UiT The Arctic University of Norway

Faculty of Biosciences, Fisheries, and Economics

Department of Arctic and Marine Biology

Effect of simulated latitudinal variation in daylength conditions on oscillations of circadian gene expression in populations of woodland strawberry (*Fragaria vesca L.*) from Northern Norway and Italy

Corine Faehn

BIO-3950 Master's Thesis in Biology, May 2021

Abstract

Almost all species on Earth possess circadian rhythms that align their physiological processes with suitable times of day and year based on predictable changes in the light cycle. While it is known that natural variations exist in photoperiodic responses regulated by the circadian clock, there are currently no studies assessing the effect of latitudinal variations in daylength on the properties of plant circadian genes. This thesis investigated the association of the properties of the circadian clock with daylength in two woodland strawberry (*Fragaria vesca L.*) clones originating from Italy (45°N) and Northern Norway (69°N). Plants were grown under two simulated daylengths (18h photosynthetic light/6h twilight and 15h photosynthetic light/1h twilight/8h dark) corresponding with the summer photoperiod at each latitude. Real-time qPCR was used to analyze the expression of eight circadian clock genes present in the leaves over a time-course of 48 hours. Six of the genes (*FvLHY, FvRVE8, FvPRR9, FvPRR7, FvPRR5,* and *FvLUX*) expressed clear circadian oscillations of transcription levels in both daylengths, with significantly longer phases of expression under the longer daylength. Interestingly, there were only differences in expression between the two clones 3.3% of the time, demonstrating a lack of specific latitudinal responses in circadian clock properties in this species. All genes maintained transcriptional rhythms in constant darkness except for two (*FvPRR9* and *FvPRDX*) where transcription was completely abolished. *FvPRDX* also had no rhythm of transcription, though other studies have shown that the oxidation cycles of peroxiredoxin proteins do exhibit circadian rhythmicity. One gene (*FvTOC1)* had an irregular rhythm that warrants further testing due to its central role in the circadian pacemaker. This study shows that the oscillations of circadian clock transcription levels in *F. vesca* from a lower latitudinal origin are highly entrainable to the long daylength conditions that are characteristic of northern latitudes. While the circadian clock is able to acclimate its oscillation timing with the external daylength, photoperiodic outputs controlled by the clock, such as flowering time, do not exhibit the same level of plasticity to sudden changes in daylength. This provides an important resource for understanding the circadian clocks contribution to adaptability.

Abbreviations

ZT	Zeitgeber
RNA	Ribonucleic Acid
mRNA	Messenger RNA
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
TTFL	Transcription-Translation Feedback Loop
PTO	Post-Translational Oscillator
LHY	Late Elongated Hypocotyl
CCA1	Circadian-Associated 1
RVE8/6/4	Reveille 8/6/4
LNK1/2	Night Light Inducible and Clock-Regulated 1/2
PRR9/7/5/3	Pseudo-Response Regulator 9/7/5/3
TOC1	Timing of Chlorophyll A/B Binding Protein 1
LUX	Lux Arrythmo
PRDX	Peroxiredoxin
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
MSI1	Multicopy Suppressor of IRA 1
ELF3/4	Early Flowering 3/4
EE	Evening Element
PPFD	Photosynthetic Photon Flux Density
CTAB	Cetyl Trimethylammonium Bromide
PVP	Polyvinylpyrrolidone
EDTA	Ethylenediaminetetraacetic Acid
cDNA	Complementary Deoxyribonucleic Acid
C_q	Cycle Quantity

Table of Contents

List of Tables

List of Figures

Acknowledgements

First, I would like to thank my amazing supervisors. Professor Laura Jaakola had so many exciting ideas when taking me on as a master student and provided such great direction and encouragement throughout the whole project. Laura, your thoughtfulness and involvement was monumental for me. I am especially thankful for your time in helping grind up all 200+ samples, I truly could not have gotten through that without you (now we have strong arm muscles!).

To my incredible co-supervisor, Jørgen Mølmann, your knowledge and familiarity with this project was tremendously helpful. The feedback and attention to details you provided, as well as your assistance in the long 48 hours of sampling, truly helped me to see the potential of this thesis from beginning to end.

I always looked forward to my meetings with the both of you, your knowledge and experience is truly inspiring to me. I could not have asked for better supervisors.

Thank you to Leidulf Lund who grew these strawberry plants, he truly has a gift with plants. Thank you to Amos Samkumar for sparing his valuable time to help sample the plants as well as sharing his knowledge in laboratory preparations. I am also so thankful to everyone at the Klimalab for dealing with my inability to speak Norwegian (I'm still learning). It has been a wonderful place to work and it is exciting to see it growing as a research facility.

The support from my mom, grandparents, and friends in Florida to follow my adventurous heart and move halfway across the world has truly allowed me to flourish these last 2 years. Every long distance phone call was cherished and motivated me to keep doing what I love.

The deepest thanks to my selfless and compassionate other half, Griffin, for never letting me doubt myself. You have always helped me stay motivated and push my boundaries through the struggles of Python, as well as taking care of me when I broke my ribs at the alpine park. Thank you for always being up for adventures and lots of great meals.

Lastly, this thesis could not have happened without my dad's support, who left this Earth 3 years ago but has stayed with me in spirit to guide me here. I am so grateful to have this network of support with me to continue on studying my passions.

INTRODUCTION

Circadian Rhythms

The Earth has been rotating on its axis around the sun since the beginning of its formation, about 4.5 billion years ago (BYA). This rotation brings about daily changes in the light cycle creating one of the most predictable seasonal cues for plants and other living organisms. Almost all species on Earth have biological rhythms that are aligned with the daily and seasonal light cycles to help predict when to perform activities such as sleep, movement, and photosynthesis (Bhadra et al., 2017; Eriksson & Millar, 2003). When these biological rhythms display an oscillation cycle of about 24 hours, they are called circadian rhythms. Circadian rhythms are present in all domains of life and control proper timing of biological functions. They regulate everything from entire cellular function in single-cell organisms to seasonal timing of flowering or tuber formation and onset of bud dormancy in preparation for winter in plants (Golden, 2003; Martínez-García et al., 2002). Animal behaviors are also largely determined by circadian timing, from daily sleep-wake cycles to hormone cycling, reproductive timing, metabolism and many immunoregulatory responses (Chaix et al., 2016; Cox & Takahashi, 2019). These occurrences are not strictly responses to the external environment. They are internally generated rhythms that align with the external light cycles to be prepared for, instead of trying to keep up with, the constant change.

Circadian rhythms are driven by an endogenous molecular clock that shares a set of distinguishing characteristics among both eukaryotes and prokaryotes (Saini et al., 2019). They are entrained by daily light cues (e.g. dawn and dusk), called zeitgebers (ZT, German for time givers) which determine the phase of the cycle. With the onset of light at dawn each morning, the timing is "reset", and the phases can stay in sync with the daily and yearly changes. Circadian rhythms are also self-sustaining, and continue even under free-running conditions (absence of a light cycle), though with a period that slightly strays from 24 hours (Bünning & Pfeffer, 1989). The clock also maintains accurate timing over a wide range of temperatures to control for daily fluctuations, called temperature compensation (Gould et al., 2006). This robust awareness of changes in the environment allows organisms to keep track of time and stay in sync with the regular diurnal and seasonal changes.

Evolutionary Origins

Since the emergence of cellular life approximately 3.5 BYA, the daylength has slowly lengthened from a roughly 6-hour cycle (due to the impact of the formation of the moon) to today's 24-hour cycle (Lathe, 2004). Therefore, even in a largely unstable climate, life on Earth has always evolved in an environment with predictably stable rhythms of light. Early life forms had to harvest and store energy from the sun as well as protect themselves from the harmful ultraviolet (UV) radiation that was not filtered by the Earth's atmosphere 3-4 BYA (Dvornyk et al., 2003; R. A. Hut & Beersma, 2011). This created a natural selection pressure to align physiological processes with certain phases of the diurnal cycle to exploit resources as well as mitigate stress.

Circadian rhythms are so conserved among lineages that they appear to have emerged very early on in life (Hurley et al., 2016). One universally conserved protein marker, peroxiredoxin (PRDX), is present among all domains of life and has been shown to exhibit rough circadian oscillations under free-running conditions (constant light), suggesting an early component in the evolution of metabolic circadian rhythms (Edgar et al., 2012). This protein arose ~ 2.5 billion years ago during the Great Oxidation Event (GOE), when species had to survive through a newly oxygenated atmosphere and needed control of intracellular peroxide levels and by-products produced by the electron transport chain (Hall et al., 2009; Loudon, 2012). There is no clear evidence of origin, but there is evidence that single-cell organisms and individual cells in multicellular plants and animals display circadian rhythms, indicating the primordial necessity of circadian timekeeping (Merrow et al., 2005).

Selective Advantages

Clocks with similar molecular systems have evolved separately in each of the four kingdoms, indicating the selective advantage to having a time-keeping mechanism (Young & Kay, 2001). The primary functions of plant circadian clock genes are to provide plants with information on the daily environmental changes, control developmental processes, predict resource availability, and be prepared for abiotic stresses (Dodd et al., 2005; Kim et al., 2017; Más & Yanovsky, 2009). Many important metabolic processes are under circadian regulation, including net carbon assimilation, starch metabolism, and the production of sugars by photosynthesis (Dodd et al., 2005; S. L. Harmer et al., 2000). Since plants are sessile and constantly exposed to environmental stresses, clock-dependent integration of these downstream physiological and developmental processes is crucial for enhanced fitness and growth (Nagel

& Kay, 2012). Having mechanisms to anticipate predictable environmental stresses helps, for example, to protect against harmful UV radiation by producing genes encoding the enzymes in phenylpropanoid biosynthesis pathways before dawn, forming a kind of "phenolic sunscreen" (S. L. Harmer et al., 2000). In higher plants, about 40% of cold-responsive genes and 50% of heat-responsive genes are under circadian control (Covington et al., 2008; Mizuno & Yamashino, 2008). Responses to abiotic stresses are so integrated with circadian timing that a stimulus given at an unusual time of the day will not produce the same response as if it were given at the expected time of day (Greenham & McClung, 2015). For example, a light signal given in the afternoon or evening doesn't induce the same degree of response as a light signal given in the morning, a term called circadian gating. Thus, having a properly functioning circadian clock provides a significant adaptive advantage to respond to the diurnal changes in light (Green et al., 2002).

Circadian Clock Research and Modern Progression

The influence of circadian rhythms on adaptation, fitness and success has become a profound area of research in the past few decades. Awareness of the impact of circadian clocks on not only physiology, but biochemistry, biophysics, and ecology has created a unifying interest among scientists (Sharma, 2006). From the earliest reports of diurnal leaf movements to present-day understanding of the preserved transcription-translation feedback complex, we are now able to investigate the plasticity of the circadian clock and how it influences behavior.

The first reports of circadian rhythms arose in the 1700's when M. de Mairan discovered the persistence of diurnal leaf movements under constant darkness (Bünning & Pfeffer, 1989). Leaf movement was the subject of all early circadian observations in plants because it was the only known function to be under circadian control. In the late 1800's to early 1900's, circadian rhythms started to receive attention as a heritable mechanism that had interesting properties which were endogenously generated and not just driven by external indicators (McClung, 2006).

The first clock gene was cloned in Drosophila in 1984, followed by identification of rhythmic expressions of light-harvesting genes in plants which began the modern-day molecular analyses of the circadian clock. (Kloppstech, 1985; Reddy et al., 1984). Genetic approaches have allowed for direct identification and characterization of clock genes as well as the mechanisms, mostly in the model plant, *Arabidopsis* (Kevei et al., 2006; Matsuo et al., 2008; Yon et al., 2012). *Arabidopsis* became the model organism for plants because of its close relation to

thousands of species and the genomic resources and methods that became available from widespread use (Somerville & Koornneef, 2002). The development of genome-wide expression profiling has allowed for novel proteins and pathways of the circadian clock to be identified, shedding light on a wide-array of regulation from physical interactions, signal transductions, protein modifications, and post-translational control (H. Huang et al., 2016; Mas, 2005). Early analyses of transcription rates of gene expression in *Arabidopsis* showed that roughly 480 mRNA (gene) levels exhibited circadian changes, which we now know to be upwards of 722 target sites being regulated by just a single circadian clock gene (Hsu et al., 2013; Adams et al., 2018).

Identification of the components and mechanisms controlling the circadian clock have led to questions of what characteristics of the environment have driven this level of complexity and variation (Troein et al., 2009). Different approaches can be employed to study circadian clock gene expression in response to different environmental conditions. An effective method is the use of quantitative real-time polymerase chain reaction (qRT-PCR) assays, which utilizes reverse-transcription of the mRNA of interest and reliable reference genes to measure changes in target gene expression (Lambret-Frotté et al., 2015; Freitas et al., 2019; Jose et al., 2020). Combined with the availability of genomic data, specific primers for individual genes can be designed to identify the molecular basis of the impact of environmental changes on the circadian clock.

The Molecular Circadian Clock

Circadian rhythms are cell-autonomous and driven by a set of core genes called the circadian clock that oscillate at the transcription and translational levels. The circadian clock shares a structural similarity across all domains of life, though genetic components vastly differ between kingdoms (Saini et al., 2019). A commonality of all circadian clocks is the use of a basic transcription-translation feedback loop (TTFL) where the accumulation of translated protein directly inhibits transcription of its own gene (Figure 1). The TTFL is regulated by post-transcriptional control of mRNA abundance as well as post-translational mechanisms which play a critical role in generating and maintaining the daily rhythm (Kojima et al., 2011; Romanowski & Yanovsky, 2015). Even prokaryotes utilize interlocking feedback loops as well as a post-translational oscillator (PTO), though the molecular mechanisms in prokaryotic circadian clocks consist of protein domains and signal transductions nonhomologous to eukaryotes (Golden, 2003; Hurley et al., 2016). The multitude of regulation is necessary to

impart temporally coordinated gene expression to outputs of physiological processes such as metabolism, hormone signaling, and responses to stresses (Chaix et al., 2016; Troncoso-Ponce & Mas, 2012).

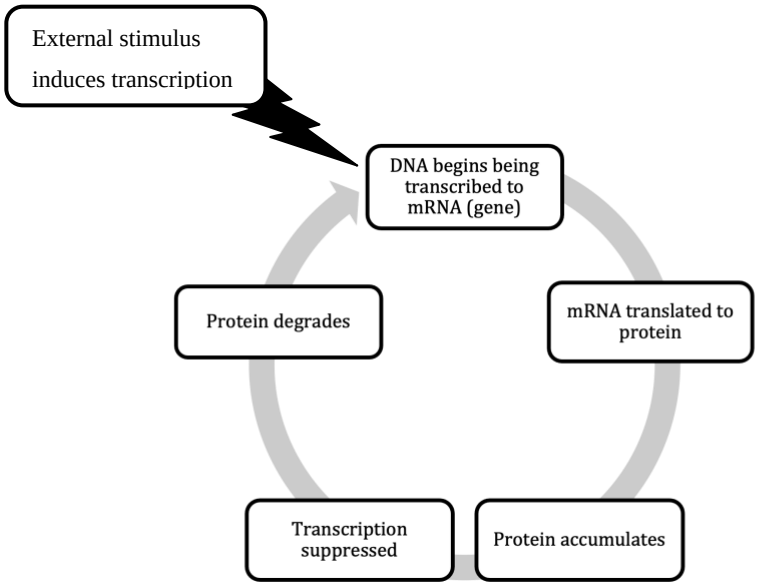

Figure 1. Simplification of the transcription-translation feedback loop (TTFL). Transcription of double-stranded DNA to single-stranded RNA by RNA polymerase is induced by a signal, of which mRNA is translated into a gene product (protein). The accumulation of protein will then suppress the transcription of the gene. Transcription will begin again either by signal or degradation of the protein, forming a "loop".

Present clock models have been derived from a few well studied model species. Due to the conserved nature, these models have provided valuable insight into the time-keeping abilities of all life on Earth. The prokaryotic clock model species, cyanobacteria (*Synechoccus elongatus*), has the oldest known clock mechanism consisting of three oscillating, multi-functional Kai proteins (A, B, and C) (Cohen & Golden, 2015). The eukaryotic clock has been widely studied in four model species: *Neurospora* (fungi), *Drosophila* (fruit flies), mouse (mammals), and *Arabidopsis* (plants) (Harmer, 2009; Andreani et al., 2015). Fungal and animal clocks share TTFL architectural similarities that are not found in plants, consisting of a positive arm activator and a negative arm inhibitor of gene expression (Hurley et al., 2016). Plant circadian clock TTFL components also differ between higher plants (angiosperms and gymnosperms) and lower plants (bryophytes and algae) mostly in complexity and number of genes involved (Karlgren et al., 2013; Linde et al., 2017). Despite differences between specific

components, circadian oscillator genes and proteins share a remarkable structural similarity across kingdoms (Saini et al., 2019).

The Plant Circadian Clock

Plant circadian clock components are defined by their oscillatory phase of expression with general morning, daytime, and evening-phased genes. Though the genes have specific times of expression, recent studies have emphasized the complexity of the circadian system with highly connected networks of regulation versus simple morning and evening feedback loops (Hsu & Harmer, 2014). The interplay of clock activators and repressors is responsible for the generation of the TTFL which keeps the clock components expressed during the right phase of day. Figure 2 represents a simplified model of the main components in the core *Arabidopsis* circadian oscillator by (Hsu & Harmer, 2014).

