and early October, while photoprotective modifications of energy quenching characteristics and
pigment composition (Fig. 4.3, Fig. 4.6) occurred later, during November and December. Our
data clearly suggests that under field conditions, an increase in temperature by 1.5°C during the
day and 3°C during the night does not extend the length of the growing season and does not
delay the downregulation of photosynthesis, the increase in photoprotective capacity,
accumulation of nonstructural carbohydrates, or development of freezing tolerance in *Pinus
strobus* seedlings.
Figure 4.9: Effect of elevated temperature on cold hardening in field-grown white pine seedlings during autumn.
Freezing tolerance was assessed in (A) August; (B) September; (C) October; and (D) November of 2013. Dotted lines, where present, indicate 95% confidence interval (CI) of model. AT and ET, seedlings grown at ambient and elevated temperature, respectively. Solid line indicates ambient temperature model; dashed line indicates elevated temperature model. Grey lines indicate LT50. Asterisk indicates significant treatment effect at LT50 (* P < 0.05).
Photosynthesis

*Elevated temperature affects photosynthetic gas exchange during the growing season*

During August and September of both years, photosynthetic gas exchange in ET seedlings was significantly different compared to Control seedlings (Table 4.3). Throughout our measurements, stomatal conductance was below 0.15 mol H₂O m⁻² s⁻¹, the threshold at which stomatal conductance begins to limit Rubisco activity in *Phaseolus vulgaris*, *Vitis vinifera* and *Rhamnus ludovici-salvatoris* (Flexas et al. 2004). In seedlings growing under elevated temperature conditions, stomatal conductance was decreased (Fig. 4.2B) and contributed to reduced assimilation (Fig. 4.2A) and evapotranspiration (Fig. 4.2D). ET seedlings from the heated plots exhibited improved water use efficiency from August through October in 2012, but this effect was not observed in 2013 (Fig. 4.2C). During August, stomatal conductance of both treatments was higher in 2013 than in 2012 (Fig. 4.2B), though assimilation for both treatments was greatly reduced in 2013 (Fig. 4.2A), suggesting that stomatal conductance did not limit assimilation in August 2013. As neither PSII activity (Fig. 4.3B) nor excitation pressure (Fig. 4.3D) differed between August 2012 and 2013, the limiting factor of assimilation was likely a decreased sink capacity of the seedlings. Measurements in September 2013 were taken after nighttime temperature fell below 5°C, and resulted in decreased stomatal conductance although assimilation remained high; again, neither PSII activity nor excitation pressure were affected.

*Elevated temperature decreases stomatal conductance and evapotranspiration even in the absence of water stress*

Seedlings exposed to elevated temperature consistently exhibited lower stomatal conductance (Fig. 4.2B) and evapotranspiration (Fig. 4.2D) during the growing season. In 2013, we assessed soil moisture and water potential in order to determine whether gas exchange during the growing season was responding to water stress imposed by the infrared heating method. We did not observe a significant decrease in soil moisture in elevated temperature plots (Fig. 4.4A). Furthermore, pre-dawn and midday water potential measurements for both control and elevated temperature treatments were greater than -0.2 MPa (Fig. 4.4B,C). Since osmotic stress is typically incurred when water potential falls below -1.0 MPa (Flexas et al. 2004, Verslues et al.
we concluded that the decrease in gas exchange observed in the elevated temperature treatment was not a result of osmotic stress.

We also assessed air temperature and humidity during July-August of 2014 and recorded a 6% increase in daytime VPD in heated plots (Fig. S4.1). A recent study which modeled water loss in response to infrared heating predicted a 12-15% increase in transpiration, but noted that certain species such as *Populus tremuloides* would exhibit reduced transpiration as a result of reduced stomatal conductance (de Boeck et al. 2012). Therefore, enhanced VPD may have contributed to the decrease in stomatal conductance observed in our elevated temperature treatment during the growing season. We conclude that when subjected to elevated temperature, *P. strobus* pre-emptively reduces stomatal conductance in an attempt to prevent excessive water loss via evapotranspiration at the cost of reduced photosynthesis.

*Downregulation of photosynthetic gas exchange is driven by both temperature and photoperiod, and is not delayed in seedlings from heated plots*

Downregulation of photosynthetic gas exchange between October and November was strongly correlated with air temperature (Table 4.4) and commenced once nighttime temperatures decreased below 10°C, irrespective of treatment (Fig. 4.2A, Fig. 4.7A). The transient downregulation of photosynthesis occurred with the decrease in temperature at a rate of 0.25 µmol CO$_2$ m$^{-2}$ s$^{-1}$/°C in Control seedlings, and at a rate of 0.11 µmol CO$_2$ m$^{-2}$ s$^{-1}$/°C in ET seedlings (Table 4.4). However, the reason for the lower rate of the downregulation of photosynthesis in ET seedlings largely reflects the fact that photosynthesis in seedlings from the heated plots was already decreased during the growing season compared to seedlings from unheated plots. No photosynthetic gas exchange was observed when temperatures decreased below -2°C, irrespective of treatment (Fig. 4.7A), but this complete downregulation occurred earlier in 2012 than in 2013 (Fig. 4.2), following the earlier occurrence of night frosts in 2012 (Fig. 4.1B). The effect of air temperature on seasonal variations in photosynthesis was previously modeled by Bergh et al. (1998) for *Picea abies* and Mäkelä et al. (2004) for *Pinus sylvestris*. Downregulation of photosynthesis following a decrease in air temperature below 0°C was also observed in *P. sylvestris* stands in northern Sweden (Strand et al. 2002) and Siberia, Russia (Lloyd et al. 2002). In addition to air temperature, our data also reveal that photosynthetic gas exchange decreased by 50% as photoperiod decreased to 10 h, irrespective of treatment, and was completely absent at a 9 h photoperiod (Fig. 4.7B). The transient response of carbon assimilation
to both temperature and photoperiod signals indicates that gas exchange is modulated in concert with decreased metabolic activity (Rossi et al. 2008) and leaf carbon export (Hoch et al. 2003). We conclude that both low temperature and photoperiod exerted a strong control on the downregulation of photosynthetic gas exchange.

*Downregulation of light reactions is preceded by downregulation of gas exchange and is not affected by elevated temperature*

$F_v/F_m$ began to decrease after photoperiod had decreased below 10 h and after occurrence of nighttime frosts (Fig. 4.1B). Despite the significant effect of the elevated temperature treatment on gas exchange in ET seedlings during the downregulation of photosynthetic CO$_2$ uptake between October and November, $F_v/F_m$ and $\Phi_{PSII}$ in seedlings from the heated plots were similar to values observed in seedlings from unheated control plots (Table 4.3). Further downregulation of $F_v/F_m$ occurred throughout December and January, while photosynthetic gas exchange had already ceased by December (Fig. 4.2A, Fig. 4.3B). The transient seasonal changes observed for $F_v/F_m$ and $\Phi_{PSII}$ result from the reorganization of thylakoid membrane-bound photosynthetic proteins. This has been demonstrated e.g. for D1 and LHCII protein content in needles of *P. strobus* (Verhoeven et al. 2009) and *P. sylvestris* (Ottander et al. 1995, Ensminger et al. 2004). These adjustments occur even when temperatures are consistently below 0°C. Given the sequence of the observed events, it therefore appears that the downregulation of photosynthetic gas exchange and hence Calvin cycle activity precedes reorganization of the photosynthetic apparatus in the thylakoid membrane during autumn.

Least squares curve fitting (Table 4.4) revealed that variation in $F_v/F_m$ was strongly correlated with both photoperiod and temperature. However, while decreases in temperature during autumn resulted in a transient decrease of $F_v/F_m$ (Fig. 4.7C), we observed an instant response of $F_v/F_m$ to photoperiod when day length decreased to 9.6 h (Fig. 4.7D). These observations indicate that the reorganization of the photosynthetic apparatus and the downregulation of the light reactions are more sensitive to photoperiod than temperature, but also that photoperiodic regulation of the light reactions operates on a threshold rather than a gradient basis.
Photoprotective Nonphotochemical Quenching and Pigment Dynamics

Sustained nonphotochemical quenching develops after the downregulation of photosynthetic gas exchange and is not delayed by elevated temperature

From August to November, excess light energy was efficiently quenched by dynamic xanthophyll cycle-mediated NPQ (Fig. 4.3C). The transition from dynamic NPQ to winter sustained NPQ occurred synchronously with the downregulation of Fv/Fm from November through January (Fig. 4.3C), as photoperiod decreased below 10 h and nighttime temperatures decreased below 0°C (Fig. 4.1B, Fig. 4.7). The development of sustained NPQ in response to low temperature (Fig. 4.3C) is correlated with the retention of antheraxanthin and zeaxanthin (Adams and Demmig-Adams 1994) and results in increased DEPS (Fig. 4.6D). In contrast to our expectations, the transition from dynamic to sustained NPQ was not significantly delayed in seedlings from the heated plots, since the sustained quenching occurred in parallel in both Control and ET seedlings (Fig. 4.3C).

