Elucidating the effects of photoperiod during gestation and lactation on mammary gene expression and function in cows and mice

by

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ABSTRACT

Photoperiod, or day length, generates a highly accurate biological calendar that allows animals to anticipate and adapt to environmental change throughout the year. Photoperiod coordinates both daily and yearly rhythms associated with sleep/wake cycles, behavior, and reproduction, including mammary development and milk production. In dairy cows, long day (summer-like) photoperiod during lactation, or short day (winter-like) photoperiod during late gestation, enhances milk production. Potential mediators of these effects include changes in mammary cell-turnover and hormonal signaling factors. The effects of photoperiod on the mammary transcriptome and subsequently the molecular mechanisms underlying the milk yield response have not been elucidated.

The aim of the work presented here is to identify genes and pathways responsive to photoperiod and associate their differential expression with functional effects of photoperiod in the mammary gland. To address this aim we employed microarray technology in two model systems, cows and mice, with the objectives of: **1.** Evaluate the effects of photoperiod on the mammary transcriptome of cows. **2.** Identify common effects of photoperiod on mammary function in cows and mice. **3.** Evaluate the effects of photoperiod on the mammary transcriptome in mice. **4.** Determine if physiological state (gestation or lactation) influences the effects of photoperiod on the mammary transcriptome. **5.** Assess common biology between the cow and mouse models.

In the bovine mammary gland, we identified 64 photoperiod responsive genes and have interpreted these genes and their associated functions in the context of the mammary gland. Differentially expressed genes were associated with mammary development and immune function consistent with the enhancement of milk production in the ensuing

lactation. The transcriptomic signatures across time relative to parturition were not consistent with those in response to photoperiod, suggesting different underlying mechanisms. Furthermore, genes identified in the interaction of photoperiod and time indicate the physiological state of the mammary gland during late gestation influences its response to photoperiod.

In the mouse, mammary cell proliferation and gene expression signatures provide substantial evidence that photoperiod can affect the ability of the mouse mammary gland to produce milk, although, we were unable to detect effects of photoperiod on litter weight. Overall, our findings provide several novel insights about the effects of photoperiod on the mammary transcriptome. Firstly, photoperiod manipulation is sufficient stimulation to affect the mouse mammary transcriptome. To that end, we have determined that long day and short day photoperiod affect very different sets of genes that are associated with distinct biological functions. In addition, photoperiod differentially affects gene expression in the mammary gland depending on the physiological state. Lastly, photoperiod can have enduring effects after the cessation of exposure on the mammary transcriptome.

Ultimately, this work reveals that photoperiod manipulation induces changes in the mammary transcriptome during both lactation and gestation. The genes and pathways identified here have been grouped into six potential mechanisms that may underlie the effects of photoperiod on mammary development and function in cows and mice.

PREFACE

This dissertation is the original work of Pamela A. P. Bentley. The use of animals was approved by the University of Alberta Animal Care and Use Committee (Protocol # 11/11/140) in accordance with the guidelines issued by the Canada Council on Animal Care.

This dissertation consists of seven chapters. **Chapter 1** gives a summary of the literature relevant to the field of study. **Chapter 2** provides the study design, objectives, and hypotheses for the work in **Chapters 3**-**6**. A version of **Chapter 3** has been submitted as "Responses of the mammary transcriptome of dairy cows to altered photoperiod during late gestation" to *Physiological Genomics* and is currently under review (PG-00112-2014). The study design and animal work for **Chapter 3** was developed and carried out by E. H. Wall, G. E. Dahl, and T. B. McFadden, who are co-authors on the manuscript. The statistical assessment and normalization of the microarray data was conducted at the University of Vermont, I was responsible for the subsequent analysis and biological interpretation of the data and manuscript preparation. E. H. Wall and T.B. McFadden edited and reviewed the manuscript.

T.B. McFadden and I conceived **Chapters 4**, **5**, and **6** at the University of Alberta. I was responsible for the study design, experimental protocols, performing experiments, data collection, analysis, interpretation, and writing of the manuscripts. The Alberta Transplant Applied Genomics Centre at the University of Alberta conducted microarray experiments including initial analysis and inter-chip normalization. I was responsible for the statistical analysis and subsequent data analysis and interpretation. **Chapter 7** provides a review of the hypotheses in light of the work presented and general discussion of the findings.

Other contributions to this work include manuscript editing by G. Oliver, F. Paradis, and V. Baracos. As a member of the Molecular Biology Service Unit, T. Locke aided in the design of qRT-PCR experiments and developed the robotic pipetting protocols. Members of the McFadden lab group assisted with animal studies.

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DEDICATION

This work is dedicated to

My Mom

For teaching me the meaning of resilience and its importance in matters of the heart and the mind

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I will love the light for it shows me the way,
yet I will endure the darkness for it shows me the stars

Og Mandino

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LISTS OF NOMENCLATURE AND ABBREVIATIONS

Abbreviation	Definition
°C	Degrees centigrade
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
bp	Base pairs
BrdU	Bromodeoxyuridine
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
DAVID	Database of Annotation, Visualization, and Integrated Discovery
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
ELISA	Enzyme-linked immunosorbent assay
g	Gram
x g	Times gravity
G17 or 19	Day 17 or 19 of gestation
h	Hour(s)
IPA	Ingenuity Pathway Analysis
IQR	Inter quartile range
L	Liters
L5, 10 or 15	Day 5, 10 or 15 of lactation
LD	Long days (specifically pertaining to 16 h light; 8 h dark)
LD_{ND}	Comparison of LD to ND photoperiod
Lux	The SI unit of illuminance; 1 lux = 1 lumen/m2
M	Molar (mol/L)
MANOVA	Multivariate analysis of variance
ND	Normal day (specifically pertaining to 12 h light: 12 h dark)
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcription-PCR
RMA	Robust Multichip Average
RNA	Ribonucleic acid
s	Second(s)
SCN	Suprachiasmatic nuclei
SD	Short days (specifically pertaining to 8 h light; 16 h dark)
sd	Standard deviation
SD_{ND}	Comparison of ND to SD photoperiod
µg or µL	Micro gram (10-6 grams) or micro liter
Gene Names	Follow species specific convention
Abc	Mouse gene
ABC	Bovine gene
IPA-specific terms	Given in italics. e.g. *upstream regulator*

CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Biological timekeeping

Nature has embedded rhythmic biology in nearly all aspects of life. Jean Jacques Ortous de Mairan initiated the study of rhythmic biology in 1729 when he noted the daily rhythmic opening and closing of *Mimosa pudica* leaves (Roenneberg and Merrow, 2005). In 1959, Franz Halberg coined the term for daily fluctuations as circadian (*circa* about, *dies* day) rhythms. Circadian rhythms are synchronized to an approximate 24-hour period by the daily light/dark cycles resulting from the earth's rotation on its axis. The circadian clock enables tracking of changes in day length, or photoperiod, over time. The yearly orbit of the earth around the sun creates the circannual rhythm. Photoperiod entrains both circadian and circannual rhythms (**Figure 1.1**). However, as de Mairan aptly indicated, these rhythms are endogenous among flora and fauna and persist under constant light and temperature for months (Rusak and Zucker, 1975; Roenneberg and Merrow, 2005; Golombek and Rosenstein, 2010).

Photoperiodism is the capacity to use day length to coordinate internal biological calendars for the anticipation of long-term physiological changes (Baker and Ranson, 1932). In the *Evolution of Animal Photoperiod*, Bradshaw and Holzapfel (2007) addressed the question: why use day length, instead of other environmental cues? They surmise that day length is highly reliable and is the same today as it was 10,000 years ago. By these standards, photoperiod acts as the optimal measure by which to anticipate, prepare for, and ultimately carry out timing of seasonal functions such as hibernation, migration and

reproduction (Bradshaw and Holzapfel, 2007). Accordingly, photoperiod has been defined as the foremost synchronizer of seasonal adaptations in mammals (Goldman, 2001).

Rhythmic biology most likely arose in photosynthetic organisms between 3-4 billion years ago. Hut and Beersma (2011) reviewed how the need to protect DNA from ultraviolet damage was set against the need for production and storage of energy in the form of ATP. Organisms, like cyanobacteria, performed photosynthesis during the light phase, whereas DNA replication and transcription occurred during the dark phase when damage from ultraviolet radiation could be minimized (Hut and Beersma, 2011). The ability to coordinate biology with environmental cues is just as important today. Bradshaw et al. (2004) evaluated the effect of photoperiod-responsiveness on fitness in mosquitos and asserted that an animal's fitness is directly related to its ability to respond to day length.

Light – setting the clock

Endogenous rhythms, or clocks, are synchronized to the environmental conditions by *zeitgebers* ('time giver' in German) (Aschoff, 1960). These exogenous cues include photoperiod, temperature, and food availability. For some animals, food is as strong a *zeitgeber* as photoperiod and may be used rather than light to entrain circadian rhythms (Stephan, 2002). For the majority of animals, photoperiod is the principle coordinator of endogenous clocks, but there are two aspects of photoperiod which can affect animal physiology, absolute day length and the direction of change in day length (Goldman, 2001). Absolute photoperiod takes into account the number of hours of light exposure per day, and the change in day length provides more long-term information.

In nature, long days occur during the summer months and range from 10-20 hours of light per day, whereas short days occur in winter with light exposure ranging from 6 to 10 hours per day. For short-lived arthropods and animals, absolute day length is the only controller of photoperiodism. For species that live multiple years, the timing of seasonal events is coordinated by two aspects of photoperiodism: circannual rhythms, and refractoriness (**Figure 1.1**) (Bradshaw and Holzapfel, 2007). The latter represents a time during which animals are not physiologically responsive to photoperiod even though the animal does continue to perceive photoperiod. Goldman (2001) provides the example of rodents that are reproductively active throughout their life while maintained on long-day photoperiod. When transferred to short day photoperiod those rodents undergo gonadal regression and are photo refractory. Subsequently, they will not be reproductively active until exposed to long-day photoperiod once again.

The intensity and the timing of light exposure can have significant effects on the physiological interpretation of photoperiod. Pittendrigh (1964) first postulated that there exists an inducible phase, during which, if light is present, the photoperiod would be interpreted as long day, while if it was dark during this inducible phase, the day would be interpreted as a short day (Pittendrigh, 1964). Therefore, short bursts of light, known as skeleton photoperiod, specifically timed during the day, can be used to mimic short or long day photoperiod. Recent work has shown skeleton photoperiod affects the expression of genes which regulate the body's internal clock (Oishi et al., 2002).

Interpreting the light

Photoperiod is the principal cue to which circadian and seasonal timekeeping is entrained. Photoperiodic information is conveyed to the body by way of endocrine signaling,

specifically melatonin, and prolactin. To follow is a review of the mechanism by which light is transduced into chemical signals and ultimately converted to physiological responses.

Melatonin

Light information captured by the retina is conveyed to the brain through the retinohypothalamic tract and converted to chemical signals in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (Reppert and Weaver, 2001) (**Figure 1.2**). The SCN translates light information to hormonal and autonomic outputs by way of the pineal gland and the paraventricular nucleus. Melatonin (N-acetyl-5-methoxtyrptamine), an indolic hormone, is secreted from the pineal gland during the dark phase. Light prevents secretion of melatonin into the blood supply by inhibiting the rate limiting enzyme in the melatonin biosynthetic pathway, arylalkylamine N-acetyltransferase (AANAT) (Lewy et al., 1980).

Rhythmic secretion of melatonin by the pineal gland in response to photoperiod is the coordinator of systemic responses and is observable in all mammalian species (Tamarkin et al., 1985). The secretion of melatonin provides the body with information on the time of day, by amplitude of melatonin concentration, and the time within the year, by duration of heightened melatonin concentrations (Lincoln et al., 2003).

Blood melatonin concentrations are commonly used as a response measure of light exposure. As reviewed by Arendt (1986), animals require varying amounts of light to inhibit melatonin secretion. Humans require relatively high levels of light intensity (1,500-2,500 lux) to suppress nocturnal melatonin secretion (Lewy et al., 1980). In contrast, in dairy heifers, the minimum intensity of light needed to depress nighttime blood melatonin concentrations to daytime levels is 400 lux whereas 50 lux is sufficient to inhibit the initial rise in plasma melatonin levels (Lawson, 2001).

Prolactin

Prolactin is a peptide hormone secreted from the *par tuberalis* of the pituitary gland (Freeman et al., 2000). Named for its predominant role in lactation, prolactin has additional actions in water/salt balance, immune function, and seasonal time-keeping. Seasonal prolactin release regulates metabolism, food intake, pelage, and reproductive functions including gonadal activity, pregnancy, and lactation (Lincoln et al., 2003). Prolactin release occurs in opposition to release of melatonin; therefore, long days enhance and short days dampen prolactin secretion. The effect of melatonin release on prolactin secretion has yet to be fully elucidated. Currently, it is proposed that melatonin has dual functions, acting at the level of the hypothalamus to drive reproductive function and secondly within the pituitary gland to regulate prolactin secretion (Johnston, 2004).

Circadian rhythms

The study of the molecular mechanisms of circadian rhythms was first undertaken in D*rosophila melanogaster*, where a set of 12 genes, known as *clock* genes were identified (Takahashi, 1992). Subsequently, homologues to these *clock* genes were identified in vertebrates (Young and Kay, 2001). The central coordination of the mammalian circadian clock is located in the SCN of the hypothalamus and is comprised of a series of feedback loops, which ultimately control daily rhythmic biological functions (**Figure 1.3**) (Reppert and Weaver, 2001).

Briefly, the circadian clock is a network of transcriptional regulators; the cycle begins with CLOCK and BMAL1 positive regulation of the expression of *Per1*, *Per2*, *Cry 1*, and *Cry 2* (**Figure 1.3a**). The products of this transcriptional regulation then dimerize and accumulate in the nucleus of the cell. In the nucleus PER1, PER2, CRY1, and CRY2 interact

with CLOCK and BMAL1, which inhibits their own expression (**Figure 1.3b**). This feedback-loop takes approximately 24 hours to complete. As depicted in **Figure 1.3**, additional feedback loops stem from this central regulatory loop and themselves contribute to circadian biology in the SCN (Mohawk et al., 2012).

Expression of these genes oscillates in response to photoperiod, not only in the SCN but also in peripheral mammalian tissues and immortalized cell culture models (Balsalobre et al., 1998; Balsalobre, 2002). The discovery of oscillating clock genes in peripheral tissues and their tissue specific effects has opened a new chapter in our understanding of biological clocks, the details of which are beyond the scope of this review.

Interaction of photoperiod with circadian rhythms

The biology of photoperiod and circadian rhythms is clearly intertwined; however, the nature of the interaction is not fully understood (Goldman, 2001). Light readily activates transcription of *clock* genes in the SCN and entrains the circadian rhythm to an approximately 24-hour period, which then controls the rhythmic secretion of melatonin (Glickman et al., 2012). It has been established that changes in photoperiod affect the circadian response to light (Pittendrigh and Daan, 1976; Wehr et al., 1993; Sumova et al., 2003). Most recently, Glickman et al. (2012) reported exposing hamsters to short day (10 h light: 14 h dark) photoperiod results in hamsters being 40-times more sensitive to light than their long day (14 h light: 10 h dark) counterparts. Manipulation of photoperiod also modulates expression of *clock* genes both in the SCN (Tournier et al., 2003) and peripheral organs (Reppert and Weaver, 2001; Carr et al., 2003; Reddy et al., 2005). Tournier et al. (2003) described substantial changes in *clock* gene expression in the SCN when hamsters were changed from long day (14 h light: 10 h dark) to a shorter photoperiod (10 h light: 14 h

dark). Although each *clock* gene is affected, the pattern of change is distinct for each gene (Tournier et al., 2003). In sheep, Andersson et al. (2005) determined that photoperiod can modify clock gene expression patterns in the liver. In male C57Bl/6 mice, long day (**LD**: 16 h light: 8 h dark) and short day (**SD**: 8 h light: 16 h dark) photoperiod results in phase shifting and reduction of amplitude of clock genes in the pituitary gland and liver when compared to normal day photoperiod (**ND**: 12 h light: 12 h dark) (Bur et al., 2010). From these studies, the physiological and molecular interactions of photoperiod with circadian rhythms are beginning to become known. The influence of altered circadian rhythms and photoperiod has drawn significant interest as the diversity and importance of their effects at the biochemical, cellular, and organismal levels come to be appreciated.

PHOTOPERIOD COORDINATES CHANGES IN IMMUNE FUNCTION

Photoperiod is used to coordinate energetically costly activities (reproduction, immune function, migration) with times of resource availability (Demas et al., 1996). The immune system, including both innate and adaptive immunity, is an energetically costly function yet all animals benefit from immune function throughout their lives (Martin et al., 2008). Therefore, trade-offs need to be made between immune function and energy usage. The capacity to fluctuate between enhanced immunological defense and weakened immunological defense, known as seasonal plasticity, is highly conserved among vertebrate species (Walton et al., 2011). Complete review of this aspect of photoperiod biology is beyond the scope of this literature review. Consequently, only a brief summary of the effects of photoperiod on immune function in cows and mice as well as the mammary gland will be presented.

Short day photoperiod enhances immune function

For animals living in the wild, photoperiod is closely tied to seasonal changes in temperature and food availability. Winter, along with short day photoperiod heightens environment-based stressors, including reduced food, and increased need for thermoregulation. Increased immune function is needed to counteract the effects of environmental stress (**Figure 1.4**) (Demas and Nelson, 1998; Walton et al., 2011). Laboratory studies are conclusive in demonstrating that short day photoperiod enhances immune function. However, for studies conducted in the field, the specific effects of photoperiod alone are not as clear; therefore, winter is considered immune enhancing (Nelson et al., 1995).

Long day photoperiod attenuates immune function

Long day photoperiod is typically accompanied by increased food resources and warmer temperatures; therefore, the environmental pressures driving increased immune function are not present. Because of this, energy is diverted away from immune function and thermoregulation and put towards growth and reproduction (**Figure 1.4**). Long day photoperiod does not have an inhibitory effect on immune function; rather, it lacks the stimulus required for activation of immune function by short day photoperiod.

Photoperiod affects mouse immune function

Even when mice are maintained in constant conditions, such as normal day (12 h light: 12 h dark, ND) photoperiod, they undergo annual changes in lymphocyte function (Brock, 1983). Indicators of immune function for laboratory animals include spleen and thymus mass, lymphocyte and white blood cell count, all of which are depressed by short day

photoperiod (Nelson et al., 1995). Work by Demas and coworkers has shown exposure of mice to short day photoperiod can increase both cell-mediated and humoral immunity (Demas et al., 1996; Demas et al., 1997; Demas and Nelson, 1998). Deer mice (*Peromyscus maniculatus*) which are relatively responsive to photoperiod have increased lymphocyte, neutrophil and white blood cell counts (Blom et al., 1994), as well as spleen mass (Vriend and Lauber, 1973) in response to short day photoperiod. Deer mice exposed to short day photoperiod along with mild ambient temperature (8°C) undergo increased splenocyte proliferation, relative to mice on LD photoperiod (Demas and Nelson, 1998). Under conditions of food restriction, to mimic winter months, the effects of short day photoperiod on immune function are sufficient to overcome the immune-suppressive effects of food restriction (**Figure 1.4**) (Demas and Nelson, 1998). In addition, unlike their LD counterparts, deer mice exposed to short day photoperiod do not develop squamous cell carcinomas after exposure to carcinogens (Nelson and Blom, 1994), indicating the effects of photoperiod on health extend beyond basic immune function.

Other species of mice, including *Mus musculus*, are not as responsive to photoperiod manipulation. Photoperiod does not greatly affect immune function in C3H, CBN, or C57Bl/6 mice (Yellon and Tran, 2002). Another investigation of the immune response of laboratory strains of mice showed that neither C3H nor C57Bl/6 mice have strong immune responses to photoperiod manipulation, as measured by leukocyte counts and delayed-type-hypersensitivity (Gatien et al., 2004). However, C57Bl/6 mice exposed to LD photoperiod have unfavorable responses to transplanted melanoma cells compared to either SD or ND mice (Lang et al., 2003); indicating photoperiod is able to elicit specific disease-related responses in some *Mus musculus* strains.

Photoperiod affects bovine immune function

Photoperiod affects bovine immune function during several stages of development. Young steers exposed to short day photoperiod have increased proliferation of peripheral blood mononuclear cells and chemotaxis in response to interleukin-8 stimulation (Palmer and Driancourt, 1983). Holstein calves exposed to LD have increased lymphocyte proliferation in response to stimulation, relative to SD photoperiod (Auchtung et al., 2003). Leading up to and shortly after parturition, cows exposed to SD photoperiod have increased neutrophil chemotaxis and lymphocyte proliferation (Auchtung et al., 2004). From this evidence, it was suggested that SD photoperiod might have a protective effect on the mammary gland, which could increase milk production in the subsequent lactation (Auchtung et al., 2004). Specifically, the increased melatonin secretion brought on by short day photoperiod may have a protective effect in the mammary gland during mastitis (Boulanger et al., 2002). This is not to say that LD photoperiod has a negative effect on immune function, as there is no evidence of increased mastitis in cows exposed to LD photoperiod (Dahl and Petitclerc, 2003).

Mechanisms of the effects of photoperiod on immune function

Melatonin

Evidence for hormonal interplay between the immune system and photoperiod is widespread. The effects of photoperiod on immune function are mediated almost entirely by secretion of melatonin from the pineal gland. Melatonin affects immune function both through direct action on immune cells and through systemic adjustments (Walton et al., 2011). Carrillo-Vico et al. (2005) conducted and summarized extensive research on the

effects of melatonin on immune function and concluded that melatonin has an enhancing effect on both acquired and innate immunity across numerous species. Exogenous treatment with melatonin increases thymus weight in gerbils and proliferation of mouse splenocytes and rat lymphocytes (Carrillo-Vico et al., 2005). In addition, melatonin regulates gene expression of immune mediators including, major histocompatibility complex class (MHC) II molecules, tumor necrosis factor and interferon γ (Carrillo-Vico et al., 2005).

Prolactin

In addition to melatonin, prolactin has been investigated as a mediator of the effect of photoperiod on immune function based on its immunomodulation properties (Leonardi and Klempau, 2003). Cows exposed to SD photoperiod, in which circulating prolactin is low, have increased expression of prolactin receptor (Auchtung et al., 2005). Treatment of cows on LD photoperiod with bromocriptine, an agonist of prolactin receptors, decreases prolactin receptor expression to that of cows on SD photoperiod (Auchtung et al., 2003). These authors propose that photoperiod modulates sensitivity to prolactin by altering the abundance of its receptor inversely to the abundance of prolactin (Auchtung and Dahl, 2004). The inter-connectedness of photoperiod on lactation and immune function makes prolactin a logical target of continued interest.

PHOTOPERIOD AFFECTS REPRODUCTION

Seasonality and photoperiod affect many aspects of reproduction, including fertility, male gonadal development, synchronization, and lactation (Woodfill et al., 1994; Ono et al., 2009). In this section, the effects of photoperiod on mammalian reproduction will be

outlined, followed by an in-depth review of the effects of photoperiod on bovine and mouse mammary development and lactation.

Overview

The environmental changes that occur across seasons create selective pressure on animals, thereby necessitating coordination of breeding. The synchronization of breeding ensures offspring are born during the most favorable conditions, when food is in abundant supply, and thus provides biological advantage to the species (Goldman, 1999; Hastings et al., 1985). Photoperiod is used as a cue to both inhibit and promote mating to ensure offspring are born during the favorable time of year.

Seasonal breeders are generally characterized as either long day or short day breeders such as Syrian hamsters and Soay sheep, respectively. Gonadal development is highly regulated by photoperiod in both of these species. Six hours of light per day is sufficient to trigger gonadal regression in Syrian hamsters, whereas 8 hours of light (SD) promotes gonadal development (**Figure 1.5**) (Hastings et al., 1985). The signal responsible for this effect on gonadal development is melatonin peak duration, such that short peaks stimulate reproduction, whereas long duration peaks inhibit reproduction.

Sexual maturity of both male and female sheep is also regulated by photoperiod. Short day photoperiod promotes earlier onset of ovulation in female sheep (Foster, 1981) and shortened light exposure stimulates sperm production in males (Lincoln and Davidson, 1977; Schanbacher, 1979). Sheep and goats are short day breeders and typically breed between October and December (Chemineau et al., 2008). Like many mammalian species

reproduction in fall ensures offspring will be born in spring, when vegetation begins to be plentiful and weather conditions favorable (Thimonier, 1981).

All of the effects of photoperiod on reproduction are regulated by the interplay of light on hormonal secretion. As photoperiod is decreasing in the fall, estradiol secretion in female sheep is accompanied by a surge of luteinizing hormone, thereby promoting ovulation and estrus. In late winter and spring, when photoperiod is increasing, estradiol inhibits the effect of luteinizing hormone and therefore ovulation is blocked (Tucker and Ringer, 1982). For details of photoperiod-induced hormonal regulation of reproduction see: (Malpaux et al., 1999; Chemineau et al., 2008; Yoshimura, 2013).

Effects of photoperiod on reproduction in mice

The effects of photoperiod in mice vary across species and by strain of mice. Because mice are a short-lived species and have short gestation lengths, it is essential they breed and rear offspring when food is most plentiful. Therefore, long day photoperiod typically increases reproductive function in mice. A great deal of the literature regarding the effects of photoperiod on reproduction in mice focuses on male reproduction. Male deer mice (*Peromyscus maniculatus*) have delayed sexual maturation (Whitsett and Lawton, 1982) and undergo a decrease in testicular mass in response to SD, relative to LD photoperiod (Demas and Nelson, 1998). Similar effects also occur in male white-footed mice (*Peromyscus leucopus*) (Pyter et al., 2005). The following will focus on the effects of photoperiod on female reproductive function.

An early study by Baker and Ranson (1932) indicated that in field mice (*Microtus agretis*) light was a sufficient stimulus to affect reproduction. Specifically, they reported that

maintaining mice on winter-like (9 h light: 15 h dark) photoperiod reduced both the number of pregnancies and the number of pups born, relative to control mice (15 h light: 9 h dark) (Baker and Ranson, 1932). Female deer mouse pups exposed to short day (6 h light: 18 h dark) from conception to weaning weigh more and undergo sexual maturation significantly later than females on long day (15 h light: 9 h dark) photoperiod (Whitsett and Miller, 1982). From these findings, Whitsett and Miller (1982) concluded there may be a period of sensitivity to photoperiods that could be lost over time. However, later in life, female deer mice maintained on SD photoperiod have reduced uterine and ovarian weight relative to LD photoperiod, indicating the effects of photoperiod on reproductive function can continue into adulthood (Nelson and Shiber, 1990). Interestingly, meadow voles (*Microtus pennsylvanicus*) exposed to short day (10 h light: 14 h dark) photoperiod for eight weeks prior to pregnancy produce 18.5% smaller litters than voles maintained on (14 h light:10 h dark) photoperiod (Lee et al., 1987), suggesting the effects of photoperiod on female reproduction may require chronic rather than acute exposure..

The effects of photoperiod are not as marked in the house mouse (*Mus musculus*). This led Bronson (1979) to conclude house mice have no reproductive response to photoperiod. However, it should be noted in Bronson's description of photoperiod experiments on mice, he does not indicate the light intensity for mice during exposure (Bronson, 1979). In this same work, Bronson cites a study using natural light 'through a window', in which the authors reported differences in weanling reproductive productivity in response to seasonal photoperiods (Pennycuik, 1972). This suggests the experimental parameters employed by Bronson (1979) may not have been sufficient to elicit a response, and therefore his conclusions may have been in error.

In later studies it was concluded that female house mice discriminate and respond to photoperiod; although, reproduction is uncoupled from photoperiodic control (Nelson, 1990; Nelson and Shiber, 1990). Yellon and Tran (2002) investigated the effects of photoperiod on three strains of house mice (C3H, CBA, and C57Bl/6) and found that males do not undergo testicular regression in response to SD photoperiod. From these findings Yellon and Tran (2002) concluded these mouse strains do not have a reproductive response to photoperiod manipulation.

More recent studies in California mice (*Peromyscus californicus*), traditionally considered a non-photoperiodic species, revealed effects of photoperiod on uterine, ovarian, oviductal and the total reproductive tract as a percentage of body weight (Steinman et al., 2012). Summa et al. (2012) described the negative effects of repeated manipulation of light/dark cycles on pregnancy rates in C57Bl/6 mice. Furthermore, photoperiod elicited differential expression of genes associated with photoperiod signal transduction in CBA mice, whereas C57Bl/6 mice, while not responsive to photoperiod itself, were responsive to exogenous melatonin (Ono et al., 2008). Taken together, the most recent literature indicates female C57Bl/6 mice are reproductively responsive to photoperiod manipulation.

Photoperiod affects maturation in young dairy cows

Exposure of young animals to different photoperiods can affect their rate of development. This is of importance to dairy producers as they want female calves (heifers) to attain sexual maturity as rapidly as possible. Peters et al., (1978) evaluated the effect of long day (>16 h light) photoperiod on weight gain in heifers and reported increased weight gain of 10-15% compared to heifers exposed to natural (~9.8 h light) photoperiod. Peters et al., (1978) also noted increased (6.1%) dry matter intake, although, the feed-to-gain ratio for

heifers on long day photoperiod was low relative to controls (Peters et al., 1980;1981). From these findings, the authors concluded that heifers on long day photoperiod were more efficient at converting feed to body mass than heifers on natural photoperiod.

Angus heifers exposed to long day (18 h light, 6 h dark) photoperiod can also achieve puberty more rapidly than when exposed to natural photoperiod (Hansen et al., 1983). Similar effects are seen in female pigs, such that supplemental lighting increases the number of pigs attaining puberty by eight months of age (Diekman and Hoagland, 1983). In dairy heifers, exposure to LD relative to SD photoperiod, also decreased the time before heifers reach puberty (Rius and Dahl, 2006). Subsequently, the heifers maintained on LD photoperiod grew more rapidly than heifers on SD photoperiod and ultimately produced more milk in their first lactation (Rius and Dahl, 2006). In summary, long day photoperiod promotes growth and maturity in dairy heifers compared to short day photoperiod and therefore can ultimately increase profit potential for dairy farmers.

Photoperiod affects mammary development in young dairy cows

Post-natal mammary development mainly consists of ductal growth. In dairy cows, the mammary gland grows isometrically relative to the rest of the body during the post-natal period. After the initiation of ovulation, fluctuations in estrogen stimulate ductal development growth and subsequently mammary growth is allometric (Akers, 2002). Mammary development prior to gestation can influence milk production in subsequent lactations; therefore, enhancement of mammary growth during this period may be advantageous. To this end, manipulation of photoperiod during development has been explored as a means to enhance mammary growth.

Initial investigations of the effects of photoperiod on mammary composition in heifers did not detect differences when comparing LD and SD photoperiod (Petitclerc et al., 1984),, subsequent studies, with increased sample size, revealed exposure to LD photoperiod increased the weight of mammary parenchyma (secretory tissue) (Petitclerc et al., 1985). The authors also noted LD photoperiod did not affect the total weight of the mammary gland, meaning the weight of extra-parenchymal tissue (fat/connective tissue) was not affected (Petitclerc et al., 1985). Working in Holstein heifers during pregnancy, Newbold et al. (1991) found exposure to LD or SD photoperiod did not affect mammary development. Taken together, these data indicate LD photoperiod, prior to but not during pregnancy, may specifically stimulate growth of parenchyma in the mammary gland.

Mechanisms of the effects of photoperiod on mammary development

The mechanisms underlying the effects of photoperiod on mammary development, although not fully understood, have been investigated since the effects were first observed. Diurnal variation in mammary cell function led researchers to investigate melatonin as a potential modulator of mammary growth.

Diurnal variation associated with the mammary gland

The rate of lipid production and milk secretion displays diurnal variation in rats (Grigor and Thompson, 1987). Milk production occurs primarily during the dark phase while rat pups consumed the majority of milk during the first half, compared to the second half, of the light phase (Grigor and Thompson, 1987). Diurnal variation in milk contents can be observed in both humans and animals. Women who deliver babies either full-term or preterm, produce milk with higher fat concentration in the evening compared to the morning

(Cregan et al., 2002; Lubetzky et al., 2006). In contrast to fat synthesis, lactose synthesis is minimal in the evening whereas synthesis is greatest during the night and early morning (Carrick and Kuhn, 1978). In dairy cows and sheep, there is diurnal variation in the somatic cell count in milk, a measure of mammary health (Smith and Schultze, 1967; Gonzalo et al., 1994). Together, these studies demonstrate the variation that occurs in mammary function within a 24-hour period, indicating variation must occur at the cellular and molecular level.

Mammary cells do not have equivalent function during a 24-hour period. In fact, several basic cellular functions display diurnal variation. Daily variations in DNA synthesis have been observed in the mammary gland of pregnant Balb/c mice (Borst and Mahoney, 1980). Specifically, ^{3}H-thymidine incorporation is 2.5-times higher in mammary secretory epithelial cells at midnight compared to 12 hours prior whereas mammary ductal epithelial cells do not show the same diurnal pattern of DNA synthesis (Borst and Mahoney, 1980). In developing mouse mammary ductal tissue, DNA synthesis and mitotic activity is ~2.5-times higher during a 12-hour dark period than during a 12-hour light period (Berger and Daniel, 1982). Although it was not established knowledge at the time, Berger and Daniel (1982) proposed that their findings may be due to diurnal variation in melatonin secretion from the pineal gland.

Melatonin affects cells in the mammary gland

Diurnal effects

Diurnal variation occurs at numerous levels in the mammary gland including sensitivity to hormonal signals (Paape et al., 1974). Melatonin, rhythmically secreted from the pineal gland, reaches maximal concentrations in the blood during the dark phase (**Figure 1.2**) (Reiter, 1991). Recio et al. (1994) used Michaelis-Menten kinetics to show that binding

of labeled melatonin (2-[^{125}I]-iodomelatonin) to its receptors in the mammary gland occurs in a diurnal fashion. Binding follows a similar rhythm to secretion, is greatest in the middle of the dark phase, and returns to basal levels during the light phase (Reiter, 1991; Recio et al., 1994). Interestingly, Coto-Montes et al. (2003) reported ^{125}I-melatonin binds mouse mammary cell nuclei with greater affinity during the light phase than the dark phase. Furthermore, the daytime affinity for melatonin is reduced by continuous light exposure (Coto-Montes et al., 2003). Using exogenous melatonin injections Eriksson et al. (1998) determined that the concentration of melatonin in milk reflects the concentration in blood, following a short post-injection delay. Together these studies suggest the diurnal variation in melatonin secretion and binding in the mammary gland must ultimately affect mammary function.

Melatonin affects mammary growth

There are two principal mechanisms by which melatonin could affect the mammary gland during development. Indirectly, by affecting the circulation of pituitary hormones (e.g. prolactin) that regulate mammogenesis, or by direct action of melatonin in mammary tissue (Coto-Montes et al., 2003). Pregnant heifers exposed to SD photoperiod have an increased duration of elevated serum melatonin concentrations, whereas LD photoperiod increases prolactin secretion prior to and during the periparturient surge (Newbold et al., 1991). Feeding melatonin to mimic short day photoperiod in pre-pubertal dairy heifers decreases circulating prolactin (Sanchez-Barcelo et al., 1991), although it cannot be used to mimic the effects of photoperiod during the dry period in dairy cows (Petitclerc et al., 1998). Together these findings highlight the opposite effects of photoperiod on melatonin and prolactin secretion.

Pre-pubertal mice, treated with sex hormones to mimic estrus cycles, administration of high doses of melatonin (100 or 200 μg/day) decreases mammary growth (Sanchez-Barcelo et al., 1990), potentially by decreasing the concentration of cAMP in the mammary gland (Cardinali et al., 1992). Similarly, during puberty and pregnancy, when the mammary gland undergoes its greatest periods of growth, melatonin inhibits normal development in mice (Mediavilla et al., 1992). Treatment of dairy heifers with melatonin mimics the effect of short day photoperiod, thereby inhibiting mammary growth (Sanchez-Barcelo et al., 1991). During development, exogenous melatonin inhibits mammary development in mice by way of its G-protein coupled receptor, MT1 (Mediavilla et al., 1992; Xiang et al., 2012). Overexpression of MT1 inhibits lobulo-alveolar development during pregnancy and early lactation (Xiang et al., 2012). Overexpression of MT1 inhibits bromodeoxyuridine (BrdU) incorporation during puberty, and pregnancy, but not early lactation (Xiang et al., 2012). Interestingly, pups born to dams carrying a melatonin receptor (MT1) transgene in the mammary epithelium have a 36% decrease in weight relative to non-transgene dam litters (Xiang et al., 2012). Taken together it is clear that both the secretion of melatonin and its reception at the cell surface can affect mammary growth.

Melatonin and aberrant mammary cell proliferation

Because of the negative effects of melatonin on mammary growth, this hormone has been investigated for its oncostatic effects (Hill et al., 2009). A connection between breast cancer and the pineal gland was first proposed by Cohen et al. (1978). Subsequently, as part of the large Nurses' Health Study, begun in the 1980s, correlations were drawn between breast cancer and women who work nightshifts, and therefore have irregular melatonin secretion patterns (Schernhammer et al., 2001). In this study of nearly 100,000 women,

investigators found premenopausal women who worked 1-14 years in rotating night shifts had an increased lifetime breast cancer incidence 23% higher than women who never worked nightshifts (Schernhammer et al., 2001). Epidemiological studies have shown similar trends in female flight attendants, who, due to extensive traveling, also have altered circadian rhythms and melatonin secretion patterns (Tokumaru et al., 2006). It is postulated that increased breast cancer risk in these groups of woman may be due to low nighttime melatonin levels (Blask et al., 2009).

In mice genetically predisposed to mammary carcinogenesis, administration of exogenous melatonin significantly reduces the incidence of mammary tumor formation, (Subramanian and Kothari, 1991). Melatonin treatment of transgenic animals expressing oncogenes (e.g. N-ras or c-neu) decreases tumor formation (Mediavilla et al., 1997; Rao et al., 2000). Overall, exogenous melatonin has oncostatic effects both *in vivo* and *in vitro*, the molecular mechanisms of which have been reviewed by Proietti and coworkers (2013).

It is thought melatonin acts as a tumor suppressor by disrupting the neuroendocrine reproductive axis, most likely by interacting with the estrogen signal transduction pathway (Sanchez-Barcelo et al., 2003). Melatonin affects mainly hormone dependent cell proliferation (Baldwin and Barrett, 1998), a central component of aberrant mammary cell growth. A recent study by Girgert et al. (2010) demonstrated the disruptive effects of electromagnetic fields on melatonin signaling in breast cancer cells by way of modulated MT1 function. Taken together, there are substantial linkages between photoperiod and aberrant mammary cell proliferation, the underlying mechanisms of which continue to be investigated.

PHOTOPERIOD MANIPULATION ALTERS LACTATION PERFORMANCE

Photoperiod has effects on the mammary gland well beyond the developmental stages. Depending on the timing of exposure relative to parturition, photoperiod can have substantial effects on milk production in cows and, to some extent, in mice. The majority of the work in this field has been conducted in dairy cows; reference to other dairy species will be included when relevant.

Photoperiod affects the mouse mammary gland during lactation

The effect of supplemental lighting on mouse lactation was first evaluated by Sorensen and Hacker (1979) using exogenous pineal extract from cows as well as LD and SD photoperiod exposure. Earlier studies in rats also reported that pinealectomy did not affect lactation (Nir et al., 1968; Mizuno and Sensui, 1970). To the contrary, purified pineal extract negatively affected milk yield in mice (n = 20), in a dose-dependent manner (Sorensen and Hacker, 1979). Finally, the litters of dams on LD photoperiod gained more weight (53.3 g) than the litters of dams on SD photoperiod (51.9) in the first 15 days of lactation (Sorensen and Hacker, 1979). The increased weight gain of LD litters was accompanied by increased maternal weight gain, which the authors suggested may account for the effect on litter weight gain (Sorensen and Hacker, 1979).

Long day photoperiod during lactation enhances milk production in dairy cows

Milk yield

First reported by Peters and coworkers in *Science*, exposure of Holstein heifers to long day (≥ 16 h) photoperiod during the first 100 days post-partum, increased milk production by 2 kg/day relative to heifers on natural day (~9.6 h) photoperiod (Peters et al.,

1978). The authors also reported that inverting the photoperiod of heifers used in this study, negated the effect on milk yield (Peters et al., 1978). Peters and coworkers (1981) went on to report a 1.4 kg/day increase in milk production in dairy cows exposed to LD photoperiod (**Figure 1.6**). This effect was consistent whether cows were in early lactation or late lactation, indicating no interaction of stage of lactation on the effects of photoperiod manipulation.

In goats, long day, compared to natural photoperiod, increased daily milk production 5-15%, regardless of external season (Garcia-Hernandez et al., 2007). Similarly milk yield was increased by 25% by exposure to long day compared to short day photoperiod (Bocquier et al., 1997). In conclusion, long day photoperiod has galactopoietic effects on milk production in lactating dairy ruminants.

To expand on the findings of Peters et al., Marcek and Swanson (1984) exposed dairy cows to continuous light (24 h light: 0 h dark), long day (18 h light: 6h dark) or natural day (9-12 h light/day) photoperiod. They reported cows previously on natural photoperiod produced more milk after exposure to long day photoperiod; however, cows previously on continuous lighting were not affected by long day photoperiod (Marcek and Swanson, 1984). Implantation of grazing dairy cows with melatonin capsules to mimic short day photoperiod decreased milk production, relative to cows on natural day photoperiod (Auldist et al., 2007). In contrast, feeding melatonin concurrent with LD photoperiod exposure does not mimic the effects of SD photoperiod during the dry period on milk production in dairy cows (Petitclerc et al., 1998). These findings indicate that long day photoperiod can increase milk production over natural photoperiod. However, decreasing daylight exposure below natural photoperiod, including by artificial means, can have detrimental effects on milk production.

Considerable research has been done to confirm the effects of photoperiod during lactation on milk production. Dahl et al. (2000) reviewed ten studies published between 1978 and 2000, all of which reported an increase in milk yield from + 0.5 to 3.3 kg/d in response to long day photoperiod exposure. An expanded summary of the effects of photoperiod during lactation on mammary function is presented in **Table 1.1**.

Effects of photoperiod during lactation on milk composition

Increasing milk production is of great interest to dairy farmers, but as milk is quantified by weight rather than volume, it is vital that photoperiod does not negatively affect milk composition. As reviewed by Dahl et al. (2000), the percentage of fat in milk is typically unchanged in cows exposed to long day photoperiod during lactation, although two studies reported small decreases (0.16-0.18%) in milk fat (**Table 1.1**) (Stanisiewski et al., 1985; Phillips and Schofield, 1989). In other dairy animals, milk components are more markedly affected by photoperiod. Fat content is increased by long day photoperiod in lactating goats, whereas milk protein concentration is slightly decreased (Garcia-Hernandez et al., 2007). However, the milk composition of goats in subtropical areas is not affected by photoperiod (Flores et al., 2011). In summary, long day photoperiod typically increases milk yield without negative effects on milk composition, making it an advisable management practice for dairy farmers.

Potential mechanisms of increased milk production during lactation

Dry matter intake

In addition to the effects on milk production, long day photoperiod also affects secretion of prolactin and dry matter intake (DMI). Both heifers and cows exposed to long

day photoperiod increase their DMI, however the effect on DMI is not sufficient to explain the increase in milk production, indicating that feed-to-milk conversion may be more efficient under long day photoperiod (Peters et al., 1978; Peters et al., 1981; Bilodeau et al., 1989). Overall, the increase in milk production under long day photoperiod is only occasionally accompanied by increases in dry matter intake (3.5-6.1%), indicating that the response may be mediated at the tissue level and not the metabolic level in cows (Dahl et al., 2000).

Hormonal regulation in response to photoperiod

Prolactin and melatonin

The effects on milk yield in response to photoperiod manipulation are likely to be hormonally controlled. The duration of elevated melatonin secretion is longer in heifers exposed to SD photoperiod during lactation (Newbold et al., 1991). In both early and late lactation dairy cows, long day photoperiod increases the secretion of prolactin (**Figure 1.6**) (Peters et al., 1981). Serum concentration of prolactin was increased to 30.9 ng/mL from 7.0 ng/mL in bull calves exposed to short day compared to long day photoperiod (Leining et al., 1979; Stanisiewski et al., 1988). Newbold et al. (1991) verified the increase in prolactin in Holstein heifers in response to LD photoperiod and reported the increase in prolactin in response to LD photoperiod is also evident during the peri-parturent prolactin surge that initiates lactation. However, unlike melatonin, prolactin secretion does not vary diurnally (Stanisiewski et al., 1988). Feeding bull calves melatonin to mimic the effects of SD photoperiod does not affect serum prolactin concentrations (Stanisiewski et al., 1988). To the contrary, Auldist et al. (2007) reported that implanting grazing dairy cows with melatonin capsules prior to lactation decreased plasma prolactin while decreasing milk yield relative to

natural day photoperiod. Collectively, these findings indicate interplay between melatonin and prolactin signaling in response to photoperiod manipulation.

Insulin-like growth factor-1

Pre-pubertal heifers exposed to LD photoperiod for 4 months had increased circulating IGF-1, relative to heifers on SD photoperiod (Spicer et al., 2007). Dahl et al. (1997) postulated the galactopoietic effects of LD photoperiod exposure during lactation may be mediated by IGF-1. Circulating IGF-1 increases in dairy cows exposed to artificial LD, compared to natural day (≤ 13 h light) photoperiod, as well as by seasonal changes in photoperiod (**Figure 1.6**) (Dahl et al., 1997). The increase in circulating IGF-1 precedes the increase in milk yield by approximately 4-weeks, suggesting IGF-1 may mediate long-term changes in mammary function (Dahl et al., 2000). The increase in IGF-1 seen in dairy cows can be prevented by feeding melatonin to mimic SD photoperiod (Smith et al., 1997). Taken together photoperiod manipulation during gestation has significant effects on milk production that may be mediated by effects on hormonal secretion.

Financial benefits for dairy farmers

Dahl (2005) outlined the financial investments and gains of using long day photoperiod on lactating animals. In a free-stall barn housing 250 cows, Dahl estimated 72 fixtures would be required at a cost of $400/fixture plus the additional $220 for the cost of timers and their installation. Per cow, this resulted in a $116 capital cost. Calculation of the payoff of implementing this lighting regimen, including the costs of additional feed and electricity, yields a net return of $0.43/cow/d. Based on the initial investment of $116/cow it would take a farmer 270 days to recover the expense. Dahl (2005), notes the returns on investment are greater in larger dairies, although it is still a profitable management strategy

for dairy farms of all sizes. Overall, exposure of cows to long day photoperiod by supplemental lighting during lactation requires a significant initial investment, but in the long-term, it may provide dairy farmers with increased profitability.

Short day photoperiod during the dry period increases milk production

Short day photoperiod increases milk production

The typical lactation cycle of a dairy cow includes a non-lactating, or dry, period of 40-60 days between successive lactations (**Figure 1.7**), that allows for diversion of nutrients to the fetus. The dry period in dairy cows is characterized by regression of the mammary gland, brought on by cessation of milking, followed by proliferation and differentiation of secretory cells (lactogenesis stage I) and finally milk production (lactogenesis stage II) (Tucker, 1981; Capuco et al., 1997; Bachman and Schairer, 2003). The dry period is thought to allow remodeling and restoration of mammary secretory tissue prior to the next lactation (Hurley and Loor, 2011). Optimal management of the dry period is of biological and economic importance because several factors, including the length of the dry period, can influence milk production in the subsequent lactation. Manipulation of photoperiod during the dry period is a management tool that affects subsequent milk yield in dairy cows.

Miller and coworkers (2000) first hypothesized that LD photoperiod during the dry period would enhance milk yield in the subsequent lactation, based on studies carried out during lactation. Contrary to their hypothesis, SD photoperiod, relative to LD, significantly increased milk yield (3.2 kg/d) in the subsequent lactation (Miller et al., 2000). This finding was later validated by Auchtung et al. (2005). Specifically, dairy cows exposed to SD photoperiod during a 60-day dry period produced ~3 kg/day (~5-10%) more milk in the

subsequent lactation than cows exposed to LD photoperiod (**Figure 1.6**) (Auchtung et al., 2005).

Subsequent studies went on to evaluate the length of the photoperiod exposure during the dry period on milk production. Velasco and coworkers (2008) tested the hypothesis that a 42-day dry period would be sufficient time in which to elicit an effect on milk production using photoperiod manipulation. The authors reported an increase of 3.5 kg/day more milk in the subsequent lactation than animals exposed to LD photoperiod (Velasco et al., 2008). These findings were consistent that shown for a 60-day dry period (Miller et al., 2000; Auchtung et al., 2005). However, in a preliminary report, reducing the photoperiod exposure time to a dry period of 21 days abolished the galactopoietic effects of SD photoperiod (Reid et al., 2004).

These photoperiodic effects on milk production are substantial and are consistent across multiple studies (summarized in **Table 1.2**) and similar effects are reported in other species. For example, pre-partum SD photoperiod increases milk production in the subsequent lactation in sheep (Mikolayunas et al., 2008) and goats (Mabjeesh et al., 2013). In summary, exposing dairy cows to SD photoperiod prior to parturition can have positive effects on milk yield in the subsequent lactation.

Effects of photoperiod during gestation on milk composition

Increasing milk production by photoperiod manipulation is beneficial assuming it does not negatively affect milk composition. Aharoni et al. (2000) reported slight changes in milk fat and protein percentages in response to SD photoperiod in cows exposed for three weeks prior to parturition. This study, however, was based on correlations between day length and milk components of dairy cows in hot climates so it may not be representative of

the typical dairy cow (Aharoni et al., 2000). Numerous other studies have reported no change in principal milk components (fat, protein, lactose) in cows exposed to photoperiod manipulation during the dry period (Miller et al., 2000; Auchtung et al., 2005; Velasco et al., 2008). Diurnal variation in somatic cell count has been reported in both cows and sheep (White and Rattray, 1965; Smith and Schultze, 1967; Gonzalo et al., 1994). However, there was no effect on somatic cell count in cows exposed to LD or SD photoperiod for 42-days during the dry period (Velasco et al., 2008). This was also the case in first-lactation heifers previously exposed to different photoperiods during the pre-pubertal stage (Rius and Dahl, 2006). Taken together, SD photoperiod exposure during the dry period increases milk production in the subsequent lactation, with little to no effect on milk composition.

Potential mechanisms of increased milk production during the dry period

Dry matter intake

Enhanced milk production in response to SD photoperiod treatment may be due to increased energy storage during the dry period. To test this hypothesis Velasco et al. (2008) quantified DMI of cows exposed to 42-days of either LD or SD photoperiod. They reported that the DMI of cows differed numerically but not statistically by photoperiod (Velasco et al., 2008). In an earlier study, a small effect of photoperiod on DMI was reported; specifically, cows treated with SD photoperiod consumed 1.3 kg/d more dry matter than cows on LD photoperiod (Miller et al., 2000). This is in agreement with other studies that report cows exposed to SD photoperiod increase DMI intake relative to cows on LD photoperiod although no effect of photoperiod on body weight was detected in cows with increased DMI (Auchtung et al., 2005; Crawford et al., 2005). These findings suggest the increase in milk yield is not due to increased energy storage prior to lactation, but rather is a result of

photoperiod affecting the mammary gland through hormonal and local regulation.

Cell turnover in the mammary gland

The number and activity of secretory cells in the mammary gland fundamentally determine milk production (Capuco et al., 2001). Therefore, photoperiod manipulation must affect one, or both, of those factors. During the dry period, the mammary gland regresses through a process called involution. Involution is characterized by increased cellular apoptosis (Hurley and Loor, 2011). To determine if short day photoperiod increases milk production by increasing cell numbers in the mammary gland during the dry period, Wall et al. (2005) investigated mammary cell proliferation and apoptosis. At day -24 relative to parturition, mammary tissue from cows exposed to SD photoperiod had significantly higher rates of ^{3}H-thymidine incorporation, a measure of mammary cell proliferation, compared to cows exposed to LD photoperiod (Wall et al., 2005b). This effect, however, was not detected on days -46, -9, or +11 relative to parturition. On days, -46 and -24, significantly more epithelial cells underwent apoptosis in cows exposed to LD relative to SD photoperiod, whereas on days -9 and +11 there was no effect of photoperiod exposure on cell proliferation or apoptosis (Wall et al., 2005b). These changes in cell proliferation and apoptosis illustrate the extensive remodeling that occurs in the mammary gland during the dry period. Ultimately, these data indicate that the dry period may include a critical time during which photoperiod can affect mammary cell proliferation and thereby alter milk production in the subsequent lactation.

Hormonal regulation in response to photoperiod

Prolactin

Although treatment of dairy cows with exogenous prolactin does not increase milk yield (Plaut et al., 1987), prolactin has been identified as a potential mediator of the effects of photoperiod on milk production. Exposure to LD photoperiod during the dry period consistently increases plasma prolactin concentrations in dairy cows (Miller et al., 2000; Auchtung et al., 2005). Cows on LD photoperiod during a 42-day dry period secrete more prolactin (16.9 ng/mL) than cows on SD photoperiod (11.2 ng/mL) (Velasco et al., 2008). This trend was consistent in the periparturient period, although after parturition the effect was negated (Velasco et al., 2008). Lacasse et al. (2014) reported that exposure to LD photoperiod and exogenous melatonin decreases circulating prolactin concentration in the blood below that of LD exposed cows, but not significantly below the level of SD exposed cows. This is in agreement with the previous report that feeding melatonin cannot be used to mimic the effects of SD photoperiod during the dry period in dairy cows (Petitclerc et al., 1998).

Efforts to understand the role of prolactin in the milk yield response to photoperiod have led to investigation of the prolactin receptor. Prolactin receptor exists in dairy cows and mice in both a long and a short form (Devi and Halperin, 2014). Investigation of the effects of photoperiod on prolactin receptor gene expression have identified an inverse relationship between the concentration of prolactin in the blood and the expression of the prolactin receptor in a variety of tissues, including the mammary gland (Auchtung et al., 2003; Auchtung et al., 2005; Wall et al., 2005b; Velasco et al., 2008). Therefore, the decrease in circulating prolactin associated with SD photoperiod exposure during the dry period is

accompanied by an increase in prolactin receptor in the mammary gland (Auchtung et al., 2005). Increased prolactin receptor abundance, in response to SD photoperiod, may decrease the expression of members of the suppressor of cytokine signaling (SOCS) gene family, thereby promoting lactogenesis (Wall et al., 2005b). These findings demonstrate the complex mechanisms involving prolactin signaling that may underlie the effects of photoperiod in the mammary gland.

Insulin-like growth factor-1

In addition to prolactin, IGF-1 has been identified as a potential mediator of the effects of photoperiod on mammary function. Circulation of IGF-1, a growth factor known to have galactopoietic effects in goats (Prosser et al., 1990), increases early in the dry period (Abribat et al., 1990), potentially inhibiting mammary cell apoptosis (Marshman and Streuli, 2002). Exposure to LD photoperiod during lactation also increases circulating IGF-1 concentrations in cows concurrently producing more milk than their SD counterparts (Dahl et al., 2000). These findings suggest circulating IGF-1 may mediate the galactopoietic response to photoperiod in lactating dairy cows. However, Miller and coworkers (2000) reported only a non-significant increase in blood IGF-1 concentrations in dry cows exposed to SD, compared to those on LD photoperiod.

This led Wall et al. (2005b) to hypothesize that local expression of IGF-1 in the mammary gland of dry cows might replace the blood-borne supply. Subsequently, a significant increase in mammary expression of IGF-2 but not IGF-1 mRNA was reported in cows exposed to SD relative to LD photoperiod (Wall et al., 2005b). In some cases, IGF-1 and IGF-2 interact with binding proteins (IGFBP1-7), which sequester the growth factors, thereby promoting apoptosis (Tonner et al., 1995). In cows exposed LD or SD photoperiod

during lactation, the concentrations in plasma of IGFBP-2 and -3 are not affected (Dahl et al., 1997). However, cows exposed to LD photoperiod during the dry period have increased expression of IGFBP-5 in the mammary gland on day 11 of lactation (Wall et al., 2005b). In rats, prolactin inhibits IGFBP-5 expression, thereby promoting cell survival in the mammary gland (Akers, 2006). When unbound, IGFBP-5 can interact with IGF-1 receptor and function as a growth-inducing mitogen (Marshman and Streuli, 2002). Therefore, the differential expression of IGFBP-5 in response to photoperiod suggests this gene may have a role function in the milk yield response to photoperiod.

Financial benefits for dairy farmers

Dahl (2005) outlined the financial investments and gains of using LD photoperiod on lactating animals. He concluded the initial investment could be recouped in 270 days, less than one typical 305-day lactation cycle of a dairy cow. Given the scenario Dahl (2005) outlined, in a herd of 250 cows, there might be ~ 50 dry cows at any given time, therefore perhaps 15 light fixtures would be needed to expose the dry-cows to SD photoperiod. In addition, it would be neccesary to exclude light form the dry-cow environment. This would result in a capital cost of at least $121 per cow. Assuming the net return is the same as Dahl (2005) predicted ($0.20/cow/day) it would take a farmer 282 days to begin to make a net profit. Although, this estimate could be refined, it demonstrates the potential for profitability of implementing SD photoperiod exposure on a dairy farm.

Summary

In summary, LD photoperiod during lactation, and SD photoperiod during the dry period, increase milk production without affecting milk composition and can be profitable for

dairy producers. Substantial work has been conducted to identify mediators of the response including prolactin and IGF-1. These hormones have potential to modulate mammary function; however, the molecular mechanisms underlying the effects of photoperiod on mammary function remain unclear.

THE MOUSE – A MODEL FOR PHOTOPERIOD RESEARCH

Photoperiod-responsiveness

Inbred laboratory mice were for some time considered non-responsive to photoperiod manipulation. In a much cited study by Goto et al. (1989), the pineal melatonin content of various laboratory mouse strains was quantified. They reported that some strains (C3H and CBA) undergo large diurnal fluctuations in pineal melatonin, whereas BALB/C and C57BL/6 mice do not (Goto et al., 1989). The absence of melatonin rhythm was attributed to a lack of the rate-limiting enzyme in melatonin synthesis (Ebihara et al., 1986; Goto et al., 1989). It was concluded that C57Bl/6 mice were non-responsive to photoperiod as they lacked detectable levels of melatonin in their blood (Ebihara et al., 1986; Vollrath et al., 1988; Goto et al., 1989). Later studies revealed that BALB/C and C57BL/6 mice display short-term peaks of melatonin in the pineal gland and blood as well as melatonin metabolites in urine (Maestroni et al., 1987; Conti et al., 1992; Conti and Maestroni, 1996).

Mice have subsequently been used as a model system to study photoperiod manipulation. Numerous studies using C57Bl/6 mice have demonstrated the responsiveness of this strain to photoperiod manipulation in the study of behavior (Comas and Hut, 2009; Adamah-Biassi et al., 2013; Otsuka et al., 2014), signal transduction (Ono et al., 2008; Yasuo et al., 2009), cancer biology (Lang et al., 2003), circadian biology (Metz et al., 2006;

Etchegaray et al., 2009; Sosniyenko et al., 2009; Bur et al., 2010; Pendergast et al., 2010; Ciarleglio et al., 2011; Summa et al., 2012; Azzi et al., 2014; Jackson et al., 2014), brain and eye function (Brooks et al., 2011; Brooks et al., 2014). Despite differences in the photoperiod transduction mechanism in C57Bl/6 mice, the molecular mechanisms of the pineal gland and the circadian clock are similar between 'melatonin-deficient' (C57Bl/6) and 'melatonin-proficient' (C3H) mice (von Gall et al., 2000). These studies support the use of C57Bl/6 mice as a model to study the molecular mechanisms of the effects of photoperiod.

