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Epigenetic effects of temperature during embryogenesis on climatic adaptation traits in Norway spruce (*Picea abies*) – studies of cold hardiness, gene expression and epigenetic marks

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Abstract

Norway spruce (*Picea abies*) is known to have an epigenetic memory of temperature during embryogenesis, which affects phenological traits in the trees more than 20 years later. This memory was first shown in trees from zygotic embryos (seeds) developed under different temperatures, and it was confirmed to be caused by epigenetics when the same memory effect was seen in genetically identical epitype trees grown from somatic embryos that had been developing under 18 and 28 °C. These studies have shown that low temperature during embryogenesis advances bud set and cold acclimation in autumn and bud burst and deacclimation in spring, compared to higher temperature. The cold epitype (CE) has been shown to have lower dehydrin expression than the warm epitype (WE) when close to bud burst. However, cold hardiness and deacclimation have only been studied in the trees from the zygotic embryogenesis, and not in the genetically identical epitypes. Also, the knowledge about expression of epigenetically related genes in the epitypes is limited, and the distribution of epigenetic marks has not been studied.

In this thesis, the aims were to investigate these issues in the two epitypes. Freeze tests were performed on twigs collected at four timepoints: March, April, May 2018 and March 2019. As expected, freezing tolerance decreased during spring, and consistent with its later bud burst, for all timepoints WE was significantly more frost tolerant than CE in at least one of the three examined tissues (needles, cambium and buds).

Expression of the cold-hardiness related genes *DEHYDRIN 6* (*PaDHN6*) and *DEHYDRIN 40* (*PaDHN40*), the bud-dormancy related gene *FLOWERING LOCUS T-TERMINAL FLOWER 1-LIKE 2* (*PaFTL2*) and the epigenetically-related genes *DNA (CYTOSINE-5)-METHYLTRANSFERASE CMT3 ISOFORM* (*PaCMT3*)*, HISTONE DEACETYLASE HDT1* (*PaHDT1*) and *HISTONE DEACETYLASE HDT2* (*PaHDT2*) in buds collected in March and May was analyzed using RT-qPCR. As expected, the expression of the *PaDHN*s and *PaFTL2* decreased from March to May, while *PaCMT3* expression increased. CE had a higher expression of both the *PaDHN*s than WE in March, which is surprising, since *DHN*s are associated with frost tolerance and WE was shown to be more frost tolerant than CE for this timepoint. The expression of the *PaHDT*s did not differ significantly between the epitypes or collection dates.

The distribution of epigenetic marks in buds was studied by immunolocalization of methylated cytosine (5-mdC) and acetylated histone 4 (AcH4). In March, some of the WE buds seemed to be more methylated in the procambium and shoot apical meristem than in CE and both epitypes in May. However, the distribution of 5-mdC varied quite much among individuals. The AcH4 was evenly distributed in all buds, regardless of epitype or collection date. Thus, such crude immunolocalizations in buds appear unable to detect differences in distribution of epigenetic marks between the two epitypes.

In conclusion, low temperature during somatic embryogenesis results in more rapid dehardening during the spring in the resulting trees than higher temperature does, as tested more than 10 years after planting in a common garden. Highest expression of *PaDHN*s in CE in March was surprising given the lower cold hardiness. Lack of clear differences in the distribution of 5-mdC and AcH4 is consistent with no significant differences in expression of *PaCMT3*, *HDT1* and *HDT2* between the epitypes, indicating that more targeted analyses of epigenetic marks in specific genes in the different cells will be required.

Sammendrag

Gran (*Picea abies*) har et epigenetisk minne om temperaturen under embryoutviklinga, som påvirker trærnes fenologiske egenskaper mer enn 20 år seinere. Dette minnet ble først påvist i trær fra zygotiske embryoer (frø) utvikla under ulike temperaturer, og det ble bekrefta at det skyldtes epigenetikk da den samme minneeffekten viste seg i genetisk identiske epitypetrær produsert fra somatiske embryoer som ble utvikla under 18 og 28 °C. Disse studiene har vist at lav temperatur under embryoutviklinga framskynder knoppdannelse og kuldeherding om høsten og knoppsprett og avherding om våren, sammenligna med høyere temperatur. Den kalde epitypen (CE) har vist seg å ha lavere uttrykk av dehydriner enn den varme epitypen (WE) nær knoppsprett. Imidlertid har kuldeherding og avherding bare vært studert i trær fra zygotisk embryogenese og ikke i genetisk identiske epityper. Det er også begrensa kunnskap om uttrykk av epigenetisk relaterte gener i epitypene, og fordelinga av epigenetiske markører har ikke blitt studert.

Målet med denne masteroppgaven var å undersøke dette nærmere i de to epitypene. Frysetester ble utført med kvister samla inn på fire tidspunkter: mars, april, mai 2018 og mars 2019. Som forventa, sank frosttoleransen utover våren, og i samsvar med dens seinere knoppsprett, var WE signifikant mer frosttolerant enn CE på alle tidspunktene i minst en av de tre delene som ble undersøkt (nåler, kambium og knopper).

Uttrykk av de kuldeherdingsrelaterte genene *DEHYDRIN 6* (*PaDHN6*) og *DEHYDRIN 40* (*PaDHN40*), det knopphvilerelaterte genet *FLOWERING LOCUS T-TERMINAL FLOWER 1-LIKE 2* (*PaFTL2*) og de epigenetikkrelaterte genene *DNA (CYTOSINE-5)-METHYLTRANSFERASE CMT3 ISOFORM* (*PaCMT3*)*, HISTONE DEACETYLASE HDT1* (*PaHDT1*) og *HISTONE DEACETYLASE HDT2* (*PaHDT2*) i knopper samla inn i mars og mai ble analysert ved hjelp av RT-qPCR. Som forventa, sank uttrykket av *PaDHN*-ene og *PaFTL2* fra mars til mai, mens uttrykket av *PaCMT3* økte. CE hadde høyere uttrykk av begge *PaDHN*-ene enn WE i mars, som er overraskende, siden *DHN*-er er knytta til frosttoleranse og WE var mer frosttolerant enn CE på dette tidspunktet. Uttrykket av *PaHDT*-ene var ikke signifikant forskjellig mellom epitypene eller tidspunktene.

Fordelinga av epigenetiske markører i knopper ble studert ved hjelp av immunolokalisering av metylert cytosin (5-mdC) og acetylert histon 4 (AcH4). I mars så noen av WE-knoppene ut til å være mer metylert i prokambiet og det apikale skuddmeristemet enn i CE og begge epitypene i mai. Imidlertid varierte fordelinga av 5-mdC ganske mye mellom individene.

AcH4 var jevnt fordelt i alle knoppene uavhengig av epitype og tidspunkt. Dermed ser en slik grov immunolokalisering ut til å være uegna til å oppdage forskjeller i fordeling av epigenetiske markører mellom de to epitypene.

Konklusjonen er at lav temperatur under somatisk embryoutvikling fører til en raskere avherding om våren i trærne enn høyere temperatur gjør, mer enn 10 år etter planting i samme felt. Høyest uttrykk av *PaDHN*-er i CE i mars var overraskende gitt den lavere frosttoleransen. Mangelen på tydelige forskjeller i fordelinga av 5-mdC og AcH4 samsvarer med manglende signifikante forskjeller i uttrykk av *PaCMT3*, *HDT1* og *HDT2* mellom epitypene, noe som tyder på at det kreves mer målretta analyser av epigenetiske markører i bestemte gener i ulike celler.

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Kaia Slågedal, 13th of May 2019

Abbreviations

CE	cold epitype
WE	warm epitype
SD	short day
LD	long day
DHN6	*DEHYDRIN 6*
DHN40	*DEHYDRIN 40*
FTL2	*FLOWERING LOCUS T-TERMINAL FLOWER 1-LIKE 2*
CMT3	*DNA (CYTOSINE-5)-METHYLTRANSFERASE CMT3 ISOFORM*
HDT1	*HISTONE DEACETYLASE HDT1*
HDT2	*HISTONE DEACETYLASE HDT2*

Key words

Norway spruce, epigenetic memory, cold hardiness, immunolocalization

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1.0 Introduction

1.1 Dormancy

Temperate and boreal areas have large variations in weather through the year, from sub-zero temperatures and snow in the winter, to warm temperatures in summer. Plants growing in these areas need to be able to survive several months of winter when conditions for growth are not met. Perennial plants like trees, become dormant in the autumn; they stop growing, set winter buds and wait until conditions become favourable to resume growth in the spring.

Lang et al. (1987) defined dormancy as "a temporary suspension of visible growth of any plant structure containing a meristem". This definition was disputed by Junttila (1988), who wanted to restrict the term dormancy to growth inhibition caused by internal factors. In line with this, Rohde and Bhalerao (2007) suggested that dormancy should be defined as "the inability to initiate growth from meristems (and other organs and cells with the capacity to resume growth) under favourable conditions". Different terms have been used to describe different types of dormancy. In 1987, Lang et al. suggested the terms endodormancy, ecodormancy and paradormancy. Endodormancy was defined as inhibition of growth caused by internal physiological factors, while paradormancy was defined as inhibition of growth caused by physiological factors outside the affected structures. Inhibition of growth caused by the environment was called ecodormancy.

The induction of dormancy starts with growth cessation and development of a terminal bud. According to the terminology of Lang et al. (1987), the first part of dormancy is ecodormancy since the buds are in a quiescent state where bud break is possible. This is followed by development of endodormancy, and in this period the plant will not grow even if the temperature and growth factors are optimal. In the winter or early spring, after a required amount of chilling, the endodormancy is broken, and the plant becomes ecodormant, which means that the buds will burst and growth will be resumed as soon as the conditions are favorable and a specific temperature sum is reached (Welling & Palva, 2006; Lang et al., 1987).

Plants respond to photoperiod, which is the length of the day or night (Garner & Allard, 1923). Many plants will only grow, flower or set winter buds if the day is longer or shorter than a critical length (Nitsch 1957; Garner & Allard, 1923). In young trees with free growth pattern (formation of leaf initials and elongation of internodes occur at the same time), dormancy is induced in autumn by short days (SD), altered light quality and reduced

irradiance (Olsen 2010, Olsen and Lee 2011 and references therein). Older trees with fixed growth patterns (formation of leaf initials and elongation occur at different times) do not induce dormancy as a response to SD, but rather seem to have an endogenous control of dormancy, and they also set buds earlier than young individuals (Junttila, 2007). Trees from different latitudes have different critical day lengths for growing. As the days in the summer are longer at more northern latitudes, trees in the north will have a critical day length for growth, growth cessation and bud set that is longer than the critical day length for trees from more southern areas. These different tree populations can be regarded as different photoperiodic ecotypes (Vaartaja, 1959; Pauley & Perry, 1954; Wareing, 1956).

Temperature also affects dormancy, but studies in growth chambers and the field have shown opposite results. In growth chamber experiments, warmer temperatures during bud set was shown to result in deeper dormancy, better tolerance for frost, earlier bud set and later bud burst than colder temperatures (Olsen et al., 2014; Søgaard et al., 2008; Tanino et al., 2010). In several field studies, on the other hand, warmer temperatures in autumn delayed bud set and advanced bud burst (Strømme et al., 2015; 2017; Rohde et al., 2011). In the field, the shortening of the photoperiod occurs gradually, the irradiance is commonly higher, and the temperature varies more between day and night and from day to day, in contrast to in growth chambers where the temperature often is constant and the plants often are transferred directly from long days (LD) to SD. It has been speculated if such differences could be an explanation for the contrasting results in indoor and field studies (Strømme et al., 2015).

1.2 Cold hardiness

The cold hardiness of a plant, i.e. how low temperatures it can tolerate, varies with season. In summer, plants adapted to cold conditions may not tolerate any freezing or only a few degrees below 0°C, while in winter they can tolerate down to extreme low freezing temperatures due to cold acclimation (Strimbeck et al., 2015). Cold hardiness, like dormancy, is induced by SD, and it is further developed by low and freezing temperatures. During cold acclimation, the water content of buds decreases and the cells store more sugars and proteins (Strimbeck et al., 2015; Welling & Palva, 2006). Dehydration is a stress factor related to overwintering, because the formation of extracellular ice pulls water out of the cells. This dehydration may affect the membrane structure and protein denaturation and give higher concentration of toxic substances (Welling & Palva, 2006). Plants have several protection

mechanisms against these stresses, like adjusting the osmotic potential, the production of compatible solutes and sugars and controlling the site of ice nucleation (Gusta & Wisniewski, 2013).

In late winter or early spring, the cold hardiness starts to decrease again, as a response to warmer temperatures (Welling & Palva, 2006). This dehardening occurs relatively fast compared to the cold hardening in the autumn, and it is dependent not only on the present temperature, but also on the temperatures the plant has been exposed to recently (Kalberer et al., 2006).

1.3 Molecular regulation of bud dormancy and cold hardiness

Phytochrome A is an important light receptor that allows trees to sense the length of the day (photoperiod) and thus initiate photoperiodic responses (Olsen et al., 1997; Howe et al., 1996). The gibberellin hormones (GA) decrease during SD and increase during bud burst. Application of these hormones will prevent growth cessation as long as the plant is not endodormant (Olsen et al., 1995a; 1995b; Moritz, 1995). The hormone abscisic acid (ABA) increases during SD and seems to be involved in bud development, but probably not in dormancy maintenance, since ABA levels in the fully dormant state are low (Rohde et al., 2002; Ruttink et al., 2007).

In woody angiosperms like *Populus*, flowering time and growth is regulated by the genes *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*). The *FT* gene stimulates growth and is downregulated in SD (Böhlenius et al., 2006). In Norway spruce, such an *FT* gene has not been found (Nystedt et el., 2013), but there was found a similar gene called *FLOWERING LOCUS T-TERMINAL FLOWER 1-LIKE 2* (*FTL2*). This gene has an opposite pattern of *FT*; it is induced by SD and increases during late summer and autumn. *FTL2* is involved in induction of growth cessation, bud set and dormancy. The expression decreases towards the break of endodormancy, and it decreases further during bud burst (Gyllenstrand et al., 2007; Karlgren et al., 2013; Asante et al., 2011; Opseth et al., 2016).

The transcription factor C-repeat binding factor (CBF) is important in regulation of cold hardiness in plants (Wisniewski et al., 2018). CBF regulates several cold responsive genes (*COR*), and it is upregulated in low temperatures, and leads to higher freezing tolerance. As in dormancy, ABA is also involved in freezing tolerance and an increase of this hormone has

been shown to result in higher tolerance for frost in trees (Welling et al., 1997; Rinne et al., 1998). Dehydrins (DHNs) are part of the LEA group of proteins (late embryogenesis abundant) and protect the plant against dehydration, which is a major stress during winter (Welling & Palva, 2006, Strimbeck et al., 2015). One of the ways DHNs protect plants against dehydration stress is by binding to macromolecules and prevent them from coagulating (Rinne et al., 1999). However, the mechanism by which dehydrins work is not fully understood (Kjellsen et al., 2013). Expression of dehydrin genes (*DHN*s) is induced by low temperatures (Renaut et al., 2005) and are upregulated in cold-acclimated trees compared to in non-acclimated trees (Strimbeck et al., 2015, Kjellsen et al., 2013, Rinne et al., 1999, Artlip et al., 1997). *DHN* expression decreases towards bud burst (Yakovlev et al., 2008).

1.4 Climate change

The ongoing climate change may affect the phenology and distribution of plants. The temperature has increased by 0,85 °C the last 150 years and will continue to increase in the future (IPCC, 2014). The temperature is rising faster at higher latitudes, and it is predicted an increase of up to 6,7 °C, 5,8 °C and 4,5 °C in North America, North Asia and northern Europe, respectively (Christensen et al., 2013). All organisms must find methods to cope with higher temperatures and altered climatic conditions. Trees have already shown changes in phenology, e.g. earlier bud burst and longer growth periods, in response to the temperature increase (Körner & Basler, 2010; Olsen & Lee, 2011). Tree populations show strong adaptation to the local environment, and the distribution of the tree populations is expected to change in the future as a response to the climate changes (Aitken et al. 2008). The rise in temperature is occurring so fast that it may be difficult to adapt through classical evolutionary mechanisms, especially for organisms with long generation times, like trees.

1.5 Epigenetics

One way that plants might be able to adapt faster than what is possible through natural selection, is by the help of epigenetic mechanisms (Bossdorf et al., 2008). Epigenetics are changes in gene expression that are not due to altered DNA sequence and which are potentially heritable either mitotically or meiotically (Iwasaki & Paszkowski, 2014). The epigenetic changes are more stable than other factors which affect gene expression (e.g. transcription factors) and they may last through the whole life of the organism and even be

inheritable from generation to generation (Bossdorf et al., 2008; Yakovlev et al. 2012). Epigenetic variations contribute to phenotypic plasticity and might be important for adaptation to a changing climate (Bräutigam et al. 2013).

Important epigenetic mechanisms are DNA methylation, histone modifications and small non-coding RNAs (Iwasaki & Paszkowski, 2014). Histones may be modified in several different ways, like methylation, acetylation and phosphorylation. Non-coding RNAs regulate gene expression by inhibiting translation of complementary mRNA, or target it for degradation, and RNAs may also regulate methylation of specific DNA sequences, via the RNA directed DNA methylation pathway (RdDM) (Grant-Downton & Dickinson, 2006).

DNA methylation and histone acetylation are two of the most studied epigenetic mechanisms. Methylation of DNA is associated with heterochromatin and inactivation of gene expression, while acetylation of histones activates the associated genes (Taiz et al., 2014).