Elevated temperature does not affect pigment pool size

Photosynthetic pigments in ET seedlings from the heated plots did not reveal any significant differences when compared to Control seedlings from unheated plots, indicating that moderately elevated temperature did not impact the pool sizes of chlorophylls or carotenoid pigments (Fig. 4.5, Table 4.3). Nonetheless, we observed major changes in pigment composition during autumn in Pinus strobus seedlings, which are consistent with development of sustained nonphotochemical quenching. Total chlorophylls (Fig. 4.5A) and α-carotene levels (Fig. 4.5D) increased from August to early October, following the transient increase in assimilation (Fig. 4.2A), and decreased again during November when assimilation decreased (Fig. 4.2A, Fig. 4.3B). While photosynthesis and total chlorophyll levels decreased from October onwards, the pool of total carotenoids showed the opposite trend and nearly doubled from October to November (Fig. 4.5C). This was mainly due to the increase of the photoprotective lutein and xanthophyll cycle pigments (Fig. 4.6A,C). Increases in these pigments during autumn have been previously observed in pine species as well as Pseudotsuga menziesii and Picea pungens (Adams and Demmig-Adams 1994, Ensminger et. al. 2004, Verhoeven et al. 2009) and contribute to alleviate the enhanced risk of photo-oxidative damage (Ensminger et al. 2004).
β-carotene, a component of both reaction centers and core antenna, serves dual functions as an accessory pigment (Trebst 2003) and also as a biosynthetic precursor to zeaxanthin (Bartley and Scolnik 1995). We observed transient accumulation of β-carotene from October to November followed by a decrease in December, concurring with results reported by Verhoeven et al. (2009). Our results suggest that the β-carotene accumulated during this period is converted to zeaxanthin during the development of winter sustained nonphotochemical quenching, as β-carotene (Fig. 4.5D) and NPQ (Fig. 4.3C) responded similarly during late autumn. The accumulation of pigments involved in photoprotective quenching of excess light (Fig. 4.6A-C,E) was completed by November and thereby also indicated the complete cessation of photosynthetic gas exchange (Fig. 4.2A, Fig. 4.3B).

Nonstructural Carbohydrates

*Elevated temperature increases leaf starch content during autumn*

Starch levels in mature needles were low (2.5-3% of leaf dry weight) during August, October and December of 2012 (Fig. 4.8A), consistent with autumn starch levels reported previously (Little 1970, Pomeroy et al. 1970, Hoch et al. 2003). Elevated autumn temperature caused a small but significant increase in needle starch content, indicated by higher starch levels in ET seedlings from the heated plots during the period October to December 2012 (Table S4.1). The reason for this increase is unclear. Typically growth at elevated temperature results in depletion of starch due to the associated increases in foliar respiration (Geigenberger 2011). However, we did not observe an increase in respiration in seedlings from heated plots. In addition, the increase in starch cannot be explained by increased assimilation, since rates of assimilation were always lower in ET seedlings than in Control seedlings in 2012. However, increases in leaf starch content resulting from elevated temperature have been reported recently by Glaubitz et al. (2014). They observed accumulation of leaf carbohydrates including starch in some *Oryza* cultivars in response to asynchronous elevated night time temperature. In another study, Zhao et al. (2012) showed an increase in leaf starch content in poplar leaves when growing under elevated temperature. At this point, the cause for the increased starch levels in seedlings from heated plots remains unclear and deserves further investigation.
Accumulation of soluble carbohydrates during autumn occurs in response to low temperature in needles of ET and Control seedlings

There was no significant difference in soluble carbohydrate content of needles from heated or control plots (Fig. 4.8; Table S4.1), indicating that soluble carbohydrate metabolism did not respond to the elevated temperature treatment. However, cold acclimation during the autumn was associated with major changes in the carbohydrates assessed in our study. In August, total soluble sugars were mainly comprised of fructose and sucrose (Fig. 4.8). During August, we also observed the presence of moderate amounts of pinitol (2% of leaf dry weight), an osmoprotectant with cryoprotective characteristics (Angelcheva et al. 2014); these levels concur with levels observed during the growing season in *P. sylvestris* (Ericsson 1979). The majority of changes in soluble sugar levels occurred between October and December. When nighttime temperatures decreased to below 10°C (Fig. 4.1B), seedlings began to adjust to low temperature and short photoperiod. Aside from raffinose, which increased by over 20-fold from October to December (Fig. 4.8C), increases in carbohydrate levels remained between the 1-2 fold range (Fig. 4.8D-G), as expected for glucose and pinitol (*P. sitchensis*, Dauwe et al. 2012; *Picea obovata*, Angelcheva et al. 2014). Raffinose is known to accumulate significantly in response to low temperature (Strimbeck et al. 2008, Dauwe et al. 2012, Angelcheva et al. 2014). Raffinose is also associated with the enhancement of freezing tolerance (Strimbeck et al. 2008), and has been shown to increase PSII stability during freeze-thaw cycles in *Arabidopsis thaliana* (Knaupp et al. 2011).

We observed a 30% increase in sucrose from October to December (Fig. 4.7D), which concurs with previously reported levels in other conifer species (Strimbeck et al. 2008, Dauwe et al. 2012) but is much lower than the 5-fold increase previously reported for *P. strobus* (Hinesley et al. 1992). The rather small changes in leaf soluble carbohydrate content observed here may be a consequence of the mild winter in 2012 (Fig. 4.1B). Even so, by December, the amount of total soluble carbohydrates had doubled (Fig. 4.8B), of which 25% were represented by raffinose, which was absent in samples from August and October (Fig. 4.8C). This shift in leaf carbohydrate composition likely improved winter freezing tolerance.
Cold Hardiness

Freezing tolerance is first induced by photoperiod, and is not impaired by elevated autumn temperature

ET seedlings from the heated plots did not exhibit delayed induction of cold hardening in August and September, and in contrast to our hypothesis, freezing tolerance was not impaired in seedlings from the heated plots. Growth at moderately elevated temperature instead appeared to enhance freezing tolerance in ET seedlings in October and November (Fig. 4.9C). This concurs with a previous study on *P. sylvestris*, which revealed that there was no effect of elevated temperature on the induction of cold hardening and freezing tolerance during midwinter (Repo et al. 1996).

In 2013, seedlings were already tolerant to freezing exposure at -10°C in August (Fig. 4.9A), and their freezing tolerance continued to increase in September to -16°C (Fig. 4.9B), which is within the ranges previously reported for freezing tolerance in summer-acclimated conifers (Strimbeck et al. 2008). Four days after the first frost in October (Fig. 4.1B), freezing tolerance further increased to -30°C (Fig. 4.9C), and a significant treatment effect (*P* < 0.05) was observed with enhanced freezing tolerance exhibited by the elevated temperature treatment. By November, following several weeks of exposure to night temperatures below 0°C (Fig. 4.1B), seedlings of both treatments were fully cold hardened, with freezing tolerance below -60°C (Fig. 4.9). We conclude that the initial stages of cold hardening during early autumn are triggered by decreasing photoperiod; similar observations have previously been reported in *Populus tremula x tremuloides* (Welling et al. 2002), *Betula pubescens* (Welling et al. 2004) and *Picea abies* (Rostad et al. 2006). The addition of the low temperature signal in October greatly increased the development of freezing tolerance.

4.6 Conclusions

In contrast to our initial hypotheses, we did not observe a significant delay in the downregulation of photosynthesis or cold hardening when seedlings were exposed to elevated temperature in heated plots, nor did these seedlings exhibit altered carbohydrate metabolism or impaired cold hardiness. Though exposure to +1.5/+3°C in heated plots was insufficient to delay autumn cold acclimation, it was sufficient to decrease photosynthesis during the growing season and enhance
nonphotochemical quenching. Our data further indicate that the downregulation of photosynthetic gas exchange occurs synchronously with the accumulation of photoprotective carotenoids, accumulation of soluble sugars and early stages of cold hardening, but its timing precedes the downregulation of the light reactions and the transition from dynamic NPQ to sustained NPQ. We also observed that the autumn physiology of P. strobus is most sensitive to elevated temperature during the transition starting at the beginning of the downregulation of photosynthesis and during the development of cold hardiness.

Based on our findings it seems unlikely that P. strobus seedlings will be significantly affected by the moderately elevated autumn temperatures used in our experiment. However, we have shown that the sensitivity of P. strobus seedlings to elevated temperature is increased under water-limited or chilling conditions. We have further demonstrated that a +1.5/+3°C increase in elevated temperature will not significantly extend the growing season or adversely affect cold acclimation. Instead it appears that moderate increases in elevated temperature will affect productivity during the growing season, when P. strobus may compromise photosynthetic CO₂ uptake under water-limiting conditions, whereas elevated temperature during autumn does not necessarily increase the carbon uptake period and extend the growing season length in this evergreen conifer.

4.7 Supporting Information

Table S4.1: Summary of two-way ANOVA analysis showing the effects of time, treatment and their interaction on leaf carbohydrate concentrations. P-values in bold indicate statistical significance (α = 0.05).