The mouse as a model of mammary biology

Unlike most organs, the mammary gland undergoes the majority of its development *ex-utero*, creating a model system in which to study cellular development, hormonal signaling, and gene expression. Some of the earliest research on mammary development and tumorigenesis was conducted using the mouse (Lathrop and Loeb, 1916; Cole, 1933). Our understanding of mammary biology and the molecular mechanisms underlying mammary function have been greatly aided by incorporation of the mouse as a research model (Hennighausen and Robinson, 2005). The mouse mammary gland has had an essential role in understanding the molecular mechanisms of lactation. Lemay et al. (2007) used the mouse to identify gene networks associated with the stages of lactation, and others have used the mouse to study the molecular process of secretion in the mammary gland (Ramanathan et al., 2007). More recently, analysis of genes associated with lactation performance in the mouse has been used to identify gene targets for further study of lactation variation in both humans and animals (Ramanathan et al., 2008; Wei et al., 2013).

MICROARRAY ANALYSIS TO UNDERSTAND MAMMARY FUNCTION

First implemented to investigate gene expression profiles between cancer cell lines (Ross et al., 2000), DNA microarray analysis has revolutionized our understanding of mammary gland biology. In simple terms, DNA microarray is used to detect changes in gene expression across entire transcriptomes (**Figure 1.8**). Microarray analysis has been used to understand all aspects of mammary biology and lactation in humans, mice, and cows. To date, PubMed returns more than 4000 publications by searching the keywords 'mammary' and 'microarray'. Recent transcriptomic studies of the bovine mammary gland include peri-partum mammary development (Finucane et al., 2008; Casey et al., 2011; Gao et al., 2013), milking frequency (Connor et al., 2008; Littlejohn et al., 2010; Wall et al., 2012), environmental stress (Collier et al., 2006), bacterial invasion (Swanson et al., 2009), hormonal response (Connor et al., 2007; Stiening et al., 2008) and immune function (Park et al., 2004; Swanson et al., 2009). These studies identified differentially expressed genes and ultimately gained insight into mammary function and physiology.

LITERATURE REVIEW SUMMARY

Photoperiod affects the majority of life on earth and serves as a consistent measure of time. Because of its ubiquitous nature, photoperiod has vast effects on the physiology of mammals including immune function and reproduction. Information on external photoperiod is captured in the brain and converted to hormonal signals that relay that information throughout an organism. Manipulation of photoperiod affects mammary development and function in cows, mice, and humans, suggesting common biological mechanisms. Although, a great deal of work has been done to elucidate the effects of photoperiod on mammary

function, many avenues remain to be explored. The following chapters detail a series of experiments designed to identify the genes underlying the molecular mechanisms of the effects of photoperiod in the mammary gland. Using cows and the mouse as models and microarray as an approach, the aim of this work is to identify genes and pathways responsive to photoperiod and associate their differential expression with functional effects of photoperiod in the mammary gland.

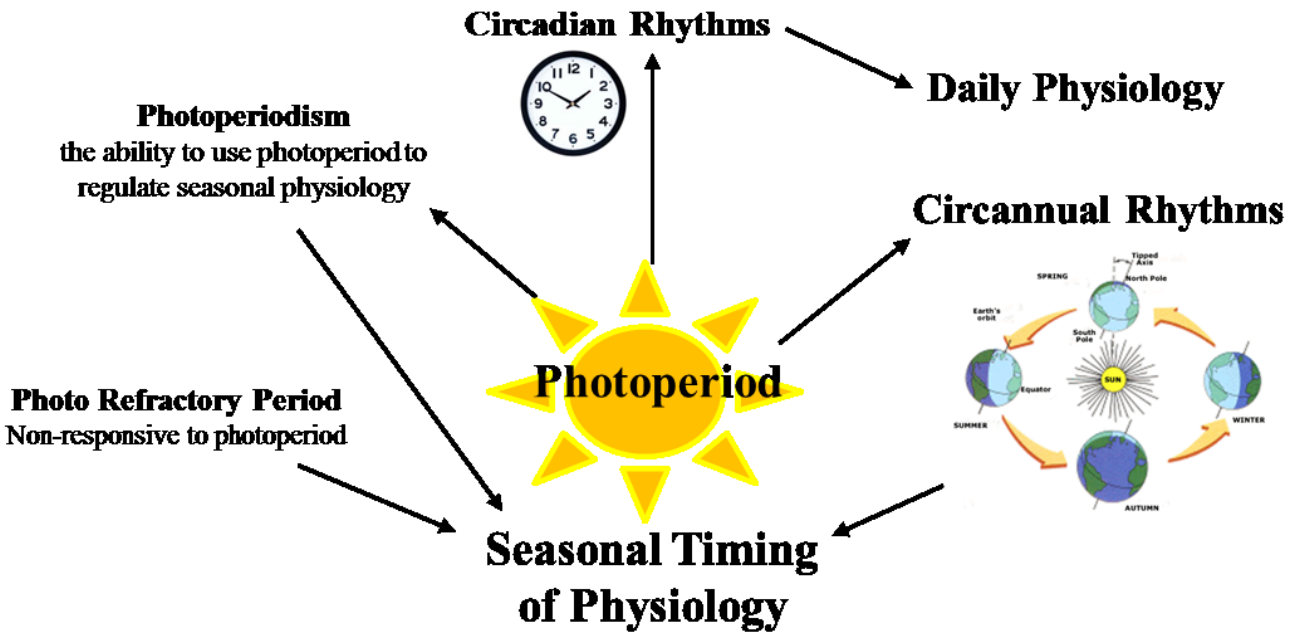


Figure 1.1. Photoperiod – a central role in rhythmic biology.

Daily light exposure coordinates many biological functions and entrains circadian and circannual rhythms.

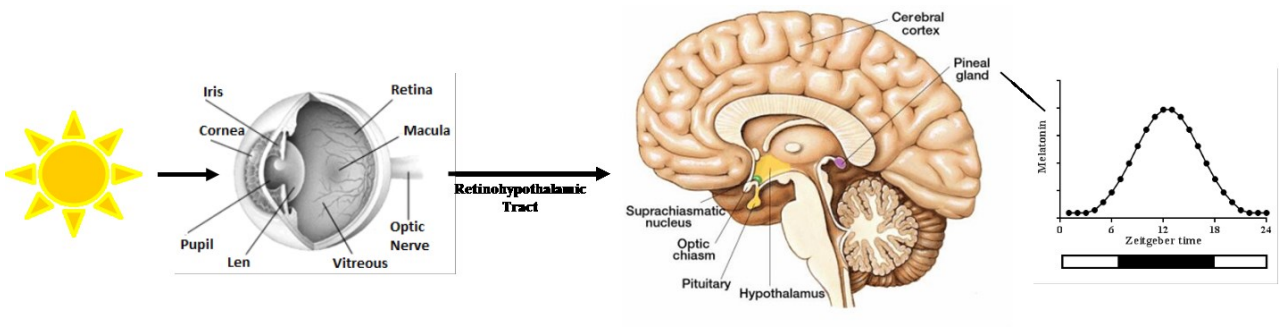


Figure 1.2. Conversion of light to hormonal information.

Photoperiodic information is detected by the eye and passes through the retinohypothalamic tract to the suprachiasmatic nucleus in the brain. From there, the information is converted into hormonal signals secreted in the form of melatonin from the pineal gland, the secretion of which is inhibited by light.

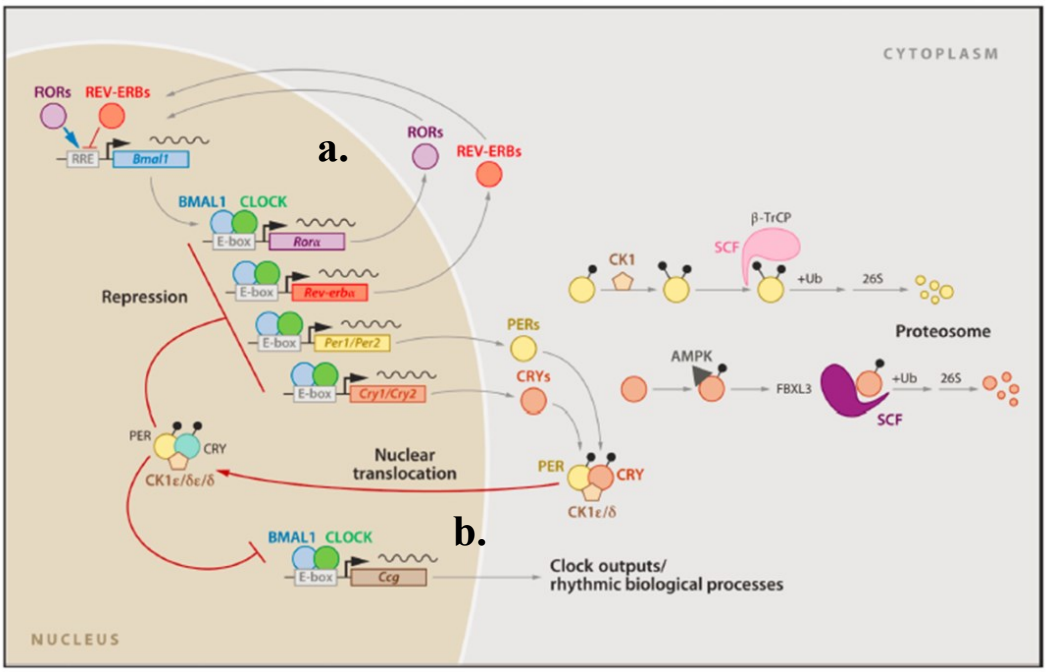


Figure 1.3. The molecular mechanisms of the circadian clock in mammals.

The circadian clock is a network of transcriptional regulators and feedback loops entrained to an approximately 24-h cycle. **a.** CLOCK and BMAL1 positively regulate of the expression of *Per1* and *Per2* and *Cry 1* and *Cry 2*. **b.** In the nucleus PER1, PER2, CRY1, and CRY2 interact with CLOCK and BMAL1 inhibiting their own expression. Figure from Mohawk et al. (2012).

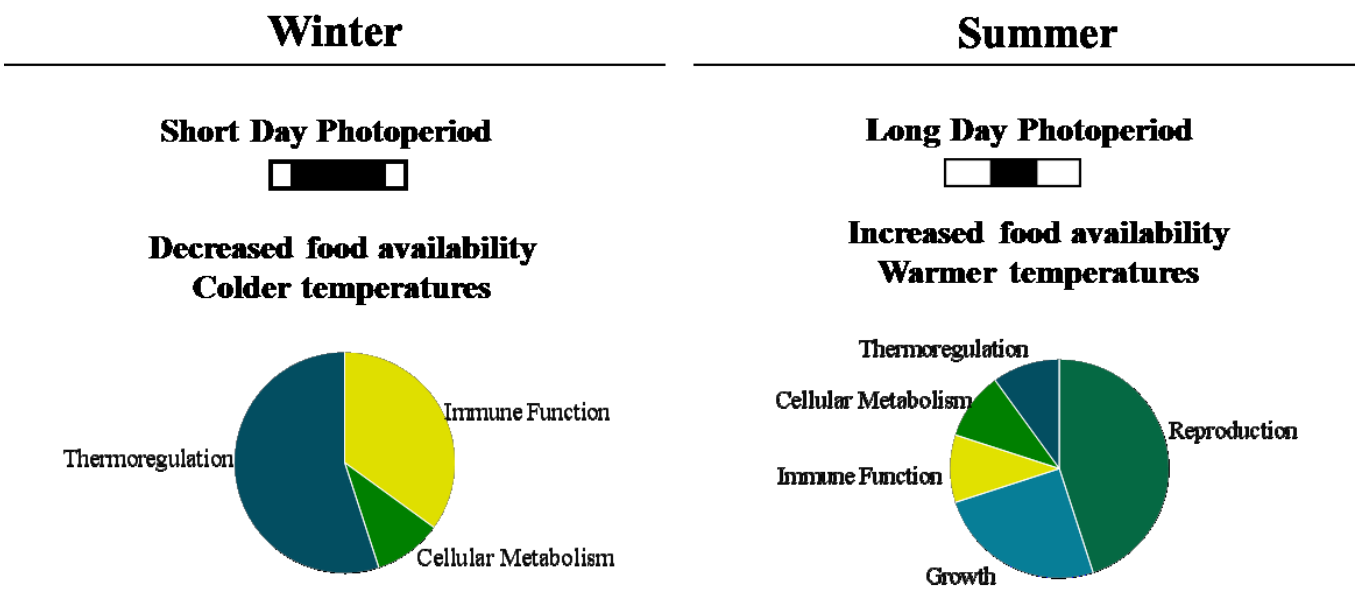


Figure 1.4. Relative energy expenditure in winter and summer.

Due to different environmental stresses, a greater percentage of energy is expended on immune function in the winter. During the summer, this energy is used for reproduction and growth. Charts redrawn from Walton et al. (2011).

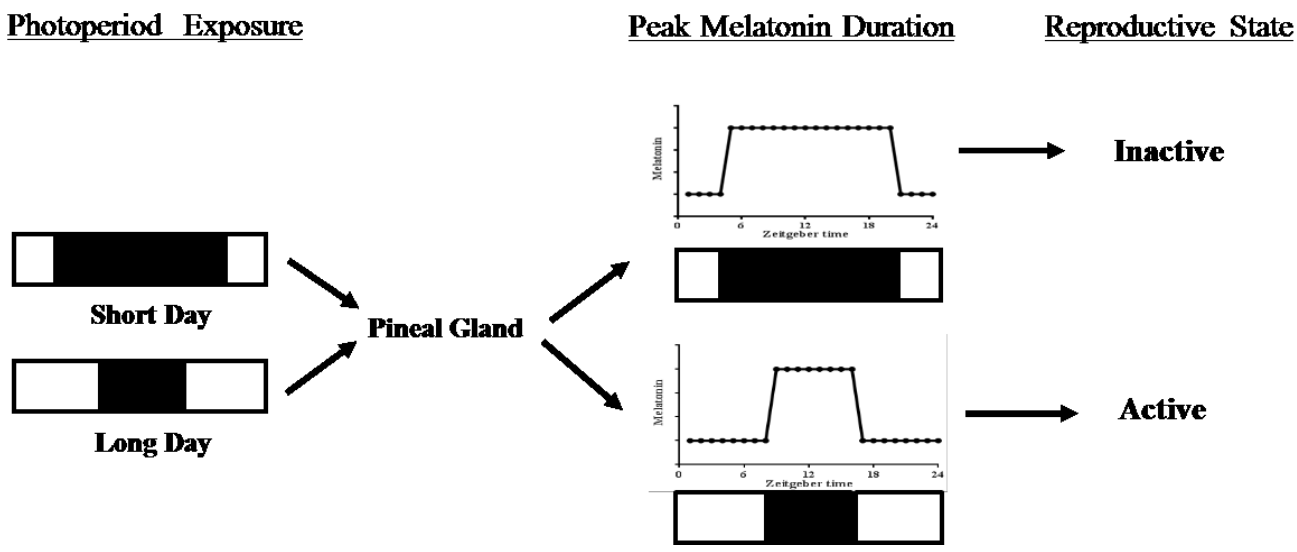


Figure 1.5. Photoperiod coordinates breeding schedules.

Short day photoperiod increases the duration of elevated melatonin secreted from the pineal gland, triggering reproductive inactivity in long day breeders (e.g.: Syrian hamster). Long day photoperiod decreases the duration of melatonin secretion and allows reproductive activity. Adapted from (Goldman, 1999).

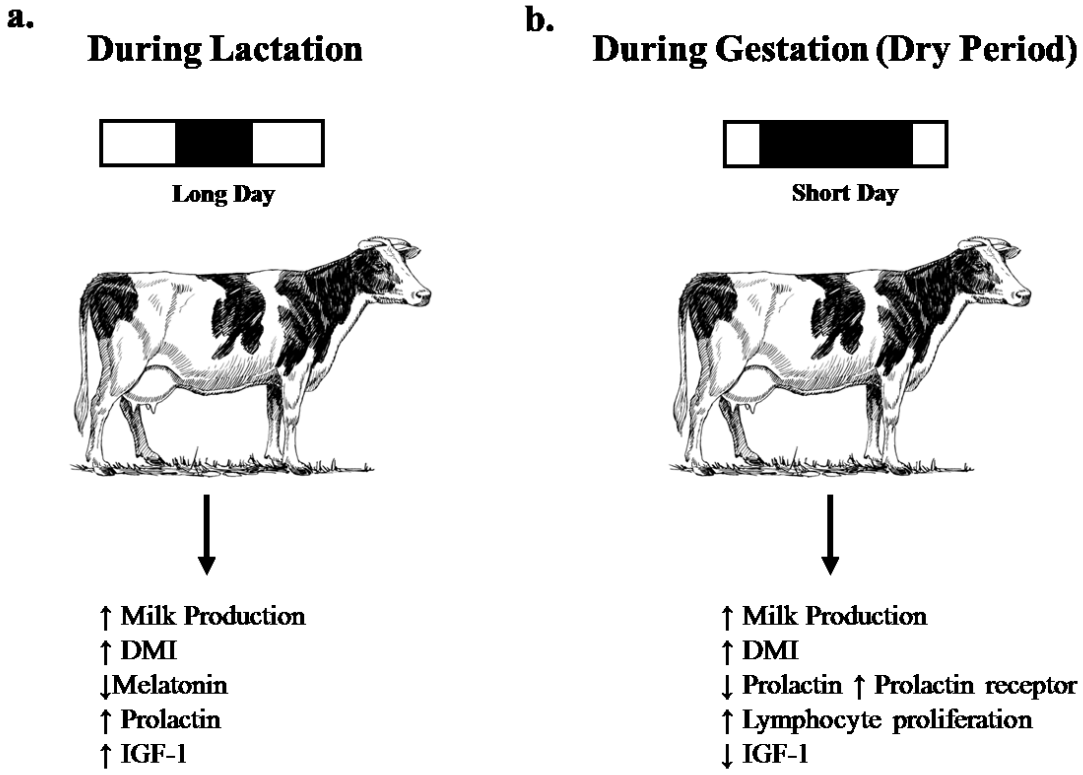


Figure 1.6. Photoperiod manipulation alters lactation performance in cows.

The effects of photoperiod manipulation **a.** during lactation, or **b.** during the dry period in dairy cows. Dry mater intake (DMI), Insulin-like growth factor-1 (IGF-1).

Table 1.1. The effects of photoperiod manipulation during lactation on milk production of lactating cows – a summary of the literature to date.

Author (year)	Principle Findings	Change in Milk Yield	Breed / Dev. Stage	Photoperiod Treatments (h light: h dark)[1]
Peters et al. (1978)	• LD photoperiod increases milk yield	↑ 2.0 kg/day on LD	Holstein cows	LD vs natural (9.8 h/day)
Peters et al. (1981)	• LD photoperiod increase prolactin se creation • LD PP increase dry mater intake • No effect of pp on % fat in milk	↑ 1.4 kg/day on LD	Holstein cows	LD vs natural (9.8 h/day)
Marcek and Swanson (1984)	• Continuous PP does not affect milk yield relative to LD	n.d ↑ 2.2 kg/day on LD	Heifers Cows	Natural (9-12) vs 18:6
Stanisiewski et al. (1985)	• LD photoperiod decreased milk fat by 0.16%	↑ 2.2 kg/day on LD	Holsteins + Jersey + Brown Swiss	16-16.25 h vs natural (9-12 h/day)
Stanisiewski et al. (1988)	• LD increased prolactin relative to SD • No difference between SD and continuous light • Melatonin feeding does not affect serum prolactin	n/a	Holstein bull calves	SD vs LD vs continuous
Bilodeau et al. (1989)	• LD photoperiod increases milk production • LD increases DMI	↑ 2.0 kg/day on LD	Holstein cows	LD vs (8light:2dark:2light:12 dark)
Evans and Hacker (1989)	• LD photoperiod increases milk yield	↑ 2.8 kg/day on LD	Holstein cows	Natural (12-13 h) vs skeletal photoperiod
Phillips and Schofield (1989)	• LD photoperiod increases milk yield • LD tends to decrease % milk fat	↑ 3.3 kg/day on LD	British Friesian Cows	18:6 vs 6:18
Newbold et al. (1991)	• LD increases serum prolactin before /during the periparturient surge • Duration of elevated melatonin was longer under SD • PP had no effect on mammary development during pregnancy	n/a	Holstein heifers	LD vs SD
Dahl et al. (1997)	• LD photoperiod increases milk yield • LD photoperiod increases plasma IGF-1	LD ↑ 2.2 kg/day	Holstein cows	Natural (<13 h) vs 18:6
Reksen et al. (1999)	• A minimum of 12 hours is necessary to stimulate	LD ↑ 2.2 kg/day	Norwegian Red Cattle	Natural vs LD
Barash et al. (2001)	• LD photoperiod increases milk yield	LD ↑ 1.2 kg/h	Holstein cows	Natural: ~10.5h – 14.5
Crawford et al. (2005)	• LD increases circulating prolactin 2-fold over SD photoperiod • SD +prolactin decrease milk yield – but not to the level of LD	SD ↑ 6.2 kg/day	Holstein cows	LD vs SD
Bernabucci et al. (2006)	• 18:6 increased plasma prolactin secretion • PP did not affect feed intake or body condition score • PP had no effect on plasma leptin, growth hormone, cortisol, NEFA, β-OH-butyrate or glucose • 18:6 increased leptin • Leptin receptors were affected by PP	↓ 2.9 under SD relative to LD	Holstein cows	6:18 vs 18:6 and 12:12
Auldist et al. (2007)	• Melatonin implants decrease plasma prolactin, but not IGF-1 • Implants decrease milk yield	Melatonin ↓ milk yield	Friesian Cows	Melatonin implant vs natural (NZ: Nov-Feb)

[1] **LD**: 16 h light:8 h dark, **SD**: 8 h light:16 h dark

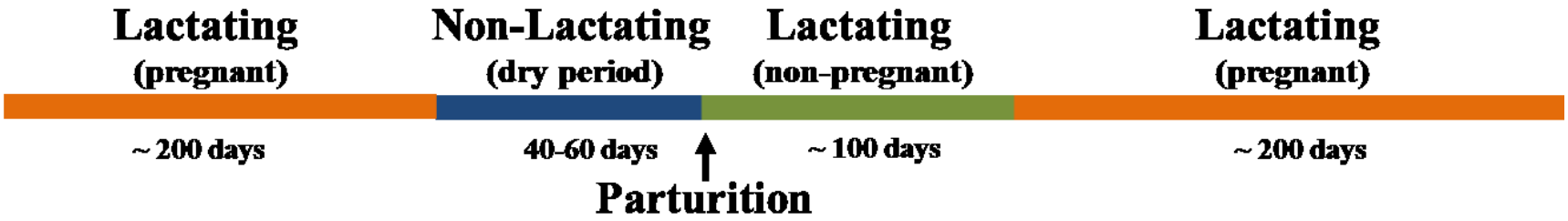


Figure 1.7. Typical lactation cycle of a dairy cow.

Table 1.2. The effects of photoperiod manipulation during gestation (dry period) on milk production in the subsequent lactation – a summary of the literature to date.

Author	Principle Findings	Change in Milk Yield	Breed / Dev. Stage	Photoperiod Treatments (h light: h dark)[1]
Aharoni et al. (2000)	• Pre-partum day length is negatively related to milk yield	LD ↓ milk yield	Holstein cows	Natural long day vs Natural short day
Miller et al. (2000)	• LD during 60 day dry period increase plasma prolactin • SD during 60 day dry period increases milk yield • PP did not affect IGF-1	SD ↑ 3.2 kg/day	Holstein cows	LD vs SD
Reid et al. (2004)	• 21 days of pre-partum SD photoperiod does not affect milk yield in the subsequent lactation.	n.d.	Holstein cows	LD vs SD
Auchtung et al. (2004)	• SD increased neutrophil chemotaxis and lymphocyte proliferation • LD increased periparturient prolactin surge	n/a	Holstein cows	LD vs SD
Auchtung et al. (2005)	• SD photoperiod during the dry period increases milk yield • SD increased DMI • LD increased plasma prolactin concentration • SD increased prolactin receptor (S and L) mRNA abundance	SD ↑ 3.1 kg/day	Holstein cows	LD vs SD
Wall et al. (2005b)	• SD photoperiod increases mammary growth (3H-thymidine) during dry period • % of apoptotic epithelium tended to be greater in LD cows • SD increased mRNA abundance of IGF-II • LD increased mRNA abundance of IGFBP5 on day 11 of lactation	n/a	Holstein cows	LD vs SD
Wall et al. (2005a)	• SD photoperiod during the dry period decreases expression of SOC2 and -3 genes	n/a	Holstein cows	LD vs SD
Rius and Dahl (2006)	• LD photoperiod during the pre-pubertal period decreases the time until heifers can be bred • LD tends to increase milk yield in first lactation	n/a	Holstein cows	LD vs SD
Velasco et al. (2008)	• SD during 42 day dry period increased milk production in subsequent lactation • SD during 42 day dry period increase DMI • Peri-parturient prolactin surge was greater in cows on LD	SD ↑ 3.6 kg/d	Holstein Cows	LD vs SD
Lacasse et al. (2014)	• Pre-partum SD photoperiod increases milk yield in early lactation, not late lactation • SD photoperiod ↓ prolactin concentrations in the blood • SD cows had increased feed efficiency relative to LD • Pre-partum melatonin treatment did not mimic SD photoperiod exposure	n.d. ↑ SD	Holstein Heifers Cows	LD vs SD

[1.] **LD**: 16 h light:8 h dark, **SD**: 8 h light:16 h dark

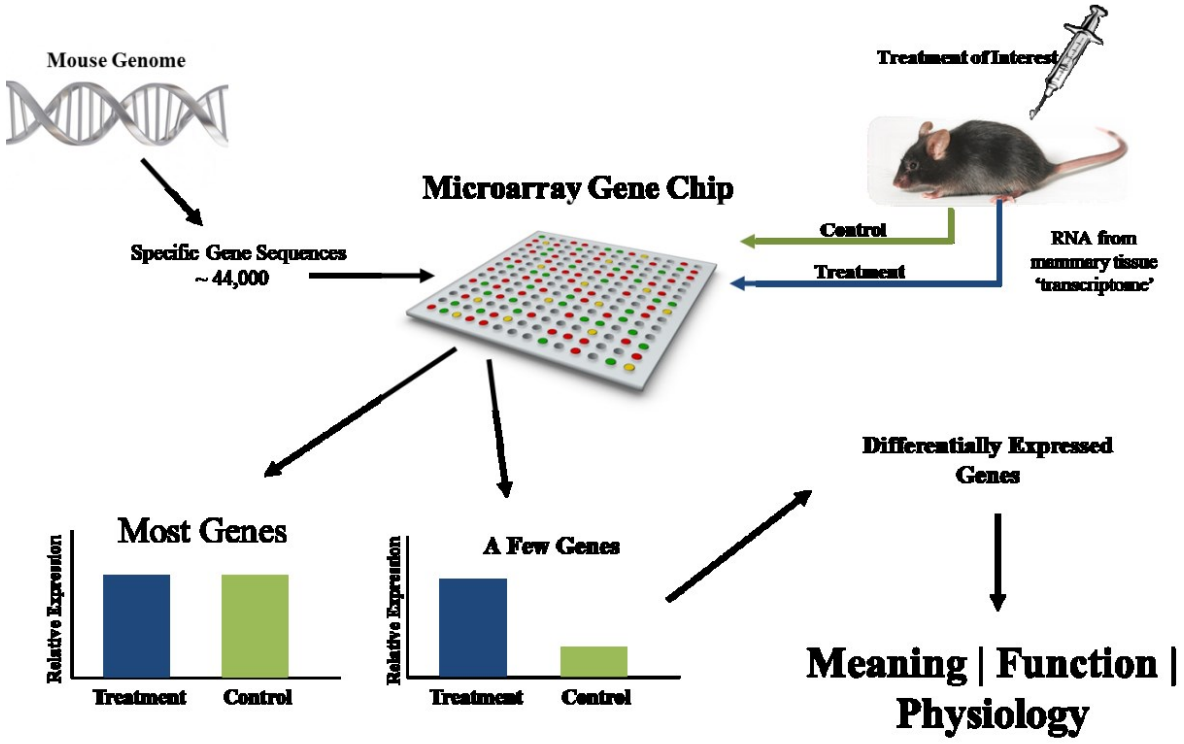


Figure 1.8. Principles of microarray analysis.

Microarray analysis enables the quantification of gene expression across entire genomes. The aim of microarray is to identify differentially expressed genes. Subsequently the importance and role of these differentially expressed genes in physiology must be determined.

REFERENCES

Abribat, T., H. Lapierre, P. Dubreuil, G. Pelletier, P. Gaudreau, P. Brazeau, and D. Petitclerc. 1990. Insulin-like growth factor-i concentration in holstein female cattle: Variations with age, stage of lactation and growth hormone-releasing factor administration. Domest Anim Endocrinol 7:93.

Adamah-Biassi, E. B., I. Stepien, R. L. Hudson, and M. L. Dubocovich. 2013. Automated video analysis system reveals distinct diurnal behaviors in c57bl/6 and c3h/hen mice. Behav Brain Res 243:306.

Aharoni, Y., A. Brosh, and E. Ezra. 2000. Short communication: Prepartum photoperiod effect on milk yield and composition in dairy cows. J Dairy Sci 83:2779.

Akers, R. M. 2002. Lactation and the mammary gland. 1st ed. Wiley-Blackwell, Ames, Iowa.

Akers, R. M. 2006. Major advances associated with hormone and growth factor regulation of mammary growth and lactation in dairy cows. J Dairy Sci 89:1222.

Andersson, H., J. D. Johnston, S. Messager, D. Hazlerigg, and G. Lincoln. 2005. Photoperiod regulates clock gene rhythms in the ovine liver. Gen Comp Endocrinol 142:357.

Arendt, J. 1986. Role of the pineal gland and melatonin in seasonal reproductive function in mammals. Oxf Rev Reprod Biol 8:266.

Aschoff, J. 1960. Exogenous and endogenous components in circadian rhythms. Cold Spring Harb Symp Quant Biol 25:11.

Auchtung, T. L. and G. E. Dahl. 2004. Prolactin mediates photoperiodic immune enhancement: Effects of administration of exogenous prolactin on circulating concentrations, receptor expression, and immune function in steers. Biol Reprod 71:1913.

Auchtung, T. L., P. E. Kendall, J. L. Salak-Johnson, T. B. McFadden, and G. E. Dahl. 2003. Photoperiod and bromocriptine treatment effects on expression of prolactin receptor mrna in bovine liver, mammary gland and peripheral blood lymphocytes. J Endocrinol 179:347.

Auchtung, T. L., A. G. Rius, P. E. Kendall, T. B. McFadden, and G. E. Dahl. 2005. Effects of photoperiod during the dry period on prolactin, prolactin receptor, and milk production of dairy cows. J Dairy Sci 88:121.

Auchtung, T. L., J. L. Salak-Johnson, D. E. Morin, C. C. Mallard, and G. E. Dahl. 2004. Effects of photoperiod during the dry period on cellular immune function of dairy cows. J Dairy Sci 87:3683.

Auldist, M. J., S. A. Turner, C. D. McMahon, and C. G. Prosser. 2007. Effects of melatonin on the yield and composition of milk from grazing dairy cows in new zealand. J Dairy Res 74:52.

Azzi, A., R. Dallmann, A. Casserly, H. Rehrauer, A. Patrignani, B. Maier, A. Kramer, and S. A. Brown. 2014. Circadian behavior is light-reprogrammed by plastic DNA methylation. Nat Neurosci 17:377.

Bachman, K. C. and M. L. Schairer. 2003. Invited review: Bovine studies on optimal lengths of dry periods. J Dairy Sci 86:3027.

Baker, J. R. and R. M. Ranson. 1932. Factors affecting the breeding of the field mouse (*microtus agrestis*). Part i.--light. Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character 110:313.

Baldwin, W. S. and J. C. Barrett. 1998. Melatonin: Receptor-mediated events that may affect breast and other steroid hormone-dependent cancers. Mol Carcinog 21:149.

Balsalobre, A. 2002. Clock genes in mammalian peripheral tissues. Cell Tissue Res 309:193.

Balsalobre, A., F. Damiola, and U. Schibler. 1998. A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell 93:929.

Barash, H., N. Silanikove, A. Shamay, and E. Ezra. 2001. Interrelationships among ambient temperature, day length, and milk yield in dairy cows under a mediterranean climate. J Dairy Sci 84:2314.

Berger, J. J. and C. W. Daniel. 1982. Diurnal rhythms in developing ducts of the mouse mammary gland. J Exp Zool 224:115.

Bernabucci, U., L. Basirico, N. Lacetera, P. Morera, B. Ronchi, P. A. Accorsi, E. Seren, and A. Nardone. 2006. Photoperiod affects gene expression of leptin and leptin receptors in adipose tissue from lactating dairy cows. J Dairy Sci 89:4678.

Bilodeau, P. P., D. Petitclerc, N. St Pierre, G. Pelletier, and G. J. St Laurent. 1989. Effects of photoperiod and pair-feeding on lactation of cows fed corn or barley grain in total mixed rations. J Dairy Sci 72:2999.

Blask, D. E., R. T. Dauchy, G. C. Brainard, and J. P. Hanifin. 2009. Circadian stage-dependent inhibition of human breast cancer metabolism and growth by the nocturnal melatonin signal: Consequences of its disruption by light at night in rats and women. Integr Cancer Ther 8:347.

Blom, J. M., J. M. Gerber, and R. J. Nelson. 1994. Day length affects immune cell numbers in deer mice: Interactions with age, sex, and prenatal photoperiod. Am J Physiol 267:R596.

Bocquier, F., S. Ligios, G. Molle, and S. Casu. 1997. Effect of photoperiod on milk yield, milk composition and voluntary food intake in lactating dairy ewes. Ann. Zootech 46:427.

Borst, D. W. and W. B. Mahoney. 1980. Diurnal changes in mouse mammary gland DNA synthesis. J Exp Zool 214:215.

Boulanger, V., X. Zhao, and P. Lacasse. 2002. Protective effect of melatonin and catalase in bovine neutrophil-induced model of mammary cell damage. J Dairy Sci 85:562.

Bradshaw, W. and C. Holzapfel. 2007. Evolution of animal photoperiodism Annu Rev Ecol Evol Syst 38:1.

Bradshaw, W. E., P. A. Zani, and C. M. Holzapfel. 2004. Adaptation to temperate climates. Evolution 58:1748.

Brock, M. A. 1983. Seasonal rhythmicity in lymphocyte blastogenic responses of mice persists in a constant environment. J Immunol 130:2586.

Bronson, F. H. 1979. The reproductive ecology of the house mouse. Q Rev Biol 54:265.

Brooks, E., D. Patel, and M. M. Canal. 2014. Programming of mice circadian photic responses by postnatal light environment. PLoS ONE 9:e97160.

Brooks, E., E. Waters, L. Farrington, and M. M. Canal. 2011. Differential hypothalamic tyrosine hydroxylase distribution and activation by light in adult mice reared under different light conditions during the suckling period. Brain Struct Funct 216:357.

Bur, I. M., S. Zouaoui, P. Fontanaud, N. Coutry, F. Molino, A. O. Martin, P. Mollard, and X. Bonnefont. 2010. The comparison between circadian oscillators in mouse liver and pituitary gland reveals different integration of feeding and light schedules. PLoS ONE 5:e15316.

Capuco, A. V., R. M. Akers, and J. J. Smith. 1997. Mammary growth in holstein cows during the dry period: Quantification of nucleic acids and histology. J Dairy Sci 80:477.

Capuco, A. V., D. L. Wood, R. Baldwin, K. McLeod, and M. J. Paape. 2001. Mammary cell number, proliferation, and apoptosis during a bovine lactation: Relation to milk production and effect of bst. J Dairy Sci 84:2177.

Cardinali, D. P., R. A. Bonanni Rey, M. D. Mediavilla, and E. Sanchez-Barcelo. 1992. Diurnal changes in cyclic nucleotide response to pineal indoles in murine mammary glands. J Pineal Res 13:111.

Carr, A. J., J. D. Johnston, A. G. Semikhodskii, T. Nolan, F. R. Cagampang, J. A. Stirland, and A. S. Loudon. 2003. Photoperiod differentially regulates circadian oscillators in central and peripheral tissues of the syrian hamster. Curr Biol 13:1543.

Carrick, D. T. and N. J. Kuhn. 1978. Diurnal variation and response to food withdrawal of lactose synthesis in lactating rats. Biochem J 174:319.

Carrillo-Vico, A., J. Guerrero, P. Lardone, and R. Reiter. 2005. A review of the multiple actions of melatonin on the immune system. Endocrine 27:189.

Casey, T., H. Dover, J. Liesman, L. DeVries, M. Kiupel, M. Vandehaar, and K. Plaut. 2011. Transcriptome analysis of epithelial and stromal contributions to mammogenesis in three week prepartum cows. PLoS ONE 6:e22541.

Chemineau, P., D. Guillaume, M. Migaud, J. C. Thiery, M. T. Pellicer-Rubio, and B. Malpaux. 2008. Seasonality of reproduction in mammals: Intimate regulatory mechanisms and practical implications. Reprod Domest Anim 43 Suppl 2:40.

Ciarleglio, C. M., J. C. Axley, B. R. Strauss, K. L. Gamble, and D. G. McMahon. 2011. Perinatal photoperiod imprints the circadian clock. Nat Neurosci 14:25.

Cohen, M., M. Lippman, and B. Chabner. 1978. Role of pineal gland in aetiology and treatment of breast cancer. Lancet 2:814.

Cole, H. A. 1933. The mammary gland of the mouse, during the oestrous cycle, pregnancy and lactation. Pages 136 in Proc. Royal Society of London The Royal Society.

Collier, R. J., C. M. Stiening, B. C. Pollard, M. J. VanBaale, L. H. Baumgard, P. C. Gentry, and P. M. Coussens. 2006. Use of gene expression microarrays for evaluating environmental stress tolerance at the cellular level in cattle. J Anim Sci 84 Suppl:E1.

Comas, M. and R. A. Hut. 2009. Twilight and photoperiod affect behavioral entrainment in the house mouse (*Mus musculus*). J Biol Rhythms 24:403.

Connor, E. E., M. J. Meyer, R. W. Li, M. E. Van Amburgh, Y. R. Boisclair, and A. V. Capuco. 2007. Regulation of gene expression in the bovine mammary gland by ovarian steroids. J Dairy Sci 90 Suppl 1:E55.

Connor, E. E., S. Siferd, T. H. Elsasser, C. M. Evock-Clover, C. P. Van Tassell, T. S. Sonstegard, V. M. Fernandes, and A. V. Capuco. 2008. Effects of increased milking frequency on gene expression in the bovine mammary gland. BMC Genomics 9:362.

Conti, A., N. Haran-Ghera, and G. J. Maestroni. 1992. Role of pineal melatonin and melatonin-induced-immuno-opioids in murine leukemogenesis. Med Oncol Tumor Pharmacother 9:87.

Conti, A. and G. J. Maestroni. 1996. Hplc validation of a circadian melatonin rhythm in the pineal gland of inbred mice. J Pineal Res 20:138.

Coto-Montes, A., C. Tomas-Zapico, G. Escames, J. Leon, M. J. Rodriguez-Colunga, D. Tolivia, and D. Acuna-Castroviejo. 2003. Specific binding of melatonin to purified cell nuclei from mammary gland of swiss mice: Day-night variations and effect of continuous light. J Pineal Res 34:297.

Crawford, H. M., J. L. Dauderman, D. E. Morin, T. B. McFadden, and G. E. Dahl. 2005 Evidence of a role of prolactin in mediating photoperiodic effects during the dry period. J Dairy Sci 88:363.

Cregan, M. D., L. R. Mitoulas, and P. E. Hartmann. 2002. Milk prolactin, feed volume and duration between feeds in women breastfeeding their full-term infants over a 24 h period. Exp Physiol 87:207.

Dahl, G. E. 2005. Let there be light: Photoperiod management of dairy cattle for performance and health in Proc. Florida Dairy Production Gainesville, Fl

Dahl, G. E., B. A. Buchanan, and H. A. Tucker. 2000. Photoperiodic effects on dairy cattle: A review. J Dairy Sci 83:885.

Dahl, G. E., T. H. Elsasser, A. V. Capuco, R. A. Erdman, and R. R. Peters. 1997. Effects of a long daily photoperiod on milk yield and circulating concentrations of insulin-like growth factor-1. J Dairy Sci 80:2784.

Dahl, G. E. and D. Petitclerc. 2003. Management of photoperiod in the dairy herd for improved production and health. J Anim Sci 81 Suppl 3:11.

Demas, G. E., V. Chefer, M. I. Talan, and R. J. Nelson. 1997. Metabolic costs of mounting an antigen-stimulated immune response in adult and aged c57bl/6j mice. Am J Physiol 273:R1631.

Demas, G. E., S. L. Klein, and R. J. Nelson. 1996. Reproductive and immune responses to photoperiod and melatonin are linked in *peromyscus* subspecies. J Comp Physiol A 179:819.

Demas, G. E. and R. J. Nelson. 1998. Photoperiod, ambient temperature, and food availability interact to affect reproductive and immune function in adult male deer mice (*Peromyscus maniculatus*). J Biol Rhythms 13:253.

Devi, Y. S. and J. Halperin. 2014. Reproductive actions of prolactin mediated through short and long receptor isoforms. Mol Cell Endocrinol 382:400.

Diekman, M. A. and T. A. Hoagland. 1983. Influence of supplemental lighting during periods of increasing or decreasing daylength on the onset of puberty in gilts. J Anim Sci 57:1235.

Ebihara, S., T. Marks, D. J. Hudson, and M. Menaker. 1986. Genetic control of melatonin synthesis in the pineal gland of the mouse. Science 231:491.

Eriksson, L., M. Valtonen, J. T. Laitinen, M. Paananen, and M. Kaikkonen. 1998. Diurnal rhythm of melatonin in bovine milk: Pharmacokinetics of exogenous melatonin in lactating cows and goats. Acta Vet Scand 39:301.

Etchegaray, J. P., K. K. Machida, E. Noton, C. M. Constance, R. Dallmann, et al. 2009. Casein kinase 1 delta regulates the pace of the mammalian circadian clock. Mol Cell Biol 29:3853.

Evans, N. M. and R. R. Hacker. 1989. Effect of chronobiological manipulation of lactation in the dairy cow. J Dairy Sci 72:2921.

Finucane, K. A., T. B. McFadden, J. P. Bond, J. J. Kennelly, and F. Q. Zhao. 2008. Onset of lactation in the bovine mammary gland: Gene expression profiling indicates a strong inhibition of gene expression in cell proliferation. Funct Integr Genomics 8:251.

Flores, M. J., J. A. Flores, J. M. Elizundia, A. Mejia, J. A. Delgadillo, and H. Hernandez. 2011. Artificial long-day photoperiod in the subtropics increases milk production in goats giving birth in late autumn. J Anim Sci 89:856.

Foster, D. 1981. Mechanism for delay of first ovulation in lambs born in the wrong season (fall). Biol Reprod 25:85.

Freeman, M. E., B. Kanyicska, A. Lerant, and G. Nagy. 2000. Prolactin: Structure, function, and regulation of secretion. Physiol Rev 80:1523.

Gao, Y., X. Lin, K. Shi, Z. Yan, and Z. Wang. 2013. Bovine mammary gene expression profiling during the onset of lactation. PLoS ONE 8:e70393.

Garcia-Hernandez, R., G. Newton, S. Horner, and L. C. Nuti. 2007. Effect of photoperiod on milk yield and quality, and reproduction in dairy goats. Livest Sci 110:214.

Gatien, M. L., A. K. Hotchkiss, G. N. Neigh, F. S. Dhabhar, and R. J. Nelson. 2004. Immune and stress responses in c57bl/6 and c3h/hen mouse strains following photoperiod manipulation. Neuro Endocrinol Lett 25:267.

Girgert, R., V. Hanf, G. Emons, and C. Grundker. 2010. Signal transduction of the melatonin receptor mt1 is disrupted in breast cancer cells by electromagnetic fields. Bioelectromagnetics 31:237.

Glickman, G., I. C. Webb, J. A. Elliott, R. M. Baltazar, M. E. Reale, M. N. Lehman, and M. R. Gorman. 2012. Photic sensitivity for circadian response to light varies with photoperiod. J Biol Rhythms 27:308.

Goldman, B. D. 1999. The circadian timing system and reproduction in mammals. Steroids 64:679.

Goldman, B. D. 2001. Mammalian photoperiodic system: Formal properties and neuroendocrine mechanisms of photoperiodic time measurement. J Biol Rhythms 16:283.

Golombek, D. A. and R. E. Rosenstein. 2010. Physiology of circadian entrainment. Physiol Rev 90:1063.

Gonzalo, C., J. A. Carriedo, J. D. Gomez, L. D. Gomez, and F. San Primitivo. 1994. Diurnal variation in the somatic cell count of ewe milk. J Dairy Sci 77:1856.

Goto, M., I. Oshima, T. Tomita, and S. Ebihara. 1989. Melatonin content of the pineal gland in different mouse strains. J Pineal Res 7:195.

Grigor, M. R. and M. P. Thompson. 1987. Diurnal regulation of milk lipid production and milk secretion in the rat: Effect of dietary protein and energy restriction. J Nutr 117:748.

Hansen, P. J., L. A. Kamwanja, and E. R. Hauser. 1983. Photoperiod influences age at puberty of heifers. J Anim Sci 57:985.

Hastings, M. H., J. Herbert, N. D. Martensz, and A. C. Roberts. 1985. Annual reproductive rhythms in mammals: Mechanisms of light synchronization. Ann NY Acad Sci 453:182.

Hennighausen, L. and G. W. Robinson. 2005. Information networks in the mammary gland. Nat Rev Mol Cell Biol 6:715.

Hill, S. M., T. Frasch, S. Xiang, L. Yuan, T. Duplessis, and L. Mao. 2009. Molecular mechanisms of melatonin anticancer effects. Integr Cancer Ther 8:337.

Hurley, W. L. and J. J. Loor. 2011. Mammary gland: Growth, development and involution. Pages 338 in Encyclopedia of dairy sciences (second edition). J. W. Fuquay, ed. Academic Press, San Diego.

Hut, R. A. and D. G. Beersma. 2011. Evolution of time-keeping mechanisms: Early emergence and adaptation to photoperiod. Philos Trans R Soc Lond B Biol Sci 366:2141.

Jackson, C. R., M. Capozzi, H. Dai, and D. G. McMahon. 2014. Circadian perinatal photoperiod has enduring effects on retinal dopamine and visual function. J Neurosci 34:4627.

Johnston, J. D. 2004. Photoperiodic regulation of prolactin secretion: Changes in intra-pituitary signalling and lactotroph heterogeneity. J Endocrinol 180:351.

Lacasse, P., C. M. Vinet, and D. Petitclerc. 2014. Effect of prepartum photoperiod and melatonin feeding on milk production and prolactin concentration in dairy heifers and cows. J Dairy Sci 97:3589.

Lang, R., H. Hintner, A. Hermann, and R. Brandstaetter. 2003. Photoperiod modulates melanoma growth in c57bl/6 mice. Exp Dermatol 12:510.

Lathrop, A. E. and L. Loeb. 1916. Further investigations on the origin of tumors in mice. III. On the part played by internal secretion in the spontaneous development of tumors. J Cancer Res 1:1.

Lawson, T. J., and A.D. Kennedy. 2001. Inhibition of nighttime melatonin secretion in cattle: Threshold light intensity for dairy heifers. Can. J. Anim. Sci. 81:153.

Lee, T. M., L. Smale, I. Zucker, and J. Dark. 1987. Role of photoperiod during pregnancy and lactation in the meadow vole, *Microtus pennsylvanicus*. J Reprod Fertil 81:343.

Leining, K. B., R. A. Bourne, and H. A. Tucker. 1979. Prolactin response to duration and wavelength of light in prepubertal bulls. Endocrinology 104:289.

Lemay, D. G., M. C. Neville, M. C. Rudolph, K. S. Pollard, and J. B. German. 2007. Gene regulatory networks in lactation: Identification of global principles using bioinformatics. BMC Syst Biol 1:56.

Leonardi, M. O. and A. E. Klempau. 2003. Artificial photoperiod influence on the immune system of juvenile rainbow trout (*Oncorhynchus mykiss*) in the southern hemisphere. Aquaculture 221:581.

Lewy, A. J., T. A. Wehr, F. K. Goodwin, D. A. Newsome, and S. P. Markey. 1980. Light suppresses melatonin secretion in humans. Science 210:1267.

Lincoln, G. A., H. Andersson, and A. Loudon. 2003. Clock genes in calendar cells as the basis of annual timekeeping in mammals--a unifying hypothesis. J Endocrinol 179:1.

Lincoln, G. A. and W. Davidson. 1977. The relationship between sexual and aggressive behaviour, and pituitary and testicular activity during the seasonal sexual cycle of rams, and the influence of photoperiod. Reproduction 49:267.

Littlejohn, M. D., C. G. Walker, H. E. Ward, K. B. Lehnert, R. G. Snell, G. A. Verkerk, R. J. Spelman, D. A. Clark, and S. R. Davis. 2010. Effects of reduced frequency of milk removal on gene expression in the bovine mammary gland. Physiol Genomics 41:21.

Lubetzky, R., Y. Littner, F. B. Mimouni, S. Dollberg, and D. Mandel. 2006. Circadian variations in fat content of expressed breast milk from mothers of preterm infants. J Am Coll Nutr 25:151.

Mabjeesh, S. J., C. Sabastian, O. Gal-Garber, and A. Shamay. 2013. Effect of photoperiod and heat stress in the third trimester of gestation on milk production and circulating hormones in dairy goats. J Dairy Sci 96:189.

Maestroni, G. J., A. Conti, and W. Pierpaoli. 1987. Role of the pineal gland in immunity: Ii. Melatonin enhances the antibody response via an opiatergic mechanism. Clin Exp Immunol 68:384.

Malpaux, B., J. C. Thiery, and P. Chemineau. 1999. Melatonin and the seasonal control of reproduction. Reprod Nutr Dev 39:355.

Marcek, J. M. and L. V. Swanson. 1984. Effect of photoperiod on milk production and prolactin of holstein dairy cows. J Dairy Sci 67:2380.

Marshman, E. and C. H. Streuli. 2002. Insulin-like growth factors and insulin-like growth factor binding proteins in mammary gland function. Breast Cancer Res 4:231.

Martin, L. B., Z. M. Weil, and R. J. Nelson. 2008. Seasonal changes in vertebrate immune activity: Mediation by physiological trade-offs. Philos Trans R Soc Lond B Biol Sci 363:321.

Mediavilla, M. D., A. Guezmez, S. Ramos, L. Kothari, F. Garijo, and E. J. Sanchez Barcelo. 1997. Effects of melatonin on mammary gland lesions in transgenic mice overexpressing n-ras proto-oncogene. J Pineal Res 22:86.

Mediavilla, M. D., M. San Martin, and E. J. Sanchez-Barcelo. 1992. Melatonin inhibits mammary gland development in female mice. J Pineal Res 13:13.

Metz, R. P., X. Qu, B. Laffin, D. Earnest, and W. W. Porter. 2006. Circadian clock and cell cycle gene expression in mouse mammary epithelial cells and in the developing mouse mammary gland. Dev Dyn 235:263.

Mikolayunas, C. M., D. L. Thomas, G. E. Dahl, T. F. Gressley, and Y. M. Berger. 2008. Effect of prepartum photoperiod on milk production and prolactin concentration of dairy ewes. J Dairy Sci 91:85.

Miller, A. R., R. A. Erdman, L. W. Douglass, and G. E. Dahl. 2000. Effects of photoperiodic manipulation during the dry period of dairy cows. J Dairy Sci 83:962.

Mizuno, H. and N. Sensui. 1970. Lack of effects of melatonin administration and pinealectomy on the milk ejection response in the rat. Endocrinol Jpn 17:417.

Mohawk, J. A., C. B. Green, and J. S. Takahashi. 2012. Central and peripheral circadian clocks in mammals. Annu Rev Neurosci 35:445.

Nelson, R. J. 1990. Photoperiodic responsiveness in house mice. Physiol Behav 48:403.

Nelson, R. J. and J. M. Blom. 1994. Photoperiodic effects on tumor development and immune function. J Biol Rhythms 9:233.

Nelson, R. J., G. E. Demas, S. L. Klein, and L. J. Kriegsfeld. 1995. The influence of season, photoperiod, and pineal melatonin on immune function. J Pineal Res 19:149.

Nelson, R. J. and J. R. Shiber. 1990. Photoperiod affects reproductive responsiveness to 6-methoxy-2-benzoxazolinone in house mice. Biol Reprod 43:586.

Newbold, J. A., L. T. Chapin, S. A. Zinn, and H. A. Tucker. 1991. Effects of photoperiod on mammary development and concentration of hormones in serum of pregnant dairy heifers. J Dairy Sci 74:100.

Nir, I., J. Mishkinsky, N. Eshchar, and F. G. Sulman. 1968. Effect of pinealectomy on milk yield in rats. J. Endocrinol. 42:161.

Oishi, K., H. Fukui, K. Sakamoto, K. Miyazaki, H. Kobayashi, and N. Ishida. 2002. Differential expressions of mper1 and mper2 mrnas under a skeleton photoperiod and a complete light-dark cycle. Brain Res Mol Brain Res 109:11.

Ono, H., Y. Hoshino, S. Yasuo, M. Watanabe, Y. Nakane, A. Murai, S. Ebihara, H. W. Korf, and T. Yoshimura. 2008. Involvement of thyrotropin in photoperiodic signal transduction in mice. Proc Natl Acad Sci U S A 105:18238.

Ono, H., N. Nakao, and T. Yoshimura. 2009. Identification of the photoperiodic signaling pathway regulating seasonal reproduction using the functional genomics approach. Gen Comp Endocrinol 163:2.

Otsuka, T., M. Kawai, Y. Togo, R. Goda, T. Kawase, et al. 2014. Photoperiodic responses of depression-like behavior, the brain serotonergic system, and peripheral metabolism in laboratory mice. Psychoneuroendocrinology 40:37.

Paape, M. J., D. W. Carroll, A. J. Kral, R. H. Miller, and C. Desjardins. 1974. Corticosteroids, circulating leukocytes, and erythrocytes in cattle: Diurnal changes and effects of bacteriologic status, stage of lactation, and milk yield on response to adrenocorticotropin. Am J Vet Res 35:355.

Palmer, E. and M. A. Driancourt. 1983. Some interactions of season of foaling, photoperiod and ovarian activity in the equine. Livest Prod Sci 10:197.

Park, Y. H., Y. S. Joo, J. Y. Park, J. S. Moon, S. H. Kim, N. H. Kwon, J. S. Ahn, W. C. Davis, and C. J. Davies. 2004. Characterization of lymphocyte subpopulations and major histocompatibility complex haplotypes of mastitis-resistant and susceptible cows. J Vet Sci 5:29.

Pendergast, J. S., R. C. Friday, and S. Yamazaki. 2010. Distinct functions of period2 and period3 in the mouse circadian system revealed by in vitro analysis. PLoS ONE 5:e8552.

Pennycuik, P. R. 1972. Seasonal changes in reproductive productivity, growth rate, and food intake in mice exposed to different regimes of day length and environmental temperature. Aust J Biol Sci 25:627.

Peters, R. R., L. T. Chapin, R. S. Emery, and H. A. Tucker. 1980. Growth and hormonal response of heifers to various photoperiods. J Anim Sci 51:1148.

Peters, R. R., L. T. Chapin, R. S. Emery, and H. A. Tucker. 1981. Milk yield, feed intake, prolactin, growth hormone, and glucocorticoid response of cows to supplemented light. J Dairy Sci 64:1671.

Peters, R. R., L. T. Chapin, K. B. Leining, and H. A. Tucker. 1978. Supplemental lighting stimulates growth and lactation in cattle. Science 199:911.

Petitclerc, D., L. T. Chapin, and H. A. Tucker. 1984. Carcass composition and mammary development responses to photoperiod and plane of nutrition in holstein heifers. J Anim Sci 58:913.

Petitclerc, D., R. D. Kineman, S. A. Zinn, and H. A. Tucker. 1985. Mammary growth response of holstein heifers to photoperiod. J Dairy Sci 68:86.

Petitclerc, D., C. M. Vinet, G. Roy, and P. Lacasse. 1998. Prepartum photoperiod and melatonin feeding on milk production and prolactin concentrations of dairy heifers and cows. J. Dairy Sci. 81:251.

Phillips, C. J. C. and S. A. Schofield. 1989. The effect of supplementary light on the production and behaviour of dairy cows. Animal Science 48:293.

Pittendrigh, C. S. 1964. The entrainment of circadian oscillations by skeleton photoperiods. Science 144:565.

Pittendrigh, C. S. and S. Daan. 1976. Functional-analysis of circadian pacemakers in nocturnal rodents .4. Entrainment - pacemaker as clock. J Comp Physiol 106:291.

Plaut, K., D. E. Bauman, N. Agergaard, and R. M. Akers. 1987. Effect of exogenous prolactin administration on lactational performance of dairy cows. Domest Anim Endocrinol 4:279.

Proietti, S., A. Cucina, R. J. Reiter, and M. Bizzarri. 2013. Molecular mechanisms of melatonin's inhibitory actions on breast cancers. Cell Mol Life Sci 70:2139.

Prosser, C. G., I. R. Fleet, A. N. Corps, E. R. Froesch, and R. B. Heap. 1990. Increase in milk secretion and mammary blood flow by intra-arterial infusion of insulin-like growth factor-i into the mammary gland of the goat. J Endocrinol 126:437.

Pyter, L. M., A. K. Hotchkiss, and R. J. Nelson. 2005. Photoperiod-induced differential expression of angiogenesis genes in testes of adult *Peromyscus leucopus*. Reproduction 129:201.

Ramanathan, P., I. Martin, P. Thomson, R. Taylor, C. Moran, and P. Williamson. 2007. Genomewide analysis of secretory activation in mouse models. J Mammary Gland Biol Neoplasia 12:305.

Ramanathan, P., I. C. Martin, M. Gardiner-Garden, P. C. Thomson, R. M. Taylor, C. J. Ormandy, C. Moran, and P. Williamson. 2008. Transcriptome analysis identifies pathways associated with enhanced maternal performance in qsi5 mice. BMC Genomics 9:197.

Rao, G. N., E. Ney, and R. A. Herbert. 2000. Effect of melatonin and linolenic acid on mammary cancer in transgenic mice with c-neu breast cancer oncogene. Breast Cancer Res Treat 64:287.

Recio, J., M. D. Mediavilla, D. P. Cardinali, and E. J. Sanchez-Barcelo. 1994. Pharmacological profile and diurnal rhythmicity of 2-[125i]-iodomelatonin binding sites in murine mammary tissue. J Pineal Res 16:10.

Reddy, A. B., G. K. Wong, J. O'Neill, E. S. Maywood, and M. H. Hastings. 2005. Circadian clocks: Neural and peripheral pacemakers that impact upon the cell division cycle. Mutat Res 574:76.

Reid, E. D., T. L. Auchtung, D. E. Morin, T. B. McFadden, and G. E. Dahl. 2004. Effects of 21-day short day photoperiod (sdpp) during the dry period on dry matter intake and subsequent milk production in dairy cows. J Dairy Sci 87:424.

Reiter, R. J. 1991. Melatonin: The chemical expression of darkness. Mol Cell Endocrinol 79:C153.

Reksen, O., A. Tverdal, K. Landsverk, E. Kommisrud, K. E. Boe, and E. Ropstad. 1999. Effects of photointensity and photoperiod on milk yield and reproductive performance of norwegian red cattle. J Dairy Sci 82:810.