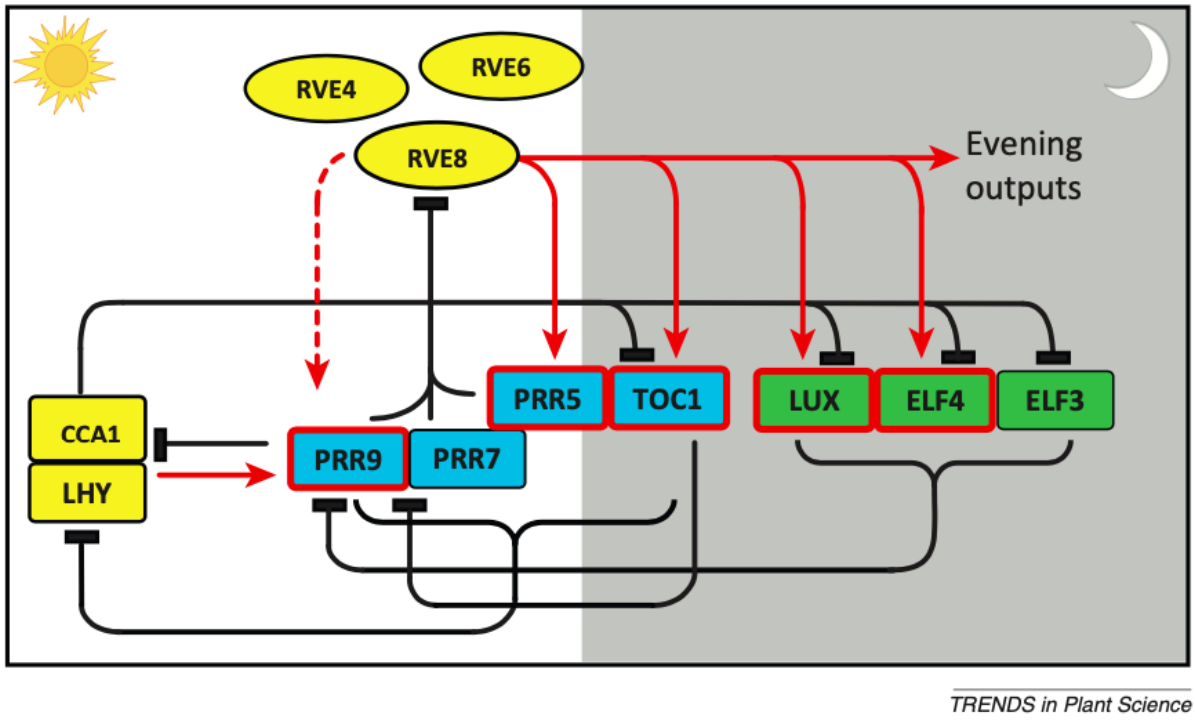

Figure 2. The plant circadian clock (Hsu & Harmer, 2014). This image shows a simplified transcriptional molecular clock mechanism in Arabidopsis thaliana. Components are grouped by family, REVEILLE family in yellow, PSEUDO-RESPONSE-REGULATOR family in blue, and evening complex (EC) in green. Grouping and placement also indicate general phase of expression, moving from left to right, morning phased (*CCA1*, *LHY*, *RVE4*, *RVE6*, and *RVE8*) are in yellow, day-time phased (*PRR9*, *7*, and *5*) are in blue, and evening phased (*TOC1*, *LUX*, *ELF4*, and *ELF3*) are in blue and green. Components containing the evening element (EE) in their promotor region are marked by a red box. Arrows indicate transcriptional activation while bars indicate transcriptional repression. White area indicates daytime, gray area indicates night-time.

Morning-Phased Genes

In *Arabidopsis*, two morning-expressed genes, *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), and *LATE ELONGATED HYPOCOTYL (LHY*), are essential components to the core clock. Accumulation of CCA1 and LHY proteins function as transcriptional repressors of their own transcription as well as many other clock genes (Alabadí et al., 2001; Adams et al., 2015). As CCA1 and LHY levels decline throughout the day, daytime and evening phased genes are then transcribed which further represses transcription of *CCA1* and *LHY* (Wang & Tobin, 1998; Alabadí et al., 2001). LHY is a crucial component to the central oscillator as well as overall plant fitness. A loss or mutation can result in a disrupted rhythm leading to delayed developmental processes such as longer hypocotyls, late flowering, and loss of viability (Schaffer et al., 1998; Wang & Tobin, 1998; Green et al., 2002). *CCA1* and *LHY* are two partially redundant MYB-domain containing transcription factors that belong to the small REVEILLE subfamily including the close homologues, *REVEILLE 8* (*RVE8*), *RVE6*, and *RVE4*. *RVE8* is a morning-expressed gene that is a necessary component of the circadian system due to its direct transcriptional activation of evening elements (Rawat et al., 2011). Less is known about the function of *RVE4* and *RVE6*, other than that they play partially redundant roles with *RVE8* (Hsu et al., 2013). Recently, two novel transcription regulators were also identified to be morning-phased genes and components of the light-signaling pathways, *NIGHT LIGHT INDUCIBLE AND CLOCK-REGULATED 1* (*LNK1*) and *LNK2* (De Leone et al., 2019).

Daytime-Phased Genes

Daytime expressed genes play roles within the regulation of both morning and evening-phased complexes of the clock. Members of the *PSEUDO-RESPONSE-REGULATOR* gene family (*PRR9, PRR7*, *PRR5*, and *PRR3*) are repressed by *LHY* and are said to express sequentially throughout the day in *Arabidopsis* (Matsushika et al., 2000; Adams et al., 2015). *PRR9, 7,* and *5* function as transcriptional repressors of the morning genes *CCA1*, *LHY*, *RVE8, LNK1*, and *LNK2* (Nakamichi, 2020).

Evening-Phased Genes

An evening-expressed *PRR, TIMING OF CHLOROPHYLL A/B BINDING PROTEIN 1* (*TOC1* or *PRR1*), is an indispensable gene to the core clock network, which functions to repress expression of morning-expressed genes at night (Huang et al., 2016). An Evening Complex (EC) composed of *LUX ARRYTHMO* (*LUX*) also known as *PHYTOCLOCK 1*(*PCL1*), and

EARLY FLOWERING 3 & *4* (*ELF3* & *ELF4*) downregulates transcription of *TOC1* after dusk, allowing transcription of *CCA1* and *LHY* to restart the next dawn (Nusinow et al., 2011; Adams et al., 2015). RVE8 is the main transcriptional activator of evening genes by targeting an evening element (EE) motif in their promoter regions (Rawat et al., 2011). Models of the complex plant clock framework have led us to understand the connection of the morning, daytime, and evening-phased genes in a three-component repressilator which acts via sequential repression of: 1) *CCA1/LHY* repress the EC (*LUX, ELF3,* and *ELF4*); 2) the EC represses *PRR* genes (*PRR9, PRR7, PRR5,* and *TOC1*); and 3) *PRR* genes repress *CCA1/LHY* (Pokhilko et al., 2012).

The Circadian Clock and Photoperiod

The rotation combined with the axial tilt of the earth gives rise to characteristic photoperiod (daylength) variations at different latitudes. The photoperiod can range from a very long daily photosynthetic light period of 24 h during summer and no photosynthetic light period during the winter at latitudes above the Arctic Circle (66°N), to a nearly constant 13 h photoperiod at the equator (Hut et al., 2013; Mølmann et al., 2021). Mid-latitudes (23°N - 66°N) experience a substantial change in photoperiod, from 15h of photosynthetic light during the summer to 10h during the winter, but that is far less extreme than the 24h sunlight/darkness experienced near the poles.

Photoperiod sensitivity determines the geographical range over which a plant can grow (Chen et al., 2020). Plant species with a large geographic spread are subject to a wide range of photoperiods throughout the year which has led to local genetic variations that influence their adaptability to different environmental conditions (Santamaría et al., 2003; Paolucci et al., 2019). This has created latitudinal variations in photoperiodic responses such as flower timing, dormancy, and germination to align with the different timing of environmental cues. The circadian clock is essential for matching these physiological processes with the photoperiod, so understanding the circadian function underlying latitudinal variations within a species can reveal important selection pressures (Hut et al., 2013; Greenham et al., 2017). Several studies have investigated latitudinal variations of circadian rhythmicity in animals such as *Drosophila*, parasitic wasps, and beetles, showing circadian rhythms have geographic variations, with life at higher latitudes losing the strong rhythmicity seen at lower latitudes, denoting clear involvement of natural selection on circadian rhythmic parameters within different environments (Bloch et al., 2013; Bertolini et al., 2019; Paolucci et al., 2019; Abe et al., 2021).

The latitudinal variation in plant circadian clock properties, however, is not well understood. Plants have a crucial necessity to be able to detect changes in the photoperiod due to their dependence on light for photosynthesis and their immobility. Variations in plant circadian periods have been observed along latitudinal gradients by differential leaf movements, but leaf movements are only one of many outputs controlled by the circadian clock (Michael et al., 2003; Greenham et al., 2017). To fully understand the evolutionary adaptation of the circadian system with latitude, studies must be done to relate its molecular function with photoperiodic adjustment.

Fragaria vesca

The genus *Fragaria,* commonly known as strawberry, belongs to the Rosaceae family and is estimated to have originated in the Pliocene to Pleistocene Epoch's (Late Tertiary to Quaternary Period) with an estimated origin of 1.0-4.1 MYA based on a fossil calibrated relaxed molecular clock analysis by Njuguna et al. (Njuguna et al., 2012). *Fragaria* contains approximately 24 known species with natural ploidy levels ranging from diploid (2n = 14) to decaploid (10n = 70) as well as a full range of sexual systems from self-compatibility to dioecy (Hummer et al., 2009; Liston et al., 2014).

The strawberry plant is a low-growing, typically 10-20 cm in height, herbaceous perennial that spreads primarily by stolons (runners) which shoot away horizontally to form new plants. The exact origin of the common name "strawberry" predates cultivation and has many theories of why they are called "strawberries", with many names in other languages such as jordbær in Norwegian, jordgubb or smultron in Swedish, and aardbei in Dutch all meaning "earth berries". The leaves, flowers, stolons, and fruit all grown from the crown, which is a short, thickened stem with the roots at the base of the plant. The leaves grow in a rosette around the crown and are comprised of 3 separate leaflets, called a "trifoliate" with toothed margins, usually 4-5 cm in width at maturity. The bright red "berry" is not a true fruit, it consists of dry achenes (seeds) on the outside of the fleshy modified receptacle. The fruit, leaves, and stem are highly nutritious, containing essential micronutrients such as vitamin C, vitamin K, folates, potassium, iron, and polyphenolic compounds such as tannins, lignans, flavonoids, and ellagic acid which have antioxidant, anti-inflammatory, antidiabetic, and anticancer properties (Muthukumaran et al., 2017; Baby et al., 2018).

Woodland (wild) strawberries, *Fragaria vesca,* are much smaller than the common strawberry crop, *Fragaria x. ananassa,* and can be found growing along the edges of wooded areas, in

fields, and even along sidewalks. *F. vesca* has a wide geographic distribution in cool, temperate climates (37°N-70°N with average temperatures below 30°C) across the northern hemisphere throughout North America, Northern Asia, Europe, and Northern Africa. The fruits of the wild plants were harvested long before they were cultivated, which began in Europe in the 1300's when the French began transplanting them from the wilderness to their garden's (Darrow, 1966). The modern strawberry crop species wasn't created until the mid-1700's by a cross between the North American strawberry, *F. virginiana*, and the Chilean strawberry, *F. chiloensi* (Hancock et al., 2010). This hybrid strawberry, *F. × ananassa*, has one of the most complex genomes among crop plants (2n=8x=56).

In addition to strawberries being a popularly consumed fruit, they are also an excellent proposed model plant for studying genetic mechanisms. *F. vesca* has one of the smallest fully sequenced genomes (2n=14, ~240 Mb) and shares a significant amount of sequence identity with crop species, *F. × ananassa* (Edger et al., 2019; Folta & Davis, 2006; Shulaev et al., 2011). Many studies defining the circadian clock framework, as well as the basic concepts of plant biology, have been done using the model species *Arabidopsis thaliana* (Shulaev et al., 2011). The diploid woodland strawberry, *F. vesca*, is an analogous model organism for testing these principles that has been largely under-studied in comparison to *A. thaliana*. *F. vesca's* small genome, compact stature, as well as it's quick generation time and ease of propagation, are all exceptionally useful for controlled laboratory experiments making it an ideal genetic model plant to understand the role that the circadian clock plays in its adaptation.

Thesis Aims

Major developments have been made in understanding the molecular mechanisms driving circadian rhythms, but studies identifying the latitudinal variation and adaptability of plant clock systems are lacking, especially under long photoperiods at high latitudes. The aim of this thesis was to identify the effect that daylength conditions have on natural variance in circadian clock gene oscillations in two populations of a single species, *Fragaria vesca*, originating from Italy (45°N) and Northern Norway (69°N). Different properties of phase, amplitude, and period of several circadian clock genes were analyzed to identify if population variation from different latitudinal origin has a role in photoentrainment of the circadian clock. Further aims of this research include discussion of the circadian clock's role in adaptation, as well as assessing how the knowledge of this relationship can be utilized to optimize agricultural activity of important related crop species.

MATERIALS AND METHODS

Plant Material

Four distinctive clones of *Fragaria vesca* were obtained from the research laboratory at the University of Helsinki (Dr. Timo Hytönen) and raised at the Klimalaboratorium at Holt, Tromsø with the Institute for Arctic and Marine Biology at the University of Tromsø, Norway. The clones originated from Italy; I1 (Tenno, Ville del Monte, Tennosee 45°93'N 10°81'E) and I4 (Da Salorno, Pochi, Alto, Adige, Italy, 46°23'N 11°23' E), and Northern Norway; N2 (Alta1, Leirbukta 69°10' N 23°67' E) and N13 (Indre Nordnes 69°53' N 20°38' E).

Experimental Set-Up

At the beginning of January 2020, plants were grown under 12-hour daylengths at +18°C to produce runners. The runners were transplanted every couple of weeks, until enough plants were produced for the experimental design to begin light treatments. In June 2020, 125 young plants from each clone were transplanted into 9-cm pots filled with peat soil and perlite 1:1 [v/v]. Plants at the beginning of treatment consisted of about 3-5 small trifoliate leaves 1-3 cm in width and 2-4 cm in height. The daylength treatments were carried out in separate phytotrons (growth chambers) at a constant temperature of 18°C from 19 June 2020 to 8 July 2020 and sampling occurred from 6 July 2020 to 8 July 2020, taking all leaves from 3 biological replicates of each clone from the 2 light treatments. At the 24-hour mark (ZT24), half of the plants from each treatment (36/clone) were moved to a dark treatment room and concurrently sampled to identify free-running periods. Sampling occurred every 4 hours for 48 hours, giving 13 time points (of which 6 include dark treatment) and 114 samples/clone. At the end of sampling, plants averaged 10 cm in height with 3-6 leave that averaged 5 cm in length. After sampling, 3 biological replicates of each clone were cut down to the soil from each growth room, dried in an oven at 60°C for 24 hours and weighed.

Daylength Conditions

Daylength conditions were applied to the plants using fluorescent tubes (PHILIPS Cool White MASTER TL-D Super 80 58W/840, Eindhoven, The Netherlands) to simulate photosynthetic light and light emitting diodes (LED, PHILIPS Softone 18W) to simulate non-photosynthetic twilight. Lighting was automatically controlled in the phytotrons using computer-controlled

timer switches. Two daylength treatments were applied to the plants, simulating a summer daylength in Northern Norway (18h photosynthetic light, 6h non-photosynthetic light/twilight, hereafter referred to as "Norway") and a summer daylength in Italy (15h photosynthetic light, 1h total non-photosynthetic light/twilight, 8h darkness) with ZT0 at 08:00, along with a dark treatment room (Figure 3).

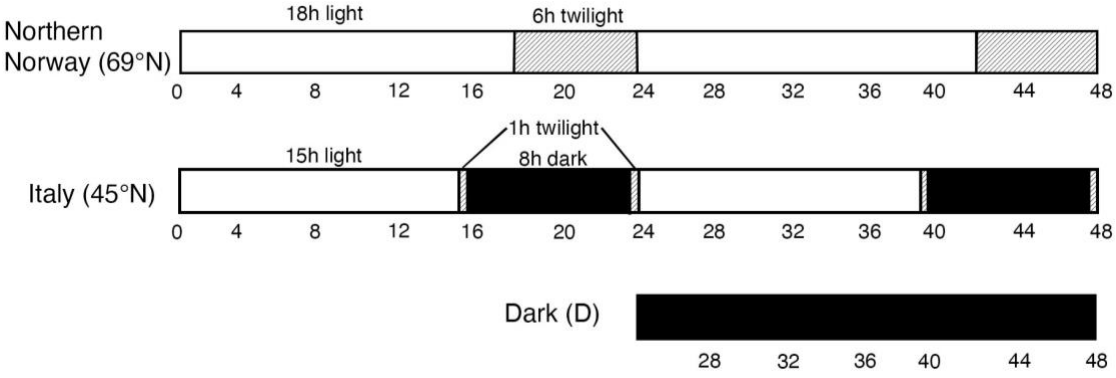

Figure 3. Daylength treatments. The two simulated daylength treatments for Northern Norway (18h photosynthetic light, 6h twilight) and Italy (15h photosynthetic light, 1h total twilight, 8h dark), along with the dark (D) treatment, all contained in separate phytotrons. The times indicate the 13 timepoints (zeitgebers, ZT) of sampling (every 4h) including the 6 sampling times in the dark treatment.

Sampling that occurred during dark phases occurred using a Heliospectra LED lamp at 550 nm (green light) and an irradiance setting of 1 to minimize light disturbance. The irradiance for each treatment room was adjusted to give similar total energy of photosynthetic light per day. The total photosynthetic photon flux density (PPFD) in each chamber was measured using a quantum sensor (LI-1000, LI-COR Inc., USA). The Norwegian growth chamber had a PPFD of 200 µmol m^{-2} s^{-} photosynthetic light (over 18h), while the Italian growth chamber had a PPFD of 243 µmol m^{-2} s^{-} photosynthetic light (over 15h). Both chambers had a PPFD of 3 µmol m^{-2} s^{-} non-photosynthetic light. Relative absorbance of the visible light spectrum was measured at the level of the plants using a spectrophotometer (Jaz, Ocean Optics Inc., USA). Spectral distributions of the photosynthetic light are shown in Figure 4.