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Figure S4.1: Effects of infrared heating on air temperature and vapor pressure deficit (VPD). (A) Temperature increment in heated plots, (B) VPD increment in heated plots, and (C) daily precipitation measured during July 17-August 26, 2014 at Koffler Scientific Reserve, King City, Ontario.
4.8 References


Chapter 5
Conclusions and Future Directions

In this thesis, I investigated the impacts of photoperiod, temperature and CO2 levels on autumn cold acclimation in the temperate evergreen conifer, *Pinus strobus*. Specifically, my research characterized the distinct responses of three major processes involved in conifer cold acclimation - photosynthesis, carbohydrate metabolism and the development of freezing tolerance - to changes in photoperiod, temperature, and CO2. The presented results establish that conifers are sensitive to elevated temperature but less sensitive to elevated CO2 during autumn. Under elevated temperature and CO2, the downregulation of photosynthesis may be delayed, while the development of freezing tolerance may be impaired; however, *P. strobus* seedlings growing in southern Ontario are unlikely to experience increased freezing damage during winter. Below, I discuss my findings in context of the three main hypotheses, as well as additional findings that advance our understanding of how conifers may respond to future climate conditions. Throughout this chapter, I also propose avenues of investigation that develop from the presented work.

5.1 Hypothesis 1: The induction and development of cold hardening, which includes downregulation of photosynthesis, accumulation of cryoprotective carbohydrates and development of freezing tolerance, requires both low temperature and short photoperiod.

*Downregulation of photosynthesis is rapidly induced by low temperature, but not short photoperiod.*

In conifers, photosynthesis is downregulated during autumn. This process is characterized by reconfiguration of the photosynthetic apparatus in the thylakoid membrane (Ottander et al. 1995, Ensminger et al. 2004, Crosatti et al. 2013) and accumulation of photoprotective pigments in order to quench excess light energy (Ensminger et al. 2006, Demmig-Adams et al. 2012). Decreasing temperature has often been considered the environmental signal that drives the downregulation of photosynthesis (Öquist and Huner 2003, Ensminger et al. 2006, Ensminger et al. 2012), though a few studies have suggested that photosynthesis may be downregulated in response to short photoperiod in trees (e.g. Busch et al. 2007, Bauerle et al. 2012).
Our findings demonstrated that downregulation of photosynthesis in *P. strobus* is driven by low temperature, not short photoperiod. Under controlled conditions, seedlings shifted from long photoperiod and warm temperature to short photoperiod and low temperature exhibited decreased photosynthetic carbon assimilation within the first 24 hours (Fig. 2.2A, Table 2.3). After three days, the same seedlings exhibited a decrease in photosynthetic quantum yield (Fig. 2.4A) and increased dissipation of excess energy in leaves by increasing both daytime and nighttime levels of zeaxanthin and de-epoxidation state of the xanthophyll cycle (DEPS), indicative of the development of sustained nonphotochemical quenching (NPQs) (Fig. 2.4, 2.6). After five weeks, levels of key PSII proteins, reaction center core protein D1 and light harvesting complex protein Lhcb1, were significantly decreased (Fig. 3.5B,C), indicating that protein composition in the thylakoid membrane was altered to direct light energy away from the photosystems. Concurrently, xanthophyll cycle pigments increased twofold, indicating that seedlings developed a much greater capacity for excess energy dissipation (Fig. 3.3D).

In contrast, *P. strobus* seedlings that were shifted to short photoperiod and warm temperature exhibited no change in photosynthetic carbon assimilation or photosynthetic quantum yield, and also did not accumulate photoprotective pigments or develop NPQs. Previously, a factorial experiment using *Pinus banksiana* showed that seedlings grown at short photoperiod and warm temperature exhibited significantly lower photosynthetic carbon assimilation than seedlings grown under long photoperiod and warm temperature, and concluded that photosynthesis is strongly influenced by photoperiod (Busch et al. 2007). However, Busch et al. reported extremely low levels of carbon assimilation that were only 25-50% of the values previously reported for other conifer species (e.g. *Pinus sylvestris*, Troeng and Linder 1982; *Pinus strobus*, Chang et al. 2015; *Picea abies*, Stinziano et al. 2015). Furthermore, photosynthetic quantum yield did not significantly decrease in response to short photoperiod alone, indicating that downregulation of the light reactions did not occur (Busch et al. 2007). In our experiments, we found no indication that short photoperiod induces downregulation of photosynthesis in *P. strobus* seedlings.

Interestingly, once initiated by exposure to low temperature, the downregulation of photosynthesis and cold hardening progressed in spite of sporadic periods of warmer temperature that occurred during late autumn (Fig. 4.1B, 4.2A, 4.3A, 3.8, 4.9). These findings suggest that exposure to the combination of low temperature and short photoperiod induces a regulated cold
response including the decrease in functionality of the light reactions, accumulation of photoprotective pigments, and accumulation of cryoprotective compounds. In their natural environments, trees experience considerably variable temperatures that require constant adjustment of photosynthesis and carbon metabolism on both diurnal and seasonal scales (Dietze et al. 2014). Thus, seasonal programming of the downregulation of photosynthesis and development of cold hardiness may be a strategy that conifers have evolved in order to avoid risking photo-oxidative stress during unpredictable autumn conditions.

Changes in starch and sucrose levels are induced by short photoperiod, while accumulation of hexose and raffinose is induced by low temperature.

As extensively characterized using herbaceous models, enzyme-mediated carbohydrate metabolism can be strongly inhibited by low temperature (Graham and Patterson 1982, Holaday et al. 2002), but can also be specifically accelerated under low temperature to enhance synthesis of soluble carbohydrates associated with increased freezing tolerance (Strand et al. 1999). In addition, short photoperiod induces cessation of growth in many trees (Downs and Borthwick 1956, Li et al. 2003) followed by translocation of NSCs from leaves to stem and root tissues (Hansen and Beck 1994, Oleksyn et al. 2000, Hoch et al. 2003). Our results show that for P. strobus seedlings, changes in carbohydrate metabolism were mainly driven by decreasing photoperiod rather than temperature. Within the first three days of exposure to short photoperiod, diurnal adjustments of leaf starch and soluble sugar pools changed as seedlings metabolized more carbohydrates over the longer night periods (Fig. 2.1, Table 2.2). After five weeks of short photoperiod and warm temperature, seedlings exhibited a 70% decrease in leaf starch and 30% increase in leaf sucrose (Fig. 3.4A,C) while photosynthesis remained highly active (Fig. 3.1A, 3.2A). Phosphoenolpyruvate carboxylase (PEPC) levels also decreased by 70% in all seedlings exposed to short photoperiod, reflecting a strong decrease in demand for photosynthates entering the citric acid cycle (Fig. 3.5D). Together, these findings indicate a shift of carbon demand from within the leaf to external tissues.

Unlike sucrose and starch, hexose and raffinose accumulated under low temperature, but only transiently increased under short photoperiod (Fig. 2.1, 3.4). In field-grown Picea sitchensis, low temperature during late autumn inhibited glycolysis (Dauwe et al. 2012). However, we observed an increase in dark respiration in P. strobus seedlings exposed to low temperature and short
photoperiod (Fig. 2.3B, 3.1C). This suggests that the accumulation of hexoses under low
temperature could serve alternate functions, possibly to enhance cellular osmotic potential
(Dietze et al. 2014) or to activate sugar signalling pathways (Rolland et al. 2006).

Further studies are needed to characterize carbon translocation in conifers by assessing carbon
sinks throughout the whole plant, rather than focusing primarily on carbon pools within leaf
tissues. Though several studies have demonstrated changes in whole-plant carbon partitioning on
a seasonal scale (e.g. Hansen and Beck 1994, Hoch et al. 2003, Dauwe et al. 2012), we still lack
understanding about how rapidly trees can mobilize NSCs under carbon limiting conditions. In
order to elucidate the mechanics of carbon transport, we recommend coupling analysis of NSC
pools in leaves, stems and roots with changes in the expression and/or activity of key metabolic
enzymes involved in sucrose and starch biosynthesis, e.g. sucrose transporters, sucrose
phosphate synthase and ADP-glucose pyrophosphorylase.

_A 16-kD dehydrin is implicated in the development of cold hardening in P. strobos._

Dehydrins are a highly diverse group of proteins that accumulate in higher plants during
exposure to abiotic stress (Graether and Boddington 2014). Dehydrins are traditionally classified
using three motifs that are highly conserved among known angiosperm dehydrins (Close 1996):
K-segment (EKKGIMDKIKEKLPG), S-segment (LHRS_n), and Y-segment (DEYGNP). The K-
segment is considered common to all dehydrins (Close 1996, Graether and Boddington 2014).
Recently, distinct structural motifs have also been discovered for gymnosperm dehydrins that are
not observed in angiosperms (Perdiguero et al. 2012), and putative dehydrins have also been
identified that lack a complete K-segment (Perdiguero et al. 2014). A number of dehydrins have
been demonstrated to have cryoprotective functions, from species including wheat (Danyluk et
al. 1998), peach (Wisniewski et al. 1999), citrus (Hara et al. 2001), barley (Bravo et al. 2003),
and strawberry (Davik et al. 2013). Additional functions include metal and ROS scavenging,
shown in citrus (Hara et al. 2004, 2005), osmoprotection, shown in maize (Koag et al. 2003), and
salt tolerance, shown using wheat dehydrins expressed in *Arabidopsis* (Brini et al. 2007). To
date, however, functional characterization of dehydrins has only been performed in angiosperms
(Hanin et al. 2011, Graether and Boddington 2014). In conifers, dehydrins are typically
upregulated during autumn (Kjellsen et al. 2013) and are downregulated during spring bud burst (Yakovlev et al. 2008).