Reppert, S. M. and D. R. Weaver. 2001. Molecular analysis of mammalian circadian rhythms. Annu Rev Physiol 63:647.

Rius, A. G. and G. E. Dahl. 2006. Exposure to long-day photoperiod prepubertally may increase milk yield in first-lactation cows. J Dairy Sci 89:2080.

Roenneberg, T. and M. Merrow. 2005. Circadian clocks - the fall and rise of physiology. Nat Rev Mol Cell Biol 6:965.

Ross, D. T., U. Scherf, M. B. Eisen, C. M. Perou, C. Rees, et al. 2000. Systematic variation in gene expression patterns in human cancer cell lines. Nat Genet 24:227.

Rusak, B. and I. Zucker. 1975. Biological rhythms and animal behavior. Annu Rev Psychol 26:137.

Sanchez-Barcelo, E. J., S. Cos, R. Fernandez, and M. D. Mediavilla. 2003. Melatonin and mammary cancer: A short review. Endocr Relat Cancer 10:153.

Sanchez-Barcelo, E. J., M. D. Mediavilla, and H. A. Tucker. 1990. Influence of melatonin on mammary gland growth: In vivo and in vitro studies. Proc Soc Exp Biol Med 194:103.

Sanchez-Barcelo, E. J., M. D. Mediavilla, S. A. Zinn, B. A. Buchanan, L. T. Chapin, and H. A. Tucker. 1991. Melatonin suppression of mammary growth in heifers. Biol Reprod 44:875.

Schanbacher, B. D. 1979. Increased lamb production with rams exposed to short daylengths during the nonbreeding season. J Anim Sci 49:927.

Schernhammer, E. S., F. Laden, F. E. Speizer, W. C. Willett, D. J. Hunter, I. Kawachi, and G. A. Colditz. 2001. Rotating night shifts and risk of breast cancer in women participating in the nurses' health study. J Natl Cancer Inst 93:1563.

Smith, J. D., L. W. Douglas, J. A. Coyne, and G. E. Dahl. 1997. Melatonin feeding that simulates a short day photoperiod (sdpp) suppresses circulating insuline-like growth factor-1 (igf-1) in pre-pubertal heifers J. Anim. Sci 75:215.

Smith, J. W. and W. D. Schultze. 1967. Variation in cell content of milk associated with time of sample collection. I. Diurnal variation. J Dairy Sci 50:1083.

Sorensen, M. T. and R. R. Hacker. 1979. Negative effect of bovine pineal gland extract and positive effect of supplemental lighting on lactation in mice. J Anim Sci 49:1270.

Sosniyenko, S., R. A. Hut, S. Daan, and A. Sumova. 2009. Influence of photoperiod duration and light-dark transitions on entrainment of per1 and per2 gene and protein expression in subdivisions of the mouse suprachiasmatic nucleus. Eur J Neurosci 30:1802.

Spicer, L. J., B. A. Buchanan, L. T. Chapin, and H. A. Tucker. 2007. Effect of exposure to various durations of light on serum insulin-like growth factor-i in prepubertal holstein heifers. Am J Anim Vet Sci 42.

Stanisiewski, E. P., L. T. Chapin, N. K. Ames, S. A. Zinn, and H. A. Tucker. 1988. Melatonin and prolactin concentrations in blood of cattle exposed to 8, 16 or 24 hours of daily light. J Anim Sci 66:727.

Stanisiewski, E. P., R. W. Mellenberger, C. R. Anderson, and H. A. Tucker. 1985. Effect of photoperiod on milk yield and milk fat in commercial dairy herds. J Dairy Sci 68:1134.

Steinman, M. Q., J. A. Knight, and B. C. Trainor. 2012. Effects of photoperiod and food restriction on the reproductive physiology of female california mice. Gen Comp Endocrinol 176:391.

Stephan, F. K. 2002. The "other" circadian system: Food as a zeitgeber. J Biol Rhythms 17:284.

Stiening, C. M., J. B. Hoying, M. B. Abdallah, A. M. Hoying, R. Pandey, K. Greer, and R. J. Collier. 2008. The effects of endocrine and mechanical stimulation on stage i lactogenesis in bovine mammary epithelial cells. J Dairy Sci 91:1053.

Subramanian, A. and L. Kothari. 1991. Melatonin, a suppressor of spontaneous murine mammary tumors. J Pineal Res 10:136.

Summa, K. C., M. H. Vitaterna, and F. W. Turek. 2012. Environmental perturbation of the circadian clock disrupts pregnancy in the mouse. PLoS ONE 7:e37668.

Sumova, A., M. Jac, M. Sladek, I. Sauman, and H. Illnerova. 2003. Clock gene daily profiles and their phase relationship in the rat suprachiasmatic nucleus are affected by photoperiod. J Biol Rhythms 18:134.

Swanson, K. M., K. Stelwagen, J. Dobson, H. V. Henderson, S. R. Davis, V. C. Farr, and K. Singh. 2009. Transcriptome profiling of *Streptococcus uberis*-induced mastitis reveals fundamental differences between immune gene expression in the mammary gland and in a primary cell culture model. J Dairy Sci 92:117.

Takahashi, J. S. 1992. Circadian clock genes are ticking. Science 258:238.

Tamarkin, L., C. J. Baird, and O. F. Almeida. 1985. Melatonin: A coordinating signal for mammalian reproduction? Science 227:714.

Thimonier, J. 1981. Control of seasonal reproduction in sheep and goats by light and hormones. J Reprod Fertil Suppl 30:33.

Tokumaru, O., K. Haruki, K. Bacal, T. Katagiri, T. Yamamoto, and Y. Sakurai. 2006. Incidence of cancer among female flight attendants: A meta-analysis. J Travel Med 13:127.

Tonner, E., L. Quarrie, M. Travers, M. Barber, A. Logan, C. Wilde, and D. Flint. 1995. Does an igf-binding protein (igfbp) present in involuting rat mammary gland regulate apoptosis? Prog Growth Factor Res 6:409.

Tournier, B. B., J. S. Menet, H. Dardente, V. J. Poirel, A. Malan, M. Masson-Pevet, P. Pevet, and P. Vuillez. 2003. Photoperiod differentially regulates clock genes' expression in the suprachiasmatic nucleus of syrian hamster. Neuroscience 118:317.

Tucker, H. A. 1981. Physiological control of mammary growth, lactogenesis, and lactation. J Dairy Sci 64:1403.

Tucker, H. A. and R. K. Ringer. 1982. Controlled photoperiodic environments for food animals. Science 216:1381.

Velasco, J. M., E. D. Reid, K. K. Fried, T. F. Gressley, R. L. Wallace, and G. E. Dahl. 2008. Short-day photoperiod increases milk yield in cows with a reduced dry period length. J Dairy Sci 91:3467.

Vollrath, L., A. Huesgen, B. Manz, and K. Pollow. 1988. Day/night serotonin levels in the pineal gland of male balb/c mice with melatonin deficiency. Acta Endocrinol (Copenh) 117:93.

von Gall, C., A. Lewy, C. Schomerus, B. Vivien-Roels, P. Pevet, H. W. Korf, and J. H. Stehle. 2000. Transcription factor dynamics and neuroendocrine signalling in the mouse pineal gland: A comparative analysis of melatonin-deficient c57bl mice and melatonin-proficient c3h mice. Eur J Neurosci 12:964.

Vriend, J. and J. K. Lauber. 1973. Letter: Effects of light intensity, wavelength and quanta on gonads and spleen of the deer mouse. Nature 244:37.

Wall, E. H., T. L. Auchtung-Montgomery, G. E. Dahl, and T. B. McFadden. 2005a. Short communication: Short-day photoperiod during the dry period decreases expression of suppressors of cytokine signaling in mammary gland of dairy cows. J Dairy Sci 88:3145.

Wall, E. H., T. L. Auchtung, G. E. Dahl, S. E. Ellis, and T. B. McFadden. 2005b. Exposure to short day photoperiod during the dry period enhances mammary growth in dairy cows. J Dairy Sci 88:1994.

Wall, E. H., J. P. Bond, and T. B. McFadden. 2012. Acute milk yield response to frequent milking during early lactation is mediated by genes transiently regulated by milk removal. Physiol Genomics 44:25.

Walton, J. C., Z. M. Weil, and R. J. Nelson. 2011. Influence of photoperiod on hormones, behavior, and immune function. Front Neuroendocrinol 32:303.

Wehr, T. A., D. E. Moul, G. Barbato, H. A. Giesen, J. A. Seidel, C. Barker, and C. Bender. 1993. Conservation of photoperiod-responsive mechanisms in humans. Am J Physiol 265:R846.

Wei, J., P. Ramanathan, I. C. Martin, C. Moran, R. M. Taylor, and P. Williamson. 2013. Identification of gene sets and pathways associated with lactation performance in mice. Physiol Genomics 45:171.

White, F. and E. A. Rattray. 1965. Diurnal variation in the cell content of cows' milk. J Comp Pathol 75:253.

Whitsett, J. M. and A. D. Lawton. 1982. Social stimulation of reproductive development in male deer mice housed on a short-day photoperiod. J Comp Physiol Psychol 96:416.

Whitsett, J. M. and L. L. Miller. 1982. Photoperiod and reproduction in female deer mice. Biol Reprod 26:296.

Woodfill, C. J., N. L. Wayne, S. M. Moenter, and F. J. Karsch. 1994. Photoperiodic synchronization of a circannual reproductive rhythm in sheep: Identification of season-specific time cues. Biol Reprod 50:965.

Xiang, S., L. Mao, L. Yuan, T. Duplessis, F. Jones, et al. 2012. Impaired mouse mammary gland growth and development is mediated by melatonin and its mt1 g protein-coupled receptor via repression of erα, akt1, and stat5. J Pineal Res 53:307.

Yasuo, S., T. Yoshimura, S. Ebihara, and H. W. Korf. 2009. Melatonin transmits photoperiodic signals through the mt1 melatonin receptor. J Neurosci 29:2885.

Yellon, S. M. and L. T. Tran. 2002. Photoperiod, reproduction, and immunity in select strains of inbred mice. J Biol Rhythms 17:65.

Yoshimura, T. 2013. Thyroid hormone and seasonal regulation of reproduction. Front Neuroendocrinol 34:157.

Young, M. W. and S. A. Kay. 2001. Time zones: A comparative genetics of circadian clocks. Nat Rev Genet 2:702.

CHAPTER 2: OBJECTIVES, STUDY DESIGN AND HYPOTHESES

OVERALL OBJECTIVES, DESIGN AND HYPOTHESIS

Overall objectives

1. Quantify the effects of photoperiod on the mammary transcriptome of cows
2. Establish whether there are common effects in the mammary gland in response to photoperiod manipulation in cows and mice
3. Quantify the effects of photoperiod manipulation on the mammary transcriptome in mice
4. Determine if physiological state (gestation or lactation) influences the effects of photoperiod on the mammary transcriptome
5. Assess common biology between the cow and mouse models

Overall design

There are 4 component studies described in this work and summarized in **Figure 2.1**.

1. LD compared to SD photoperiod exposure during the dry period in dairy cows.
2. LD_{ND} and SD_{ND} photoperiod exposure during lactation in mice
3. LD_{ND} and SD_{ND} photoperiod exposure during gestation in mice – concurrent effects
4. LD_{ND} and SD_{ND} photoperiod exposure during gestation in mice – carryover effects

Overall study design

Four studies were undertaken to elucidate the effects of photoperiod on the mammary gland. The effect of photoperiod on milk production in dairy cows (grey) has previously been established (**Figure 2.1**)

Overall hypothesis

Exposure to short day photoperiod treatment during gestation and long day photoperiod during lactation will affect transcription of genes in the mammary gland that support lactation.

PHOTOPERIOD EXPOSURE DURING THE DRY PERIOD IN DAIRY COWS

Objectives

1. Identify genes differentially expressed between SD and LD photoperiod
2. Identify genes differentially expressed between -24 and -9 days pre-partum
3. Determine associated *signaling pathways*, *biofunction*s and *upstream regulators* with genes identified
4. Draw functional associations to explain how their differential expression may influence subsequent milk production

Study design

See **Figure 2.2**.

Hypotheses

1. **Photoperiod manipulation during the dry period affects genes associated with lactation performance. More specifically:**
    a. Short day photoperiod regulates the expression of genes that promote cell proliferation.
    b. Short day photoperiod regulates genes associated with mammary health and immune function.
2. **Differential expression of genes between day -24 and -9 relative to parturition reflect the physiological change in the mammary gland between stage I and stage II lactogenesis. More specifically:**
    a. Genes differentially expressed on -9 are associated with initiation of milk synthesis, whereas on day -24 they are not.

3. **Genes identified in the effect of time will be different from those identified in the effect of photoperiod.**

PHOTOPERIOD EXPOSURE DURING LACTATION IN MICE

Objectives

1. Determine if photoperiod affects litter weight gain in mice
2. Quantify changes in mammary cell proliferation in response to photoperiod
3. Identify photoperiod-responsive genes in the mouse mammary gland
4. Associate differentially expressed genes with mammary function

Study design

See **Figure 2.3.**

Hypotheses

It was hypothesized that:

1) **Photoperiod manipulation during lactation will alter milk production as measured by litter weight gain. More specifically:**

   a) LD photoperiod will increase milk production relative to SD photoperiod

   b) ND photoperiod will increase milk production relative to SD photoperiod

2) **Photoperiod manipulation during lactation will affect the proliferation of mammary cells as measured by BrdU incorporation. More specifically:**

   a) LD photoperiod will increase cell proliferation relative to SD photoperiod

   b) ND photoperiod will increase cell proliferation relative to SD photoperiod

3) **Photoperiod manipulation during lactation will affect mouse body and organ weights. More specifically:**

a) Body weight

i) Mice exposed to LD photoperiod will have higher body weights than mice on SD photoperiod

ii) Mice exposed to ND photoperiod will have higher body weights than mice on SD photoperiod

b) Spleen weight

i) Mice exposed to SD photoperiod will have higher spleen weight than mice on LD photoperiod

ii) Mice exposed to ND photoperiod will have higher spleen weight than mice on LD photoperiod

c) Thymus weight

i) Mice exposed to SD photoperiod will have higher thymus weight than mice on LD photoperiod

ii) Mice exposed to ND photoperiod will have higher thymus weight than mice on LD photoperiod

d) Liver weight

i) Mice exposed to LD photoperiod will have higher liver weight than mice on SD photoperiod

ii) Mice exposed to ND photoperiod will have higher liver weight than mice on SD photoperiod

4) Photoperiod manipulation during lactation will affect expression of genes in the mammary transcriptome. More specifically:

a) The comparison of LD_{ND} will identify differential expression of genes associated with increased lactation performance

b) The comparison of SD_{ND} will identify differential expression of genes associated with cell proliferation

c) The comparisons of LD_{ND} and SD_{ND} will identify different sets of genes.

PHOTOPERIOD EXPOSURE DURING GESTATION IN MICE – CONCURRENT EFFECTS

Objectives

1. Quantify changes in cellular proliferation in response to photoperiod
2. Identify photoperiod-responsive genes in the mouse mammary gland
3. Associate differentially expressed genes with mammary function

Study design

See **Figure 2.4.**

Hypotheses

1) Photoperiod manipulation during gestation will affect the proliferation of mammary cells as measured by BrdU incorporation. More specifically:

a) The comparison of SD_{ND} photoperiod will identify differential expression of genes associated with cell proliferation, relative to the comparison of LD_{ND}

2) Photoperiod manipulation during gestation will affect mouse body weight and organ weights. More specifically:

a) Body weight

i) Mice exposed to SD photoperiod will have higher body weights than mice exposed to LD photoperiod

ii) Mice exposed to ND photoperiod will have higher body weights than mice exposed to LD photoperiod

b) Spleen weight

i) Mice exposed to SD photoperiod will have higher spleen mass than mice on LD photoperiod

ii) Mice exposed to SD photoperiod will have higher spleen mass than mice on ND photoperiod

c) Thymus weight

i) Mice exposed to SD photoperiod will have higher thymus weights than mice exposed to LD photoperiod

ii) Mice exposed to SD photoperiod will have higher thymus weights than mice exposed to ND photoperiod

d) Liver weight

i) Mice exposed to LD photoperiod will have heavier livers than mice on SD photoperiod

ii) Mice exposed to LD photoperiod will have heavier livers than mice on ND photoperiod

e) Pups *in utero*

i) Mice exposed to LD photoperiod will have heavier pups *in utero* than mice on SD photoperiod

ii) Mice exposed to LD photoperiod will have heavier pups *in utero* than mice on ND photoperiod

3) Photoperiod manipulation during gestation will affect expression of genes in the mammary transcriptome. More specifically:

a) Genes identified in the comparison of SD_{ND} photoperiod will be associated with increased lactation performance, compared to genes identified in the comparison of LD_{ND} photoperiod

b) Genes identified in the comparisons of LD_{ND} and SD_{ND} will identify different sets of genes

PHOTOPERIOD EXPOSURE DURING GESTATION IN MICE – CARRYOVER EFFECTS

Objectives

1. Identify photoperiod-programmed genes differentially expressed on L10 in the mouse mammary gland
2. Associate differentially expressed genes with mammary function

Study design

See **Figure 2.5**.

Hypotheses

It was hypothesized that:

1) **Photoperiod manipulation during gestation will have carry over effects on litter weight gain during lactation More specifically:**
    - **a.** SD photoperiod during gestation will increase litter weight gain relative to LD photoperiod.
    - **b.** ND photoperiod during gestation will increase litter weight gain relative to LD photoperiod.
2) **Photoperiod manipulation during gestation will affect pregnancy outcomes. More specifically:**
    - **a. Litter weight**
        - i. SD photoperiod during gestation will decrease the weight of litters at time of birth relative to LD photoperiod.

ii. SD photoperiod during gestation will decrease the weight of litters at time of birth relative to ND photoperiod.

b. Pup numbers

i. Mice exposed to SD photoperiod during gestation will have fewer pups than mice on LD photoperiod.

ii. Mice exposed to ND photoperiod during gestation will have fewer pups than mice on LD photoperiod.

3) Photoperiod manipulation during gestation will not have a carryover effect on the mammary transcriptome on L10. More specifically:

a. No genes will be differentially expressed in the comparison of SD_{ND}

b. No genes will be differentially expressed in the comparison of LD_{ND}

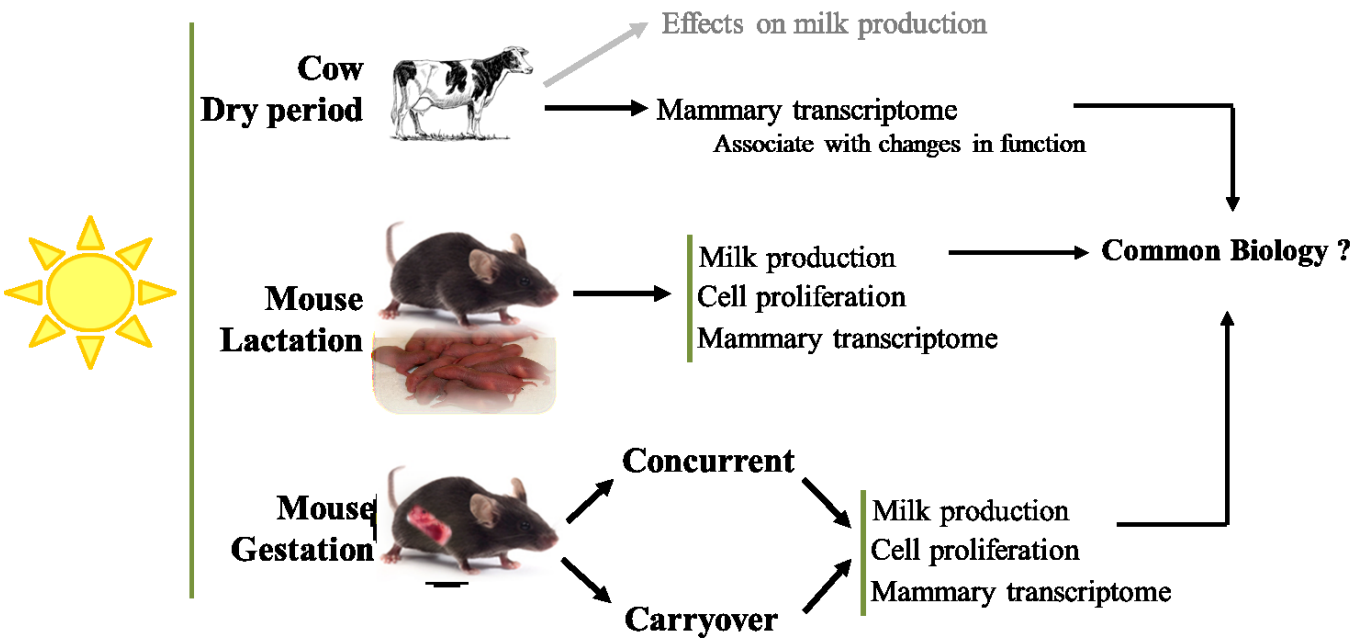


Figure 2.1. Overall study design.

Four studies were undertaken to elucidate the effects of photoperiod on the mammary gland. The effect of photoperiod on milk production in dairy cows (grey) has previously been established.

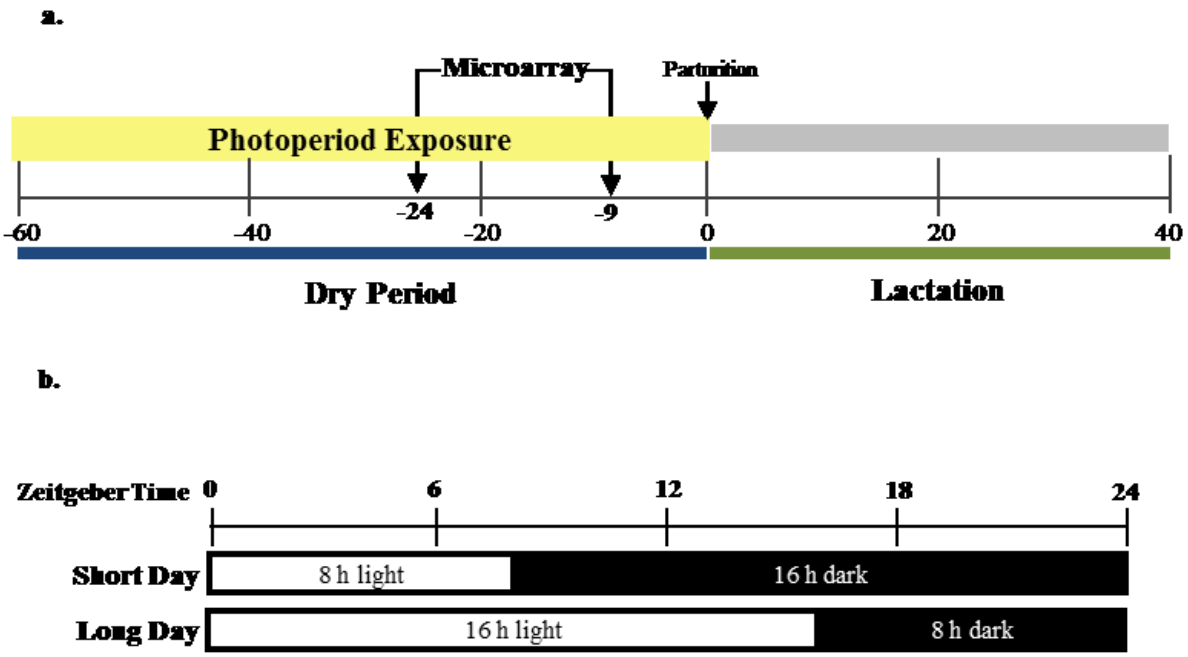


Figure 2.2. Manipulation of photoperiod during the dry period in dairy cows.

a. Study design, **b.** photoperiod treatments

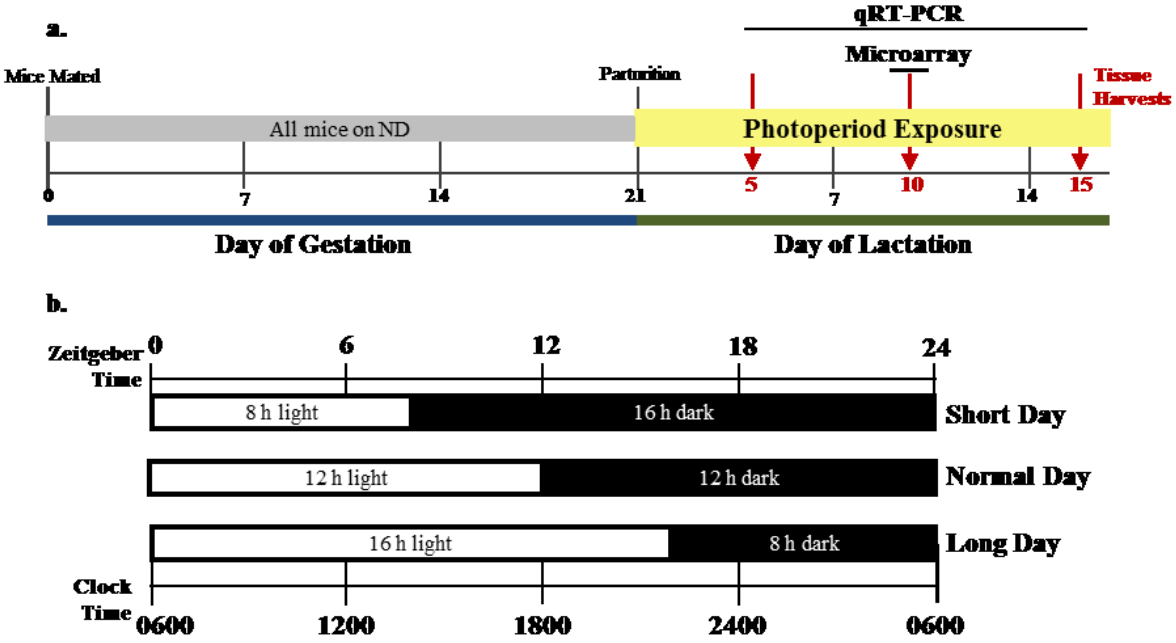


Figure 2.3. Manipulation of photoperiod during lactation in mice.

a. Study design, **b**. Photoperiod treatments

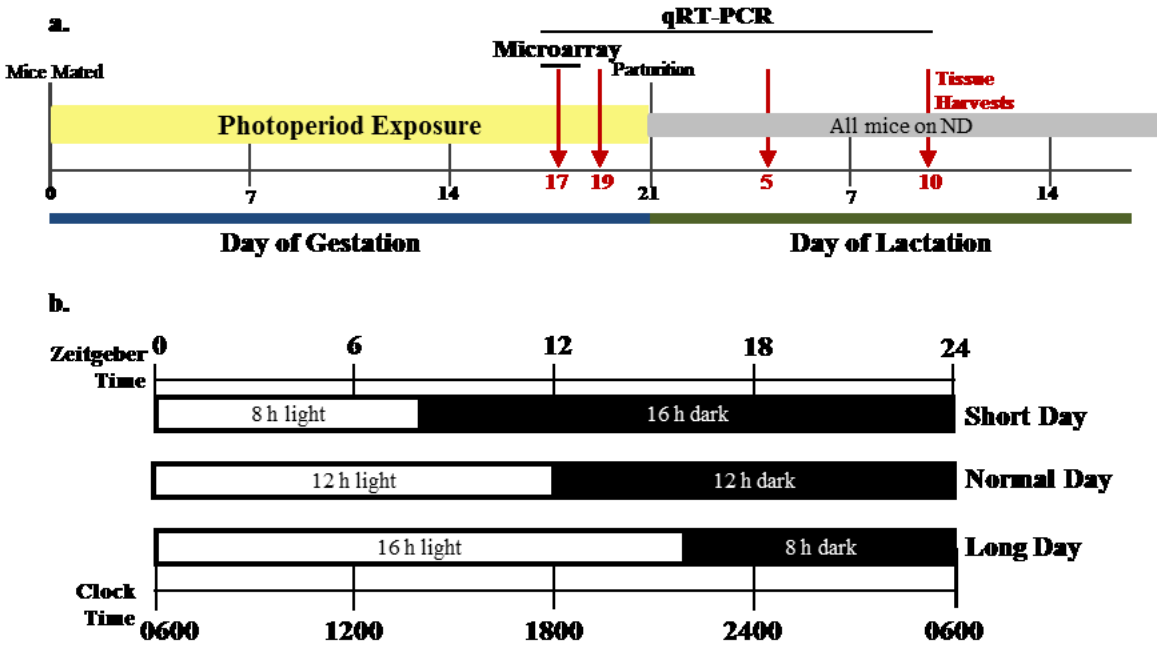


Figure 2.4. Manipulation of photoperiod during gestation in mice – concurrent effects.

a. Study design, **b.** photoperiod treatments

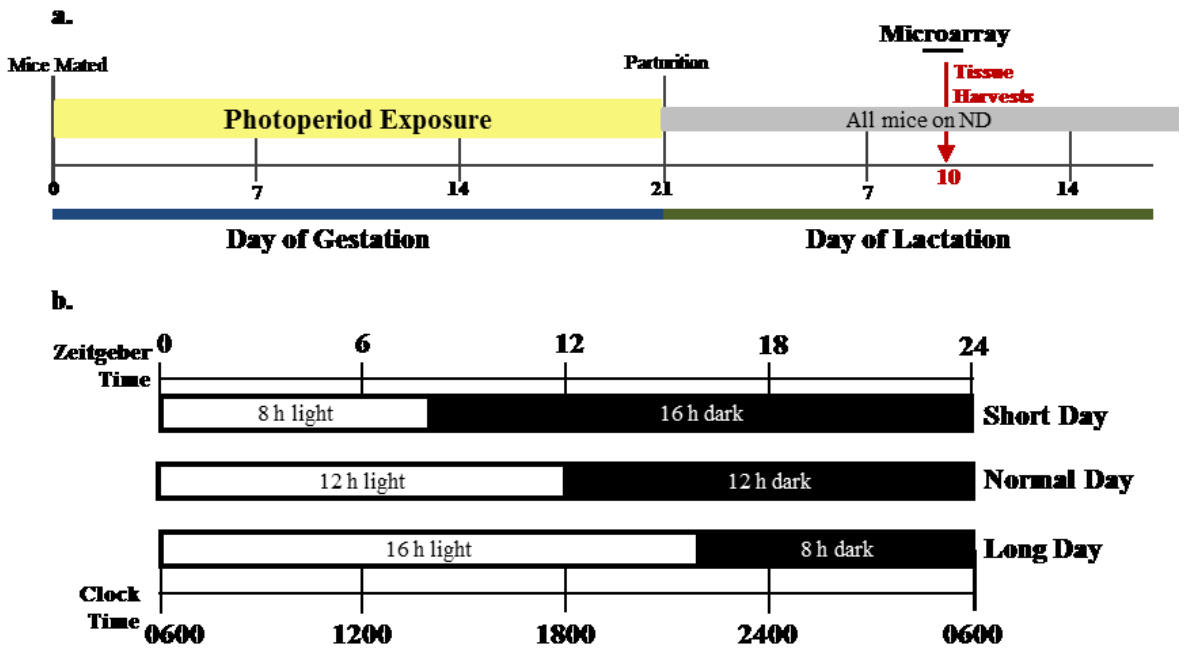


Figure 2.5. Manipulation of photoperiod during gestation in mice – carryover effects.

a. Study design, **b.** photoperiod treatments

CHAPTER 3: RESPONSES OF THE MAMMARY TRANSCRIPTOME OF DAIRY COWS TO ALTERED PHOTOPERIOD DURING LATE GESTATION[1]

[1] A version of this chapter has been submitted: Bentley, P. A., E. H. Wall, G. E. Dahl, and T. B. McFadden. 2014. Responses of the mammary transcriptome of dairy cows to altered photoperiod during late gestation. Physiol Genomics Submitted: PG-00112-2014.

ABSTRACT

Cows exposed to short day photoperiod (SD, 8 h light:16 h dark) during the 60-day non-lactating period prior to parturition produce more milk in their subsequent lactation compared to cows exposed to long day photoperiod (LD,16 h light: 8 h dark). Although this response is well-established in dairy cows, the underlying mechanisms are not understood. We hypothesized that differential gene expression in cows exposed to SD or LD photoperiods during the dry period could be used to identify the functional basis for the subsequent increase in milk production during lactation. Pregnant, multiparous cows were maintained on a SD or LD photoperiod for 60-days prior to parturition. Mammary biopsies were obtained on days -24 and -9 relative to parturition and Affymetrix GeneChip® Bovine Genome Arrays were used to quantify gene expression. Sixty-four genes were differentially expressed ($p \leq 0.05$ and fold-change $\geq |1.5|$) between SD and LD treatments. Many of these genes were associated with cell growth and proliferation, or immune function. Ingenuity Pathway Analysis predicted *upstream regulators* to include TNF, TGFβ1, interferon γ and several interleukins. In addition, expression of 125 genes was significantly different between day -24 and day -9; those genes were associated with milk component metabolism and immune function. The interaction of photoperiod and time affected 32 genes associated with insulin-like growth factor 1 (IGF-1) signaling. Genes differentially expressed in response to photoperiod were associated with mammary development and immune function consistent with the enhancement of milk yield in the ensuing lactation. Our results provide insight into the mechanisms by which photoperiod affects the mammary gland and subsequently lactation.

INTRODUCTION

The typical lactation cycle of a dairy cow includes a non-lactating, or dry, period of 40-60 days between successive lactations. This dry-period promotes remodeling and restoration of mammary secretory tissue prior to the next lactation (Hurley and Loor, 2011). Manipulation of photoperiod, or the duration of daily light exposure, during the dry period is a management tool to increase milk yield in the subsequent lactation. Specifically, cows exposed to a short day (**SD**; 8 h light: 16 h dark) photoperiod during the dry period produce about 3 kg/day (~5-10%) more milk in the subsequent lactation than cows exposed to long day (**LD**; 16 h light: 8 h dark) photoperiod (Auchtung et al., 2005). This photoperiodic effect on milk production is substantial and consistent across multiple studies and species, including sheep and goats (Dahl et al., 2012). The increased milk yield must result from enhanced function of the mammary gland; yet the underlying mechanisms have not been elucidated.

Milk production is fundamentally determined by the number and activity of secretory cells in the mammary gland (Capuco et al., 2001), suggesting that photoperiod manipulation must affect one, or both, of those factors. Indeed, mammary epithelial cells of cows exposed to SD during the dry period had higher proliferation rates at three weeks prior to parturition and reduced apoptosis rates overall, relative to cows on LD (Wall et al., 2005b). Photoperiod manipulation also affects mammary parenchymal growth in pre-pubertal calves, such that mammogenesis is enhanced by LD treatment (Dahl et al., 2012). Together, these studies clearly demonstrate that photoperiod can influence mammary development and function; however, data on the effects of photoperiod on mammary gene expression are limited.

To identify potential mediators of photoperiodic effects on mammary gene expression and function most studies have focused on investigating the role of two hormones, insulin-like growth factor 1 (IGF-1) and prolactin. Relative to LD, exposure to SD photoperiod, during either the dry period or lactation, decreases circulating prolactin (Dahl et al., 2012). Pre-partum SD exposure is also accompanied by an increase in mammary expression of prolactin receptor (Auchtung et al., 2005; Dahl et al., 2012). The increase in receptor abundance could promote growth through signaling pathways that include members of the suppressor of cytokine signaling (SOCS) gene family (Wall et al., 2005b), although additional transcriptional regulation seems likely. Circulation of IGF-1, a growth factor known to have galactopoietic effects in goats (Prosser et al., 1990), increases early in the dry period (Abribat et al., 1990), potentially inhibiting mammary cell apoptosis (Marshman and Streuli, 2002). Exposure to LD photoperiod during lactation also increases circulating IGF-1 concentrations in cows concurrently producing more milk than their SD counterparts (Dahl et al., 2000). These findings suggest that circulating IGF-1 may mediate the galactopoietic response to photoperiod in lactating dairy cows. To the contrary, Miller and coworkers reported only a non-significant increase in blood IGF-1 concentrations in cows exposed to SD, compared to those on LD, while dry (Miller et. al., 2000). This led Wall and coworkers (2005b) to hypothesize that local expression of IGF-1 in the mammary gland of dry cows might replace the blood-borne supply. However, they reported a significant increase in mammary expression of IGF-2 but not IGF-1 in cows on SD relative to those on LD photoperiod (Wall et al., 2005b).

As indicated above, very few genes have been investigated as potential mediators of the milk yield response to photoperiod treatment in dairy cows (Dahl et al., 2012). Recent

transcriptomic studies of peri-partum mammary development (Finucane et al., 2008; Casey et al., 2011; Gao et al., 2013), milking frequency (Connor et al., 2008; Littlejohn et al., 2010; Wall et al., 2012), environmental stress (Collier et al., 2006), and bacterial invasion (Swanson et al., 2009) have identified changes in gene expression related to functional outcomes in the bovine mammary gland. Genes identified in those studies are good candidate modulators of mammary function in response to external cues. To identify photoperiod responsive genes, we have compared the mammary transcriptome of dairy cows exposed to LD or SD photoperiod during the dry period. We also compared gene expression at two time points to identify genes differentially expressed during mammary differentiation pre-partum. We hypothesized that differential gene expression in cows exposed to LD or SD photoperiods during the dry period could be used to identify the functional basis for the subsequent difference in milk production during lactation. Specifically, we hypothesized that exposure to SD photoperiod would alter the expression of genes associated with enhanced mammary development and functional support of milk production. The objectives of this study were to identify genes differentially expressed between SD and LD photoperiod, and between 24- and 9-days pre-partum, to determine associated *signaling pathways*, *biofunction*s and *upstream regulators*, and to draw functional associations to explain how their differential expression may influence subsequent milk production.

MATERIALS AND METHODS

Animals, photoperiod treatment, mammary biopsies

Mammary gland samples used in this experiment were derived from a study described previously (Auchtung et al., 2005; Wall et al., 2005a; Wall et al., 2005b). Briefly,

multiparous Holstein cows were dried-off approximately 60-days before their predicted parturition date, and randomly assigned to LD or SD photoperiod treatment for the duration of the dry period. Lights were turned on at 0800 h for both groups, and off at 1600 h for SD or at 2400 h for LD. Mammary biopsies were collected on day -24 ± 2 and -9 ± 2 relative to calving, as described by Farr and coworkers (1996). Biopsies were trimmed of non-parenchymal tissue then immediately snap-frozen in liquid nitrogen and stored at -80°C pending subsequent RNA isolation.

RNA isolation and reverse transcription

RNA was isolated from biopsied mammary tissues as previously described (Wall et al., 2005b) using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was further purified using the RNeasy Mini Kit (Qiagen). Nucleic acid concentration was quantified using a NanoDrop ND1000 spectrophotometer and RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). Samples meeting the criteria of RIN > 7.0 were used for microarray analysis.

Microarray analysis – Affymetrix GeneChip® bovine genome arrays

Microarray analysis was conducted on samples from four cows for each photoperiod treatment and time (total $n = 16$ cows). Preparation of RNA and microarray procedures were performed at the University of Vermont Microarray Core Facility using previously described protocols (Affymetrix, 2005-2006). Briefly, 2 μg of total RNA were reverse transcribed to single-stranded cDNA using T7 oligo dT primer. T4 DNA polymerase was used to synthesize double-stranded cDNA, which served as a template for *in vitro* transcription using T7 RNA polymerase to produce biotinylated cRNA. The biotinylated cRNAs were

fragmented into 50- to 200-base fragments and then hybridized to GeneChip® Bovine Genome Arrays (Affymetrix) for 16 h at 45°C in a rotating Affymetrix GeneChip® Hybridization Oven 320. After hybridization, arrays were washed and stained with streptavidin-phycoerythrin on an automated Affymetrix GeneChip® Fluidic Station F450. The arrays were scanned with an Affymetrix GeneChip® Scanner 2700 and the images quantified using Affymetrix GeneChip® Operating Software.

Data and statistical analysis

The signal intensity for each probe was calculated from scanned images using GeneChip® Operating Software (Affymetrix). Signal intensities were analyzed using BioConductor (http://www.bioconductor.org), background corrected, normalized using the loess method, and summarized as robust multichip averages (RMA) (Bolstad et al., 2003; Irizarry et al., 2003). Two samples, one from each photoperiod treatment at the day -24 time point, were excluded from further analysis due to large variation in average signal intensities. This resulted in $n = 3$/treatment for day -24 and $n = 4$/treatment for day -9 (total $n = 14$). Data were analyzed for the effect of photoperiod treatment (LD minus SD), time relative to parturition (day -24 minus day -9) and the interaction of photoperiod treatment and time. Individual probes meeting the criteria fold-change $\geq |1.5|$ and p-value ≤ 0.05, were considered differentially expressed (Patterson et al., 2006). To visualize the effect of photoperiod and time relative to parturition on specific genes, heat maps were generated from average RMA values using JMP® 10 Pro.

Gene function and pathway analysis

Probe set information and GO Biological and Molecular functions were obtained on Affymetrix NetAffx™ Analysis Center (www.affymetrix.com). Ingenuity Pathway Analysis (**IPA**; Ingenuity® Systems, www.ingenuity.com) was used to identify *biofunctions*, *canonical pathways* and *upstream regulators* enriched in our data sets. Differentially expressed probes (fold-change ≥ |1.5|, $p \leq 0.05$) for each effect (treatment: $n = 131$, time: $n = 177$, interaction: $n = 956$) were imported to IPA for analysis. When multiple probes for a single gene were differentially expressed, data were consolidated within IPA; probes with no current annotation were excluded from further analysis. Mapped IDs (treatment: $n = 64$, time: $n = 125$, interaction: $n = 601$), were identified from the Ingenuity Knowledge Base® at the time of analysis (December 2013). 'Analysis ready IDs' (treatment: $n = 44$, time: $n = 104$, interaction: $n = 556$) were subjected to *Core Analysis*. The Affymetrix GeneChip® Bovine Genome Array was identified as a reference platform in IPA. We excluded *biofunctions* and *canonical pathways* specifically relating to *diseases, disorders* and *cancer* from the analyses to obtain results most applicable to the bovine system. All other default settings were maintained. Statistical significance of enriched *biofunctions* and *pathways* was corrected for multiple comparisons within IPA using Benjamini-Hochberg (B-H) p-value test correction threshold of $p \leq 0.05$ (Benjamini and Hochberg, 1995). The *z-score of activation*, generated in IPA, provided insight into the *functional effects* of differentially expressed genes. The *z-score* denotes the predicted relationship between experimentally observed gene expression that is either activating ($z > 0$) or inhibiting ($z < 0$), as compiled in the Ingenuity® Knowledge Base. The two *functional effects* with the largest |*z-score*| were considered representative of the *biofunctional* category. *Canonical pathway* ratios represent the number

of differentially expressed genes relative to total number of genes in the IPA *pathway*. Comparison of our data set to gene lists reported by Lemay and coworkers (bovine lactation genome: milk protein, pregnancy, lactation and involution) (Lemay et al., 2009) was conducted within IPA.

Analysis of *upstream regulators* included all IPA-defined molecule types. Two metrics, *p-value of overlap* and predicted *activation state* were used to understand the relationship among *upstream regulators* and differentially expressed genes. Fisher's exact *p-value of overlap* ($p \leq 0.05$) assesses the significance of the expression data for genes downstream of an *upstream regulator*. Given the fold-change of the differentially expressed genes, the predicted *activation state* indicates whether the *upstream regulator* would have activated, inhibited or affected (unknown direction) the differentially expressed gene. In the analysis of photoperiod, *upstream regulators* affecting ≥ 6 genes are presented, whereas in the analysis of time relative to parturition, the top five *upstream regulators* by lowest *p-value of overlap* are shown.

RESULTS

The physiological responses to photoperiod treatment of cows used in this study were reported previously (Auchtung et al., 2005; Wall et al., 2005a). Briefly, cows exposed to SD photoperiod during the dry period produced significantly more milk compared to cows exposed to LD photoperiod for the first 16 weeks of lactation (Auchtung et al., 2005; Wall et al., 2005a).

Photoperiod induces differential expression of genes in the mammary gland

Microarray analysis was used to identify differentially expressed genes in response to photoperiod and time relative to parturition and the interaction of photoperiod and time. For complete lists of differentially expressed probes, see **Suppl. T3.1**. Among the 2 main effects and interaction 757 mapped genes (1264 probes) met our criteria for differential expression.

In response to photoperiod, 131 probes were differentially expressed (LD minus SD; **Suppl. T3.1**). Of the 131 differentially expressed probes, 64 unique genes were annotated by Affymetrix, 35 of which had lower expression in cows exposed to SD, relative to LD photoperiod (**Table 3.1)**. These enriched the *biofunctions: cell-to-cell interactions*, *small molecule biochemistry*, *cell movement*, *hematological system development, and immune cell trafficking* (**Table 3.1**, **Suppl. T3.2**). Notably, nine members of the bovine lymphocyte antigen (BoLA) family were differentially expressed, including BoLA-DQA5 which underwent the largest (28.5) fold-change. Genes associated with *immune cell trafficking* (CCR1, S100A12 and S100A8) were primarily decreased in response to SD photoperiod. Of the differentially expressed genes in response to photoperiod, 15 were in common with the bovine lactation genome (Lemay et al., 2009) (**Table 3.1**).

To gain further insight into the functions of these genes, we examined the top 5 *canonical pathways* enriched by genes differentially expressed in response to photoperiod (**Table 3.2**). Four of the top 5 pathways were associated with immune function, largely due to the high fold-change of three BoLA genes. Genes associated with fatty acid oxidation (**Table 3.2**, **Suppl. T3.3**) enriched the fifth pathway. The ratios of differentially expressed genes to total number of genes in these *canonical pathways* were generally low (average:

0.043) which can be attributed to the relatively small number of differentially expressed genes in response to photoperiod.

Of the *upstream regulators* identified (n = 368, **Suppl. T3.4**), nearly all (97%) were predicted to affect < 6 of the photoperiod responsive genes. *Upstream regulators* predicted to effect ≥ 6 differentially expressed genes were selected for further investigation and included 2 factors involved in tumor necrosis factor (TNF) signaling (TNF and FAS, a member of the TNF receptor super-family), four interleukins (IL), interferon γ (IFNG), transforming growth factor β1 (TGFβ1), oncostatin M, and the hormone β-estradiol (**Table 3.3**). Prolactin was predicted to regulate 3 genes and was included as a reference *upstream regulator*. Together, these *upstream regulators* were predicted to affect 26 of the photoperiod responsive genes, the majority of which were activated. Complete lists of *upstream regulators* are available in **Suppl. T3.4**.

Expression of genes related to milk synthesis increases with approaching parturition

Time relative to parturition significantly affected the expression of 125 mapped genes (171 probes, **Suppl. T3.1**). Of the probe sets, 146 were more highly expressed on day -9 than day -24. These differentially expressed genes aligned with 28 unique (38 total) significantly enriched (B-H p ≤ 0.05) *biofunctions* (**Table 3.4**). *Biofunctions* predicted to increase (*activation z-score* ≥ 2.0) include: *lipid synthesis*, *migration of granulocytes and neutrophils*, and *metabolism of carbohydrate*. The *functional effect, organismal death*, had a *z-score of activation* < -2.0, and thereby was predicted to decrease. Genes associated with all *biofunctional* categories are available in **Suppl. T3.2**.

A central theme in the time relative to parturition data set was altered metabolism, including 45 differentially expressed genes that enriched 5 *biofunctions* highly relevant to lactogenesis: *lipid metabolism*, *protein synthesis*, *carbohydrate metabolism*, *vitamin and mineral metabolism* and *reproductive system development and function* (**Tables 3.4** and **3.5**). Of these differentially expressed genes, 41 were more highly expressed at day -9 compared to day -24 relative to parturition, with an average 2-fold increase in expression. Genes associated with these functions also enriched the *canonical pathway* supporting lipid metabolism (*LXR/RXR Activation*) and immune function (**Table 3.2** and **Suppl. T3.3**). Several of these differentially expressed genes are established markers of mammary function, including AQPI, LALBA, LPL, LPO, and NT5E. In addition to the milk synthesis-related *biofunctions*, 7 genes were functionally associated with *involution of the mammary gland* as part of the *reproductive system development and function biofunction* (**Table 3.5**, **Suppl. T3.2**). Comparison to the lactation-related data set identified 23 genes in common with the bovine lactation genome (Lemay et al., 2009) (**Table 3.5**).

Genes differentially expressed in response to time were associated with 1373 *upstream regulators* in IPA, the top 5 by *p-value of overlap* were: β-estradiol, lipopolysaccharide (LPS), TNF, dexamethasone and TGFβ1 (**Table 3.6**). Together, these *upstream regulators* were predicted to affect 68 differentially expressed genes (**Suppl. T3.4**).

Interactive effects of photoperiod and time on mammary gene expression

Analysis of the interaction of photoperiod and time relative to parturition identified differential expression of 601 genes (965 probes, **Suppl. T3.1**). There was overlap among differentially expressed probes in the effects of photoperiod (9 common probes) and time (16 common probes) with those identified in the interaction. Functional analysis identified

organismal death (activation score: 3.53) as the most highly enriched functional effect of the differentially expressed genes. The complete list of *biofunctions* and associated differentially expressed genes are in **Suppl. T3.2**. One *canonical pathway*, *IGF-1 signaling*, was enriched by 11 genes (**Table 3.2**, **Suppl. T3.3**). Additionally, IGF-1 was a predicted *upstream regulator* (**Suppl. T3.3**) of 24 differentially expressed genes. Three genes were present in both lists, resulting in 32 differentially expressed genes associated with *IGF-1 signaling* (**Figure 3.1**). Cows exposed to LD photoperiod had the lowest level of expression of 5 of these genes on day -24 and 21 genes on day -9 relative to calving. The expression of 6 genes was lowest in cows exposed to SD on day -9, all of which were most highly expressed in LD treated cows on the same day (**Figure 3.1**).

DISCUSSION

Effect of photoperiod on mammary gene expression

The cessation of milking at the onset of the dry period promotes mammary regression. This is followed by the proliferation and differentiation of secretory cells (lactogenesis stage I) and finally milk production (lactogenesis stage II), approximately one week prior to parturition (Tucker, 1981; Capuco et al., 1997; Bachman and Schairer, 2003). Cows exposed to SD photoperiod, during the dry period, subsequently produce more milk than LD exposed cows (Auchtung et al., 2005). Wall and coworkers went on to show these same SD cows had increased mammary cell proliferation, as measured by tritiated thymidine uptake, on day -24 prior to parturition (Wall et al., 2005b). Comparison of the transcriptome of cows on SD or LD photoperiod identified 64 genes that were affected by photoperiod, averaged over days -24 and -9 relative to parturition. Four of these genes function in cell

growth and proliferation (AKR1C3, GPNMP, TMEM183A, and TRAF3IP3), suggesting they may have a role in mediating the effects of photoperiod on mammary cell proliferation. *Upstream regulators* of differentially expressed genes provided additional insight into the functional outcomes in response to photoperiod. We have identified two lactogenic hormones (prolactin and dexamethasone) and well-known mammary growth factors, TGFβ1, IFNG, and interleukin -13,-1B, -10 (Hynes and Watson, 2010), as *upstream regulators* of these differentially expressed genes. This suggests genes associated with these *upstream regulators* may mediate changes in mammary cell proliferation in response to photoperiod reported by Wall and coworkers (Wall et al., 2005b).

The mammary gland is thought to have evolved as part of the innate immune system, such that milk served as both nourishment and immunological protection for newborns (Vorbach et al., 2006). Therefore, lactation and inflammatory responses share many common mechanisms (Vorbach et al., 2006; Lemay et al., 2009). Here, we report photoperiod responsive expression of genes with known immune regulatory function including anti-microbial and pro-inflammatory factors: granzyme (gzm) A, secretory leukocyte peptidase inhibitor (SLPI), butyrylcholinesterase (BCHE), and butyrophilin (BTN3A2) (Das, 2007; Lieberman, 2010; Wilkinson et al., 2011). The effect of photoperiod on immune function has been well established in mammals (Nelson and Demas, 1996; Walton et al., 2011) including dairy cows during the dry period (Auchtung et al., 2004) and specifically the mammary gland defense system (Goldman, 2002). Among the differentially expressed genes identified here, members of the BoLA gene family have also been associated with mastitis resistance (Park et al., 2004), the transition from non-lactating to lactating states (Lemay et al., 2007) and in response to milking frequency in dairy cows (Connor et al., 2008;

Wall et al., 2012). Taken together, differential expression of numerous BoLA and immune-supportive factors provides evidence that photoperiod, through common mechanisms, is affecting both the mammary defense system as well as lactation.

The effects of photoperiod on lactation may be mediated by the secretion of prolactin as has been previously suggested (Auchtung et al., 2005; Crawford et al., 2005; Dahl, 2008). Calendar cells of the *pars tuberalis* of the pituitary gland regulate the secretion of prolactin to align with seasonal changes in day length (Lincoln et al., 2003). Specifically, LD photoperiod increases and SD photoperiod decreased prolactin secretion. In dairy cows, changes in immune function resulting from photoperiod manipulation are also mediated by prolactin secretion (Lincoln et al., 2003; Dahl et al., 2012). Therefore, it is plausible that prolactin signaling is a common mechanism underlying the effects of photoperiod on immune function and lactation in the mammary gland. In the effect of photoperiod, three differentially expressed genes (AKR1C3, GPNMB and SERPINA3) were associated with prolactin signaling, in the effect of time there were eight genes, and in the broader data set of the interaction, 16 genes were associated with prolactin as an *upstream regulator* (Table S4). These genes provide targets for additional study of the role of prolactin in mediating the effects of photoperiod in the mammary gland.

Two additional *upstream regulators* identified here, TNF and IFNG, have recently been reported as photoperiod responsive. McFarlane and coworkers (2012), working with captive baboons, identified seasonal fluctuations in expression of TNF gene family members and IL-6 release in response to LPS challenge. Bilbo and coworkers reported a similar response in hamsters (2002). SD photoperiod also affects TNF gene expression in the testes of mice, thereby promoting angiogenesis (Pyter et al., 2005). In the mammary gland,

expression of TNF super-family member-12A is higher in cows milked once daily compared to those milked twice daily (Littlejohn et al., 2010). The expression of IFNG, another immune-related *upstream regulator*, is responsive to melatonin in human peripheral blood mononuclear cells (Garcia-Maurino et al., 1997), and is associated with circadian release of hormones from the pituitary gland (Cano et al., 2005). Taken together, there is a growing body of evidence that TNF and IFNG alter immune function in response to photoperiod. Building on this knowledge, we have identified genes associated with these *upstream regulators* that may modulate mammary defense mechanisms in response to photoperiod manipulation.

Here, we report genes in the mammary gland that are responsive to photoperiod manipulation and describe how their differential expression may affect mammary function. The potential for common mechanisms between mammary immune function and lactation provides logical explanations for the effects of photoperiod in the mammary gland. However, we suggest that many of these genes have as-yet undescribed mammary-specific functions, in addition to their immune-related functions listed in the current annotation. Study of these genes and their *upstream regulators*, in the context of the mammary gland, may clarify their importance in mammary development and lactation as well as the roles they play in the response of the mammary gland to photoperiod manipulation.

Time relative to parturition affects mammary gene expression

The comparison of gene expression on day -9 to day -24 served to both validate our method of detecting differentially expressed genes and provide insight into similarities in the mechanisms involved in the mammary response to photoperiod and the initiation of lactation. Of the 125 mapped genes we identified, 45 were associated with lactation promoting

biofunctions. Many of the genes in this data set were common with the findings of other lactation-related transcriptional studies of the mammary gland. Twenty-three differentially expressed genes in our study were common with those identified in the bovine lactation genome (Lemay et al., 2009). These included α-lactalbumin (LALBA), which is the regulatory element of lactose synthase, as well as aquaporin (AQPI), lactoperoxidase (LPO) and 5'-nucleotidase, ecto (NT5E), all of which are established markers of mammary function. In our previous study of mammary gene expression during the transition from pregnancy to lactation (Finucane et al., 2008), we reported differential expression of parathyroid hormone-like hormone (PTHLH), pyruvate dehydrogenase kinase isozyme 4 (PDK4), solute carrier (zinc transporter) family 39 member 12 (SLC39A12), carbonic anhydrase VI (CA6), along with 18 other probes common with our current data. Our present dataset also overlaps with our previously reported changes in gene expression in response to milking frequency, which included complement component 3 (C3), mucin 1 (MUC1), chemokine ligand 10 (CXCL10), NT5E, myostatin (MSTN), and chitinase 3-like 1 (CHI3L-1) (Wall et al., 2012). The commonality between our current and past datasets and those of others supports our conclusion that genes identified here as being affected by time are, indeed, associated with the onset of lactation. Further, the consistent effects of external stimuli on expression of these genes suggest that they play an essential role in modulating mammary gland development and function. These genes may be markers of mammary function and as such could serve as targets for future efforts to maximize milk production.

Immune signature of lactation

Similar to our findings in response to photoperiod, numerous genes differentially expressed in response to time were associated with immune function and inflammation.

These genes enriched *biofunctions* including *migration of granulocytes*, *adhesion of immune cells* and *stimulation of leukocytes* as well as including *acute phase response* and *IL-10 signaling pathways*. However, classification of these genes as 'immune' may obscure what are actually mammary-specific functions.

The products of several genes identified here and associated with immune function are found in milk, including acute phase response proteins, complement factors, LALBA, LPO and mucins (Vorbach et al., 2006). As milk components, these proteins serve both nutritional and protective functions for neonates (Vorbach et al., 2006). In addition, nearly all of these immune-related genes are up-regulated during late gestation, implying potential roles in regulation of mammary functions such as colostrogenesis and/or lactogenesis. In goats, changes in the mammary transcriptome during late pregnancy (day 90 vs day 110) correspond to immune response signatures (Faucon et al., 2009). Faucon and coworkers proposed that the differential expression of these 'immune-related' genes was essential for functional differentiation of mammary secretory cells (Faucon et al., 2009). The authors also noted down-regulation of these genes early in lactation, and suggested that they have a role in mammary development rather than solely immune protection (Faucon et al., 2009). Others have noted the expression of IL-10 and acute phase response proteins in the mammary gland of healthy cows and mice, respectively (Stein et al., 2004; Britti et al., 2005), supporting our supposition that these immune factors may have alternate functions in the mammary gland.

Overall, time relative to parturition affected numerous markers of lactation as well as genes associated with metabolism. However, the effects of photoperiod on mammary gene expression were not consistent with the responses to time, implying these effectors operate through distinct mechanisms. Also, photoperiod did not enrich the same lactation-supportive

biofunctions that responded to time. Therefore, we surmise that LD and SD photoperiod do not directly influence the developmental pathways associated with the onset of lactation. There is, however, commonality in the immune-signature associated with both sets of differentially expressed genes, further suggesting genes annotated as immune-related may have mammary specific functions that support lactation.

Interdependent effect of time and photoperiod on mammary gene expression

Genes identified in the interaction represent those that were differently affected by photoperiod depending on the time relative to parturition. This may indicate that photoperiod differentially affects genes important in stage I or stage II of lactogenesis and/or that photoperiod may alter the timing of mammary development. Although this analysis was inherently less robust than that of the main effects (due to lower sample size), it proved useful for identifying functional pathways not significantly enriched by the smaller data sets generated by analyzing the main effects of photoperiod and time.