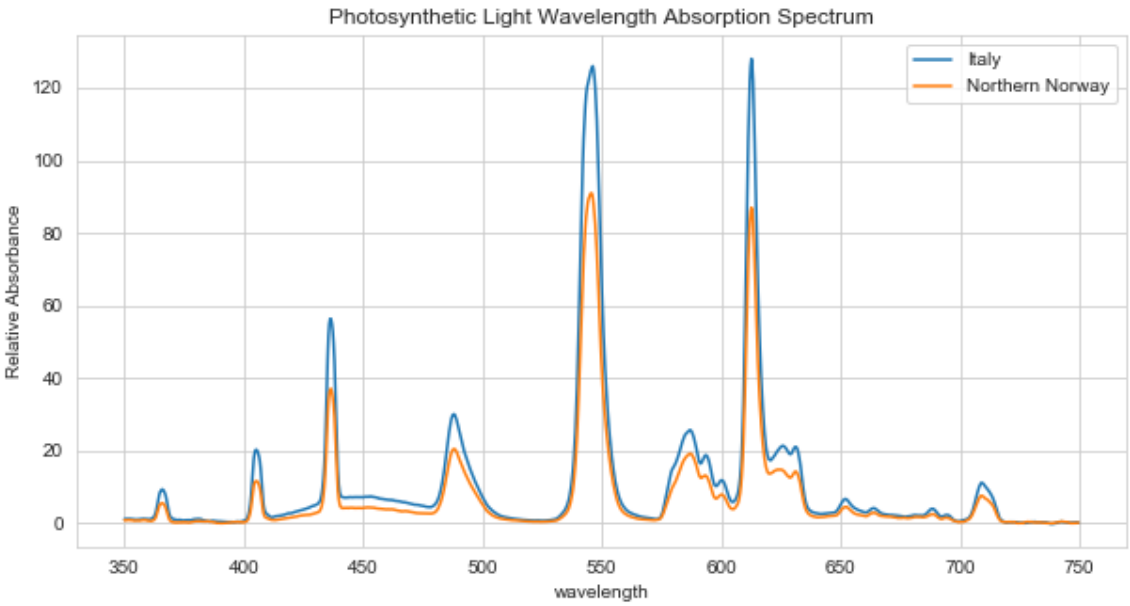

Figure 4. Relative absorbance spectrum. Spectrum of photosynthetic light in the two growth chambers. Total growth light was equal for each treatment, with the Norwegian growth chamber having a PPFD of 200 µmol m^{-2} s^{-} photosynthetic light over 18 hours, and the Italian growth chamber having a PPFD of 243 µmol m^{-2} s^{-} photosynthetic light over 15 hours.

RNA Extraction and Reverse-Transcription

All samples were collected in liquid nitrogen and stored at -80°C. Mortars and pestles were autoclaved before use, and all plasticwares were autoclaved before use. Frozen leaf tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle, and then stored again at -80°C.

Total RNA was extracted from all samples for two clones (N2 and I1, 114 samples each) using the E.Z.N.A Total RNA Kit I (Omega Bio-tek Inc., USA, 2020) following a modified protocol from Ouyang et al. (Ouyang et al., 2014). An amount of 150-200 mg of ground leaf tissue were transferred into individual 2 mL RNase-free Eppendorf tubes. Samples were incubated at 65°C for 10 min. in 1 mL of extraction buffer (2% CTAB, 2% PVP, 100 mM Tris-HCl (pH 8), 25 mM EDTA (pH 8), 2.0 M NaCl, 0.5g/L Spermidin (mixed and autoclaved), 2% β-mercaptoethanol added just before use). Chloroform:isoamyl alcohol (24:1, Sigma-aldrich) was added, mixed, and centrifuged (10000 g, 4°C, 10 min.) twice, transferring the supernatants to new tubes each time by pipetting, aiming not to disturb the lower suspension. Samples were precipitated in 10 M $LiCl_2$ (1/4 v) at -20°C overnight. The following day, samples were centrifuged (4°C, 20 min., 15000 g) and washed with 70% ice-cold ethanol. DNase mix (40 µL

nuclease-free H_2O, 5 µL DNase I, and 5 µL DNase I digestion buffer) was added and incubated for 10 min. at room temperature. 150 µL of H_2O, 200 µL Total RNA Kit I buffer, and 200 µL ice-cold absolute ethanol were added, respectively, and gently mixed. Samples were pipetted onto Hi-Bind® columns and centrifuged (4°C, 1 min., 13000 rpm). The collection tube liquid was discarded, 500 µL RNA Wash Buffer II was added, centrifuged (4°C, 1 min., 13000 rpm), and repeated. Columns were transferred to clean collection tubes and centrifuged (4°C, 1 min., 13000 rpm). The tubes were incubated in 40 µL pre-heated H_2O (70°C) for 1 min., centrifuged (4°C, 1 min., 13000 rpm) and repeated with the eluate. 1 µL of sample was tested for purity and concentration on a NanoDrop™ 2000 spectrophotometer (RNA-40 mode; A_{260}/A_{280} , A_{260}/A_{230}) (ThermoFisher, USA).

cDNA was synthesized in 10uL reactions from 0.75 ug RNA, by using 50 uM Oligo $d(T)_{20}$ primer, and nuclease-free water following the Superscript IV First-Strand cDNA Synthesis Reaction protocol (Invitrogen, Carlsbad, CA, USA), omitting the RNAseOUT Recombinant RNase Inhibitor. The reaction mixtures were incubated at 55°C for 30 minutes, and inactivated at 80°C for 10 minutes, then stored at -80°C.

Primer Design

Sequences for the genes *LHY, PRR9, PRR7, PRR5, RVE8, TOC1/APRR1,* and *LUX/PCL1* were selected from the recently published paper that characterized the circadian clock genes in *F. vesca* (Chen et al., 2018). Five of the gene primer sequences (*LHY, PRR9, PRR7, PRR5,* and *RVE8*) and one reference gene primer sequence (*GAPDH*) were provided in the paper and ordered from ThermoFisher. For genes without primer sequences listed, gene ID's were searched in the *F. vesca* genome database (*Fragaria vesca* Whole Genome v1.0 (build 8) Assembly & Annotation) at the *Rosaceae* genome database (GDR, https:/www.rosaceae.org) to get the genomic sequences. Entire gene sequences were uploaded to the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to design specific primers according to the standard primer parameters: Melting temperature (Tm) of 58-60°C, primer length of 20-24 nt, and a GC content of 50-60%. Gene primers were designed for *TOC1* and *LUX* using the NCBI Primer-Blast tool and ordered from ThermoFisher. The primer sequence for *PRDX* had recently undergone primer design testing in the Klimalab at UiT in an unpublished scientific report by Arpine Ayvazyan. An additional reference gene, *MSI1*, was also previously tested at the Klimalab which had been proven to be a stably expressed gene

under various experimental conditions (Mouhu et al., 2013). All primers and source are listed in Supplementary Table 1.

Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR assays were performed using a CFX96 Touch Real-Time PCR System (Bio-Rad, Hercules, CA, USA) to analyze gene expression. Each 15 uL reaction mixture contained 1 uL of cDNA template (10-fold dilution in sterile H_2O), 7.5 uL of SsoFast^{TM} EvaGreen Supermix (Bio-Rad), 1.5 uL (500 nM) of each primer, and 3.5 uL of sterile H_2O. qRT-PCR and melt curve analyses were performed using the amplification cycling conditions: 1 denaturation cycle of 95°C for 2 m, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, ending with 1 cycle for the melt curve analysis from 65-95°C in 0.5°C increments for 5 s. qRT-PCR quantification values (C_q) are defined as the PCR cycle number that crosses an automatically detected threshold signal.

A no-template control (NTC) was included for each gene assay to account for contamination or non-gene specific products. The sample-maximization method (Hellemans et al., 2007) was used for the experimental set up for qRT-PCR relative quantification, which minimizes technical (run-to-tun) variation between the samples (Supplementary Figure 1 for example 96-well plate set-up). In addition, inter-run calibrators (IRC's) were included in every run to calculate a calibration factor, if necessary, to proceed as if all samples were analyzed in the same run (Hellemans et al., 2007).

Primer-Pair Efficiency

Each primer pair was tested via qRT-PCR following the conditions in the previous section. To determine efficiency, a standard curve was created using 10-fold dilutions over 5 orders of magnitude (10^0-10^{-4}) of pooled cDNA. PCR amplification efficiency was calculated in excel using the equation:

$$Efficiency\ (\%)\ =\ 10^{(-1/Slope)} - 1)\ x\ 100$$

If standard curve efficiency of the primer was low or melt peaks indicated more than one RT-PCR-amplicon, new primers were designed using the NCBI Primer-Blast tool with the same parameters and re-tested via qRT-PCR.

Statistical Analysis

C_q values for each gene over the thirteen timepoints were measured with the qPCR system. All real-time qRT-PCR data was statistically analyzed in Jupyter Notebook; a Python language based open-source web application. To determine if the control genes varied under experimental conditions the candidate reference genes are presented as average C_q values grouped by light treatment. A one-way ANOVA (analysis of variance) and post-hoc Tukey HSD test was used to determine if there were statistically significant differences in candidate reference gene expression under the different treatments. Target gene expression was then normalized to MSI1 and presented as $2^{-\Delta Cq}$ values following a modification of the ΔC_q method (Schmittgen & Livak, 2008). A Students *t*-Test was applied to compare statistical significances between clones under each treatment, between overall phase and amplitude between the daylength treatments, as well as between daylength and dark treatments. Tables of significance are listed in Appendix D. The real-time qRT-PCR results were confirmed by three biological repetitions. The qPCR data (CSV files) as well as the Jupyter notebook are available through GitHub in Appendix E.

RESULTS

Plant Biomass

To directly compare gene expression between individual plants, they need to be generally in the same developmental stage. The dry weight of each of the clones indicated no significant difference in developmental stage due to treatment (Table 1). For sake of time along with labor intensity for RNA extraction and genetic analyses for a large number of samples (114/clone), two of the four clones (N2 and I1) were selected for analysis for this thesis. The other two clone samples (N13 and I4) are stored in a -80°C freezer for future testing.

Table 1. Comparative plant biomass. Dry weight of each clone measured on a gram scale for each treatment at the end of sampling. I1 and I4 clones originated from Italy, N2 and N13 clones originated from Northern Norway. Clones in bold were used for analysis in this thesis. Values represent mean ± s.d. of 3 biological replicates.

	Daylength Treatment	
Clone	Italy (15L:8D)	Northern Norway (18L:6h twilight)
I1	**1.02 ± 0.14 g**	**0.96 ± 0.23 g**
I4	0.62 ± 0.20 g	0.62 ± 0.23 g
N2	**0.64 ± 0.22 g**	**0.84 ± 0.37 g**
N13	0.62 ± 0.15 g	0.39 ± 0.13 g

Sample Preparation Validation

Using the qRT-PCR method to measure gene expression requires a set of carefully chosen parameters beginning with extraction of high-quality RNA with no contamination of DNA or other metabolites produced by the plant. Total RNA yields measured by Nanodrop averaged 309.8 ± 175.4 ng/µl (n= 110) and 246.2 ± 98.1 ng/µl (n= 112) for N2 and I1, respectively. Samples that had yields < 15 ng/µl were omitted from analysis (Supplementary Table 2). Absorbance ratios measured by Nanodrop averaged 1.96 ± 0.21, and 2.10 ± 0.14 for 260/280 and 1.24 ± 0.57, 1.57 ± 0.54 for 260/230, for N2 and I1, respectively. Low 260/230 absorbance

ratios presented potential concern due to indication of possible contamination of proteins or polyphenols that could inhibit downstream PCR amplification, but analysis via gel electrophoresis showed clear bands of 28S and 18S RNA bands, indicating intact RNA fragments (Figure 5).

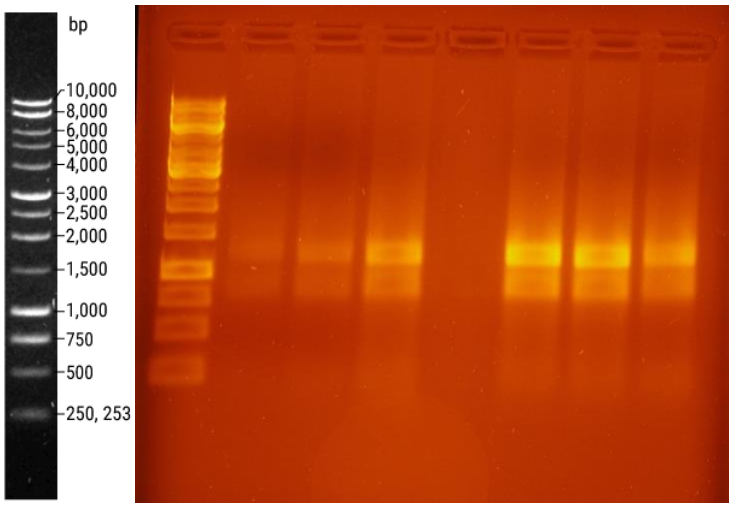

Figure 5. Gel electrophoresis results for RNA extraction. Double bands indicate intact 28S (top) and 18S (bottom) RNA fragments.

Primer-Pair Efficiency

Optimal primer design is essential for successful qRT-PCR reactions. Primer pairs for each of the genes gave single peaks on the melt curve analysis, indicating singular amplified fragments. Primer efficiency (E%) values averaged 116 ± 9.8%. Regression coefficients (R^2) averaged 0.98 ± 0.02 (Supplementary Table 1). From standard curve analysis, a 10x dilution of cDNA was selected as optimal (C_q value between 20 and 30 cycles) for all genes and was used for all qRT-PCR assays.

Reference Gene Stability and Expression Validation

Choosing a stably expressed reference gene that is unaffected by the treatment is one of the most crucial aspects when performing comparative gene expression studies to provide the most accurate and reliable results. The average amplification of C_q values varied between the candidate genes, Figure 6 and Figure 7 show their distributions. The average C_q value for *MSI1* was 28.17 ± 0.68, and 21.68 ± 1.00 for *GAPDH*. The ANOVA tests indicated significant differences only in *GAPDH* expression in N2, between Italian and D-Italian treatments (p=0.001), Norwegian and D-Italian treatments (p=0.001), and Italian and D-Norwegian treatments (p=0.04) (Supplementary Table 7). For all gene expression analysis, MSI1 was chosen as the reference gene. All no-template controls (NTC) in the qPCR assays confirmed no contamination or non-gene specific products of the primer mix. Inter-run calibrators (IRC's) also confirmed no significant difference in a one-way ANOVA (p=0.06) of C_q measurements between any run, allowing direct comparison of gene expression between plates.

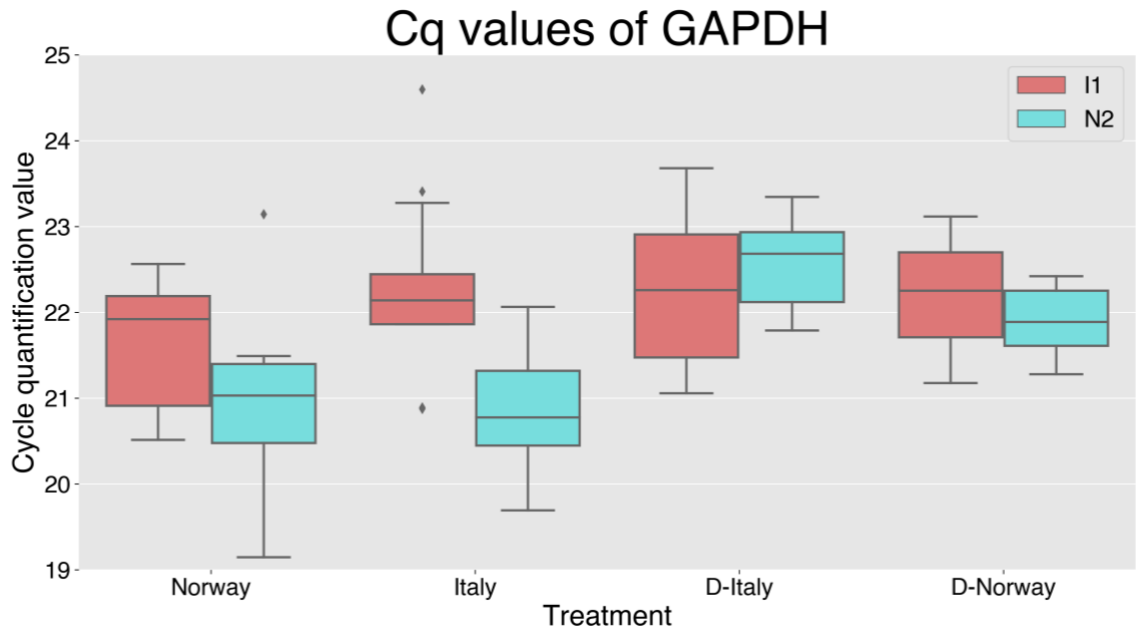

Figure 6. *GAPDH* expression by treatment. Comparison of cycle quantification values for *GAPDH* expression in all samples. D-Italy indicates plants moved to dark from Italian treatment, and D-Norway indicates plants moved to dark from Norwegian treatment.