Methylation of DNA in plants are performed by three groups of DNA methyltransferases: METHYLTRANSFERASE1 (MET1), CHROMOMETHYLASE2 and 3 (CMT2 and CMT3) and DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2). MET1, CMT2 and CMT3 maintain methylation at CG, CHH and CHG sites, respectively, i.e. they methylate the new unmethylated strand of hemimethylated DNA (Iwasaki & Paszkowski, 2014). DRM2 establish new methylation (*de novo* methylation) at all three sites (Chen et al. 2010; Bewick et al. 2016; Ausin et al. 2016; Lindroth et al. 2001; Finnegan & Kovac, 2000). Removal of methyl groups from DNA – demethylation – can occur passively or actively. Passive demethylation occurs when DNA is replicated, and the new strand is not methylated. Active demethylation occurs when the methyl groups are removed by glycosylases (Valledor et al., 2007; Bartels et al., 2018).

Histones are the proteins that make up the core of the nucleosomes. A nucleosome consists of an octamer of four different histones (H1, H2, H3 and H4) with 146 bp of DNA coiled around (Lusser et al. 2001). If the histones are tightly bound to the DNA, expression of the genes in this region will be downregulated, because of low accessibility of DNA transcription components to the DNA. Different epigenetic modifications of the histones, like methylation and acetylation can affect the binding between histones and DNA, and thus alter gene expression. Acetylated histones have a lower affinity for the DNA, because of the negative charge of the acetyl group, which will repel the also negatively charged DNA. Thus, the binding between the acetylated histones and the DNA will be weaker, and the DNA will be

more accessible for transcription. Acetylated histones are therefore associated with gene expression, while non-acetylated histones are associated with gene silencing. Histone acetylation is regulated by specific enzymes that either add acetyl groups to the histones (histone acetyltransferases) or remove them (histone deacetylases) (Chen et al., 2010).

The distribution of epigenetic marks is associated with different developmental stages. Tissues that are more differentiated or have less organogenic capability have been found to have more DNA methylation and less histone acetylation than less differentiated tissues (Valledor et al., 2010; Fraga et al., 2002; Alakarppa et al., 2018). Changes in epigenetic marks may also be induced by environmental factors, and has been shown to vary through the growth-dormancy cycle (Conde et al., 2013). Epigenetic changes may be long-lasting and thus form an epigenetic memory. This may help organisms to adapt to different environments (Yakovlev 2012; Bräutigam et al., 2013).

Epitypes are genes or genomes that are identical in genotype and which differ in epigenetic marks (Meagher, 2010). Different epitypes might thus differ in phenotypic traits even if they are genetically identical.

1.6 Norway spruce

Norway spruce (*Picea abies* (L.) H. Karst.) is an important tree species in Europe, both ecologically and economically (Jansson et al., 2013). It is native to Northern and Eastern Europe, as well as mountain areas in Central Europe (Boratynska, 2007). In addition, it is cultivated outside of its natural ranges, as it is an important species for production of timber, paper and Christmas trees (Jansson et al., 2013). Norway spruce was the first gymnosperm which genome was sequenced, and like other conifers, its genome is extremely large (De La Torre et al., 2014), containing 20 billion base pairs (20 Gbp) (Nystedt et al., 2013).

1.7 Epigenetic memory in Norway spruce

An epigenetic memory has been shown to exist in Norway spruce, which makes the trees "remember" the temperature they were exposed to during embryogenesis (Yakovlev et al., 2012). This epigenetic memory in Norway spruce was starting to be discovered when Bjørnstad (1981) found that progenies from the same mother trees showed differences in bud set when the seeds had developed in northern versus southern areas. Grafts that had been

transferred from northern areas to a southern seed orchard produced progenies that had delayed bud set compared to plants from seeds produced by the same mother trees in northern areas, and thus was more similar to the southern ecotype. Johnsen (1989) did a similar study and found that trees grown from seeds produced in northern areas had higher freezing tolerance than trees from seeds produced in the more southern seed orchard. Both Bjørnstad (1981) and Johnsen (1989) suggested several explanations for this, among others that the parental environment affects traits in the progenies. Later studies of progenies from crosses performed at different latitudes and altitudes and inside and outside greenhouses confirmed that there was an effect of the environment during reproduction on the phenology and freezing tolerance of the trees growing from these seeds (Johnsen et al., 1996; 2005a; 2005b; Skrøppa et al., 2007; 2010). It was also shown that the effect was caused by the environment of the mother tree during the maturation of the embryo (Johnsen et al., 1996; 2005a). This effect has been shown to last more than 20 years and is probably life-long (Skrøppa et al., 2007).

To further investigate this effect, somatic embryogenesis was used as a tool to make genetically identical plants. During the embryogenesis, the developing embryos were exposed to different temperatures (18, 23 and 28 °C), giving rise to different epitypes (Kvaalen & Johnsen, 2008). These epitypes have shown differences in timing of bud burst and bud set; higher temperatures during embryogenesis give later bud burst and bud set than lower temperatures (Kvaalen & Johnsen, 2008; Carneros et al., 2017). The epitypes were shown to have a difference in timing of bud set equivalent to the difference between populations separated by 4-6° of latitude (Kvaalen & Johnsen, 2008). This indicates that the epigenetic effect of temperature during embryogenesis may be important for the formation of different ecotypes in natural populations. Temperature-induced epitypes also differ in gene expression; dehydrin genes and *FTL2* were downregulated in the cold epitype (CE) compared to the warm (WE) (Carneros et al., 2017). Studies of gene expression in the epitype trees have only been done close to bud burst, thus the expression pattern of these genes earlier in spring or winter when the cold hardiness is stronger is unknown. Several epigenetically related genes (DNA methyltransferases and histone deacetylases, among others) and micro RNAs were differentially expressed during the somatic embryogenesis and the formation of temperature-induced epitypes (Yakovlev et al., 2014; 2016; Yakovlev & Fossdal, 2017). Epigenetically related genes, like *DNA (CYTOSINE-5)-METHYLTRANSFERASE CMT3 ISOFORM* (*PaCMT3*)*, HISTONE DEACETYLASE HDT1* (*PaHDT1*) and *HISTONE DEACETYLASE*

HDT2 (*PaHDT2*) have also been shown to be differentially expressed in the epitype trees (Viejo et al., unpublished). *PaCMT3* and *PaHDT1* were upregulated, while *PaHDT2* was downregulated in CE compared to WE at one timepoint in spring (20 April).

1.8 Aims

The overall aim of this study was to find out if different temperatures during embryogenesis influence the freezing tolerance and the expression of genes related to freezing tolerance and dormancy, and to study possible mechanisms of this epigenetic memory.

The specific aims were to:

- Investigate freezing tolerance of twigs collected in March, April and May. Freeze tests have not earlier been performed on genetically identical epitypes.
- Investigate expression of genes related to overwintering (*DHN*s and *FTL2*) and epigenetics (*PaCMT3*, *PaHDT1* and *PaHDT2*) during dehardening in spring (March and May), and if the expression differs between the epitypes. Expression of these genes has not earlier been analyzed in March.
- Investigate differences between the epitypes in distribution of epigenetic marks (DNA methylation and histone 4 acetylation) in different cell types and tissues in the buds, and if these patterns change during the dehardening (from March to May). The epigenetic memory is thought to be formed in the central mother cells, so that differences in epigenetic marks between the epitypes in these cells are of interest.

2.0 Materials and methods

2.1 Study species

The samples used in this study were harvested from genetically identical epitype trees of Norway spruce (*Picea abies* (L.) H. Karst) grown from embryos produced by somatic embryogenesis as described in Kvaalen & Johnsen, 2008. The embryos were originated from the clone B10W, which was obtained by the crossing of ♀ #2650 and ♂ #2707 inside a heated greenhouse. Some embryos were exposed to 18 °C during embryo development and some were exposed to 28 °C, giving rise to a cold epitype (CE) and a warm epitype (WE), respectively (Kvaalen & Johnsen, 2008). The trees were planted in a field at Hoxmark, Ås (59°40´07,5N/10°43´7,7E) in 2007. The individual ID numbers of the trees that were harvested in 2018 were 3653, 3669, 3644, 3667 and 3656 from CE and 3679, 3677, 3663, 3665 and 3685 from WE. In March 2019, the same WE individuals were harvested in addition to two more (3678 and 3662). Two of the CE individuals (3653 and 3656) were replaced by 3668, because they were found to not be genetically identical with the others (see below).

2.2 Collection of samples

Twigs and buds were collected at three different time points in spring (12 March, 23 April and 7 May 2018). Harvesting was done from approximately the 5th-7th whorl of five individual trees of each of the two epitypes. Later it was discovered by microsattelite analyses that two of the trees from CE (ID numbers 3653 and 3656) were not genetically identical to the other eight individuals, probably due to mislabeling before planting (M. Viejo, personal communication). Thus, the analyses were performed on 3 individuals of the CE and 5 individuals of the WE. Twigs for an additional freeze experiment were harvested on the 8 March 2019. These twigs were collected from further down on the trees than the three first collection dates, approximately the 7th-8th whorl.

2.3 Cold hardiness testing

Cold hardiness was tested by controlled freezing experiments, followed by visual assessment of injury, using a method based on Olsen et al. (1997), Strømme et al. (2018) and Johnsen et al. (2005b).

2.3.1 Freezing and damage development

Twigs of approximately 15-20 cm length were collected. The twigs were put in metal boxes in between two layers of moist paper, with moist sand above and below. The boxes were put in freeze chambers (custom made, Weiss Umwelttechnik simulationsanlagen, Reiskirchen-Lindenstruth, Germany) at different temperatures, one control with non-freezing temperature at 5 °C and 5 freezing temperatures (-5, -15, -25, -35 and -45 °C for the March experiments (both years), -5, -10, -15, -20, -25 °C in April and -3, -6, -9, -12, -15 °C in May). The higher test temperatures for the April and May experiments were chosen since the trees were expected to become less frost tolerant during the spring, because of a gradual dehardening. Three twigs from each individual were used for each temperature. The freezing program was as follows:

- 8 hours at -0.5 ºC for controlled ice nucleation.
- -3 ºC per hour down to -17 ºC (or target temperature)
- -10 ºC per hour down to target temperature (if lower than -17 ºC)
- 4 hours at target temperature
- 2 ºC per hour up to 5 ºC
- Minimum 2-3 hours at 5 ºC (until thawing of the moist sand).

When the freezing program was finished, the twigs were put in transparent, closed plastic boxes with moist paper on the bottom. They were sprayed with water every day to keep them moist. The boxes were put in a room with normal room temperatures (approximately 20-23 °C) and day light (not direct sunlight). For the twigs collected and frozen in May 2018, the temperature was higher than the other time points (28-35 °C during the day) because of unusual warm weather (Appendix 1). This was also the case the last week of the damage development for the twigs collected in April.

2.3.2 Evaluation of freeze damage

One week after freezing, damage to the needles was evaluated. Based on this evaluation and Johnsen et al. (2005), it was decided to wait until three weeks after freezing for the full evaluation. After three weeks, damage to the needles, cambium and buds was evaluated. The main branch of the twig and all the buds were cut in the middle longitudinally to be able to inspect the cambium and bud damage. For needle damage and cambium damage a scale with 5 steps was used (0 = 0 % damage, 1 = 1-33 % damage, 2 = 34-66 % damage, 3 = 67-99 %

damage, 4 = dead; 100% damage), with brown tissue counting as damage. Buds were scored as either dead or alive (alive = some green tissue, dead = completely brown). The number of buds in each test temperature and epitype is listed in table 1.

Lethal temperature 50 % (LT50) was estimated based on the frost damage graphs. For needle and cambium, category 2 was used as an indication of 50 % damage, while for buds, 50 % dead buds was used. LT50 was calculated as the temperature at which the frost damage graphs crossed the line of 50 % damage.

Table 1. Number of buds in each test temperature for each epitype for twigs collected 12 March, 23 April, 7 May 2018 and 8 March 2019. Average number of buds per individual in each epitype and collection date are also shown. In 2018, twigs were collected from three individuals of the cold epitype (CE) and five individuals of the warm epitype (WE). In 2019, twigs were collected from four individuals of CE and seven individuals of WE.

12 March	Test temperature (°C)						Average
2018	5	-5	-15	-25	-35	-45	individual^{-1}
CE	49	43	44	51	71	51	103,0
WE	175	187	147	181	132	150	194,4
23 April 2018	Test temperature (°C)						
	5	-5	-10	-15	-20	-25	
CE	143	112	99	158	170	153	278,3
WE	263	226	196	183	188	199	251,0
7 May 2018	Test temperature (°C)						
	5	-3	-6	-9	-12	-15	
CE	70	91	36	46	41	40	108
WE	113	154	125	146	164	119	164,2
8 March 2019	Test temperature (°C)						
	5	-5	-15	-25	-35	-45	
CE	100	98	138	118	130	137	180,3
WE	175	170	147	145	111	143	127,3

2.3.3 Bud burst recording

At the same time as the freezing damage was evaluated, the number of buds that had burst in the twigs was also recorded. The buds were scored as either burst or not burst, where buds that had visible green leaf primordia sticking out between the bud scales were counted as burst.

2.4 Gene expression analysis

Transcript levels of the genes *DNA (CYTOSINE-5)-METHYLTRANSFERASE CMT3 ISOFORM* (*CMT3*)*, HISTONE DEACETYLASE HDT1* (*HDT1*), *HISTONE DEACETYLASE HDT2* (*HDT2*), *DEHYDRIN 6* (*DHN6*), *DEHYDRIN 40* (*DHN40*) and *FLOWERING LOCUS T-TERMINAL FLOWER 1-LIKE 2* (*FTL2*) in buds were analyzed by reverse transcription quantitative real-time PCR (RT-qPCR).

Three individuals from each epitype from the collection in March and May were used. After harvest, the bud scales were removed, and the buds were frozen in liquid nitrogen before storage in a freezer at -80 °C until RNA isolation.

2.4.1 RNA isolation

The frozen bud tissue was crushed in a mixer mill (MM301, Retsch, Haan, Germany) with 5 mm beads for 30 seconds at 24 Hz. Subsamples of approximately 4-10 mg were used for the following RNA isolation. The MasterPure Complete DNA and RNA Purification Kit (Epicentre, Wisconsin, USA) was used to extract the RNA from the cells according to the protocol of MasterPure Plant RNA Purification Kit, with some modifications to the protocol. Tissue lysis was done with a solution containing 600 µl Tissue and Cell Lysis Solution and 1 µl Proteinase K from the kit in addition to 3 µl β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) and 0,5 % polyvinylpyrrolidone (PVP, Sigma-Aldrich) for each sample. The two latter ingredients were used instead of DTT (dithiothreitol) from the manufacturer`s protocol. The samples were mixed for 1 min, and then incubated at 56 °C for 15 min, with mixing every 5 min. After incubation, the samples were centrifuged at 10 000 g (Centrifuge 5415 R, Eppendorf, Hamburg, Germany) for 5 min in room temperature and then the supernatant of each sample was transferred to a new tube (1,5 ml Eppendorf tube) and placed on ice for 3-5 min.

To each sample, 250 µl of MPC Protein Precipitation Reagent from the MasterPure kit was added and the samples vortexed for 5 sec. They were then centrifuged at 10 000 g for 10 min at 4 °C. The supernatant was transferred to a new tube, and 500 µl isopropanol (Arcus, Oslo, Norway) was added to this before inverting the tubes 30-40 times, to precipitate the nucleic acids. Then the precipitated nucleic acids were pelleted by centrifugation at 10 000 g for 10 min at 4 °C, and the isopropanol removed.

2.4.2 Removal of contaminating DNA

Contaminating or genomic DNA was removed with 5 µl DNase I in 195 µl 1x DNase buffer from the MasterPure kit for each sample, incubating at 37 °C for 30 min. Then 200 µl of 2x T and C Lysis Solution and 200 µl of MPC Protein Precipitation Reagent were added and mixed by tapping, before placed on ice for 3-5 min. The samples were then centrifuged at 10 000 g (Centrifuge 5417 R, Eppendorf) for 10 min at 4 °C and the supernatant transferred to a new tube. This centrifugation and supernatant transfer were done two times, after which 500 µl of isopropanol (Arcus) was added and the tubes inverted 30-40 times, to precipitate the RNA. The precipitated RNA was pelleted by centrifugation at 10 000 g for 10 min at 4 °C, and the isopropanol removed. The RNA pellet was washed twice with 70 % ethanol. After washing, all the ethanol was removed, and the RNA pellet resuspended in 20 µl RNase free water, and 1 µl RiboGuard RNase Inhibitor were added. The samples were stored at -80 °C for 2 days (samples harvested in March 2018) and 25 days (samples harvested in May 2018) until cDNA synthesis.

2.4.3 Synthesis of cDNA

The concentration of the isolated RNA was measured with Nanodrop ND-1000 (Nanodrop Technologies, North Carolina, USA), and the quality was analyzed with a bioanalyzer (2100 Bioanalyzer, Agilent, California, USA). 1 µg of RNA from each sample was used to synthesize cDNA in a 20 µl reaction volume with random primers and reverse transcriptase using SuperScript VILO cDNA Synthesis Kit (Invitrogen, Thermo fisher Scientific, Massachusetts, USA), according to the manufacturer's protocol. Each sample contained 4 µl 5X VILO Reaction Mix, 2 µl 10X Superscript Enzyme Mix and 1000 ng of RNA. RNase free water was added so that the total volume became 20 µl. Reactions without reverse transcriptase (-RT) were also made for each sample (500 ng RNA in 10 µl reaction volume). The program for the cDNA synthesis was as follows: 25 °C for 10 min, 42 °C for 60 min, and

85 °C for 5 min (DNA Engine Tetrad 2 Peltier Thermal Cycler, Bio-Rad Laboratories, California, USA). After the cDNA synthesis, 80 µl nuclease-free water were added to each sample (40 µl for the -RT samples), diluting it 1:5.