The (IGF-1) signaling pathway was the only *canonical pathway* significantly enriched by genes differentially expressed in the interaction of photoperiod treatment and time. IGF-I stimulates growth and proliferation of mammary secretory cells in preparation for milk secretion (Akers et al., 2005) and has been investigated as a potential mediator of photoperiodic effects on the mammary gland (Dahl et al., 1997; Wall et al., 2005b; Dahl et al., 2012). Specifically, Dahl and coworkers reported an increase in plasma IGF-1 concentrations in cows exposed to LD compared to natural photoperiod (≤ 13 hours of light) during lactation (Dahl et al., 1997). In the same cows used in our current study, Wall and coworkers (Wall et al., 2005b) did not detect local differences in mammary expression of IGF-1, although IGF-2 expression was higher in cows exposed to SD photoperiod.

We report that the interaction of photoperiod and time affected expression of thirty-two genes associated with IGF-1, including growth hormone receptor (GHR), IGF-1 receptor (IGF1R), and IGF-1 binding proteins. Functional effects of circulating IGF-1 are regulated by binding proteins, including IGFBP1-7/nephroblastoma over expressed 1 (NOV1), which was differentially expressed in our data set. This gene is highly up-regulated in rat mammary gland during lactogenesis (Patel et al., 2010), and its absence in null mice accelerates the onset of involution (Chatterjee et al., 2014), suggesting expression of this gene may be required for the initiation of copious milk secretion. Plasma concentrations of IGFBP-2 and -3 are not affected by photoperiod (Dahl et al., 1997), but cows exposed to LD photoperiod during the dry period have increased expression of IGFBP-5 in the mammary gland on day 11 of lactation (Wall et al., 2005b) and differential expression from day -24 to day -9 of the dry period. IGFBP-5 is known to sequester IGF-1 and 2, promoting mammary apoptosis (Marshman and Streuli, 2002). However, when unbound, IGFBP-5 can interact with IGF1R and function as a growth-inducing mitogen (Marshman and Streuli, 2002). Differential expression of these genes in the interaction indicates the effects of photoperiod on IGF-1 signaling differ by time relative to parturition. We interpret this to mean that the physiological state of the gland influences its response to photoperiod. Because Auchtung and coworkers (2005) reported an increase in milk production in cows exposed to SD photoperiod during the dry period, we suggest that IGF-1 signaling may, in part, mediate the effects of SD photoperiod on enhanced mammary development and function in the ensuing lactation.

In conclusion, photoperiod manipulation during the dry period induces differential expression of the mammary transcriptome in dairy cows. Photoperiod-responsive genes were

associated with mammary development and immune function consistent with the enhancement of milk production in the ensuing lactation. Furthermore, we propose these genes may have mammary-specific functional roles, in addition to their immune-related annotation. Overall, the transcriptomic signatures of photoperiod and time were distinct, suggesting these effectors utilize different mechanisms of action. Molecular signatures identified in the interaction of photoperiod and time implicate IGF-1 signaling as a potential mediator of the physiological changes in the mammary gland in response to photoperiod. Further study of gene targets identified here may elucidate mammary-specific gene functions and will ultimately expand our understanding of photoperiodic effects on mammary development and function.

Table 3.1. Effect of photoperiod on differentially expressed (DE) genes in the mammary gland of dry cows [1,2].

DE Gene Symbol	DE Gene Name	Fold Change	p-value	Probe Set ID	IPA *Biofunctions*[4]	GO Molecular Function[5]	GO Biological Function[5]
Expression Higher in Short Day Photoperiod							
ADAM1B	A disintegrin and metalloproteinase 1B	-1.54	0.038	Bt.12799.1.S1_at	CI, CM	oxidoreductase activity	
AGA	Aspartylglucosaminidase	-1.85	< 0.001	Bt.3115.1.A1_at			
ALDH5A1	Aldehyde dehydrogenase 5 member A1	-1.91	0.003	Bt.2173.1.S1_at	CI, SMB		nucleic acid/ amino acid metabolic process
AKR1C3[6]	Aldo-keto reductase family 1, member C3	-1.61	0.003	Bt.23094.4.S1_at	SMB	proliferation, differentiation	
BCHE[6]	Butyrylcholinesterase	-1.85	0.05	Bt.28385.1.A1_at	CI, SMB	hydrolase activity	
BoLA[3,6]	Major histocompatibility complex (MHC) I	-5.15	< 0.001	Bt.29823.1.S1_x_at		receptor activity	antigen processing/presentation, cellular defense
BoLA-DQB1[3,6]	MHC class II, DQ β1	-3.48	< 0.001	Bt.350.1.S1_at	CI, HSD, ICT		antigen processing and presentation, cellular defense
BoLA-DQA1[3]	MHC class II DQ α1	-7.07	< 0.001	Bt.22867.2.A1_at	CI, HSD, ICT		
BoLA-DQA2[3]	MHC, class II, DQ α2	-6.29	< 0.001	Bt.4751.2.S1_at			
BoLA-DQA3[3]	MHC, class II DQ α3	-3.20	<0.001	Bt.4751.2.S1_at			
BoLA-N[3]	MHC class I antigen	-7.95	< 0.001	Bt.5324.1.S1_s_at			
BoLA-NC1	MHC class I antigen	-3.72	< 0.001	Bt.28022.1.A1_at			
BTN3A2	Butyrophilin, sub-family 3A	-2.17	0.002	Bt.28475.1.A1_at		ubiquitin-protein ligase activity	immune system process, synaptic vesicle exocytosis
DMP1	Dentin matrix acidic phosphoprotein 1	-2.27	0.028	Bt.554.1.S1_at	CM		
GBP1[6]	Guanylate binding protein 1	-1.54	< 0.001	Bt.24012.1.A1_at		invasion, tubulation, proliferation	
gzmA	Granzyme A	-1.58	0.023	Bt.29672.1.S1_at		serine-type peptidase activity	
IgCgamma	IgG2a heavy chain constant region	-1.61	0.013	Bt.12490.2.A1_x_at			
KERA	Keratocan	-1.98	0.002	Bt.5391.1.S1_at	HSD	receptor activity	visual perception; GCPR/ cytokine-mediated signaling
KIR2DS1	Killer cell immunoglobulin-like receptor	-1.56	0.003	Bt.11174.2.S1_at			
MAL	Mal, T-cell differentiation protein	-1.53	0.010	Bt.4060.1.S1_at			
MAN1C1	Mannosidase, alpha, class 1C, member 1	-1.52	0.019	Bt.10587.1.S1_at		hydrolase activity	metabolic process, glycosylation, proteolysis
MAP1LC3C	Microtubule-associated protein 1 light chain 3 γ	-1.63	0.006	Bt.3000.2.S1_a_at		cytoskeleton, microtubule binding	
MCPH1	Microcephalin 1	-1.56	0.002	Bt.11907.1.A1_at			
MOXD1	Monooxygenase, DBH-like 1	-1.56	0.025	Bt.20519.1.A1_at		oxidoreductase activity	neurological system, metabolic process
OAS1X	2',5'-Oligoadenylate synthetase 1, 40/46kDa	-1.75	< 0.001	Bt.20922.1.S1_at			
PRSS2	Protease, serine, 2	-11.1	< 0.001	Bt.4404.1.A1_at			
SEPT2	Septin 2	-1.81	< 0.001	Bt.20352.1.S1_at			
SLC27A6[6]	Solute carrier family 27 (fatty acid transporter), member 6	-1.63	0.010	Bt.26921.1.A1_at	SMB	ligase activity, transporter activity	immune system process, fatty acid metabolic process
SLPI	Secretory leukocyte peptidase inhibitor	-1.61	0.018	Bt.9736.1.S1_at		protein binding, endopeptidase inhibitor	proteolysis

Table 3.1 continued

Expression Lower in Short Day Photoperiod							
ABCC4	ATP-binding cassette, sub-family C 4	2.10	< 0.001	Bt.27292.1.S1_at			
BEX4	Brain expressed, X-linked 4	1.65	0.003	Bt.3698.1.S1_at			
BoLA-DQA5[6]	MHC class II, DQ α5	28.53	< 0.001	Bt.215.1.S1_at	CI, HSD, ICT		
BoLA-DRB3[6]	MHC class II, DRB3	8.38	0.003	Bt.20925.1.S1_at			
CCR1[6]	Chemokine (C-C motif) receptor 1	1.58	0.001	Bt.1377.1.S1_at	CI,SMB,CM, HSD, ICT	G-protein coupled receptor activity	immune/ stimulus response, cytokine-mediated signaling
CD68[6]	CD68 molecule	1.65	0.011	Bt.2334.1.S1_at	CI		lysosomal transport, proteolysis
CLEC4E	C-type lectin domain family 4, member E	1.86	0.016	Bt.16271.2.A1_at	CI, CM, SD, ICT		
CSTB	Cystatin B (stefin B)	1.54	0.007	Bt.24354.1.S1_at		protein binding, endopeptidase inhibitor activity	proteolysis
DDC	Dopa decarboxylase	1.75	0.003	Bt.115.1.S1_at	CI, SMB		
DEFB1	Defensin, beta 1	2.14	0.001	Bt.13125.1.S1_s_at			immune system/metabolic process, response to stress
DEFB5[3]	Defensin beta 5	4.53	0.001	Bt.13125.1.S1_at			immune system/metabolic process, response to stress
DRD1	Dopamine receptor D1	1.6	0.017	Bt.9029.1.S1_at	CI, SMB, CM	G-protein coupled receptor activity	neurological system , GPCR / cell-cell signaling
F13A1[3]	Coagulation factor XIII, A1 polypeptide	1.64	0.012	Bt.19845.2.A1_at	CI,CM, HSD, ICT	acyltransferase activity	immune system, protein modification, blood coagulation
FXYD3	FXYD domain containing ion transport regulator 3	1.53	0.027	Bt.9573.1.S1_a_at		ion channel activity, protein binding	ion transport, signal transduction
GIMAP8	GTPase IMAP family member 8-like	1.81	< 0.001	Bt.26769.1.S1_at			
GPNMB[3,6]	Glycoprotein (transmembrane) nmb	1.94	0.011	Bt.9807.1.S1_at			
IGL@	immunoglobulin light chain, λ gene cluster	1.52	0.008				
IL17RB[6]	Interleukin 17 receptor B	1.64	0.012	Bt.24532.1.A1_at	CM, HSD, ICT		
IL8	Interleukin 8	1.67	0.002	Bt.155.1.S1_at	CI,SMB, CM,HSD, ICT	chemokine activity	macrophage activation, cytokine-mediated signaling
KIR2DL5A	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 5A	1.89	0.002	Bt.11174.1.S1_at			
NPL	N-acetylneuraminate pyruvate lyase	1.64	< 0.001	Bt.26155.1.A1_at		lyase activity	cellular amino acid biosynthetic process
PEBP4	Phosphatidylethanolamine-binding protein 4	2.93	< 0.001	Bt.14398.1.S1_at		protein binding, kinase inhibitor activity; kinase regulator activity	signal transduction
PTI[3]	Pancreatic trypsin inhibitor	2.96	0.011	Bt.28518.1.S1_at			
RARRES1	Retinoic acid receptor responder	1.61	0.039	Bt.24933.1.S1_at	CM		
S100A12[6]	S100 calcium binding protein A12	1.91	0.019	Bt.357.1.S1_at	CI, ICT	calcium ion/calmodulin binding	immune response, macrophage activation, cell cycle
S100A8	S100 calcium binding protein A8	1.91	0.014	Bt.9360.1.S1_at	CI,CM, ICT	calcium ion/calmodulin binding	macrophage activation, cell cycle
SDS	Serine dehydratase	1.61	0.039	Bt.5878.1.A1_at	SMB		
SERPINA3	Serpin peptidase inhibitor A3	1.56	0.004	Bt.5362.1.S1_at	CM, HSD, ICT	protein binding, endopeptidase inhibitor activity	proteolysis
SFRP1	Secreted frizzled-related protein 1	1.55	0.028	Bt.5226.1.S1_at	CI CM,HSD, ICT		
SPADH1	Spermadhesin 1	2.24	0.03	Bt.457.1.S1_at			

Table 3.1 continued

TMEM183A[6]	Transmembrane protein 183A	1.55	< 0.001	Bt.17595.3.A1_at			
TRAF3IP3[6]	TRAF3 interacting protein 3	1.56	0.024	Bt.2266.3.S1_at			
VNN1	Vanin 1	1.84	0.045	Bt.28243.1.S1_a_at	CI	hydrolase activity	signal transduction, adhesion, vitamin metabolism
VNN2	Vanin 2	1.72	0.006	Bt.19160.1.A1_at			
VSTM1	V-set and transmembrane domain 1	1.59	0.007	Bt.9131.1.S1_at			

[1.] Biopsies were obtained from cows exposed to long or short day photoperiod. Microarray analysis was conducted on purified RNA using Affymetrix GeneChip® Bovine Genome Arrays.

[2.] Genes identified in response to photoperiod treatment (long day-short day). Differential expression was attributed to genes meeting the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$.

[3.] Multiple probes were differentially expressed; average fold-change and p-values are shown.

[4.] Top five Ingenuity pathway analysis (IPA) *biofunctions* and corresponding genes identified as differentially expressed in response to photoperiod. **CI:** *Cell-to-cell interactions*, **SMB:** *Small molecule biochemistry*, **CM:** *Cell movement,* ***H*SD:** *Hematological system development***, ICT:** *Immune cell trafficking*.

[5.] GO Molecular and Biological Functions were assigned using Affymetrix NetAffx™ Analysis Center (www.affymetrix.com).

[6.] Genes in common with the bovine lactation genome (Lemay, 2009).

Table 3.2. Top five canonical pathways enriched by genes differentially expressed in response to photoperiod, time relative to parturition and the interaction[1,2].

Effect	*Canonical Pathway*	B-H p-value[3]	Ratio[4]	Differentially Expressed Genes
Photoperiod	Cytotoxic T Lymphocyte mediated Apoptosis of cells	0.01	0.03	↑BoLA, ↓BoLA-DQA5, BoLA-DRB3
	OX40 Signaling	0.01	0.03	↑BoLA, ↓BoLA-DQA5, BoLA-DRB3
	B Cell Development	0.04	0.06	↓BoLA-DQA5, BoLA-DRB3
	Antigen Presentation	0.06	0.05	↑BoLA, ↓BoLA-DQA5
	Fatty Acid Beta Oxidation I	0.06	0.04	↓SDS, ↑SLC27A6
Time	Acute Phase Response Signaling	<0.001	0.04	↓C3,C4BPA,CFB,CP,HP, IL6, IL33, LBP, RBP1
	LXR/RXR Activation	<0.001	0.04	↓C3, CD14, IL6, IL33, LBP, LPL
	VDR/RXR Activation	<0.001	0.06	↑CAMP, CXCL10, ↓CD14, IGFBP5, THBD
	Granulocyte Adhesion and Diapedesis	0.02	0.03	↑CXCL10,CXCL13,CLDN1, ↓IL33, SDC2,CXCL2
	IL-10	0.02	0.05	↑CAMP, CXCL10, ↓CD14, IGFBP5, THBD
Interaction	IGF-1 Signaling	0.01	0.10	↓NOV, SOCS1, ↑FOS, IGF1R, JUN, KRAS, NEDD4, PRKACB, PRKAR2B, PTK2, YWHAG
	CDK5 Signaling	0.06[5]	0.09	↑ ITGA2, KRAS, PPM1L, PPP1CB, PRKACB, PRKAR2B ↓ADCY3, PPP1R10, PPP1R3C
	Dopamine Receptor Signaling	0.08[5]	0.08	↑ PPM1L, PPP1CB, PRKACB, PRKAR2B ↓ADCY3, GCH1, PPP1R10, PPP1R3C
	Sertoli Cell Junction Signaling	0.08 [5]	0.05	↑ACTN4, ATF2, CDH1, CLDN1, ITGA2 JUN, KRAS, PRKACB, PRKAR2B, PVRL3, SPTBN1 ↓ACTA1, ACTG2, TGFB3
	Protein Kinase A Signaling	0.09 [5]	0.05	↑PPP1R3C, ADCY3, PPP1R10, TGFB3, HIST1H1C, TTN, PYGM, ↓PRKACB, PPP1CB, PDE1A, AKAP5, DUSP10, PRKAR2B, ATF2, TGFBR1, YWHAG, BRAF, PTK2, EYA3, PTPLA, PTPN2

[1.] Biopsies were obtained from cows exposed to either long or short day photoperiod on days -24 and -9 relative to parturition. Microarray analysis was conducted on purified RNA using Affymetrix GeneChip® Bovine Genome Arrays. Genes were identified in the effect of photoperiod treatment, time relative to parturition and the interaction. Differential expression was attributed to genes meeting the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$.

[2.] Ingenuity pathway analysis (IPA) was used to identify *canonical pathways*.

[3.] B-H p-value test correction to identify the probability of gene associations by chance (Benjamini and Hochberg, 1995).

[4.] Ratio of differentially expressed genes to the total number of genes in the *canonical pathway* as defined by IPA.

[5.] *Canonical pathways* that did not meet the B-H threshold criteria of $p \leq 0.5$

Table 3.3. ***Upstream regulators*** **predicted to affect ≥ 6 genes differentially expressed in the mammary gland of dairy cows in response to photoperiod treatment during the dry period[1,2].**

	Upstream Regulators[3,4]												
Gene Symbol	**PRL[5]**	**DEX**	**IFNG**	**LPS**	**TGFβ1**	**TNF**	**E_2**	**OSM**	**IL13**	**FAS**	**IL6**	**IL1B**	**IL10**
AKR1C3													
ALDH5A1													
BoLA													
BoLA-DQA5													
BoLA-DRB3													
CCR1													
CD68													
CLEC4E													
CSTB													
DMP1													
DRD1													
F13A1													
GBP1													
GPNMB													
IL17RB													
IL8													
MAL													
MAN1C1													
PEBP4													
RARRES1													
S100A12													
S100A8													
SDS													
SERPINA3													
SFRP1													
VNN1													
# of genes affected	**3**	**11**	**11**	**11**	**11**	**10**	**8**	**8**	**7**	**6**	**6**	**6**	**6**

[1.] Biopsies were obtained from cows exposed to long or short day photoperiod. Microarray analysis was conducted on purified RNA using Affymetrix GeneChip® Bovine Genome Arrays.

[2.] Genes were identified in the effect of photoperiod treatment (long day-short day). Differential expression was attributed to genes meeting the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$.

[3.] Predicted *Upstream Regulators* **- PRL:** Prolactin**, DEX:** Dexamethasone, **IFNG:** Interferon γ**, LPS:** Lipopolysaccharide**, TGFβ1:** transforming growth factor -β1, **TNF:** Tumor necrosis factor, **E_2:** Beta-estradiol**, OSM:** Oncostatin M, **IL: Interleukin members:** 1B, 6, 10, 13, **FAS:** TNF receptor superfamily member 6.

[4.] Identified using Ingenuity pathway analysis (IPA) *upstream regulator* function. IPA predictions of the *upstream regulators*' activation state are based on the direction of gene expression change. Green: predicted activation, yellow: affected, red: predicted inhibition.

[5.] Prolactin was included based on its role in lactation and photoperiod biology.

Table 3.4. IPA *Biofunctions* enriched by genes differentially expressed in the mammary gland of cows in response to time (day -9 minus day -24) relative to parturition[1,2].

IPA *Biofunction*[3]	# of Genes	*Functional Effect*[4]	*z-score of activation*[5]	p-value[6]	Associated *Biofunctions*[7]
Lipid Metabolism	32	↑Synthesis of Lipid	3.4	0.003	Small molecule biochemistry
		↑Fatty Acid metabolism	2.6	0.027	
Molecular Transport	42	↑Transport of Molecules	2.4	0.019	
		Secretion of molecules	1.7	0.023	
Cellular Movement	38	↑Migration of Granulocytes	2.8	0.000	Hematological sys. devel. and funct., immune cell trafficking, tissue devel.
		↑Migration of neutrophils	2.2	0.005	
Cell-To-Cell Signaling and Interaction	34	↑Binding of Phagocytes	2.6	0.000	Hematological sys. devel. and funct., cell signaling, cell growth and prolif.
		↑Stimulation of cells	2.6	0.003	
Immune Cell Trafficking	24	↑Adhesion of immune cells	2.5	0.000	
		↑Binding of granulocytes	2.2	0.001	
Carbohydrate Metabolism	25	↑Metabolism of Carbohydrate	2.6	0.010	
		Quantity of Carbohydrate	1.8	0.000	
Connective Tissue Devel. and Funct.	26	↑Quantity of Adipose tissue	2.2	0.000	Tissue morphology
		Quantity of White adipose	2.0	0.016	
Organismal Survival	36	↓Organismal death	-3.6	0.004	
		Survival or organism	1.5	0.000	
Protein Synthesis	21	Quantity of IgE	1.1	0.004	Humoral immune response
		Quantity of TNF in blood	0.4	0.002	
Cardiovascular System Devel. and Funct.	28	↑Binding of endothelial cells	2.2	0.006	
		Cell movement of endothelial cells	1.7	0.019	
Cellular Growth and Proliferation	45	↑Stimulation of Leukocytes	2.4	0.000	
		↑Stimulation of phagocytes	2.2	0.001	
Cell Death and Survival	44	Cell death of breast cancer cell line	-1.7	0.027	
		Fragmentation of DNA	-1.6	0.009	
Cellular Development	35	↑Prolif. of smooth muscle cells	2.4	0.014	Cell morphology
		Prolif. of vascular smooth muscle cells	2.0	0.016	
Cellular Assembly and Organization	19	Formation of Filopodia	1.2	0.019	Cellular funct. and maintenance
		Organization of cytoskeleton	1.5	0.027	
Digestive Sys. Devel. and Funct.	22	Mass of liver	1.0	0.002	Hepatic sys. devel. and funct., organ devel, organ morphology
		Inflammation of liver	0.3	0.004	
Organismal Development	29	Development of blood vessels	1.4	0.005	
		Endothelial cell development	1.2	0.005	
Cellular Compromise	17	Degranulation of mast cells	-0.3	0.009	
		Degranulation of cells	0.2	0.001	

Table 3.4 continued

Organ Morphology	22	Size of bone	-1.0	0.027
Energy Production	9	Oxidation of fatty acid	0.7	0.006
Cell Cycle	15	Mitogenesis	0.7	0.027
Vitamin and Mineral Metabolism	12	Mobilization of Ca2+	-0.7	0.007
		Flux of Ca2+	0.1	0.017
Renal and Urological Sys. Devel. and Funct.	12	Proliferation of glomerular cells	0.3	0.009
DNA Replication, Recombination, and Repair	9	Fragmentation of DNA	-1.6	0.009
		Metabolism of DNA	1.0	0.014
Tumor Morphology	11	Proliferation of tumor cells	0.6	0.010
Cell-mediated Immune Response	6	Cell movement of T lymphocytes	-1.4	0.016
Free Radical Scavenging	10	Metabolism of reactive oxygen species	0.4	0.014
Hair and Skin Devel. and Funct.	6	Proliferation of endothelial cells	0.1	0.017
Organismal Functions	10	Thermoregulation	1.1	0.027

[1.] Biopsies were obtained from cows during the dry period on day -24 and -9 relative to parturition. Microarray analysis was conducted on purified RNA using Affymetrix GeneChip® Bovine Genome Arrays.

[2.] Genes were identified in the of time relative to parturition (day -9 minus day –24). Differential expression was attributed to genes meeting the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$.

[3.] All *biofunctions* met the Benjamini- Hochberg threshold criteria of $p \leq 0.05$.

[4.] The top two functional effects as determined by |*z-score of activation*|.

[5.] *Biofunctions* with identical functional effects are listed as associated functions.

[6.] *z-score of activation*: IPA prediction of the relationship between experimentally observed gene expression that is either activating or inhibiting (as compiled in the Ingenuity® Knowledge Base), *z-score* > 0 are activating, < 0 are inhibiting. IPA required a *z-scores* ≤ -2.0 or > 2.0 to form a prediction (indicated by arrows).

[7.] Measure of the probability genes were randomly associated with a *biofunction*, $p \leq 0.05$ was the threshold for significance.

Table 3.5. Genes differentially expressed (day -9 minus day -24) in the mammary gland during the dry period enriching *biofunctions* related to milk production[1,2].

Gene Symbol	Gene Name	Fold-change	p-value	IPA *Biofunctions*[3]				
				LM	PS	VM	CM	RS
ACSM1[4]	Acyl-CoA synthetase medium-chain 1	2.0	0.001	■				
ALOX15	Arachidonate 15-lipoxygenase	2.4	<0.001	■				
ANGPTL4[4]	Angiopoietin-like 4	2.1	0.019	■	■			
AQP1[4]	Aquaporin 1 (Colton blood group)	1.6	0.003				■	
C3[4]	Complement component 3	1.8	0.038	■	■	■	■	
CAMP[4]	Cathelicidin antimicrobial peptide	-2.8	0.003	■		■	■	
CD14[4]	CD14 molecule	1.6	0.012	■	■		■	
CEBPD[4]	CCAAT/enhancer binding protein (C/EBP), delta	1.7	0.036	■				■
CFB	Complement factor B	2.4	0.025		■			
CHI3L1[4]	Chitinase 3-like 1 (cartilage glycoprotein-39)	2.5	0.040		■			
CIDEA[4]	Cell death-inducing DFFA-like effector a	2.3	0.004	■			■	
CP	Ceruloplasmin (ferroxidase)	2.7	0.002	■				
CXCL10	Chemokine (C-X-C motif) ligand 10	-1.7	0.008	■	■	■	■	
CXCL13	Chemokine (C-X-C motif) ligand 13	-2.1	0.033			■		
CXCL2[4]	Chemokine (C-X-C motif) ligand 2	3.0	0.018			■		
CYP11A1	Cytochrome P450, family 11, subfamily A1	3.2	0.016	■	■			■
DARC	Duffy blood group, chemokine receptor	1.7	0.008			■		
DUSP1[4]	Dual specificity phosphatase 1	1.6	0.002	■	■			■
FABP3[4]	Fatty acid binding protein 3	3.0	0.001	■			■	
GK[4]	Glycerol kinase	1.6	<0.001	■			■	
HP	Haptoglobin	4.5	0.025	■				
IGFBP5	Insulin-like growth factor binding protein 5	1.6	0.029		■		■	■
IL13RA1	Interleukin 13 receptor, alpha 1	1.5	0.007		■			
IL17RB[4]	Interleukin 17 receptor B	-1.5	0.022		■			
IL33	Interleukin 33	2.1	0.001	■	■			
IL6	Interleukin 6	1.7	0.002	■	■	■	■	■
KLF6[4]	Kruppel-like factor 6	1.5	0.011					■
LALBA[4]	α-Lactalbumin,	1.5	0.005	■			■	
LBP[4]	Lipopolysaccharide binding protein	3.4	0.005	■			■	
LPIN1	Lipin 1	1.7	0.023	■				
LPL[4]	Lipoprotein lipase	1.9	<0.001	■	■		■	
MSTN	Myostatin	1.6	<0.001	■	■			
MUC1[4]	Mucin 1, cell surface associated	2.0	0.003	■				
NT5E[4]	5'-Nucleotidase, ecto (CD73)	1.6	0.031		■			
NTS	Neurotensin	1.7	0.002	■		■		
PDK4[4]	Pyruvate dehydrogenase kinase, isozyme 4	1.9	0.014	■	■		■	
PLVAP	Plasmalemma vesicle	1.6	<0.001	■	■			
PTHLH	Parathyroid hormone-like hormone	2.5	0.002	■		■	■	
RAB35[4]	Rab 35, member ras	1.5	0.005	■			■	
RBP1	Retinol binding protein 1, cellular	1.8	0.042			■		■
S100A9[4]	S100 calcium binding protein A9	2.4	0.013		■			
SDC2	Syndecan 2	1.5	0.005	■			■	
SFN	Stratifin	1.9	0.021	■	■			
SULF2	Sulfatase 2	1.6	0.027				■	
THBD	Thrombomodulin	1.9	0.005		■	■		

[1.] Biopsies were obtained from cows during the dry period on day -24 and -9 relative to parturition. Microarray analysis was conducted on purified RNA using Affymetrix GeneChip® Bovine Genome Arrays.

[2.] Genes were identified in the effect of time relative to parturition (day -9 minus day –24). Differential expression was attributed to genes meeting the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$.

[3.] **LM:** *Lipid metabolism*, **PS:** *Protein synthesis*, **VM:** *Vitamin and mineral metabolism*, **CM:** *Carbohydrate metabolism,* **RS:** *Reproductive System Development and Function.*

[4.] Genes in common with the bovine lactation genome (Lemay, 2009).

Table 3.6. *Upstream regulators* predicted to affect genes differentially expressed in response to time relative to parturition in the mammary gland of cows during the dry period[1].

Upstream Regulator[2]	Molecule Type	*Activation Score*[3]	p-value of Overlap	# of Genes[4]
β-Estradiol	Chemical - endogenous mammalian	0.81	<0.0001	37
Lipopolysaccharide	Chemical drug	2.568	<0.0001	36
TNF	Cytokine	1.734	<0.0001	35
Dexamethasone	Chemical drug	1.156	<0.0001	35
TGFβ1	Growth factor	1.416	<0.0001	31

[1.] Biopsies were obtained from cows during the dry period on day -24 and -9 relative to parturition. Microarray analysis was conducted on purified RNA using Affymetrix GeneChip® Bovine Genome Arrays. Genes were identified in the effect of time relative to parturition (day -9 minus day –24). Differential expression was attributed to genes meeting the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$.

[2.] Identified using Ingenuity pathway analysis (IPA) *upstream regulator* function. The top five *upstream regulators* by lowest p-value of overlap.

[3.] *z-score of activation*: IPA prediction of the relationship between experimentally observed gene expression that is either activating or inhibiting (as compiled in the Ingenuity® Knowledge Base), *z-score* > 0 are activating, < 0 are inhibiting.

[4.] The number of differentially expressed genes predicted to be affected by *upstream regulator.*

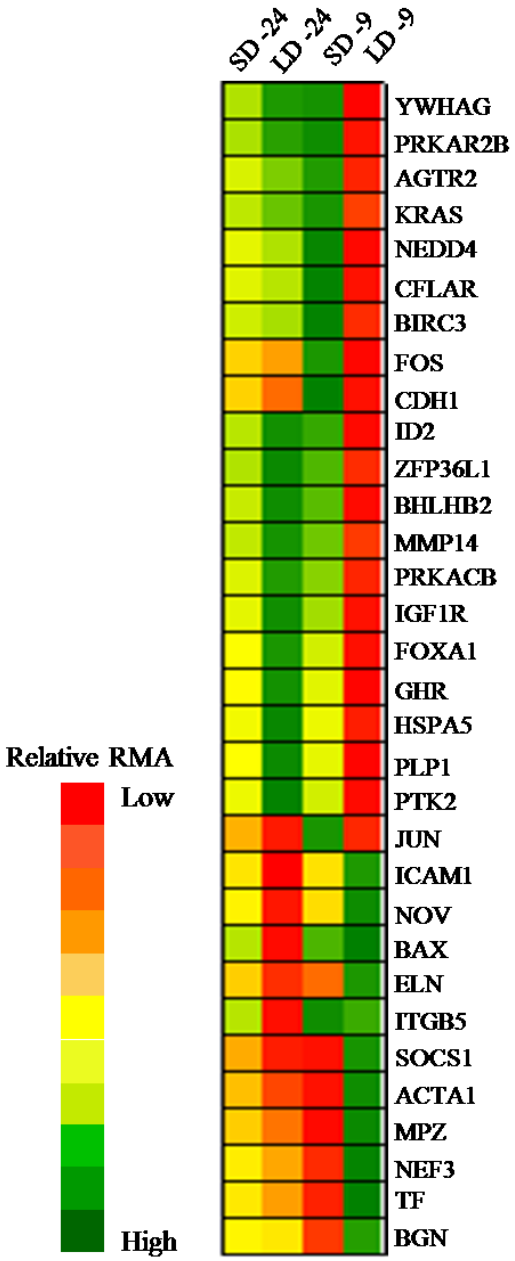


Figure 3.1. The relative robust multi-chip average (RMA) values of genes differentially expressed in bovine mammary gland that are associated with IGF-1 signaling.

Ingenuity pathway analysis (IPA) associated thirty-two genes, differentially expressed in the interaction of photoperiod and time relative to parturition, with the *IGF-1 signaling pathway* or IGF-1 as predicted *upstream regulator*. Relative RMA values indicate the change in expression in response to long day (LD) and short day (SD) photoperiod on day -9 and -24 and relative to parturition.

REFERENCES

Abribat, T., H. Lapierre, P. Dubreuil, G. Pelletier, P. Gaudreau, P. Brazeau, and D. Petitclerc. 1990. Insulin-like growth factor-i concentration in holstein female cattle: Variations with age, stage of lactation and growth hormone-releasing factor administration. Domest Anim Endocrinol 7:93.

Affymetrix. 2005-2006. Genechip® expression analysis technical manual.

Akers, R. M., S. E. Ellis, and S. D. Berry. 2005. Ovarian and igf-i axis control of mammary development in prepubertal heifers. Domest Anim Endocrinol 29:259.

Auchtung, T. L., A. G. Rius, P. E. Kendall, T. B. McFadden, and G. E. Dahl. 2005. Effects of photoperiod during the dry period on prolactin, prolactin receptor, and milk production of dairy cows. J Dairy Sci 88:121.

Auchtung, T. L., J. L. Salak-Johnson, D. E. Morin, C. C. Mallard, and G. E. Dahl. 2004. Effects of photoperiod during the dry period on cellular immune function of dairy cows. J Dairy Sci 87:3683.

Bachman, K. C. and M. L. Schairer. 2003. Invited review: Bovine studies on optimal lengths of dry periods. J Dairy Sci 86:3027.

Benjamini, Y. and Y. Hochberg. 1995. Controlling the false discovery rate - a practical and powerful approach to multiple testing. J Roy Stat Soc B Met 57:289.

Bilbo, S. D., D. L. Drazen, N. Quan, L. He, and R. J. Nelson. 2002. Short day lengths attenuate the symptoms of infection in siberian hamsters. Proc Biol Sci 269:447.

Bolstad, B. M., R. A. Irizarry, M. Astrand, and T. P. Speed. 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19:185.

Britti, D., A. Peli, G. Massimini, A. Polci, A. Luciani, and P. Famigli-Bergamini. 2005. Evaluation of tnf-alpha, il-8 and il-10 transcriptional activity in milk from healthy dairy cows during lactation period. Vet Res Commun 29 Suppl 2:281.

Cano, P., D. P. Cardinali, V. Jimenez, M. P. Alvarez, R. A. Cutrera, and A. I. Esquifino. 2005. Effect of interferon-gamma treatment on 24-hour variations in plasma acth, growth hormone, prolactin, luteinizing hormone and follicle-stimulating hormone of male rats. Neuroimmunodulat 146.

Capuco, A. V., R. M. Akers, and J. J. Smith. 1997. Mammary growth in holstein cows during the dry period: Quantification of nucleic acids and histology. J Dairy Sci 80:477.

Capuco, A. V., D. L. Wood, R. Baldwin, K. McLeod, and M. J. Paape. 2001. Mammary cell number, proliferation, and apoptosis during a bovine lactation: Relation to milk production and effect of bst. J Dairy Sci 84:2177.

Casey, T., H. Dover, J. Liesman, L. DeVries, M. Kiupel, M. Vandehaar, and K. Plaut. 2011. Transcriptome analysis of epithelial and stromal contributions to mammogenesis in three week prepartum cows. PLoS ONE 6:e22541.

Chatterjee, S., S. Bacopulos, W. Yang, Y. Amemiya, D. Spyropoulos, A. Raouf, and A. Seth. 2014. Loss of igfbp7 causes precocious involution in lactating mouse mammary gland. PLoS ONE 9:e87858.

Collier, R. J., C. M. Stiening, B. C. Pollard, M. J. VanBaale, L. H. Baumgard, P. C. Gentry, and P. M. Coussens. 2006. Use of gene expression microarrays for evaluating environmental stress tolerance at the cellular level in cattle. J Anim Sci 84 Suppl:E1.

Connor, E. E., S. Siferd, T. H. Elsasser, C. M. Evock-Clover, C. P. Van Tassell, T. S. Sonstegard, V. M. Fernandes, and A. V. Capuco. 2008. Effects of increased milking frequency on gene expression in the bovine mammary gland. BMC Genomics 9:362.

Crawford, H. M., J. L. Dauderman, D. E. Morin, T. B. McFadden, and G. E. Dahl. 2005 Evidence of a role of prolactin in mediating photoperiodic effects during the dry period. J Dairy Sci 88:363.

Dahl, G. E. 2008. Effects of short day photoperiod on prolactin signaling in dry cows: A common mechanism among tissues and environments? J Anim Sci 86:10.

Dahl, G. E., B. A. Buchanan, and H. A. Tucker. 2000. Photoperiodic effects on dairy cattle: A review. J Dairy Sci 83:885.

Dahl, G. E., T. H. Elsasser, A. V. Capuco, R. A. Erdman, and R. R. Peters. 1997. Effects of a long daily photoperiod on milk yield and circulating concentrations of insulin-like growth factor-1. J Dairy Sci 80:2784.

Dahl, G. E., S. Tao, and I. M. Thompson. 2012. Effects of photoperiod on mammary gland development and lactation. J Anim Sci 90:755.

Das, U. N. 2007. Acetylcholinesterase and butyrylcholinesterase as possible markers of low-grade systemic inflammation. Med Sci Monit 13:RA214.

Farr, V. C., K. Stelwagen, L. R. Cate, A. J. Molenaar, T. B. McFadden, and S. R. Davis. 1996. An improved method for the routine biopsy of bovine mammary tissue. J Dairy Sci 79:543.

Faucon, F., E. Rebours, C. Bevilacqua, J. C. Helbling, J. Aubert, S. Makhzami, S. Dhorne-Pollet, S. Robin, and P. Martin. 2009. Terminal differentiation of goat mammary tissue during pregnancy requires the expression of genes involved in immune functions. Physiol Genomics 40:61.

Finucane, K. A., T. B. McFadden, J. P. Bond, J. J. Kennelly, and F. Q. Zhao. 2008. Onset of lactation in the bovine mammary gland: Gene expression profiling indicates a strong inhibition of gene expression in cell proliferation. Funct Integr Genomics 8:251.

Gao, Y., X. Lin, K. Shi, Z. Yan, and Z. Wang. 2013. Bovine mammary gene expression profiling during the onset of lactation. PLoS ONE 8:e70393.

Garcia-Maurino, S., M. G. Gonzalez-Haba, J. R. Calvo, M. Rafii-El-Idrissi, V. Sanchez-Margalet, R. Goberna, and J. M. Guerrero. 1997. Melatonin enhances il-2, il-6, and ifn-gamma production by human circulating cd4+ cells: A possible nuclear receptor-mediated mechanism involving t helper type 1 lymphocytes and monocytes. J Immunol 159:574.

Goldman, A. S. 2002. Evolution of the mammary gland defense system and the ontogeny of the immune system. J Mammary Gland Biol Neoplasia 7:277.

Hurley, W. L. and J. J. Loor. 2011. Mammary gland: Growth, development and involution. Pages 338 in Encyclopedia of dairy sciences (second edition). J. W. Fuquay, ed. Academic Press, San Diego.

Hynes, N. E. and C. J. Watson. 2010. Mammary gland growth factors: Roles in normal development and in cancer. Cold Spring Harb Perspect Biol 2:a003186.

Irizarry, R. A., B. M. Bolstad, F. Collin, L. M. Cope, B. Hobbs, and T. P. Speed. 2003. Summaries of affymetrix genechip probe level data. Nucleic Acids Res 31:e15.

Lemay, D. G., D. J. Lynn, W. F. Martin, M. C. Neville, T. M. Casey, et al. 2009. The bovine lactation genome: Insights into the evolution of mammalian milk. Genome Biol 10:R43.

Lemay, D. G., M. C. Neville, M. C. Rudolph, K. S. Pollard, and J. B. German. 2007. Gene regulatory networks in lactation: Identification of global principles using bioinformatics. BMC Syst Biol 1:56.

Lieberman, J. 2010. Granzyme a activates another way to die. Immunol Rev 235:93.

Lincoln, G. A., H. Andersson, and A. Loudon. 2003. Clock genes in calendar cells as the basis of annual timekeeping in mammals--a unifying hypothesis. J Endocrinol 179:1.

Littlejohn, M. D., C. G. Walker, H. E. Ward, K. B. Lehnert, R. G. Snell, G. A. Verkerk, R. J. Spelman, D. A. Clark, and S. R. Davis. 2010. Effects of reduced frequency of milk removal on gene expression in the bovine mammary gland. Physiol Genomics 41:21.

Marshman, E. and C. H. Streuli. 2002. Insulin-like growth factors and insulin-like growth factor binding proteins in mammary gland function. Breast Cancer Res 4:231.

McFarlane, D., R. F. Wolf, K. A. McDaniel, and G. L. White. 2012. The effect of season on inflammatory response in captive baboons. J Med Primatol 41:341.

Miller, A. R., R. A. Erdman, L. W. Douglass, and G. E. Dahl. 2000. Effects of photoperiodic manipulation during the dry period of dairy cows. J Dairy Sci 83:962.

Nelson, R. J. and G. E. Demas. 1996. Seasonal changes in immune function. Q Rev Biol 71:511.

Park, Y. H., Y. S. Joo, J. Y. Park, J. S. Moon, S. H. Kim, N. H. Kwon, J. S. Ahn, W. C. Davis, and C. J. Davies. 2004. Characterization of lymphocyte subpopulations and major histocompatibility complex haplotypes of mastitis-resistant and susceptible cows. J Vet Sci 5:29.

Patel, O. V., T. Casey, H. Dover, and K. Plaut. 2010. Homeorhetic adaptation to lactation: Comparative transcriptome analysis of mammary, liver, and adipose tissue during the transition from pregnancy to lactation in rats. Funct Integr Genomics 11:193.

Patterson, T. A., E. K. Lobenhofer, S. B. Fulmer-Smentek, P. J. Collins, T. M. Chu, et al. 2006. Performance comparison of one-color and two-color platforms within the microarray quality control (maqc) project. Nat Biotechnol 24:1140.

Prosser, C. G., I. R. Fleet, A. N. Corps, E. R. Froesch, and R. B. Heap. 1990. Increase in milk secretion and mammary blood flow by intra-arterial infusion of insulin-like growth factor-i into the mammary gland of the goat. J Endocrinol 126:437.

Pyter, L. M., A. K. Hotchkiss, and R. J. Nelson. 2005. Photoperiod-induced differential expression of angiogenesis genes in testes of adult *Peromyscus leucopus*. Reproduction 129:201.

Stein, T., J. S. Morris, C. R. Davies, S. J. Weber-Hall, M. A. Duffy, et al. 2004. Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving lbp, cd14 and stat3. Breast Cancer Res 6:R75.

Swanson, K. M., K. Stelwagen, J. Dobson, H. V. Henderson, S. R. Davis, V. C. Farr, and K. Singh. 2009. Transcriptome profiling of *Streptococcus uberis*-induced mastitis reveals fundamental differences between immune gene expression in the mammary gland and in a primary cell culture model. J Dairy Sci 92:117.

Tucker, H. A. 1981. Physiological control of mammary growth, lactogenesis, and lactation. J Dairy Sci 64:1403.

Vorbach, C., M. R. Capecchi, and J. M. Penninger. 2006. Evolution of the mammary gland from the innate immune system? Bioessays 28:606.

Wall, E. H., T. L. Auchtung-Montgomery, G. E. Dahl, and T. B. McFadden. 2005a. Short communication: Short-day photoperiod during the dry period decreases expression of suppressors of cytokine signaling in mammary gland of dairy cows. J Dairy Sci 88:3145.

Wall, E. H., T. L. Auchtung, G. E. Dahl, S. E. Ellis, and T. B. McFadden. 2005b. Exposure to short day photoperiod during the dry period enhances mammary growth in dairy cows. J Dairy Sci 88:1994.

Wall, E. H., J. P. Bond, and T. B. McFadden. 2012. Acute milk yield response to frequent milking during early lactation is mediated by genes transiently regulated by milk removal. Physiol Genomics 44:25.

Walton, J. C., Z. M. Weil, and R. J. Nelson. 2011. Influence of photoperiod on hormones, behavior, and immune function. Front Neuroendocrinol 32:303.

Wilkinson, T. S., A. Roghanian, A. J. Simpson, and J. M. Sallenave. 2011. Wap domain proteins as modulators of mucosal immunity. Biochem Soc Trans 39:1409.

CHAPTER 4: RESPONSES OF THE MOUSE MAMMARY TRANSCRIPTOME TO ALTERED PHOTOPERIOD DURING LACTATION

ABSTRACT

The photoperiod, or duration of light, an animal is exposed to can influence reproduction, including gonadal development, timing of mating and milk production. The effects of manipulating photoperiod on lactation have been described in dairy animals; however, the molecular mechanisms are not well understood. We hypothesized that altering photoperiod induces differential expression of genes associated with lactation that result in changes in mammary physiology and function. The objective of this study was to quantify the effects of photoperiod manipulation on mammary function, cell proliferation, and transcriptome. Mice were exposed to one of three photoperiods, long day (LD), normal day (ND), or short day (SD) for 5, 10, or 15 days of lactation. Photoperiod manipulation affected body, spleen and liver weights of lactating dams. There was no effect of photoperiod on litter weight, a proxy for milk production, during the first 15 days of lactation. Using microarray analysis, we quantified the effects of photoperiod on the mammary transcriptome of mice on day 10 of lactation. Relative to ND, we detected differential expression of 723 genes in response to SD photoperiod and 195 genes in response to LD photoperiod. Genes responsive to LD photoperiod enriched *lipid metabolism* and included clock genes (*Tef, Cry2, Per3, Dbp, and Nr1d1)*, whereas SD photoperiod affected genes associated with *immune function* and *cell proliferation*. Many, photoperiod responsive genes were associated with lactation and thyroid signaling (*Tshr*). Using qRT-PCR we have further investigated the role of thyroid signaling and clock gene expression in mediating the response of the mouse mammary gland to photoperiod early in lactation. In summary, we report effects of photoperiod on the mammary transcriptome have identified key genes and pathways that may coordinate the effects of photoperiod on the mammary gland during lactation.

INTRODUCTION

Photoperiodism enables animals to measure day length, or photoperiod, to coordinate their internal biological calendar. Seasonal changes in photoperiod affect numerous aspects of physiology including reproduction, immune function, and behavior (Walton et al., 2011). Timing of reproduction based on photoperiodic cues ensures offspring are born when food is most plentiful, thereby increasing the survival of young (Hastings et al., 1985). In addition to timing of mating and subsequently parturition, photoperiod has substantial effects on the mammary gland and lactation.

As first reported by Peters and co-workers (Peters et al., 1978), and in many studies since, exposure of dairy cows to long day photoperiod (**LD**: 16 h light: 8 h dark) during lactation, increases milk production by 10-15% (~2 kg/d) (Dahl et al., 2000). Additionally, photoperiod manipulation alters mammary immune function, mammary cell-turnover and gene expression in dairy cows (Auchtung et al., 2005; Wall et al., 2005a; Wall et al., 2005b; Bentley et al., 2014).

Photoperiodic information is transmitted from the brain to the body through secretion of hormones, several of which also modulate mammary function. Melatonin, secreted from the pineal gland in response to light/dark cycles, has received substantial attention for its roles in coordination of circadian rhythms and breast cancer biology (Arendt, 1988; Blask et al., 2009). Prolactin, although best known for triggering the onset of lactation (Trott et al., 2012), is responsive to photoperiod and coordinates seasonal changes in physiology (Duncan, 2007). Thyroid hormones, which coordinate peripheral metabolism in support of lactation (Neville et al., 2002) have been identified as mediators of the reproductive response to photoperiod in Japanese quail (Yasuo et al., 2003). More recently, the molecular

mechanisms of thyroid signaling in response to photoperiod have been described in mice (Ono et al., 2008).

The mouse has been an essential model in understanding the molecular mechanisms of lactation (Lemay et al., 2007; Ramanathan et al., 2007; Wei et al., 2013). Previously, some laboratory mouse strains, specifically C57Bl/6, were considered unresponsive to photoperiod (Goto et al., 1989); however, more recent studies using C57Bl/6 mice have clearly demonstrated the responsiveness of this strain to photoperiod manipulation (Lang et al., 2003; Metz et al., 2006; Bur et al., 2010; Ciarleglio et al., 2011; Otsuka et al., 2012; Otsuka et al., 2014). Because of the wealth of lactation-related knowledge gleaned from mouse models, we chose to use C57Bl/6 mice as our model in which to study the functional molecular effects of photoperiod in the mammary gland.

Despite long-standing knowledge of seasonality of reproduction, the effects of photoperiod on mammary function and gene expression during lactation, a critical component of reproduction, have not been fully explored. Our aim was to identify photoperiod-responsive genes and pathways and relate effects on expression to changes in mammary gland function. We hypothesized that LD, relative to ND and SD, photoperiod would induce differential expression of genes that promote milk production. Here, we report the effects of photoperiod exposure during lactation on cell proliferation, the mammary transcriptome and have identified differentially expressed genes with functional importance to the mammary gland during lactation.

MATERIALS AND METHODS

Animal care

The University of Alberta Animal Care and Use Committee approved all animal procedures. Female C57BL/6 mice (n = 54) were obtained at 5 weeks of age from Charles River Laboratories. Mice were maintained on normal day (**ND**, 12 h light: 12 h dark) photoperiod and provided a diet of 9% (w/v) fat mouse chow (Diet Labs) and water for *ad libitum* consumption. Mice were housed (3/cage) in wire-top cages on wire racks to allow for unobstructed light exposure. At 7 weeks of age, females were mated in a 3:1 ratio with males and they remained together until vaginal plugs were detected or females were visibly pregnant. Females were housed together (n = 3/cage) until three days prior to expected parturition, at which point dams were transferred to individual cages. Nests were checked twice daily (8 am and 6 pm) for pups. When first observed, initial litter weights were recorded and litters were standardized to 8 pups (mean ± sd: 7.9 ± 1.2), after which there was no further adjustment of litters. Dams with their litters were then randomly assigned (n = 18 dams/photoperiod) to photoperiod treatments, each administered in a separate room, for the remainder of lactation.

Photoperiod treatments

Three lighting treatments were used in this study, LD, ND, and short day (**SD**: 8 h light: 16 h dark), all of which included rectangular light-dark transitions. Each photoperiod treatment was applied in one of three adjacent animal rooms that were continuously monitored for light intensity, relative humidity, and temperature using Hobo® data loggers. The photic phase began at 0600 h and ended at 2200, 1800, and 1400 hours, respectively.

The light intensity in treatment rooms was > 300 lux during the photic phase and < 20 lux during the scotophase. Staff entered treatment rooms only during the photic period.

Euthanasia and tissue collection

Mice were killed by CO_2 inhalation on days 5 (L5), 10 (L10) and 15 (L15) of lactation during the photic phase between 0600 and 1200 hrs. Mammary tissue from both 4^{th}-inguinal glands was dissected away from surrounding tissue and the supra-mammary lymph nodes were removed. The 4^{th}-right inguinal mammary gland was frozen in liquid nitrogen and stored at −80°C for subsequent use. Liver and spleen wet-weights were quantified directly after excision.

Bromodeoxyuridine injection and quantification

Lactating dams (n = 6/photoperiod treatment) were injected intraperitoneally with 10 μL/g of body weight of bromodeoxyuridine (BrdU) solution (10 mM, Amersham Biosciences) 2 h prior to sacrifice. The 4^{th}-left inguinal mammary gland was removed and frozen on dry ice for BrdU analysis. Samples were homogenized in 50 mM NaCl and 10 mM Tris HCl, pH 7.5 and frozen at -80°C. Homogenates were thawed and sonicated on ice for 30 seconds (s) followed by centrifugation at 1,500 x g for 15 minutes (min). Supernatant was retained and diluted 1:100 in water and stored at -20°C prior to analysis. Incorporated BrdU was detected using the mouse BrdU ELISA (Behl et al., 2006) kit following manufacturer's instructions for tissue homogenate (Bluegene Biotech). The concentration of double-stranded DNA was assessed using the Quant-iT dsDNA Assay Kit, Broad Range (Invitrogen™) following manufacturer's instructions. The concentration of incorporated

BrdU was normalized to the concentration of total dsDNA present in the 1:100 dilution of supernatant.

RNA isolation and microarray sample preparation

The entire 4th-right inguinal mammary gland was pulverized in liquid nitrogen using a mortar and pestle and a portion was used for RNA isolation using TRIzol Reagent (Invitrogen^{TM}) according to the manufacturer's instruction. Microarray preparation and analysis was conducted on samples collected on L10. Assays were done at the Alberta Transplant Applied Genomics Centre at the University of Alberta. Nucleic acid concentration was quantified using a NanoDrop ND1000 spectrophotometer and RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). Eighteen RNA samples (n = 6/photoperiod treatment, RNA integrity number $\geq$ 7, mean ± sd: 8.03 ± 0.86) were prepared for analysis using Roche Nimblegen 12x135K arrays following the manufacturer's instructions (for details http://www.nimblegen.com/support/dna-microarray-support.html). Briefly, cDNA was synthesized using dT primer and SuperScript II Reverse Transcriptase. Second strand cDNA was prepared using T4 DNA polymerase. Samples were labelled using Cy3 random primers and the Klenow fragment ('3 -> 5'exo-). Labelled samples were hybridized to the array chips using the Nimblegen Hybridization System. Arrays were scanned using MS 200 Microarray Scanner and a MS 200 Data Collection Software. Images were collected using Roche Nimblegen DEVA software and data were normalized by the quantile normalization method (Bolstad et al., 2003). Gene calls were generated using the Robust Multichip Average (RMA) method (Irizarry et al., 2003a; Irizarry et al., 2003b). Analysis of RMA values was conducted using Partek® Software. An IQR filter (IQR > 0.5) was used to eliminate probe sets with little variation. Remaining probe sets

were subjected to the following t-test comparisons: LD vs ND (LD_{ND}) and ND vs SD (SD_{ND}). Fold-change was identified from the mean differences of LD−ND and ND−SD; the resulting positive fold-change values indicated higher expression in LD and ND, respectively. Genes meeting the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$ were considered differentially expressed and were used in functional analysis of microarray data.

Quantitative real-time PCR

Total RNA (1 μg) was treated with one unit of DNase 1 (Invitrogen™) for 15 min at RT. The DNase was heat inactivated at 65°C for 10 min following the addition of 25 mM EDTA. Sufficient cDNA was prepared for the investigation of all genes described here using the Superscript Kit (Invitrogen™). Synthesis of cDNA from the DNase-treated RNA required l μL of 10 mM dNTP mix, 1 μL of oligo (dT) and nuclease-free water to 10 μL. This mixture was incubated at 65°C for 5 min then chilled on ice for 1 minute. A master mix of 2 μL of 10X RT buffer, 4 μL 25 mM $MgCl_2$ 2 μL 0.1 M DTT and 40 units of RNAase OUT was combined with the RNA/primer mixture and heated to 42°C for 2 min. The addition of 200 units of SuperScript™ II RT (Invitrogen™) was followed by incubation at 42°C for 50 min. The reaction was terminated at 70°C for 15 mins. Resulting cDNA was diluted 1:5 in nuclease free water for use in amplification reactions. A pool of undiluted cDNA from all samples was used to create a standard curve.

Primer sequences (**Table 4.1**) for genes of interest were obtained from the mouse qPrimerDepot (http://mouseprimerdepot.nci.nih.gov/) and were selected to have products ~100 bp in length, melting temperature ~60°C and to span exon-exon junctions. Reference gene primer sequences were selected based on previous assessment as reference genes in the mouse mammary gland (Han et al., 2010). The predicted PCR products were compared to

the *Mus musculus* genome database using Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/BLAST/) to confirm specificity of the primers to the mouse transcript sequence.

Quantitative real-time PCR reactions were set up in MicroAmp™ Optical 384-Well Reaction Plates (Applied Biosystems) in 10 µL reaction volumes. 5 µL of 2X Fast SYBR® Green Master Mix (Applied Biosystems) were combined with 2.5 µL of 0.10 µM forward and reverse primer mix and 2.5 µL diluted cDNA. Each sample was run in duplicate along with a five point standard curve and the no-template control. Reactions were performed using the ViiA™ 7 Real-Time PCR System (Applied Biosystems) under the following conditions: 20 s at 95°C, 40 cycles of 1 s at 95°C, 20 s at 60°C. A single PCR product was verified using the dissociation method. Data were analysed using the ViiA™ 7 Software v1.1. Expression of genes of interest was normalized to the BestKeeper© value based on the geometric mean generated from six stable reference genes (**Table 4.1)** using the method of Pfaffl and co-workers (Pfaffl et al., 2004).

Statistical analysis

Litter weights on the day of euthanasia, organ weights, BrdU incorporation and qPCR data ($n \geq 5$ dams/photoperiod) were analyzed using the ANOVA procedure in JMP® Pro 10, using the standard least squares method to detect effects of photoperiod, time and the interaction of photoperiod and time. Within-day comparisons were conducted using ANOVA procedure by day followed by means separation by Tukey-Kramer HSD test. Significance was declared at $p \leq 0.05$. Daily litter weight was assessed for the effect of photoperiod, time and the interaction using MANOVA procedure with the repeated measure of time in JMP® Pro 10.

Gene functions and network analysis

Gene function and network analysis was conducted using Ingenuity Pathway Analysis (**IPA**) software (Ingenuity® Systems, www.ingenuity.com). Probe sets from the comparisons of LD_{ND} and SD_{ND} that met the criteria for differential expression were uploaded to the software. Probe sets were evaluated using the Ingenuity Knowledge Base® and known genes were included in further analysis. Core analysis was conducted using the Ingenuity Knowledge Base® with the parameters set to include genes only, direct and indirect relationships, endogenous chemicals, and information from rat, mouse, and human species. Within IPA, we used the Benjamini-Hochberg corrected p-value score to account for multiple testing (Benjamini and Hochberg, 1995) when determining the significance of *biofunctions*. The grouping of genes into functional networks was done in IPA. The top three *networks* from each comparison, LD_{ND} and SD_{ND}, were identified based on significance scores and the number of focus molecules from our data sets. These scores, derived from p-values, represent the likelihood that focus genes are assigned to *networks* by random chance based on the number of focus genes and the number of genes in the entire network.

Intersection of genes identified in the comparisons of LD_{ND} and SD_{ND} to those published in three lactation-focused microarray studies (Lemay et al., 2007; Lemay et al., 2009; Wei et al., 2013) was conducted in IPA and common genes are reported. This analysis provided additional mammary-specific annotation for differentially expressed genes. The top five *molecular and cellular biofunctions* enriched by differentially expressed genes in our datasets were identified using *p-value of overlap* resulting from Fisher Exact t-test. The top five unique *functional annotations,* with > 1 associated gene, for each *biofunction* are presented.

RESULTS

Animal and organ weights

Dam body weight was affected by the day of lactation ($p = 0.01$) and photoperiod treatment ($p < 0.03$) **(Table 4.2)**. On average, dams exposed to LD photoperiod weighed more than those exposed to ND, but not SD photoperiod. Photoperiod affected spleen ($p = 0.02$) weight, mice exposed to SD photoperiod had heavier spleens than mice exposed to LD photoperiod, whereas ND exposed mice did not differ from SD or LD (**Table 4.2**). Photoperiod affected liver ($p = 0.01$) weight (**Table 4.2**). Liver weight was highest in mice exposed to LD photoperiod and was significantly heavier than mice exposed to SD, but not ND photoperiod. Liver weight increased from L5 to L10 ($p \leq 0.001$), although, not from L10 to L15. There was no interaction of photoperiod and day of lactation on dam, spleen, or liver weight.