We identified a small 16-kD protein containing the conserved K-segment region characteristic to the majority of dehydrins in higher plants (Fig. S3.2). Notably, this 16-kD protein was completely absent under long photoperiod, and was induced upon shift to short photoperiod (Fig. 3.5). Expression of the 16-kD protein was greatly enhanced by low temperature exposure. We also observed two more proteins, 52 kD and 46 kD in size, that reacted to the K-segment specific dehydrin antibody but did not respond to photoperiod, temperature or CO2 level. Expression of the 16-kD protein was highly correlated with photoperiod, minimum daily air temperature, and freezing tolerance in field-grown seedlings (Table 3.2, Fig. 3.8).

We attempted to sequence the 16-kD dehydrin using mass spectrometry on the 16-kD fraction of total leaf protein, and produced a heterogenous mixture of peptides that included fragments of key dehydrin motifs, S- and K-segments. These results indicate that the 16-kD protein is likely a SK$_n$-type dehydrin, similar to the majority of known putative gymnosperm dehydrins (Perdiguero et al. 2012). However, further research efforts are needed to obtain the complete protein sequence. Since genomic data is unavailable for this species, we recommend first purifying the dehydrin protein using SDS-PAGE followed by immunoaffinity chromatography or 2D gel electrophoresis, digesting the dehydrin using multiple enzymes, and finally MS/MS analysis.

**Development of freezing tolerance is induced by both short photoperiod and low temperature.**

Extensive work in *Arabidopsis* and other model species has revealed that cold response is achieved via multiple regulatory pathways that respond to different stimuli. Decreasing photoperiod induces photoreceptor-mediated pathways regulating cold response (Catalá et al. 2011) and growth cessation (Petterle et al. 2013), while low temperature activates pathways that regulate drought and cold response, including development of freezing tolerance (Shinozaki et al. 2003, Catalá et al. 2011).

Our results demonstrated that short photoperiod was sufficient to induce development of freezing tolerance in *P. strobus* seedlings. Cryoprotective compounds, including sucrose, raffinose and
the 16-kD dehydrin, accumulated at low or moderate levels under short photoperiod alone and high levels with the addition of low temperature (Fig. 3.4, 3.5). After five weeks of exposure to short photoperiod alone, seedlings developed freezing tolerance to -25°C, while seedlings acclimated to short photoperiod and chilling temperatures exhibited tolerance to -30°C (Fig. 3.6). In the field, seedlings developed freezing tolerance to -10°C during August when photoperiod was decreasing but temperatures remained warm; by November, after several weeks of night frost exposure, seedlings achieved freezing tolerance of more than -60°C (Fig. 3.7, 4.9). In southern Ontario, where the experiment seedlings originated, minimum winter temperatures have averaged -10°C from 1981 to 2010, with a record minimum of -35°C (Environment Canada, 2016). Our findings indicate that short photoperiod enables sufficient development of freezing tolerance in *P. strobus* to protect against typical winter temperatures. The addition of low temperature enhances freezing tolerance and protects against extreme freezing episodes. In one-year-old *Pinus sylvestris* and *Picea abies* seedlings, short photoperiod induced freezing tolerance of -25°C, while the combination of photoperiod and low temperature induced freezing tolerance of -33°C after six weeks of treatment (Christersson 1978). Together, these findings suggest that evergreen conifers rely upon photoperiod signals to establish high levels of freezing tolerance and provide a strong defense against unpredictable freezing events. However, considerably more investigation is needed to elucidate the molecular pathways that control the downregulation of photosynthesis and cold hardening in evergreen conifers. Exploration of these molecular pathways will be facilitated by the recent availability of conifer genome sequences, including *Pinus taeda* (Neale et al. 2014), *Picea abies* (Nystedt et al. 2013) and *Picea glauca* (Birol et al. 2013).

5.2 Hypothesis 2: Elevated autumn temperature extends the growing season by delaying the downregulation of photosynthesis and associated changes in carbohydrate metabolism, and impairs the development of freezing tolerance.

In a natural environment, response to elevated temperature may be masked by response to large diurnal or seasonal temperature variations.

Over the last half century, rising temperatures have affected the duration of the growing season in forests across the Northern hemisphere (Piao et al. 2007, Menzel et al. 2006). Growth cessation was induced earlier by warming in trees with strong photoperiod sensitivity, e.g. *Betula*

As discussed in the previous section, the downregulation of photosynthesis and development of freezing tolerance are impaired in *P. strobus* seedlings that are exposed to short photoperiod but not low temperature. However, under field conditions, seedlings exposed to +1.5°C above ambient during the day and +3°C above ambient during the night did not delay the downregulation of photosynthesis (Fig. 4.2, 4.3). Seedlings acclimated to elevated temperature also did not exhibit less freezing tolerance than control seedlings (Fig. 4.9). At our field site, daily minimum temperatures suddenly fell by 5-10°C during mid-autumn (Fig. 4.1B). Consequently, seedlings in both control and elevated temperature treatments experienced chilling and freezing exposure at similar time points. These findings indicate that responses to small temperature increments may be masked by responses to larger diurnal and seasonal changes in temperature experienced by field-grown plants during autumn.

Admittedly, the findings presented in Chapter 4 are limited by the low temporal resolution of the study. Historical records from 1980 to 2002 describe a 0.14 day year⁻¹ delay of autumn leaf senescence that correspond with a +0.06°C average increase in annual temperature across the Northern hemisphere (Piao et al. 2007). In the native region for the seedlings used in our experiments (Ontario seed zone 34), the growing season extended by 4.7 days from 1950 to 2003 (McKenney et al. 2009). Such incremental changes in the length of the growing season may have been missed by our monthly measurements. However, significant differences in stomatal conductance, evapotranspiration, water use efficiency and freezing tolerance were detected between treatments during late September and early October (Fig. 4.2, 4.9). These measurements were taken shortly after first chilling exposure, when the downregulation of photosynthesis (Fig. 4.1, 4.2), the accumulation of dehydrins (Fig. 3.5) and the enhancement of freezing tolerance (Fig. 3.8, 4.9) commenced. This early transitional period may be particularly sensitive to elevated temperature. Further studies are recommended to assess the impact of elevated temperature on the activation of cold-response pathways and adjustments in photosynthesis and water relations that occur shortly after first chilling or frost exposure.
5.3 Hypothesis 3: The combination of elevated temperature and CO$_2$ enhances photosynthesis in addition to extending the growing season, but further exacerbates vulnerability to freezing damage.

The combination of elevated temperature and CO$_2$ enhances photosynthesis but does not further increase vulnerability to freezing.

Numerous studies concur that elevated CO$_2$ levels enhance photosynthetic carbon uptake in plants during the growing season (Ceulemans and Mousseau 1994, Ainsworth and Long 2005, Norby et al. 2005, Leakey et al. 2009). However, the reported impact of elevated CO$_2$ on cold hardiness is inconsistent among different evergreen species (Guak et al. 1998, Bigras and Bertrand 2006, Loveys et al. 2006), and the factors underlying its influence on freezing tolerance remain unclear. Elevated CO$_2$ can stimulate vegetative growth (Kirschbaum 2011) instead of growth cessation, but can also stimulate accumulation of nonstructural carbohydrates (Ainsworth and Long 2005), which may act as cryoprotectants.

As hypothesized, the downregulation of photosynthesis was suppressed in seedlings exposed to the combination of elevated temperature and elevated CO$_2$, and photosynthetic carbon assimilation rates were enhanced (Fig. 2.3A, 3.1A). No significant difference in glucose, fructose, raffinose, or pinitol occurred in seedlings grown under elevated temperature and CO$_2$ in comparison with seedlings grown at elevated temperature and ambient CO$_2$, although elevated CO$_2$ slightly enhanced accumulation of leaf sucrose (Fig. 3.4). 16-kD dehydrin expression and freezing tolerance were both slightly but not significantly lower in seedlings grown under elevated CO$_2$ in comparison with ambient CO$_2$ counterparts (Fig. 3.5G). We conclude that elevated CO$_2$ does not exacerbate sensitivity to freezing in *Pinus strobus* seedlings. Further metabolomic and/or transcriptomic studies are required to identify the factors which increase vulnerability to freezing under elevated CO$_2$ in some conifers, e.g. *Pseudotsuga menziesii* (Guak et al. 1998), but not others, e.g. *Picea mariana* (Bertrand and Bigras 2006) or *P. strobus*.

Under long photoperiod, elevated CO$_2$ causes seedlings to exceed carbon sink capacity and increases their susceptibility to photo-oxidative stress.