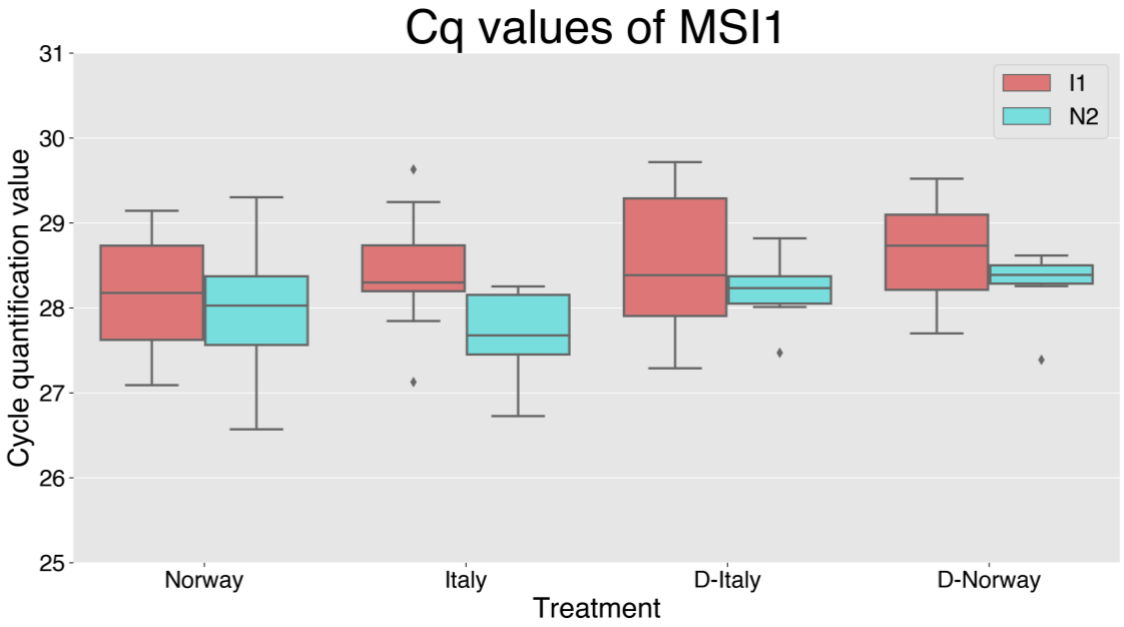

Figure 7. *MSI1* expression by treatment. Comparison of cycle quantification values for *MSI1* expression in all samples. D-Italy indicates plants moved to dark from Italian treatment, and D-Norway indicates plants moved to dark from Norwegian treatment.

Expression Profiles of Circadian Clock Genes

A total of eight *Fragaria vesca* genes, *FvLHY*, *FvPRR9, FvPRR7, FvPRR5*, *FvRVE8, FvLUX*, *FvTOC1*, and *FvPRDX*, were selected to identify circadian rhythm expression levels in woodland strawberries using qRT-PCR. Gene expression is shown as relative expression ($2^{-\Delta Cq}$ analysis) over the course of 48 hours under the various treatments. During analysis, samples that had consistently high C_q values (greater than 30 cycles) for multiple genes were removed from qRT-PCR analysis due to likely skewed gene expression from lower total RNA (10 samples, Supplementary Table 3). Of the eight genes analyzed, six expressed clear circadian oscillations of transcription levels in both treatments, while two (*FvTOC1* and *FvPRDX*) had variable rhythms. The same six genes also had significantly longer phases of expression under the longer daylength (Supplementary Table 6). *FvLHY, FvRVE8, FvPRR7, FvPRR5, FvTOC1*, and *FvLUX* maintained transcriptional rhythms in constant darkness, with significant shifts in the phase of expression in all but *FvTOC1* (Supplementary Table 5).

FvLHY (Figure 8), and *FvRVE8* (Figure 9), two morning-expressed genes in the REVEILLE family that act in opposing ways on other components in the circadian clock, had only 3 total timepoints where gene expression was significantly different between the clones. *FvLHY*

expression was significantly different between the clones in the Norwegian daylength at ZT0 (p=0.02), and in the Italian daylength, ZT32 (p=0.03), while *FvRVE8* was only significantly different between the clones in the Italian daylength at ZT40 (p=0.03).

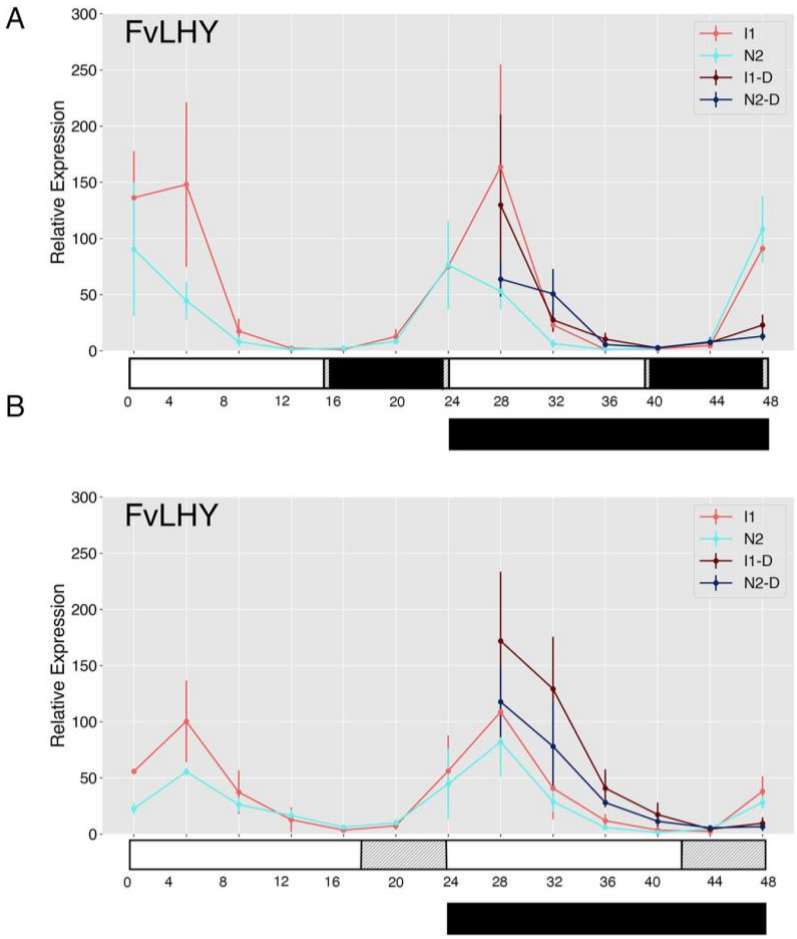

Figure 8. Relative expression of *FvLHY*. A: Italian (15:8) daylength. B: Norwegian (18:6) daylength. White bars indicate photosynthetic light, striped bars indicate non-photosynthetic light (twilight), and black bars indicate total night. Black bar across bottom indicates dark (D) treatment. Each datapoint represents mean ± S.E. of three biological replicates.

Response to dark treatment varied between clonal origin as well as between the two daylength treatments. Expression of *FvLHY* underwent a phase shift in N2 when moved to dark from the Norwegian daylength, with significantly different expression at ZT36 (p=0.001), ZT40 (p=0.01), and ZT48 (0.01), but had only one significantly different expressed timepoint when moved to dark from the Italian daylength at ZT48 (p=0.01). I1 only had one significant differential expression of *FvLHY* when moved to dark from the Italian daylength, at ZT48

(p=0.02). A longer phase of expression was seen in the Norwegian daylength with significant differences in amplitudes of expression at ZT8 (p=0.04), ZT12 (p=0.01), ZT16 (p=0.01), ZT36 (p=0.01), and ZT48 (p=0.0003).

Expression of *FvRVE8* was only significantly different in N2 when moved to dark from the Norwegian treatment at ZT32 (p=0.04), and ZT40 (p=0.007). A longer phase of expression was seen in *FvRVE8* as well with significant differences in amplitudes of expression at ZT12 (p=0.03), ZT16 (p=0.002) and ZT36 (p=0.02).

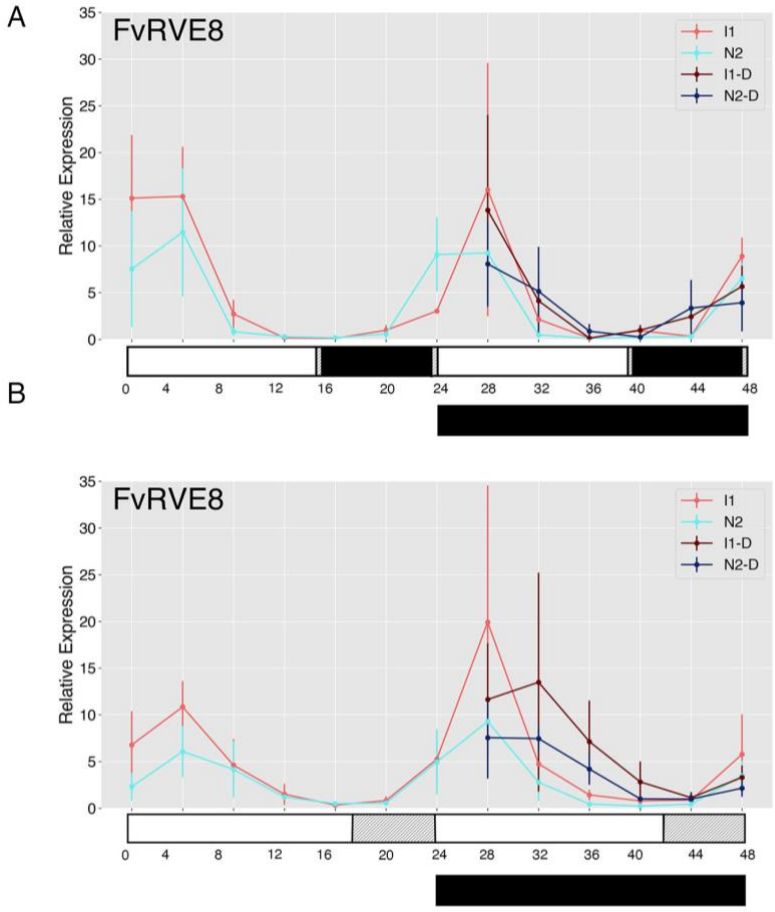

Figure 9. Relative expression of *FvRVE8*. A: Italian (15:8) daylength. B: Norwegian (18:6) daylength. White bars indicate photosynthetic light, striped bars indicate non-photosynthetic light (twilight), and black bars indicate total night. Black bar across bottom indicates dark (D) treatment. Each datapoint represents mean ± S.E. of three biological replicates.

FvPRR9 (Figure 10), a member of the *PSEUDO-RESPONSE-REGULATOR* family, showed only 1 timepoint where gene expression was significantly different between the clones, in the Italian daylength at ZT36 (p=0.01). There was a large difference between amplitude of

expression as well as phase between the daylength treatments with significant differences at 9 timepoints, ZT4 (p=0.0002), ZT8 (p=0.007), ZT16 (p=0.01), ZT20 (p=0.0001), ZT28 (p=0.04), ZT36 (p=0.02), ZT44 (p=0.004), and ZT48 (p=0.04).

FvPRR9 transcription was completely abolished under both dark treatments for both clones. N2 had significant differences for 4 of the timepoints when moved to dark from the Norwegian daylength, ZT28 (p=0.008), ZT32 (p=0.04), ZT36 (p=0.03), and ZT48 (0.007), as well as 5 of the timepoints when moved to dark from the Italian daylength, ZT28 (12:00, p=0.006), ZT32 (p=0.006), ZT36 (p=0.003), ZT44 (p=0.012), and ZT48 (p=0.02). I1 also had significant differences for 3 of the timepoints when moved to dark from the Norwegian daylength, ZT32 (p=0.0004), ZT36 (12:00, p=0.009), ZT44 (p=0.01), and 3 of the timepoints when moved to dark from the Italian daylength, ZT32 (p=0.009), ZT36 (p=0.003), ZT48 (p=0.03).

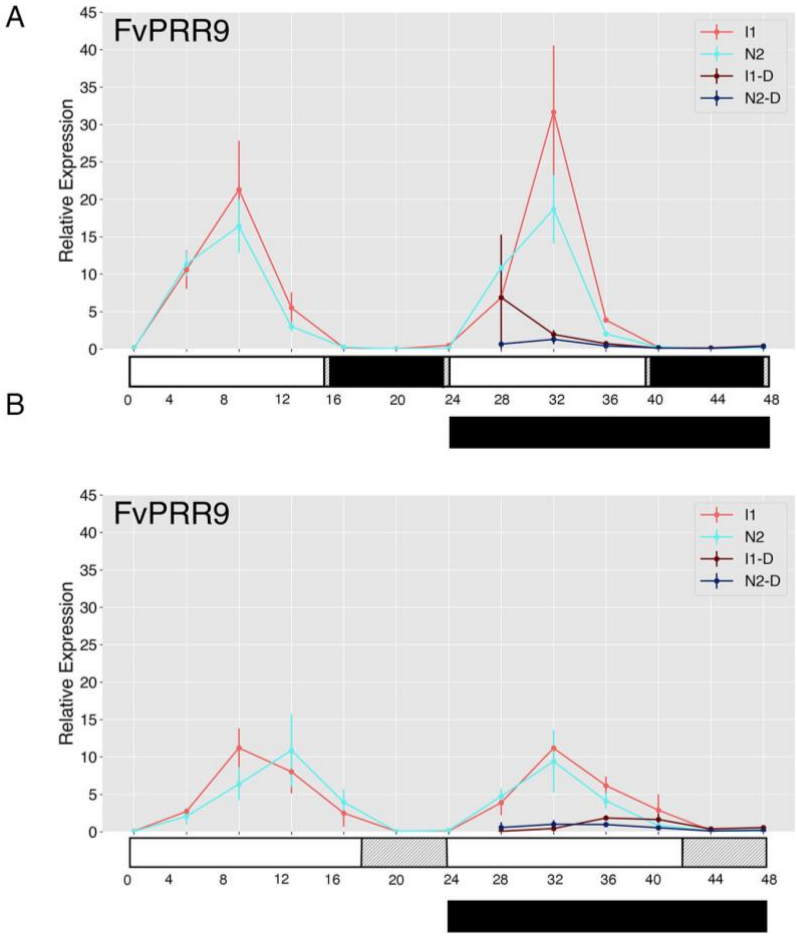

Figure 10. Relative expression of *FvPRR9*. A: Italian (15:8) daylength. B: Norwegian (18:6) daylength. White bars indicate photosynthetic light, striped bars indicate non-photosynthetic light (twilight), and black bars indicate total night. Black bar across bottom indicates dark (D) treatment. Each datapoint represents mean ± S.E. of three biological replicates.

Another member of the *PRR* family, *FvPRR7* (Figure 11), had no significant differential expressions between clones for either of the daylengths. Movement to dark caused significant differences at some timepoints in both clones. When moved to dark from the Norwegian daylength, both clones had significant differential expression at ZT48 (p=0.03 for N2, and p=0.0003 for I1). When moved to dark from the Italian daylength, N2 had significant differential expression at ZT28 (p=0.02), and ZT44 (p=0.005), while I1 only had significant differential expression at ZT48 (p=0.02). The difference in amplitude of expression was only significant between daylength treatments when peak expression was lowest at ZT20 (p=0.004), ZT24 (p=0.04), ZT44 (p=0.006), and ZT48 (p=0.006).

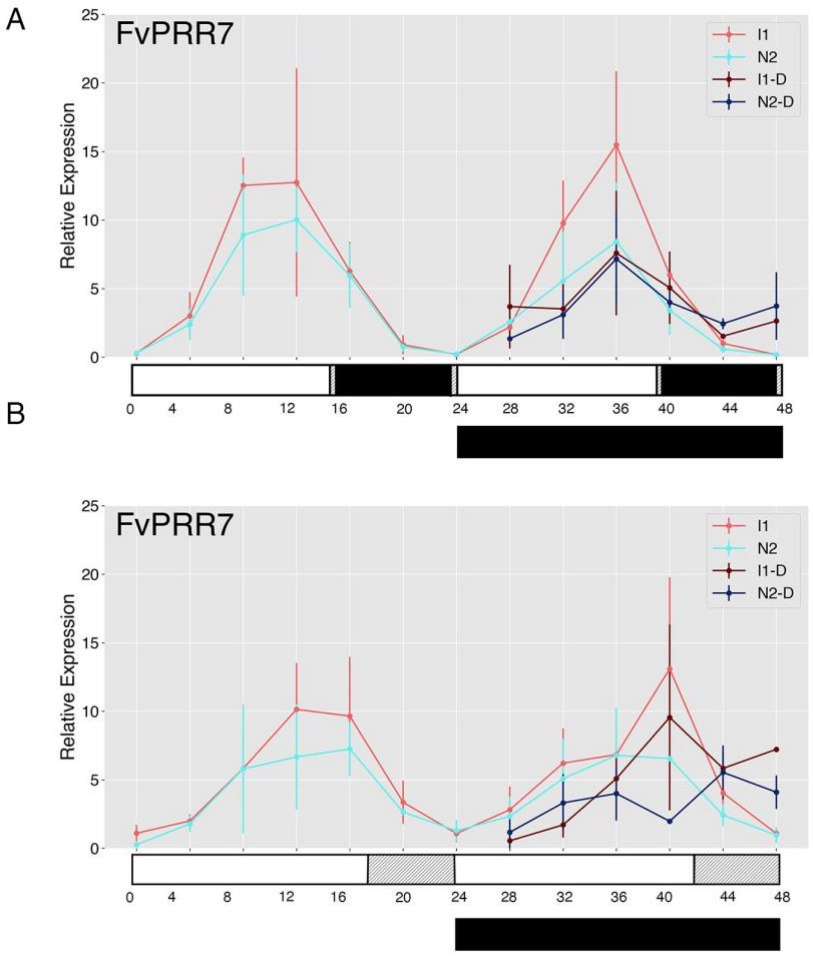

Figure 11. Relative expression of *FvPRR7*. A: Italian (15:8) daylength. B: Norwegian (18:6) daylength. White bars indicate photosynthetic light, striped bars indicate non-photosynthetic light (twilight), and black bars indicate total night. Black bar across bottom indicates dark (D) treatment. Each datapoint represents mean ± S.E. of three biological replicates.