Litter weight

Photoperiod affected ($p < 0.004$) litter weight prior to sacrifice on L5 (**Figure 4.1a**). Specifically, mice exposed to SD or LD photoperiod had heavier litters (SD_{ND}: $p = 0.02$; LD_{ND}: $p = 0.005$) than mice maintained on ND photoperiod. There was no difference between SD and LD exposed mice (LD vs SD: $p = 0.69$) on L5. There was no effect of photoperiod on L1, L10, or L15 (**Figure 4.1a**). There was no overall effect ($p = 0.87$) of photoperiod on litter weight during lactation but there was an effect of time on litter weight ($p \leq 0.001$) (**Figure 4.1b**).

BrdU incorporation

Within-day comparisons identified an effect of photoperiod on BrdU incorporation into DNA on L5 **(Figure 4.2)**. Dams exposed to LD photoperiod incorporated less BrdU ($p < 0.03$) than mice exposed to ND photoperiod, whereas dams exposed to SD did not differ from either LD or ND exposed mice. There was no significant effect of photoperiod in within-day comparisons on L10 or L15. Overall, day of lactation affected ($p \leq 0.001$) the incorporation of BrdU. Incorporation peaked on L10 and decreased by half on L15 **(Figure 4.2)**. Photoperiod did not have an overall effect ($p = 0.34$) on incorporation of BrdU during 15 days of lactation. There was also no interaction of photoperiod and day of lactation ($p = 0.25$).

Effects of photoperiod on mammary gland transcriptome

Microarray analysis revealed differential expression of 249 probe sets in the comparison LD_{ND} photoperiod (**Suppl. T4.1**). Of these, 230 were IPA-mapped genes, 195 of them were *analysis ready* and included in pathway analysis. Relative differential expression ranged from –3.1 to 3.3 fold. Of 195 genes, 135 were more abundant (negative fold-change) in mice exposed to ND, compared to LD photoperiod. The remaining 60 had relatively higher expression (positive fold-change) in mice exposed to LD, compared to ND photoperiod. Pathway analysis revealed gene networks enriched in the comparison of LD_{ND} photoperiod (**Table 4.3**, **Suppl. T4.3).** The top three IPA *networks* of differentially expressed genes in LD_{ND} include *lipid metabolism*, *small molecule biochemistry*, and *cell morphology*. We further investigated the expression of six of these genes by qPCR analysis (**Figure 4.3a-f**). During the first 15 days of lactation, photoperiod had an overall effect ($p \leq 0.05$) on *Tef*, *Cry2*, *Nr1d1*, *Per3*, *Dbp*, and *Sgk1*. The within-day effects of photoperiod are

shown individually on graphs (**Figure 4.3a-f**). Day of lactation affected ($p \leq 0.05$), *Tef*, *Cry2*, *Nr1d1*, and *Dbp*, but *Sgk1* was not affected by time ($p = 0.05$) nor was *Per3* expression ($p \leq 0.39$) on. Within IPA, 10 differentially expressed genes in the comparison of LD_{ND} were associated with thyroid-related functions or molecules (**Table 4.4**).

Our analysis identified differential expression of 866 probe sets in the comparison of SD_{ND} photoperiod (**Suppl. T4.2**). Of these, 787 were IPA-mapped genes, and 723 were *analysis ready* and included in pathway analysis. The relative differential expression ranged from -3.5 to 4.8 fold. The majority ($n = 430$) of these genes were more abundantly expressed (positive fold-change values) in mice exposed to ND photoperiod compared to SD; the remaining 293 had higher expression (negative fold-change) in SD compared to ND photoperiod. Gene networks associated with the comparison of SD_{ND} photoperiod included *humoral immune and inflammatory responses*, *haematological system development,* and *cellular growth and proliferation* (**Table 4.3, Suppl. T4.3**). Gene expression analysis of two genes, *Rab37* and *Tnf* by qRT-PCR did not recapitulate the microarray findings (**Figure 4.3g**, **4.4b**). Overall, *Tnf* was not affected ($p = 0.07$) by photoperiod and there were no within-day differences in *Tnf* expression evident from the qRT-PCR analysis. Within IPA, 38 differentially expressed genes in the comparison of SD_{ND} were associated with thyroid-related functions or molecules (**Table 4.4**).

There were 14 differentially expressed genes common in the comparisons of LD_{ND} and SD_{ND} **(Table 4.5).** Of these, 7 genes (*1700092C02Rik*, *Drd3*, *Jun*, *Phf20l1*, *Rab37*, *Spock2*, *Tbx1*) were consistently down-regulated by LD and SD, compared to ND, whereas 3 genes (*Crisp1*, *Ereg*, *Nipsnap3a*) were up-regulated by LD and SD photoperiod. Finally, 4 genes (*Efcab7*, *Glip2*, *Tshr* and *Zfp932*) were down-regulated by SD, but up-regulated by

LD, compared to ND photoperiod (**Table 4.5**). We further studied the expression of GLI pathogenesis-related 2 (*Glipr2*), a member of the RAS oncogene family (*Rab37*), and thyroid stimulating hormone receptor (*Tshr*) (**Figure 4.4**) based on their potential functional effects. Overall, there was an effect of photoperiod ($p < 0.004$) on *Glipr2* expression. Mice exposed to LD expressed more *Glipr2* than mice on SD photoperiod, whereas mice on ND did not differ from SD or LD exposed mice. *Glipr2 expression* was affected by time ($p < 0.001$), with expression being highest in all treatment groups on L5 (**Figure 4.4a**). There was no interaction ($p = 0.07$) of photoperiod and day of lactation on *Glipr2* expression. Within-day comparisons showed expression of *Glipr2* was higher ($p < 0.001$) in mice exposed to LD than SD photoperiod on L5 and L10, but there was no effect of photoperiod on L15 (**Figure 4.4a**). Although not significantly different from ND, the direction of change of LD and SD photoperiod relative to ND is in agreement with the microarray findings. Overall, *Rab37* expression did not differ ($p \leq 0.1$) by photoperiod, or by time ($p \leq 0.45$), nor was there a significant interaction ($p = 0.18$). Within-day comparisons on L10 showed no effect ($p = 0.07$) of photoperiod on *Rab37* expression (**Figure 4.4b**), although the direction of change relative to ND is in agreement with the microarray data for both SD_{ND} and LD_{ND}. Overall, expression of *Tshr* was affected by photoperiod ($p \leq 0.001$) with mice exposed to LD photoperiod expressing more *Tshr* than mice on SD photoperiod (**Figure 4.4c**). Expression of *Tshr* was not affected by day of lactation ($p \leq 0.84$) nor was there an interaction ($p \leq 0.31$). Within day comparisons showed LD photoperiod increased the expression of *Tshr* over SD photoperiod on L5 and L10 although this effect diminished by L15 (**Figure 4.4c**). Although not significantly different from ND, the direction of change of LD and SD photoperiod relative to ND is in agreement with the microarray findings.

Lactation-related gene expression

Genes (n = 95) responsive to photoperiod manipulation were common with the lactation-related genes (Lemay et al., 2007; Lemay et al., 2009; Wei et al., 2013). Analysis of the *biofunctions* enriched by these data sets indicated differing effects on mammary function through gene expression. In the comparison of $LD_{ND,}$ 31 lactation-related genes were functionally annotated with *lipid metabolism*, the *transport of ions* and *cell movement* (**Figure 4.5, Table 4.6**). In the comparison of SD_{ND}, 64 genes were in common the lactation-related genes and genes identified in the comparison of SD_{ND} (**Table 4.7**). These genes were functionally associated with *gene expression*, and *immune function* including *proliferation of lymphocytes* and *activation of leukocytes* (**Figure 4.5**).

DISCUSSION

Photoperiod, an external cue, provides an accurate measure of time of year, thereby facilitating anticipation of, and adaptation to, seasonal conditions. Photoperiod affects numerous aspects of metabolism, immune function and reproduction (Walton et al., 2011). Here, we report photoperiodic effects on body, liver, and spleen weight in lactating mice. A similar effect on liver weight in response to photoperiod, was recently reported in hamsters (Petri et al., 2014). Photoperiod affects metabolism through neuroendocrine signaling pathways and adjustment of circulating thyroid hormone concentrations. Typically, LD photoperiod increases food intake and body weight, whereas SD photoperiod has the inverse effect (Ebling and Barrett, 2008). Lactation, a metabolically demanding condition, is supported by changes in nutrient partitioning coordinated, in part, by thyroid hormone signaling (Neville et al., 2002). Our findings indicate mice exposed to LD photoperiod may

be more metabolically active than mice exposed to ND photoperiod, an effect that may be mediated by thyroid hormone signaling.

In laboratory studies, immune functional markers including spleen mass are typically increased in response to SD photoperiod (Nelson et al., 1995). Demas et al. (1996) reported that the enhanced immune cell function in deer mice exposed to SD photoperiod is linked to the reproductive responsiveness to photoperiod manipulation. The mammary gland, having evolved as an immune organ (Vorbach et al., 2006) is also photoperiod responsive. The reproductive response of dairy cows to photoperiod manipulation includes changes in milk yield, cell proliferation and the transcriptome of the mammary gland (Auchtung et al., 2005; Wall et al., 2005b; Bentley et al., 2014). Here, we show mice exposed to SD photoperiod which have increased spleen weight also undergo changes in mammary cell proliferation and transcriptional regulation, suggesting the responsiveness to photoperiod of the spleen and mammary gland may be linked in C57Bl/6 mice.

In mice, lactation occurs in three phases; milk production increases from L1 to L6, reaches a plateau, and finally decreases as weaning is approached (Knight and Peaker, 1982). Sorensen and Hacker (1979) reported the litters of 20 dams exposed to LD photoperiod gained significantly more weight during the first 15 days of lactation, compared to dams on SD photoperiod. In the current study, photoperiod affected terminal litter weight on L5. Daily litter weights from all dams included in the study (n = 18 on L5) did not support this finding. Although not all mice responded similarly, we surmise mammary function may be most susceptible to photoperiod manipulation during the phase of increasing milk production.

The quantity of milk produced is dependent on the number and activity of secretory cells in the mammary gland (Capuco et al., 2001). We report no overall effect of

photoperiod on litter weight over 15 days of lactation. Although, on L5, LD photoperiod had an inhibitory effect on mammary cell proliferation. During development, melatonin, which is more abundant under SD conditions, has inhibitory effects on mammary growth (Mediavilla et al., 1992). However, in mature dairy cows, exposure to SD photoperiod increased mammary cell proliferation 3 weeks prior to parturition, compared to LD exposed cows (Wall et al., 2005b). In women, suppression of melatonin secretion by light exposure during the dark phase is thought to promote carcinogenesis (Blask et al., 2002). Together with our findings, it appears LD and SD photoperiod do not each have a discrete stimulatory or inhibitory effect on mammary cell proliferation. Rather, it appears the effect of photoperiod on mammary cell proliferation depends on the stage of development and possibly time relative to lactation.

We report, for the first time, the effects of photoperiod manipulation during lactation on the mammary transcriptome of mice. Among the genes identified in the comparison of LD_{ND}, five genes: *Per3*, *Dbp*, *Tef*, *Nr1d1 (Rev-erbα)* and *Cry2*, are of key interest for their role in circadian biology. Substantial work in the last twenty years has elucidated the molecular mechanisms of the circadian clock, and its role as an intrinsic daily timekeeper (Takahashi, 1992; Reppert and Weaver, 2001; Duffield, 2003; Hastings et al., 2007). Recent investigations have established the expression of clock genes in the mammary gland and their impact on mammary function. Metz and co-workers (2006) characterized the expression of clock genes in HC-11 cells and the developing mouse mammary gland. Their work shows clock gene expression rhythm is dependent on developmental stage, suggesting clock genes may have mammary-specific functions during development and differentiation. In the mammary gland, 7% of all expressed genes have circadian patterns of expression, including

the core clock genes *Arntl*, *Cry1*, *Cry2*, *Clock*, *Csnk1ε* and *Per1-3* (Maningat et al., 2009; Maningat et al., 2011). Casey and co-workers (Casey et al., 2009), working in rats, reported that a number of core clock genes were differentially regulated in the mammary gland, liver and adipose tissue, between the end of pregnancy and early lactation. They went on to suggest differential expression of clock genes might affect hormonal signaling pathways to coordinate the onset of lactation (Plaut and Casey, 2012). Given the importance of timing reproduction with the seasons, the connection between photoperiod, circadian gene expression, and lactation is logical.

Differential expression of gene common to in both SD_{ND} and LD_{ND} comparisons suggests those 14 genes (**Table 4.5**) may have core roles in mediating the mammary response to photoperiod manipulation. Of the common genes, *Glipr2* and *Tshr*, were affected by photoperiod on L5 and L10, but not on L15. Thyroid signaling, which was associated with a total of 48 photoperiod-responsive genes in our data set, is closely tied to seasonal changes in physiology in many species (Walton et al., 2011; Dardente et al., 2014). In mice, expression of thyroid stimulating hormone (*Tsh*) in the *pars tuberalis* is increased in LD and decreases in SD photoperiod (Ono et al., 2008). During lactation, reduced concentrations of thyroid hormones in the blood may decrease metabolism in other peripheral tissues to support the metabolic demands of milk production, for review see (Neville et al., 2002). In dairy cows, Capuco and co-workers (Capuco et al., 2008) reported differential expression of deiodinase 2 (DIO2), deiodinase 3 (DIO3) and thyroid hormone receptors during the initiation of lactation. They proposed that increased local conversion of the inactive (T4) to the active (T3) form would increase the sensitivity of the mammary gland to thyroid hormones (Capuco et al., 2008). Here, *Tshr*, *Dio3*, and 36 other thyroid-related genes were differentially expressed in

the comparison of SD_{ND}. Two genes, thyroid embryonic factor (*Tef*) and D site of albumin promoter binding protein (*Dbp*), which are not annotated as thyroid-related in IPA, were also responsive to photoperiod. Dardente and coworkers proposed that Tef along with Dbp make up a molecular switch for photoperiod responsiveness (Dardente et al., 2010). In the mammary gland, others have reported *Tef* and *Dbp* are up-regulated during lactation (Lemay et al., 2007). Taken together, these data provide further evidence supporting a key role of thyroid signaling in mediating the effects of photoperiod in the mammary gland.

To overcome the limited lactation-related annotation, we identified those genes that have previously been associated with lactation in selected microarray studies (Lemay et al., 2007; Lemay et al., 2009; Wei et al., 2013). These three studies provided additional mammary-specific annotation for differentially expressed genes. Notable genes from the comparison of LD_{ND} function in water transport (*Aqp3*), lipid metabolism and transport (*Abcg2*, *Fads1*, *Mpst*) and ion transport (*Cysltr1*, *Sgk1*, *Slc16a1*). These functions align with the top IPA networks enriched by the complete LD_{ND} data set. Fatty acid desaturase 1 (*Fads1*) has a role in the synthesis of very-long-chain fatty acids for milk secretion and is highly expressed during the first half of lactation in dairy cows (Bionaz and Loor, 2008). In the same study, Bionaz and Loor reported that *Abcg2* (ATP-binding cassette, sub family G, member 2) undergoes a 30-fold increase in mRNA abundance during lactation, relative to 15 days prior to parturition; suggesting a lactation-supportive function (Bionaz and Loor, 2008).

Lactation-related genes identified in the comparison of SD_{ND} were broadly associated with *cell cycle* (e.g. *Anapc5*, *Cdkn2c*, *Dnajc2*, *Dsn1*), or *activation of immune cells* (e.g. *Cd44*,*-48*, *-96*, *Fcgr2b*, *Hpse*, *Srsf5*, *Vamp7*). Fc gamma receptor IIB (*Fcgr2b*) is a negative regulator of immune cell activation and is required for the pro-apoptotic activity of death-

receptor 5, a key target of breast cancer therapy (Li and Ravetch, 2012). Heparanase (HPSE), is an enzyme that, in addition to facilitating cell invasion by way of inflammation and angiogenesis, promotes cell proliferation, including tumor metastasis (Parish et al., 2001; Vlodavsky and Friedmann, 2001; Cohen et al., 2006). Virgin mice over-expressing heparanase have extensive alveolar and ductal development relative to their wild type counterparts (Zcharia et al., 2004). Differential expression of these genes further suggests that SD photoperiod may affect mammary immune function and proliferation. Although it is not currently clear how these genes may function in the mammary gland during lactation, their responsiveness to photoperiod may relevance in both dairy cows and humans.

Most prior studies of the effects of photoperiod on lactation included only two photoperiod treatments, typically SD and LD. Although the subsequent comparison (LD vs SD) provides valuable information, it does not distinguish the effects of either from ND photoperiod. Here, our inclusion of ND photoperiod permits the comparisons of LD_{ND} and SD_{ND}. Subsequently, we have determined that SD and LD photoperiod do not simply affect the mammary gland in opposite ways; rather, the effects on gene expression are far more complex. More broadly, summer does not simply have the opposite effect of winter. The comparisons of SD_{ND} and LD_{ND} resulted in gene lists that had a small number of genes in common but mostly differ both in specific differentially expressed genes and predicted functional outcomes. Here we have shown that SD and LD, relative to ND, affect body, spleen, liver weight, and mammary gene expression in different same ways.

In conclusion, our findings support our initial hypothesis that altered photoperiod would induce differential expression of genes that could result in altered mammary physiology and function, Although, we did not detect an effect of photoperiod on litter

weight, many of the photoperiod-responsive genes identified here have known mammary functions and are associated with lactation. The response of the mouse mammary gland to photoperiod includes effects on the transcriptome, with LD and SD photoperiod affecting gene expression in markedly different ways. We provide evidence that physiological adaptation to photoperiod during lactation is coordinated through circadian and thyroid-related gene expression. Genes identified here are targets for future studies of the mechanisms underlying the effects of photoperiod on lactation and the mammary gland.

Table 4.1. Primer pairs used in quantitative real-time PCR of mouse mammary tissue[1].

Gene Symbol	Ref. Sequence	Sense Primer (5' – 3')	Anti-Sense Primer (5' – 3')	Amplicon (bp)
Cry2 (variant1)	NM_009963	CTCGTCTGTGGGCATCAAC	TCCCCGGACTACAAACAGAC	103
Dbp	NM_016974	TCTTGCAGCTCCTCTTCCC	GTGTCTGGGTCCACAGGACT	128
Glipr2	NM_027450	AGGCCATGGGCAAATCAG	TTCTTACAAAGCTTCAGGGGC	106
Nr1d1	NM_145434	CCAGTTTGAATGACCGCTTT	AGGAGCCACTAGAGCCAATG	101
Per3	NM_011067	GTGAAGCCAGTGGCAGAGA	CCAGTATCCGTGGTGCTTTT	104
Rab37	NM_021411	AACTACGATCTCACCGGCAA	CTATGAAGGTTCCGGACAGG	109
Sgk1	NM_011361	GTCCTCCATAAGCAGCCGTA	CCGTGTTCCGGCTATAAAAC	106
Tef (variant 1)	NM_017376	GCAGAGCTTGAAGGAAAGGA	AGGACGATTCTGTGCTGGAC	109
Tnf	NM_013693	CCACCACGCTCTTCTGTCTAC	AGGGTCTGGGCCATAGAACT	103
Tshr	NM_011648	CAAGGAGCTCCACCGAATC	ATTGGGCAGACTCGAAAATG	109
Reference Genes[2]				
Actb	NM_007393	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA	171
B2m	NM_009735	CATGGCTCGCTCGGTGACC	AATGTGAGGCGGGTGGAACTG	166
Cyc1	NM_025567	CCAGGTATACAAGCAGGTGTGCTC	CATCATTAGGGCCATCCTGGAC	140
Gapdh	NM_008084	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG	150
Tuba1a	NM_011653	AAGGAGGATGCTGCCAATAA	GCTGTGGAAAACCAAGAAGC	135
Ubc	NM_019639	AGCCCAGTGTTACCACCAAG	ACCCAAGAACAAGCACAAGG	97

[1.] Primer sequences were obtained from the mouse qPrimerDepot (http://mouseprimerdepot.nci.nih.gov/).
[2.] Reference gene sequences were obtained from (Han et al., 2010).

Table 4.2. Exposure to photoperiod[1] during lactation affects dam body, spleen and liver weight[2].

		Long Day		Normal Day		Short Day		
	Day of Lactation	**Weight (g) ± sd**		**Weight (g) ± sd**		**Weight (g) ± sd**		**Means by Time[3]**
Body	**5**	33.5	±2.2	27.9	±3.6	30.6	±2.9	**30.5[b]**
	10	33.2	±4.1	32.7	±1.2	31.9	±2.3	**32.6[ab]**
	15	34.1	±1.9	33.0	±1.7	33.0	±1.9	**33.4[a]**
Means by Photoperiod[3]		**33.60[a]**		**31.21[b]**		**31.83[ab]**		
		Weight (mg) ± sd		**Weight (mg) ± sd**		**Weight (mg) ± sd**		
Spleen	**5**	3.98	±0.4	4.07	±0.7	4.42	±0.5	**4.17**
	10	4.20	±0.4	3.74	±0.3	4.50	±0.5	**4.15**
	15	3.90	±0.2	4.73	±1.0	4.89	±0.8	**4.52**
Means by Photoperiod[3]		**4.04[b]**		**4.19[ab]**		**4.59[a]**		
Liver	**5**	66.6	±3.7	67.6	±10.8	60.7	±3.0	**64.9[b]**
	10	72.3	±5.4	73.2	±1.77	67.8	±8.7	**71.1[a]**
	15	79.0	±3.3	74.9	±3.6	70.5	±9.7	**74.8[a]**
Means by Photoperiod[3]		**73.0[a]**		**71.9[ab]**		**66.3[b]**		

[1.] Photoperiods: long day (16 h light: 8 h dark), normal day (12 h light: 12 h dark) and short day (8 h light: 16 h dark).

[2.] The effects of time, photoperiod, and the interaction of photoperiod and time were determined using the ANOVA procedure in JMP® Pro 10.

[3.] Separation of means by photoperiod or time were conducted using Tukey-Kramer HSD test, differences were characterized as significant at $p \leq 0.05$ and are indicated by differing superscript letters (a, b).

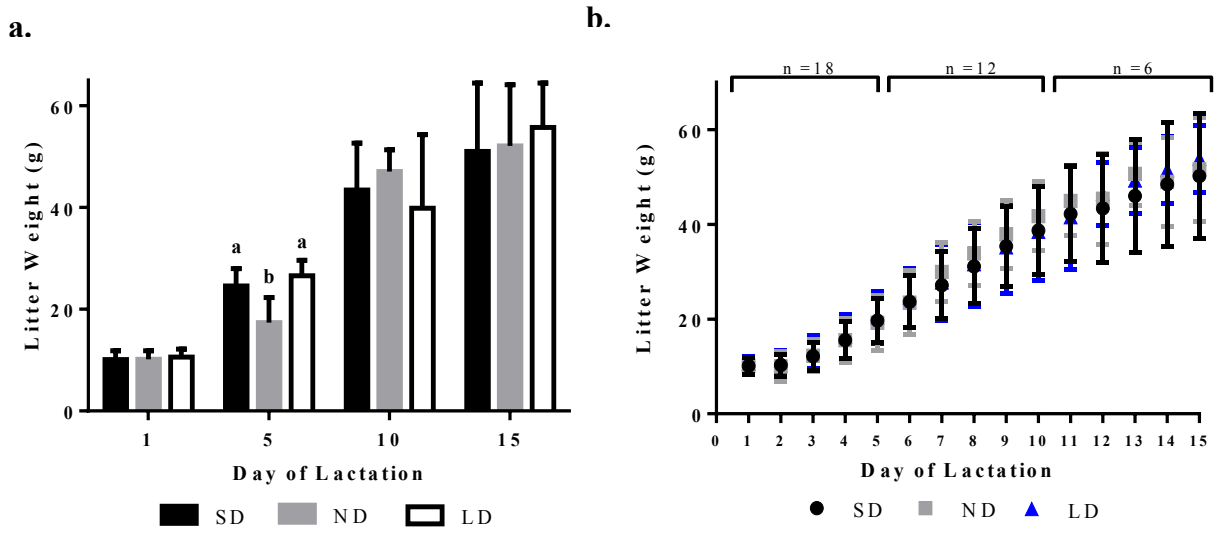


Figure 4.1. Photoperiod manipulation during lactation does not affect litter weight.

a. Initial (day 1 of lactation) and terminal litter weights of mice exposed to long day (LD: 16 h light: 8 h dark), normal day (ND: 12 h light: 12 h dark) or short day (SD: 8 h light: 16 h dark) photoperiod. The mean ± sd ($n \geq 5$ litters) is plotted, distinct letters denote significant differences for within-day comparisons at $p \leq 0.05$. **b.** Daily litter weights (mean ± sd) of all mice included in study. Respective *n* are indicated by brackets.

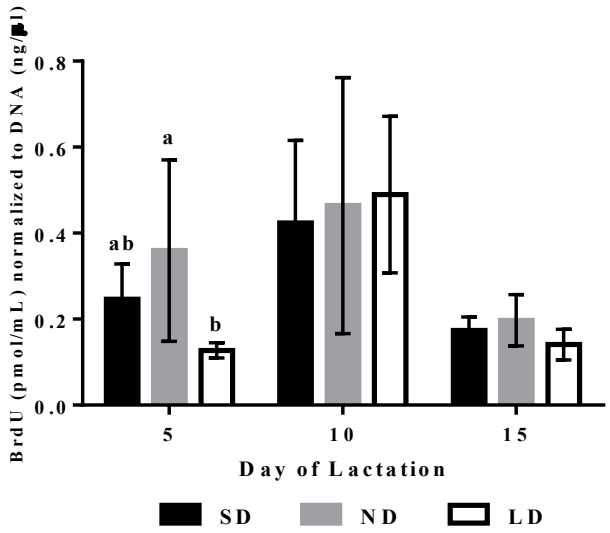


Figure 4.2. Photoperiod affects the incorporation of BrdU into mouse mammary cells early in lactation.

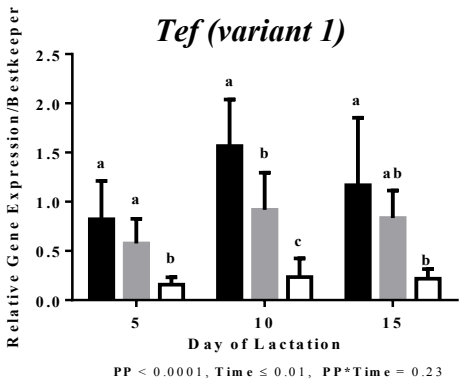
Mice were exposed to long day (LD: 16 h light: 8 h dark), normal day (ND: 12 h light: 12 h dark) or short day (SD: 8 h light: 16 h dark) photoperiod for 5, 10, or 15 days of lactation. Cell proliferation was quantified by analysis of BrdU incorporation normalized to the total double-stranded DNA content. Within-day differences were considered significant at $p \leq 0.05$ and are indicated by distinct letters (a, b).

Table 4.3. Top three IPA networks of differentially expressed[1] (DE) genes in the comparison of LD_{ND} and SD_{ND} in the mammary gland of lactating mice[2].

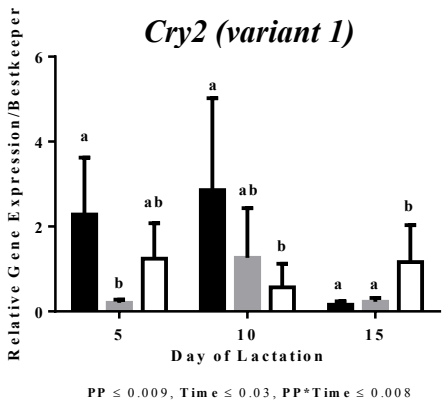
	Network	IPA Score[4]	DE Genes	Focus Genes[5]
LD_{ND}[3]				
1	Lipid Metabolism, Small Molecule Biochemistry, Gene Expression	44	23	↓ ***Per3***, *Nppb*, ***Dbp***, ***Tef***, *Nr1d2*, ***Nr1d1***, *Tbx1, Svs5, Trib3, St3gal1, Adcy9,* ***Sgk1***, *Foxs1, Sftpd, Rnasel,* ***Cry2*** ↑ *Cdkn1a, Hif3a, Gcnt1, Dhx58, Atr, Prl, Igha1*
2	Cell Movement, Reproductive System Development and Function, Cell Morphology	34	19	↓ *Lamb1, Aqp3, Gng4, Rasgrp1, Plce1, Ca4, Bcar1, Stk17b, Prox1, Chat, Tpo, Tcl1a, Lrp8* ↑ *Msr1, Lamc2, Pde8b, Mmp8, Rgs7, Igkc*
3	Nucleic Acid Metabolism Small Molecule Biochemistry, Cell Signalling	29	17	↓ *Mchr1, Ramp1, Drd4, Jun, Drd3, Lphn2, Ccr8, Gnal, Angptl2, Lpar6* ↑ *Ppp1r9a, Efcab7, Cysltr1, Trim5, Gpr98, Avpr1a,* ***Tshr***
SD_{ND}[3]				
1	Humoral Immune Response, Inflammatory Disease, Inflammatory Response	38	26	↓ *Adh4, Srsf5, Elpla2g16* ↑ *Cd48,* ***Rab37****, Calcb, Ier2, Scara5, Banp, Tbxas1, Tial1, Kif20a, Glis2 Ltbp2, Mgst2, Nfkbie, Crlf1, St3gal6, Kynu, Pstpip1, Pmpca,* ***Tnf****, Il27ra, Cd209b, Cd2avl2*
2	Hematological System Development and Function, Hematopoiesis, Tissue Morphology	30	23	↓ *Rag2, Magi2, Grb14* ↑ *Tcrb-J, Flt3lg, Magi1, Lax1, Il2rg, Cmahp, Pag1, B4galt5, Lat, Tcf7, Acap1, Pik3cd, Il2rb, Skap1, Il7r, Cd3g, Cd37, Lck, Cd8a, Cd3d*
3	Cellular Growth and Proliferation, Cellular Development,	28	21	↓ *Ifna1/Ifna13* ↑ *Nfkbid, Clip2, Pdcd11, Sp100, Senp6, Pdlim2, Tlr13, Ltc4s, Tlr1, Bnip2, Eomes, Fgl2, Txk, Adamts9, Il10ra, Madd, Icoslg, Rhoh, Cd180, Cd83*

1. Microarray analysis was used to quantify relative expression of genes in the mammary gland. Genes were considered differentially expressed if they met the criteria of fold-change ≥ |1.5| and $p \leq 0.05$.
2. Photoperiods: long day (LD: 16 h light: 8 h dark), normal day (ND: 12 h light: 12 h dark) and short day (SD: 8 h light: 16 h dark).
3. LD_{ND} ↓ = negative fold-change (expression higher in ND than LD photoperiod); SD_{ND} ↑ = positive fold-change (expression higher in ND than SD photoperiod).
4. IPA Score: derived from p-values, represent the likelihood that focus genes are assigned to networks by random chance based on the number of focus genes and the number of genes in the entire network (IPA, 2005).
5. **Bolded** molecules were further analysed using quantitative real-time PCR.

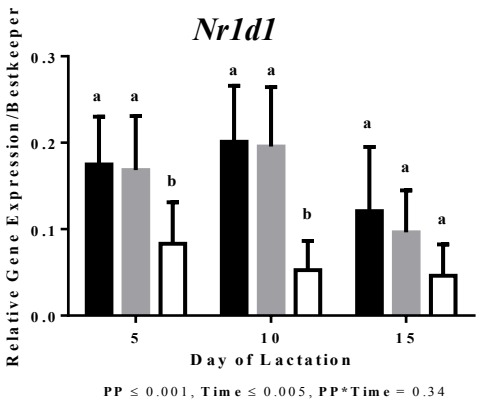
a.


Tef (variant 1)
Relative Gene Expression/Bestkeeper
Day of Lactation
PP < 0.0001, Time ≤ 0.01, PP*Time = 0.23

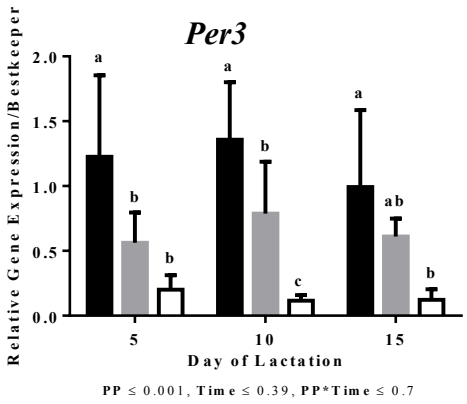
b.


Cry2 (variant 1)
Relative Gene Expression/Bestkeeper
Day of Lactation
PP ≤ 0.009, Time ≤ 0.03, PP*Time ≤ 0.008

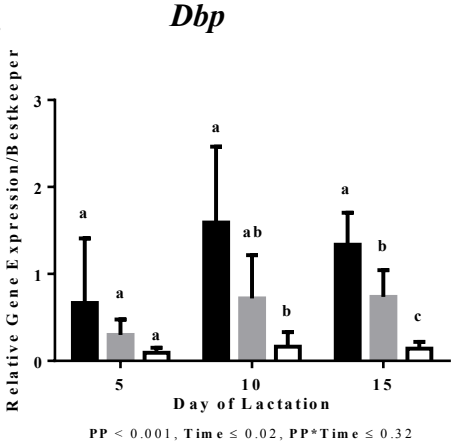
c.


Nr1d1
Relative Gene Expression/Bestkeeper
Day of Lactation
PP ≤ 0.001, Time ≤ 0.005, PP*Time = 0.34

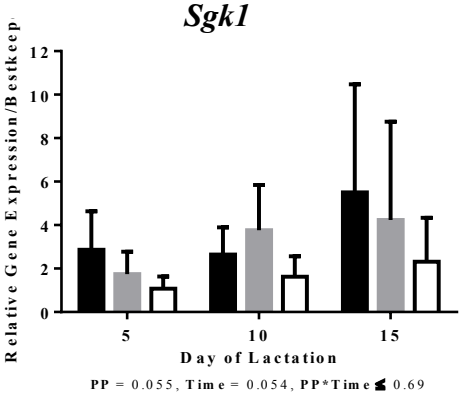
d.


Per3
Relative Gene Expression/Bestkeeper
Day of Lactation
PP ≤ 0.001, Time ≤ 0.39, PP*Time ≤ 0.7

e.


Dbp
Relative Gene Expression/Bestkeeper
Day of Lactation
PP < 0.001, Time ≤ 0.02, PP*Time ≤ 0.32

f.


Sgk1
Relative Gene Expression/Bestkeep
Day of Lactation
PP = 0.055, Time = 0.054, PP*Time ≤ 0.69

g.

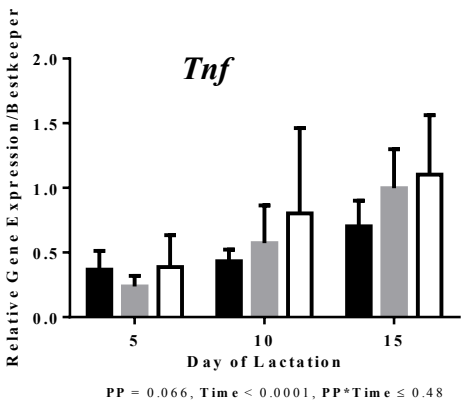

Tnf
Relative Gene Expression/Bestkeeper
Day of Lactation
PP = 0.066, Time < 0.0001, PP*Time ≤ 0.48

Figure 4.3. Expression of genes identified in the functional analysis of photoperiod exposure during lactation in mice.

Mice were exposed to long day (LD: 16 h light: 8 h dark), normal day (ND: 12 h light: 12 h dark) or short day (SD: 8 h light: 16 h dark) for 5, 10 or 15 days of lactation. Mammary tissue was excised post-mortem and gene expression measured using quantitative real-time PCR for seven genes **a.** *Tef var 1*, **b.** *Cry2 var1*, **c.** *Nr1d1*, **d.** *Per3*, **e.** *Dbp*, **f.** *Sgk1*, **g.** *Tnf*. The p-values for the effects of photoperiod (PP), time and the interaction of photoperiod and time are shown below each graph. Distinct letters (a, b, c) above bars indicate significant differences of within-day comparisons at $p \leq 0.05$.

Table 4.4. Differentially expressed[1] genes associated with thyroid signalling in the comparisons of LD_{ND} and SD_{ND} photoperiod[2].

Gene Symbol	Entrez Gene Name	Fold-Change	p-value
LD_{ND}[3]			
Atp1a3	ATPase Na+/K+ transporting, alpha 3 polypeptide	2.14	0.036
Ccnb1	Cyclin B1	1.54	0.008
Cdkn1a	Cyclin-dependent kinase inhibitor 1A	1.52	0.046
Csf2	Colony stimulating factor 2	-1.81	0.000
Il7	Interleukin 7	1.96	0.007
Lamc2	Laminin, gamma 2	1.55	0.013
Mchr1	Melanin-concentrating hormone receptor 1	-2.15	0.043
Tbx1	T-box 1	-1.86	0.021
Tpo	Thyroid peroxidase	-1.56	0.014
Tshr	Thyroid stimulating hormone receptor	2.26	0.001
SD_{ND}[5]			
Agrp	Agouti related protein homolog (mouse)	1.96	0.014
Atp1a3	ATPase, Na+/K+ transporting, alpha 3 polypeptide	-2.89	0.010
Cd44	CD44 molecule (Indian blood group)	1.78	0.002
Cd48	CD48 molecule	1.51	0.008
Cd83	CD83 molecule	1.91	0.006
Cdk5r1	Cyclin-dependent kinase 5, regulatory subunit 1	1.56	0.030
Cdkn2c	Cyclin-dependent kinase inhibitor 2C	1.85	0.011
Crym	Crystallin, mu	-1.57	0.043
Cxcl2	Chemokine (C-X-C motif) ligand 2	2.04	0.030
Dio3	Deiodinase, iodothyronine, type III	1.60	0.033
Duox2	Dual oxidase 2	-2.44	0.049
Ets1	v-Ets oncogene homolog 1	1.60	0.036
Flt3lg	Fms-related tyrosine kinase 3 ligand	1.53	0.002
Glis2	GLIS family zinc finger 2	1.65	0.042
Hhex	Hematopoietically expressed homeobox	1.77	0.005
Hif1a	Hypoxia inducible factor 1, alpha subunit	2.83	0.007
Ifi16	Interferon, gamma-inducible protein 16	-1.91	0.047
Il1a	Interleukin 1, alpha	1.57	0.017
Itga5	Integrin, alpha	1.52	0.042
Lck	Lymphocyte-specific protein tyrosine kinase	3.53	0.005
Lep	Leptin	1.52	0.045
Myh7	Myosin, heavy chain 7	-2.51	0.021
Npy	Neuropeptide Y	-1.73	0.000
Pcsk2	Proprotein convertase subtilisin/kexin type 2	-1.79	0.029
Ppargc1a	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	-1.63	0.026
Ptgds	Prostaglandin D2 synthase 21kDa	-1.53	0.041
Ptprc	Protein tyrosine phosphatase, receptor type, C	1.60	0.023
Rarb	Retinoic acid receptor, beta	1.53	0.047
Rassf2	Ras association domain family member 2	1.79	0.009
Runx2	Runt-related transcription factor 2	1.55	0.002
Slco4a1	Solute carrier organic anion transporter family 4A1	-1.94	0.020
Tbx1	T-box 1	1.64	0.035
Tnf	Tumor necrosis factor	2.38	0.001
Trpv5	Transient receptor potential cation channel, subfamily V, member 5	-1.98	0.030
Tshr	Thyroid stimulating hormone receptor	1.60	0.013
Ttr	Transthyretin	-2.23	0.015

Table 4.4 continued

Wif1	WNT inhibitory factor 1	-1.82	0.027
Wnt11	Wingless-type MMTV integration site family 11	-1.54	0.017

[1.] Microarray analysis using the Nimblegen 12x132K mouse microarray platform was used to quantify relative expression of genes in the mammary gland. Genes were considered differentially expressed if they met the criteria of fold-change $\geq |1.5|$ and $p \leq 0.05$.

[2.] Photoperiods: long day (LD: 16 h light: 8 h dark), normal day (ND: 12 h light: 12 h dark), short day (SD: 8 h light: 16 h dark).

[3.] LD_{ND}: negative fold-change (expression higher in ND than LD photoperiod).

[4.] SD_{ND}: positive fold-change (expression higher in ND than SD photoperiod).

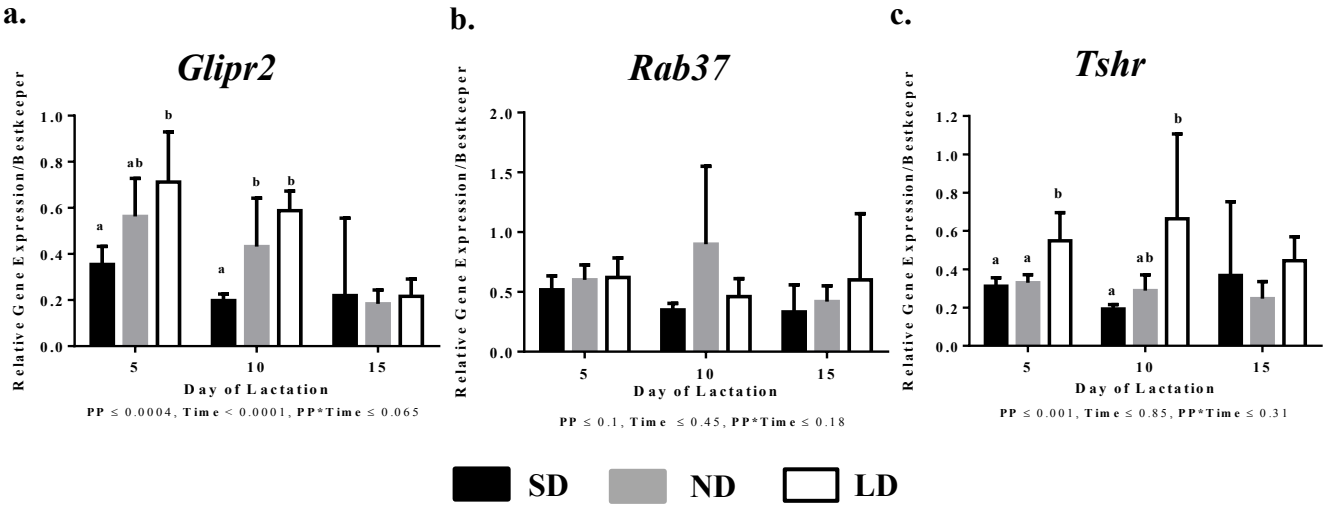


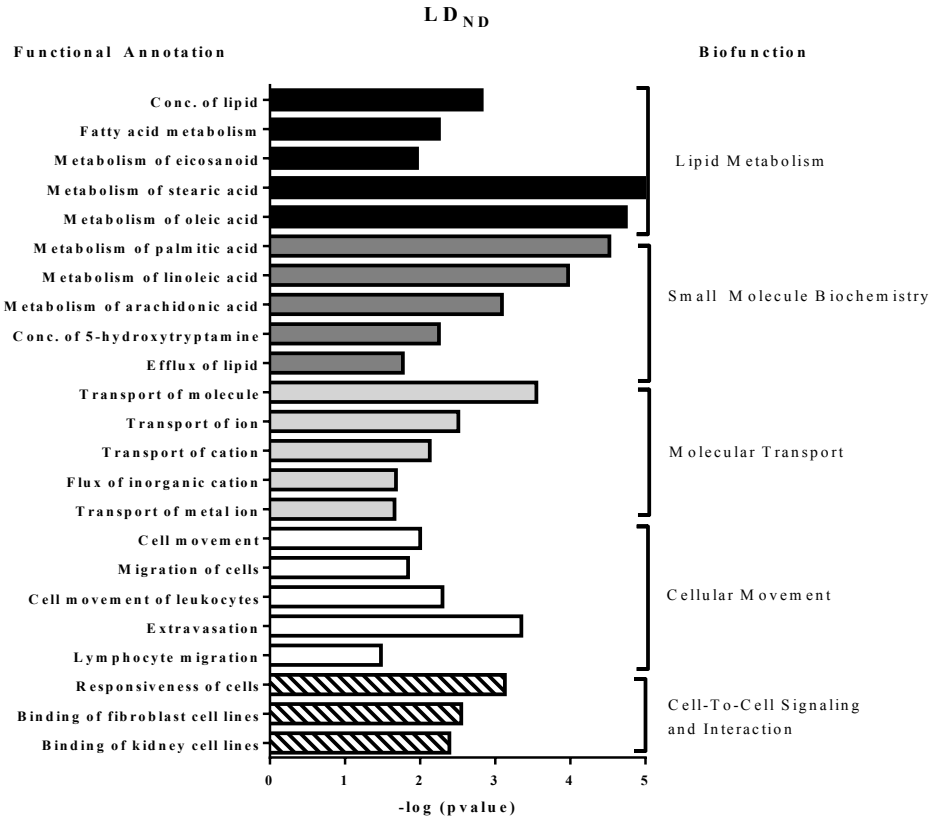
Figure 4.4. Quantitative RT-PCR of genes common in the comparisons of LD_{ND} and SD_{ND} photoperiod.

Mice were exposed to long day (LD: 16 h light: 8 h dark), normal day (ND: 12 h light: 12 h dark) or short day (SD: 8 h light: 16 h dark) for 5, 10, or 15 days of lactation. Mammary tissue was excised post-mortem and gene expression measured using quantitative real-time PCR for three genes **a.** *Glipr2* **b.** *Rab37* and **c.** *Tshr.* The p-values for the effects of photoperiod (PP), time and the interaction of photoperiod and time are shown below each graph. Distinct letters (a, b) above bars indicate significant differences of within-day comparisons at $p \leq 0.05$.

Table 4.5. Differentially expressed[1] genes common in the comparisons LD_{ND} and SD_{ND} photoperiod[2].

Gene Symbol	Entrez Gene Name	Relative Expression[3]		
		LD	ND	SD
1700092C02Rik	RIKEN cDNA 1700092C02 gene			
Crisp1	Cysteine-rich secretory protein 1			
Drd3	Dopamine receptor D3			
Efcab7	EF-hand calcium binding domain 7			
Ereg	Epiregulin			
Glipr2	GLI pathogenesis-related 2			
Jun	Jun proto-oncogene			
Nipsnap3a	Nipsnap homolog 3A			
Phf20l1	PHD finger protein 20-like 1			
Rab37	*Rab37*, member RAS oncogene family			
Spock2	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan 2			
Tbx1	T-box 1			
Tshr	Thyroid stimulating hormone receptor			
Zfp932	Zinc finger protein 932			

1. Microarray analyses using the Nimblegen 12x132K mouse microarray platform was used to quantify the relative expression of genes in the mammary gland. Genes were considered differentially expressed if they met the criteria of fold-change $\geq |1.5|$ and $p \leq 0.05$.
2. Photoperiods: long day (LD: 16 h light: 8 h dark), normal day (ND: 12 h light: 12 h dark), short day (SD: 8 h light: 16 h dark).
3. Heat map indicating relative robust multichip averages. Green = low, Yellow = medium, Red = high.


LD ND
Functional Annotation
Biofunction
Conc. of lipid
Fatty acid metabolism
Metabolism of eicosanoid
Metabolism of stearic acid
Metabolism of oleic acid
Metabolism of palmitic acid
Metabolism of linoleic acid
Metabolism of arachidonic acid
Conc. of 5-hydroxytryptamine
Efflux of lipid
Transport of molecule
Transport of ion
Transport of cation
Flux of inorganic cation
Transport of metal ion
Cell movement
Migration of cells
Cell movement of leukocytes
Extravasation
Lymphocyte migration
Responsiveness of cells
Binding of fibroblast cell lines
Binding of kidney cell lines
Lipid Metabolism
Small Molecule Biochemistry
Molecular Transport
Cellular Movement
Cell-To-Cell Signaling and Interaction
0
1
2
3
4
5
-log (pvalue)

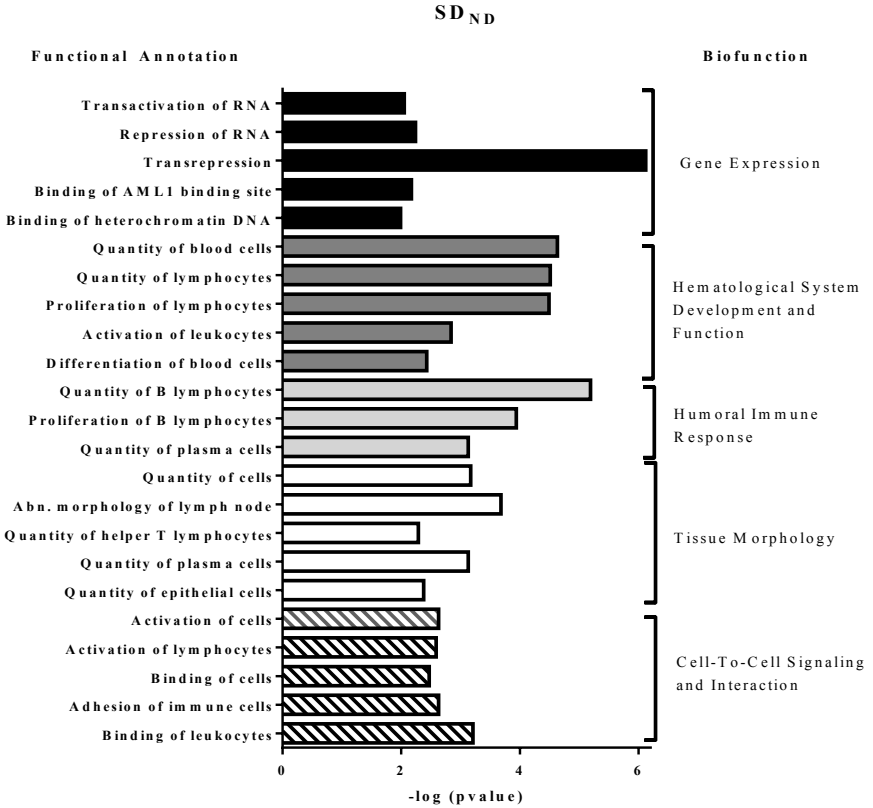

SD ND
Functional Annotation
Biofunction
Transactivation of RNA
Repression of RNA
Transrepression
Binding of AML1 binding site
Binding of heterochromatin DNA
Quantity of blood cells
Quantity of lymphocytes
Proliferation of lymphocytes
Activation of leukocytes
Differentiation of blood cells
Quantity of B lymphocytes
Proliferation of B lymphocytes
Quantity of plasma cells
Quantity of cells
Abn. morphology of lymph node
Quantity of helper T lymphocytes
Quantity of plasma cells
Quantity of epithelial cells
Activation of cells
Activation of lymphocytes
Binding of cells
Adhesion of immune cells
Binding of leukocytes
Gene Expression
Hematological System Development and Function
Humoral Immune Response
Tissue Morphology
Cell-To-Cell Signaling and Interaction
0
2
4
6
-log (pvalue)

Figure 4.5. IPA *Biofunctions* and *functional annotation* for lactation-related genes differentially expressed in response to photoperiod.

Microarray analysis was used to quantify relative gene expression in the mammary gland of mice exposed to long day (LD; 16 h light: 8 h dark), normal day (ND; 12 h light: 12 h dark), or short day (SD; 8 h light: 16 h dark) for the duration of lactation. Genes were considered differentially expressed in the comparison of **a.** LD_{ND} or **b.** SD_{ND} if they met the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$. IPA *biofunctions* and associated *functional annotation* with > 1 associated differentially expressed gene were ranked by –log (p-value), an estimate of whether the group is over-represented in the data set. The top five *biofunctions* by p-value are shown; redundant *functional annotations* are not shown.

Table 4.6. Differentially expressed[1] genes in comparison of LD_{ND} photoperiod[2] in mice that are associated with lactation[3].

Gene Symbol	Entrez Gene Name	Genbank ID	Fold-Change[4]	p-value
Abcg2	ATP-binding cassette, sub-family G2	AK142528	-1.65	0.037
Angptl2	Angiopoietin-like 2	AK196015	-1.54	0.013
Aqp3	Aquaporin 3	BC027400	-2.02	0.032
Ca4	Carbonic anhydrase IV	BC012704	-1.61	0.048
Cysltr1	Cysteinyl leukotriene receptor 1	BC027102	1.54	0.030
Cytip	Cytohesin 1 interacting protein	AK164790	-1.93	0.041
Dbp	D site of albumin promoter binding protein	BC018323	-2.35	0.006
Dhodh	Dihydroorotate dehydrogenase	BC019542	-1.56	0.004
Eno1	Enolase 1	AK014335	-1.55	0.035
Fads1	Fatty acid desaturase 1	AK080706	-1.72	0.023
Fdx1	Ferredoxin 1	AK148115	-1.51	0.039
Fkbp1	FK506 binding protein 11, 19 kDa	BC022900	-2.86	0.024
Foxs1	Forkhead box S1	BC131937	-1.60	0.026
Frmd4b	FERM domain containing 4B	AK051779	-1.78	0.043
Gnb4	Guanine nucleotide binding protein β4	AK165084	-1.68	0.034
Golph3	Golgi phosphoprotein 3	AK167805	-1.53	0.049
Lamb11	Laminin, beta 1	AK051131	-2.17	0.003
Lamc22	Laminin, gamma 2	AK147105	1.55	0.013
Lrp8	LDL receptor.-related protein 8	AK030143	-1.51	0.038
Mchr1	Melanin-concentrating hormone recept. 1	BC128286	-2.15	0.043
Mpp1	Membrane protein, palmitoylated 1	AK036415	-1.82	0.029
Mpst	Mercaptopyruvate sulfurtransferase	AK136571	-1.51	0.043
Msr1	Macrophage scavenger receptor 1	L04274	1.51	0.044
Nr1d2	Nuclear receptor subfamily 1, D2	AK054522	-2.02	0.008
Ramp1	Receptor activity modifying protein 1	BC012644	-1.79	0.034
Slc22a16	Organic cation transporter, member 16	BC100473	-1.50	0.028
Tef	Thyrotrophic embryonic factor	AK189278	-1.59	0.019
Tmem144	Transmembrane protein 144	BC018493	-1.57	0.012
Tor3a	Torsin family 3, member A	BC052851	-1.57	0.050
Sgk1	Serum/glucocorticoid regulated kinase 1	BC005720	-1.53	0.050
Slc16a1	Monocarboxylate transporter	BC014777	-1.62	0.037

[1.] Microarray analysis using the Nimblegen 12x132K mouse microarray platform was used to quantify relative expression of genes in the mammary gland. Genes were considered differentially expressed if they met the criteria of fold-change $\geq |1.5|$ and $p \leq 0.05$.

[2.] Photoperiods: long day (LD: 16 h light: 8 h dark), normal day (ND: 12 h light: 12 h dark), short day (SD: 8 h light: 16 h dark).

[3.] Differentially expressed genes in common those identified in Lemay, 2007; Lemay 2009 or Wei, 2013 are listed here

[4.] LD_{ND}: negative fold-change (expression higher in ND than LD photoperiod). Fold-change was identified from mean differences of LD-ND.

Table 4.7. Differentially expressed[1] genes in the comparison of SD_{ND} photoperiod[2] in mice that are associated with lactation[3].

Gene Symbol	Entrez Gene Name	Genbank ID	Fold-Change[4]	p-value
Anapc5	Anaphase promoting complex subunit 5	AK003821	1.92	0.031
Ap2b1	Adaptor-related protein complex 2β1 subunit	AK030505	1.69	0.050
Bnip2	BCL2/adenovirus E1B 19kDa interacting protein 2	AK014659	1.75	0.012
Ccdc82	Coiled-coil domain containing 82	BC098496	1.76	0.034
Cd44	CD44 molecule	X66081	1.65	0.008
Cd48	CD48 molecule	BC060977	1.51	0.008
Cd96	CD96 molecule	BC052865	2.32	0.002
Cdk14	Cyclin-dependent kinase 14	AK045083	-1.96	0.002
Cdkn2C	Cyclin-dependent kinase inhibitor 2C	U19596	1.85	0.011
Clcn1	Chloride channel, voltage-sensitive 1	BC114336	-1.56	0.047
Comtd1	Catechol-O-methyltransferase domain containing 1	BC049670	1.52	0.044
Crip1	Cysteine-rich protein 1	BC031922	1.82	0.020
Dlst	Dihydrolipoamide S-succinyltransferase	AK005477	1.58	0.024
Dnajc2	DnaJ (Hsp40) homolog, subfamily C, member 2	AK162409	2.58	0.005
Dsn1	MIS12 kinetochore complex component	BC046807	1.67	0.014
Dtx1	Deltex homolog 1	BC053055	1.60	0.042
Fbxw2	F-box and WD repeat domain containing 2	AK045743	1.70	0.018
Fcgr2b	Fc fragment of IgG, low affinity IIb, receptor	BC038070	1.83	0.002
Fgl2	Fibrinogen-like 2	BC028893	1.82	0.034
Fhl1	Four and a half LIM domains 1	BC029024	1.61	0.034
Gadd45b	Growth arrest and DNA-damage-inducible β	BC023815	1.58	0.007
Ganab	Glucosidase, alpha; neutral AB	AK043606	1.53	0.030
Grin3a	Glutamate receptor, N-methyl-D-aspartate 3A	AK138366	-1.74	0.030
Grm8	Glutamate receptor, metabotropic 8	AY673682	-2.12	0.033
H3f3a	H3 histone, family 3A	AK160635	1.52	0.021
Hif1a	Hypoxia inducible factor 1, alpha subunit	AK048798	1.53	0.033
Hla-Dqa1	Major histocompatibility complex, class II, DQ α1	AK170844	1.86	0.002
Hmgcr	3-hydroxy-3-methylglutaryl-CoA reductase	BC019782	1.54	0.023
Hpse	Heparanase	AK087283	1.56	0.020
Ier2	Immediate early response 2	BC002067	1.55	0.019
Ifi204	Interferon activated gene 204	XM_001474715	-1.91	0.047
Il2rg	Interleukin 2 receptor γ	BC014720	1.62	0.035
Jun	Jun proto-oncogene	BC021888	1.80	0.033
Kcna1	Potassium voltage-gated channel, member 1	BC112970	1.61	0.027
Ltbp2	Latent transforming growth factor β binding protein 2	BC119785	1.69	0.016
Mcm6	Minichromosome maintenance complex component 6	AK145520	1.50	0.039
Mgst2	Microsomal glutathione S-transferase 2	BC132234	1.70	0.026
Morc3	MORC family CW-type zinc finger 3	BC145705	1.57	0.046
Nfkbie	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor ε	BC030923	1.72	0.012
Nnat	Neuronatin	BC036984	1.64	0.011
Nop56	NOP56 ribonucleoprotein	AK037405	1.53	0.047
Oprm1	Opioid receptor, mu 1	AF346812	-2.05	0.022
Pabpc1	Poly(A) binding protein, cytoplasmic 1	AK005009	1.87	0.011
Pdcd11	Programmed cell death 11	BC051231	1.56	0.019
Pmpca	Peptidase alpha	AK032081	1.96	0.036
Prc1	Protein regulator of cytokinesis 1	BC005475	1.66	0.028
Prkacb	Protein kinase, cAMP-dependent, catalytic, β	BC054533	1.56	0.011
Rac2	Ras-related C3 botulinum toxin substrate 2	BC005455	2.32	0.005

Table 4.7 continued

Rhof	Ras homolog family member F	BC096597	2.02	0.007
Rnase6	Ribonuclease, RNase A family, k6	BC094892	1.89	0.005
Sdad1	SDA1 domain containing 1	AK164608	1.72	0.027
Srsf5	Serine/arginine-rich splicing factor 5	AK155141	-1.91	0.040
Srsf7	Serine/arginine-rich splicing factor 7	AK045884	1.51	0.031
Suv39h1	Suppressor of variegation 3-9 homolog 1	AF193862	1.63	0.022
Tbccd1	TBCC domain containing 1	AK035909	1.53	0.019
Tial1	TIA1 cytotoxic granule-associated RNA binding protein-like 1	AK132677	1.61	0.044
Traf6	Tnf receptor-associated factor 6	AK041172	2.03	0.017
Trappc12	Trafficking protein particle complex 12	AK084171	1.52	0.022
Trip4	Thyroid hormone receptor interactor 4	AK166192	1.59	0.050
Tsfm	Ts translation elongation factor, mitochondrial	AK020437	2.32	0.025
Use1	Unconventional SNARE in the ER 1 homolog	BC075695	2.54	0.006
Vamp7	Vesicle-associated membrane protein 7	NM_011515	1.53	0.049
Vps37a	Vacuolar protein sorting 37 homolog A	AK054055	1.55	0.024
Zc3h14	Zinc finger CCCH-type containing 14	AK006009	1.67	0.004

[1.] Microarray analysis using the Nimblegen 12x132K mouse microarray platform was used to quantify relative expression of genes in the mammary gland. Genes were considered differentially expressed if they met the criteria of fold-change $\geq |1.5|$ and $p \leq 0.05$.