Seedlings exhibited symptoms of saturated carbon sink capacity when grown at twice ambient CO$_2$ concentrations. Under long photoperiod, seedlings grown at elevated CO$_2$ accumulated
significantly higher leaf starch than seedlings grown at ambient CO₂ (Fig. 2.1B, 3.4A). Seedlings grown at elevated CO₂ also exhibited low PEPC content under both long and short photoperiods, suggesting that the influx of carbon into the citric acid cycle via pyruvate dehydrogenase was sufficiently meeting metabolic demand (Fig. 3.5D; O’Leary et al. 2011). Seedlings grown at ambient CO₂ dynamically adjusted leaf carbon pools from high nighttime hexose-to-sucrose ratio and low sucrose-to-starch ratio to the reverse during the day. In contrast, seedlings grown at elevated CO₂ and long photoperiod contained low hexose but high starch and sucrose during both night and day, and did not dynamically adjust of carbon pools until three days after transfer to short photoperiod, when nighttime starch and sucrose levels began to decrease (Fig. 2.1).

Seedlings grown at elevated CO₂ and long photoperiod also exhibited elevated excitation pressure at PSII (1-qP), indicating an increase in the proportion of light entering versus exiting the photochemical pathway (Fig. 3.2E). Furthermore, these seedlings expressed several characteristic symptoms of photo-oxidative stress response, including decreased leaf chlorophyll and higher leaf carotenoid contents, as well as increased de-epoxidation of the xanthophyll cycle (DEPS) and enhanced daytime accumulation of zeaxanthin (Fig. 2.5A, 2.6B, 2.6G, 3.3). Together, these findings suggest that *Pinus strobus* seedlings grown at elevated CO₂ levels accumulate a surplus of leaf carbon during long days, restricting photosynthetic carbon assimilation and increasing their susceptibility to photo-oxidative stress. Following shift to short photoperiod, 1-qP and DEPS decreased as carbon demand was restored in the leaf.

Several limitations of this experiment indicate pertinent directions for future study. These experiments were performed using seedlings that were acclimated to elevated CO₂ in climate-controlled chambers that provided a constant warm, high light environment with abundant water supply. Under field conditions, temperature and water availability can be highly unpredictable; altering these variables will affect seedling responses to elevated CO₂. We recommend conducting elevated CO₂ experiments under field conditions to determine whether conifer seedlings subjected to fluctuating temperature, light, and water availability are more prone to photo-oxidative stress during the summer when exposed to elevated CO₂.

Furthermore, needles in the presented experiments were developed under ambient CO₂; the mature needles were acclimated for 6 weeks to elevated CO₂ prior to the beginning of each experiment. Our data suggest that the bulk of responses to elevated CO₂ occurs when the plant is
exposed to long photoperiod (observed in Chapters 2 and 3) and that differences between EC and AC plants disappear upon shift to short photoperiod (shown clearly in Chapter 3). However, our results reflect the response of needles developed under ambient, not elevated, CO2. Previous studies that have reported increased NPQ in response to elevated CO2 were similarly performed using mature leaves exposed to elevated CO2 treatments (Roden and Ball 1996, Wang and Kellomäki 1997, Bigras and Bertrand 2006). Therefore, it is possible that the anatomical constraints that limited carbon sink capacity in our studies may not be present in needles developed under elevated CO2. We therefore recommend using a longer acclimation period prior to commencing measurements in order to enable target tissues to develop in the elevated CO2 environment. Comparison of mature trees and seedlings may also reveal whether seedlings are more susceptible to photo-oxidative stress under elevated CO2 due to smaller carbon sink capacity.

5.4 Conclusions

The results presented in this thesis reveal that for *P. strobus*, elevated temperature and elevated CO2 can delay the downregulation of photosynthesis and inhibit accumulation of cryoprotectants. However, the timing of carbohydrate export from leaves during growth cessation and the development of freezing tolerance remains unaffected by elevated temperature and CO2 due to photoperiodic control. *P. strobus* photosynthesizes until exposed to freezing temperatures, which rapidly induce sustained nonphotochemical quenching to dissipate excess light energy and mitigating photo-oxidative stress. Elevated temperature can impair the development of freezing tolerance; however, decreasing photoperiod induces *P. strobus* to develop sufficient freezing tolerance to protect against typical winter temperatures at its native origin. We have also shown that response to a small elevation in temperature may be superseded by response to large diurnal temperature variation in a complex natural environment. Consequently, rising temperature and CO2 may allow *P. strobus* to benefit from increased carbon uptake when photosynthesis is active, but impaired freezing tolerance is not likely to pose a significant threat to its winter survival.

5.5 References


Appendix
Experimental Conditions and Methods

A1 Experimental conditions

Plant materials (Chapters 2-4)

Three-year-old (3+0) bare-rooted Eastern white pine (*Pinus strobus*) seedlings were obtained from a local seed orchard during April of 2012 and 2013 (Ontario seed zone 37, Somerville Seedlings, Everett, Ontario, Canada).

For growth chamber experiments (Chapters 2 and 3), seedlings were planted in deep conifer pots with a volume of 3 L, filled with a mixture of sand and sphagnum peat moss (1:2 v/v) and fertilized with 28:10:10 (N:P:K) Water Soluble Evergreen & Acid Loving Plant Food (Miracle-Gro, Scotts, Maryville, OH, USA). Potted seedlings were then kept outside in an experimental garden and transferred to growth chambers (Biochambers, Winnipeg, Canada) in early June of 2012 and 2013.

For the field experiment (Chapter 4), seedlings were planted in May of 2012 in prepared plots which were filled to a depth of 30 cm with a mixture of one-third peat, one-third sand and one-third local soil. Ninety seedlings were evenly distributed in each plot.

Growth chamber environment (Chapters 2 and 3)

The growth chambers used in Chapters 2 and 3 were Biochambers models GC20-BDAF (1 chamber) and GC20-BDAFLT (2 chambers). The BDAFLT chamber model was designed for freezing temperature experiments without interruption for the defrost cycle, with a temperature range from -10°C to +40°C irrespective of light status; the BDAF model operated with a temperature range from +5°C to +40°C with lights off and +10°C to +40°C with lights on. All chambers were equipped with evenly distributed 400W high pressure sodium and 400W metal halide lamps which were enclosed in a light bank compartment and separately cooled at a constant set temperature of 20°C. Temperature was maintained within ±0.25°C of the set temperature, with brief temperature spikes of approximately 1°C when the door was opened for equipment setup and measurements; CO₂ was maintained within ±50 ppm of the setpoint during
the experiment (Fig A1.1). Light intensity was regulated by a light sensor that was adjusted to the height of the top of the canopy in the chamber. Light distribution across the chamber varied by approximately ±50 µmol m⁻² s⁻¹ quanta. Needle temperature was maintained within ±0.2°C of the ambient chamber temperature (Table A1.1).

![Figure A1.1: Temperature and CO₂ changes during experiment.](image)

Table A1.1: Ambient and canopy leaf temperature within BDAFLT chamber.
Chamber temperature was registered by the chamber temperature sensor. \( T_{\text{amb}} \) and \( T_{\text{leaf}} \) were measured using the Junior-PAM monitoring Junior-BD leaf clip, which measured temperature using a Ni-CrNi thermocouple. Measurements on three seedlings were performed for each temperature.

<table>
<thead>
<tr>
<th>Set temp. (°C)</th>
<th>Chamber temp. (°C)</th>
<th>( T_{\text{amb}} ) (°C)</th>
<th>( T_{\text{leaf}} ) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>22.1 ± 0.1</td>
<td>22.9 ± 0.3</td>
<td>23.1 ± 0.3</td>
</tr>
<tr>
<td>15</td>
<td>15.2 ± 0.1</td>
<td>15.1 ± 0.1</td>
<td>15.1 ± 0.1</td>
</tr>
<tr>
<td>12</td>
<td>12.3 ± 0.1</td>
<td>12.2 ± 0.1</td>
<td>12.2 ± 0.2</td>
</tr>
<tr>
<td>5</td>
<td>5.2 ± 0.1</td>
<td>5.1 ± 0.1</td>
<td>5.0 ± 0.1</td>
</tr>
</tbody>
</table>
Growth chamber settings during experiments (Chapters 2 and 3)

Seedlings were acclimated to long day (LD, 14 h day length) conditions at either ambient (400 ppm) or elevated (800 ppm) CO₂. After 6 weeks, seedlings were shifted to short day (SD, 8 h day length) treatments as follows: seedlings grown at ambient CO₂ were shifted either low temperature/ambient CO₂ (12°C day/5°C night, 400 ppm CO₂) or high temperature/ambient CO₂ (22°C day/15°C night, 400 ppm CO₂) conditions, while seedlings grown at elevated CO₂ were shifted to high temperature/elevated CO₂ (22°C day/15°C night, 800 ppm CO₂) conditions. Photosynthetic active radiation at the top of the canopy was maintained at 1400 µmol quanta m⁻² s⁻¹ during the day and at 400 µmol quanta m⁻² s⁻¹ during the first and last half hour of each day. Light was provided using an equal combination of metal halide and high pressure sodium lamps. Humidity was set to 60% RH. Treatments were rotated between chambers during experimental replicates.