FvPRR5 (Figure 12) had no significant differential expression between the clones in either daylength treatment. However, there was a significant difference between amplitude of expression between the treatments, at ZT8 (p=0.01), ZT16 (p=0.002), ZT20 (p=0.002), ZT44 (p=0.01), and ZT48 (p=0.02), exhibiting a longer phase of expression under the Norwegian daylength for both clones. Both clones had sustained expression levels of *FvPRR5* when moved to dark from the Norwegian treatment at ZT48 (p=0.01 for N2, p=0.004 for I1), as well as in the Italian daylength, N2 had significantly different expression at ZT44 (p=0.005), while I1 had significantly different expression at ZT36 (p=0.04), and ZT48 (p=0.03).

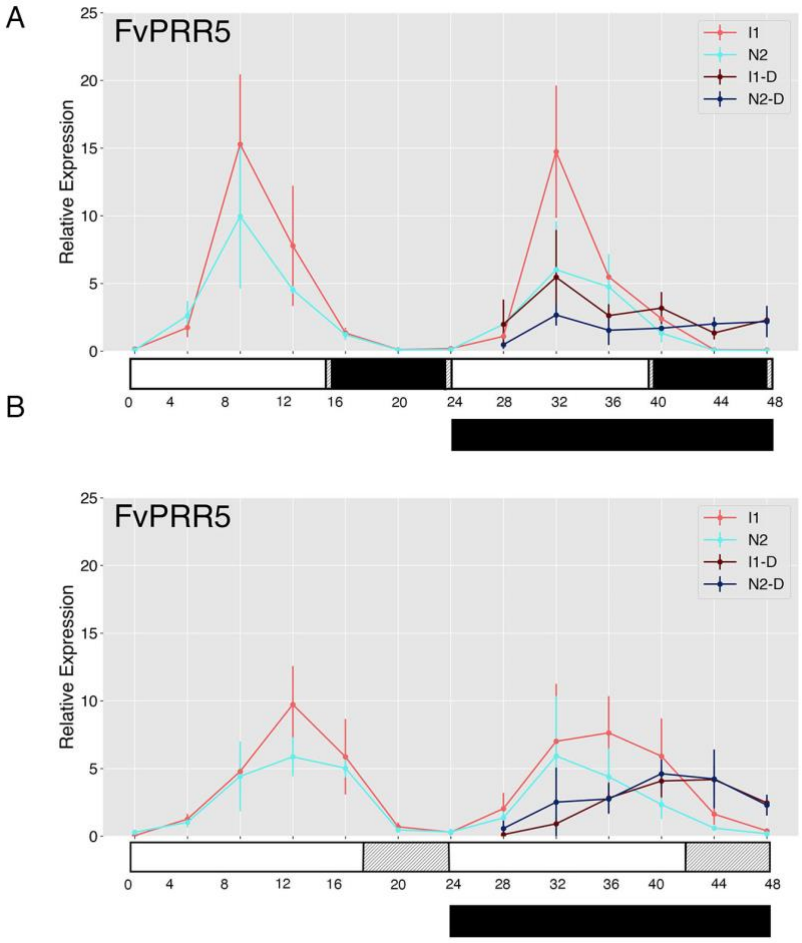

Figure 12. Relative expression of *FvPRR5*. A: Italian (15:8) daylength. B: Norwegian (18:6) daylength. White bars indicate photosynthetic light, striped bars indicate non-photosynthetic light (twilight), and black bars indicate total night. Black bar across bottom indicates dark (D) treatment. Each datapoint represents mean ± S.E. of three biological replicates.

FvTOC1 (Figure 13), a critical gene in the core transcription network of the clock, exhibited a rough, long phase of expression that occurred throughout the evening and into the morning, with lowest levels of expression 4h after dawn. There were no significant differences in expression between the clones, between the daylength treatments, or when moved to the dark treatment due to large standard errors.

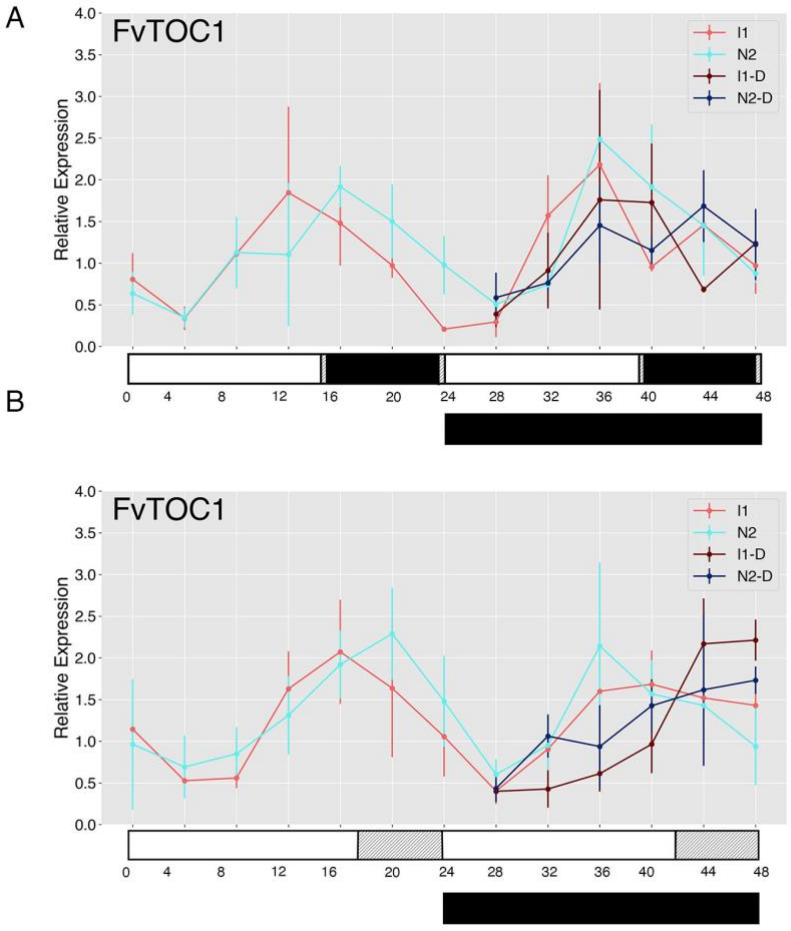

Figure 13. Relative expression of *FvTOC1*. A: Italian (15:8) daylength. B: Norwegian (18:6) daylength. White bars indicate photosynthetic light, striped bars indicate non-photosynthetic light (twilight), and black bars indicate total night. Black bar across bottom indicates dark (D) treatment. Each datapoint represents mean ± S.E. of three biological replicates.

FvLUX (Figure 14), a part of the EC, had only one significant difference in expression between the clones in the Italian daylength at ZT0 ($p=0.02$), though the average relative expression values were still quite close (0.101 for I1 and 0.058 for N2). Movement to dark caused significant differences in expression in the Italian daylength for N2 at ZT44 ($p=0.04$) and for I1 at ZT32 ($p=0.04$), while in the Norwegian daylength N2 had a significant difference at ZT48

(p=0.01) and for I1 at ZT40 (p=0.02), signifying some difference in phase and amplitude of expression in the dark treatment. Phases between the two treatments were significantly different at ZT8 (p=0.01), ZT44 (p=0.03), and ZT48 (p=0.007), signifying a slight longer phase in the Norwegian daylength.

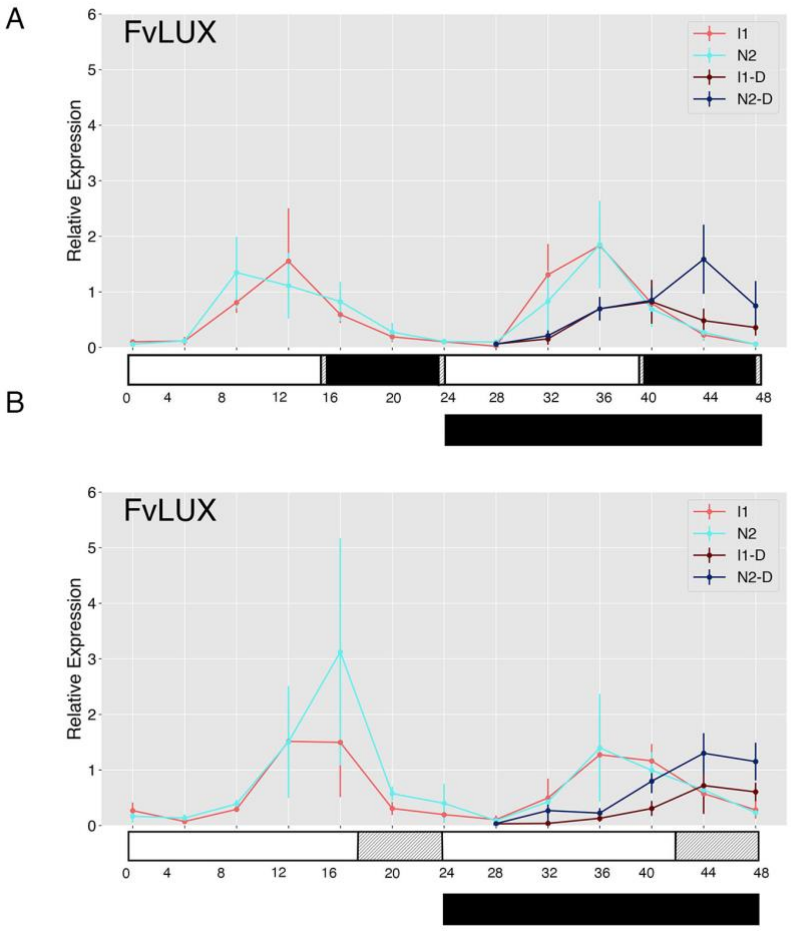

Figure 14. Relative expression of *FvLUX*. A: Italian (15:8) daylength. B: Norwegian (18:6) daylength. White bars indicate photosynthetic light, striped bars indicate non-photosynthetic light (twilight), and black bars indicate total night. Black bar across bottom indicates dark (D) treatment. Each datapoint represents mean ± S.E. of three biological replicates.

FvPRDX (Figure 15), a gene encoding a peroxiredoxin protein, did not show any clear phase or rhythm of mRNA transcription levels, with no significant differences between the treatments, and only one significantly different expression timepoint between clones in the Italian daylength at ZT44 (p=0.04). Movement to dark, however, did cause significant differential expression in both treatments. In N2, when moved to dark from the Norwegian daylength there

was a significant difference at ZT44 (p=0.01) and ZT48 (p=0.007), and when moved to dark from the Italian daylength there was a significant difference at ZT40 (p=0.0006) and ZT48 (p=0.0005), corresponding with a decrease in expression without light. I1 only had a significant difference in expression when moved to dark from Italian at ZT32 (p=0.04), and ZT40 (p=0.04).

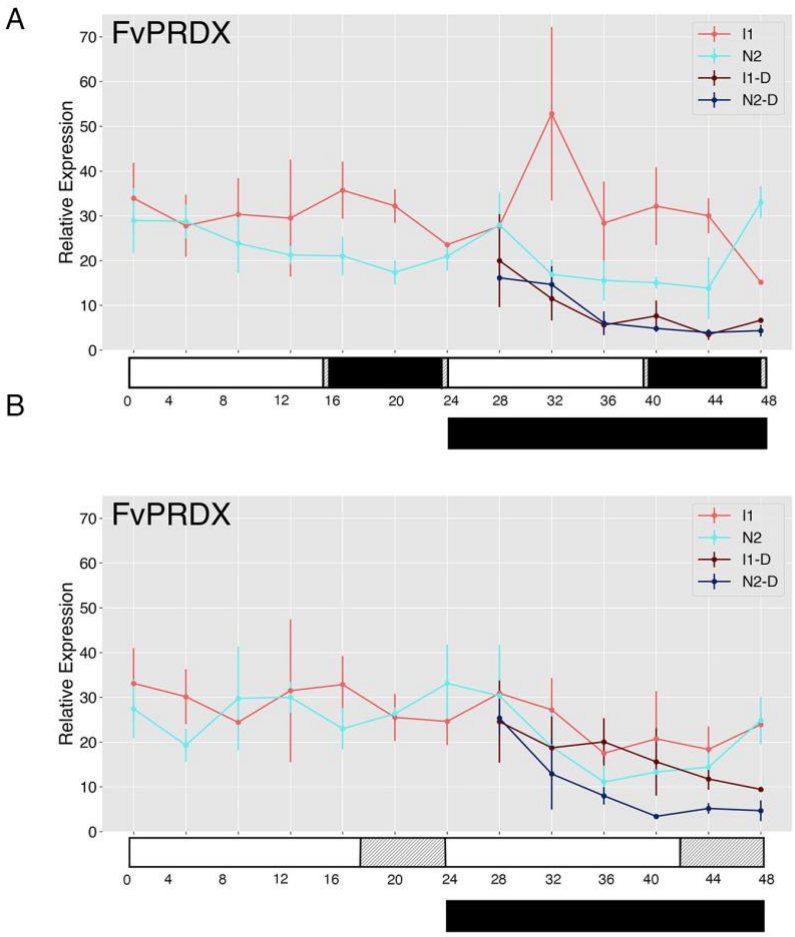

Figure 15. Relative expression of *FvPRDX*. A: Italian (15:8) daylength. B: Norwegian (18:6) daylength. White bars indicate photosynthetic light, striped bars indicate non-photosynthetic light (twilight), and black bars indicate total night. Black bar across bottom indicates dark (D) treatment. Each datapoint represents mean ± S.E. of three biological replicates.

DISCUSSION

Photoperiodic Entrainment

Latitudinal differences in photoperiod have led to natural variations in circadian clock outputs such as period of leaf movement and distinct daylength response phenotypes observed in flowering (Michael et al., 2003; Giakountis et al., 2010; Greenham et al., 2017). In this study, six of the circadian clock genes had significant differences in phase of expression between the two simulated summer daylengths. This demonstrates distinctive circadian responses to the different lengths of photosynthetic light, as well as light conditions at night. Remarkably, there were only significant differences in expression between the clones 3.3% of the time (Supplementary Table 4). Thus, latitudinal origin did not have a significant effect on photoentrainment, suggesting that plant clock systems have the capacity to adapt to photoperiods outside of their original range of daylength conditions. This is especially noteworthy for the Italian clone, which has never been subject to the very long daylength conditions at northern latitudes.

All genes maintained transcriptional rhythms in constant darkness, except for two (*FvPRR9* and FvPRDX) where transcription was completely abolished. The observation of maintained rhythms under free-running conditions (constant light or darkness) has been well established in studies dating back to the 1700's (Bünning & Pfeffer, 1989). Free-running transcriptional rhythms of clock genes in *Arabidopsis* have been published previously, with different effects under light vs. dark conditions (Wang & Tobin, 1998; Schaffer et al., 1998; Strayer, 2000; Matsushika et al., 2000; Alabadí et al., 2001; Rawat et al., 2011). Here, however, *FvPRR9* expression levels and rhythm were lost in conjunction with the loss of the light cycle. This is consistent with a previous study in *Arabidopsis* that found very low *PRR9* mRNA levels in dark-grown etiolated plants (Makino et al., 2001). In addition to regulating the central oscillator, *PRR9* plays a large role in modulating light input to the circadian clock, hence explaining the loss of transcription in the absence of light (Eriksson et al., 2003; Farré et al., 2005). This also confirms that observations of free-running periods are different in constant light versus constant darkness.

Importance of Clock Genes

The circadian genes here were chosen for their specific roles in the plant clock system. Both *FvRVE8* and *FvLHY* had a strong morning phase of expression, even in the Norwegian

daylength which had low levels of non-photosynthetic light at night, emphasizing the dependence on the predictable onset of photosynthetic light at dawn for timing of expression. LHY directly interacts with abscisic acid (ABA) pathways, which play a large part in tolerance of drought and osmotic stress (Adams et al., 2018). This could explain why transcription begins before dawn, to accumulate the particular genes needed to be prepared for a hot or dry day. While LHY is a key repressor of all other genes in the clock (Adams et al., 2015), RVE8 is a direct transcriptional activator of evening-phased genes as well as involved in temperature compensation and light signaling (Hsu et al., 2013). The transcription profiles of the morning-phased genes are very similar, but their protein accumulation patterns are quite different, signifying their differential roles in maintaining proper clock timing (Rawat et al., 2011).

In *Arabidopsis,* the *PRR*'s have been identified to express in sequential waves after dawn starting with *PRR9,* followed by *PRR7, PRR5, PRR3,* and ending with *TOC1* in the evening (Makino et al., 2001). A recent study characterizing the circadian clock genes in *Fragaria vesca* discovered that *PRR9, PRR7,* and *PRR5* have general afternoon phases instead of sequential waves of expression (Chen et al., 2018). Chen et al. also discovered that *F. vesca* doesn't have homologues of the *Arabidopsis* genes *AtPRR3, AtRVE4,* or *AtCCA1*. Here, *PRR9, PRR7,* and *PRR5* were confirmed to have roughly similar phases of expression in the afternoon in *F. vesca* with corresponding shifts in the phase with the later afternoon in the Norwegian daylength. These afternoon-phased genes act as transcriptional repressors of *CCA1* and *LHY* in *Arabidopsis* (Nakamichi et al., 2010), and coincided with the timing of decreased *LHY* expression in *F. vesca* here, confirming similarities in clock gene functions across higher plants (Song et al., 2010).

FvTOC1's phase of expression occurred throughout the evening and had lowest expression in late morning when *FvLHY* had the highest expression, providing supporting evidence of the association of reciprocal repression between *TOC1* and morning genes (Huang et al., 2012). This gene also had the largest variance in expression, a possible attribute of suboptimal primer design that requires further testing. TOC1 is central to the pacemaker function and also plays a part in reciprocal regulation with promoters of ABA pathways to maintain cellular homeostasis (Legnaioli et al., 2009). *FvTOC1*'s extended phase of expression from afternoon into early morning has been reported in previous studies and aligns with TOC1's close association with ABA to regulate drought stress via processes such as stomatal closure, which largely occur at night (Strayer, 2000; Makino et al., 2001; Pokhilko et al., 2013).