2.4.4 Check for contaminating DNA

cDNA from all the samples, including the -RT samples, were run through real time qPCR (7500 Fast Real-time PCR System, Applied Biosystems, Thermo fisher Scientific, Massachusetts, USA) with primers for a reference gene (α-tubulin) to check for contaminating or genomic DNA, following the same method as described below in the section 2.4.5, but with only one technical replicate for each sample.

2.4.5 Real time quantitative PCR

For the real time qPCR assay, 96-well reaction plates were used (MicroAmp Fast Optical 96-Well Reaction Plate, Applied Biosystems, Thermo fisher Scientific, Massachusetts, USA). In each well, there were 7 µl nuclease free water, 10 µl SYBR Select Master Mix (Applied Biosystems, Thermo fisher Scientific) and 0,5 µl of each of the forward and reverse primers, with a primer concentration in the total reaction volume of 250 nM. To each well, 2 µl of the template cDNA were added. Each sample had 4 technical replicates for each gene tested. There were also 3 no template controls (NTC) for each gene, which contained water instead of template. The program for the qPCR was as follows: 2 min at 50 °C, followed by 2 min at 95 °C and then 40 cycles of 15 sec at 95 °C and 1 min at 60 °C.

2.4.6 Primer design and primer sequences

Primers were designed to span over exon-exon junctions, to avoid potential DNA contamination. This was done for all the primers, except the primers for *DHN6* and *DHN40*, as the sequences for these genes in the database (congenie.org) did not include information about exons and introns. Because of this, another set of primers that did not span over exon-exon junctions was used for the reference genes for the dehydrins. All the primer sequences were checked with the OligoAnalyzer Tool from Integrated DNA Technologies and ordered from Invitrogen. The primer sequences and access numbers are listed in table 2.

Table 2. Primer sequences for the 6 genes analyzed by RT-qPCR in epitypes of Norway spruce: *DNA (CYTOSINE-5)-METHYLTRANSFERASE CMT3 ISOFORM* (*CMT3*), *HISTONE DEACETYLASE HDT1* (*HDT1*), *HISTONE DEACETYLASE HDT2* (*HDT2*), *DEHYDRIN 6* (*DHN6*), *DEHYDRIN 40* (*DHN40*) and *FLOWERING LOCUS T-TERMINAL FLOWER 1-LIKE 2* (*FTL2*) as well as the reference genes *α-TUBULIN* (*α-TUB*), *ACTIN, TRANSLATION INITIATION FACTOR-5-α* (*elF5α*) and *ELONGATION FACTOR-1-α* (*EF1α*).

Gene	Access no.	Forward primer Reverse primer	Product length (bp)
PaDHN6	MA_747559g0010	CGTGGATCAAGTCAAAGAGAAGCTGC GCCTTCTCAGTTGGATTACTCTGAGC	150
PaDHN40	MA_10257300g0010	AGTAGAGTCCGTTCAGGGTGAGC CAGCCCCATCTTCTGAGTTGGATTC	102
PaFTL2	MA_720135g0010	GCTACAACAGCTGCTTCCTTTGGACG GCTGAAGTTGACGCGGGACTGTG	147
PaCMT3	MA_173651g0020	GTGACTGTGCTTCTGTCAAGGGTG CTCAAGTCTTACTGTGTCCTCTGCTCTG	140
PaHDT1	MA_3905g0020	GCAGCTGCAAAGGCAAAGCCAG CGTCACTTGATCCTTCCATGCCCTC	166
PaHDT2	MA_3905g0010	GTGTGGAGGTAAAACCAGGGGAGG TCGCATTTTGAATCTCACCCAGAGTTGC	99
PaelF5α	MA_103714g0010	CATTAAGGCCAGGCCCTGCAAG CAGTGCGAGTAACATGTGGGACGTC	167
*PaEF1α**	MA_434977g0010	GGATTGCCACACTTGCCACA CTTGGGTTCCTTCTCCAGTTCC	94
PaαTUB	MA_93486g0010	TCAGCGAGACAGGAGCTGGGAAG GTCCACAATCTCCTTCCCCACAGTG	191
*PaαTUB**	MA_93486g0010	GTCCACAATCTCCTTCCCCACAGTG ACCACGAGCGAAGTTGTTG	111
PaACTIN	MA_10427661g0030	TGAGCTTCGAGTTGCTCCAGAAGAAC ACCATCCCCAGAATCTAGCACAATACCAG	199
*PaACTIN**	MA_10427661g0030	TGAGCTCCCTGATGGGCAGGTGA TGGATACCAGCTTCCATCCCAAT	105

* Reference genes used for the *DHNs*, primers do not contain exon-exon junctions

2.4.7 Calculating relative transcript levels

Relative transcript levels were calculated by the delta delta Ct method, which normalizes the transcript levels of the target genes to the reference genes and quantifies the transcript levels relative to a calibrator group.

For each sample and gene, the following formulas were used:

1. $\Delta Ct(s) = Ct(GOI\ s) - Ct(ref.\ s)$
2. $\Delta Ct(c) = Ct(GOI\ c) - Ct(ref.\ c)$
3. $\Delta\Delta Ct = \Delta Ct(s) - \Delta Ct(c)$
4. Fold change = $2^{-\Delta\Delta Ct}$

GOI is gene of interest, i.e. the gene which transcript level is being measured. Ct(ref.) is the average of the Ct values for the three reference genes. s is the sample of interest, c is the average of the calibrator samples. For each gene, the group with the lowest transcript level was chosen to be the calibrator, to avoid fold changes below 1, for easier comparisons.

To get the relative transcript levels of each group, the fold changes of the three samples in each group were averaged. The fold change shows the transcript level of each group relative to the calibrator group. A fold change of 10 means that this group has transcript levels 10 times higher than the calibrator group.

2.5 In situ localization of DNA-methylation and histone acetylation

Buds from three CE individuals and five WE individuals collected on the 12 March and 7 May 2018 were used for the *in situ* localization. DNA methylation and histone 4 acetylation were localized in the buds by immunolabeling with primary antibodies against 5-methyldeoxycytidine (5-mdc) and acetylated histone 4 (AcH4), and fluorescent secondary antibodies.

2.5.1 Fixation and wax embedding

Relatively big apical buds were dissected (removal of bud scales) and fixed with 4 % paraformaldehyde in phosphate buffer saline (PBS) with 0,1 % v/v Tween 20, vacuum infiltrated for 30 minutes and incubated at 4 °C overnight. The next day they were dehydrated with increasing concentrations of ethanol (30 %, 50 % and 70 %, 60 min at each step). Further dehydration (up to 100 % ethanol) and clearing with xylene was done in a tissue

processing machine (Tissue-Tek VIP 5 Jr, Sakura Finetek, Tokyo, Japan). Then the buds were embedded in paraffin wax and made into blocks using the Tissue-Tek TEC (Sakura Finetek) and stored at 4 °C until sectioning (several months).

2.5.2 Immunolabeling

The paraffin-embedded buds were cut into 10 µm thick sections using a rotary microtome (Leica RM2255, Leica Biosystems, Wetzlar, Germany). The sections were deparaffinized with Histo-Clear (National Diagnostics, Atlanta, Georgia, USA) and rehydrated with decreasing concentrations of ethanol (100 %, 75 %, 50 % and 25 %, 5 min each). They were then permeabilized with 2 % cellulase (Onozuka R-10, Duchefa Biochemie, Haarlem, The Netherlands) in PBS for 30 min at 45 °C. The sections that were going to be localized with the 5-mdC antibody were incubated with HCl 2N for 30 min to denaturalize the DNA.

Blocking was done with 10 % bovine serum albumin (BSA) in PBS for 30 min.
Two different primary antibodies were used, one against 5-mdc (MABE146, Millipore, Burlington, Massachusetts, USA) and one against AcH4 (06-866, Millipore). The working concentration of the 5-mdC antibody was 1:100 in 1 % BSA in PBS, and the incubation was done for 60 min in room temperature. For the AcH4 antibody the concentration was also 1:100, but the BSA concentration was 5 % and the incubation was done at 37 °C. The increased BSA concentration and incubation temperature for the H4ac antibody was done to try to reduce unspecific binding. The secondary antibody Alexa Fluor 488 anti-mouse (Life Technologies, Thermo fisher Scientific) was used for the 5-mdC sections and Alexa Fluor 488 anti-rabbit (Life Technologies, Thermo fisher Scientific) for the H4ac sections. The concentrations were 1:50 in 1 % BSA in PBS for both the secondary antibodies, and the incubation lasted for 45 min in darkness. The sections were then stained with DAPI (4′,6-diamidino-2-phenylindole 0,6 µg/ml in PBS 1x with 2 % Tween 20) for 35 min in darkness. In between each step the sections were washed with PBS 1x or 0,1 % Tween 20 in PBS. The sections were mounted with DAKO Fluorescent Mounting Medium (Agilent, Santa Clara, California, USA) and covered with cover glasses (No. 631-0137, VWR, Radnor, Pennsylvania, USA).

2.5.3 Microscopy

The *in situ* localization of the epigenetic marks were done in a confocal laser scanning microscope (Leica TCS SP5, CLSM, Leica Microsystems, Wetzlar, Germany). Pictures were taken with a 20x objective with immersion oil. The function Tile Scan was used to get a picture of the whole bud, sequential scanning was used to avoid the overlap in emission for the DAPI and the Alexa Fluor 488. Z-stacks with 5 levels were also included. In addition, transmission pictures (regular bright field) were taken to be able to see the structure of the bud and cells. Detection ranges for DAPI was set to 420-470 nm and for Alexa Fluor 488 it was set to 510-545 nm.

The pictures were taken of representative sections for each individual, three from the cold epitype and five from the warm epitype.

2.6 Statistical analysis

The cold hardiness data was analyzed using R, version 3.5.1. Needle and cambium damage were tested with the cumulative link mixed models (clmm) from the ordinal package (Christensen 2015), because the data were ordinal. Needle damage and cambium damage were tested as response variables with the categorical epitypes and the numerical test temperatures as factors. A binomial model was used for the bud damage, as it only had two levels (dead/alive). The same binomial model was used to analyze bud burst.

The effects of epitype and collection date on the transcript levels (fold change values) of the 6 genes were analyzed with repeated measures ANOVA, including individual as a random factor nested in epitype. The analysis was done in Minitab 18.1 (Minitab Inc., State College, PA, USA). Repeated measurements ANOVA were used because the data were not independent, as the same trees were harvested in for the two timepoints. In addition (to find out if the difference between the epitypes was significant for one or both timepoints) a regular one-way ANOVA with transcript level as response variable and epitype as factor was performed for each timepoint.

The significance level was set to $p \leq 0.05$ for all the statistical tests.

3.0 Results

3.1 Cold hardiness testing

3.1.1 One week after freezing

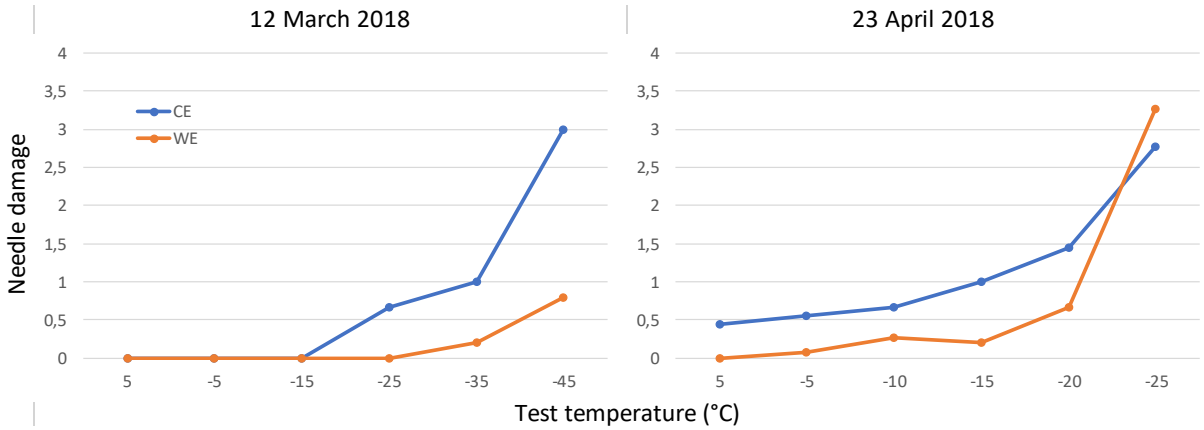

Figure 1. Needle damage one week after freezing of Norway spruce twigs collected in March and April 2018. The damage scale ranges from no damage to dead (0=no damage/completely green, 1=1-33 % brown, 2=34-66 % brown, 3=67-99 % brown, 4=dead/completely brown). The data was calculated as averages of three biological replicates for the cold epitype (CE) and five biological replicates for the warm epitype (WE).

Needle damage was evaluated one week after freezing for the twigs collected 12 March and 23 April 2018. The twigs had then developed visible damage to some extent. Twigs from the lower freeze temperatures had more damage than twigs from higher freeze temperatures and the control temperature (Fig. 1). For both time points, CE was significantly more damaged than WE (p=0,00159 in March, p=4.20e-06 in April) (Tables 3 and 4).

Table 3. Summary of a cumulative link model run to test the effect of epitype and freeze temperature on needle damage in twigs of Norway spruce collected 12 March 2018, one week after freezing. Three individuals from the cold epitype (CE) and five individuals from the warm epitype (WE). Positive coefficients mean more damage. Negative coefficients mean less damage.

Treatment	Coefficient	SE	z value	Pr (>|z|)
EpitypeWE	-4,52714	1,43343	-3,158	0,00159 **
Temperature	-0,28320	0,08149	-3,475	0,00051 ***

*Significance codes: ***P<0.001 **P<0.01 *P<0.05*

Table 4. Summary of a cumulative link model run to test the effect of epitype and freeze temperature on needle damage in twigs of Norway spruce collected 23 April 2018, one week after freezing. Three individuals from the cold epitype (CE) and five individuals from the warm epitype (WE). Positive coefficients mean more damage. Negative coefficients mean less damage.

Treatment	Coefficient	SE	z value	Pr (>|z|)
EpitypeWE	-7,05391	1,53298	-4,601	4.20e-06 ***
Temperature	-0,13244	0,03041	-4,356	1.33e-05 ***
EpitypeWE:Temperature	-0,32953	0,07498	-4,395	1.11e-05 ***

*Significance codes: ***P<0.001 **P<0.01 *P<0.05*

3.1.2 Three weeks after freezing

Three weeks after freezing, the damage had developed further. Twigs from lower test temperatures had clearly more damage than twigs from higher test temperatures (Fig. 2-6), and generally, CE was significantly more damaged than WE (Fig. 2). There were quite large variations in damage among the individuals, especially for the buds (Appendix 2).

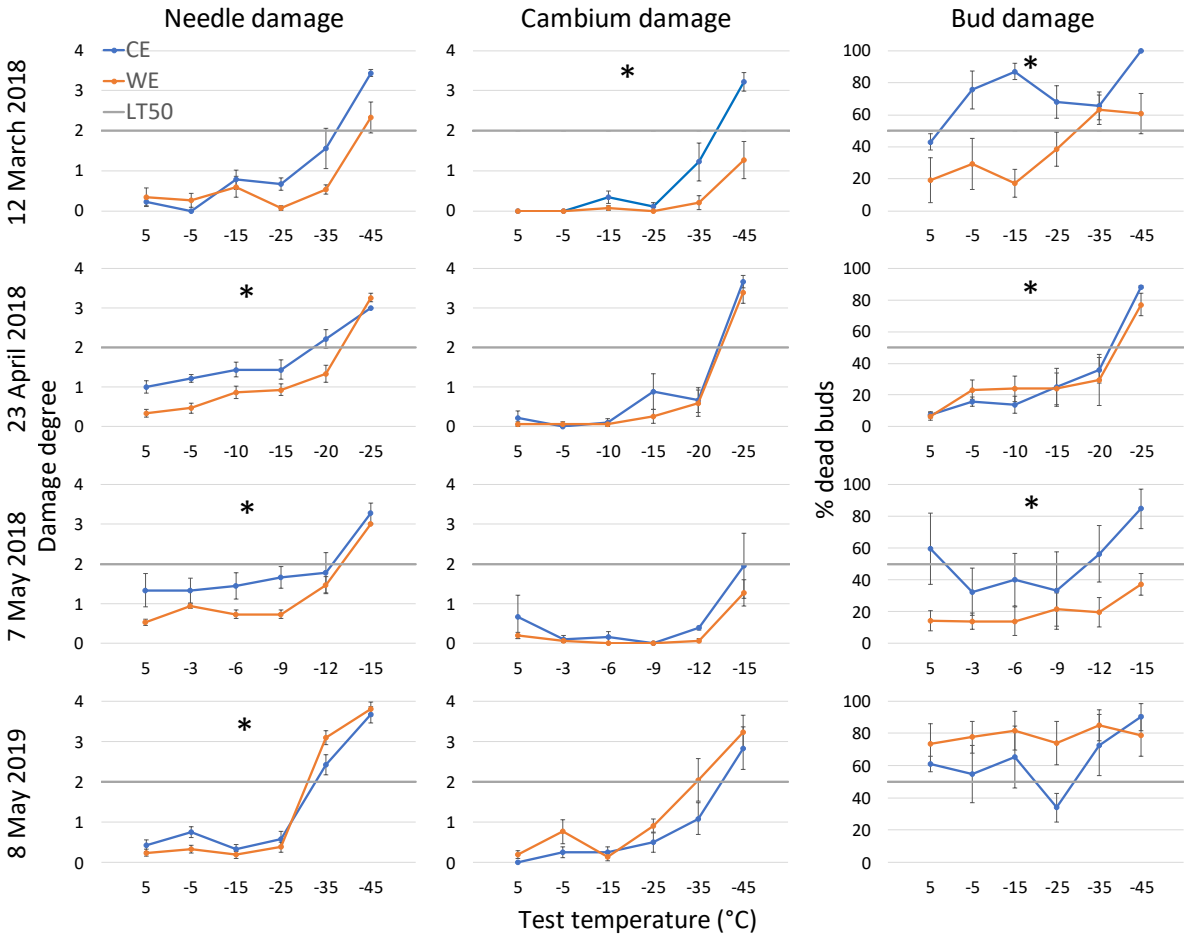

Fig. 2. Damage in the needles, cambium and buds of Norway spruce epitypes after freezing at different temperatures, for twigs collected in a common garden in Ås, Norway, at four different time points. The damage scale for needles and cambium goes from no damage to dead (0=no damage/completely green, 1=1-33 % brown, 2=34-66 % brown, 3=67-99 % brown, 4=dead/completely brown). Bud damage is shown as percent dead buds. The data was calculated as averages of three biological replicates for the cold epitype (CE) and five biological replicates for the warm epitype (WE) ± SE for the collections in 2018. In 2019, there were four biological replicates for CE and seven biological replicates for WE. * indicates an overall significant difference between the epitypes.