T-FACE array settings (Chapter 4)

A Temperature Free-Air-Controlled Enhancement (T-FACE) system was set up at the Koffler Scientific Reserve of the University of Toronto, located near King City, Ontario (44°050’N, 79°483’W) according to Kimball et al. (2008), consisting of 10 experimental plots, each with a diameter of 3 m. Ambient canopy temperature (AT) was recorded using infrared sensors (Model IRT-P5, Apogee Instruments, Logan, UT, USA) in five unheated control plots. For the elevated temperature (ET) treatment, five plots were arranged with six 1000 W infrared heaters (Mor Electric Heating Association, Comstock Park, MI, USA) per plot in a hexagonal array, where leaf temperature was raised by +1.5°C during the day and +3°C during the night, according to Kimball et al. (2008), and again the temperature was recorded using infrared sensors. The sensor outputs were periodically checked over the course of the field experiment and adjusted to focus on seedlings at the center of the plot. Maintenance was also performed as required to clear the sensor aperture of insect nests, e.g. wasps and spiders.

Ambient air and canopy temperatures were recorded using a CR1000 datalogger (Campbell Scientific Inc., Edmonton, AB, Canada). Precipitation data were obtained from the Buttonville Airport weather station in Newmarket, ON (Environment Canada, 2014), located 25 km from the field site.
Vapor pressure deficit (VPD), as a consequence of infrared heating, may increase the loss of water from needles (Kimball et al. 2005). We measured 0-0.6 kPa VPD in our field experiment (Fig. S4.1), and no change in needle water status according to leaf water potential measurements (Fig. 4.4); however, trends in stomatal conductance (gs; Fig. 4.2B) and evapotranspiration (E; Fig. 4.2D) differed during August, September and October measurements. Since stomatal conductance is calculated as E/VPD, the measurements indicate that VPD did vary during the experiment. Future experiments are recommended to account for effects of water loss; Kimball et al. (2005) suggest using drip irrigation.

**A2 Photosynthesis protocols**

**Gas exchange using the Walz GFS-3000 (Chapters 2 and 3)**

GFS-3000 settings were as follows:
- Flow: 750 µmol min⁻¹
- CO₂: 400 ppm
- H₂O: 15000 ppm or 5000 ppm, according to treatment measured (equivalent to ~60% RH at 22 or 12°C, respectively)
- Tcuv: 22 or 12°C, according to treatment measured
- Impeller: 7
- PARtop: 0 or 1400 µmol m⁻² s⁻¹, according to measurement taken

Gas exchange was measured at days 0, 1 and 4 inside the growth chambers on a single, flat layer of attached, current-year needles after ≥2 h of exposure to growth light using a GFS-3000 (Walz, Effeltrich, Germany). Dark respiration (Rd) was measured after 40 minutes of dark adaptation. Net photosynthetic carbon assimilation (Anet) was subsequently measured at growth light intensity (1400 µmol quanta m⁻² s⁻¹) once steady state assimilation was achieved.

For Chapter 3, A/Cᵢ curves were measured on days 0 and 36. Assimilation was assessed after 2-3 minutes of exposure to CO₂ levels of 400, 300, 250, 200, 150, 100, 50; 400, 550, 650, 800 and 1000 ppm CO₂, based on a protocol by Long and Bernacchi (2003). Measurements were taken at 25°C and 1400 µmol quanta m⁻² s⁻¹. The initial slope of the A/Cᵢ curve, which represents the Rubisco-limited rate of carboxylation (Wₖ), was used to calculate the maximum substrate-saturated rate of Rubisco carboxylation (Vₖₘₐₓ) at 25°C using the following equation:
\[ W_c = \frac{V_{cmax}(C_i)}{C_i + K_c(1 + \frac{O}{K_o})} \]

where, \( C_i \) represents the intracellular partial pressure of CO2, \( O \) represents the partial pressure of oxygen at 25°C, and \( K_c \) and \( K_o \) represent the Michaelis-Menten constants of Rubisco for the competing carboxylation and oxygenation reactions, respectively (Farquhar et al. 1980, Sage 1990).

Simultaneous gas exchange and chlorophyll fluorescence using the Li-Cor 6400XT equipped with Leaf Chamber Fluorometer (Chapter 4)

6400XT+LCF settings were as follows:
- Flow: 400 µmol s\(^{-1}\)
- Reference CO2: 400 ppm
- \( T_{cuv} \): 30-year historical average daily temperature of measurement date, ranging from 0 to 26°C
- Humidity: ~60%
- PARtop: 0 or 1200 µmol m\(^{-2}\) s\(^{-1}\), according to measurement taken
- Blue portion: 10%
- Intensity: 0.1
- Rate: 0.25 kHz
- Filter: 5 Hz
- Gain: 10

Gas exchange and chlorophyll fluorescence measurements were performed simultaneously using a portable photosynthesis system (LI-6400 XT; Li-Cor Biosciences, Lincoln, NE, USA) with attached leaf chamber fluorometer (6400-40). Topmost, south-facing needles of the primary shoot were arranged in a flat single-needle layer and placed into the cuvette. The cuvette was set to maintain a level of 400 ppm CO2 and ambient temperature.

Dark-adapted minimum PSII fluorescence (\( F_o \)), and dark-adapted maximum PSII fluorescence (\( F_m \)) were determined after 40 minutes of dark adaptation. Subsequently, plants were exposed to 1200 µmol quanta m\(^{-2}\) s\(^{-1}\) for 7-12 minutes to obtain measurements of steady-state photosynthesis. Measured parameters included photosynthetic CO2 assimilation (\( A \)), stomatal conductance (\( g_s \)), evapotranspiration (\( E \)), light-adapted minimum PSII fluorescence (\( F_{o'} \)), light-adapted maximum fluorescence (\( F_{m'} \)), and transient fluorescence (\( F_t \)), which were used to calculate the following gas exchange and fluorescence parameters:
Intrinsic water use efficiency was calculated as IWUE = A/gs (Silva and Horwath 2013). Maximum quantum yield of PSII was calculated as $F_v/F_m = (F_m - F_o)/F_m$, and effective quantum yield of PSII was calculated as $\Phi_{PSII} = (F_m' - F_t)/F_m'$ (Genty et al. 1989). The excitation pressure at PSII was calculated as $1 - qP = 1 - (F_m' - F_t)/(F_m' - F_o')$ (Maxwell and Johnson 2000). Nonphotochemical quenching was calculated as $NPQ = (F_{mrec}/F_m') - 1$ (Bilger and Björkman 1990). Sustained nonphotochemical quenching was calculated as $NPQS = (F_{mrec}/F_m) - 1$ (Ensminger et al. 2004, Maxwell and Johnson 2000, Porcar-Castell 2011). For the calculation of non-photochemical quenching (NPQ) and sustained non-photochemical quenching (NPQS), fully recovered $F_m$ ($F_{mrec}$) was estimated as $F_o \times 5$, according to Schreiber et al. (1995) and Ensminger et al. (2004). This estimation is based on two assumptions: firstly, the ratio of fully recovered $F_m/F_o$ is equal to 5, which has been demonstrated in multiple plant species (Björkman and Demmig 1987), including conifers (Adams and Demmig-Adams 1994); and secondly, unlike $F_m$, $F_o$ shows little seasonal variation (Ottander et al. 1995). However, this approach might occasionally underestimate NPQ when $F_o$ is strongly decreased.

Each measurement took approximately 15 minutes and were taken from 2 hours after dawn until 2 hours prior to sunset. Prior to each measurement, the gas exchange system was started to allow the infrared gas analyzer to warm up for 30-60 minutes. Prior to measurements, analyzer readings were assessed with an empty cuvette to ensure that the baseline was zero. During initial measurement, needle area was arbitrarily set to 1 cm². All measurements were performed on attached needles that were arranged into a flat surface and secured using breathable tape prior to insertion in the cuvette. After the cuvette was closed, a puff of air was applied by mouth on the cuvette seal to test for leaks, the presence of which would be indicated by a spike in CO₂.

All measurements were performed on attached needles from the topmost branch. The needles were arranged in a flat, single-needle layer and secured with breathable tape before being inserted into the cuvette. Following measurement, the measured region of needles within the cuvette was harvested and scanned to estimate the light-exposed needle surface area, using WinSeedle software (Regent Instruments Inc., Québec, QC, Canada). The correct surface area was then used to recalculate the original measurements using the GFS-Win software (Walz, Effeltrich, Germany).
Chlorophyll fluorescence using the Walz Dual-PAM-100 (Chapters 2-4)

Dual-PAM-100 settings were as follows:
- Fluo: Gain High, Damping Low, ML 12
- Red light intensity: 20
- Far-red intensity: 10
- Blue light intensity: 8
- Sat Pulse: Intensity 10, Width 400 ms

Chlorophyll fluorescence was measured on attached, current-year needles using the Dual-PAM-100 (Walz, Effeltrich, Germany). During night measurements, minimum PSII fluorescence ($F_o$) and maximum PSII fluorescence ($F_m$) were measured. During day measurements, $F_o$ and $F_m$ were measured during the day after 40 min of dark adaptation in the leaf clip, followed by assessment of light-adapted minimum PSII fluorescence ($F'_o$), light-adapted maximum PSII fluorescence ($F'_m$), and transient fluorescence ($F_t$) after exposure to 1400 µmol quanta m$^{-2}$ s$^{-1}$ of actinic light for 3-5 minutes. $F_v/F_m$, $\Delta F/F_m'$ ($\Phi_{PSII}$), 1-qP, NPQ and NPQs were calculated as described above.