Lastly, *LUX* along with the other components of the Evening Complex (EC), directly regulate *PRR9* in *Arabidopsis*, as well as play an important role in growth in the early evening, corresponding with *FvLUX*'s short phase of expression right before dusk (Helfer et al., 2011; Nusinow et al., 2011). The EC regulates the circadian gating of hypocotyl growth in the early evening by repressing the expression of phytochrome interacting factors *PIF4* and *PIF5,* which help regulate plant responses to differential red (R), far-red (FR), and blue light throughout the day, and are not activated again until the following morning by interacting with LHY (Nusinow et al., 2011; Sun et al., 2019). Thus, the components in the clock system are directly dependent on the proper transcriptional timing of all other transcription factors in the TTFL to stay in sync with the external photoperiod.

Gene Regulation

Rhythmic transcription of mRNA is necessary for the molecular clock to maintain the rhythm of its core oscillator, however this is not the case for the PRDX protein. (Kojima et al., 2011). PRDX is not a specific clock component, but has been proposed as a possible link of the evolutionary origin of circadian rhythms due to its conserved nature across taxa (Edgar et al., 2012). The oxidation cycles of PRDX exhibit circadian rhythmicity that persist under constant darkness, but are independent of RNA transcription or protein synthesis (O'Neill et al., 2011; Edgar et al., 2012). Evidence of this was supported here by 1) no clear rhythm of *PRDX* transcription levels, and 2) the loss of *PRDX* transcription in constant darkness. This steady level of transcription in *PRDX* under different daylengths was also seen in a recent study in potato cultivars where *LHY* exhibited the same strong diurnal pattern (Mølmann et al. unpublished). This highlights the fact that mRNA levels do not always correlate with changes in protein levels due to multiple levels of regulation through post-transcriptional control, protein-protein interaction, and post-translational modifications such as phosphorylation of proteins (Leloup 2009; Pruneda-Paz and Kay, 2010; Cox and Takahashi 2019; Saini et al., 2019). Post-transcriptional control is imperative for the circadian clock to maintain its robust rhythms of mRNA expression as well as controlling other circadian regulated genes (Kojima et al., 2011). Very recent analyses have identified the role of small RNA's (sRNA) in epigenetic gene-silencing pathways and the role of intrinsically disordered proteins in maintaining robustness of the circadian system (Pelham et al., 2020; Tiwari et al., 2020; Zhou et al., 2021). Micro RNA's (miRNA) modulate gene expression either at the post-transcriptional level by degrading RNA or at the translation level by blocking protein biosynthesis, and have been associated with differing light intensities (Tripathi et al., 2019). The development of methods

to analyze post-transcriptional regulations will continue to lead us to a greater understanding of the intricate complexities of gene regulatory processes (Kojima et al., 2011).

Expression Validation

Recent studies have highlighted the importance of carefully validating reference gene candidates because novel reference genes may perform better than the traditionally used housekeeping genes (Lambret-Frotté et al., 2015). The candidate reference genes chosen for this study were *GAPDH* and *MSI1*. *GAPDH* is a commonly used reference gene due to its role in glycolysis and subsequent stable expression levels but has received scrutiny in recent years as an unsuitable reference gene because its transcription levels significantly differed under experimental settings (Radonić et al., 2004). MSI1 is a conserved WD-40 repeat protein involved in histone deacetylase complexes which control chromatin metabolism and abscisic acid levels that has been successfully used as the normalization gene in expression studies in *F. vesca* (Mouhu et al., 2009, 2013; Mehdi et al., 2016). This study found significantly different expression levels of *GAPDH* under different light treatments, further emphasizing the need to validate reference genes for each assay and not to choose a candidate based on its popularity.

Circadian Variation in Crop Species

While *Arabidopsis thaliana*, the typical plant model, can be used to represent the Brassicaceae family, an economically and agriculturally important crop family, *Fragaria vesca* can be used to represent the Rosaceae family, an economically significant family containing *Prunus* (peach, cherry, apricot, almond, plum), *Rubus* (blackberry, raspberry), *Malus* (apple), *Pyrus* (pear) and many species of ornamental trees and shrubs (rose, hawthorn, potentilla, cotoneaster, pyracantha) (Shulaev et al., 2008). Studies identifying how species adapt to northern latitudes are becoming necessary due to a warming climate making these areas more available for plant production (Mueller et al., 2015; Ettinger et al., 2021). The wild species of strawberry, *F. vesca,* is a valuable potential model organism due to their natural wide geographic range and shared sequence identity with the highly valued crop species, *F x. ananassa*. Observing the natural entrainment of the circadian clock in a clone from Italy to a Northern Norwegian summer daylength provides meaningful insight to the general adaptability of this species. Due to the conserved nature of the circadian clock and relatively recent formation of *F x. ananassa*, it can be hypothesized that the cultivated strawberry should also exhibit a similar level of entrainment in its circadian clock.

However, in contrast to the entrainment of the clock observed here, shifts in experienced photoperiod often impose constraints on phenological responses such as flowering time, one of the most important traits in crop yield (Ettinger et al., 2021). In strawberries, flowering occurs when *CONSTANS (CO)* mRNA expression levels coincide with light in the afternoon of long days, leading to the expression of *FLOWERING LOCUS T (FT)* (Kurokura et al., 2017). Even though *CO* expression is regulated by the circadian clock and the circadian clock is able to entrain to changes in the photoperiod, photoperiodic responses are complex traits with many loci that have stricter responses to changes in daylength (Yanovksy & Kay, 2003; Giakountis et al., 2010). Still, the circadian clock should be taken into account when choosing and modifying future crop species. Having a robust circadian rhythm will likely contribute to better adaptation to higher latitudes. Artificial selection in crop species to be more productive at different latitudes through modified flowering time, maturation time, and yield has indirectly been selecting for modifications of circadian clocks for years without any previous knowledge of circadian rhythms (Li and Lam, 2000). Now, with our current understanding of the circadian clock's importance and pliability, we can make informed decisions for the future of production.

Future Research

A key aspect of the circadian clock function is its plasticity under a wide range of temperatures (Gil & Park, 2019). Keeping the temperature constant (18°C) enabled us to isolate the impact that photoperiod has on mRNA transcriptional entrainment of the core circadian clock genes in woodland strawberries. On the other hand, a controlled temperature of 18°C doesn't reflect natural fluctuations of daily temperatures. Though the clock exhibits temperature compensation, it does not mean that small fluctuations will not have some effect on rhythmic output. Removing environmental noise was relevant for the purpose of this study to identify the specific effect of photoperiod on circadian entrainment. Additionally, using fluorescent lights as a light source is also different from daily variations in solar radiation. These results would benefit from a parallel study in the field to identify differences in laboratory vs environmental conditions on circadian clock entrainment. Perhaps the natural, cooler summer climate along with very long photosynthetic days in northern Norway would not be as suitable for entrainment of the Italian clone.

The plants of *F. vesca* have been moved around by humans for hundreds of years which may have obscured natural selection of photoperiodic ecotypes (Heide & Sønsteby, 2007). The clonal nature of this plant can also raise speculation, as the observations in this study are

assuming each of the clones represent their original locations. Despite this, genome sequencing has provided evidence that these clones are are more evolutionarily similar with the clones in their original regions (Hytönen, unpublished), thus the similarities between the clones observed here shed light on the adaptability of the clock. The two other clones that were not analyzed for this thesis are planned to be analyzed in the future, which will give information about replicability of this study, as well two more sources of data from each origin.

An interesting feature of this study was how quickly the clock was able to entrain to the new photoperiod. It would be interesting to know if it took the full two weeks to establish, or if it only takes a few days to adjust. Further studies involving transcriptomics comparing clock performance before and after introduction to a new daylength can identify deeper genetic involvement and the expression changes that occur during the transition.

CONCLUSIONS

In this study, latitudinal origin did not affect photoentrainment of circadian gene expression in the woodland strawberry, *Fragaria vesca*. Six of the eight genes analyzed expressed clear circadian oscillations of transcript levels where the phase and rhythm were nearly identical in both the Italian and Northern Norwegian clone under each simulated daylength. These genes also had significantly longer phases of expression under the longer daylength that is a characteristic of summers at northern latitudes. This highlights the remarkable plasticity of the circadian clock mechanism, even when the processes it regulates, such as flowering time, develop a strict daylength response phenotype. While it was previously known that circadian rhythms are advantageous, this direct gene expression analysis has improved our understanding of the adaptation potential of the plant circadian oscillator. Future work involving parallel field studies as well as analogous studies in different species will further our knowledge of photoperiodic entrainment in plants.

Works Cited

Abe, M. S., Matsumura, K., Yoshii, T., & Miyatake, T. (2021). Amplitude of circadian rhythms becomes weaken in the north, but there is no cline in the period of rhythm in a beetle. *PloS One*, *16*(1), e0245115. https://doi.org/10.1371/journal.pone.0245115

Adams, S., Grundy, J., Veflingstad, S. R., Dyer, N. P., Hannah, M. A., Ott, S., & Carré, I. A. (2018). Circadian control of abscisic acid biosynthesis and signalling pathways revealed by genome-wide analysis of LHY binding targets. *New Phytologist*, *220*(3), 893–907. https://doi.org/10.1111/nph.15415

Adams, S., Manfield, I., Stockley, P., & Carré, I. A. (2015). Revised Morning Loops of the Arabidopsis Circadian Clock Based on Analyses of Direct Regulatory Interactions. *PLOS ONE*, *10*(12), e0143943. https://doi.org/10.1371/journal.pone.0143943

Alabadí, D., Oyama, T., Yanovsky, M. J., Harmon, F. G., Más, P., & Kay, S. A. (2001). Reciprocal regulation between TOC1 and LHY/CCA1 within the Arabidopsis circadian clock. *Science (New York, N.Y.)*, *293*(5531), 880–883. https://doi.org/10.1126/science.1061320

Andreani, T. S., Itoh, T. Q., Yildirim, E., Hwangbo, D.-S., & Allada, R. (2015). Genetics of Circadian Rhythms. *Sleep Medicine Clinics*, *10*(4), 413–421. https://doi.org/10.1016/j.jsmc.2015.08.007

Baby, B., Antony, P., & Vijayan, R. (2018). Antioxidant and anticancer properties of berries. *Critical Reviews in Food Science and Nutrition*, *58*(15), 2491–2507. https://doi.org/10.1080/10408398.2017.1329198

Bertolini, E., Schubert, F. K., Zanini, D., Sehadová, H., Helfrich-Förster, C., & Menegazzi, P. (2019). Life at High Latitudes Does Not Require Circadian Behavioral Rhythmicity

under Constant Darkness. *Current Biology*, *29*(22), 3928-3936.e3. https://doi.org/10.1016/j.cub.2019.09.032

Bhadra, U., Thakkar, N., Das, P., & Pal Bhadra, M. (2017). Evolution of circadian rhythms: From bacteria to human. *Sleep Medicine*, *35*, 49–61. https://doi.org/10.1016/j.sleep.2017.04.008

Bloch, G., Barnes, B. M., Gerkema, M. P., & Helm, B. (2013). Animal activity around the clock with no overt circadian rhythms: Patterns, mechanisms and adaptive value. *Proceedings of the Royal Society B: Biological Sciences*, *280*(1765), 20130019. https://doi.org/10.1098/rspb.2013.0019

Bünning, E., & Pfeffer, H. W. (1989). *Wilhelm Pfeffer: Early Advances in Plant Biology*. McGill-Queen's University Press. http://ebookcentral.proquest.com/lib/tromsoub-ebooks/detail.action?docID=3331223

Chaix, A., Zarrinpar, A., & Panda, S. (2016). The circadian coordination of cell biology. *The Journal of Cell Biology*, *215*(1), 15–25. https://doi.org/10.1083/jcb.201603076

Chen, L., Cai, Y., Qu, M., Wang, L., Sun, H., Jiang, B., Wu, T., Liu, L., Sun, S., Wu, C., Yao, W., Yuan, S., Han, T., & Hou, W. (2020). Soybean adaption to high-latitude regions is associated with natural variations of GmFT2b, an ortholog of FLOWERING LOCUS T. *Plant, Cell & Environment*, *43*(4), 934–944. https://doi.org/10.1111/pce.13695

Chen, X. D., Wang, J., Zhao, M. Z., & Zhao, F. (2018). Characterization and expression analysis of circadian clock genes in the diploid woodland strawberry Fragaria vesca. *Biologia Plantarum*, *62*(3), 451–461. https://doi.org/10.1007/s10535-018-0793-4

Cohen, S. E., & Golden, S. S. (2015). Circadian Rhythms in Cyanobacteria. *Microbiology and Molecular Biology Reviews*, *79*(4), 373–385. https://doi.org/10.1128/MMBR.00036-15

Covington, M. F., Maloof, J. N., Straume, M., Kay, S. A., & Harmer, S. L. (2008). Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. *Genome Biology*, *9*(8), R130. https://doi.org/10.1186/gb-2008-9-8-r130

Cox, K. H., & Takahashi, J. S. (2019). Circadian clock genes and the transcriptional architecture of the clock mechanism. *Journal of Molecular Endocrinology*, *63*(4), R93–R102. https://doi.org/10.1530/JME-19-0153

Darrow, G. M. (1966). *The Strawberry; History, Breeding, and Physiology* (1st ed.). New York, Holt, Rinehart and Winston. https://www.uvm.edu/vtvegandberry/factsheets/strawberryhistory.html

De Leone, M. J., Hernando, C. E., Romanowski, A., García-Hourquet, M., Careno, D., Casal, J., Rugnone, M., Mora-García, S., & Yanovsky, M. J. (2019). The LNK Gene Family: At the Crossroad between Light Signaling and the Circadian Clock. *Genes*, *10*(1), 2. https://doi.org/10.3390/genes10010002

Dodd, A. N., Salathia, N., Hall, A., Kévei, E., Tóth, R., Nagy, F., Hibberd, J. M., Millar, A. J., & Webb, A. A. R. (2005). Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science (New York, N.Y.)*, *309*(5734), 630–633. https://doi.org/10.1126/science.1115581

Dvornyk, V., Vinogradova, O., & Nevo, E. (2003). Origin and evolution of circadian clock genes in prokaryotes. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(5), 2495–2500. https://doi.org/10.1073/pnas.0130099100

Edgar, R. S., Green, E. W., Zhao, Y., van Ooijen, G., Olmedo, M., Qin, X., Xu, Y., Pan, M., Valekunja, U. K., Feeney, K. A., Maywood, E. S., Hastings, M. H., Baliga, N. S., Merrow, M., Millar, A. J., Johnson, C. H., Kyriacou, C. P., O'Neill, J. S., & Reddy, A.

B. (2012). Peroxiredoxins are conserved markers of circadian rhythms. *Nature*, *485*(7399), 459–464. https://doi.org/10.1038/nature11088

Edger, P. P., Poorten, T. J., VanBuren, R., Hardigan, M. A., Colle, M., McKain, M. R., Smith, R. D., Teresi, S. J., Nelson, A. D. L., Wai, C. M., Alger, E. I., Bird, K. A., Yocca, A. E., Pumplin, N., Ou, S., Ben-Zvi, G., Brodt, A., Baruch, K., Swale, T., … Knapp, S. J. (2019). Origin and evolution of the octoploid strawberry genome. *Nature Genetics*, *51*(3), 541–547. https://doi.org/10.1038/s41588-019-0356-4

Eriksson, M. E., Hanano, S., Southern, M. M., Hall, A., & Millar, A. J. (2003). Response regulator homologues have complementary, light-dependent functions in the Arabidopsis circadian clock. *Planta*, *218*(1), 159–162. https://doi.org/10.1007/s00425-003-1106-4

Eriksson, M. E., & Millar, A. J. (2003). The Circadian Clock. A Plant's Best Friend in a Spinning World. *Plant Physiology*, *132*(2), 732–738. https://doi.org/10.1104/pp.103.022343

Ettinger, A. K., Buonaiuto, D. M., Chamberlain, C. J., Morales-Castilla, I., & Wolkovich, E. M. (2021). Spatial and temporal shifts in photoperiod with climate change. *New Phytologist*, *230*(2), 462–474. https://doi.org/10.1111/nph.17172

Farré, E. M., Harmer, S. L., Harmon, F. G., Yanovsky, M. J., & Kay, S. A. (2005). Overlapping and Distinct Roles of PRR7 and PRR9 in the Arabidopsis Circadian Clock. *Current Biology*, *15*(1), 47–54. https://doi.org/10.1016/j.cub.2004.12.067

Folta, K. M., & Davis, T. M. (2006). Strawberry Genes and Genomics. *Critical Reviews in Plant Sciences*, *25*(5), 399–415. https://doi.org/10.1080/07352680600824831

Freitas, F. C. P., Depintor, T. S., Agostini, L. T., Luna-Lucena, D., Nunes, F. M. F., Bitondi, M. M. G., Simões, Z. L. P., & Lourenço, A. P. (2019). Evaluation of reference genes

for gene expression analysis by real-time quantitative PCR (qPCR) in three stingless bee species (Hymenoptera: Apidae: Meliponini). *Scientific Reports*, *9*(1), 17692. https://doi.org/10.1038/s41598-019-53544-0

Giakountis, A., Cremer, F., Sim, S., Reymond, M., Schmitt, J., & Coupland, G. (2010). Distinct Patterns of Genetic Variation Alter Flowering Responses of Arabidopsis Accessions to Different Daylengths. *Plant Physiology*, *152*(1), 177–191. https://doi.org/10.1104/pp.109.140772

Gil, K.-E., & Park, C.-M. (2019). Thermal adaptation and plasticity of the plant circadian clock. *New Phytologist*, *221*(3), 1215–1229. https://doi.org/10.1111/nph.15518

Golden, S. S. (2003). Timekeeping in bacteria: The cyanobacterial circadian clock. *Current Opinion in Microbiology*, *6*(6), 535–540. https://doi.org/10.1016/j.mib.2003.10.012

Gould, P. D., Locke, J. C. W., Larue, C., Southern, M. M., Davis, S. J., Hanano, S., Moyle, R., Milich, R., Putterill, J., Millar, A. J., & Hall, A. (2006). The Molecular Basis of Temperature Compensation in the Arabidopsis Circadian Clock. *The Plant Cell*, *18*(5), 1177–1187. https://doi.org/10.1105/tpc.105.039990