The twigs collected in March 2018 had significantly less damage in the cambium (p=0,00002) and buds (p=0,00001) for WE compared to CE (Table 5). There was no significant difference in needle damage, but there was a trend of more damage in CE than WE for the three lowest test temperatures (-25, -35 and -45 °C) (Fig. 2). There was a significant interaction between epitype and test temperature for bud damage (p=0.000648).

Table 5. Summary of statistical models run to test the effect of epitype and freeze temperature on frost damage in twigs of Norway spruce epitypes collected 12 March 2018, three weeks after freezing. A) Needle damage and B) cambium damage tested by a cumulative link model. C) Bud damage tested by a binomial generalized linear model. Three individuals from the cold epitype (CE) and five individuals from the warm epitype (WE). For the needle and cambium damage, positive coefficients mean more damage and negative coefficients mean less damage. For the bud damage, positive coefficients mean less damage and negative coefficients mean more damage.

Treatment	Coefficient	SE	z value	Pr (>|z|)
A				
EpitypeWE	0,15998	0,76677	0,209	0,8347
Temperature	-0,11712	0,02152	-5,442	5.28e-08 ***
EpitypeWE:Temperature	0,04413	0,02539	1,738	0,0822
B				
EpitypeWE	-2,38421	0,55978	-4,259	2.05e-05 ***
Temperature	-0,17334	0,02986	-5,806	6.41e-09 ***
C				
Intercept	-0,361875	0,446335	-0,811	0,417499
EpitypeWE	2,488796	0,564081	4,412	1.02e-05 ***
Temperature	0,030556	0,008010	3,815	0,000136 ***
EpitypeWE:Temperature	0,032933	0,009656	3,410	0,000648 ***

*Significance codes: ***P<0.001 **P<0.01 *P<0.05*

The twigs collected in April 2018 had significantly less needle damage (p=0,000005) for WE compared to CE (Table 6). WE had significantly more damage in the buds than CE (p=0,0201), but this seemed to apply only for the higher test temperatures (-5 and -10 °C) (Fig. 2). There was no significant difference in cambium damage. There were significant interactions between epitype and test temperature for needle damage (p=0.0029) and bud damage (p=2.53e-05).

Table 6. Summary of statistical models run to test the effect of epitype and freeze temperature on frost damage in twigs of Norway spruce epitypes collected 23 April 2018, three weeks after freezing. A) Needle damage and B) cambium damage tested by a cumulative link model. C) Bud damage tested by a binomial generalized linear model. Three individuals from the cold epitype (CE) and five individuals from the warm epitype (WE). For the needle and cambium damage, positive coefficients mean more damage and negative coefficients mean less damage. For the bud damage, positive coefficients mean less damage and negative coefficients mean more damage.

Treatment	Coefficient	SE	z value	Pr (>|z|)
A				
EpitypeWE	-3,44626	0,75390	-4,571	4.85e-06 ***
Temperature	-0,16526	0,03255	-5,078	3.82e-07 ***
EpitypeWE:Temperature	-0,12520	0,04204	-2,978	0,0029**
B				
EpitypeWE	-0,44445	0,41198	-1,079	0,281
Temperature	-0,22898	0,03728	-6,142	8.17e-10 ***
C				
Intercept	3,42063	0,39756	8,604	< 2e-16 ***
EpitypeWE	-1,10271	0,47750	-2,309	0,0209 *
Temperature	0,17023	0,01435	11,866	< 2e-16 ***
EpitypeWE:Temperature	-0,06957	0,01652	-4,212	2.53e-05 ***

*Significance codes: ***P<0.001 **P<0.01 *P<0.05*

For the twigs collected in May 2018, WE had significantly less damage in needles (p=0,0027) and buds (p=0,0228) (Table 7). There was no significant difference in cambium damage. There were also no significant interactions between epitype and test temperatures.

Table 7. Summary of statistical models run to test the effect of epitype and freeze temperature on frost damage in twigs of Norway spruce epitypes collected 7 May 2018, three weeks after freezing. A) Needle damage tested by a cumulative link mixed model. B) Cambium damage tested by a cumulative link model. C) Bud damage tested by a binomial generalized linear model. Three individuals from the cold epitype (CE) and five individuals from the warm epitype (WE). For the needle and cambium damage, positive coefficients mean more damage and negative coefficients mean less damage. For the bud damage, positive coefficients mean less damage and negative coefficients mean more damage.

Treatment	Coefficient	SE	z value	Pr (>|z|)
A				
Intercept	3,42063	0,39756	8,604	< 2e-16 ***
EpitypeWE	-1,10271	0,47750	-2,309	0,0209 *
Temperature	0,17023	0,01435	11,866	< 2e-16 ***
EpitypeWE:Temperature	-0,06957	0,01652	-4,212	2.53e-05 ***
B				
EpitypeWE	-1,79645	0,59879	-3,000	0,002699 **
Temperature	-0,19346	0,05183	-3,733	0,000189 ***
EpitypeWE:Temperature	-0,06431	0,06024	-1,068	0,285665
C				
EpitypeWE	-0,45051	0,45416	-0,992	0,32121
Test temp.	-0,14947	0,04659	-3,208	0,00134**

*Significance codes: ***P<0.001 **P<0.01 *P<0.05*

The twigs collected in March 2019 had significantly less damage in WE compared to CE for needles (p=0.00253) (Table 8), but for the lowest test temperatures (-35 and -45 °C) WE seemed to be more damaged than CE (Fig. 2). There was no significant difference between the epitypes for cambium and bud damage. There were significant interactions between epitype and test temperatures for needle (p=0.00246) and bud damage (p=0.0239), but not for cambium damage.

Table 8. Summary of statistical models run to test the effect of epitype and freeze temperature on frost damage in twigs of Norway spruce epitypes collected 8 March 2019, three weeks after freezing. A) Needle damage tested by a cumulative link model. B) Cambium damage tested by a cumulative link mixed model. C) Bud damage tested by a binomial generalized linear model. Four individuals from the cold epitype (CE) and seven individuals from the warm epitype (WE). For the needle and cambium damage, positive coefficients mean more damage and negative coefficients mean less damage. For the bud damage, positive coefficients mean less damage and negative coefficients mean more damage.

Treatment	Coefficient	SE	z value	Pr (>|z|)
A				
EpitypeWE	-1,74796	0,57887	-3,020	0,00253
Temperature	-0,09933	0,01545	-6,431	1.27e-10 ***
EpitypeWE:Temperature	-0,06006	0,01983	-3,029	0,00246
B				
EpitypeWE	0,9649	0,6262	1,541	0,123
Temperature	-0,1104	0,0128	-8,625	<2e-16 ***
C				
Intercept	0,155083	1,027599	0,151	0,8800
EpitypeWE	-2,168352	1,320432	-1,642	0,1006
Temperature	0,032601	0,005556	5,868	4.42e-09 ***
EpitypeWE:Temperature	-0,018194	0,008055	-2,259	0,0239*

*Significance codes: ***P<0.001 **P<0.01 *P<0.05*

The estimated temperature at which 50 % of the needles, cambium and buds were dead (LT50) was calculated for all the four timepoints (Table 9).

In March 2018, the estimated LT50 was higher in CE than in WE for needles (7 °C) and cambium (>6 °C). In CE, LT50 for buds could not be estimated, since more than 50 % of the buds were dead for all test temperatures except the control.

In April, the estimated LT50 was higher in CE than in WE for needles (4 °C) and buds (1 °C), while for cambium it was the same for both epitypes.

In May, the estimated LT50 for needles was almost the same (0,5 °C higher in CE) for both epitypes, LT50 for cambium was the same and LT50 for buds were higher in CE than in WE (>4 °C). The curve for percent dead buds for CE crossed the LT50 line two times (at +2 and -11 °C), due to more of the control buds being dead than the buds in the highest freezing temperatures.

In March 2019, the estimated LT50 was lower in CE than in WE for both needles (2 °C) and cambium (5 °C). LT50 for buds could not be estimated since more than 50 % of the buds were dead for all temperatures including the control for WE, and for all temperatures except -25 °C for CE.

Table 9. The estimated test temperatures (based on the results in Fig. 2) at which 50 % of the biomass of the needles and cambium and 50 % of the buds were dead (LT50) for the two epitypes of Norway spruce harvested in a common garden in Ås, Norway, on four collection dates. CE = cold epitype, WE = warm epitype.

12 March 2018

	Needles	Cambium	Buds
CE	-37	-39	—
WE	-44	< -45	-30

23 April 2018

	Needles	Cambium	Buds
CE	-18	-22,5	-21,5
WE	-22	-22,5	-22,5

7 May 2018

	Needles	Cambium	Buds
CE	-12,5	< -15	-11
WE	-13	< -15	< -15

8 May 2019

	Needles	Cambium	Buds
CE	-32	-40	-
WE	-30	-35	-

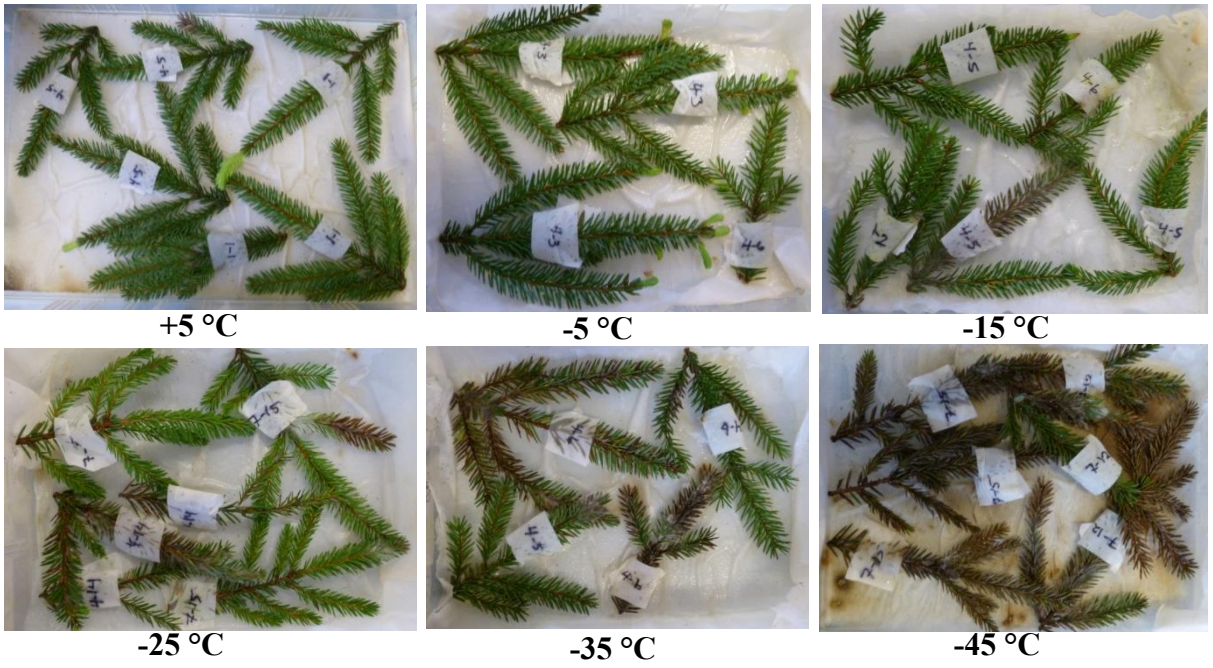

Fig. 3. Twigs of Norway spruce epitypes harvested in a common garden in Ås, Norway, 12 March 2018 and frozen at 5 different freeze temperatures and one control temperature (5 °C), three weeks after freezing. The pictures show a random mix of individuals and epitypes.

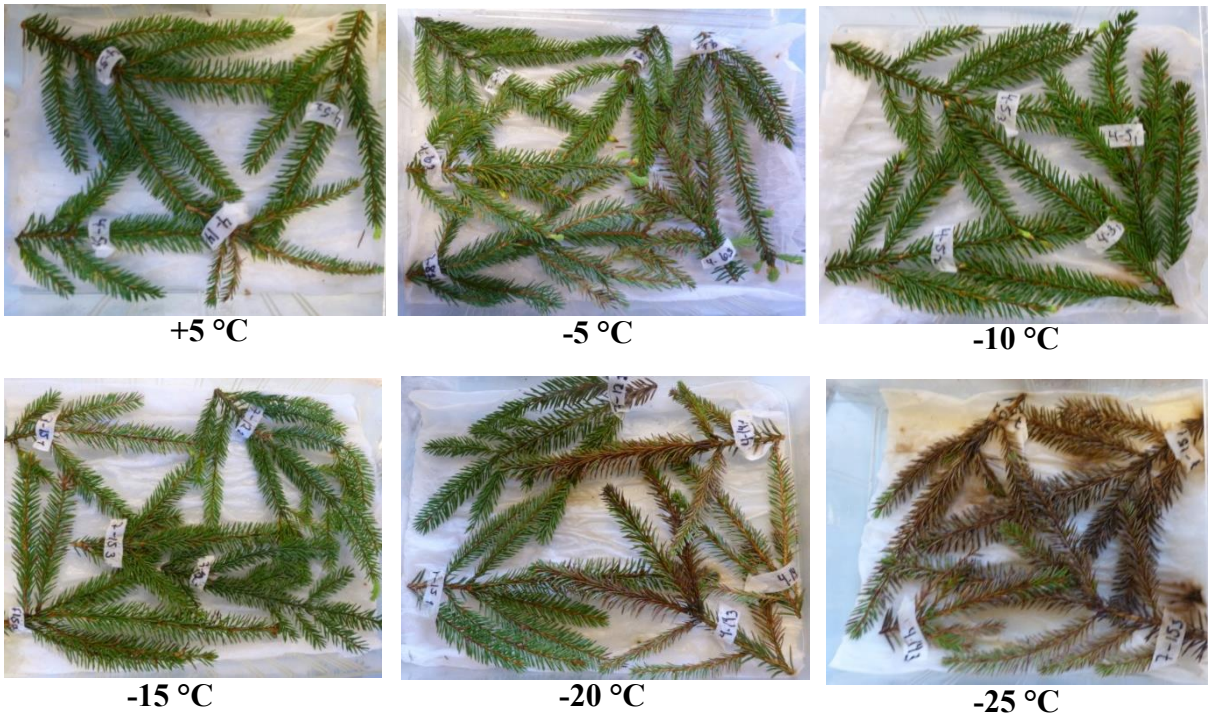

Fig. 4. Twigs of Norway spruce epitypes harvested in a common garden in Ås, Norway, 23 April 2018 and frozen at 5 different freeze temperatures and one control temperature (5 °C), three weeks after freezing. The pictures show a random mix of individuals and epitypes.