A3 Water status protocols

Shoot water potential using the PMS Model 1505D Pressure Chamber Instrument (Chapter 4)

Water potential measurements were performed from August to October, 2013. Pre-dawn and midday (noon) water potential ($\Psi_w$) were assessed on individual current-year needles using a Model 1505D Pressure Chamber Instrument (PMS Instrument Company, Albany, OR, USA). At each time point, measurements were taken from three needles per seedling on three seedlings per plot, five plots per treatment. During November and January water potential was not assessed because the system did not operate at sub-freezing temperatures.

Soil moisture content using the Hydrosense™ moisture probe (Chapter 4)

Soil moisture was measured using a HydroSense™ soil water content sensor (Campbell Scientific Inc., Edmonton, AB, Canada). Soil moisture, measured as percent volumetric water content, was assessed at a depth of 15 cm at three points, 10 cm from the base of each seedling.
A4 Photosynthetic pigment protocols

Recipes

Pigment extraction buffer (0.01M ammonium acetate in methanol)
2 ml 0.5 M ammonium acetate, pH 7.1
98 ml methanol

Photosynthetic pigment extraction and HPLC analysis (Chapters 2-4)

Pigments were extracted according to Junker and Ensminger (2016). Roughly 50-60 mg homogenized frozen needle tissue was extracted at 4°C in 98% methanol buffered with 2% 0.5 M ammonium acetate for 2 h in the dark. The extract was centrifuged at 4°C at 14,000 rpm for 5 min and the supernatant collected. The pellet then was washed twice with 100% methanol at 4°C followed by centrifugation. Finally, the total supernatant was filtered using 0.2 µm pore PTFE syringe filters (Thermo Scientific, Rockwood, TN, USA). Photosynthetic pigments were separated using a reverse-phase C30 column (5 µm, 250×4.6mm; YMC Co., Ltd., Kyoto, Japan) and analyzed with an Infinity 1260 series high performance liquid chromatography (HPLC) system equipped with a UV-diode array detector (Agilent Technologies, Santa Clara, USA). Pigments were eluted using a gradient created using three solvents: A) 100% methanol, B) water buffered with 0.2% ammonium acetate, and C) tert-butyl methyl ether at a flow rate of 1 ml min⁻¹ with a column temperature of 25°C. The initial solvent concentration was 92% A, 5% B and 3% C. During each run, solvent A was gradually replaced by solvent B to a minimum of 5% A, while C was kept constant. Every run was followed by a 5 min reconditioning phase with initial solvent concentrations. Wavelengths were scanned from 250 nm to 680 nm. Chromatograms at 290 nm, 450 nm and 656 nm were analyzed using Chemstation software (Agilent Technologies). Peak quantification was performed via Chemstation software, using standards for chlorophyll a and chlorophyll b from Sigma Aldrich (St. Louis, MO, USA), and antheraxanthin, α-carotene, β-carotene, lutein, neoxanthin, violaxanthin and zeaxanthin from DHI Lab products (Hørsholm, Denmark).

A5 Carbohydrate protocols

Recipes

Galactitol stock (5 mg/ml)
50 mg in 10 ml H₂O
Methanol:chloroform:water (12:5:3 v/v)
600 ml methanol
250 ml chloroform
150 ml water

2% H$_2$SO$_4$ (v/v)
2 ml H$_2$SO$_4$ per 100 ml H$_2$O

Fucose stock (1 mg/ml)
10 mg in 10 ml H$_2$O

Analysis of soluble sugars (Chapters 2-4)

Leaf soluble carbohydrates were extracted according to Park et al. (2009). A total of 250 µg galactitol internal standard was added to 30-40 mg homogenized, lyophilized needle tissue. The mixture was incubated in 4 ml of extraction buffer (methanol:chloroform:water, 12:5:3 v/v), overnight at 4°C. The mixture was centrifuged at 6,000 rpm for 10 min and the supernatant collected. The pellet was washed twice in extraction buffer followed by centrifugation. A total of 5 ml of water was added to the total supernatant, mixed, and centrifuged at 4000 rpm for 4 min. Lastly, the upper aqueous phase was collected.

An aliquot of 2 ml of the soluble carbohydrate extract were dried using a vacuum centrifuge and re-suspended in 1 ml of nanopure water. The re-suspended extract was filtered through a 0.45 µm pore nylon syringe filter (Chromatographic Specialties Inc., Brockville, ON, Canada) and analyzed using an ICS-5000 anion-exchange HPIC system (Dionex, Sunnyvale, CA, USA) equipped with an electrochemical pulse amperometric detector (EC-PAD). Glucose, sucrose, fructose and pinitol were eluted using a Hi-Plex Ca column (Agilent Technologies, Santa Clara, CA, USA) with water at a flow rate of 0.170 ml min$^{-1}$ with a column temperature of 70°C. Raffinose was eluted using a Carbo-Pac PA1 column (Dionex, Sunnyvale, CA, USA) with 150 mM NaOH (isocratic) at a flow rate of 1 ml min$^{-1}$. For all soluble carbohydrates, post-column detection was performed using NaOH at a rate of 100 mM min$^{-1}$.

Analysis of starch (Chapters 2-4)

Leaf starch was determined using the residual tissue pellet from the soluble sugar extraction according to Park et al. (2009). The pellet was dried overnight at 55°C. Roughly 20-30 mg of
pellet was re-suspended in 5 ml of 2% H$_2$SO$_4$ and autoclaved for 5 min at 120°C. Once cooled, the extract was spun at 500 rpm for 5 min and the supernatant was collected, filtered using a 0.45 µm nylon filter and analyzed using a DX-600 anion-exchange IC/HPLC system (Dionex, Sunnyvale, CA, USA) equipped with a Carbo-Pac PA1 column (Dionex, Sunnyvale, CA, USA) and EC-PAD. The extract was eluted with water at a flow rate of 1 ml min$^{-1}$ with a column temperature of 30°C. Post-column detection was performed using NaOH at a rate of 100 mM min$^{-1}$. Peak detection and quantification of all nonstructural carbohydrates were performed using PeakNet software (Dionex).

A6 Protein protocols

Recipes

Protein extraction buffer
6 ml 1M Tris-HCl, pH 6.8
20 ml 10% SDS
15 g sucrose
4 ml 0.5M DTT
Add H$_2$O to 100 ml
Add one tablet of Complete, EDTA-free per 50 ml extraction buffer

1x MES-SDS running buffer
50 ml 20x NuPAGE MES-SDS running buffer
950 ml H$_2$O

Transfer buffer
100 ml 10x Tris-glycine buffer
200 ml methanol
700 ml H$_2$O

10x stock TBS washing buffer
200 ml 1M Tris-HCl pH 7.5
300 ml 5M NaCl
50 ml 10% (v/v) Tween 20
Add H$_2$O to 1L

Blocking solution (5% w/v non-fat milk)
2.5 g non-fat milk powder
50 ml 1x washing buffer

Antibody solution (2% w/v non-fat milk)
1 g non-fat milk powder
50 ml 1x washing buffer
Add antibody at recommended concentration
Coomassie stain  
5 g Coomassie Brilliant Blue G  
500 ml methanol  
400 ml H$_2$O  
100 ml acetic acid

Coomassie destain  
500 ml methanol  
400 ml H$_2$O  
100 ml acetic acid

Gel storage buffer (1% v/v acetic acid)  
1 ml acetic acid  
99 ml H$_2$O

Protein extraction, SDS-PAGE, and Western blot (Chapter 3)

Proteins were extracted from 50-60 mg homogenized frozen needle tissue obtained from chamber-grown and field-grown seedlings according to Busch et al. (2007). The extraction buffer consisted of 60 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 15% (w/v) sucrose, 20 mM dithiothreitol, and Complete EDTA-free proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentration was assessed using the RC/DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Precisely 5 µg total protein per lane was loaded on 10% NuPage Novex Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and separated at 120V for 90 min at room temperature using the XCell Mini gel system. Following separation, proteins were transferred to a PVDF membrane (0.2 µm pore size, Bio-Rad) using the Xcell II Blot Module (Invitrogen) for 1 h at 30V on ice. The membrane was blocked using 5% nonfat milk in TBS for 45 minutes at room temperature (RT), then probed with antibodies against PsbA (1:5000, AS05084), Lhcb1 (1:5000, AS09522), RbcL (1:5000, AS01017), PEPC (1:1000, A209458), or Dhn (1:1000, AS07206). The Dhn antibody was raised against the K-segment peptide sequence from the dehydrin C-terminal, EKKGIMDKIKEKLPG. Goat anti-rabbit (AS09602) or chicken anti-rabbit (AS10833) IgG conjugated with horseradish peroxidase (1:75,000) were used as secondary antibodies for chemiluminescent protein detection using the Amersham ECL Prime kit (GE Healthcare, Buckinghamshire, UK) and a ChemiDoc MP (Bio-Rad) for visualization. All antibodies were obtained from Agrisera (Vännäs, Sweden). Optical band density was quantified using Image Lab software (Bio-Rad).
Sequencing of 16-kD dehydrin using mass spectrometry (Chapter 3)

Precisely 40 µg of total protein from a sample containing high levels of the 16-kD dehydrin were loaded on a 12% Bolt Bis-Tris gel (Invitrogen) and separated at 100 V for 2 h 45 min at room temperature using the Bolt Mini Gel Tank (Invitrogen). Presence of dehydrins in the 16-kD band was confirmed on a replicate gel using Western blot. Following separation, the gel was stained using Coomassie Blue for 1 h, then destained overnight. The 16-kD band was subsequently excised and immersed in 1% acetic acid prior to analysis. Trypsin digest of the sample, followed by LC-MS/MS analysis, was performed at the SPARC BioCentre mass spectrometry facility of the Hospital for Sick Children (Toronto, ON, Canada). Peptide sequences were predicted by protein BLAST against nr and Uniprot databases using PEAKS Studio v7.5 (Bioinformatics Solutions Inc., Ma et al. 2003). Peptides containing fragments of the dehydrin K-segment were aligned using MUSCLE (www.ebi.ac.uk/Tools/msa/muscle/, Edgar 2004) and manually reviewed. The aligned peptides were then used to generate a consensus K-segment peptide using Jalview v2.9.0b2 (www.jalview.org/, Waterhouse et al. 2009).