[2.] Photoperiods: long day (LD: 16 h light: 8 h dark), normal day (ND: 12 h light: 12 h dark), short day (SD: 8 h light: 16 h dark)

[3.] Genes in common those identified in Lemay, 2007; Lemay 2009 or Wei, 2013 are listed here

[4.] SD_{ND}: positive fold-change (expression higher in ND than SD photoperiod). Fold-change was identified from mean differences of ND-SD.

REFERENCES

Arendt, J. 1988. Melatonin. Clin Endocrinol (Oxf) 29:205.

Auchtung, T. L., A. G. Rius, P. E. Kendall, T. B. McFadden, and G. E. Dahl. 2005. Effects of photoperiod during the dry period on prolactin, prolactin receptor, and milk production of dairy cows. J Dairy Sci 88:121.

Behl, B., M. Klos, M. Serr, U. Ebert, B. Janson, K. Drescher, G. Gross, and H. Schoemaker. 2006. An elisa-based method for the quantification of incorporated brdu as a measure of cell proliferation in vivo. J Neurosci Methods 158:37.

Benjamini, Y. and Y. Hochberg. 1995. Controlling the false discovery rate - a practical and powerful approach to multiple testing. J Roy Stat Soc B Met 57:289.

Bentley, P. A., E. H. Wall, G. E. Dahl, and T. B. McFadden. 2014. Responses of the mammary transcriptome of dairy cows to altered photoperiod during late gestation. Physiol Genomics Submitted: PG-00112-2014

Bionaz, M. and J. J. Loor. 2008. Gene networks driving bovine milk fat synthesis during the lactation cycle. BMC Genomics 9:366.

Blask, D. E., R. T. Dauchy, G. C. Brainard, and J. P. Hanifin. 2009. Circadian stage-dependent inhibition of human breast cancer metabolism and growth by the nocturnal melatonin signal: Consequences of its disruption by light at night in rats and women. Integr Cancer Ther 8:347.

Blask, D. E., R. T. Dauchy, L. A. Sauer, J. A. Krause, and G. C. Brainard. 2002. Light during darkness, melatonin suppression and cancer progression. Neuro Endocrinol Lett 23 Suppl 2:52.

Bolstad, B. M., R. A. Irizarry, M. Astrand, and T. P. Speed. 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19:185.

Bur, I. M., S. Zouaoui, P. Fontanaud, N. Coutry, F. Molino, A. O. Martin, P. Mollard, and X. Bonnefont. 2010. The comparison between circadian oscillators in mouse liver and pituitary gland reveals different integration of feeding and light schedules. PLoS ONE 5:e15316.

Capuco, A. V., E. E. Connor, and D. L. Wood. 2008. Regulation of mammary gland sensitivity to thyroid hormones during the transition from pregnancy to lactation. Exp Biol Med (Maywood) 233:1309.

Capuco, A. V., D. L. Wood, R. Baldwin, K. McLeod, and M. J. Paape. 2001. Mammary cell number, proliferation, and apoptosis during a bovine lactation: Relation to milk production and effect of bst. J Dairy Sci 84:2177.

Casey, T., O. Patel, K. Dykema, H. Dover, K. Furge, and K. Plaut. 2009. Molecular signatures reveal circadian clocks may orchestrate the homeorhetic response to lactation. PLoS ONE 4:e7395.

Ciarleglio, C. M., J. C. Axley, B. R. Strauss, K. L. Gamble, and D. G. McMahon. 2011. Perinatal photoperiod imprints the circadian clock. Nat Neurosci 14:25.

Cohen, I., O. Pappo, M. Elkin, T. San, R. Bar-Shavit, R. Hazan, T. Peretz, I. Vlodavsky, and R. Abramovitch. 2006. Heparanase promotes growth, angiogenesis and survival of primary breast tumors. Int J Cancer 118:1609.

Dahl, G. E., B. A. Buchanan, and H. A. Tucker. 2000. Photoperiodic effects on dairy cattle: A review. J Dairy Sci 83:885.

Dardente, H., D. G. Hazlerigg, and F. J. Ebling. 2014. Thyroid hormone and seasonal rhythmicity. Front Endocrinol (Lausanne) 5:19.

Dardente, H., C. A. Wyse, M. J. Birnie, S. M. Dupre, A. S. Loudon, G. A. Lincoln, and D. G. Hazlerigg. 2010. A molecular switch for photoperiod responsiveness in mammals. Curr Biol 20:2193.

Demas, G. E., S. L. Klein, and R. J. Nelson. 1996. Reproductive and immune responses to photoperiod and melatonin are linked in *peromyscus* subspecies. J Comp Physiol A 179:819.

Duffield, G. E. 2003. DNA microarray analyses of circadian timing: The genomic basis of biological time. J Neuroendocrinol 15:991.

Duncan, M. J. 2007. Circannual prolactin rhythms: Calendar-like timer revealed in the pituitary gland. Trends Endocrinol Metab 18:259.

Ebling, F. J. and P. Barrett. 2008. The regulation of seasonal changes in food intake and body weight. J Neuroendocrinol 20:827.

Goto, M., I. Oshima, T. Tomita, and S. Ebihara. 1989. Melatonin content of the pineal gland in different mouse strains. J Pineal Res 7:195.

Han, L. Q., G. Y. Yang, H. S. Zhu, Y. Y. Wang, L. F. Wang, Y. J. Guo, W. F. Lu, H. J. Li, and Y. L. Wang. 2010. Selection and use of reference genes in mouse mammary glands. Genet Mol Res 9:449.

Hastings, M., J. S. O'Neill, and E. S. Maywood. 2007. Circadian clocks: Regulators of endocrine and metabolic rhythms. J Endocrinol 195:187.

Hastings, M. H., J. Herbert, N. D. Martensz, and A. C. Roberts. 1985. Annual reproductive rhythms in mammals: Mechanisms of light synchronization. Ann NY Acad Sci 453:182.

IPA. 2005. Ipa network generation algorithm white paper.

Irizarry, R. A., B. M. Bolstad, F. Collin, L. M. Cope, B. Hobbs, and T. P. Speed. 2003a. Summaries of affymetrix genechip probe level data. Nucleic Acids Res 31:e15.

Irizarry, R. A., B. Hobbs, F. Collin, Y. D. Beazer-Barclay, K. J. Antonellis, U. Scherf, and T. P. Speed. 2003b. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4:249.

Knight, C. H. and M. Peaker. 1982. Mammary cell proliferation in mice during pregnancy and lactation in relation to milk yield. Q J Exp Physiol 67:165.

Lang, R., H. Hintner, A. Hermann, and R. Brandstaetter. 2003. Photoperiod modulates melanoma growth in c57bl/6 mice. Exp Dermatol 12:510.

Lemay, D. G., D. J. Lynn, W. F. Martin, M. C. Neville, T. M. Casey, et al. 2009. The bovine lactation genome: Insights into the evolution of mammalian milk. Genome Biol 10:R43.

Lemay, D. G., M. C. Neville, M. C. Rudolph, K. S. Pollard, and J. B. German. 2007. Gene regulatory networks in lactation: Identification of global principles using bioinformatics. BMC Syst Biol 1:56.

Li, F. and J. V. Ravetch. 2012. Apoptotic and antitumor activity of death receptor antibodies require inhibitory fcgamma receptor engagement. Proc Natl Acad Sci U S A 109:10966.

Maningat, P. D., P. Sen, M. Rijnkels, D. L. Hadsell, M. S. Bray, and M. W. Haymond. 2011. Short-term administration of rhgh increases markers of cellular proliferation but not milk protein gene expression in normal lactating women. Physiol Genomics 43:381.

Maningat, P. D., P. Sen, M. Rijnkels, A. L. Sunehag, D. L. Hadsell, M. Bray, and M. W. Haymond. 2009. Gene expression in the human mammary epithelium during lactation: The milk fat globule transcriptome. Physiol Genomics 37:12.

Mediavilla, M. D., M. San Martin, and E. J. Sanchez-Barcelo. 1992. Melatonin inhibits mammary gland development in female mice. J Pineal Res 13:13.

Metz, R. P., X. Qu, B. Laffin, D. Earnest, and W. W. Porter. 2006. Circadian clock and cell cycle gene expression in mouse mammary epithelial cells and in the developing mouse mammary gland. Dev Dyn 235:263.

Nelson, R. J., G. E. Demas, S. L. Klein, and L. J. Kriegsfeld. 1995. The influence of season, photoperiod, and pineal melatonin on immune function. J Pineal Res 19:149.

Neville, M. C., T. B. McFadden, and I. Forsyth. 2002. Hormonal regulation of mammary differentiation and milk secretion. J Mammary Gland Biol Neoplasia 7:49.

Ono, H., Y. Hoshino, S. Yasuo, M. Watanabe, Y. Nakane, A. Murai, S. Ebihara, H. W. Korf, and T. Yoshimura. 2008. Involvement of thyrotropin in photoperiodic signal transduction in mice. Proc Natl Acad Sci U S A 105:18238.

Otsuka, T., M. Goto, M. Kawai, Y. Togo, K. Sato, K. Katoh, M. Furuse, and S. Yasuo. 2012. Photoperiod regulates corticosterone rhythms by altered adrenal sensitivity via melatonin-independent mechanisms in fischer 344 rats and c57bl/6j mice. PLoS ONE 7:e39090.

Otsuka, T., M. Kawai, Y. Togo, R. Goda, T. Kawase, et al. 2014. Photoperiodic responses of depression-like behavior, the brain serotonergic system, and peripheral metabolism in laboratory mice. Psychoneuroendocrinology 40:37.

Parish, C. R., C. Freeman, and M. D. Hulett. 2001. Heparanase: A key enzyme involved in cell invasion. Biochim Biophys Acta 1471:M99.

Peters, R. R., L. T. Chapin, K. B. Leining, and H. A. Tucker. 1978. Supplemental lighting stimulates growth and lactation in cattle. Science 199:911.

Petri, I., R. Dumbell, F. Scherbarth, S. Steinlechner, and P. Barrett. 2014. Effect of exercise on photoperiod-regulated hypothalamic gene expression and peripheral hormones in the seasonal dwarf hamster phodopus sungorus. PLoS ONE 9:e90253.

Pfaffl, M. W., A. Tichopad, C. Prgomet, and T. P. Neuvians. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: Bestkeeper--excel-based tool using pair-wise correlations. Biotechnol Lett 26:509.

Plaut, K. and T. Casey. 2012. Does the circadian system regulate lactation? Animal 6:394.

Ramanathan, P., I. Martin, P. Thomson, R. Taylor, C. Moran, and P. Williamson. 2007. Genomewide analysis of secretory activation in mouse models. J Mammary Gland Biol Neoplasia 12:305.

Reppert, S. M. and D. R. Weaver. 2001. Molecular analysis of mammalian circadian rhythms. Annu Rev Physiol 63:647.

Sorensen, M. T. and R. R. Hacker. 1979. Negative effect of bovine pineal gland extract and positive effect of supplemental lighting on lactation in mice. J Anim Sci 49:1270.

Takahashi, J. S. 1992. Circadian clock genes are ticking. Science 258:238.

Trott, J. F., A. Schennink, W. K. Petrie, R. Manjarin, M. K. VanKlompenberg, and R. C. Hovey. 2012. Triennial lactation symposium: Prolactin: The multifaceted potentiator of mammary growth and function. J Anim Sci 90:1674.

Vlodavsky, I. and Y. Friedmann. 2001. Molecular properties and involvement of heparanase in cancer metastasis and angiogenesis. J Clin Invest 108:341.

Vorbach, C., M. R. Capecchi, and J. M. Penninger. 2006. Evolution of the mammary gland from the innate immune system? Bioessays 28:606.

Wall, E. H., T. L. Auchtung-Montgomery, G. E. Dahl, and T. B. McFadden. 2005a. Short communication: Short-day photoperiod during the dry period decreases expression of suppressors of cytokine signaling in mammary gland of dairy cows. J Dairy Sci 88:3145.

Wall, E. H., T. L. Auchtung, G. E. Dahl, S. E. Ellis, and T. B. McFadden. 2005b. Exposure to short day photoperiod during the dry period enhances mammary growth in dairy cows. J Dairy Sci 88:1994.

Walton, J. C., Z. M. Weil, and R. J. Nelson. 2011. Influence of photoperiod on hormones, behavior, and immune function. Front Neuroendocrinol 32:303.

Wei, J., P. Ramanathan, I. C. Martin, C. Moran, R. M. Taylor, and P. Williamson. 2013. Identification of gene sets and pathways associated with lactation performance in mice. Physiol Genomics 45:171.

Yasuo, S., M. Watanabe, N. Okabayashi, S. Ebihara, and T. Yoshimura. 2003. Circadian clock genes and photoperiodism: Comprehensive analysis of clock gene expression in the mediobasal hypothalamus, the suprachiasmatic nucleus, and the pineal gland of japanese quail under various light schedules. Endocrinology 144:3742.

Zcharia, E., S. Metzger, T. Chajek-Shaul, H. Aingorn, M. Elkin, et al. 2004. Transgenic expression of mammalian heparanase uncovers physiological functions of heparan sulfate in tissue morphogenesis, vascularization, and feeding behavior. FASEB J 18:252.

CHAPTER 5: LONG AND SHORT DAY PHOTOPERIOD EXPOSURE DURING GESTATION ALTERS THE MOUSE MAMMARY TRANSCRIPTOME WITH EFFECTS ON MAMMARY DEVELOPMENT AND CELL PROLIFERATION

ABSTRACT

Daily light exposure, or photoperiod, provides an accurate measure of season and thereby coordinates reproduction, including mammary development and lactation. The effects of photoperiod on lactation have been described in dairy cows but the molecular mechanisms underlying the response are unknown. We hypothesizes that exposure of mice to different photoperiods during gestation would induce differential expression of genes associated with mammary development and the initiation of lactation leading to altered mammary function. The objectives of this study were to quantify the effects of photoperiod exposure during gestation on cell proliferation and the mammary transcriptome, and to identify differentially expressed genes and *upstream regulators* with functional importance to the mammary gland during early the onset of lactation. Pregnant mice were exposed to one of three photoperiods, long day (**LD**), normal day (**ND**), or short day (**SD**) for the duration of gestation. On day 17 of gestation (G17) mice exposed to SD photoperiod had higher levels of cell proliferation, compared to mice on LD photoperiod. Using microarray analysis, we quantified changes in the mammary transcriptome of mice on G17. We identified differential expression of 520 genes in response to LD, and 123 genes in response to SD, relative to ND photoperiod. Functional annotation using IPA associated SD-responsive genes and *upstream regulators* with cellular proliferation. Thyroid-stimulating hormone receptor (*Tshr*) was among the genes responsive to SD photoperiod. We report that *Tshr* expression was lower in mice on SD relative to LD photoperiod before parturition, but not after the cessation of exposure on day 5 (L5), or day 10 (L10) of lactation. In contrast, LD photoperiod affected genes that were associated with mammary development and differentiation, which we infer is indicative of the effects of photoperiod on mammary remodeling necessary for the onset of

lactation. These data identify the genes and pathways responsive to photoperiod manipulation during gestation and give insight into how their expression may affect the onset of lactation.

INTRODUCTION

Photoperiod, or day length, generates a highly accurate biological calendar that allows animals to anticipate and adapt to environmental changes throughout the year. Photoperiod coordinates circannual adjustments associated with reproduction, hibernation, migration, and behavior (Tamarkin et al., 1985). The effects of photoperiod on reproduction include development and functionality of reproductive organs, the time of year when mating occurs and thereby when offspring are born, and regulation of mammary development and lactation (Hastings et al., 1985; Dahl et al., 2012).

Photoperiodic information is conveyed to target cells through the secretion of hormones, on both a daily and circannual timescale. Daily light/dark cycles result in rhythmic secretion of melatonin from the pineal gland during the scotophase (Bartness et al., 1993). In addition, the length of the scotophase also affects the duration of elevated melatonin concentrations in the blood (Malpaux et al., 2001). Circulating melatonin also affects cells in the mammary gland. Exogenous melatonin inhibits post-natal mammary gland development in mice (Mediavilla et al., 1992). Furthermore, as reviewed by Cos and Sanchez-Barcelo (2000), numerous *in vivo* and *in vitro* studies report that melatonin has oncostatic properties; whereas aberrant pineal function or reduced melatonin secretion may promote oncogenesis. Prolactin, a key hormone in mammary development and the onset of lactation (Trott et al., 2012), is photoperiod-responsive and drives numerous aspects of seasonal physiology including gonadal activity and pelage cycle (Lincoln et al., 2003; Duncan, 2007). Taken together, there is considerable overlap between photoperiod and mammary hormone signaling pathways.

The most widely known effect of photoperiod manipulation on lactation in dairy cows was first reported by Peters et al. (1978). Specifically, exposing dairy cows to LD (**LD**, 16 h light: 8 h dark) photoperiod during lactation increased milk production by 10-15% (Peters et al., 1978). Later studies confirmed these findings in dairy cows, sheep, goats, and pigs (for reviews see: Dahl (2005); Dahl et al. (2012)). Contrary to exposure during lactation, cows exposed to short day (**SD**, 8 h light: 16 h dark) photoperiod during the last 40-days of gestation, known as the dry period, went on to produce more milk than their LD counterparts (Auchtung et al., 2005). Investigation of the mechanisms underlying these effects has focused on cell proliferation, immune cell function, and small-scale gene expression in the mammary gland (Auchtung et al., 2005; Wall et al., 2005a; Wall et al., 2005b). Potential mediators of the photoperiodic response in the mammary gland include prolactin (*Prl*), its receptor (*Prlr*), and insulin-like growth factor-1(*Igf1*); however, the effects of photoperiod on the mammary transcriptome have yet to be fully elucidated.

We sought to identify the effects of photoperiod on the mammary transcriptome of the mouse, a well-established model of mammary development and function (Lemay et al., 2007; Wei et al., 2013) that respond to photoperiod (Ciarleglio et al., 2011; Otsuka et al., 2014). We hypothesized that exposure of mice to different day lengths during gestation would induce both functional responses and differential expression of genes associated with mammary development and initiation of lactation. The objectives of this study were to quantify the effects of photoperiod exposure during gestation on cell proliferation and the mammary transcriptome to identify differentially expressed genes, pathways and *upstream regulators* with functional importance to the mammary gland during the onset of lactation.

MATERIALS AND METHODS

Animal care and breeding

Procedures reported here were approved by the University of Alberta Animal Care and Use Committee. Female C57Bl/6 mice (n = 72) were obtained from Charles River Laboratories. Virgin female mice were maintained on normal day (**ND**, 12 h light: 12 h dark) photoperiod and fed a diet of 9% (w/v) fat mouse chow (Diet Labs) and water *ad libitum* prior to mating. Mice were housed (3/cage) in wire-top cages on wire racks to allow unobstructed light exposure. At seven weeks of age, females were mated in a 3:1 ratio with male C57Bl/6 mice. Each morning, female mice were examined for the presence of vaginal plugs. When vaginal plugs were observed, female mice were randomly assigned to adjacent photoperiod treatment rooms until day 17 (G17) or 19 (G19) of gestation (**Figure 5.1a**). Mice to be euthanized on day 5 (L5) or 10 (L10) of lactation remained on photoperiod treatment for remainder of gestation but were transferred back to ND photoperiod when pups were observed in nests.

Photoperiod treatments

Three photoperiod treatments, LD, ND, and SD, with rectangular light-dark transitions were used in this study (**Figure 5.1b**). The photic phase was initiated at 0600 h and ended at 2200, 1800, and 1400 h, respectively. Mice were exposed to lighting treatments in adjacent rooms that were monitored for light intensity, relative humidity, and temperature using Hobo® data loggers. The light intensity in treatment rooms was > 300 lux during the photic phase and < 20 lux during the scotophase. Photoperiod treatment rooms were entered

only during the photic phase and contact with animals was minimized to minimize associations with external cues.

Euthanasia and tissue collection

Mice (n = 6/photoperiod treatment) and fetal pups were killed on G17 and G19 of gestation and L5 and day 10 L10 of lactation by CO_2 overdose. Mice were sacrificed during the photic phase, between 0600 and 1200 h, and alternating between photoperiod treatment groups to balance for time effects. Immediately post-sacrifice, the 4th-right inguinal mammary gland was excised and the supra-mammary lymph node was removed. Mammary tissue was frozen in liquid nitrogen and stored at -80°C for RNA extraction.

BrdU injection and quantification

Bromodeoxyuridine (BrdU) injection and quantification was carried out using the previously described method (**Chapter 4**). Briefly, pregnant females and lactating dams (n = 6/photoperiod treatment) were injected intraperitoneally with 10 μL/g of body weight of BrdU solution (10 mM, Amersham Biosciences), 2 h prior to sacrifice. At the time of sacrifice, the 4th-left inguinal mammary gland was excised and frozen on dry ice. Samples were later homogenized in buffer (50 mM NaCl and 10 mM Tris HCl, pH 7.5) and frozen at -80°C. Homogenates were thawed and sonicated on ice for 30 s followed by centrifugation at 1,500 x g for 15 minutes. Supernatant was retained and diluted 1:100 in water and stored at -20°C for later analysis. Cell proliferation was quantified using the mouse anti-BrdU ELISA (Behl et al., 2006) kit (Bluegene Biotech), following the manufacturer's instructions. The concentration of double-stranded (ds) DNA was assessed using the Quant-iT dsDNA Assay

Kit, Broad Range (Invitrogen™). The concentration of incorporated BrdU was normalized to the concentration of dsDNA in each sample.

RNA isolation and microarray preparation

RNA extraction and microarray sample preparation was carried out as previously described (**Chapter 4**). Briefly, RNA was isolated from the whole 4th -right inguinal mammary gland by TRIzol Reagent (Invitrogen™) according to the manufacturer's instructions. Microarray sample preparation and analysis was conducted on G17 samples at the Alberta Transplant Applied Genomics Centre, University of Alberta, following standard procedures. First-strand cDNA was synthesized using oligo dT primer and SuperScript II Reverse Transcriptase. Second strand cDNA was prepared using T4 DNA polymerase. Samples were labelled using Cy3 random primers and the Klenow fragment ('3 -> 5'exo-). Labelled samples were hybridized to Roche Nimblegen 12x135K array chips using the Nimblegen Hybridization System. Arrays were scanned using the MS 200 Microarray Scanner and MS 200 Data Collection Software. Images were collected and normalized using Roche Nimblegen DEVA software by the quantile normalization method (Bolstad et al., 2003). An IQR filter (> 0.5) eliminated probe sets with little variation. Gene calls were generated using the Robust Multichip Average (RMA) method (Irizarry et al., 2003a; Irizarry et al., 2003b) and analysis of RMA values was conducted using Partek® Software. The remaining probe sets were subjected to the following t-test comparisons: LD vs ND (**LD_{ND}**) and ND vs SD (**SD_{ND}**). Fold-change was calculated as LD – ND and ND – SD, such that resulting positive fold-change values indicated higher relative expression in LD in the comparison of $LD_{ND,}$ or ND in the comparison of SD_{ND}. Genes meeting the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$ were considered differentially expressed and were included in

functional analysis. To visualize how photoperiod and time relative to parturition affected specific genes, RMA values from the microarray data were plotted using the cell plot function of JMP® Pro 10.0 to generate heat maps.

Gene functional analysis

Gene annotation, *biofunction* and *upstream regulator* analysis was conducted using Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com). Probe sets from the comparisons of LD_{ND} and SD_{ND} that met our criteria for differential expression were uploaded. Probe sets were evaluated using the Ingenuity Knowledge Base® and mapped genes were included in further analysis. *Core analysis* was conducted with the default parameters set to include genes only, direct and indirect relationships, endogenous chemicals, and information from rat, mouse, and human species. The top 5 *molecular and cellular biofunctions* enriched by differentially expressed genes in our datasets were identified using *p-value of overlap* resulting from Fisher Exact t-test. The top 5 unique *functional annotations,* with > 1 associated gene, for each *biofunction* are presented herein.

Identification of potential *upstream regulators* of genes differentially expressed in response to photoperiod was completed using IPA default settings. *Upstream regulators* predicted to affect ≥ 5 differentially expressed genes or with a significant *p-value of overlap* ($p \leq 0.05$), as determined by a Fisher's Exact t-test, are reported. The *activation z-score* was used to interpret potential functional effects in the mammary gland of selected *upstream regulators*. The *activation z-score* is based on IPA comparison models; we considered *z-scores* ≤ 0 as inhibitory and > 0 as activating. Gene clustering was done using the Functional Annotation tool of the Database of Annotation, Visualization, and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/). Differentially expressed genes were used to

identify enriched gene set clusters from which representative GO Terms with *enrichment scores > 1.0* are reported.

Quantitative real-time PCR

Methods for qRT-PCR were previously described (**Chapter 4**). Briefly, total RNA (1 μg) was treated with DNase 1 prior to cDNA synthesis using SuperScript™ II RT (Invitrogen™) following the manufacturer's instructions. Reactions were carried out using 2X Fast SYBR® Green Master Mix (Applied Biosystems) on the ViiA™ 7 Real-Time PCR System (Applied Biosystems) under standard conditions. Data were analyzed using the ViiA™ 7 Software v1.1. Expression of genes of interest was normalized to the BestKeeper (Pfaffl et al., 2004) value based on the geometric mean generated from four stable reference genes (*Actb*, *Cyc*1, *B2M*, *Ubc*). Primer sequences were previously reported (**Chapter 4**).

Statistical analysis

Dam and organ weights, BrdU incorporation and qPCR data ($n \geq 5$ dams/photoperiod) were analyzed by the ANOVA procedure in JMP® Pro 10, using the standard least squares method to detect effects of photoperiod, time and the interaction of photoperiod and time. Pre-planned within-day comparisons were made using the ANOVA procedure to determine the effects of photoperiod at each time. Means separation was conducted using the Tukey-Kramer HSD test and significance was declared at $p \leq 0.05$.

RESULTS

Organ weight

Photoperiod did not have an overall effect on dam body, spleen thymus or liver weight (**Table 5.1**) during late gestation and mid-lactation. Time relative to parturition did significantly affect body, spleen, thymus and liver weight. Spleen and thymus weight were lowest on G19, whereas, liver weight increased from pregnancy into lactation (**Table 5.1**). There was no interaction of photoperiod and time on body or organ weights.

BrdU incorporation

There was an overall tendency ($p = 0.10$) for decreasing photoperiod to increase BrdU uptake (**Figure 5.2a**), such that mammary glands of mice exposed to SD photoperiod took up nearly twice the BrdU of mice on LD photoperiod. Uptake of BrdU changed markedly over time ($p< 0.0001$), declining from G17 to G19, then increasing from L5 to L10 (**Figure 5.2b**). There was a tendency for an interaction ($p= 0.083$) between the effects of photoperiod and time relative to parturition. Within-day comparisons showed an effect ($p = 0.01$) of photoperiod on G17, such that mice exposed to SD photoperiod took up more BrdU into mammary cells than LD exposed mice (**Figure 5.2b**). There were no significant within-day effects of photoperiod on G19, L5, or L10.

Differential gene expression by microarray analysis

In the comparison of LD_{ND}, 520 genes were differentially expressed (**Table 5.2**, **Suppl. T5.1**). Clustering within DAVID grouped these genes into 84 functional groups, 9 with enrichment scores > 1 (**Table 5.3**). These clusters included extracellular activity,

phosphorylation, peptidase activity, regulation of cell proliferation, cytokine, and growth factor activity. Functional analysis in IPA associated many of these genes with reproductive, cellular, and embryonic development (**Figure 5.3a**, **Suppl. T5.2**). Mammary gland development was enriched by differentially expressed genes (n = 8): LIM domain only 4 (*Lmo4*), neuralized-like homolog (*Neurl1*), oxytocin receptor (*Oxtr*), prostaglandin-endoperoxide synthase 2 (*Ptgs2*), relaxin 1 (*Rln1*), relaxin/insulin-like family peptide receptor 2 (*Rxfp*), teratocarcinoma-derived growth factor 1 (*Tdgf1*), transforming growth factor alpha (*Tgfa*) (**Figure 5.4**). Differentiation of cells was also enriched by differentially expressed genes (n = 46). *Upstream regulator* analysis revealed 49 factors predicted to affect ≥ 5 differentially expressed genes in the comparison of LD_{ND} (**Figure 5.5**, **Suppl. T5.3**). Factors predicted to affect the greatest number of genes were lipopolysaccharide (LPS), catenin (cadherin-associated protein) β1 (*Ctnnb1*) and estrogen receptor 1(*Esr1*).

In the comparison of SD_{ND}, 123 genes were differentially expressed in the mammary gland in response to SD photoperiod (**Table 5.2**, **Suppl.T5.1**). Clustering within DAVID identified 24 associated clusters, 5 with enrichment scores ≥1.0 (**Table 5.3**), including chromosome organization, cell cycle and cell junctions. IPA functional analysis associated genes with *cellular proliferation* (n = 25 genes), *cell cycle progression* (n = 11 genes), *lipid metabolism* (n = 2 genes) and *concentration of hormones* (n = 6 genes) (**Figure 5.3b**, **Suppl. T5.2**). IPA *upstream regulator* analysis identified 29 factors predicted to affect ≥ 5 differentially expressed genes in the comparison of SD_{ND} (**Figure 5.5**, **Suppl. T5.3**). The *upstream regulators* predicted to affect the highest number of genes were Hnf4a, β-estradiol, *Tp53*, dexamethasone and, transforming growth factor β1 (*Tgfβ1*). Together, these *upstream regulators* were predicted to affect 36 genes that were functionally associated with the

proliferation of cells (**Figure 5.6**). Of these overlapping genes, the expression of *Tshr* was further analyzed. Photoperiod did not have an overall effect on the expression of *Tshr* ($p = 0.31$), there was, however, an effect of time ($p < 0.0001$) and an interaction ($p < 0.02$) of photoperiod and time on the expression of *Tshr* (**Figure 5.7**). Within-day comparisons showed a significant effect of photoperiod on G17, such that mice exposed to SD photoperiod expressed significantly less Tshr than mice on LD photoperiod. There were no significant effects of photoperiod on G19, L5, or L10 (**Figure 5.7**). There were 39 differentially expressed genes common to the comparisons of LD_{ND} and SD_{ND} (**Figure 5.8**, **Suppl. T5.4**).

DISCUSSION

Short day photoperiod increases mammary cell proliferation

Mouse mammary cell proliferation, as measured by tritiated thymidine incorporation into DNA increased from G12 to L5 (Knight and Peaker, 1982). We report mice exposed to SD photoperiod took up significantly more BrdU on G17 than mice exposed to LD photoperiod. Photoperiod-induced differential incorporation of tritiated-thymidine during gestation in dairy cows was previously reported by Wall and co-workers (2005b). Cows exposed to SD underwent a 3-fold increase in tritiated-thymidine incorporation on day -24 relative to parturition. Similar to our findings, no effect of photoperiod on proliferation was observed after the cessation of photoperiod treatment (Wall et al., 2005b). Together, these data imply the mammary gland is susceptible to photoperiodic effects on cell proliferation at specific times during gestation in both mice and cows.

Analysis of the mammary transcriptome on G17 provided additional evidence of the effects of SD photoperiod on cell proliferation. Among the differentially expressed genes associated with cellular proliferation, we have previously identified seperase (*Espl1*) as being responsive to photoperiod in the bovine mammary gland during gestation (**Chapter 3**). As an endopeptidase *Espl1* functions in the detachment of sister chromatids during cell replication. When over-expressed, *Espl1* can induce mammary cell tumorigenesis (Pati et al., 2004). In addition, 16 other differentially expressed genes were associated with chromosome and chromatin organization consistent with the differences in proliferation observed on G17.

Among the genes associated with cellular proliferation, we show *Tshr* expression is photoperiod-responsive in the mammary gland on G17. Thyroid-related hormones are essential for lactation in rodents and dairy cows, acting through both local and systemic effects (Neville et al., 2002). In agreement with data presented here, we recently reported mammary Tshr expression was higher on L5 and L10 in mice exposed to LD photoperiod during lactation than those on SD or ND photoperiod (**Chapter 4**). These findings add to a growing body of evidence that thyroid signaling mediates physiological effects of photoperiod (Dahl et al., 1994; Barrett et al., 2007; Ono et al., 2008; Ono et al., 2009; Ross et al., 2011; Kampf-Lassin and Prendergast, 2013).

Upstream regulators associated with SD_{ND} photoperiod-responsive genes further supports an effect on cellular proliferation. Dexamethasone, a synthetic glucocorticoid, *Tgfβ1*, and β-estradiol were also among predicted *upstream regulators* of photoperiod-responsive genes in the mammary gland of cows during late gestation (Bentley et al., 2014). Glucocorticoids, like many hormones, undergo diurnal secretory rhythms. Exposure to LD photoperiod disrupts the rhythmic secretion of corticosterone in C57Bl/6 mice, compared to

SD exposure (Otsuka et al., 2012). Short day length induces testicular regression in hamsters, an effect mediated by local expression of *Tgfβ1* (Gonzalez et al., 2012). Another *upstream regulator*, *Hnf4*, is a member of the steroid/thyroid nuclear receptor superfamily (Sladek et al., 1990) and is regulated by *Tgfβ1* in mouse mammary epithelial cells (Ishikawa et al., 2008). Taken together, we surmise these *upstream regulators* may coordinate the differential expression of genes that regulate mammary cell proliferation in response to photoperiod.

Long day photoperiod affects remodeling of the mammary gland

In the comparison of LD_{ND} identified distinct effects on the mammary transcriptome. Differentially expressed genes were associated with the extracellular matrix, and developmental processes, including *mammary development*. Three of the genes associated with mammary development were more down-regulated in dams exposed to LD photoperiod (**Figure 5.4**). Two of them, *Oxtr* and *Lmo4*, have roles in regulating milk ejection and mammary cell proliferation, respectively (Politowska et al., 1999; Sum et al., 2005). The third, *Neurl1*, is an ubiquitin ligase that was shown to be vital in terminal maturation of the mammary gland as mice lacking $Neurl1^{(-/-)}$ display defects in mammary development and maternal behavior (Vollrath et al., 1988). Taken together, the decreased expression of these genes suggests LD photoperiod may suppress the onset of lactation, relative to ND photoperiod.

Long day photoperiod increased the expression of several other genes with mammary-specific functions. Specifically, *Tgfa* is a mammary differentiation factor (Smith et al., 1995), and *Ptgs2* (*Cox-2*), promotes angiogenesis associated with aberrant mammary cell growth (Hoellen et al., 2011). Furthermore, LD photoperiod increased the expression of

Tdgf1, which induces branching morphogenesis of the mammary gland, and is known to inhibit the expression of milk proteins (Salomon et al., 1999). In rodents, *Rln1* functions in reproductive tissue remodeling and nipple development (Sherwood, 2004). In pigs, *Rln1* expression is required for pre-partum mammary development (Hurley et al., 1991). Together, *Rln1* and its receptor *Rxfp2* may promote tissue remodeling in the mammary gland by affecting the extracellular matrix. Ultimately, the molecular signatures of genes in the comparison of LD_{ND} suggest mammary development is affected by photoperiod exposure during gestation.

In addition to the above differentially expressed genes related to development, we further report differential expression in the comparison of LD_{ND} of 38 photoperiod-responsive genes associated with the extracellular matrix, including 5 serine proteases. β-catenin was identified as an *upstream regulator* (**Figure 5.5**) of 13 differentially expressed, photoperiod-responsive genes including *Cdh8* and *Cdh26*. These cadherins interact with β -catenin in the extracellular matrix to mediate cell-cell-interactions required for mammary function (Knudsen and Wheelock, 2005). In summary, it appears that LD photoperiod may affect terminal differentiation and remodeling of the mammary gland prior to the onset of lactation. Along with our cell proliferation findings, these data suggest LD photoperiod me hinder mammary development and potentially milk production relative to ND and SD photoperiod.

Most studies of the effects of photoperiod on gene expression in the mammary gland were carried out using the comparison of LD vs SD (Auchtung et al., 2005; Wall et al., 2005a; Wall et al., 2005b; Bentley et al., 2014). We have included a third photoperiod, 'normal day,' which allowed us to evaluate whether LD or SD, per se, affected gene

expression relative to ND. We found that 39 genes were common between the comparisons of LD_{ND} and SD_{ND} (**Figure 5.8**). These genes may encompass a fundamental mechanism underlying the response of the mammary gland to any change in photoperiod. Despite this commonality, our findings are in agreement with those reported in **Chapter 4** and show that LD and SD photoperiod generally trigger dissimilar effects on the mammary transcriptome.

In conclusion, the mouse mammary transcriptome is responsive to both LD and SD photoperiod during gestation. Short day photoperiod stimulated cellular proliferation relative to LD on G17. The transcriptomic signature in the comparison of SD_{ND} is consistent with effects of SD photoperiod on cell proliferation and suggest thyroid-hormone signaling, and *upstream regulators* we identified may mediate the effects of SD photoperiod in the mammary gland. In contrast, LD photoperiod affected genes associated with *mammary development* and differentiation, which hinder the final stages of mammary remodeling necessary for the onset of lactation. More broadly, LD and SD had largely dissimilar effects on gene expression; we have identified some genes that may be part of a common mechanism that mediates the response to photoperiod in the mammary gland. The genes and *upstream regulators* identified here provide targets for further study of the mechanisms underlying the effects of photoperiod on mammary function.

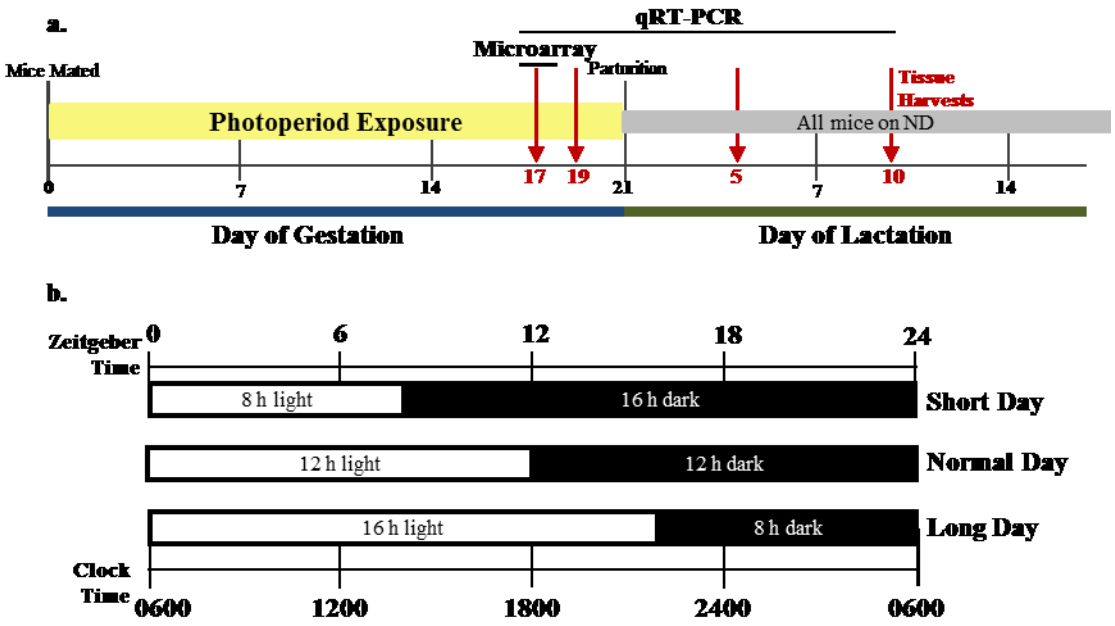


Figure 5.1. Study design of mice exposed to photoperiods during gestation

a. Female mice were maintained on normal day (12 h light:12 h dark) photoperiod for at least 2 weeks prior to mating. Upon detection of a vaginal plug, mice were randomly assigned one of three photoperiod treatment rooms for the remainder of gestation. **b.** long day (16 h light: 8 h dark), normal day, or short day (8 h light: 16 h dark). Upon parturition, mice were returned to normal day photoperiod. Tissue harvests were conducted on day G17, G19, L5 or L10. Microarray analysis of mammary tissue RNA extracts was conducted on samples from G17.

Table 5.1. Body and organ weights of pregnant mice exposed to photoperiod[1] throughout gestation[2].

		Long Day		Normal Day		Short Day			P value[3]		
	Day of Gestation / Lactation	Weight (g) ± sd		Weight (g) ± sd		Weight (g) ± sd		Means by Time[4]	Time	PP	Time *PP
Body	17	33.7	±3.0	34.6	±3.3	32.0	±4.3	**33.6[a]**	<0.001	0.23	0.52
	19	38.8	±1.5	38.4	±3.9	35.0	±4.5	**37.4[b]**			
	5	31.6	±1.7	32.4	±1.9	31.9	±2.0	**31.9[a]**			
	10	33.3	±1.5	34.4	±2.7	34.4	±3.0	**34.0[a]**			
Means by Photoperiod[5]		**34.33**		**34.95**		**33.42**					
		Weight (mg) ± sd		**Weight (mg) ± sd**		**Weight (mg) ± sd**					
Spleen	17	3.7	±0.4	4.1	±0.9	3.8	±0.4	**3.9[a]**	<0.001	0.22	0.32
	19	3.0	±0.3	3.5	±0.8	3.2	±0.7	**3.2[a]**			
	5	4.1	±0.6	4.3	±1.0	5.8	±2.9	**4.8[b]**			
	10	4.0	±0.7	3.3	±0.4	3.8	±0.6	**3.7[a]**			
Means by Photoperiod		**3.71**		**3.79**		**4.17**					
Thymus	17	0.91	±0.5	0.98	±0.3	1.2	±0.3	**1.0[a]**	<0.001	0.20	0.68
	19	0.53	±0.2	0.69	±0.2	0.8	±0.3	**0.7[b]**			
	5	0.86	±0.4	1.0	±0.2	1.0	±0.2	**0.9[ab]**			
	10	1.3	±0.3	1.5	±0.5	1.3	±0.1	**1.4[c]**			
Means by Photoperiod		**0.9**		**1.0**		**1.1**					
Liver	17	57.5	±1.9	56.9	±2.9	51.9	±4.0	**55.4[a]**	<0.001	0.43	0.47
	19	52.1	±3.7	49.8	±4.3	52.5	±6.2	**55.5[a]**			
	5	61.5	±8.1	61.7	±2.6	61.3	±5.8	**61.5[b]**			
	10	67.4	±4.3	64.7	±6.3	66.0	±3.2	**66.0[c]**			
Means by Photoperiod		**59.6**		**58.3**		**57.9**					

[1.] Photoperiods (h light:h dark): long day (16 h light:8 h dark), normal day (12 h light:12 h dark) and short day (8 h light:16 h dark)

[2.] Organs weights were normalized to body wt for each mouse; mean weights and standard deviations from mice ($n \geq 5$) are shown.

[3.] The effects of time, photoperiod and the interaction of photoperiod and time were determined using the ANOVA procedure in JMP® Pro 10.

[4, 5.] Separation of means by time or photoperiod were conducted using Tukey-Kramer HSD test, differences were characterized as significant at $p \leq 0.05$ and are indicated by differing superscript letters.

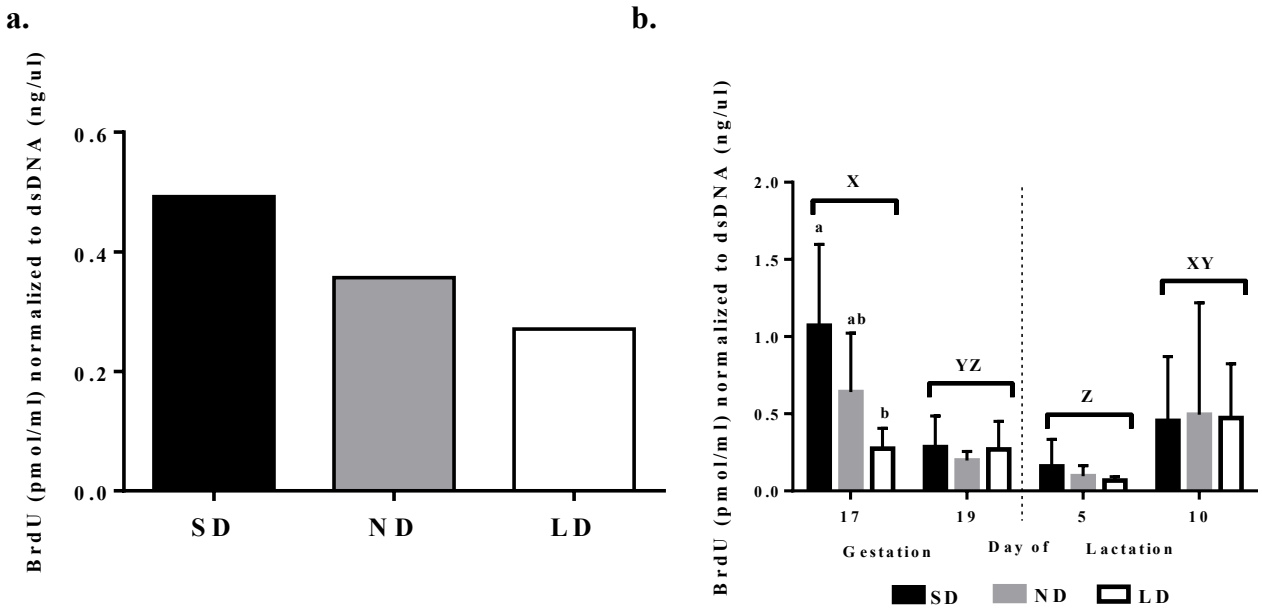


Figure 5.2. Photoperiod during gestation affects the uptake of BrdU into mouse mammary cells.

Pregnant female mice were exposed to long day (LD; 16 h light: 8 h dark), normal day (ND; 12 h light:12 h dark), or short day (SD; 8 h light:16 h dark) photoperiod for the duration of gestation and were sacrificed on G17 or G19. The morning of parturition, dams and their litters were returned to ND photoperiod until sacrifice on L5 or L10. BrdU concentrations were normalized to total double-stranded DNA in the tissue homogenate. Resulting BrdU values were analyzed for the overall effect of **a.** photoperiod and **b.** time. Distinct letters (**X, Y, Z**) above bars indicate overall differences in the effect of time at $p < 0.05$. Distinct letters above bars (**a**, **b**) indicate significant differences of within-day comparisons of the effect of photoperiod at $p < 0.05$.

Table 5.2. Differentially expressed (DE)[1] genes in the mammary gland of mice exposed to photoperiod throughout gestation.

	Microarray Comparison	
	LD_{ND} [2]	**SD_{ND} [3]**
Negative Fold-Change	181	6
Positive Fold-Change	339	117
Total DE genes	520	123

[1.] Microarray analysis using the Nimblegen 12x135K mouse array platform was used to quantify relative gene expression in the mammary gland. Genes were considered DE if they met the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$.

[2.] LD_{ND}: negative fold-change indicates expression was higher in mice exposed to ND photoperiod.

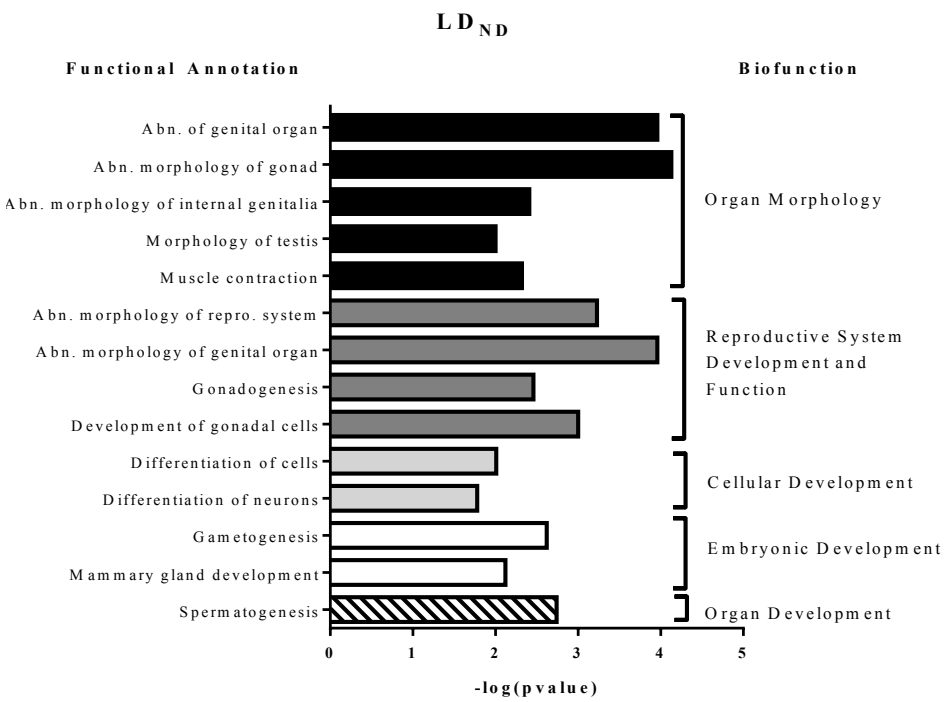
[3.] SD_{ND}: negative fold-change indicates expression was higher in mice exposed to SD photoperiod.

Table 5.3. Representative GO Terms[1] of clusters enriched by differentially expressed[2] (DE) genes in response to LD or SD photoperiod on G17.

GO Term[3]		GO ID	# DE genes	Score[4]
LD_{ND}				
MF	Peptide binding	GO:0042277	8	1.74
BP	Multicellular organism reproduction	GO:0032504	14	1.74
CC	Extracellular region	GO:0005576	38	1.67
BP	Protein amino acid phosphorylation	GO:0006468	15	1.26
MF	Antigen binding	GO:0003823	5	1.26
MF	Growth factor activity	GO:0008083	7	1.18
MF	Cytokine activity	GO:0005125	8	1.12
MF	Peptidase activity	GO:0008233	15	1.09
BP	Regulation of cell proliferation	GO:0042127	13	1.01
SD_{ND}				
BP	Chromosome organization	GO:0051276	10	2.28
BP	Regulation of cell cycle	GO:0051726	5	1.51
BP	Chromatin organization	GO:0006325	7	1.31
MF	Hydrogen ion transmembrane transporter activity	GO:0015078	3	1.17
CC	Cell junction	GO:0030054	6	1.03

1. Representative GO terms for functional clusters identified using Functional Clustering in DAVID (http://david.abcc.ncifcrf.gov/).
2. Microarray analysis using the Nimblegen 12x135K mouse array platform was used to quantify relative gene expression in the mammary gland. Genes were considered DE if they met the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$.
3. **MF**: molecular function; **BP**: biological process; **CC**: cellular component.
4. Score is DAVID's enrichment score; only clusters with scores > 1.0 are shown.

a.


LD ND
Functional Annotation
Biofunction
Abn. of genital organ
Abn. morphology of gonad
Abn. morphology of internal genitalia
Morphology of testis
Muscle contraction
Abn. morphology of repro. system
Abn. morphology of genital organ
Gonadogenesis
Development of gonadal cells
Differentiation of cells
Differentiation of neurons
Gametogenesis
Mammary gland development
Spermatogenesis
Organ Morphology
Reproductive System Development and Function
Cellular Development
Embryonic Development
Organ Development
0
1
2
3
4
5
-log(pvalue)

b.

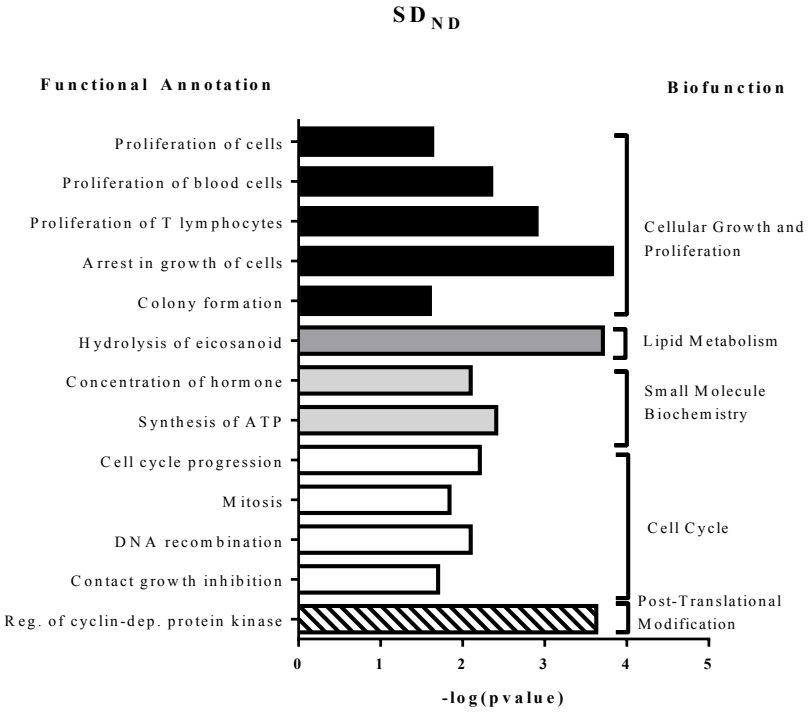

SD ND
Functional Annotation
Biofunction
Proliferation of cells
Proliferation of blood cells
Proliferation of T lymphocytes
Arrest in growth of cells
Colony formation
Hydrolysis of eicosanoid
Concentration of hormone
Synthesis of ATP
Cell cycle progression
Mitosis
DNA recombination
Contact growth inhibition
Reg. of cyclin-dep. protein kinase
Cellular Growth and Proliferation
Lipid Metabolism
Small Molecule Biochemistry
Cell Cycle
Post-Translational Modification
0
1
2
3
4
5
-log(pvalue)

Figure 5.3. IPA *Biofunctions* and functional annotation for genes differentially expressed genes in response to photoperiod on G17.

Microarray analysis was used to quantify relative gene expression in the mammary gland of mice exposed to long day (LD; 16 h light: 8 h dark), normal day (ND; 12 h light: 12 h dark), or short day (SD; 8 h light: 16 h dark) for the duration of gestation. Genes were considered differentially expressed in the comparison of **a.** LD_{ND} or **b.** SD_{ND} if they met the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$. IPA *biofunctions* and associated *functional annotation* with > 1 associated differentially expressed gene were ranked by –log (p-value), an estimate of whether the group is over-represented in the data set. The top five *biofunctions* by p-value are shown; redundant *functional annotations* are not shown.

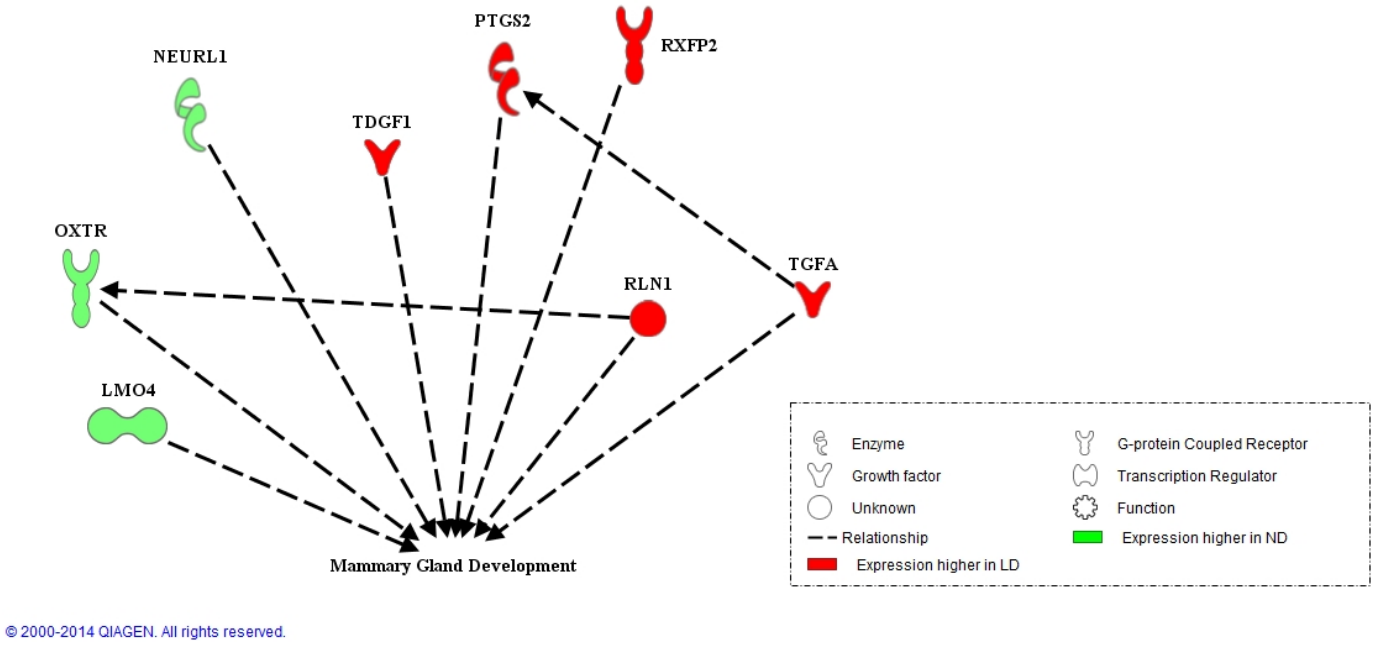


Figure 5.4. Genes differentially expressed in the comparison of LD_{ND} are associated with mammary development.

Microarray analysis was used to quantify relative gene expression in the mammary gland of mice exposed to long day (LD, 16 h light: 8 h dark), normal day (ND, 12 h light: 12 h dark) for the duration of gestation. Eight genes were associated with mammary gland development: LIM domain only 4 (*Lmo4*), neuralized-like homolog (*Neurl1*), oxytocin receptor (*Oxtr*), prostaglandin-endoperoxide synthase 2 (*Ptgs2*), relaxin 1 (*Rln1*), relaxin/insulin-like family peptide receptor 2 (*Rxfp*), teratocarcinoma-derived growth factor 1 (*Tdgf1*), transforming growth factor alpha (*Tgfa*).