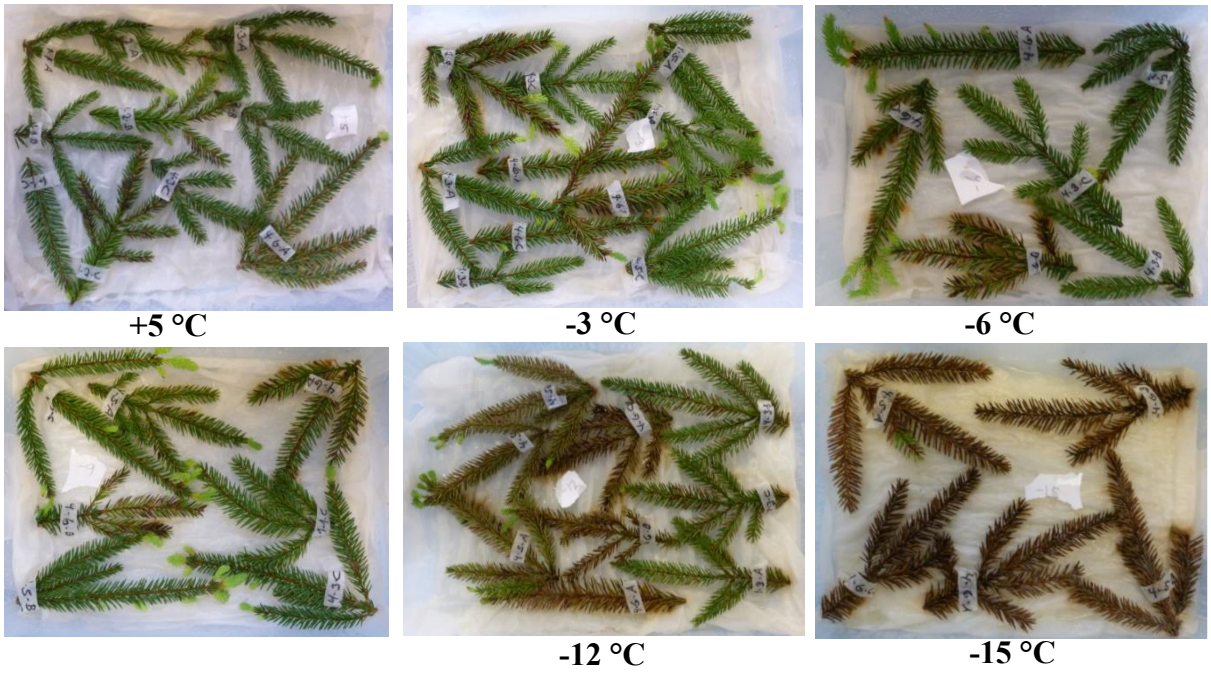

Fig. 5. Twigs of Norway spruce epitypes harvested in a common garden in Ås, Norway, 7 May 2018 and frozen at 5 different freeze temperatures and one control temperature (5 °C), three weeks after freezing. The pictures show a random mix of individuals and epitypes.

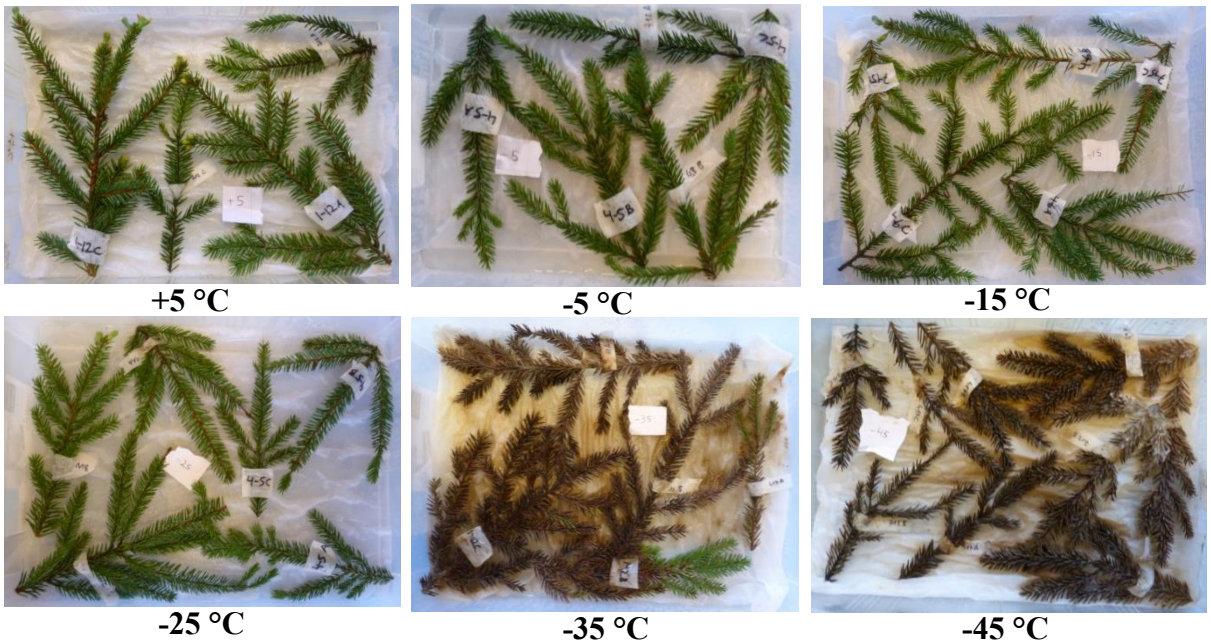

Fig. 6. Twigs of Norway spruce epitypes harvested in a common garden in Ås, Norway, 8 March 2019 and frozen at 5 different freeze temperatures and one control temperature (5 °C), three weeks after freezing. The pictures show a random mix of individuals and epitypes.

3.1.3 Bud burst

Some of the buds had burst in the twigs from the freeze test after three weeks. The number of buds that had burst in the control twigs was recorded and analyzed with a binomial model for each of the collection dates. There was not any significant difference in number of burst buds between the epitypes for any of the timepoints.

3.2 Gene expression analysis

PaDHN6 transcript levels differed significantly between the epitypes (p=0,015), (Fig. 7). There was also a significant interaction present between collection date and epitype (p=0,018); the effect of epitype was present only in March (p=0,017). CE had 1,6 times higher transcript levels than WE. The effect of collection date was highly significant (p≤0,001), in CE the transcript level was 122 times higher in March than in May and in WE the transcript level was 119 times higher in March than in May.

PaDHN40 transcript levels differed significantly between the epitypes (p=0,031). There was also a significant interaction present between collection date and epitype (p=0,031); the effect of epitype was present only in March (p=0,031). CE had 1,9 times higher transcript levels than WE. The effect of collection date was highly significant (p≤0,001), in CE the transcript level was 420 times higher in March than in May and in WE the transcript level was 159 times higher in March than in May.

PaFTL2 transcript levels showed no significant difference between the epitypes, and there was no significant interaction between epitype and collection date. The transcript levels were significantly affected by collection date (p=0,001). In CE the transcript level was 22 times higher in March than in May and in WE the transcript level was 26 times higher in March than in May.

PaCMT3 transcript levels did not differ significantly between the epitypes and there was no significant interaction between epitype and collection date. Collection date had a significant effect on the transcript levels (p=0,001). In CE the transcript level was 65 times higher in May than in March and in WE the transcript level was 44 times higher in May than in March.

PaHDT1 transcript levels showed no significant differences between the epitypes or between the collection dates and there was no significant interaction.

PaHDT2 transcript levels were not significantly affected by epitype or collection date, and there was no significant interaction, but there was a trend of higher transcript levels in March than in May (p=0,097).

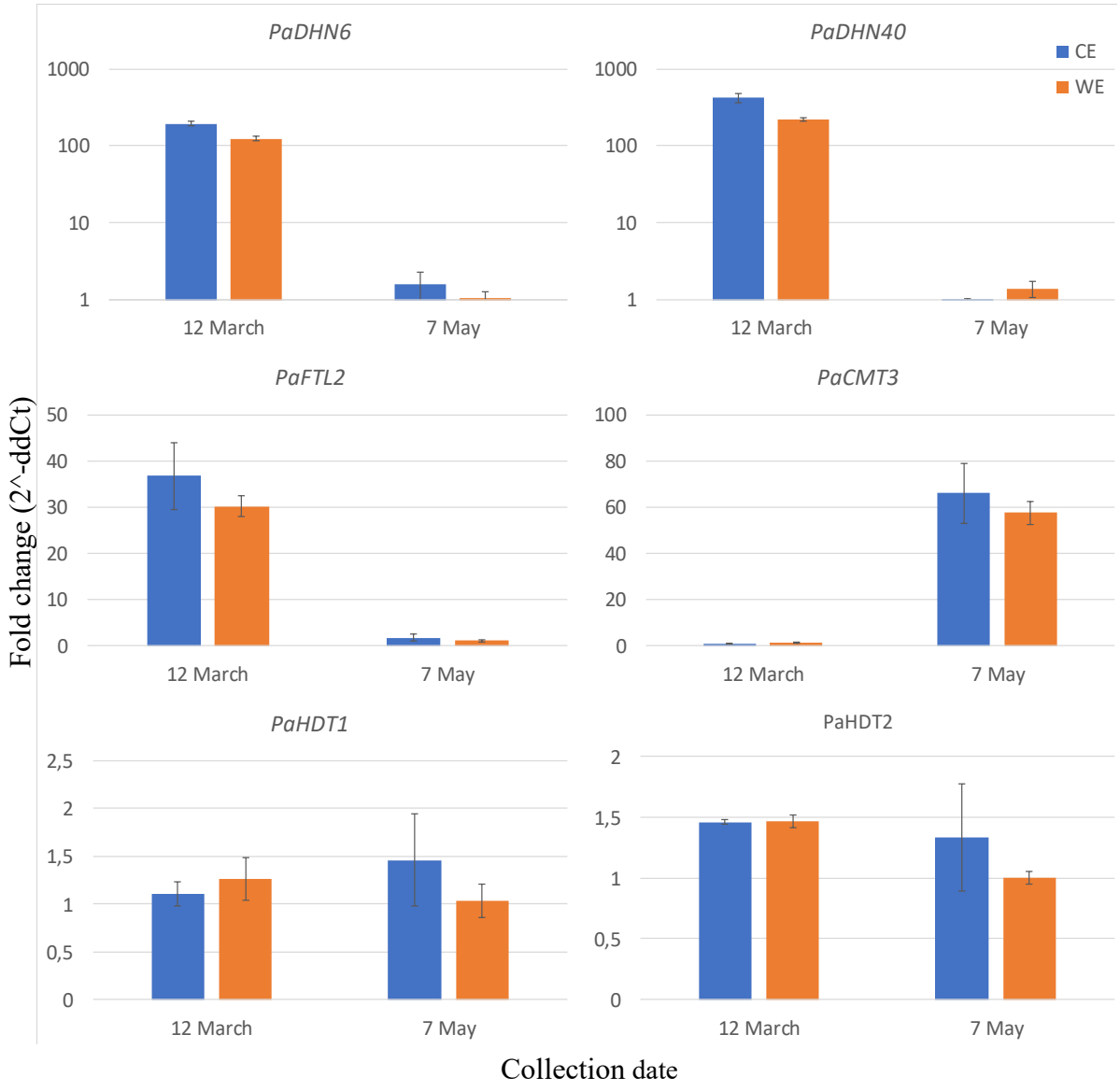

Fig. 7. Relative transcript levels of the genes *DEHYDRIN 6* (*PaDHN6*), *DEHYDRIN 40* (*PaDHN40*), *FLOWERING LOCUS T-TERMINAL FLOWER 1-LIKE 2* (*PaFTL2*), *DNA (CYTOSINE-5)-METHYLTRANSFERASE CMT3 ISOFORM* (*PaCMT3*), *HISTONE DEACETYLASE HDT1* (*PaHDT1*) and *HISTONE DEACETYLASE HDT2* (*PaHDT2*) in buds from the warm epitype (WE) and the cold epitype (CE) of Norway spruce, collected in March and May 2018. The results are average of three biological replicates (individuals) ± SE.

3.3 Immunolocalization of epigenetic marks

All the buds, both those marked with AcH4 and with 5-mdC antibodies, had lower intensity of the fluorescence signal in the pith than in the rest of the tissues of the bud. The DAPI signal followed the same pattern of intensity (Fig. 8-11). There was fewer cells in the pith than in the rest of the bud.

3.3.1 DNA methylation

DNA methylation was present in all parts of the bud, but the intensity was different among different tissues. The pattern differed slightly between the two collection dates and the epitypes (Fig. 8-9; all collected in 2018).

Generally, the WE buds collected in March were more methylated in the procambium than in the rest of the bud (Fig. 8B.). Three individuals (3679, 3663 and 3677) showed this pattern quite clear, one had a less clear pattern (3685) and one individual did not have such a pattern at all (3665). At least two of the individuals (3663 and 3679) were more methylated in the shoot apical meristem (SAM) than in the rest of the bud, in addition to one individual (3665) with more methylation in the whole upper part of the bud (shoot apex).

The CE buds collected in March also had a pattern of more methylation in the procambium (individuals 3644 and 3669) (Fig. 8A), but it was weaker than in WE (Fig. 9B). Two of the individuals were more methylated in the base of the bud (3644 and 3667), while the third individual was more methylated in the top (3669).

The WE buds collected in May varied in their pattern of DNA methylation (Fig. 9B). One individual (3679) had a quite weak pattern of more methylation in the procambium and in the SAM than in the rest of the bud, in addition to a little more methylation in the tips of the leaf primordia. Two of the other individuals (3677 and 3663) also had a weak pattern of more methylation in the tips of the leaf primordia, 3677 was cut so that it lacked the upper part. Two individuals (3663 and 3665) seemed to be less methylated in the central zone of SAM than in the peripheral zone.

The CE buds collected in May had quite evenly distributed methylation, except for one individual (3644), which had a little more methylation in the tips of the leaf primordia (Fig. 9A.).

3.3.2 Histone 4 acetylation

There were no clear differences in the pattern of histone 4 acetylation (AcH4) between the epitypes or between the two collection dates (Fig. 10-11). The acetylation signal was evenly distributed and present in all tissues of the buds, but it was quite nonspecific, with some fluorescence signal also outside of the nuclei.

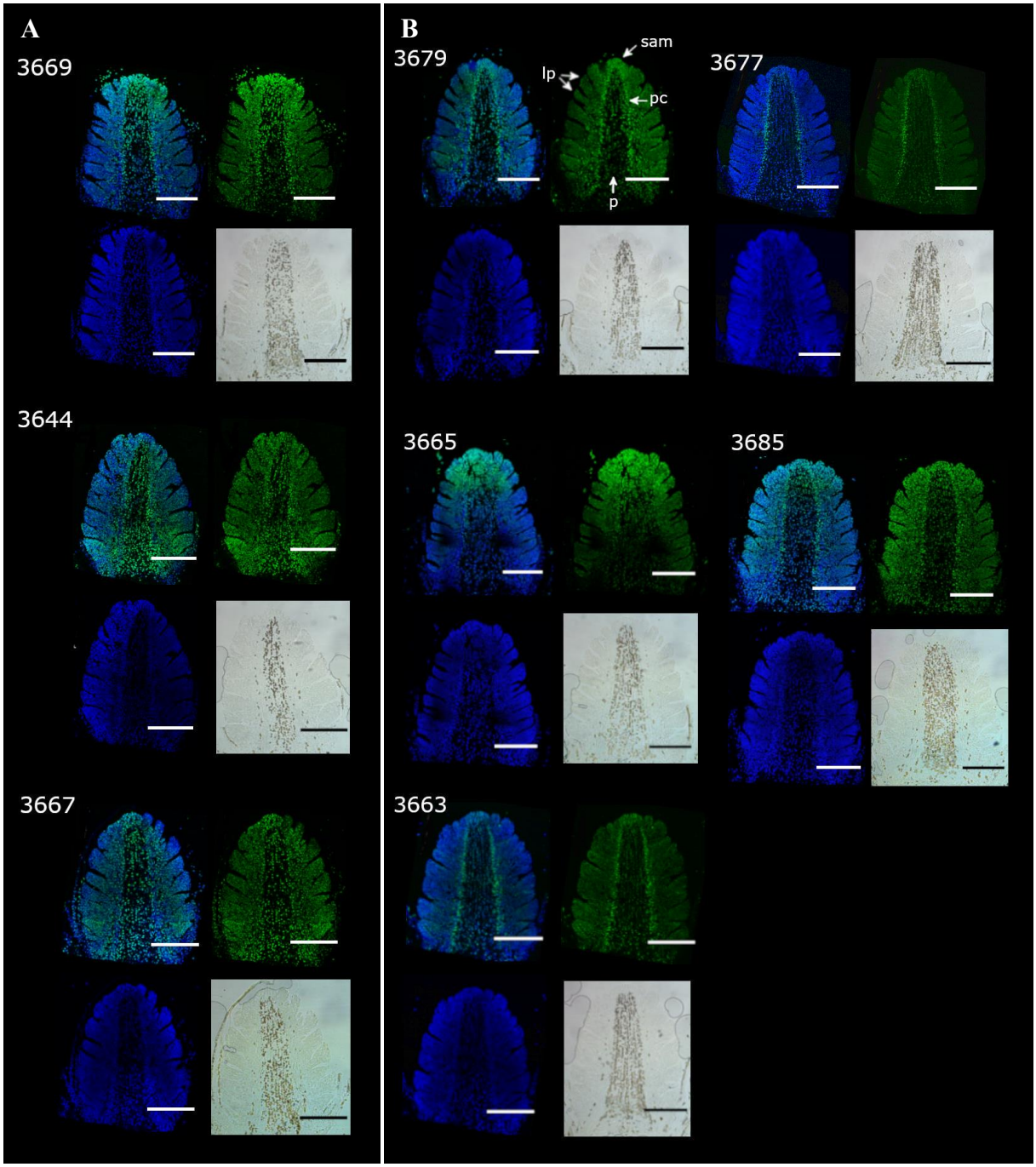

Fig. 8. Immunolocalization of 5-methyl-2'-deoxycytidine (5-mdc) in buds of Norway spruce epitypes collected 12 March 2018. A) Three individuals from the cold epitype (CE). B) Five individuals from the warm epitype (WE). Each individual is represented in four pictures: one brigth field picture showing the structure and three fluorescence pictures; one showing the nuclei stained with DAPI (blue), one showing 5-mdc (green) and one picture showing the combination of DAPI and 5-mdc (blue and green). Numbers are individual ID. The scale bars are 500 µm. sam = shoot apical meristem, lp = leaf primordia, pc = procambium, p = pith.