A7 Freezing tolerance protocol

Chlorophyll fluorescence using the Walz Dual-PAM-100 (Chapters 3 and 4)

Determination of the percentage of electrolyte leakage has been established as a method of assessing chilling and freezing tolerance in plants (Burr et al. 2001). Freezing exposure also damages thylakoid membranes and inhibits the turnover rate of PSII reaction core protein D1, therefore impairing the light reactions. An alternate, non-invasive method uses chlorophyll fluorescence to assess freezing damage to the chloroplast (Burr et al. 2001, Thalhammer et al. 2014). The chlorophyll fluorescence method assumes that freezing-tolerant leaf tissues are able to fully resume photosynthesis upon recovery at room temperature following freezing exposure, and assesses the functionality of PSII.

Dual-PAM-100 settings were as follows:

- Fluo: Gain High, Damping High, ML 12
- Red light intensity: 20
- Far-red intensity: 10
- Blue light intensity: 8
- Sat Pulse: Intensity 10, Width 400 ms
Chlorophyll fluorescence was used to assess freezing tolerance using a modified protocol after Sutinen et al. (1992). Sections of current-year shoots were cut, dark-adapted for 40 minutes, and Fv/Fm was measured. Shoots were then individually wrapped in moist paper towel and aluminum foil and sealed in plastic bags. Shoots were exposed to a range of freezing temperatures at 5°C intervals from 0 to -40°C using a Thermotron SM-16-8200 environmental test chamber (Thermotron Industries, Holland, MI, USA). The initial decrease from 0 to -1°C was achieved over 1 hour, followed by a maximum cooling rate of 5°C h⁻¹ to reach target temperature. Each target temperature was held for 6-12 h. At the end of each interval, shoot sections were transferred to a 4°C refrigerator for 24 h, followed by transfer to RT, where the shoots were allowed to recover for 24 h. Following the 24 h recovery period, shoots were unwrapped and exposed to 1 h light exposure at 800 µmol quanta m⁻² s⁻¹ in order to stimulate PSII, then dark-adapted for 40 minutes (Burr et al. 2001). Fv/Fm was then recorded. Since we used chlorophyll fluorescence to evaluate freezing injury at PSII, we defined LT50 as the temperature required to reduce maximum Fv/Fm by 50%. Maximum Fv/Fm was assessed by subjecting non-frozen shoots to the same protocol of 24 h recovery period, 1h of light exposure, 40 minutes of dark adaptation and measurement.

The temperature at which 50% of seedlings were damaged by freezing (LT50) was estimated as a 50% reduction in the post-recovery Fv/Fm, which was used as a proxy to indicate damage to the photosynthetic apparatus. LT50 values were calculated by fitting Fv/Fm values measured from freezing-recovered shoots using a modified Richards curve model (von Fircks and Verwijst 1993):

\[ f(x) = \frac{K}{1 + e^{-B(x-M)}} \]

where K represents the upper asymptote, or pre-freezing Fv/Fm; B represents the maximum slope at LT50 and M represents LT50. Data was tested for normality using the D’Agostino-Pearson omnibus normality test. The curve for each treatment (elevated vs. ambient temperature) was fitted using the least squares method. LT50 values were compared between treatments using an extra sum-of-squares F test with a P-value cutoff of 0.05. Analysis was performed using Graphpad Prism v6.04 (Graphpad Software, Inc., La Jolla, CA, USA).
A8 Statistics methods

Analysis of variance (Chapters 2-4)

For data presented in Chapter 2, the effects of treatment and time on nonstructural carbohydrates, gas exchange, chlorophyll fluorescence, and photosynthetic pigments were assessed using two-way ANOVA. Statistics were performed in R v3.1.1 (http://www.r-project.org/) using the lmer function of the lme4 package (Bates et al. 2014). The ANOVA model used was: Variable ~ Treatment * Time + (1|Trial) + (1|Individual), where treatment and time represented categorical fixed factors, and trial (2 experiment replicates) and individual (5 biological replicates) represented random factors. Tukey’s HSD post-hoc tests were used to contrast 1) between treatments at each time point, 2) between night and day for each experiment day, and 3) between days of the experiment. The Tukey contrasts were performed in R using the glht function of the multcomp package (Hothorn et al. 2008).

For data presented in Chapter 3, the effects of treatment and time on photosynthesis, pigment content, nonstructural carbohydrates and freezing tolerance were assessed by two-way ANOVA, using the lmer function of the lme4 package in R v3.1.1. The ANOVA model used was: Variable ~ Treatment * Time + (1|Trial) + (1|Individual), where treatment and time represented categorical fixed factors, and trial (2 experiment replicates) and individual (5 biological replicates) represented random factors. Tukey’s HSD post-hoc tests were used to contrast between treatments at each time point, and were performed in R using the glht function of the multcomp package. The effects of treatment on protein content were assessed by one-way ANOVA, using GraphPad Prism v6.04 (GraphPad Software, Inc., La Jolla, CA, USA).

For data in presented in Chapter 4, the effects of treatment and time on starch and soluble sugars were analyzed using two-way ANOVA. Tukey’s HSD post-hoc tests were used to identify significantly different groups. The statistical analyses for sugars were performed using GraphPad Prism v6.04.

Analysis of covariance (Chapter 4)

For data presented in Chapter 4, two-way ANCOVA was used to assess the effect of the elevated temperature treatment and time on gas exchange, fluorescence and photosynthetic pigments,
while accounting for the effect of seasonal variation introduced by photoperiod and daily temperature. The ANCOVA model used was: Variable ~ Treatment * DOY + Photoperiod + T_min + (1|Plot) + (1|Year), where treatment and day of year (DOY) represented categorical fixed factors, photoperiod and minimum temperature (T_min) represented continuous numeric covariates, and plot (5 replicates) and year (2 replicates) represented random factors. Analysis was performed using the \textit{lme4} package in R v3.1.1. Multiple comparisons were used to contrast treatments within each time point, and were performed using the \textit{multcomp} package in R v3.1.1. \(P\)-values for multiple comparisons were adjusted using Bonferroni correction.

\textbf{Nonlinear regression curve fitting (Chapters 3 and 4)}

For data presented in Chapter 3, the strength of the relationships among Dhn-16, LT\textsubscript{50}, photoperiod and minimum air temperature were evaluated by linear regression of log-transformed values, and the slope was considered significantly different from zero when \(p < 0.05\). \(R^2\) values were calculated from nonlinear regression of the data using the exponential growth equation \(Y = Y_0 e^{kX}\), where \(k\) is the rate constant. Regressions were performed using GraphPad Prism v6.04.

For data presented in Chapter 4, treatment responses of A, \(F_v/F_m\), and NPQ\(s\) from both years were pooled, independently plotted against minimum daily temperature and photoperiod, and fitted using the least squares method with a 4-parametric sigmoidal curve function:

\[ f(x) = A + \frac{K - A}{1 + e^{-B(x - M)}} \]

where \(K\) represents the maximal parameter value; \(A\) represents the minimal parameter value; \(B\) represents the maximum slope and \(M\) represents the midpoint of the curve at which estimated values represent 50\% of the maximum value of the parameter. \(R^2\) and 95\% confidence intervals were calculated. Midpoints were compared between treatments using a sum-of-squares F test. Modelling and analyses of the sigmoid curves were performed using Graphpad Prism v6.04.1.
Generalized linear mixed modelling (Chapter 3)

Linear mixed models were used to estimate the best predictor for seasonal changes in 16-kD levels and freezing tolerance assessed in field-grown seedlings using the lmer function of the lme4 package (Bates et al. 2014) in R v3.1.1. Plot was included as a random factor in all models. Models were scored using the Akaike information criterion (AIC) to evaluate goodness-of-fit. ΔAIC was calculated by subtracting the AIC score of each predictor from that of the null model. Significance of predictors was calculated by comparing the null model with the predictor using ANOVA.

A9 References

Copyright Acknowledgements