Green, R. M., Tingay, S., Wang, Z.-Y., & Tobin, E. M. (2002). Circadian Rhythms Confer a Higher Level of Fitness to Arabidopsis Plants. *Plant Physiology*, *129*(2), 576–584. https://doi.org/10.1104/pp.004374

Greenham, K., Lou, P., Puzey, J. R., Kumar, G., Arnevik, C., Farid, H., Willis, J. H., & McClung, C. R. (2017). Geographic Variation of Plant Circadian Clock Function in Natural and Agricultural Settings. *Journal of Biological Rhythms*, *32*(1), 26–34. https://doi.org/10.1177/0748730416679307

Greenham, K., & McClung, C. R. (2015). Integrating circadian dynamics with physiological processes in plants. *Nature Reviews Genetics*, *16*(10), 598–610. https://doi.org/10.1038/nrg3976

Hall, A., Karplus, P. A., & Poole, L. B. (2009). Typical 2-Cys peroxiredoxins—Structures, mechanisms and functions. *The FEBS Journal*, *276*(9), 2469–2477. https://doi.org/10.1111/j.1742-4658.2009.06985.x

Hancock, J., Finn, C. E., Luby, J. J., Callow, P. W., & Serce, S. (2010, July). *Reconstruction of the Strawberry, Fragaria ×ananassa, Using Genotypes of F. virginiana and F. chiloensis in: HortScience Volume 45 Issue 7 (2010)*. https://journals-ashs-org.mime.uit.no/hortsci/view/journals/hortsci/45/7/article-p1006.xml

Harmer, S. L., Hogenesch, J. B., Straume, M., Chang, H. S., Han, B., Zhu, T., Wang, X., Kreps, J. A., & Kay, S. A. (2000). Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. *Science (New York, N.Y.)*, *290*(5499), 2110–2113. https://doi.org/10.1126/science.290.5499.2110

Harmer, Stacey L. (2009). The Circadian System in Higher Plants. *Annual Review of Plant Biology*, *60*(1), 357–377. https://doi.org/10.1146/annurev.arplant.043008.092054

Heide, O. M., & Sønsteby, A. (2007). Interactions of temperature and photoperiod in the control of flowering of latitudinal and altitudinal populations of wild strawberry (Fragaria vesca). *Physiologia Plantarum*, *130*(2), 280–289. https://doi.org/10.1111/j.1399-3054.2007.00906.x

Helfer, A., Nusinow, D. A., Chow, B. Y., Gehrke, A. R., Bulyk, M. L., & Kay, S. A. (2011). LUX ARRHYTHMO Encodes a Nighttime Repressor of Circadian Gene Expression in the Arabidopsis Core Clock. *Current Biology*, *21*(2), 126–133. https://doi.org/10.1016/j.cub.2010.12.021

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., & Vandesompele, J. (2007). QBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology*, *8*(2), R19. https://doi.org/10.1186/gb-2007-8-2-r19

Hsu, P. Y., Devisetty, U. K., & Harmer, S. L. (2013). Accurate timekeeping is controlled by a cycling activator in Arabidopsis. *ELife*, *2*, e00473. https://doi.org/10.7554/eLife.00473

Hsu, P. Y., & Harmer, S. L. (2014). Wheels within wheels: The plant circadian system. *Trends in Plant Science*, *19*(4), 240–249. https://doi.org/10.1016/j.tplants.2013.11.007

Huang, H., Alvarez, S., Bindbeutel, R., Shen, Z., Naldrett, M. J., Evans, B. S., Briggs, S. P., Hicks, L. M., Kay, S. A., & Nusinow, D. A. (2016). Identification of Evening Complex Associated Proteins in Arabidopsis by Affinity Purification and Mass Spectrometry*. *Molecular & Cellular Proteomics*, *15*(1), 201–217. https://doi.org/10.1074/mcp.M115.054064

Huang, W., Pérez-García, P., Pokhilko, A., Millar, A. J., Antoshechkin, I., Riechmann, J. L., & Mas, P. (2012). Mapping the Core of the Arabidopsis Circadian Clock Defines the Network Structure of the Oscillator. *Science*, *336*(6077), 75–79. https://doi.org/10.1126/science.1219075

Hummer, K., Nathewet, P., & Yanagi, T. (2009). Decaploidy in Fragaria iturupensis (Rosaceae). *American Journal of Botany*, *96*, 713–716. https://doi.org/10.3732/ajb.0800285

Hurley, J. M., Loros, J. J., & Dunlap, J. C. (2016). Circadian Oscillators: Around the Transcription–Translation Feedback Loop and on to Output. *Trends in Biochemical Sciences*, *41*(10), 834–846. https://doi.org/10.1016/j.tibs.2016.07.009

Hut, R. A., & Beersma, D. G. M. (2011). Evolution of time-keeping mechanisms: Early emergence and adaptation to photoperiod. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *366*(1574), 2141–2154. https://doi.org/10.1098/rstb.2010.0409

Hut, Roelof A., Paolucci, S., Dor, R., Kyriacou, C. P., & Daan, S. (2013). Latitudinal clines: An evolutionary view on biological rhythms,. *Proceedings of the Royal Society B: Biological Sciences*, *280*(1765). https://doi.org/10.1098/rspb.2013.0433

Jose, S., Abbey, J., Jaakola, L., & Percival, D. (2020). Selection and validation of reliable reference genes for gene expression studies from Monilinia vaccinii-corymbosi infected wild blueberry phenotypes. *Scientific Reports*, *10*(1), 11688. https://doi.org/10.1038/s41598-020-68597-9

Karlgren, A., Gyllenstrand, N., Källman, T., & Lagercrantz, U. (2013). Conserved Function of Core Clock Proteins in the Gymnosperm Norway Spruce (Picea abies L. Karst). *PLOS ONE*, *8*(3), e60110. https://doi.org/10.1371/journal.pone.0060110

Kevei, É., Gyula, P., Hall, A., Kozma-Bognár, L., Kim, W.-Y., Eriksson, M. E., Tóth, R., Hanano, S., Fehér, B., Southern, M. M., Bastow, R. M., Viczián, A., Hibberd, V., Davis, S. J., Somers, D. E., Nagy, F., & Millar, A. J. (2006). Forward Genetic Analysis of the Circadian Clock Separates the Multiple Functions of ZEITLUPE. *Plant Physiology*, *140*(3), 933–945. https://doi.org/10.1104/pp.105.074864

Kim, J. A., Kim, H.-S., Choi, S.-H., Jang, J.-Y., Jeong, M.-J., & Lee, S. I. (2017). The Importance of the Circadian Clock in Regulating Plant Metabolism. *International Journal of Molecular Sciences*, *18*(12). https://doi.org/10.3390/ijms18122680

Kloppstech, K. (1985). Diurnal and circadian rhythmicity in the expression of light-induced plant nuclear messenger RNAs. *Planta*, *165*(4), 502–506. https://doi.org/10.1007/BF00398095

Kojima, S., Shingle, D. L., & Green, C. B. (2011a). Post-transcriptional control of circadian rhythms. *Journal of Cell Science*, *124*(3), 311–320. https://doi.org/10.1242/jcs.065771

Kurokura, T., Samad, S., Koskela, E., Mouhu, K., & Hytönen, T. (2017). Fragaria vesca CONSTANS controls photoperiodic flowering and vegetative development. *Journal of Experimental Botany*, *68*(17), 4839–4850. https://doi.org/10.1093/jxb/erx301

Lambret-Frotté, J., Almeida, L. C. S. de, Moura, S. M. de, Souza, F. L. F., Linhares, F. S., & Alves-Ferreira, M. (2015). Validating Internal Control Genes for the Accurate Normalization of qPCR Expression Analysis of the Novel Model Plant Setaria viridis. *PLOS ONE*, *10*(8), e0135006. https://doi.org/10.1371/journal.pone.0135006

Lathe, R. (2004). Fast tidal cycling and the origin of life. *Icarus*, *168*(1), 18–22. https://doi.org/10.1016/j.icarus.2003.10.018

Legnaioli, T., Cuevas, J., & Mas, P. (2009). TOC1 functions as a molecular switch connecting the circadian clock with plant responses to drought. *The EMBO Journal*, *28*(23), 3745–3757. https://doi.org/10.1038/emboj.2009.297

Linde, A.-M., Eklund, D. M., Kubota, A., Pederson, E. R. A., Holm, K., Gyllenstrand, N., Nishihama, R., Cronberg, N., Muranaka, T., Oyama, T., Kohchi, T., & Lagercrantz, U. (2017). Early evolution of the land plant circadian clock. *New Phytologist*, *216*(2), 576–590. https://doi.org/10.1111/nph.14487

Liston, A., Cronn, R., & Ashman, T.-L. (2014). Fragaria: A genus with deep historical roots and ripe for evolutionary and ecological insights. *American Journal of Botany*, *101*(10), 1686–1699. https://doi.org/10.3732/ajb.1400140

Loudon, A. S. I. (2012). Circadian Biology: A 2.5 Billion Year Old Clock. *Current Biology*, *22*(14), R570–R571. https://doi.org/10.1016/j.cub.2012.06.023

Makino, S., Matsushika, A., Kojima, M., Oda, Y., & Mizuno, T. (2001). Light Response of the Circadian Waves of the APRR1/TOC1 Quintet: When Does the Quintet Start Singing Rhythmically in Arabidopsis? *Plant and Cell Physiology*, *42*(3), 334–339. https://doi.org/10.1093/pcp/pce036

Martínez-García, J., Virgós-Soler, A., & Prat, S. (2002). Control of photoperiod-regulated tuberization in potato by the Arabidopsis flowering-time gene CONSTANS. *Proceedings of the National Academy of Sciences of the United States of America*, *99*, 15211–15216. https://doi.org/10.1073/pnas.222390599

Más, P. (2005). Circadian clock signaling in Arabidopsis thaliana: From gene expression to physiology and development. *The International Journal of Developmental Biology*, *49*(5–6), 491–500. https://doi.org/10.1387/ijdb.041968pm

Más, P., & Yanovsky, M. J. (2009). Time for circadian rhythms: Plants get synchronized. *Current Opinion in Plant Biology*, *12*(5), 574–579. https://doi.org/10.1016/j.pbi.2009.07.010

Matsuo, T., Okamoto, K., Onai, K., Niwa, Y., Shimogawara, K., & Ishiura, M. (2008). A systematic forward genetic analysis identified components of the Chlamydomonas circadian system. *Genes & Development*, *22*(7), 918–930. https://doi.org/10.1101/gad.1650408

Matsushika, A., Makino, S., Kojima, M., & Mizuno, T. (2000). Circadian Waves of Expression of the APRR1/TOC1 Family of Pseudo-Response Regulators in Arabidopsis thaliana: Insight into the Plant Circadian Clock. *Plant and Cell Physiology*, *41*(9), 1002–1012. https://doi.org/10.1093/pcp/pcd043

McClung, C. R. (2006). Plant Circadian Rhythms. *The Plant Cell*, *18*(4), 792–803. https://doi.org/10.1105/tpc.106.040980

Mehdi, S., Derkacheva, M., Ramström, M., Kralemann, L., Bergquist, J., & Hennig, L. (2016). The WD40 Domain Protein MSI1 Functions in a Histone Deacetylase Complex to Fine-Tune Abscisic Acid Signaling. *The Plant Cell*, *28*(1), 42–54. https://doi.org/10.1105/tpc.15.00763

Merrow, M., Spoelstra, K., & Roenneberg, T. (2005). The circadian cycle: Daily rhythms from behaviour to genes. *EMBO Reports*, *6*(10), 930–935. https://doi.org/10.1038/sj.embor.7400541

Michael, T. P., Salomé, P. A., Yu, H. J., Spencer, T. R., Sharp, E. L., McPeek, M. A., Alonso, J. M., Ecker, J. R., & McClung, C. R. (2003). Enhanced fitness conferred by naturally occurring variation in the circadian clock. *Science (New York, N.Y.)*, *302*(5647), 1049–1053. https://doi.org/10.1126/science.1082971

Mizuno, T., & Yamashino, T. (2008). Comparative Transcriptome of Diurnally Oscillating Genes and Hormone-Responsive Genes in Arabidopsis thaliana: Insight into Circadian Clock-Controlled Daily Responses to Common Ambient Stresses in Plants. *Plant and Cell Physiology*, *49*(3), 481–487. https://doi.org/10.1093/pcp/pcn008

Mølmann, J., Dalmannsdottir, S., Hykkerud, A. L., Hytönen, T., Samkumar, A., & Jaakola, L. (2021). Influence of the arctic light conditions on the crop production and quality. *Physiologia Plantarum*

Mouhu, K., Hytönen, T., Folta, K., Rantanen, M., Paulin, L., Auvinen, P., & Elomaa, P. (2009). Identification of flowering genes in strawberry, a perennial SD plant. *BMC Plant Biology*, *9*(1), 122. https://doi.org/10.1186/1471-2229-9-122

Mouhu, K., Kurokura, T., Koskela, E. A., Albert, V. A., Elomaa, P., & Hytönen, T. (2013). The Fragaria vesca Homolog of SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 Represses Flowering and Promotes Vegetative Growth. *The Plant Cell*, *25*(9), 3296–3310. https://doi.org/10.1105/tpc.113.115055

Mueller, B., Hauser, M., Iles, C., Rimi, R. H., Zwiers, F. W., & Wan, H. (2015). Lengthening of the growing season in wheat and maize producing regions. *Weather and Climate Extremes*, *9*, 47–56. https://doi.org/10.1016/j.wace.2015.04.001

Muthukumaran, S., Tranchant, C., Shi, J., Ye, X., & Xue, S. J. (2017). Ellagic acid in strawberry (Fragaria spp.): Biological, technological, stability, and human health aspects. *Food Quality and Safety*, *1*(4), 227–252. https://doi.org/10.1093/fqsafe/fyx023

Nagel, D. H., & Kay, S. A. (2012). Complexity in the Wiring and Regulation of Plant Circadian Networks. *Current Biology*, *22*(16), R648–R657. https://doi.org/10.1016/j.cub.2012.07.025

Nakamichi, N. (2020). The Transcriptional Network in the Arabidopsis Circadian Clock System. *Genes*, *11*(11), 1284. https://doi.org/10.3390/genes11111284

Nakamichi, N., Kiba, T., Henriques, R., Mizuno, T., Chua, N.-H., & Sakakibara, H. (2010). PSEUDO-RESPONSE REGULATORS 9, 7, and 5 Are Transcriptional Repressors in the Arabidopsis Circadian Clock[W][OA]. *The Plant Cell*, *22*(3), 594–605. https://doi.org/10.1105/tpc.109.072892

Njuguna, W., Liston, A., Cronn, R., Ashman, T.-L., & Bassil, N. (2012). Insights into phylogeny, sex function and age of Fragaria based on whole chloroplast genome sequencing. *Molecular Phylogenetics and Evolution*, *66*. https://doi.org/10.1016/j.ympev.2012.08.026

Nusinow, D. A., Helfer, A., Hamilton, E. E., King, J. J., Imaizumi, T., Schultz, T. F., Farre, E. M., & Kay, S. A. (2011). The ELF4-ELF3-LUX complex links the circadian clock to diurnal control of hypocotyl growth. *Nature*, *475*(7356), 398–405. https://doi.org/10.1038/nature10182

O'Neill, J. S., van Ooijen, G., Dixon, L. E., Troein, C., Corellou, F., Bouget, F.-Y., Reddy, A. B., & Millar, A. J. (2011). Circadian rhythms persist without transcription in a eukaryote. *Nature*, *469*(7331), 554–558. https://doi.org/10.1038/nature09654

Ouyang, K., Li, J., Huang, H., Que, Q., Li, P., & Chen, X. (2014). A simple method for RNA isolation from various tissues of the tree Neolamarckia cadamba. *Biotechnology, Biotechnological Equipment*, *28*(6), 1008–1013. https://doi.org/10.1080/13102818.2014.981086

Paolucci, S., Dalla Benetta, E., Salis, L., Doležel, D., van de Zande, L., & Beukeboom, L. W. (2019). Latitudinal Variation in Circadian Rhythmicity in Nasonia vitripennis. *Behavioral Sciences (Basel, Switzerland)*, *9*(11). https://doi.org/10.3390/bs9110115

Pelham, J. F., Dunlap, J. C., & Hurley, J. M. (2020). Intrinsic disorder is an essential characteristic of components in the conserved circadian circuit. *Cell Communication and Signaling*, *18*(1), 181. https://doi.org/10.1186/s12964-020-00658-y

Pokhilko, A., Fernández, A. P., Edwards, K. D., Southern, M. M., Halliday, K. J., & Millar, A. J. (2012). The clock gene circuit in Arabidopsis includes a repressilator with additional feedback loops. *Molecular Systems Biology*, *8*, 574. https://doi.org/10.1038/msb.2012.6

Pokhilko, A., Más, P., & Millar, A. (2013). Modelling the widespread effects of TOC1 signalling on the plant circadian clock and its outputs. *BMC Systems Biology*, *7*, 23. https://doi.org/10.1186/1752-0509-7-23

Radonić, A., Thulke, S., Mackay, I. M., Landt, O., Siegert, W., & Nitsche, A. (2004). Guideline to reference gene selection for quantitative real-time PCR. *Biochemical and Biophysical Research Communications*, *313*(4), 856–862. https://doi.org/10.1016/j.bbrc.2003.11.177

Rawat, R., Takahashi, N., Hsu, P. Y., Jones, M. A., Schwartz, J., Salemi, M. R., Phinney, B. S., & Harmer, S. L. (2011). REVEILLE8 and PSEUDO-REPONSE REGULATOR5 Form a Negative Feedback Loop within the Arabidopsis Circadian Clock. *PLOS Genetics*, *7*(3), e1001350. https://doi.org/10.1371/journal.pgen.1001350

Reddy, P., Zehring, W. A., Wheeler, D. A., Pirrotta, V., Hadfield, C., Hall, J. C., & Rosbash, M. (1984). Molecular analysis of the period locus in Drosophila melanogaster and identification of a transcript involved in biological rhythms. *Cell*, *38*(3), 701–710. https://doi.org/10.1016/0092-8674(84)90265-4

Romanowski, A., & Yanovsky, M. J. (2015). Circadian rhythms and post-transcriptional regulation in higher plants. *Frontiers in Plant Science*, *6*. https://doi.org/10.3389/fpls.2015.00437

Saini, R., Jaskolski, M., & Davis, S. J. (2019). Circadian oscillator proteins across the kingdoms of life: Structural aspects. *BMC Biology*, *17*. https://doi.org/10.1186/s12915-018-0623-3

Santamaría, L., Figuerola, J., Pilon, J. J., Mjelde, M., Green, A. J., de Boer, T., King, R. A., & Gornall, R. J. (2003). Plant Performance across Latitude: The Role of Plasticity and Local Adaptation in an Aquatic Plant. *Ecology*, *84*(9), 2454–2461.