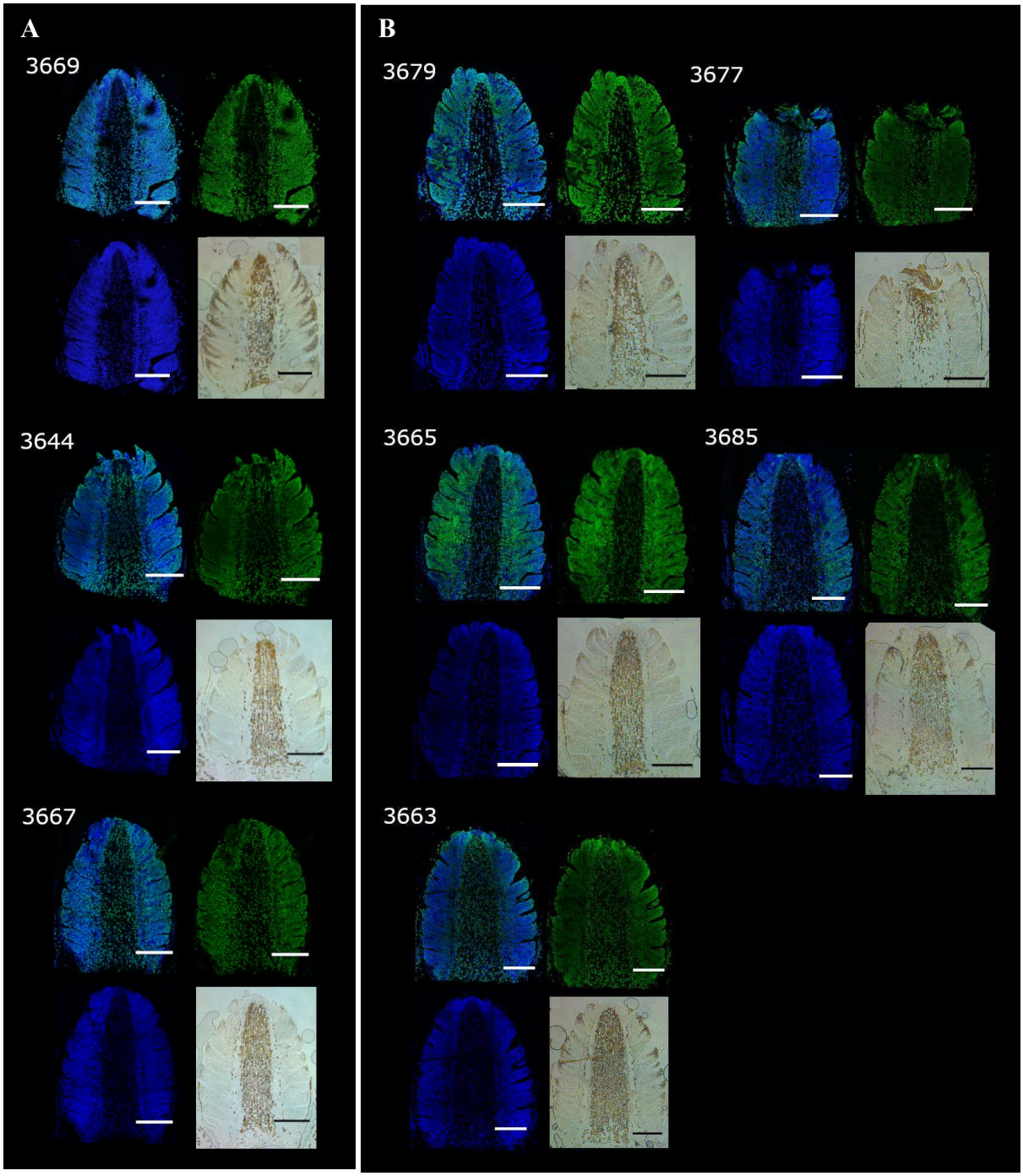

Fig. 9. Immunolocalization of 5-methyl-2'-deoxycytidine (5-mdc) in buds of Norway spruce epitypes collected 7 May 2018. A) Three individuals from the cold epitype (CE). B) Five individuals from the warm epitype (WE). Each individual is represented in four pictures: one brigth field picture showing the structure and three fluorescence pictures; one showing the nuclei stained with DAPI (blue), one showing 5-mdc (green) and one picture showing the combination of DAPI and 5-mdc (blue and green). Numbers are individual ID. The scale bars are 500 μm.

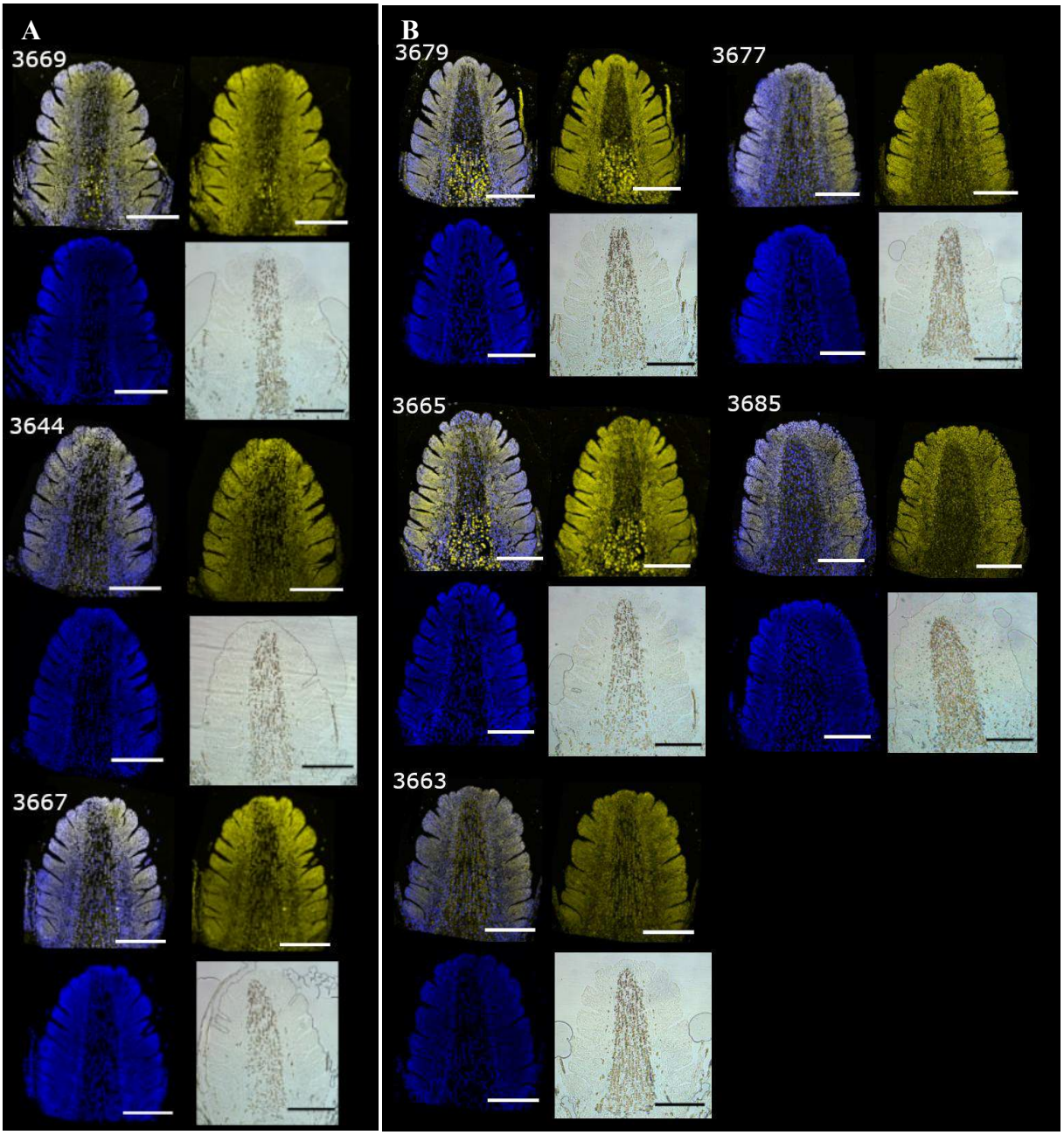

Fig. 10. Immunolocalization of histone 4 acetylation (AcH4) in buds of Norway spruce epitypes collected 12 March 2018. A) Three individuals from the cold epitype (CE). B) Five individuals from the warm epitype (WE). Each individual is represented in four pictures: one brigth field picture showing the structure and three fluorescence pictures; one showing the nuclei stained with DAPI (blue), one showing H4ac (yellow) and one picture showing the combination of DAPI and AcH4 (blue and yellow). Numbers are individual ID. The scale bars are 500 µm.

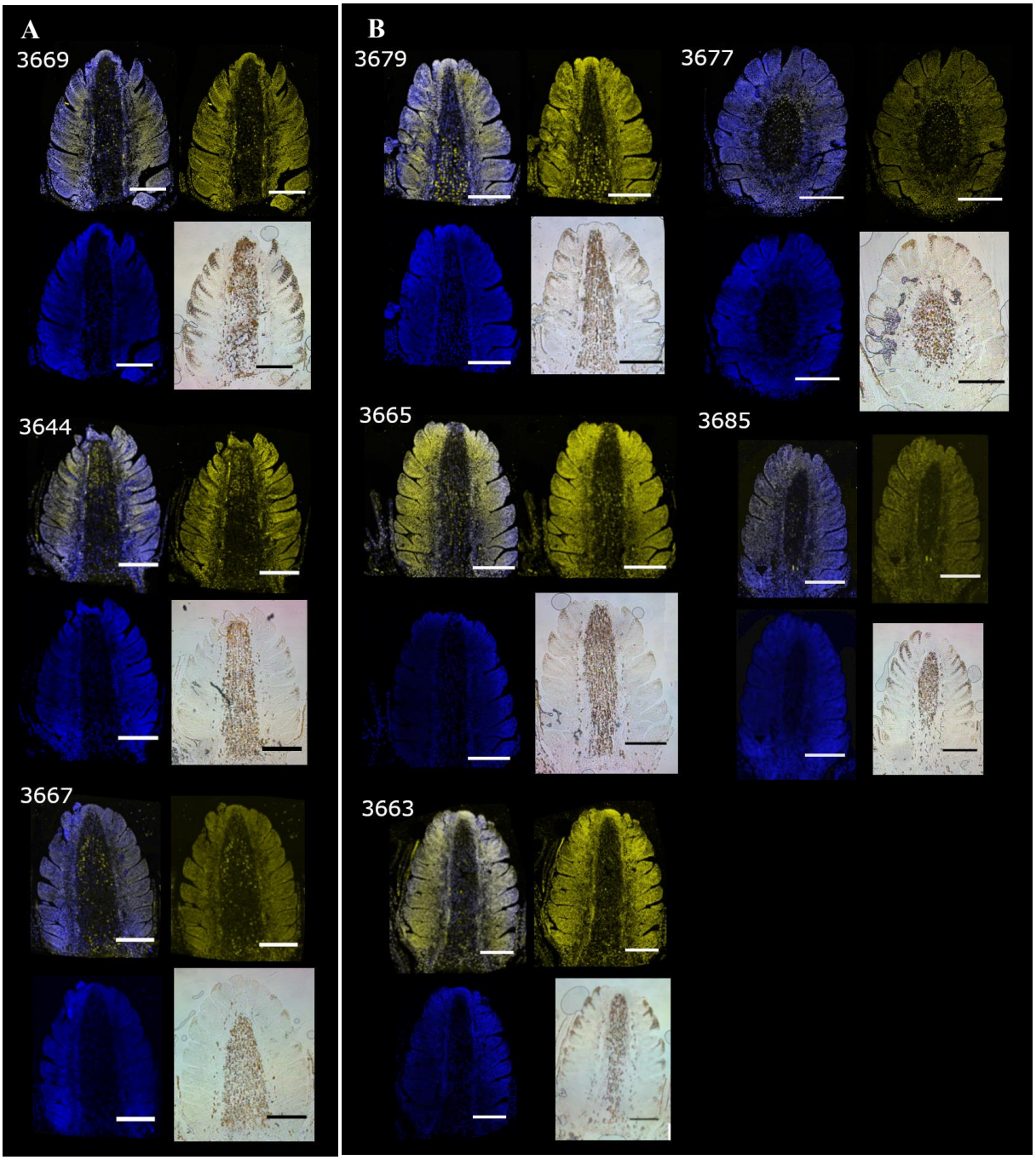

Fig. 11. Immunolocalization of histone 4 acetylation (AcH4) in buds of Norway spruce epitypes collected 7 May 2018. A) Three individuals from the cold epitype (CE). B) Five individuals from the warm epitype (WE). Each individual is represented in four pictures: one brigth field picture showing the structure and three fluorescence pictures; one showing the nuclei stained with DAPI (blue), one showing H4ac (yellow) and one picture showing the combination of DAPI and AcH4 (blue and yellow). Numbers are individual ID. The scale bars are 500 µm.

3.4 Daily mean temperatures

The daily mean temperatures one month prior to the collection date was higher for the collection 8 March 2019 than for 12 March 2018. Prior to the collection in March 2018 every day except one had a mean temperature below 0 °C (Fig. 12), while prior to the collection in March 2019, every day except five had a mean temperature above 0 °C (Fig. 13).

Prior to the collection 23 April 2018, the daily mean temperatures were rising from below 0 °C in the end of March to around 8 °C in the middle of April, were it stayed until the collection day.

The daily mean temperatures were above 0 °C for all the days one month prior to the collection 7 May 2018. The mean temperature was rising rapidly from around 8 °C three days before collection to 13 °C on the collection day.

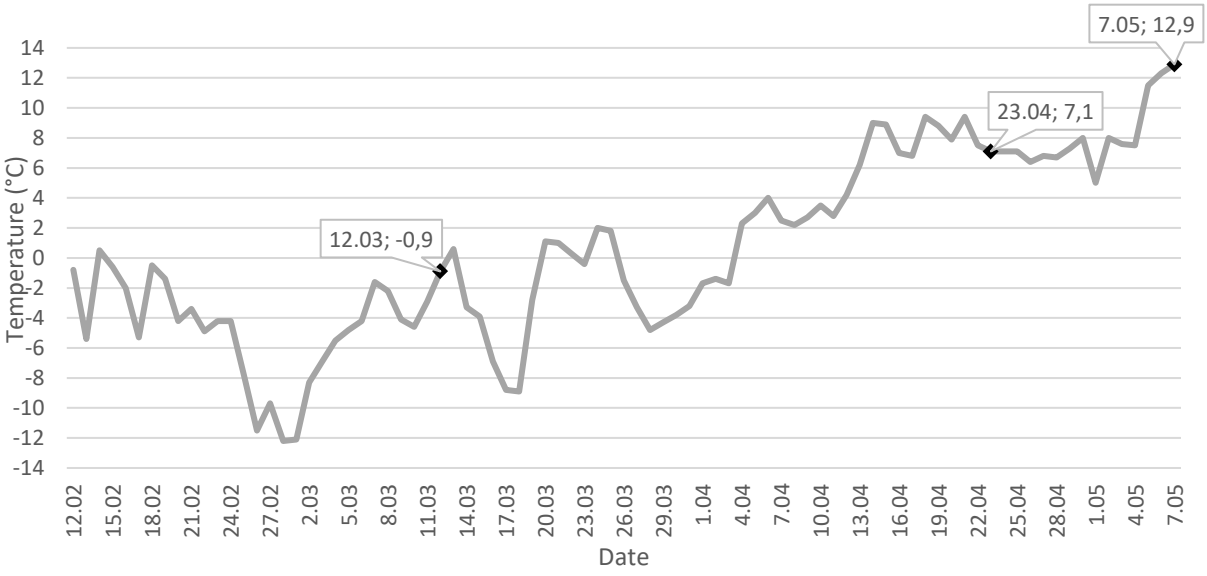

Fig. 12. Daily mean temperatures in Ås, Norway during the collection period of samples of Norway spruce epitypes in 2018, from one month prior to the first collection until the day of the last collection. The collection days (12 March, 23 April and 7 May 2018) are marked with black dots. The temperature data comes from the Sørås Field Station for Agroclimatic studies, Norwegian University of Life Sciences in Ås, Norway (N 59° 39' 37", Ø 10 ° 46' 54", 93.3 MOH), which is situated approximately 4 km from the collection site.