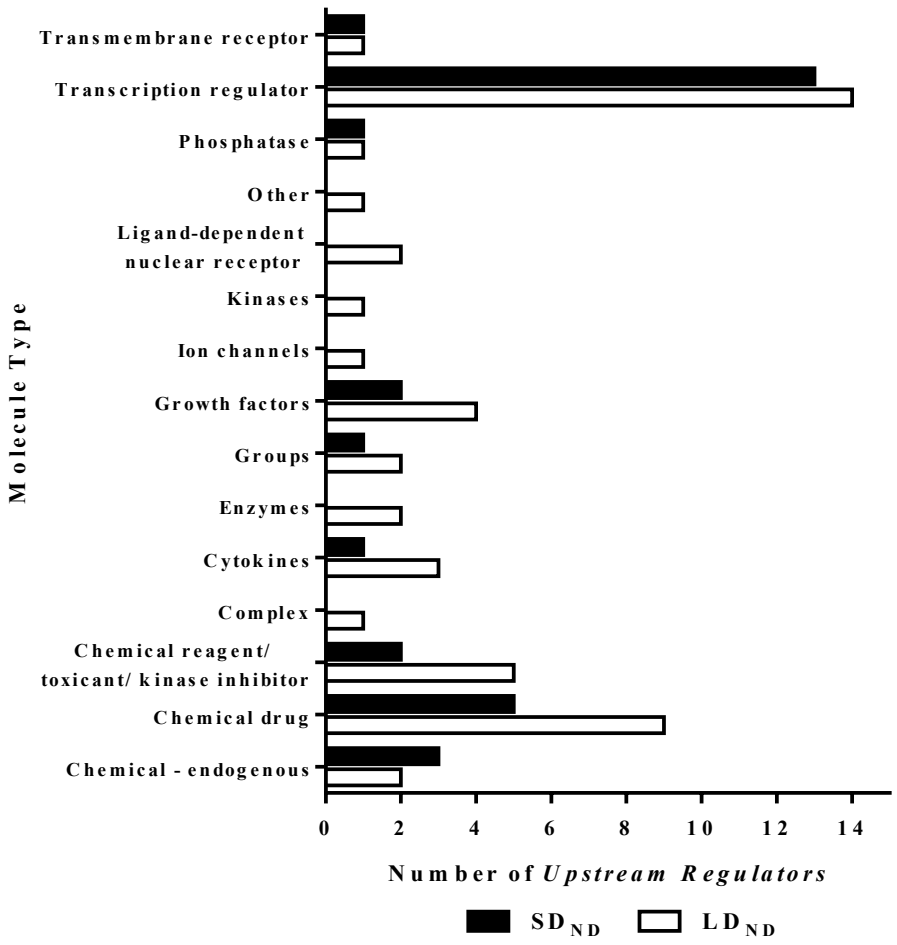


Figure 5.5. Predicted *upstream regulators* of differentially expressed genes in the mammary gland of mice exposed to different photoperiods during gestation.

Microarray analysis was used to quantify relative gene expression in the mammary gland of mice exposed to long day (LD, 16 h light: 8h dark), normal day (ND, 12 h light: 12 h dark), or short day (SD, 8 h light: 16 h dark) for the duration of gestation. Genes were considered differentially expressed if they met the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$. The resulting gene list was uploaded to IPA and *upstream regulators* predicted based on the IPA Knowledge Base®. *Upstream regulators* predicted to affect ≥ 5 genes are summarized by molecule type.

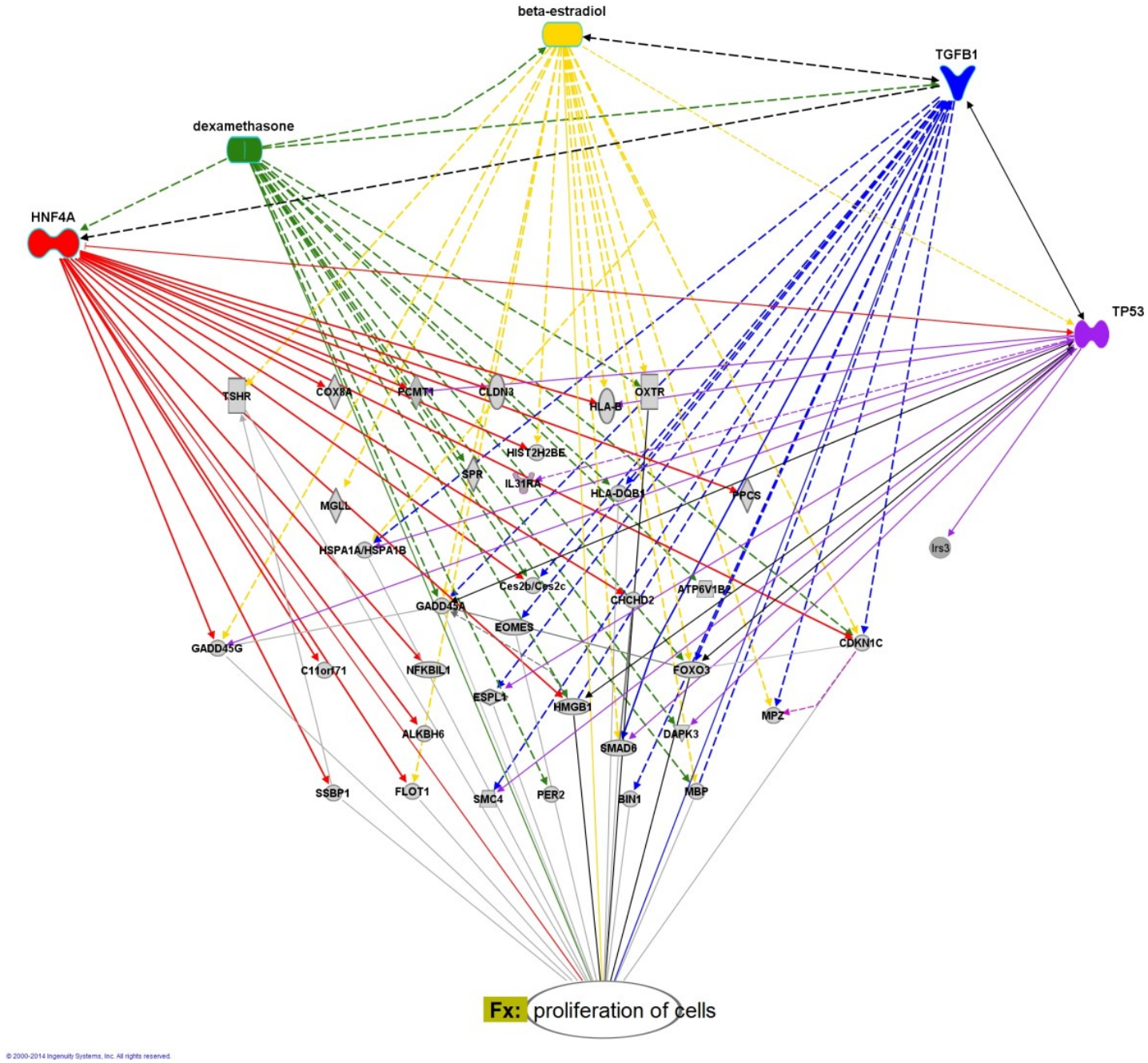


Figure 5.6. Genes differentially expressed in the comparison of SD_{ND} have common *upstream regulators* and functional outcomes.

Microarray analysis was used to quantify relative gene expression in the mammary gland of mice exposed to normal day (ND, 12 h light: 12 h dark), or short day (SD, 8 h light: 16 h dark) for the duration of gestation. Genes were considered differentially expressed in the comparison of SD_{ND} if they met the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$. The resulting gene list was uploaded to IPA and *upstream regulators* predicted based on the IPA Knowledge Base®. Five predicted *upstream regulators,* Hepatocyte nuclear factor 4 α- (*Hnf4a)*, dexamethasone, beta-estradiol, transforming growth factor β1 (*Tgfb1*), and tumor protein 53 (*Tp53*), the downstream genes, and the common functional effect are shown here.

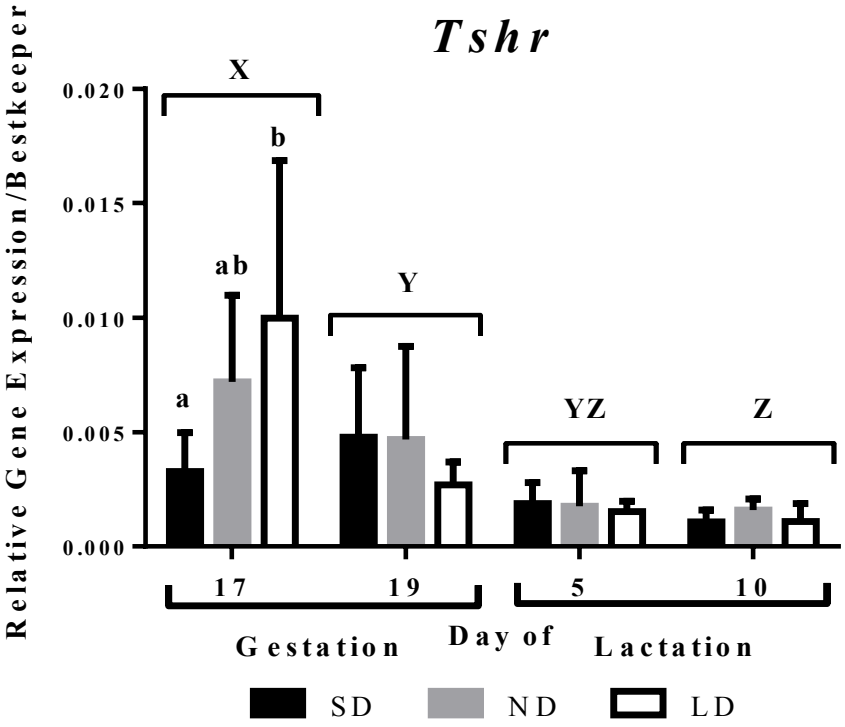


Figure 5.7. Quantitative RT-PCR of *Tshr* in the mammary gland of mice exposed to different photoperiods for the duration of gestation.

Mice were exposed to long day (LD; 16 h light: 8 h dark), normal day (ND; 12 h light:12 h dark), or short day (SD; 8 h light:16 h dark) for the duration of gestation and were sacrificed on G17 or G19. The morning of parturition, dams and their litters were returned to ND photoperiod until sacrifice on L5 or L10. Gene expression values were analyzed for the overall effect of photoperiod ($p = 0.31$), time ($p \leq 0.001$), and the interaction of photoperiod and time ($p = 0.01$). Distinct letters above bars indicate overall differences by time (X, Y, Z) at $p \leq 0.05$. Distinct letters above bars indicates significant differences of within-day comparisons of the effects of photoperiod (a, b) at $p \leq 0.05$.

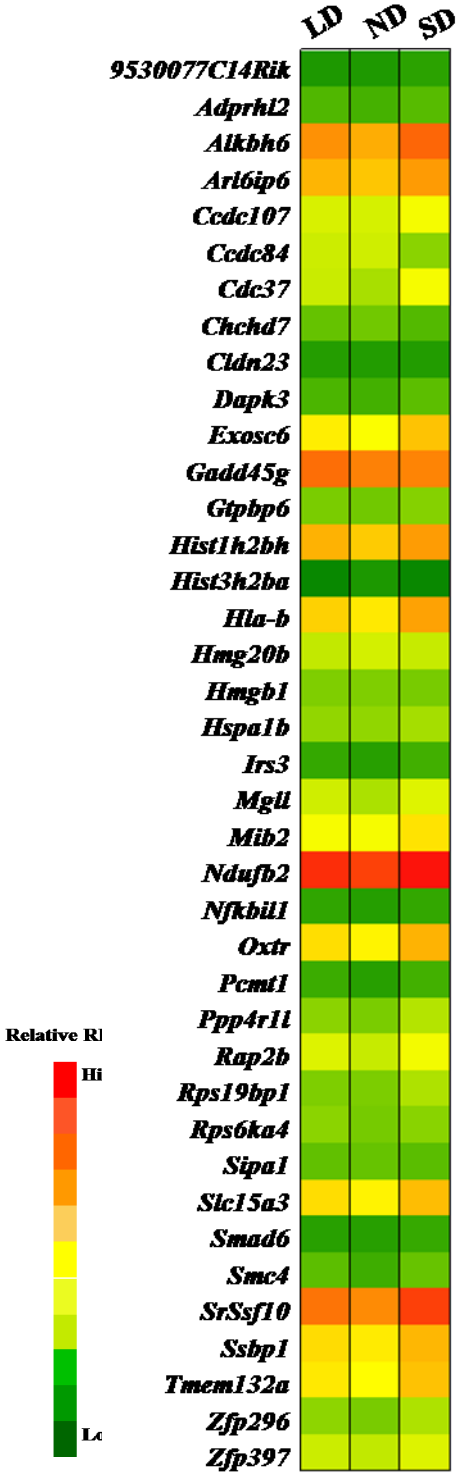


Figure 5.8. The relative robust multi-chip average (RMA) values of genes common in the comparisons of LD_{ND} and SD_{ND} photoperiod in mouse mammary gland.

Mice were exposed to long day (LD; 16 h light: 8 h dark), normal day (ND; 12 h light: 12 h dark), or short day (SD; 8 h light: 16 h dark) for the duration of gestation and were sacrificed on G17. Microarray analyses using the Nimblegen 12x132K mouse microarray platform was used to quantify the relative expression of genes in the mammary gland. Genes were considered differentially expressed if they met the criteria of fold-change $\geq |1.5|$ and $p \leq 0.05$. The heat map shows genes (n = 39) commonly differentially expressed in the comparison of LD_{ND} and SD_{ND} on G17. Each box represents the mean RMA value for mice ($n \geq 4$) exposed to LD, ND or SD photoperiod.

REFERENCES

Auchtung, T. L., A. G. Rius, P. E. Kendall, T. B. McFadden, and G. E. Dahl. 2005. Effects of photoperiod during the dry period on prolactin, prolactin receptor, and milk production of dairy cows. J Dairy Sci 88:121.

Barrett, P., F. J. Ebling, S. Schuhler, D. Wilson, A. W. Ross, et al. 2007. Hypothalamic thyroid hormone catabolism acts as a gatekeeper for the seasonal control of body weight and reproduction. Endocrinology 148:3608.

Bartness, T. J., J. B. Powers, M. H. Hastings, E. L. Bittman, and B. D. Goldman. 1993. The timed infusion paradigm for melatonin delivery: What has it taught us about the melatonin signal, its reception, and the photoperiodic control of seasonal responses? J Pineal Res 15:161.

Behl, B., M. Klos, M. Serr, U. Ebert, B. Janson, K. Drescher, G. Gross, and H. Schoemaker. 2006. An elisa-based method for the quantification of incorporated brdu as a measure of cell proliferation in vivo. J Neurosci Methods 158:37.

Bentley, P. A., E. H. Wall, G. E. Dahl, and T. B. McFadden. 2014. Responses of the mammary transcriptome of dairy cows to altered photoperiod during late gestation. Physiol Genomics Submitted: PG-00112-2014

Bolstad, B. M., R. A. Irizarry, M. Astrand, and T. P. Speed. 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19:185.

Ciarleglio, C. M., J. C. Axley, B. R. Strauss, K. L. Gamble, and D. G. McMahon. 2011. Perinatal photoperiod imprints the circadian clock. Nat Neurosci 14:25.

Cos, S. and E. J. Sanchez-Barcelo. 2000. Melatonin and mammary pathological growth. Front Neuroendocrinol 21:133.

Dahl, G. E. 2005. Let there be light: Photoperiod management of dairy cattle for performance and health in Proc. Florida Dairy Production Gainesville, Fl.

Dahl, G. E., N. P. Evans, S. M. Moenter, and F. J. Karsch. 1994. The thyroid gland is required for reproductive neuroendocrine responses to photoperiod in the ewe. Endocrinology 135:10.

Dahl, G. E., S. Tao, and I. M. Thompson. 2012. Lactation biology symposium: Effects of photoperiod on mammary gland development and lactation. J Anim Sci 90:755.

Duncan, M. J. 2007. Circannual prolactin rhythms: Calendar-like timer revealed in the pituitary gland. Trends Endocrinol Metab 18:259.

Gonzalez, C. R., R. S. Calandra, and S. I. Gonzalez-Calvar. 2012. Influence of the photoperiod on tgf-beta1 and p15 expression in hamster leydig cells. Reprod Biol 12:201.

Hastings, M. H., J. Herbert, N. D. Martensz, and A. C. Roberts. 1985. Annual reproductive rhythms in mammals: Mechanisms of light synchronization. Ann NY Acad Sci 453:182.

Hoellen, F., K. Kelling, C. Dittmer, K. Diedrich, M. Friedrich, and M. Thill. 2011. Impact of cyclooxygenase-2 in breast cancer. Anticancer Res 31:4359.

Hurley, W. L., R. M. Doane, M. B. O'Day-Bowman, R. J. Winn, L. E. Mojonnier, and O. D. Sherwood. 1991. Effect of relaxin on mammary development in ovariectomized pregnant gilts. Endocrinology 128:1285.

Irizarry, R. A., B. M. Bolstad, F. Collin, L. M. Cope, B. Hobbs, and T. P. Speed. 2003a. Summaries of affymetrix genechip probe level data. Nucleic Acids Res 31:e15.

Irizarry, R. A., B. Hobbs, F. Collin, Y. D. Beazer-Barclay, K. J. Antonellis, U. Scherf, and T. P. Speed. 2003b. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4:249.

Ishikawa, F., K. Nose, and M. Shibanuma. 2008. Downregulation of hepatocyte nuclear factor-4alpha and its role in regulation of gene expression by tgf-beta in mammary epithelial cells. Exp Cell Res 314:2131.

Kampf-Lassin, A. and B. J. Prendergast. 2013. Photoperiod history-dependent responses to intermediate day lengths engage hypothalamic iodothyronine deiodinase type iii mrna expression. Am J Physiol Regul Integr Comp Physiol.

Knight, C. H. and M. Peaker. 1982. Mammary cell proliferation in mice during pregnancy and lactation in relation to milk yield. Q J Exp Physiol 67:165.

Knudsen, K. A. and M. J. Wheelock. 2005. Cadherins and the mammary gland. J Cell Biochem 95:488.

Lemay, D. G., M. C. Neville, M. C. Rudolph, K. S. Pollard, and J. B. German. 2007. Gene regulatory networks in lactation: Identification of global principles using bioinformatics. BMC Syst Biol 1:56.

Lincoln, G. A., H. Andersson, and A. Loudon. 2003. Clock genes in calendar cells as the basis of annual timekeeping in mammals--a unifying hypothesis. J Endocrinol 179:1.

Malpaux, B., M. Migaud, H. Tricoire, and P. Chemineau. 2001. Biology of mammalian photoperiodism and the critical role of the pineal gland and melatonin. J Biol Rhythms 16:336.

Mediavilla, M. D., M. San Martin, and E. J. Sanchez-Barcelo. 1992. Melatonin inhibits mammary gland development in female mice. J Pineal Res 13:13.

Neville, M. C., T. B. McFadden, and I. Forsyth. 2002. Hormonal regulation of mammary differentiation and milk secretion. J Mammary Gland Biol Neoplasia 7:49.

Ono, H., Y. Hoshino, S. Yasuo, M. Watanabe, Y. Nakane, A. Murai, S. Ebihara, H. W. Korf, and T. Yoshimura. 2008. Involvement of thyrotropin in photoperiodic signal transduction in mice. Proc Natl Acad Sci U S A 105:18238.

Ono, H., N. Nakao, and T. Yoshimura. 2009. Identification of the photoperiodic signaling pathway regulating seasonal reproduction using the functional genomics approach. Gen Comp Endocrinol 163:2.

Otsuka, T., M. Goto, M. Kawai, Y. Togo, K. Sato, K. Katoh, M. Furuse, and S. Yasuo. 2012. Photoperiod regulates corticosterone rhythms by altered adrenal sensitivity via melatonin-independent mechanisms in fischer 344 rats and c57bl/6j mice. PLoS ONE 7:e39090.

Otsuka, T., M. Kawai, Y. Togo, R. Goda, T. Kawase, et al. 2014. Photoperiodic responses of depression-like behavior, the brain serotonergic system, and peripheral metabolism in laboratory mice. Psychoneuroendocrinology 40:37.

Pati, D., B. R. Haddad, A. Haegele, H. Thompson, F. S. Kittrell, et al. 2004. Hormone-induced chromosomal instability in p53-null mammary epithelium. Cancer Res 64:5608.

Peters, R. R., L. T. Chapin, K. B. Leining, and H. A. Tucker. 1978. Supplemental lighting stimulates growth and lactation in cattle. Science 199:911.

Pfaffl, M. W., A. Tichopad, C. Prgomet, and T. P. Neuvians. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: Bestkeeper--excel-based tool using pair-wise correlations. Biotechnol Lett 26:509.

Politowska, E., C. Czaplewski, and J. Ciarkowski. 1999. Molecular modeling of the oxytocin receptor/bioligand interactions. Acta Biochim Pol 46:581.

Ross, A. W., G. Helfer, L. Russell, V. M. Darras, and P. J. Morgan. 2011. Thyroid hormone signalling genes are regulated by photoperiod in the hypothalamus of f344 rats. PLoS ONE 6:e21351.

Salomon, D. S., C. Bianco, and M. De Santis. 1999. Cripto: A novel epidermal growth factor (egf)-related peptide in mammary gland development and neoplasia. Bioessays 21:61.

Sherwood, O. D. 2004. Relaxin's physiological roles and other diverse actions. Endocr Rev 25:205.

Sladek, F. M., W. M. Zhong, E. Lai, and J. E. Darnell, Jr. 1990. Liver-enriched transcription factor hnf-4 is a novel member of the steroid hormone receptor superfamily. Genes Dev 4:2353.

Smith, G. H., R. Sharp, E. C. Kordon, C. Jhappan, and G. Merlino. 1995. Transforming growth factor-alpha promotes mammary tumorigenesis through selective survival and growth of secretory epithelial cells. Am J Pathol 147:1081.

Sum, E. Y., M. Shackleton, K. Hahm, R. M. Thomas, L. A. O'Reilly, K. U. Wagner, G. J. Lindeman, and J. E. Visvader. 2005. Loss of the lim domain protein lmo4 in the mammary gland during pregnancy impedes lobuloalveolar development. Oncogene 24:4820.

Tamarkin, L., C. J. Baird, and O. F. Almeida. 1985. Melatonin: A coordinating signal for mammalian reproduction? Science 227:714.

Trott, J. F., A. Schennink, W. K. Petrie, R. Manjarin, M. K. VanKlompenberg, and R. C. Hovey. 2012. Triennial lactation symposium: Prolactin: The multifaceted potentiator of mammary growth and function. J Anim Sci 90:1674.

Vollrath, L., A. Huesgen, B. Manz, and K. Pollow. 1988. Day/night serotonin levels in the pineal gland of male balb/c mice with melatonin deficiency. Acta Endocrinol (Copenh) 117:93.

Wall, E. H., T. L. Auchtung-Montgomery, G. E. Dahl, and T. B. McFadden. 2005a. Short communication: Short-day photoperiod during the dry period decreases expression of suppressors of cytokine signaling in mammary gland of dairy cows. J Dairy Sci 88:3145.

Wall, E. H., T. L. Auchtung, G. E. Dahl, S. E. Ellis, and T. B. McFadden. 2005b. Exposure to short day photoperiod during the dry period enhances mammary growth in dairy cows. J Dairy Sci 88:1994.

Wei, J., P. Ramanathan, I. C. Martin, C. Moran, R. M. Taylor, and P. Williamson. 2013. Identification of gene sets and pathways associated with lactation performance in mice. Physiol Genomics 45:171.

CHAPTER 6: PHOTOPERIOD EXPOSURE DURING GESTATION HAS PERSISTENT EFFECTS ON THE MAMMARY TRANSCRIPTOME DURING LACTATION

ABSTRACT

Photoperiod, or daily light exposure, is used by animals to synchronize their physiology including reproduction. Some effects of photoperiod manipulation on mammary function and gene expression are known; however, possible long-term effects of different photoperiods on the mammary transcriptome have not been elucidated. Our aim was to identify photoperiod-responsive genes on day 10 of lactation (L10) in the mammary gland of mice previously exposed to different photoperiods during gestation. We hypothesized that altered photoperiod would have a lasting effect on the expression of genes associated with mammary physiology and function. Pregnant female mice were exposed to one of three photoperiod treatments, long day (LD), normal day (ND), or short day (SD) for 21 days of gestation and were returned to ND photoperiod after parturition. Photoperiod exposure during gestation did not affect litter weight, as a proxy for mil production, during the first 10 days of lactation. Using microarray analysis, we quantified changes in the mammary transcriptome of mice on L10. Relative to ND, we identified differential expression of 1182 genes in response to LD photoperiod and 380 genes in response to SD photoperiod. Both LD and SD photoperiod affected genes associated with cell cycle progression and lactation performance and suggest that the effects of photoperiod in the mammary gland may be coordinated by thyroid hormone-related gene expression. Our findings provide evidence that exposure to different photoperiods during gestation has enduring effects on the mammary transcriptome and suggests long-term effects on mammary function.

INTRODUCTION

Photoperiod, or daily light exposure, affects numerous aspects of physiology including behavior, hormone secretion and reproduction (Walton et al., 2011). Although the effects of photoperiod on reproduction have been widely described (Hastings et al., 1985; Chemineau et al., 2008; Rani and Kumar, 2014) the effects of photoperiod on lactation, a critical component of reproduction, are not fully understood.

First reported in dairy cows by Peters et al. (1978), exposure to long day (**LD**, 16 h light: 8 h dark) photoperiod during lactation increases milk production. The galactopoietic effects of LD photoperiod during lactation are now well established in dairy cows (Dahl et al., 2000) and similar responses have been reported in mice, as measured by litter weight gain (Sorensen and Hacker, 1979), pigs (Stevenson et al., 1983) and goats (Garcia-Hernandez et al., 2007). In contrast to exposure during lactation, exposure to LD photoperiod during gestation does not have the same galactopoietic effects. Rather, exposure to short day (**SD**, 8 h light: 16 h dark) photoperiod during gestation increases milk production (3 kg/d) in the subsequent lactation, relative to cows on LD photoperiod (Auchtung et al., 2005). Similar findings have been reported in dairy sheep (Mikolayunas et al., 2008) and goats (Mabjeesh et al., 2013) indicating the mechanism underlying the effects may be conserved across species.

Milk production is determined by the number and activity of secretory cells in the mammary gland (Capuco et al., 2001); therefore, photoperiod manipulation must affect one or both of these factors to directly or indirectly alter milk production. Cows exposed to SD photoperiod during late gestation went on to produce more milk than their LD counterparts and had increased mammary cell proliferation three weeks prior to parturition (Wall et al., 2005). In mice, LD photoperiod during lactation decreased mammary cell proliferation on

day 10 of lactation (L10), relative to mice on ND photoperiod (**Chapter 4**). During gestation, SD photoperiod increased mammary cell proliferation on day 17 of gestation, relative to LD photoperiod (**Chapter 5**). Together, these studies demonstrate that photoperiod influence mammary cell proliferation and function.

The molecular mechanisms underlying the effects of photoperiod on mammary function involve both systemic hormonal regulation and local gene expression in the mammary gland. To date, investigations have focused on four hormonal factors as potential mediators of the effect of photoperiod on mammary function. Melatonin, a hormone rhythmically secreted during darkness, is the central communicator of photoperiodic information to peripheral tissues, including progeny *in utero* (Davis and Mannion, 1988). Prolactin, critical for initiation of lactation (Trott et al., 2012), is responsive to photoperiod and coordinates seasonal changes in physiology (Duncan, 2007). Insulin-like growth factor 1 (*Igf1*), which is increased in dairy cows exposed to LD photoperiod (Dahl et al., 2000) is a mitogen necessary for mammary development (Rosfjord and Dickson, 1999). Genes associated with *Igf1* signaling are differentially expressed in response to photoperiod and time relative to parturition in dairy cows (**Chapter 3**). Lastly, thyroid hormone signaling coordinates peripheral metabolism in support of lactation (Neville et al., 2002) and controls seasonal changes in physiology and reproduction (Dardente et al., 2010; Yoshimura, 2013). We recently reported the effects of photoperiod manipulation during lactation on thyroid hormone gene expression in the mouse mammary gland (**Chapter 4**). Despite the well-established knowledge of the effects of photoperiod on the mammary gland and lactation, little is known about the long-term effects of photoperiod on mammary gene expression.

Our aim was to identify genes differentially expressed on L10 in the mammary gland of mice after exposure to different photoperiods during gestation. We hypothesized that altered photoperiod during gestation would induce differential expression of genes associated with mammary physiology and function even ten days post-treament. Here, we report photoperiod exposure during gestation indeed affects the mammary transcriptome 10 days after the cessation of exposure. We have identified differentially expressed genes, pathways and *upstream regulators* with functional importance to the mammary gland during lactation.

MATERIALS AND METHODS

Animal care and breeding

Procedures reported here were approved by the University of Alberta Animal Care and Use Committee and were previously detailed (**Chapter 5**). Female C57BL/6 mice (n = 16) were obtained at 5 weeks of age from Charles River Laboratories. Mice were maintained on normal day (**ND,** 12 h light: 12 h dark) photoperiod and provided a diet of 9% (w/v) fat mouse chow (Diet Labs) and water for *ad libitum* consumption. Mice were housed (3/cage) in wire-top cages on wire racks to allow unobstructed light exposure. At 7 weeks of age, females were mated in a 3:1 ratio with male C57BL/6 mice. Each morning, female mice were examined for the presence of vaginal plugs; when observed (day 1) female mice were randomly assigned to adjacent photoperiod treatment rooms for the remainder of gestation (**Figure 6.1a**). The morning of parturition, dams and their pups were transferred back to ND photoperiod for the remainder of lactation. Initial litter weight and number of pups were recorded the morning of parturition. Litter weights were quantified daily and no adjustments were made in the case of pup attrition.

Photoperiod treatments

Three photoperiod treatments (LD, ND, and SD) with rectangular light-dark transitions were used in this study (**Figure 6.1b**). Each photoperiod treatment was applied in one of three adjacent rooms that were continuously monitored for light intensity, relative humidity, and temperature using Hobo® data loggers. The photic phase was initiated at 0600 h and ended at 2200, 1800, and 1400 h, respectively (**Figure 6.1b**). The light intensity in treatment rooms was > 300 lux during the photic period and < 20 lux during the scotophase. Staff entered photoperiod treatment rooms only during the photic period.

Euthanasia and tissue collection

Lactating dams (n = 6/photoperiod treatment) were killed on L10 by CO_2 overdose during the photic phase between 0600 and 1200 h. Dams from each of the photoperiod groups were alternately killed to balance timing effects on tissue. Immediately post sacrifice, the 4th-right inguinal mammary gland was excised and the supra-mammary lymph node was removed. Mammary tissue was frozen in liquid nitrogen and stored at -80°C for RNA extraction.

RNA isolation and microarray preparation

RNA extraction and microarray sample preparation was carried out as previously described (**Chapters 4** and **5**). Briefly, the whole 4th-right inguinal mammary gland was pulverized in liquid nitrogen using a mortar and pestle and a portion was used for RNA isolation by TRIzol Reagent (Invitrogen^{TM}) according to the manufacturer's instruction. Microarray sample preparation and analysis was conducted at the Alberta Transplant Applied Genomics Centre, University of Alberta, following standard procedures. Nucleic acid

concentration was quantified using the NanoDrop ND1000 spectrophotometer and RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA samples (LD: n = 6, ND: n = 4, SD: n = 6) with RNA integrity number ≥ 7 were prepared for analysis following the manufacturer's instructions (for details: http://www.nimblegen.com/support/dna-microarray-support.html). First-strand cDNA was synthesized using oligo dT primer and SuperScript II Reverse Transcriptase. Second strand cDNA was prepared using T4 DNA polymerase. Samples were labelled using Cy3 random primers and the Klenow fragment ('3 -> 5'exo-). Labelled samples were hybridized to Roche Nimblegen 12x135K array chips using the Nimblegen Hybridization System. Arrays were scanned using the MS 200 Microarray Scanner and MS 200 Data Collection Software. Images were collected and normalized using Roche Nimblegen DEVA software by the quantile normalization method (Bolstad et al., 2003). Gene calls were generated using the Robust Multichip Average (RMA) method (Irizarry et al., 2003a; Irizarry et al., 2003b). Analysis of RMA values was conducted using Partek® Software. An IQR filter (> 0.5) was used to eliminate probe sets with little variation. The remaining probe sets were subjected to the following t-test comparisons: LD vs ND (LD_{ND}) and ND vs SD (SD_{ND}). Fold-change was calculated as LD –ND and ND – SD; the resulting positive fold-change values indicate higher expression in mice exposed LD and ND photoperiod, respectively. Genes meeting the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$ were considered differentially expressed and included in functional analysis.

Gene functional analysis

Gene annotation, *biofunction* and *upstream regulator* analysis was conducted using Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com).

Probe sets from the comparisons of LD_{ND} and SD_{ND} that met our criteria for differential expression were uploaded. Probe sets were evaluated using the Ingenuity Knowledge Base® and IPA mapped genes underwent further analysis. *Core analysis* was conducted with the parameters set to defaults including genes only, direct and indirect relationships, endogenous chemicals, and information from rat, mouse, and human species. The top 5 *molecular and cellular biofunctions* by number of associated differentially expressed genes were identified and ordered using *p-value of overlap* resulting from Fisher Exact t-test. The top 5 unique *functional annotations,* with > 1 associated gene, for each *biofunction* are presented herein. Gene lists from the comparisons of LD_{ND} and SD_{ND} were compared to those associated with thyroid signaling (n = 791 factors in IPA). To annotate genes in the context of lactation, we compared differentially expressed and those reported as correlated with lactation performance in mice (n = 1435) (Wei et al., 2013). Common genes are presented.

Pathway relationships were developed in IPA and the top 5 IPA *functions* (excluding cancer-related functions) for corresponding gene sets are shown. Identification of potential *upstream regulators* of differentially expressed genes was completed using IPA default settings. *Upstream regulators* predicted to affect ≥ 10 differentially expressed with a significant *p-value of overlap* ($p \leq 0.05$), as determined by a Fisher's Exact t-test, were further explored. Functional clustering was conducted using the Functional Annotation tool of the Database of Annotation, Visualization, and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/). Differentially expressed genes were used to identify enriched gene set clusters from which representative GO Terms with *enrichment scores > 1.0* are reported.

Statistical analysis

The overall effects of photoperiod, time and the interaction of photoperiod and time on litter weight were assessed using the repeated measures MANOVA procedure in JMP® Pro 10.0.

RESULTS

Photoperiod exposure during gestation did not affect litter weight in the first 10 days of lactation (**Figure 6.2**). There was no interaction of photoperiod and time on litter weight, whereas day of lactation (time) significantly affected litter weight.

In comparing LD_{ND}, 867 genes were more highly expressed in mice exposed to LD photoperiod, and 315 genes were more highly expressed in mice exposed to ND photoperiod (**Table 6.1**, **Suppl. T6.1**). Genes identified in the comparison of LD_{ND} were arranged into 129 clusters, 12 with enrichment scores > 1 (**Table 6.2**). *Cell surface receptor linked signal transduction* was the most highly enriched cluster (n = 108 genes). Functional analysis associated differentially expressed genes with *nervous system development*, specifically *olfactory response*, and *synaptic transmission* (**Figure 6.3a**, **Suppl. T6.2**), as well as *cell cycle* including *mitosis*, *cytokinesis,* and *segregation of chrom*osomes. *Upstream regulators* (n = 74) were predicted to affect ≥ 10 differentially expressed genes in the comparison of LD_{ND} (**Suppl. T6.3**). The molecule type corresponding to the greatest number of *upstream regulators* was *transcription factors* (n = 26), *chemical drug* (n = 16) and *ligand dependent nuclear receptors* (n = 6) (**Figure 6.4**). The *upstream regulators* predicted to affect the greatest number of genes (n > 40) were dexamethasone, tumor protein 53 (*TP53*) and lipopolysaccharide (LPS). In the comparison of LD_{ND}, 36 photoperiod-responsive genes

were previously correlated with lactation performance in mice (**Table 6.3**), and 15 genes were associated with thyroid signaling (**Figure 6.6a**).

In the comparison of SD_{ND}, 380 genes were differentially expressed (**Table 6.1**, **Suppl. T6.1**). These genes aligned into 86 functional clusters, 17 with enrichment scores > 1 (**Table 6.2**). E*xtracellular region* was enriched by the greatest number of genes (n = 44). Functional analysis further associated these differentially expressed genes with *cell replication*, including *cell cycle progression, M* and *G1 phase* and *condensation of chromosomes* (**Figure 6.3b**, **Suppl. T6.2**). *Cell cycle progression* was enriched by 39 differentially expressed genes (**Figure 6.5**). There were 61 *upstream regulators* predicted to affect ≥ 10 differentially expressed genes in the comparison of SD_{ND} (**Figure 6.4**, **Suppl. T6.3**). The molecule type corresponding to the greatest number of *upstream regulators* was *transcription factors* (n = 16), *chemical drug* (n = 14) and *cytokine signaling factors* (n =7). Among the *upstream regulators*, transforming growth factor β 1 (*Tgfβ1)*, LPS, *Tp53*, β-estradiol, dexamethasone, and tumor necrosis factor (*Tnf*) were predicted to affect the greatest number of genes (n ≥ 30). Of the 380 differentially expressed genes, 23 were observed to be in common with genes correlated with lactation performance in mice (Wei et al., 2013) (**Table 6.3**), and 6 genes were associated with thyroid signaling (**Figure 6.6b**).

DISCUSSION

Photoperiod during gestation has lasting effects on mammary gene expression

We report that the photoperiod to which a pregnant mouse is exposed during gestation affects the mouse mammary transcriptome 10 days after the cessation of exposure. Our findings are in agreement with other recent reports of long-term and epigenetic effects of

photoperiod. Azzi et al. (2014) recently reported the circadian clock can be modified by photoperiod through DNA methylation, resulting in day length having lasting effects on physiology. In mice, perinatal photoperiod has an imprinting effect on behavior and circadian clock gene expression in the suprachiasmatic nuclei (SCN) (Ciarleglio et al., 2011). Perinatal photoperiod also has long-term effects on how circadian gene expression is altered by light (Brooks et al., 2014). Retinal and visual function can also be modulated by photoperiod during the perinatal period, such that mice exposed to SD photoperiod are deficient in their response to light, compared to their counterparts on LD photoperiod (Jackson et al., 2014). Together with our findings, it is clear that photoperiod during gestation and the perinatal period can have long-term effects on gene expression and physiology.

SD and LD photoperiod affect genes associated with cell cycle progression

Cell cycle progression was identified as the top *biological function* associated with differentially expressed genes in the comparison of SD_{ND}. Among the genes associated with this *biofunction*, breast cancer anti-estrogen resistance 1 (*Bcar1/p130Cas* mouse analogue) was increased in mice exposed to ND photoperiod. *Bcar1* is involved in migration, cellular survival, and transformation; when overexpressed in lactating mice, *Bcar1* increases mammary cell proliferation (Cabodi et al., 2006). *Biofunctions* associated with cellular division were enriched by, Aurora (*Aurka*, *Aurkb*), members of the cyclin family (*Ccnb1*,C*ccnb2*, *Ccnf*,) cell division cycle (*Cdc20* and *Cdc25c*), cell division associated (*Cdca2, -5, -8*), cyclin dependent kinases and cyclin dependent kinase inhibitors. The majority of these genes were down-regulated in mice exposed to SD compared to ND photoperiod. Similar genes were detected in the comparison of LD_{ND} and were also down-

regulated in mice exposed to LD compared to ND photoperiod, suggesting SD and LD photoperiod had similar effects on expression of *cell cycle progression* genes, relative to ND photoperiod.

Contrary to the transcriptomic data, we have previously reported no effect of photoperiod exposure during gestation on BrdU incorporation in mammary homogenate on L10 (**Chapter 5**), although there was an overall tendency of SD photoperiod to increase BrdU incorporation, relative to LD photoperiod between day 17 and 19 of gestation and days 5 and 10 of lactation. The lack of agreement in these data suggest transcriptional regulation of cell cycle machinery was underway on L10, but that DNA replication had not yet been initiated. Ultimately this suggests that photoperiod manipulation during gestation may affect mid-lactation cell proliferation and subsequently lactation persistency, rather than during early lactation.

There were 8 genes, abnormal spindle-like microcephaly associated (*Aspm*), *Aurkb*, *Cdc20, Cenpa*, *Cenpf*, kinesin family member 11 (*Kif11*), Rac GTPase activating protein 1 (*Racgap1*) and topoisomerase (DNA) II alpha (*Top2a*), common in the comparisons of LD_{ND} and SD_{ND} and these are associated with *cell cycle progression.* *Aspm*, *Aurkb*, *Cenpf*, and a member of the kinesin family have previously been identified as responsive to photoperiod in the bovine mammary gland during late gestation (**Chapter 3**). *Aspm*, localizes to mitotic spindle poles during cell division, and its expression is up-regulated by aurora kinases, two of which (*Aurka*, *Aurkb*) were differentially expressed in the comparison of SD_{ND}. Aurora kinases interact with centrosomes during interphase and have a critical role in cell division. Aberrant expression of aurora kinases and *Aspm* is detected in numerous cancers, including breast cancer (Katayama et al., 2003; Kouprina et al., 2005). Taken together, LD and SD

relative to ND photoperiod decrease the expression of genes associated with cell division, suggesting change away from ND photoperiod may have negative effects on mammary cell cycle progression during lactation.

More than 100 cell-surface linked receptors were differentially expressed in the comparison of LD_{ND}, many of which are associated with olfactory response (e.g. olfactory receptor 1). We have previously detected similar differential expression of olfactory-related genes on G17, concurrent with photoperiod exposure (**Chapter 5**); however, it is unclear what role these receptors may have in mammary function. We also report differential expression of melatonin receptor 1a *(Mtnr1a),* which encodes the protein MT_1. Yasuo et al. (2009) showed photoperiodic signals conveyed by melatonin affect gene expression in target cells by way of MT_1. Melatonin acting through the MT_1 receptor has anti-proliferative function in human breast cancer cells as well as other cancer types (Blask et al., 2011). Differential expression of this receptor long after the cessation of photoperiod exposure suggests alteration in melatonin signaling in the mammary gland that may elicit long-term effects on mammary function.

Upstream regulators of genes differentially expressed in the comparison of SD_{ND} included *Tgfb1, Tnf* and interferon γ (*Ifng*), all of which were previously identified as *upstream regulators* of genes responsive to photoperiod in the bovine mammary gland (**Chapter 3**). *Tgfb1* targets the mammary gland during lactation and local expression of *Tgfb1* peaks during lactation (Lamote et al., 2004). The expression of the immune modulator, *Tnf* is increased in the testes of white-footed mice exposed to SD photoperiod (Pyter et al., 2005). The expression of *Tnf* gene family members undergo seasonal changes in response to lipopolysaccharide challenge in captive baboons (McFarlane et al., 2012) as

well as hamsters (Bilbo et al., 2002). The expression of *Ifng*, another immune-related *upstream regulator*, is responsive to melatonin in human peripheral blood mononuclear cells (Garcia-Maurino et al., 1997), and is associated with circadian release of hormones from the pituitary gland (Cano et al., 2005). In the present study, numerous secreted factors were differentially expressed including secreted frizzled-related protein 1 (*Sfrp1*) and vanin 1 (*Vnn1*), retinol binding protein 4 (*Rbp4*), and butyrylcholinesterase (*Bche*), and S100 calcium binding protein A8 (*S100A8*). These secreted factors have been previously shown (**Chapter 3**) to be responsive to photoperiod in the mammary gland and are thought to enhance immune function in the mammary gland in response to SD photoperiod (Goldman, 2002; Watson, 2009). Taken together, our data add to the growing body of evidence that *Tgfb1*, *Tnf* and *Ifng* may transduce photoperiodic information signaling in peripheral tissues and regulate genes associated with cell proliferation and immune function.

Upstream regulators of genes differentially expressed in the comparison of LD_{ND} included *Igf1* which has previously been identified as a potential mediator of the effects of photoperiod in the bovine mammary gland (**Chapter 3**) (Dahl et al., 1997; Wall et al., 2005; Dahl et al., 2012). *Igf1* was predicted to affect 11 differentially expressed genes (*Acaca, Birc5, Col2a1, Ctgf, H2afx, Hsd3b1, Lep, Ly6a, Myb, Ntrk1, Ntrk2, Pbk, Serpine1, Sncg, Spp1, Th*). The *Igf1* axis in the mammary gland is affected by prolactin in mice (Hovey et al., 2003). Among these genes, leptin (*Lep*) interacts with the prolactin-regulated Jak-Stat5 signaling pathway in the mammary gland to enhance expression of β-casein, the principal protein in milk (Lin and Li, 2007). Prolactin is a lactogenic hormone also identified as a potential mediator of the effect of photoperiod on mammary function (Auchtung et al., 2005; Dahl, 2008). Members of the prolactin family were differentially expressed in the mammary

gland in both photoperiod comparisons (SD_{ND}: *Prl*, *Pr3d1*; LD_{ND}: *Pr3d1*). Collectively, the evidence for the involvement of *Igf1,* and to a lesser extent prolactin, in the response of the mammary gland to photoperiod is accumulating. Genes identified here should serve as targets for further study into the molecular mechanisms of the lasting effects of photoperiod on mammary physiology.

Photoperiod affects genes associated with thyroid signaling

Thyroid signaling has a key role in the transmission of photoperiodic information within the central clock and to peripheral clocks. In response to LD photoperiod, increased thyroid stimulating hormone (*Tsh*) in the *par tuberalis* of the pituitary gland increases the expression of activating deiodinase (*Dio2*) and decreases the expression of inactivating deiodinase (*Dio3*) (Ono et al., 2008). Thyroid hormones also function to establish metabolic priority of the mammary gland during lactation (Neville et al., 2002). We have previously reported differential expression of thyroid signaling-related genes in the mammary gland of mice exposed to LD or SD photoperiod during lactation (**Chapter 4**). Here, we provide further evidence of a role for thyroid signaling in transmitting photoperiodic signals to the mammary gland.

In the comparison of LD_{ND}, differentially expressed genes were associated with changes in cell quantity, morphology and differentiation. Neurotrophin receptors, *Ntrk1* and *Ntrk2* (aka: Trka and Trkb) interact with growth factors including brain-derived neurotrophic factor (*Bdnf*), vascular endothelial growth factor (*Vegf*) and fibroblast growth factor (*Fgf*), to enhance proliferation, angiogenesis and growth in the mammary gland (Hondermarck, 2012). Wall et al. (2012) reported differential expression of NTRK1 and NTRK2 in the mammary gland of cows milked 4-times rather than 2-times daily, suggesting these receptors may

function in the mammary response to external stimuli. The paired box family of transcription factors are regulators of tissue development (Robson et al., 2006). *Pax2* is required for mammary gland development, whereas *Pax8*, which was differentially expressed in our study, is required for thyroid development, and is over-expressed in thyroid carcinomas (Robson et al., 2006). The ligand-dependent nuclear receptor, retinoic acid receptor γ (*Rxrg*), is a thyroid hormone receptor that was down-regulated in mice exposed to LD photoperiod, and although it does not undergo large changes in expression between late gestation and early lactation in dairy cows (Capuco et al., 2008), it may have a role in the physiological response of the mouse mammary gland to photoperiod.

In the comparison of SD_{ND} we detected differential expression of *Eya1*, a member of the Eyes absent gene family (*Eya1-4*) that are involved in innate immunity, angiogenesis, organ development and photoperiodism (Tadjuidje and Hegde, 2013). *Eya3* is an *upstream regulator* of *Tsh* subunit β (*Tshβ*), which is a central component of the molecular switch for photoperiod responsiveness in the *par tuberalis* (Dardente et al., 2010). Eya proteins are themselves regulated by *Pax* transcription factors and potentially by the circadian related genes, CLOCK and BMAL1, in the *par tuberalis* (Wood and Loudon, 2014). *Foxm1*, which was observed to be common to the comparison of LD_{ND} and SD_{ND} is a cell proliferation-promoting transcription factor, that has roles in both mammary and thyroid carcinomas (Teh, 2012). However, its function in the context of lactation has not been described. Our findings provide evidence that thyroid-related signaling is a mediator of photoperiodic effects on the mammary gland. These data further suggest involvement of clock genes in the response of the mammary gland to photoperiod. Additional work is needed to understand how altered expression of these genes specifically affects mammary function.

Differentially expressed genes associated with lactation performance

We did not detect differences in litter weight gain, as a proxy for milk production, in response to different photoperiods during gestation. However, comparison of photoperiod-responsive genes to those correlated with lactation performance (Wei et al., 2013) suggest that photoperiod may affect mammary function by regulating some of those genes. Among the genes identified in the comparison of SD_{ND}, higher expression of 4 of them (*Gabrq*, *Gipr*, *Grik4* and *Htr4*) is positively correlated with lactation (Wei et al., 2013). Gamma-aminobutyric acid (GABA) A receptor θ (*Gabrq*) and glutamate receptor, ionotropic, kainate 4 (*Grik4*) were more highly expressed in mice exposed to SD photoperiod, whereas gastric inhibitory polypeptide receptor (*Gipr*) and 5-hydroxytryptamine (serotonin) receptor 4 (*Htr4*) were more highly expressed in mice on ND photoperiod. In addition to *Htr4*, *Htr5a* was differentially expressed in the comparison of SD_{ND}, whereas *Htr6* was differentially expressed in the comparison of LD_{ND}. Serotonin is a proposed regulator of lactation in mouse, human, and the bovine mammary gland (Hernandez et al., 2009; Collier et al., 2012). More broadly, serotonin is involved in regulation of sleep-wake cycles and circadian rhythms (Otsuka et al., 2014). Specifically, serotonin regulates tissue metabolism and function in the entrainment of circadian rhythms (Weber et al., 1998; Morin, 1999; Collier et al., 2012). Recently, Otsuka et al. (2014) reported the serotonergic system in the brain of C57Bl/6 mice is affected by photoperiod. During the light phase, mice on LD photoperiod have higher levels of serotonin in the amygdala compared to their SD counterparts (Otsuka et al., 2014). Photoperiod also affects the activity of tyrosine hydroxylase (*Th)* in dopaminergic terminals in the hypothalamus (Malpaux et al., 2001). Both *Th* and dopamine decarboxylase (*Ddc*), which are involved in serotonin synthesis, were differentially expressed in the comparison of

LD_{ND}. We have also previously reported *Ddc* as responsive to photoperiod in the bovine mammary gland (**Chapter 3**). Taken together, our findings provide evidence that photoperiod may modulate mammary function by way of serotonin signaling.

In the comparison of LD_{ND}, LD photoperiod generally activated gene expression in the mammary gland with nearly three times the number of differentially expressed genes having higher expression in mice exposed to LD, compared to ND, photoperiod. This finding is in agreement with our findings on G17 concurrent to photoperiod exposure (**Chapter 5**) as well as L10 during photoperiod exposure (**Chapter 4**). Up-regulation of differentially expressed genes *Adra1a*, *Grpr*, *Kcna6*, *Oprm1*, *P2rx1*, *P2rx3*, *Ptgfr*, and *Trpm1* is positively correlated with lactation (Wei et al., 2013), suggesting these genes may promote milk production in response to LD photoperiod. There were 8 genes (*Aspm*, *Aurkb*, *Cdc20, Cenpa*, *Cenpf*, *Kif11*, *Racgap1*, *Top2a*) differentially expressed in the comparisons of LD_{ND} and SD_{ND}, which when down-regulated are positively correlated with lactation performance in mice (Wei et al., 2013). All of these genes were more highly expressed in mice exposed to ND photoperiod, indicating their lower expression in LD and SD exposed mice may potentially alter lactation performance.

In conclusion, microarray analysis supported our hypothesis that altered photoperiod during gestation would have lasting effects on the mammary transcriptome during lactation. Many of the photoperiod-responsive genes reported here are known to affect cell cycle progression and have been correlated with lactation performance in mice. We provide evidence that physiological adaptation to changes in photoperiod during gestation is coordinated through thyroid-related signaling and potentially genes associated with serotonin signaling. Genes identified here provide targets for future studies of the effects of

photoperiod in the mammary gland. Finally, our findings suggest photoperiod manipulation during gestation may have long-term effects on mammary function in mice.

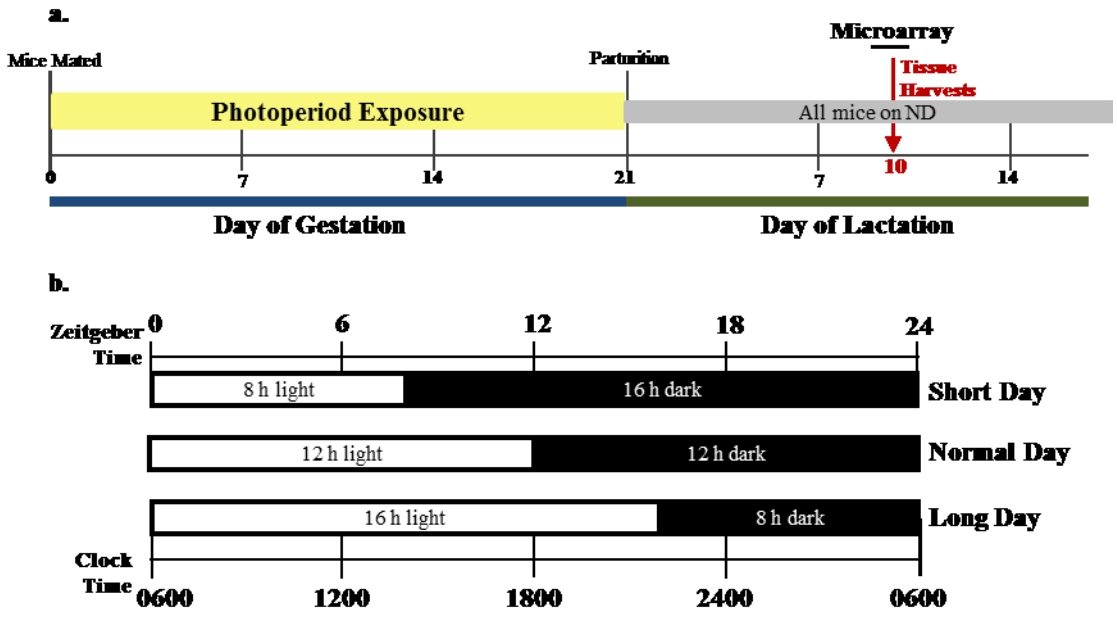


Figure 6.1. Study design of mice exposed to photoperiods during gestation.

a. Female mice were maintained on normal day (12 h light:12 h dark) photoperiod for at least 2 weeks prior to mating and subsequent pregnancy detection by presence of vaginal plugs. Mice were then randomly assigned one of three photoperiods treatment rooms **b.** long day (16 h light: 8 h dark), normal day, or short day (8 h light: 16 h dark) until parturition at which point mice were returned to normal day photoperiod. Tissue harvests were conducted on L10 followed by microarray analysis of the mammary gland.

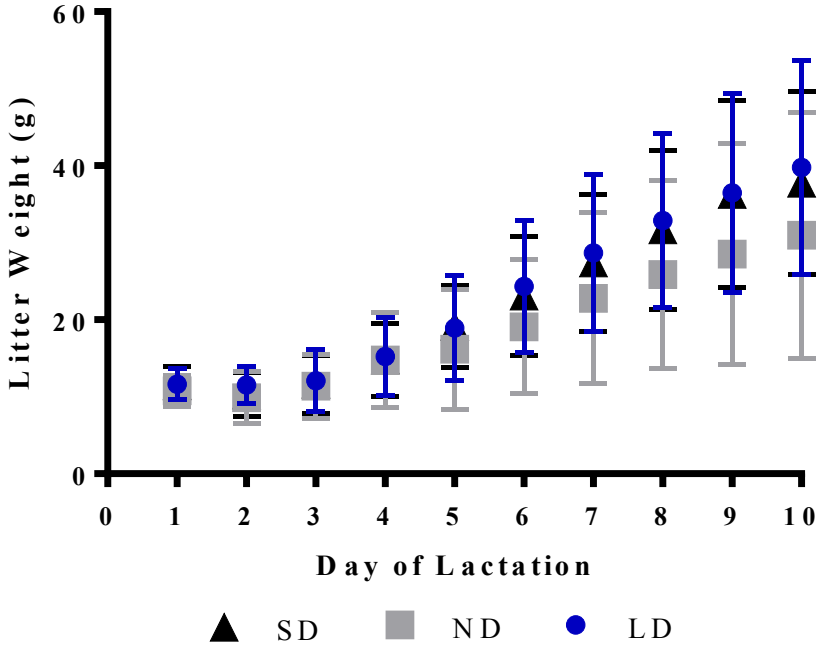


Figure 6.2. Photoperiod exposure during gestation does not affect litter weight during lactation.

Mice were exposed to short day (SD; 8 h light: 16 h dark), normal day (ND; 12 h light: 12 h dark) or long day (LD: 16 h light: 8 h dark) for the duration of gestation. Upon parturition, dams and their litters were maintained on ND photoperiod and daily litter weights were recorded for ten days of lactation. The mean ± sd ($n \geq 5$ litters) are plotted. The overall effect of photoperiod on litter weight was tested using the repeated measures MANOVA procedure. There was no overall effect of photoperiod on litter weight; however, time did significantly affect litter weight ($p \leq 0.001$).

Table 6.1. Differentially expressed[1] genes on L10 in the mammary gland of mice exposed to photoperiod throughout gestation.

Microarray Comparison	LD_{ND} [2]	SD_{ND} [3]
Positive Fold-Change	315	146
Positive Fold-Change	867	234
Total # of genes	1182	380

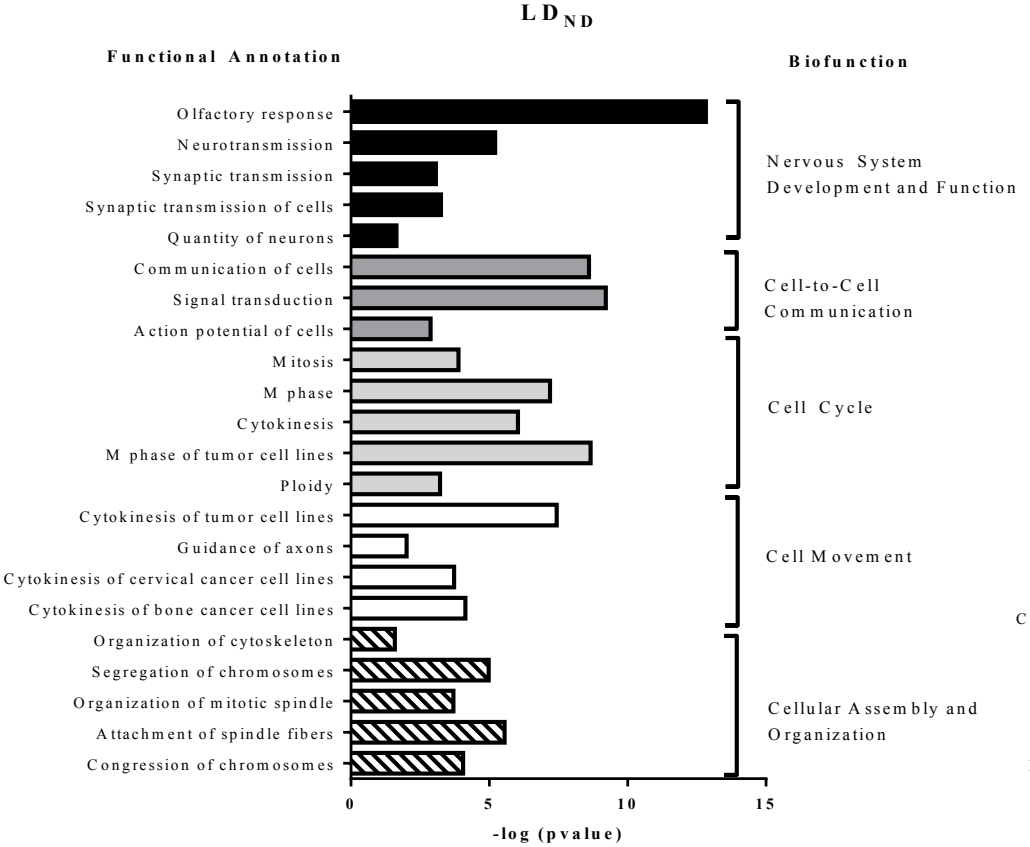
[1.] Microarray analysis using the Nimblegen 12 x 135K mouse array platforms was used to quantify relative gene expression in the mammary gland. Genes were considered differentially expressed if they met the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$.
[2.] LD_{ND}: negative fold-change indicates expression was higher in mice exposed to ND photoperiod.
[3.] SD_{ND}: negative fold-change indicates expression was higher in mice exposed to SD photoperiod.