Schaffer, R., Ramsay, N., Samach, A., Corden, S., Putterill, J., Carré, I. A., & Coupland, G. (1998). The late elongated hypocotyl Mutation of Arabidopsis Disrupts Circadian

Rhythms and the Photoperiodic Control of Flowering. *Cell*, *93*(7), 1219–1229. https://doi.org/10.1016/S0092-8674(00)81465-8

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C T method. *Nature Protocols*, *3*(6), 1101–1108. https://doi.org/10.1038/nprot.2008.73

Sharma, V. K. (2006). Pittendrigh: The darwinian clock-watcher. *Resonance*, *11*(5), 32–41. https://doi.org/10.1007/BF02839369

Shulaev, V., Korban, S. S., Sosinski, B., Abbott, A. G., Aldwinckle, H. S., Folta, K. M., Iezzoni, A., Main, D., Arús, P., Dandekar, A. M., Lewers, K., Brown, S. K., Davis, T. M., Gardiner, S. E., Potter, D., & Veilleux, R. E. (2008). Multiple models for Rosaceae genomics. *Plant Physiology*, *147*(3), 985–1003. http://dx.doi.org/10.1104/pp.107.115618

Shulaev, V., Sargent, D. J., Crowhurst, R. N., Mockler, T. C., Folkerts, O., Delcher, A. L., Jaiswal, P., Mockaitis, K., Liston, A., Mane, S. P., Burns, P., Davis, T. M., Slovin, J. P., Bassil, N., Hellens, R. P., Evans, C., Harkins, T., Kodira, C., Desany, B., … Folta, K. M. (2011). The genome of woodland strawberry (Fragaria vesca). *Nature Genetics*, *43*(2), 109–116. https://doi.org/10.1038/ng.740

Somerville, C., & Koornneef, M. (2002). Timeline - A fortunate choice: The history of Arabidopsis as a model plant. *Nature Reviews Genetics 3 (2002) 11*.

Song, Y. H., Ito, S., & Imaizumi, T. (2010). Similarities in the circadian clock and photoperiodism in plants. *Current Opinion in Plant Biology*, *13*(5), 594–603. https://doi.org/10.1016/j.pbi.2010.05.004

Strayer, C. (2000). Cloning of the Arabidopsis Clock Gene TOC1, an Autoregulatory Response Regulator Homolog. *Science*, *289*(5480), 768–771. https://doi.org/10.1126/science.289.5480.768

Sun, Q., Wang, S., Xu, G., Kang, X., Zhang, M., & Ni, M. (2019). SHB1 and CCA1 interaction desensitizes light responses and enhances thermomorphogenesis. *Nature Communications*, *10*(1), 3110. https://doi.org/10.1038/s41467-019-11071-6

Tiwari, B., Habermann, K., Arif, M. A., Weil, H. L., Garcia-Molina, A., Kleine, T., Mühlhaus, T., & Frank, W. (2020). Identification of small RNAs during cold acclimation in Arabidopsis thaliana. *BMC Plant Biology*, *20*(1), 298. https://doi.org/10.1186/s12870-020-02511-3

Tripathi, A. M., Singh, A., Singh, R., Verma, A. K., & Roy, S. (2019). Modulation of miRNA expression in natural populations of A. thaliana along a wide altitudinal gradient of Indian Himalayas. *Scientific Reports*, *9*(1), 441. https://doi.org/10.1038/s41598-018-37465-y

Troein, C., Locke, J. C. W., Turner, M. S., & Millar, A. J. (2009). Weather and Seasons Together Demand Complex Biological Clocks. *Current Biology*, *19*(22), 1961–1964. https://doi.org/10.1016/j.cub.2009.09.024

Troncoso-Ponce, M. A., & Mas, P. (2012). Newly Described Components and Regulatory Mechanisms of Circadian Clock Function in Arabidopsis thaliana. *Molecular Plant*, *5*(3), 545–553. https://doi.org/10.1093/mp/ssr117

Wang, Z.-Y., & Tobin, E. M. (1998). Constitutive Expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) Gene Disrupts Circadian Rhythms and Suppresses Its Own Expression. *Cell*, *93*(7), 1207–1217. https://doi.org/10.1016/S0092-8674(00)81464-6

Yanovsky, M. J., & Kay, S. A. (2003). Living by the calendar: How plants know when to flower. *Nature Reviews Molecular Cell Biology*, *4*(4), 265–276. https://doi.org/10.1038/nrm1077

Yon, F., Seo, P.-J., Ryu, J. Y., Park, C.-M., Baldwin, I. T., & Kim, S.-G. (2012). Identification and characterization of circadian clock genes in a native tobacco, Nicotiana attenuata. *BMC Plant Biology*, *12*(1), 172. https://doi.org/10.1186/1471-2229-12-172

Young, M. W., & Kay, S. A. (2001). Time zones: A comparative genetics of circadian clocks. *Nature Reviews Genetics*, *2*(9), 702–716. https://doi.org/10.1038/35088576

Zhou, L., Miller, C., Miraglia, L. J., Romero, A., Mure, L. S., Panda, S., & Kay, S. A. (2021). A genome-wide microRNA screen identifies the microRNA-183/96/182 cluster as a modulator of circadian rhythms. *Proceedings of the National Academy of Sciences*, *118*(1). https://doi.org/10.1073/pnas.2020454118

Appendix A – Primers

Supplementary Table 1. Primer sequences and efficiency (E%). Genes: Full names, Gene ID's (GDR, http:/www.rosaceae.org; *Fragaria vesca* Whole Genome v1.0 (build 8), gene primer sequences, and standard curve data: Slope, y-intercept, coefficient of determination (R^2) and E%.

Gene	Gene ID	Full Name	Forward Primer (5'-3')	Reverse Primer (3'-5')	Slope	Y-intercept	R^2	Efficieny (%)	Source
Target Genes									
FvLHY	gene18601	Late Elongated Hypocotyl	CACTCAGAAGCTGTGCAAGC	GTAACTGGACTCAGAAGCTG	-2.9	20.43	0.96	121.2	Chen et al. 2018
FvPRR9	gene18611	Two-component response regulator-like PRR95	GTAGCAGGTGAGATGGCCAC	CAATGTGAGCTCTATGCACG	-2.8	23.34	0.99	127.2	Chen et al. 2018
FvPRR7	gene18151	Two-component response regulator-like APRR 7	CTGCAGCAGCTCCACATTGC	CGAGGTCGCTGTTCTGCTAG	-2.9	23.62	0.99	116.1	Chen et al. 2018
FvRVE8	gene18104	Reveille 8 protein	GTCACAAGCTCATGTGCGCCA	CTGCTGAGCTATAGTCCGAAG	-2.7	23.81	0.97	131.8	Chen et al. 2018
FvPRR5	gene12454	Two-component response regulator-like APRR 5	CTGCAATGGCAATGCAAGTC	GAAGGATCAGTAACTTGACG	-3	23.02	0.99	112	Chen et al. 2018
FvTOC1/ APRR1	gene26055	Timing of CAB 1/ Two-component response regulator-like APRR1	CCGTCCGATCAGTCCTCTTC	CTCGACTCGTCGTTATCGCA	-2.96	24.7	0.99	117.6	NCBI Primer-Blast
FvLUX/ PCL1	gene30204	Transcription factor Lux arrhythmo/ Phytoclock 1	ACTTGCAGAAGTACCGCCTC	CGCAACATGTGGTTGGTACG	-3.15	28.96	0.92	107.6	NCBI Primer-Blast
FvPRDX	gene15307	2-Cys peroxiredoxin	TCAGCGATCGTCATGGTGAG	CCAAGACCGCCTGATTTCCT	-3.4	18.59	0.98	96.6	Klimalab
Reference Genes									
FvGAPDH	gene07104	Glyceraldehyde-3-phosphate dehydrogenase	CAGAAGACTGTTGATGGACC	GCAGCCTTAATCTGGTCATAG	-2.98	17.4	1	116.5	Chen et al. 2018
FvMSI1	gene03001	WD-40 repeat protein Multicopy suppressor of IRA1	TCCCCACACCTTTGATTGCCA	ACACCATCAGTCTCCTGCCAAG	-2.97	23.75	0.99	117.2	Mouhu et al. 2013

Appendix B – qRT-PCR 96-well plate set-up

PLATE	1	2	3	4	5	6	7	8	9	10	11	12
GENE												
A	N2 1A	N2 1B	N2 2A	N2 2B	N2 3A	N2 3B	N2 4A	N2 4B	N2 5A	N2 5B	N2 6A	N2 6B
	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE
B	N2 7A	N2 7B	N2 8A	N2 8B	N2 8C	N2 8D	N2 9A	N2 9B	N2 9C	N2 9D	N2 10A	N2 10B
	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE
C	N2 10C	N2 10D	N2 11A	N2 11B	N2 11C	N2 11D	N2 12A	N2 12B	N2 12C	N2 12D	N2 13A	N2 13B
	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE
D	N2 13C	N2 13D	NTC		I1 1A	I1 1B	I1 2A	I1 2B	I1 3A	I1 3B	I1 4A	I1 4B
	GENE	GENE	GENE		GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE
E	I1 5A	I1 5B	I1 6A	I1 6B	I1 7A	I1 7B	I1 8A	I1 8B	I1 8C	I1 8D	I1 9A	I1 9B
	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE
F	I1 9C	I1 9D	I1 10A	I1 10B	I1 10C	I1 10D	I1 11A	I1 11B	I1 11C	I1 11D	I1 12A	I1 12B
	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE	GENE
G	I1 12C	I1 12D	I1 13A	I1 13B	I1 13C	I1 13D	NTC					
	GENE	GENE	GENE	GENE	GENE	GENE	GENE					
H										IRC_1_1	IRC_2_1	IRC_3_1
										GAPDH	GAPDH	GAPDH

Supplementary Figure 1. 96-well plate set-up. Example used for each gene tested via qRT-PCR. "IRC1_1" represents the inter-run calibrators, which used the same samples (I1 5A_1, I1 5A_2, I1 6A_3) and gene (*GAPDH*) on every plate.

Appendix C – Omitted Samples

Supplementary Table 2. Omitted samples from cDNA synthesis. Omitted samples due to low concentration (<15 ng/uL).

Sample ID	Nucleic Acid Conc.	Unit	260/280	260/230
N2_1A_2	4	ng/µl	1.27	0.15
N2_4B_3	9.6	ng/µl	2.08	0.03
N2_10A_3	3.2	ng/µl	1.86	0.09
N2_11C_2	5	ng/µl	1.66	0.08
I1_2B_2	11.2	ng/µl	1.21	0.25
I1_10B_2	0	ng/µl	0	0

Supplementary Table 3. Omitted samples from qPCR analysis.

Sample ID	GAPDH	LHY	LUX	MSI1	PRDX	PRR5	PRR7	PRR9	RVE8	TOC1	Nucleic Acid Conc.	Unit	260/280	260/230
N2_5A_1		32.63	32.48	31.68		33.13	32.33	31.19	35.33	34.77	20.3	ng/µl	1.8	0.24
I1_1A_2	30.2	31.79	31.85	31.23	34.46	31.63	32.56	31.27	38.65	32.55	74.8	ng/µl	2.3	1.44
I1_1B_2	32.81	35.37	39.16	37.29	34.56				34.75	37.61	214.9	ng/µl	2.05	1.72
I1_7B_2		32.64	39.02	38.12	30.78	38.39		38.19	32.94		357	ng/µl	2.16	1.65
I1_7B_3			33.37	30.84		33.42	32.34	33.87		31.48	468.9	ng/µl	2.14	1.31
I1_8A_1		33.18	38.85	34.16		34.52	34.26	32.56	31.54	39.31	282	ng/µl	2.15	1.84
I1_11B_1		31.18	32.05	31.06		30.66		32.47			235.4	ng/µl	2.15	1.43
I1_13B_3			33.78	30.75		33.39	31.94	36.08		30.48	99.3	ng/µl	2.09	1.26
I1_13C_1		36.82		39.12	39.78	35.51	37.28	35.88	39.01		228.6	ng/µl	2.12	1.98
I1_13D_1			30.88	35.85				31.39			131.4	ng/µl	2	0.58

Appendix D – Significance Data

Supplementary Table 4. Students T-test results between clones. Significant differences in expression of target genes between clones under each treatment.

treatment	zeitgeber	Target	Ttest_ind	p-value
Norway	0	LHY	-6.686	0.022
Italy	0	LUX	-4.247	0.024
	20	PRDX	-4.543	0.01
	32	LHY	-3.095	0.036
	36	PRR9	-5.126	0.014
	40	RVE8	-3.763	0.033
	44	PRDX	-2.87	0.045

Supplementary Table 5. Students T-test results between dark (D) treatments. Significant differences in expression of target genes (by clone) between treatments and respective dark treatments.

Target	treatment	Clone	zeitgeber	Ttest_ind	p-value
FvLHY	D-Italy	I1	**48**	6.8536	0.0206
		N2	**48**	4.49	0.0109
	D-Norway	N2	**36**	-12.8514	0.001
			40	-5.0725	0.0148
			48	4.535	0.0105
FvRVE8	D-Norway	N2	**32**	-2.7786	0.0499
			40	-6.3776	0.0078
FvPRR9	D-Italy	I1	**36**	8.6858	0.0032
			32	4.7192	0.0092
			48	-4.9319	0.0387
		N2	**48**	-3.342	0.0288
			44	-4.9195	0.0079
			36	6.324	0.0032
			28	5.2141	0.0065
			32	5.3077	0.0061
	D-Norway	I1	**44**	-4.3486	0.0122
			36	4.7323	0.0091
			32	10.8471	0.0004
		N2	**28**	4.7624	0.0089
			32	2.8271	0.0475
			48	-5.0853	0.0071
			36	3.856	0.0308

Target	treatment	Clone	zeitgeber	Ttest_ind	p-value
FvPRR7	D-Italy	I1	**48**	-5.8716	0.0278
		N2	**44**	-5.4255	0.0056
			28	3.713	0.0206
	D-Norway	I1	**48**	-18.4676	0.0003
		N2	**48**	-3.2555	0.0312
FvPRR5	D-Italy	I1	**48**	-5.5906	0.0305
			36	3.3612	0.0437
		N2	**44**	-5.4963	0.0053
	D-Norway	I1	**48**	-7.5601	0.0048
		N2	**48**	-3.8816	0.0178
FvLUX	D-Italy	I1	**32**	2.8892	0.0446
		N2	**44**	-2.923	0.0431
	D-Norway	I1	**40**	3.6383	0.022
		N2	**48**	-3.8238	0.0187
FvPRDX	D-Italy	I1	**32**	2.9229	0.0431
			40	3.4094	0.0422
		N2	**48**	10.5395	0.0005
			40	9.7167	0.0006
	D-Norway	N2	**48**	4.9222	0.0079
			44	3.9211	0.0172

Supplementary Table 6. Students T-test results between daylength treatments. Significant differences in expression of target gene amplitude or phase between the two light treatments.

Gene	zeitgeber	Ttest_ind	p-value	Gene	zeitgeber	Ttest_ind	p-value
FvLHY	8	2.3242	0.0425	FvPRR7	20	3.7201	0.004
	12	2.9196	0.017		24	2.3543	0.0464
	16	3.1949	0.0109		44	3.4203	0.0065
	36	2.9292	0.019		48	3.5099	0.0066
	48	-5.4745	0.0004				
				FvPRR5	8	-2.8674	0.0167
FvRVE8	12	2.5412	0.0346		16	4.1263	0.0026
	16	4.0806	0.0028		20	3.9519	0.0027
	36	2.7482	0.0251		44	2.9618	0.0142
					48	2.6438	0.0267
FvPRR9	4	-8.31704	2E-05				
	8	-3.3344	0.00756	FvLUX	8	-3.0084	0.0148
	16	3.25111	0.00998		44	2.4922	0.0319
	20	6.53468	7E-05		48	3.4755	0.007
	28	-2.28108	0.04848				
	32	-3.28663	0.0082				
	36	2.85512	0.02131				
	44	3.78312	0.00433				
	48	2.29321	0.04752				

Supplementary Table 7. Tukey-HSD results for *GAPDH* expression between treatments in N2.

	group1	group2	meandiff	p-adj	lower	upper	reject
0	D-Italy	D-Norway	-0.6812	0.4309	-1.881	0.5187	FALSE
1	D-Italy	Italy	-1.7095	0.001	-2.7352	-0.6838	TRUE
2	D-Italy	Norway	-1.6073	0.001	-2.633	-0.5816	TRUE
3	D-Norway	Italy	-1.0283	0.0492	-2.054	-0.0027	TRUE
4	D-Norway	Norway	-0.9261	0.0887	-1.9518	0.0996	FALSE
5	Italy	Norway	0.1022	0.9	-0.7129	0.9173	FALSE

Appendix E – Jupyter Notebook

https://github.com/corinef/qPCR-analysis