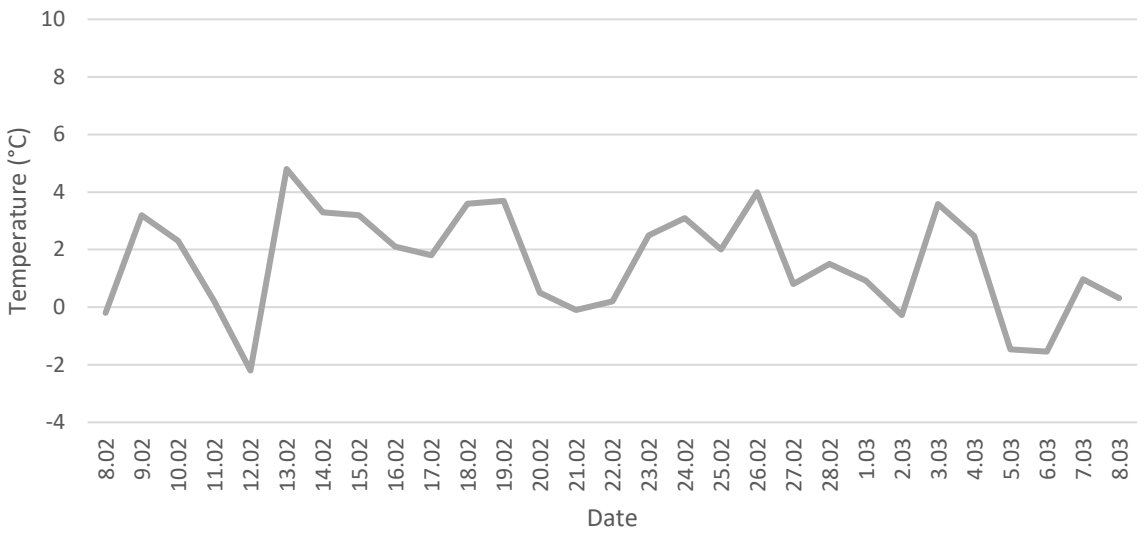

Fig. 13. Daily mean temperatures in Ås, Norway, one month prior to the collection of samples of Norway spruce epitypes on 8 March 2019. The temperature data comes from the Sørås Field Station for Agroclimatic studies, Norwegian University of Life Sciences in Ås, Norway (N 59° 39' 37", Ø 10 ° 46' 54", 93.3 MOH), which is situated approximately 4 km from the collection site.

4.0 Discussion

Different temperatures during embryogenesis in Norway spruce have been shown to affect traits in the trees grown from these embryos (Yakovlev et al., 2012). In this study, individuals of two genetically identical epitypes grown from somatic embryos developed under 18 °C and 28 °C were tested for differences in freeze tolerance. Such freeze tests have not earlier been performed on the mentioned epitypes of Norway spruce. It was hypothesized that WE would be more frost tolerant than CE because plants from seeds (zygotic embryos) developed in warmer temperatures have been shown to be more frost tolerant in the spring than plants from seeds developed in colder temperatures (Skrøppa et al. 2007). Expression of genes related to cold hardiness (dehydrins), growth cessation, bud set and bud burst (*PaFTL2*) and epigenetics (*PaCMT3*, *PaHDT1* and *PaHDT2*) were studied and *in situ* localization of DNA methylation and histone 4 acetylation were performed to investigate if the two epitypes showed any difference in gene expression and distribution of key epigenetic marks.

4.1 Cold hardiness testing

One week after freezing, the difference in needle damage between the epitypes was significant for both March and April. After three weeks, the difference was no longer significant for the twigs collected in March. This is probably because WE developed more damage between the two evaluations than CE did for the lowest freeze temperature (Fig. 1 and 2). CE in March and both CE and WE in April showed a quite small difference in damage development between one week and three weeks after freezing (Fig. 1 and 2). This may indicate that it could have been sufficient or better to do the complete damage evaluation after only one week, or some time between one and three weeks after freezing. Only needle damage was evaluated after one week, so it is not known how the cambium and bud damage were developing between the two evaluations.

As expected, cold hardiness decreased during spring (Fig. 2 and Table 9), as the daily mean temperature increased (Fig. 12). Some of the twigs collected 12 March 2018 survived -45 °C, and most of the twigs had little damage at freeze temperatures of -15 °C (Fig. 3), while the twigs collected 23 April 2018 were quite damaged at -25 °C (Fig. 4), and many of the twigs collected 7 May 2018 did not survive -15 °C (Fig. 5). This is because the trees experience dehardening in spring to prepare for the growing season. This dehardening is induced by

warmer temperatures after a cold period that breaks the endodormancy (Welling and Palva 2006, Lang et al. 1987).

Twigs collected 8 March 2019 seemed to be less frost tolerant than the twigs collected in March 2018 since those collected in 2019 were more damaged at the two lowest freezing temperatures (Fig. 1 and 6). This is probably due to the warmer temperatures the weeks before collection in 2019 than in 2018 (Fig. 12-13), since warmer temperatures are known to induce dehardening.

WE tolerated lower temperatures than CE for all collection dates, the difference was significant for at least one of the three tissues examined for each timepoint (Fig. 2). The only exception was bud damage in April, where WE had significant more damage than CE, but the difference seemed to be quite small and only applied to the test temperatures higher than -15 °C. Also, in March 2019, there seemed to be more cambium and bud damage in WE than in CE, but the difference was not significant. Cambium damage in April and May, and needle damage in March 2018, did not show significant differences between the epitypes, but there was a trend in March 2018 for needles to be more damaged in CE than WE for the test temperature -25 °C and lower.

Less frost damage in WE than in CE was as expected, since CE starts bud burst earlier than WE (Carneros et al. 2017), and thus probably also dehardens earlier than WE. It is also consistent with studies of frost tolerance in Norway spruce plants, where plants from seeds developed in warmer areas had less frost damage in spring than plants from seeds developed in colder areas (Skrøppa et al. 2007; Yakovlev et al. 2012).

The pattern of more damage at lower freezing temperatures than at higher temperatures was not as clear for the buds. Some of the twigs had high percentages of dead buds also for the control temperature. The reason these buds were dead could not be freeze damage as they had only been exposed to 5 °C. These buds were probably dead already before collection. One reason for the high amount of dead buds at non-freezing temperature might be that some of the lower branches of the trees were dying from lack of light because of high tree density in the plantation.

The high number of dead buds before freezing might have disturbed the analysis since it cannot be known how many of the buds in the different freeze temperatures died because of the freezing or how many were already dead. The LT50 values for buds could not be

estimated for all the collection dates since in some cases more than 50 % of the buds were dead for the control or for different test temperatures (Table 9). The LT50 values for buds would probably have been lower if more of the buds had been alive before freezing.

In the last half of May 2018, the weather got unusually warm (Fig. 12), which affected the temperature in the room where the twigs were developing damage. The two last weeks before evaluation of the freeze damage for the twigs collected 7 May, the room temperature was monitored. This showed that the temperature was between 28 and 35 °C every day of these two weeks (Appendix 1). It is possible that this might have made the twigs develop damage faster, but since all the twigs were affected, and the damage was seen in relation to the control twigs, the high temperature probably did not affect the results very much.

It should be noted that short-term controlled tests in freeze chambers might not be representative of the actual frost tolerance in nature (Waalen et al. 2011, Gusta & Wisniewski, 2013). Trees in the field are more exposed to variations in temperature and faster or slower cooling, and they might also experience longer periods of low freezing temperatures. Waalen et al. (2011) found that freeze tests over longer periods give better estimates of actual freeze tolerance than shorter tests. The cutting wound might also make the twigs more vulnerable to damage.

4.1 Bud burst

In contrast to what has been observed in the field, there was not any difference in number of burst buds between the epitypes in the control twigs from the freeze test. The buds were only recorded as burst/not burst, where burst meant that the green leaf initials were sticking out between the bud scales. It might be possible that more detailed recording of different bud burst stages would have given other results.

4.2 Gene expression analysis

The only genes that showed significant differences in transcript levels between the epitypes were the two dehydrins, *PaDHN6* and *PaDHN40*. For both, transcript levels were higher in CE than in WE in March. *PaDHN6* was expressed 1,6 times more, and *PaDHN40* 1,9 times more in CE than in WE. Higher expression of dehydrins in CE than WE was the opposite of expected, as dehydrins are associated with frost tolerance (Welling & Palva, 2006), and WE was shown to be more frost tolerant than CE. Carneros et al. (2017) showed that *PaDHN6*,

PaDHN40 and other dehydrins were expressed at a higher level in WE than in CE for several timepoints in spring. There were also some timepoints when the difference was not significant, and the first collection date was 20 April. Thus, there is no previous information of the expression of *DHN*s in these two epitypes in March. Yakovlev et al. (2008) studied *DHN* expression in Norway spruce in relation to bud burst. They found that *DHN* expression decreased towards bud burst, and that late flushing families had higher expression than early flushing families at the same timepoint. The results of the present study contrast this, as the early flushing epitype (CE) had higher *DHN* transcript levels than the late flushing epitype (WE) in March. This is an earlier timepoint than was studied in Yakovlev et al. (2008), where the first collection date was 1 April, and on this date the difference in transcript level (of *DHN6*) between the late flushing and early flushing families was small. In early May, the difference was at its maximum, which contrasts with the present study, where the difference in *DHN6* transcript level between the epitypes was insignificant in May.

Expression of both the *DHN*s decreased from March to May; *PaDHN6* was expressed about 120 times higher in March than in May for both epitypes, while *PaDHN40* had more than 400 times higher expression in March than in May in CE and 160 times more in WE. For *PaDHN6*, this is a somewhat smaller decrease than in Yakovlev et al. (2008), where *PaDHN6* was found to have a 250-fold decrease from 1 April until the middle of June. Carneros et al. (2017) found that expression of *PaDHN6* and *PaDHN40* were more stable in CE than WE, as there was no significant difference between the collection dates in CE. WE on the other hand, did first increase its expression of *PaDHN6* and *PaDHN40*, which peaked 3-4 weeks before bud burst, before it decreased again until bud burst. In the present study, both epitypes showed a large decrease in *DHN* expression from March to May, but for *DHN40*, the decrease was smaller in WE than in CE. Since only two timepoints were studied, and bud burst time was not recorded, the peak in expression 3-4 weeks before bud burst cannot be confirmed or rejected.

PaFTL2 showed significantly higher transcript levels in the buds collected in March compared to the buds collected in May. In WE, the transcript level was 28 times higher in March than in May, while in CE, the transcript level was 22 times higher in March than in May. This is in consistent with the action of the FTL2 protein as a growth inhibitor, which is known to increase in SD and during growth cessation and bud set, and then decrease again during bud burst (Gyllenstrand et al. 2007, Asante et al. 2011; Karlgren et al. 2013, Opseth et

al. 2016). Gyllenstrand et al. (2007) found that *PaFTL2* was expressed around twice as much in January than in April, and more than 200 times more in September than in April.

There were no significant differences between the epitypes in transcript levels of *PaFTL2*. This is partly consistent with Carneros et al. (2017), where differences in *PaFTL2* expression between the epitypes were non-significant, except for the two last collection dates in May. For these two dates, WE had higher *PaFTL2* expression than CE, which probably is because CE show bud burst earlier than WE, and *PaFTL2* is known to decrease towards bud burst. The second last collection date was one week after CE experienced bud burst and the last collection date was the same day WE experienced bud burst, thus it seems like the difference in *PaFTL2* expression between the epitypes is largest after bud burst in one of the epitypes. In the present study, the last collection date was before bud burst in both epitypes, which may explain that there was no difference between the epitypes.

PaCMT3 had significantly higher transcript levels in May compared to March. DNA methyltransferases are inactivators of gene expression, and it could be expected that the transcript levels would decrease when dormancy is released and the plant starts growing, since it needs to activate many genes that are involved in bud burst, growing, metabolism, etc. On the other hand, *PaCMT3* is a chromomethylase, which maintains methylation, i.e. methylates the new DNA strand that is made during replication (Chen et al. 2010, Law and Jacobsen 2010). Thus, it might be more expressed during cell division, since there will be many newly synthesized strands that needs to be methylated. This could explain the increase in *PaCMT3* transcript level from March to May, as the cells in the buds might have started to divide in preparation for bud burst. There were no significant differences between the epitypes in *PaCMT3* transcript level. It could have been expected that CE should have higher transcript levels of this gene than WE, since it is more advanced towards bud burst. This is supported by data from an unpublished RNAseq analysis (Viejo et al., unpublished), which showed that *PaCMT3* was upregulated in buds from CE compared to WE. The buds used in the RNA seq analysis were collected 20 April. Maybe on this timepoint, CE had started cell division and thus upregulated *PaCMT3*, while WE was still dormant, while in March and in May, when the buds for the present study were collected both epitypes were dormant (March) or dividing (May), and thus had the same level of *PaCMT3* at each timepoint.

Neither of the histone deacetylases, *PaHDT1* and *PaHDT2*, showed any significant differences in transcript levels between the epitypes or the collection dates. Histone

deacetylases are known to inactivate genes by removing acetyl groups from the histones. Removal of acetyl groups leads to tighter binding between histones and DNA, which prevents transcription (Hollender and Liu 2008, Ruijter et al. 2003). Because of this, it may be hypothesized that the expression would decrease during spring, and that WE would have higher transcript levels than CE, since WE experiences bud burst later than CE. Consistent with this, Yakovlev et al. (2006) found that a histone deacetylase (*HDAC*) was downregulated in Norway spruce plants from an early flushing family compared to a late flushing family. Unpublished data from RNAseq of buds of the two epitypes show that *PaHDT1* was downregulated in WE compared to CE, while *PaHDT2* was upregulated in WE compared to CE in buds (Viejo et al., unpublished). Different histone deacetylases might remove acetyl groups from different genes. If a histone deacetylase is associated with silencing genes involved in maintaining dormancy and cold hardiness, it might be expected to have higher expression towards bud burst, while de opposite would be true for histone deacetylases associated with genes involved in bud burst and growth.

The results show little differences in transcript levels between the epitypes, the only significant difference was for the *DHN*s in March. This could in part be due to large variance between the samples. For example, transcript levels of *PaDHN6* in May had the same average difference between the epitypes (1,6 times higher in CE than WE) as in March, but the variance was larger, so the difference was not significant. It is possible that the results could have been more precise if oligodT primers had been used instead of random hexamer primers for the cDNA synthesis. OligodT primers will only synthesize cDNA from mRNA, and the problem of genomic DNA disturbing the results are avoided. Instead, to avoid this problem, the primers used for the qPCR were designed to include exon-exon junctions, since only cDNA derived from mRNA contains these. However, for the two *DHN*s, it was not possible to design exon-exon junction primers, since there was no information on introns and exons in the database sequence (congenie.org). Thus, it is possible that genomic DNA might have disturbed the results for the *DHNs.* The samples were checked for genomic DNA using samples without reverse transcriptase (-RT) for the cDNA synthesis. Some genomic DNA was then found, as the difference between the -RT and RT samples was 5-10 Ct values, while ideally, the difference should be >10 Ct values. Also, after the qPCR had been done, it was discovered that the *DHN6* primers were quite unspecific, as both had two matching sites (with some base differences) in the *DHN6* sequence. However, regardless where they matched, they would result in amplification of a sequence from *DHN6*.

4.3 *In situ* localization of epigenetic marks

4.3.1 DNA methylation

The pattern of 5-mdc was slightly different between the epitypes and the collection dates, but the difference was not very clear. The buds collected in March had a pattern of more methylation in the procambium and in the SAM than in other parts of the bud, especially for WE (Fig. 8). CE had only one bud (3669) with this pattern in March, while other CE buds had more methylation in the base of the buds.

In May, WE had a pattern of more methylation in the tips of the leaf primordia, one CE individual (3644) also had this pattern (Fig. 10). Some WE buds in May also seemed to be less methylated in the central zone of SAM and more in the peripheral zone.

The pattern varied between the different buds in each epitype and collection date. For example, one WE individual (3665) in March did not have the same pattern as the others, instead it had more methylation in the whole of the upper third part of the bud. One individual (3669) also stood out from the other CE individuals in March; it had more methylation in the upper part of the bud, while the others had more in the lower part of the buds.

Conde et al. (2017) found that in buds of *Castanea sativa* the central zone had increased DNA methylation in SD, while in long days, the rest of the SAM was more methylated. Buds of *Castanea sativa* have also been shown to have less 5-mdC in the SAM than in the rest of the bud during bud burst, while during bud set, they had more 5-mdC in the SAM, procambium and leaf primordia (Santamaria et al. 2009). Less DNA methylation in the SAM during bud burst is similar to the results of the present study, as the buds that were closer to bud burst (collected in May) did have less methylation in parts of the SAM than in the rest of the buds. However, this only applied to some of the WE buds, which contradicts the findings of Santamaria et al., (2009), since CE probably was closer to bud burst than WE. Meijon et al. (2009) found higher levels of 5-mdC in the tunica, procambium and leaf primordia than in the corpus in vegetative buds of *Azalea*. This is partly consistent with the buds collected in March, as they also showed more methylation in the procambium and the SAM.

4.3.2 Histone 4 acetylation

The H4-acetylation (AcH4) was evenly distributed in the buds (Fig. 11-12). There was no clear difference in the pattern between the epitypes or collection dates. This might be at least

partly because the protocol and/or solutions were not optimal. The sections seemed to have some nonspecific binding, since there was fluorescence signal also inbetween the nuclei. The original protocol was modified to try to get rid of the problem of nonspecific binding. The primary antibody concentration was reduced and the slides were incubated with the primary antibody at 37 °C instead of in room temperature. Higher concentrations of BSA were also used for blocking. This helped somewhat, but did not get rid of all the nonspecific binding. It is possible that some of the sections might have dried during the incubation at 37 °C, which could have led to binding of the primary antibody to the tissue, so that it would not be washed away. Other modifications that were not tried but might have improved the specificity, are to reduce the incubation time of the antibodies, or to incubate overnight at 4 °C.

Both Meijon et al. (2009) and Santamaria et al. (2009) found that the pattern of AcH4 in vegetative buds was the opposite of the pattern of 5-mdc. Meijon et al. (2009) found higher levels of AcH4 in the corpus of the SAM, where the 5-mdc was low. Santamaria (2009) found that bursting buds had higher levels of AcH4 in the SAM, while during bud set there was less AcH4 in the SAM, and more in the subapical central zone. This is not what was found in the present study, where the histone acetylation was evenly distributed, and the pattern did not change between the epitypes or the collection dates.

4.4 Other factors that might have affected the results

In the field, the trees looked quite different from each other. Some were tall with long green twigs, while others were small with several dead branches. For example, one individual (3644) was short and with most of the lower twigs dead, and the green twigs were small and weak compared to some of the other trees. Another individual (3665) was bent over and was almost lying horizontally on the ground.

In 2018, many of the trees had some yellow needles. This was probably caused by infection by the fungi Spruce needle rust (*Chrysomyxa abietis*) (Roll-Hansen, 1981; Roll-Hansen & Roll-Hansen, 1987). The amount of yellow needles in the collected twigs was recorded before freezing, using categories of increasing amount, from 0 to 3 (0=no yellow needles, 1=some yellow needles, 2=medium amount of yellow needles, 3=high amount of yellow needles). To test if one of the epitypes had significantly more yellow needles than the other, a cumulative link model test was performed. The results showed that for the twigs collected in April and May, CE had significantly more yellow needles than WE (Appendix 3, 5-6). The twigs

collected in March did not have a significant difference in amount of yellow needles between the epitypes (Appendix 4). The twigs collected in March 2019 had no yellow needles.

Norway spruce needles are susceptible to the spores of Spruce needle rust only when the needles are new and have a thin epidermis (Roll-Hansen, 1981), which means that trees that have bud burst too early or too late compared to the spore dispersal, may not be infected (Phillips & Burdekin, 1992). This may explain why CE was more infected than WE, since bud burst occurs earlier in CE than in WE (Carneros et al., 2017).

5.0 Conclusions

Overall, WE was more frost tolerant than CE in March, April and May, in at least one of the three examined tissues (needles, cambium and buds). The lower frost tolerance in CE was not reflected in the gene expression, as this epitype had a higher expression of *PaDHN6* and *PaDHN40* than WE in March. This is surprising, as *DHN*s are associated with frost tolerance. The other genes did not differ in transcript levels between the epitypes. As expected, the dormancy related gene *PaFTL2* decreased in expression from March to May, while *PaCMT3*, which is involved in methylation of new DNA strands, increased in expression. There was not found any difference in expression of the two *HDT*s between the epitypes or the collection dates. Consistent with the lack of differences in expression of the epigenetically related genes, the distribution of the epigenetic marks in buds did also not differ in a clear way between the epitypes, except possibly more methylation in the procambium in WE than in CE in March. This immunolocalization method might be too crude to be able to detect differences between the epitype in distribution of epigenetic marks.

6.0 Suggestions for further research

The mechanism behind the formation of the epigenetic memory during the embryogenesis and how this memory is maintained during the life of the trees are still not completely understood.

Epigenetic marks show a dynamic pattern through the seasons and might change as a response to environmental factors. The challenge is to separate these changes in epigenetic marks from the changes that are caused by the epigenetic memory of temperature during the embryogenesis. To study this, the stem cells in the SAM should be investigated for epigenetic

changes, as they are the initial cells which all the other cells in the shoot are derived from. Thus, it is probably in these cells that the epigenetic memory is established and. These cells should be studied both in embryos during the formation of the epigenetic memory and in the epitype trees. Genes that are known or thought to be differentially expressed between the epitypes (e.g. related to timekeeping) should be analyzed for changes in epigenetic marks, like DNA methylation or histone modifications.

Recording of bud burst and bud set should be continued to find out more about the duration of the epigenetic memory. The long generation times of trees make it a challenge to study the transgenerational heredity of the epigenetic changes. However, the trees have now been treated with gibberellin, and have made reproductive buds. Thus, in the coming years, the progenies should be studied, to find out if they also show the same differences in phenology as the mother trees do.

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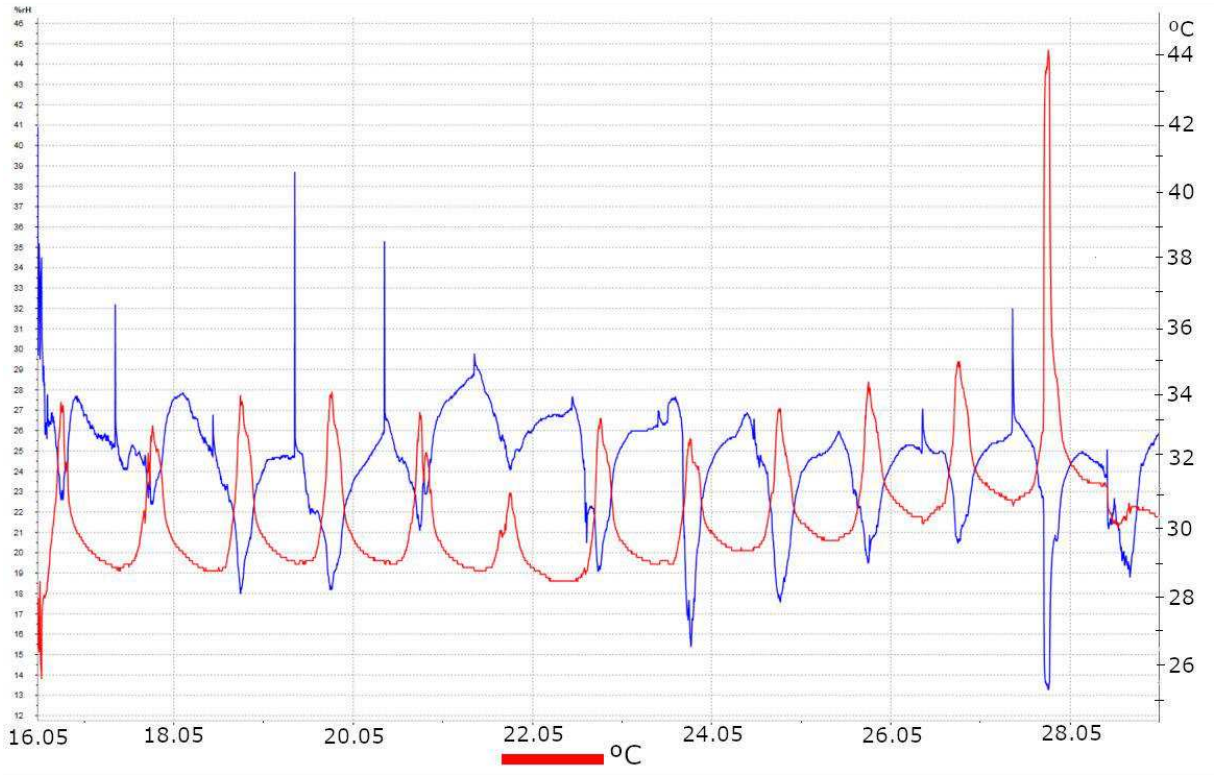
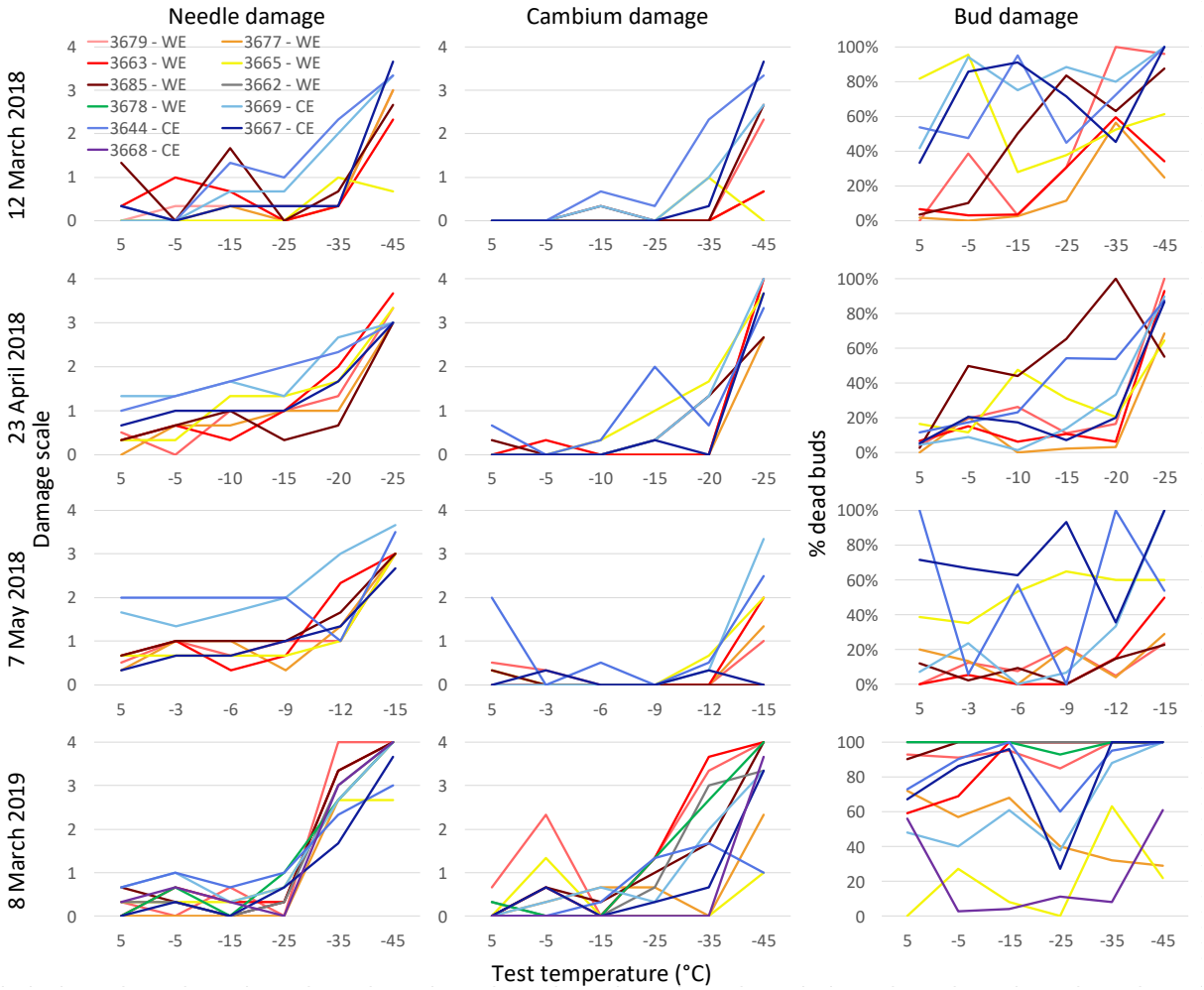
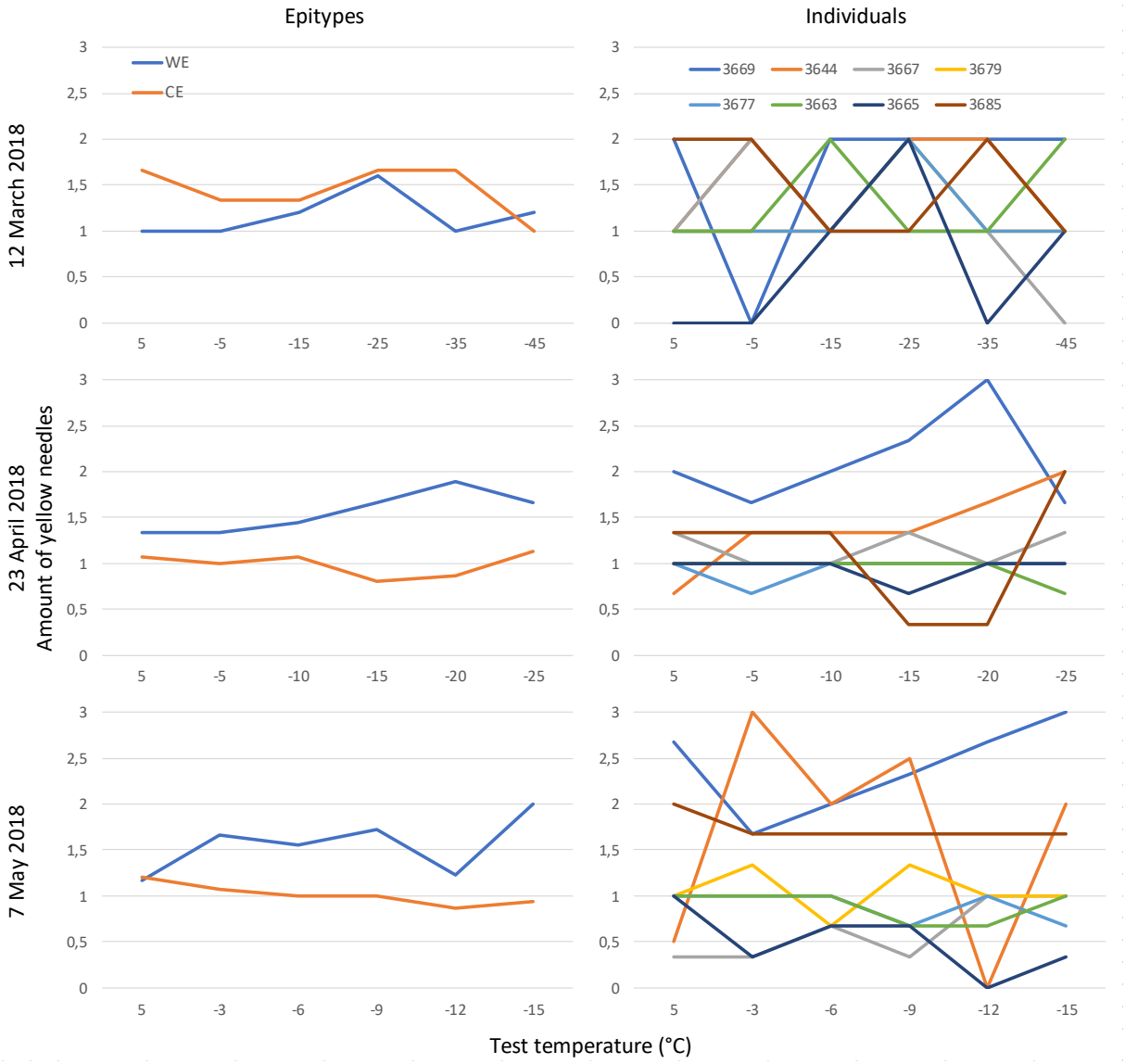
8.0 Appendix



Appendix 1. Temperature in the room where the twigs of the Norway spruce epitypes were developing damage after freezing, from 16. May to 28. May 2018, i.e. the two weeks before the damage evaluation of the twigs collected 7. May, which were done 29 and 30 May. The blue line indicates air humidity in the room, which probably did not affect the twigs, as they were in closed boxes and were sprayed with water. It is probably not true that the room temperature was 44 °C 27 May, as the outdoor temperature this day was just 0,4 °C higher than the day before. The windows were covered with fabric to avoid direct sunlight on the twigs, but it might be possible that a gap was made so that the sun shone on the temperature logger, which may explain the high temperature registered.



Appendix 2. Damage in the needles, cambium and buds of different individuals of Norway spruce epitypes after freezing at different temperatures, for twigs collected in a common garden in Ås, Norway, at four different time points. The damage scale for needles and cambium ranges from no damage to dead (0=no damage/completely green, 1=1-33 % brown, 2=34-66 % brown, 3=67-99 % brown, 4=dead/completely brown). Bud damage is shown as percent dead buds. The data is shown for each individual tree. Individuals 3679, 3677, 3663, 3665 and 3685 from the warm epitype (WE) and individuals 3669, 3644 and 3667 from the cold epitype (CE). In March 2019, the individuals 3662 and 3678 from WE and 3668 from CE were collected in addition to the others.



Appendix 3. Amount of yellow needles in twigs of Norway spruce epitypes collected in March, April and May in 2018. In each individual and the average of each epitype. The scale ranges from 0 to 3 (0=no yellow needles, 1=some yellow needles, 2=medium amount of yellow needles, 3=many yellow needles). The data is shown for each individual tree. Individuals 3679, 3677, 3663, 3665 and 3685 from the warm epitype (WE) and individuals 3669, 3644 and 3667 from the cold epitype (CE).

Appendix 4. Summary of a cumulative link model run to test the effect of epitype on amount of yellow needles in twigs of Norway spruce collected 12 March 2018. Three individuals from the cold epitype (CE) and five individuals from the warm epitype (WE). Positive coefficients mean more yellow needles. Negative coefficients mean less yellow needles.

Treatment	Coefficient	SE	z value	Pr (>|z|)
EpitypeWE	-0,7901	0,5966	-1,324	0,185

*Significance codes: ***P<0.001 **P<0.01 *P<0.05*

Appendix 5. Summary of a cumulative link model run to test the effect of epitype on amount of yellow needles in twigs of Norway spruce collected 23 April 2018. Three individuals from the cold epitype (CE) and five individuals from the warm epitype (WE). Positive coefficients mean more yellow needles. Negative coefficients mean less yellow needles.

Treatment	Coefficient	SE	z value	Pr (>|z|)
EpitypeWE	-2,5057	0,7452	-3,363	0,000772***

*Significance codes: ***P<0.001 **P<0.01 *P<0.05*

Appendix 6. Summary of a cumulative link model run to test the effect of epitype on amount of yellow needles in twigs of Norway spruce collected 7 May 2018. Three individuals from the cold epitype (CE) and five individuals from the warm epitype (WE). Positive coefficients mean more yellow needles. Negative coefficients mean less yellow needles.

Treatment	Coefficient	SE	z value	Pr (>|z|)
EpitypeWE	-1,0422	0,3683	-2,83	0,00466**

*Significance codes: ***P<0.001 **P<0.01 *P<0.05*

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