Table 6.2. Representative GO Terms[1] of clusters enriched by differentially expressed[2] genes in response to LD or SD photoperiod on L10.

GO Term[3]		GO ID	# of Genes	Score[4]
LD_{ND}				
BP	Cell surface receptor linked signal transduction	GO:0007166	108	4.58
BP	Cell division	GO:0051301	24	3.82
MF	Motor activity	GO:0003774	12	2.18
CC	Cytoskeleton	GO:0005856	53	2.09
BP	Microtubule-based process	GO:0007017	16	1.70
BP	DNA packaging	GO:0006323	10	1.65
BP	Localization of cell	GO:0051674	12	1.58
CC	Extracellular region part	GO:0044421	33	1.39
BP	Muscle system process	GO:0003012	6	1.38
MF	Cyclic nucleotide binding	GO:0030551	3	1.12
MF	Protein kinase activity	GO:0004672	28	1.11
BP	Extracellular matrix organization	GO:0030198	4	1.01
SD_{ND}				
BP	Cell cycle process	GO:0022402	28	6.86
BP	Chromosome segregation	GO:0007059	7	3.32
CC	Extracellular region	GO:0005576	44	3.04
CC	Chromosome, centromeric region	GO:0000775	10	2.79
BP	Chromosome segregation	GO:0007059	7	2.18
CC	Microtubule cytoskeleton	GO:0015630	19	2.14
CC	Membrane fraction	GO:0005624	16	1.59
BP	Cytoskeleton organization	GO:0007010	11	1.41
BP	Defense response	GO:0006952	10	1.22
BP	Cytoskeleton organization	GO:0007010	11	1.21
CC	Anchored to membrane	GO:0031225	7	1.20
MF	Extracellular matrix structural constituent	GO:0005201	3	1.18
MF	Protein domain specific binding	GO:0019904	6	1.18
BP	Chromosome organization	GO:0051276	12	1.15
CC	Cell junction	GO:0030054	13	1.14
BP	Localization of cell	GO:0051674	9	1.10
BP	Microtubule-based process	GO:0007017	8	1.01

1. Representative GO terms for functional clusters identified using Functional Clustering in DAVID (http://david.abcc.ncifcrf.gov/).
2. Microarray analysis using the Nimblegen 12x135K mouse array platform was used to quantify relative gene expression in the mammary gland. Genes were considered differentially expressed if they met the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$.
3. MF: molecular function; BP: biological process; CC: cellular component.
4. DAVID enrichment score; only clusters with scores over 1.0 are shown.

a.


LD ND
Functional Annotation
Biofunction
Olfactory response
Neurotransmission
Synaptic transmission
Synaptic transmission of cells
Quantity of neurons
Communication of cells
Signal transduction
Action potential of cells
Mitosis
M phase
Cytokinesis
M phase of tumor cell lines
Ploidy
Cytokinesis of tumor cell lines
Guidance of axons
Cytokinesis of cervical cancer cell lines
Cytokinesis of bone cancer cell lines
Organization of cytoskeleton
Segregation of chromosomes
Organization of mitotic spindle
Attachment of spindle fibers
Congression of chromosomes
Nervous System Development and Function
Cell-to-Cell Communication
Cell Cycle
Cell Movement
Cellular Assembly and Organization
0
5
10
15
-log (pvalue)

b.

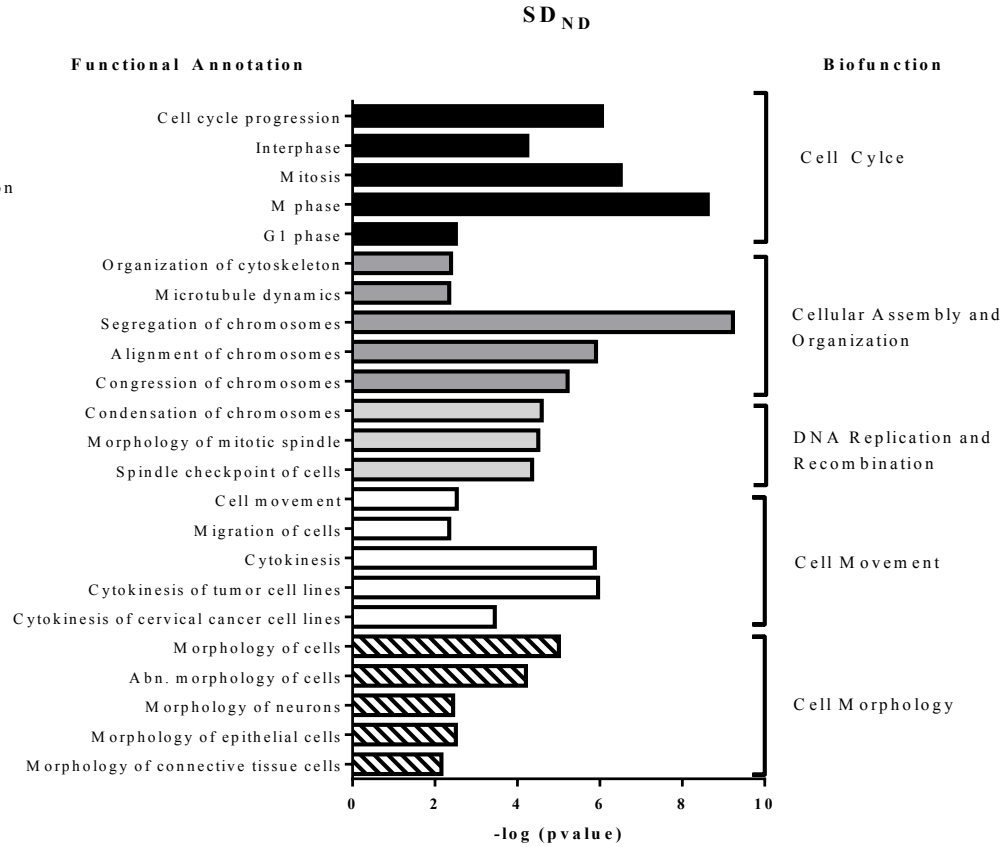

SD ND
Functional Annotation
Biofunction
Cell cycle progression
Interphase
Mitosis
M phase
G1 phase
Organization of cytoskeleton
Microtubule dynamics
Segregation of chromosomes
Alignment of chromosomes
Congression of chromosomes
Condensation of chromosomes
Morphology of mitotic spindle
Spindle checkpoint of cells
Cell movement
Migration of cells
Cytokinesis
Cytokinesis of tumor cell lines
Cytokinesis of cervical cancer cell lines
Morphology of cells
Abn. morphology of cells
Morphology of neurons
Morphology of epithelial cells
Morphology of connective tissue cells
Cell Cylce
Cellular Assembly and Organization
DNA Replication and Recombination
Cell Movement
Cell Morphology
0
2
4
6
8
10
-log (pvalue)

Figure 6.3. IPA *Biofunctions* and functional annotation for genes differentially expressed on L10 in response to photoperiod exposure throughout gestation.

Microarray analysis was used to quantify relative gene expression in the mammary gland of mice exposed to long day (LD; 16 h light: 8 h dark), normal day (ND; 12 h light: 12 h dark), or short day (SD; 8 h light: 16 h dark) for the duration of gestation. Upon parturition, dams and their litters were maintained on ND photoperiod. Genes were considered differentially expressed in the comparison of **a.** LD_{ND} or **b.** SD_{ND} if they met the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$. IPA *biofunctions* and associated *functional annotation* with > 1 associated differentially expressed genes were ranked by –log (p-value), an estimate of whether the group is over-represented in the data set. The top 5 *biofunctions* by p-value are shown; redundant *functional annotations* are not shown.

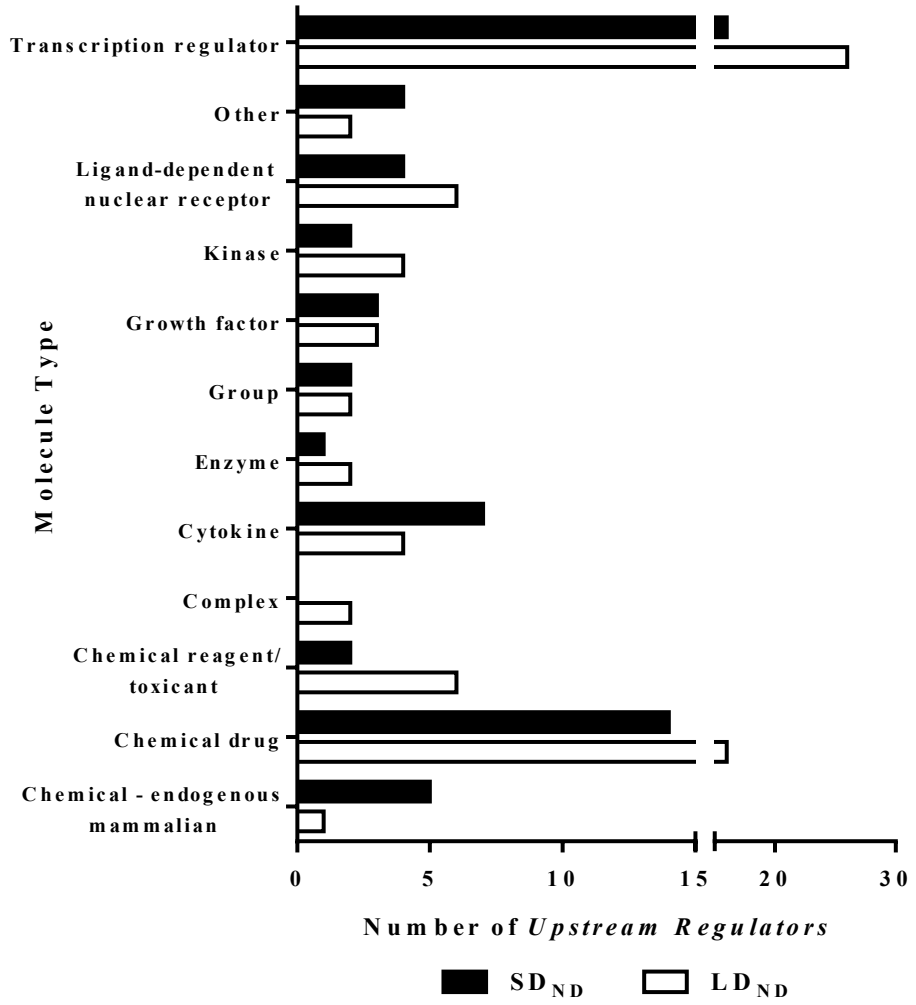


Figure 6.4. Predicted *upstream regulators* of differentially expressed genes on L10 in mice exposed to photoperiod throughout gestation.

Microarray analysis was used to quantify relative gene expression in the mammary gland of mice exposed to long day (LD, 16 h light: 8 h dark), normal day (ND, 12 h light: 12 h dark), or short day (SD, 8 h light: 16 h dark) photoperiod for the duration of gestation. Upon parturition, dams and their litters were maintained on ND photoperiod. *Upstream regulators* predicted to affect ≥ 10 genes are summarized by molecule type for genes identified in the comparison of LD_{ND} (white bars) or SD_{ND} (black bars).

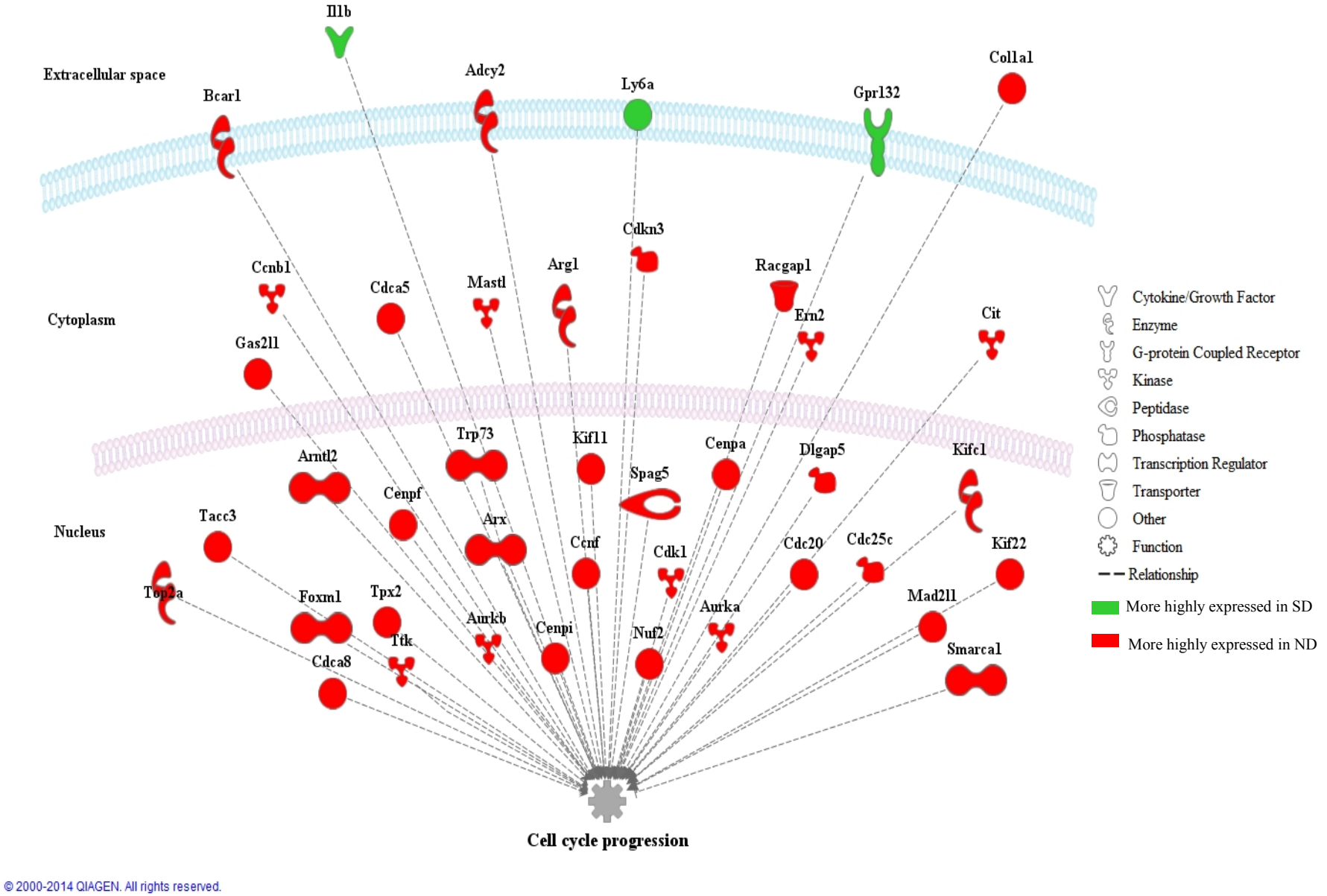


Figure 6.5. Genes differentially expressed on L10 in the comparison of SD_{ND} have roles in cell cycle progression.

Microarray analysis was used to quantify relative gene expression in the mammary gland of mice exposed to normal day (12 h light: 12 h dark), or short day (8 h light: 16 h dark) for the duration of gestation. Upon parturition, dams and their litters were maintained on ND photoperiod. Genes were considered differentially expressed in the comparison of SD_{ND} if they met the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$. Thirty-nine genes were associated with cell cycle progression. Those genes more highly expressed in mice exposed to ND photoperiod (red), genes more highly expressed in mice exposed to SD photoperiod (green).

Table 6.3. Differentially expressed[1] photoperiod-responsive genes associated with lactation performance in mice[2].

Symbol	Entrez Gene Name	GenBank ID	LD_{ND}[3]		SD_{ND}[4]	
			Fold Change	p-value	Fold Change	p-value
LD_{ND}						
Adra1a	Adrenoceptor alpha 1A	AK138919	2.0	0.02	-	-
Anxa3	Annexin A3	AK169423	-1.6	0.04	-	-
Birc5	Baculoviral IAP repeat containing 5	BC004702	-2.0	0.02	-	-
Btf3	Basic transcription factor 3	BC064010	-1.7	< 0.01	-	-
Bub1b	BUB1 mitotic checkpoint serine/threonine kinase B	BC031577	-1.7	0.01	-	-
Ctgf	Bonnective tissue growth factor	BC006783	-1.6	0.04	-	-
Fgl2	Fibrinogen-like 2	BC028893	-1.5	0.05	-	-
Grpr	Gastrin-releasing peptide receptor	BC113145	1.6	< 0.01	-	-
Hnrnpr	Heterogeneous nuclear ribonucleoprotein R	AK144992	-1.7	0.01	-	-
Kcna6	Potassium voltage-gated channel, shaker-related subfamily, member 6	AK134477	1.7	< 0.01	-	-
Klhl7	Kelch-like family member 7	AK082520	-1.6	0.01	-	-
Ndc80	NDC80 kinetochore complex component	BC020131	-2.0	0.03	-	-
Oprm1	Opioid receptor, mu 1	AF074972	-1.6	0.04	-	-
P2rx1	Purinergic receptor P2X, ligand-gated ion channel, 1	BC015084	-1.7	0.03	-	-
P2rx3	Purinergic receptor P2X, ligand-gated ion channel, 3	AK019679	1.5	0.021	-	-
Pon2	Paraoxonase 2	AK210368	-2.0	0.05	-	-
Ptgfr	Prostaglandin F receptor	BC064794	-1.6	< 0.01	-	-
Rnf13	Ring finger protein 13	AK034135	-1.5	0.03	-	-
Serpine2	Serpin peptidase inhibitor, clade E member 2	BC010675	-1.5	< 0.01	-	-
Slc4a4	Solute carrier family 4 (	AF141934	-1.5	0.01	-	-
Stat1	Signal transducer and activator of transcription 1, 91kDa	U06924	-2.2	< 0.01	-	-
Trpm1	Transient receptor potential cation channel, subfamily M, member 1	BC082560	1.8	0.02	-	-
Usp9x	Ubiquitin specific peptidase 9, X-linked	AK028443	-1.6	0.05	-	-
Wfdc2	WAP four-disulfide core domain 2	BC099427	-1.5	0.03	-	-
LD_{ND} and SD_{ND}						
Aspm	Abnormal spindle homolog, microcephaly associated	AY971958	-2.0	0.02	2.5	< 0.01
Aurkb	Aurora kinase B	BC003261	-1.7	< 0.01	1.7	0.02
Cdc20	Cell division cycle 20	BC003215	-1.6	< 0.01	1.6	< 0.01
Cenpa	Centromere protein A	BC012280	-1.7	< 0.01	1.5	< 0.01
Cenpf	Centromere protein F, 350/400kDa	AK165236	-2.0	0.05	1.6	0.03
Col1a2	Collagen, type I, alpha 2	AK142111	-1.6	0.01	1.7	< 0.01
Hspa13	Heat shock protein 70kDa family, member 13	AK021006	-1.6	0.01	1.6	< 0.01
Kif11	Kinesin family member 11	BC060670	-1.9	0.01	1.8	< 0.01
Mest	Mesoderm specific transcript	AF482999	-1.7	0.01	1.7	< 0.01
Nuf2	NDC80 kinetochore complex component	BC020026	-1.8	0.05	1.9	0.03
Racgap1	Rac GTPase activating protein 1	AF212320	-1.7	0.02	1.8	< 0.01
Top2a	Topoisomerase (DNA) II alpha 170kDa	AK033321	-1.7	0.03	1.5	0.04

Table 6.3 continued

SD_{ND}						
Arif5b	AT rich interactive domain 5B	AK162717	-	-	-1.5	0.02
Aurka	Aurora kinase A	BC005425	-	-	1.5	< 0.01
Ccnb1	Cyclin B1	BC085238	-	-	1.7	< 0.01
Cdca8	Cell division cycle associated 8	BC068181	-	-	1.7	< 0.01
Gabrq	Gamma-aminobutyric acid (GABA) A receptor, theta	AK038859	-	-	-1.5	0.02
Gipr	Gastric inhibitory polypeptide receptor	BC120671	-	-	1.6	0.02
Grik4	Glutamate receptor, ionotropic, kainate 4	BC118010	-	-	-1.6	0.04
Htr4	5-hydroxytryptamine receptor 4	BC148470	-	-	1.8	0.03
Sfrp1	Secreted frizzled-related protein 1	BC094662	-	-	1.6	0.03
Tmem53	Transmembrane protein 53	BC039805	-	-	1.5	0.010
Zmiz1	Zinc finger, MIZ-type containing 1	AK054366	-	-	-1.7	0.01

[1.] Microarray analysis using the Nimblegen 12 x 135K mouse array platforms was used to quantify relative gene expression in the mammary gland. Genes were considered differentially expressed if they met the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$

[2.] Based on gene lists published in (Wei et al., 2013).

[3.] LD_{ND}: negative fold-change indicates expression was higher in mice exposed to ND photoperiod.

[4.] SD_{ND}: negative fold-change indicates expression was higher in mice exposed to SD photoperiod.

b.

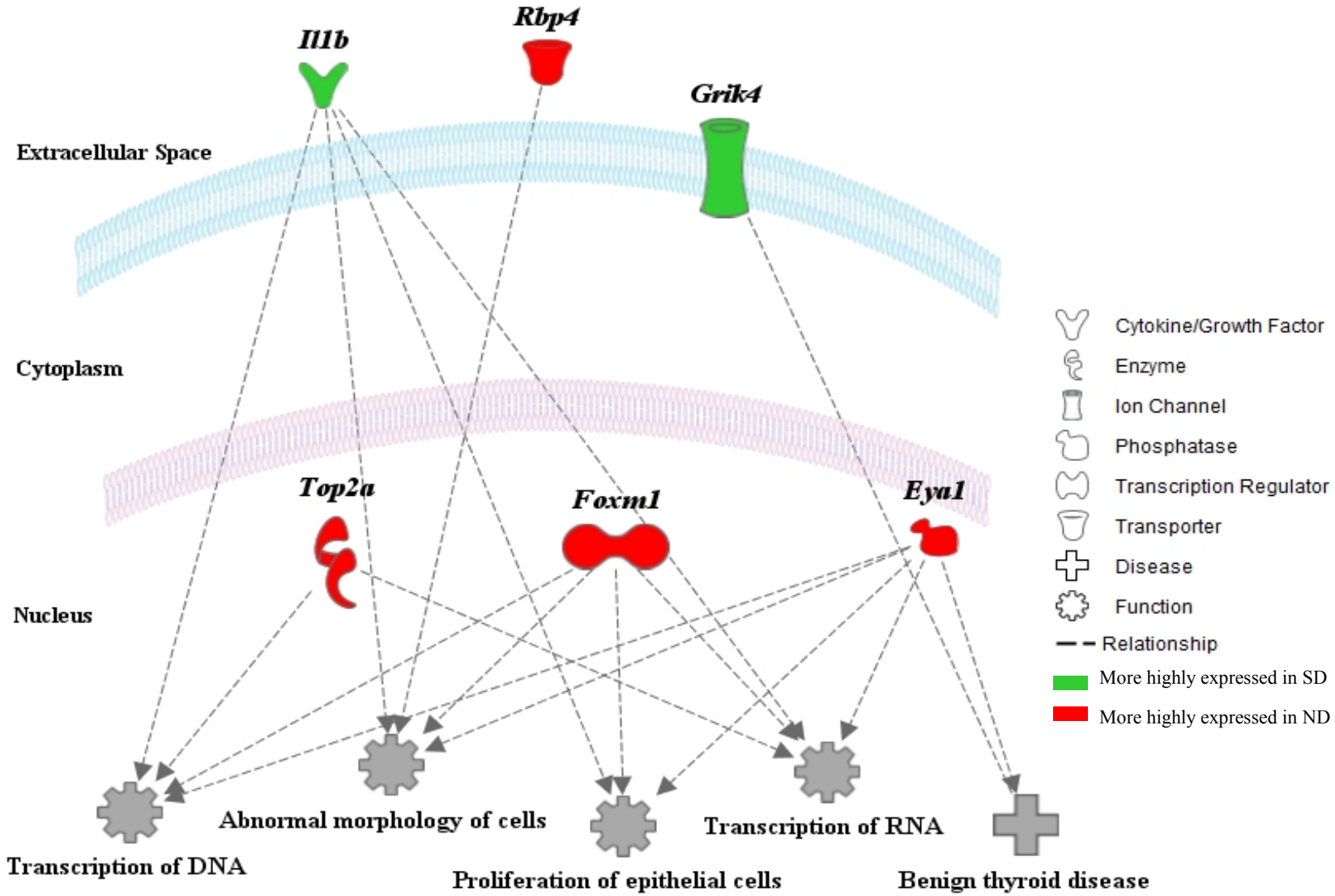


Figure 6.6. Differentially expressed genes associated with thyroid signaling.

Microarray analysis was used to quantify relative gene expression in the mammary gland of mice exposed to long day (16 h light: 8 h dark), normal day (12 h light: 12 h dark), or short day (8 h light: 16 h dark) for the duration of gestation. Upon parturition, dams and their litters were maintained on ND photoperiod. Genes were considered differentially expressed in the comparison of LD_{ND} or SD_{ND} if they met the criteria of $p \leq 0.05$ and fold-change $\geq |1.5|$. Genes associated with thyroid signaling in the comparison of **a.** LD_{ND} and **b.** SD_{ND} along with the top IPA functions associated with each gene set are shown.

REFERENCES

Auchtung, T. L., A. G. Rius, P. E. Kendall, T. B. McFadden, and G. E. Dahl. 2005. Effects of photoperiod during the dry period on prolactin, prolactin receptor, and milk production of dairy cows. J Dairy Sci 88:121.

Azzi, A., R. Dallmann, A. Casserly, H. Rehrauer, A. Patrignani, B. Maier, A. Kramer, and S. A. Brown. 2014. Circadian behavior is light-reprogrammed by plastic DNA methylation. Nat Neurosci 17:377.

Bilbo, S. D., D. L. Drazen, N. Quan, L. He, and R. J. Nelson. 2002. Short day lengths attenuate the symptoms of infection in siberian hamsters. Proc Biol Sci 269:447.

Blask, D. E., S. M. Hill, R. T. Dauchy, S. Xiang, L. Yuan, T. Duplessis, L. Mao, E. Dauchy, and L. A. Sauer. 2011. Circadian regulation of molecular, dietary, and metabolic signaling mechanisms of human breast cancer growth by the nocturnal melatonin signal and the consequences of its disruption by light at night. J Pineal Res 51:259.

Bolstad, B. M., R. A. Irizarry, M. Astrand, and T. P. Speed. 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19:185.

Brooks, E., D. Patel, and M. M. Canal. 2014. Programming of mice circadian photic responses by postnatal light environment. PLoS ONE 9:e97160.

Cabodi, S., A. Tinnirello, P. Di Stefano, B. Bisaro, E. Ambrosino, et al. 2006. P130cas as a new regulator of mammary epithelial cell proliferation, survival, and her2-neu oncogene-dependent breast tumorigenesis. Cancer Res 66:4672.

Cano, P., D. P. Cardinali, V. Jimenez, M. P. Alvarez, R. A. Cutrera, and A. I. Esquifino. 2005. Effect of interferon-gamma treatment on 24-hour variations in plasma acth, growth hormone, prolactin, luteinizing hormone and follicle-stimulating hormone of male rats. Neuroimmunomodulation 12:146.

Capuco, A. V., E. E. Connor, and D. L. Wood. 2008. Regulation of mammary gland sensitivity to thyroid hormones during the transition from pregnancy to lactation. Exp Biol Med (Maywood) 233:1309.

Capuco, A. V., D. L. Wood, R. Baldwin, K. McLeod, and M. J. Paape. 2001. Mammary cell number, proliferation, and apoptosis during a bovine lactation: Relation to milk production and effect of bst. J Dairy Sci 84:2177.

Chemineau, P., D. Guillaume, M. Migaud, J. C. Thiery, M. T. Pellicer-Rubio, and B. Malpaux. 2008. Seasonality of reproduction in mammals: Intimate regulatory mechanisms and practical implications. Reprod Domest Anim 43 Suppl 2:40.

Ciarleglio, C. M., J. C. Axley, B. R. Strauss, K. L. Gamble, and D. G. McMahon. 2011. Perinatal photoperiod imprints the circadian clock. Nat Neurosci 14:25.

Collier, R. J., L. L. Hernandez, and N. D. Horseman. 2012. Serotonin as a homeostatic regulator of lactation. Domest Anim Endocrinol 43:161.

Dahl, G. E. 2008. Effects of short day photoperiod on prolactin signaling in dry cows: A common mechanism among tissues and environments? J Anim Sci 86:10.

Dahl, G. E., B. A. Buchanan, and H. A. Tucker. 2000. Photoperiodic effects on dairy cattle: A review. J Dairy Sci 83:885.

Dahl, G. E., T. H. Elsasser, A. V. Capuco, R. A. Erdman, and R. R. Peters. 1997. Effects of a long daily photoperiod on milk yield and circulating concentrations of insulin-like growth factor-1. J Dairy Sci 80:2784.

Dahl, G. E., S. Tao, and I. M. Thompson. 2012. Effects of photoperiod on mammary gland development and lactation. J Anim Sci.
Dardente, H., C. A. Wyse, M. J. Birnie, S. M. Dupre, A. S. Loudon, G. A. Lincoln, and D. G. Hazlerigg. 2010. A molecular switch for photoperiod responsiveness in mammals. Curr Biol 20:2193.
Davis, F. C. and J. Mannion. 1988. Entrainment of hamster pup circadian rhythms by prenatal melatonin injections to the mother. Am J Physiol 255:R439.
Duncan, M. J. 2007. Circannual prolactin rhythms: Calendar-like timer revealed in the pituitary gland. Trends Endocrinol Metab 18:259.
Garcia-Hernandez, R., G. Newton, S. Horner, and L. C. Nuti. 2007. Effect of photoperiod on milk yield and quality, and reproduction in dairy goats. Livest Sci 110:214.
Garcia-Maurino, S., M. G. Gonzalez-Haba, J. R. Calvo, M. Rafii-El-Idrissi, V. Sanchez-Margalet, R. Goberna, and J. M. Guerrero. 1997. Melatonin enhances il-2, il-6, and ifn-gamma production by human circulating cd4+ cells: A possible nuclear receptor-mediated mechanism involving t helper type 1 lymphocytes and monocytes. J Immunol 159:574.
Goldman, A. S. 2002. Evolution of the mammary gland defense system and the ontogeny of the immune system. J Mammary Gland Biol Neoplasia 7:277.
Hastings, M. H., J. Herbert, N. D. Martensz, and A. C. Roberts. 1985. Annual reproductive rhythms in mammals: Mechanisms of light synchronization. Ann NY Acad Sci 453:182.
Hernandez, L. L., S. W. Limesand, J. L. Collier, N. D. Horseman, and R. J. Collier. 2009. The bovine mammary gland expresses multiple functional isoforms of serotonin receptors. J Endocrinol 203:123.
Hondermarck, H. 2012. Neurotrophins and their receptors in breast cancer. Cytokine Growth Factor Rev 23:357.
Hovey, R. C., J. Harris, D. L. Hadsell, A. V. Lee, C. J. Ormandy, and B. K. Vonderhaar. 2003. Local insulin-like growth factor-ii mediates prolactin-induced mammary gland development. Mol Endocrinol 17:460.
Irizarry, R. A., B. M. Bolstad, F. Collin, L. M. Cope, B. Hobbs, and T. P. Speed. 2003a. Summaries of affymetrix genechip probe level data. Nucleic Acids Res 31:e15.
Irizarry, R. A., B. Hobbs, F. Collin, Y. D. Beazer-Barclay, K. J. Antonellis, U. Scherf, and T. P. Speed. 2003b. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4:249.
Jackson, C. R., M. Capozzi, H. Dai, and D. G. McMahon. 2014. Circadian perinatal photoperiod has enduring effects on retinal dopamine and visual function. J Neurosci 34:4627.
Katayama, H., W. Brinkley, and S. Sen. 2003. The aurora kinases: Role in cell transformation and tumorigenesis. Cancer Metastasis Rev 22:451.
Kouprina, N., A. Pavlicek, N. K. Collins, M. Nakano, V. N. Noskov, et al. 2005. The microcephaly aspm gene is expressed in proliferating tissues and encodes for a mitotic spindle protein. Hum Mol Genet 14:2155.
Lamote, I., E. Meyer, A. M. Massart-Leen, and C. Burvenich. 2004. Sex steroids and growth factors in the regulation of mammary gland proliferation, differentiation, and involution. Steroids 69:145.

Lin, Y. and Q. Li. 2007. Expression and function of leptin and its receptor in mouse mammary gland. Sci China C Life Sci 50:669.
Mabjeesh, S. J., C. Sabastian, O. Gal-Garber, and A. Shamay. 2013. Effect of photoperiod and heat stress in the third trimester of gestation on milk production and circulating hormones in dairy goats. J Dairy Sci 96:189.
Malpaux, B., M. Migaud, H. Tricoire, and P. Chemineau. 2001. Biology of mammalian photoperiodism and the critical role of the pineal gland and melatonin. J Biol Rhythms 16:336.
McFarlane, D., R. F. Wolf, K. A. McDaniel, and G. L. White. 2012. The effect of season on inflammatory response in captive baboons. J Med Primatol 41:341.
Mikolayunas, C. M., D. L. Thomas, G. E. Dahl, T. F. Gressley, and Y. M. Berger. 2008. Effect of prepartum photoperiod on milk production and prolactin concentration of dairy ewes. J Dairy Sci 91:85.
Morin, L. P. 1999. Serotonin and the regulation of mammalian circadian rhythmicity. Ann Med 31:12.
Neville, M. C., T. B. McFadden, and I. Forsyth. 2002. Hormonal regulation of mammary differentiation and milk secretion. J Mammary Gland Biol Neoplasia 7:49.
Ono, H., Y. Hoshino, S. Yasuo, M. Watanabe, Y. Nakane, A. Murai, S. Ebihara, H. W. Korf, and T. Yoshimura. 2008. Involvement of thyrotropin in photoperiodic signal transduction in mice. Proc Natl Acad Sci U S A 105:18238.
Otsuka, T., M. Kawai, Y. Togo, R. Goda, T. Kawase, et al. 2014. Photoperiodic responses of depression-like behavior, the brain serotonergic system, and peripheral metabolism in laboratory mice. Psychoneuroendocrinology 40:37.
Peters, R. R., L. T. Chapin, K. B. Leining, and H. A. Tucker. 1978. Supplemental lighting stimulates growth and lactation in cattle. Science 199:911.
Pyter, L. M., A. K. Hotchkiss, and R. J. Nelson. 2005. Photoperiod-induced differential expression of angiogenesis genes in testes of adult *Peromyscus leucopus*. Reproduction 129:201.
Rani, S. and V. Kumar. 2014. Photoperiodic regulation of seasonal reproduction in higher vertebrates. Indian J Exp Biol 52:413.
Robson, E. J., S. J. He, and M. R. Eccles. 2006. A panorama of pax genes in cancer and development. Nat Rev Cancer 6:52.
Rosfjord, E. C. and R. B. Dickson. 1999. Growth factors, apoptosis, and survival of mammary epithelial cells. J Mammary Gland Biol Neoplasia 4:229.
Sorensen, M. T. and R. R. Hacker. 1979. Negative effect of bovine pineal gland extract and positive effect of supplemental lighting on lactation in mice. J Anim Sci 49:1270.
Stevenson, J. S., D. S. Pollmann, D. L. Davis, and J. P. Murphy. 1983. Influence of supplemental light on sow performance during and after lactation. J Anim Sci 56:1282.
Tadjuidje, E. and R. S. Hegde. 2013. The eyes absent proteins in development and disease. Cell Mol Life Sci 70:1897.
Teh, M. T. 2012. Foxm1 coming of age: Time for translation into clinical benefits? Front Oncol 2:146.
Trott, J. F., A. Schennink, W. K. Petrie, R. Manjarin, M. K. VanKlompenberg, and R. C. Hovey. 2012. Triennial lactation symposium: Prolactin: The multifaceted potentiator of mammary growth and function. J Anim Sci 90:1674.

Wall, E. H., T. L. Auchtung, G. E. Dahl, S. E. Ellis, and T. B. McFadden. 2005. Exposure to short day photoperiod during the dry period enhances mammary growth in dairy cows. J Dairy Sci 88:1994.
Wall, E. H., J. P. Bond, and T. B. McFadden. 2012. Acute milk yield response to frequent milking during early lactation is mediated by genes transiently regulated by milk removal. Physiol Genomics 44:25.
Walton, J. C., Z. M. Weil, and R. J. Nelson. 2011. Influence of photoperiod on hormones, behavior, and immune function. Front Neuroendocrinol 32:303.
Watson, C. J. 2009. Immune cell regulators in mouse mammary development and involution. J Anim Sci 87:35.
Weber, E. T., R. L. Gannon, and M. A. Rea. 1998. Local administration of serotonin agonists blocks light-induced phase advances of the circadian activity rhythm in the hamster. J Biol Rhythms 13:209.
Wei, J., P. Ramanathan, I. C. Martin, C. Moran, R. M. Taylor, and P. Williamson. 2013. Identification of gene sets and pathways associated with lactation performance in mice. Physiol Genomics 45:171.
Wood, S. and A. Loudon. 2014. Clocks for all seasons: Unwinding the roles and mechanisms of circadian and interval timers in the hypothalamus and pituitary. J Endocrinol 222:R39.
Yasuo, S., T. Yoshimura, S. Ebihara, and H. W. Korf. 2009. Melatonin transmits photoperiodic signals through the mt1 melatonin receptor. J Neurosci 29:2885.
Yoshimura, T. 2013. Thyroid hormone and seasonal regulation of reproduction. Front Neuroendocrinol 34:157.

CHAPTER 7: HYPOTHESES REVISITED AND GENERAL DISCUSSION

HYPOTHESES REVISITED

Overall hypothesis

It was hypothesized that:

1. Exposure to short day photoperiod treatment during gestation and long day photoperiod during lactation will affect transcription of genes in the mammary gland that support lactation.

In the bovine mammary gland, the expression of genes associated with cell proliferation and immune function were differentially expressed in the comparison of LD vs SD. Based on previous findings that SD photoperiod in these cows increases milk production in the subsequent lactation, we infer the direction of change corresponding to SD photoperiod of differentially expressed genes, supports lactation.

In the lactating mouse mammary gland, more genes were differentially expressed in the comparison of SD_{ND} than LD_{ND}. In addition, more SD_{ND} genes were associated with lactation and thyroid signaling. Analysis of qRT-PCR data revealed that relative to LD photoperiod, the majority of circadian-related genes were more highly expressed in SD photoperiod, suggesting these may play a role in enhanced mammary function. However, it should be noted that the majority of genes differentially expressed in the comparison of SD_{ND} and LD_{ND} were more abundant in mice on ND photoperiod, suggesting the change away from ND photoperiod may have negative effects on the mammary gland during lactation.

The mammary transcriptome, during gestation, was affected by LD more than SD photoperiod. The majority of genes were more highly expressed in mice exposed to LD than ND photoperiod, suggesting LD photoperiod enhanced mammary function. To the contrary,

genes identified in the comparison of SD_{ND} were more highly expressed in mice on ND photoperiod, suggesting that relative to SD, ND photoperiod has potential to enhance mammary function.

On L10, after exposure to photoperiod treatments for the duration of gestation, LD affected more genes than SD photoperiod. There were also more genes associated with lactation performance in mice in the comparison of LD_{ND} than SD_{ND} photoperiod. In the comparison of LD_{ND}, the majority of genes were more highly expressed in mice exposed to LD photoperiod. In the comparison of SD_{ND}, more genes had higher expression in mice on ND photoperiod.

The preceding interpretation of the gene expression findings is based on the assumption that higher expression of genes enhances lactation. This assumption was made based on the biosynthetic nature of lactation. In addition, Wei et al. (2013) in their study of lactation performance in mice showed that the majority of down-regulated genes were negatively correlated with lactation performance. This suggests higher expression would be supportive of lactation. However, it is also possible that lower expression of some genes will promote lactation. Although, the methods of analysis (IPA, DAVID) used in these studies provide some information on the effect based on direction of change, the specific effect of each gene on promoting/inhibiting lactation is difficult to ascertain.

Ultimately, our findings support our hypothesis that SD photoperiod during gestation affects genes that support lactation in cows; however, this is not the case in mice. Our findings indicate that LD enhances mammary function more than SD photoperiod, but depending on when photoperiod manipulation occurs, ND may more positively affect mammary function and lactation.

PHOTOPERIOD EXPOSURE DURING THE DRY PERIOD IN DAIRY COWS.

It was hypothesized that:

1) **Photoperiod manipulation during the dry period affects genes associated lactation performance. More specifically:**
   a) **SD photoperiod regulates the expression of genes that promote cell proliferation.**
   - Our findings support this hypothesis. In the comparison of SD and LD photoperiod, genes associated with cell proliferation were up and down regulated (**Chapter 3**). Because we know SD increases cell proliferation during the dry period, we interpret the differential expression of these genes as supportive of cell proliferation (Figure 7.1)

   b) **SD photoperiod regulates genes associated with mammary health and immune function.**
   - Our findings support this hypothesis. In the comparison of SD and LD photoperiod, the expression of numerous genes associated with immune function and mammary health was affected (Figure 7.1).

2) **Differential expression of genes between day -24 and -9 relative to parturition reflect the physiological change in the mammary gland between stage 1 and stage 2 lactogenesis. More specifically:**
   a) **Genes differentially expressed on day -9 are associated with initiation of milk synthesis, whereas on day -24 they are not.**

- Our findings support this hypothesis as the majority of genes differentially expressed by time had increased expression on day -9 relative to -24. In addition, these genes enriched *biofunctions* associated with milk production (Figure 7.1)

3) **Genes identified in the effect of time will be different from those identified in the effect of photoperiod.**
   - Our findings support this hypothesis, as there were very few common genes (n = 12) between the effect of photoperiod and time.

PHOTOPERIOD EXPOSURE DURING LACTATION IN MICE

It was hypothesized that:

1) **Photoperiod manipulation during lactation will alter milk production as measured by litter weight gain. More specifically:**

   a) LD photoperiod will increase milk production relative to SD photoperiod.

   - The data do not support this hypothesis. No effect of photoperiod was observed in response to photoperiod exposure during lactation (**Chapter 4**).

   b) ND photoperiod will increase milk production relative to SD photoperiod

   - The data do not support this hypothesis. No effect of photoperiod was observed in response to photoperiod exposure during lactation (**Chapter 4**).

2) **Photoperiod manipulation during lactation will affect the proliferation of mammary cells as measured by BrdU incorporation. More specifically:**

   a) LD photoperiod will increase cell proliferation relative to SD photoperiod.

   - The data we report do not support this hypothesis. To the contrary, on L5, mice exposed to LD photoperiod had less BrdU incorporation than SD mice, and significantly less than ND exposed mice (**Figure 4.2**).

   b) ND photoperiod will increase cell proliferation relative to SD photoperiod.

   - The data we report do not support this hypothesis. On L5, mice exposed to ND photoperiod did not take up more BrdU than mice on SD photoperiod (**Figure 4.2**).

3) **Photoperiod manipulation during lactation will affect mouse body weight and organ weights. More specifically:**

   a) Body weight

i) Mice exposed to LD photoperiod will have higher body weights than mice on SD photoperiod.

- This hypothesis was not supported by our findings. Mice exposed to LD photoperiod did not weighed significantly more than mice on SD photoperiod (**Table 4.2**).

ii) Mice exposed to ND photoperiod will have higher body weights than mice on SD photoperiod

- This hypothesis was not supported by our findings. Mice exposed to ND photoperiod did not weighed significantly more than mice on SD photoperiod (**Table 4.2**).

b) Spleen weight

i) Mice exposed to SD photoperiod will have higher spleen weight than mice on LD photoperiod

- This hypothesis was supported by our findings. Mice exposed to SD photoperiod had significantly heavier spleens than mice on LD photoperiod (**Table 4.2**).

ii) Mice exposed to ND photoperiod will have higher spleen weight than mice on LD photoperiod.

- This hypothesis was supported by our findings. Mice exposed to ND photoperiod did not have significantly heavier spleens than mice on LD photoperiod (**Table 4.2**).

c) Thymus weight

i) Mice exposed to SD photoperiod will have higher thymus weight than mice on LD photoperiod

- This hypothesis was not supported by our findings. There was no effect of photoperiod on thymus weight (**Table A1**).

ii) Mice exposed to ND photoperiod will have higher thymus weight than mice on LD photoperiod

- This hypothesis was not supported by our findings. There was no effect of photoperiod on thymus weight (**Table A1**).

d) Liver weight

i) Mice exposed to LD photoperiod will have higher liver weight than mice on SD photoperiod

- This hypothesis was supported by our findings. Mice exposed to LD photoperiod had significantly heavier livers than mice on SD (**Table 4.2**).

ii) Mice exposed to ND photoperiod will have higher liver weight than mice on SD photoperiod

- This hypothesis was not supported by our findings. Mice exposed to ND photoperiod did not have significantly heavier livers than mice on SD (**Table 4.2**).

2) Photoperiod manipulation during lactation will affect expression of genes in the mammary transcriptome. More specifically:

a) The comparison of LD_{ND} will identify differential expression of genes associated with increased lactation performance

- This hypothesis was supported by our data. Numerous differentially expressed genes in the comparison of LD_{ND} were associated with lactation performance (**Table 4.6**)

b) The comparison of SD_{ND} will identify differential expression of genes associated with cell proliferation

- This hypothesis was supported by our data. Numerous differentially expressed genes in the comparison of SD_{ND} were associated with cell proliferation (**Table 4.3**)

c) The comparisons of LD_{ND} and SD_{ND} will identify different sets of genes.

- This hypothesis was supported by our data. In the lactating mouse mammary gland, more genes were differentially expressed in the comparison of SD_{ND} than LD_{ND}. However, the majority of genes differentially expressed in the comparison of SD_{ND} and LD_{ND} were more abundant in mice on ND photoperiod (**Table 4.5**)

PHOTOPERIOD EXPOSURE DURING GESTATION IN MICE – CONCURRENT EFFECTS

It was hypothesized that:

1) **Photoperiod manipulation during gestation will affect the proliferation of mammary cells. More specifically:**
   - **a)** Mice exposed to SD photoperiod will uptake more BrdU than mice on LD or ND photoperiod.
   - ➢ The results of our experiments support this hypothesis on G17, but not on G19, L5, or L10. On G17, the incorporation of BrdU was significantly higher in mice exposed to SD photoperiod relative to LD photoperiod. Over all 4 time points there was no significant effect of photoperiod, although SD photoperiod did numerically raise BrdU incorporation relative to LD (**Figure 5.2**)
   - **b)** The comparison of SD_{ND} photoperiod will identify differential expression of genes associated with cell proliferation, relative to the comparison of LD_{ND}.
   - ➢ Our findings support this hypothesis. Numerous genes and their *upstream regulators* in the comparison of SD_{ND} were associated with cell proliferation (**Figure 5.6**)
2) **Photoperiod manipulation during gestation will affect mouse body weight and organ weights. More specifically:**
   - **a) Body weight**
     - i) Mice exposed to SD photoperiod will have higher body weights than mice exposed to LD photoperiod

- Our findings do not support this hypothesis. Body weight was not affected by photoperiod (**Table 5.1**).

ii) Mice exposed to ND photoperiod will have higher body weights than mice exposed to LD photoperiod.

- Our findings do not support this hypothesis. Body weight was not affected by photoperiod (**Table 5.1**).

b) Spleen weight

i) Mice exposed to SD photoperiod will have higher spleen mass than mice on LD photoperiod

- Our findings do not support this hypothesis. Spleen weight was not affected by photoperiod (**Table 5.1**).

ii) Mice exposed to SD photoperiod will have higher spleen mass than mice on ND photoperiod.

- Our findings do not support this hypothesis. Spleen weight was not affected by photoperiod (**Table 5.1**).

c) Thymus weight

i) Mice exposed to SD photoperiod will have higher thymus weights than mice exposed to LD photoperiod.

- Our findings do not support this hypothesis. Thymus weight was not affected by photoperiod (**Table 5.1**).

ii) Mice exposed to SD photoperiod will have higher thymus weights than mice exposed to ND photoperiod.

- Our findings do not support this hypothesis. Thymus weight was not affected by photoperiod (**Table 5.1**).

d) Liver weight

i) Mice exposed to LD photoperiod will have heavier livers than mice on SD photoperiod

- Our findings do not support this hypothesis. Liver weight was not affected by photoperiod (**Table 5.1**).

ii) Mice exposed to LD photoperiod will have heavier livers than mice on ND photoperiod.

- Our findings do not support this hypothesis. Liver weight was not affected by photoperiod (**Table 5.1**).

e) Pups *in utero*

i) Mice exposed to LD photoperiod will have heavier pups *in utero* than mice on SD photoperiod

- Our findings do not support this hypothesis. There was no effect of photoperiod on conceptus weight on total weight of pups and the number of pups *in utero* was not affect by photoperiod (**Table A2**).

ii) Mice exposed to LD photoperiod will have heavier pups *in utero* than mice on ND photoperiod.

- Our findings do not support this hypothesis. There was no effect of photoperiod on conceptus weight on total weight of pups and the number of pups *in utero* was not affect by photoperiod (**Table A2**).

3) **Photoperiod manipulation during gestation will affect expression of genes in the mammary transcriptome. More specifically:**

a) Genes identified in the comparison of SD_{ND} photoperiod will be associated with increased lactation performance, compared to genes identified in the comparison of LD_{ND} photoperiod.

- This hypothesis was supported by our findings. Genes identified in the comparison of SDND we more closely associated with lactation performance than genes in the comparison of LDND.

b) Genes identified in the comparisons of LD_{ND} and SD_{ND} will identify different sets of genes.

- This hypothesis was supported by our findings. Only 39 genes were observed to be common between the comparison of LD_{ND} and SD_{ND}. The biological functions these genes enriched also suggest the two photoperiods had differing effects on the mammary gland.

PHOTOPERIOD EXPOSURE DURING GESTATION IN MICE – CARRY OVER EFFECTS

It was hypothesized that:

1) **Photoperiod manipulation during gestation will have carry over effects on litter weight gain during lactation More specifically:**

   a) SD photoperiod during gestation will increase litter weight gain relative to LD photoperiod.

   - Our findings do not support this hypothesis. There was no effect of photoperiod on litter weight gain in mice exposed to photoperiod during gestation.

   b) ND photoperiod during gestation will increase litter weight gain relative to LD photoperiod.

   - Our findings do not support this hypothesis. There was no effect of photoperiod on litter weight gain in mice exposed to photoperiod during gestation.

1) **Photoperiod manipulation during gestation will affect pregnancy outcomes. More specifically:**

   a) Litter weight

   i) SD photoperiod during gestation will decrease the weight of litters at time of birth relative to LD photoperiod.

   - Our findings do not support this hypothesis. There was no effect of photoperiod on litter weight at the time of birth, or individual pup weight at birth (**Table A2**).

ii) SD photoperiod during gestation will decrease the weight of litters at time of birth relative to ND photoperiod.

- Our findings do not support this hypothesis. There was no effect of photoperiod on litter weight gain in mice exposed to photoperiod during gestation (**Table A2**).

b) Pup numbers

i) Mice exposed to SD photoperiod during gestation will have fewer pups than mice on LD photoperiod.

- Our findings do not support this hypothesis. There was no effect of photoperiod on the number of pups per litter (**Table A2**).

ii) Mice exposed to ND photoperiod during gestation will have fewer pups than mice on LD photoperiod.

- Our findings do not support this hypothesis. There was no effect of photoperiod on the number of pups per litter (**Table A2**).

2) Photoperiod manipulation during gestation will not have a carryover effect on the mammary transcriptome on L10. More specifically:

a) No genes will be differentially expressed in the comparison of SD_{ND}

- Our findings do not support this hypothesis. There were a large number of genes differentially expressed in the comparison of LD_{ND} and SD_{ND}.

b) No genes will be differentially expressed in the comparison of LD_{ND}

- Our findings do not support this hypothesis. There were a large number of genes differentially expressed in the comparison of LD_{ND} and SD_{ND}.

GENERAL DISCUSSION

The preceding work describes experiments conducted to address the question of how does photoperiod affect the mammary gland. Although it has been known since 1978 that photoperiod can affect lactation, the molecular mechanisms underlying the response remain unclear. Much like previous studies of the bovine mammary gland (Auchtung et al., 2005; Wall et al., 2005b), we focused on milk production, cell proliferation and gene expression to understand how photoperiod may affect mammary function.

Overall the objective of this research was to determine if common biology exists in the response of the mammary gland to photoperiod manipulation. To summarize our findings in the context of the objectives, I have assessed commonalities on three levels: function, genes and mechanisms.

Common function - milk production

An objective of this work was to determine if there is a common functional response of the mammary gland to photoperiod manipulation. In cows, it has been well established that LD during lactation and SD during the dry period promote milk production. We were unable to identify the same effect in mice using litter weight gain as a proxy for milk production. This may be due to the confounding effect of photoperiod on pups, both *in utero* (gestation studies) and after parturition (lactation study). In Siberian hamsters, a classic model of mammalian photoperiod, the effects of maternal photoperiod exposure during gestation affects the ability of offspring to respond to photoperiod (Horton and Stetson, 1990) and reproductive development in peri-pubertal offspring (Horton et al., 1990). More recent studies suggest that perinatal photoperiod exposure has significant effects on the circadian

clock, visual function, and light responsiveness (Ciarleglio et al., 2011; Brooks et al., 2014; Jackson et al., 2014). The transfer of photoperiodic information from mother to fetus is thought to be through melatonin signaling (Goldman, 2003). Research in this field has expanded to humans and investigations have been carried out on the effects of season of birth on mood and eating disorders, mental health and disease risk factors (Buckles and Hungerman, 2013; Pantazatos, 2013). In future studies it will be important to account for the effects of photoperiod on pups possibly by having a larger population of dams in order to allow cross fostering.

Despite the lack of a common response between the bovine and mouse models, our cell proliferation and gene expression analyses provides substantial evidence that photoperiod can affect the potential of the mouse mammary gland to express genes which may support milk production.

The effects of photoperiod on mammary gene expression

To understand the effects of photoperiod on mammary function and lactation, we conducted microarray experiments to identify changes in the mammary transcriptome and have highlighted genes and pathways with the potential to affect mammary function. In this investigation, we first determined the effects of photoperiod manipulation during the dry period on mammary gene expression in dairy cows. Subsequently, a mouse model was used to explore further the response of the mammary gland to photoperiod during gestation and lactation. The data reported in this work constitutes the first investigation, to our knowledge, of the effects of photoperiod on the global mammary transcriptome in either cows or mice. Our findings provide several novel overall insights about the effects of photoperiod on the mammary transcriptome. Firstly, photoperiod manipulation is sufficient stimulation to affect

the mammary transcriptome. To that end, we have determined that LD and SD photoperiod affect very different sets of genes that are associated with different biological functions. In addition, photoperiod differentially affects gene expression in the mammary gland depending on the physiological state. Lastly, photoperiod can have enduring effects after the cessation of exposure on the mammary transcriptome.

Common photoperiod-responsive genes – within study

The use of three photoperiod treatments was advantageous as it allowed for comparison between two sets of differentially expressed genes in each study and therefore identification of common genes affected by photoperiod. Most broadly, we can conclude that LD and SD photoperiod affect different sets of genes. This is true at both the individual gene level, as well as the functions affect by those genes. **Table 7.1** shows the number and percentage of common genes that were differentially expressed within each of the three mouse studies.

Two-way hierarchical analysis was used to determine the relative distance of clusters by photoperiod in expression patterns of the commonly differentially expressed gene within each study (**Figure 7.2**). In the mouse lactation day 10L study, the two clusters were more similar indicating the expression patterns were similar between the two clusters (**Figure 7.3a**). In the mouse gestation Day 17G study, the expression patterns of common differentially expressed genes were more similar between LD and ND, than LD and SD (**Figure 7.3b**). Lastly, in the mouse gestation Day 10L study, the expression patterns of common differentially expressed genes were more similar between LD and SD, than LD and ND (**Figure 7.3c**). Overall, it appears that the expression pattern of common genes is

dependent on the physiological state of the mammary gland either when photoperiod is applied or when the mammary gland is samples.

Previously, we might have hypothesized that LD and SD photoperiod would have opposite effects on gene expression relative to ND photoperiod. However, these common genes show that this is not the case. The non-consistent direction of the effects of photoperiod has been reported in other studies. For example, in their investigation of perinatal photoperiod and circadian clock imprinting, Ciarleglio et al. (2011) did not find the direction of the effect of SD and LD to always be consistent relative to ND. In a more recent study of retinal function, mice exposed to ND photoperiod did not have consistent responses relative to mice on LD or SD photoperiod (Jackson et al., 2014). Together with our findings, LD and SD are not endpoints on a spectrum of effects with ND at the middle; rather the direction of change in response to LD or SD, relative to ND photoperiod, is measurement or gene-specific.

Common mechanisms in the response of the mammary gland to photoperiod

One of the objectives of this research was to determine if there is commonality in the underlying biology of the response of the mammary gland to photoperiod. At the level of mechanisms, our findings support the presence of common biology between the bovine and mouse mammary gland, as well as between physiological states. **Figure 7.4** summarizes the study findings, six mechanisms, and their relative potential effects on altering mammary function. The following will detail common mechanisms observed to vary extents among our data sets.

Photoperiod affects cell proliferation

A central theme in our findings was the effect of photoperiod on cell proliferation as measured by BrdU incorporation and gene expression associated with cell proliferation. Wall et al. (2005b) had previously reported SD photoperiod increases mammary cell proliferation on day -24 in the same cows used in our microarray study. Our gene expression data support these findings. Differentially expressed genes, as well as predicted *upstream regulators,* were associated with cell proliferation in the bovine microarray data. In conjunction with the cell proliferation findings of Wall et al. (2005b) we infer that the effects of SD photoperiod on gene expression promote mammary cell proliferation. Based on data from the interaction of photoperiod and time, the effects on cell proliferation may be mediated through the IGF-1 signaling pathway (discussed below).

In the mouse lactation study, differential BrdU incorporation was detected early in lactation, with LD photoperiod negatively affecting BrdU incorporation (**Chapter 4**). The transcriptomic data supported these findings as *cellular growth and proliferation* was a top network associated with genes identified in the comparison of SD_{ND}. On day 17 of the mouse gestation study, cell proliferation was a key *biofunction* associated with the gene set (**Chapter 5**) and the incorporation of BrdU was higher in mice exposed to SD, relative to LD photoperiod. In addition, *upstream regulators* of genes identified in the comparison of SD_{ND}, were predicted to affect 36 genes functionally associated with proliferation of cells, a theme that was also reflected in cluster and *biofunction* analysis. In the gestation study, on L10, cell cycle progression was an enriched *biofunction* most notably in the comparison of SD_{ND}, but also present in the comparison of LD_{ND}. Although, we did not detect differential

incorporation of BrdU on L10 after photoperiod exposure during gestation, potentially due to the higher levels of variation among mice (Chapter 4).

Ultimately, these findings provide substantial evidence that photoperiod manipulation both during gestation and during lactation, can affect mammary cell proliferation. Specifically, our findings indicate SD photoperiod may enhance cell proliferation relative to LD photoperiod.

Genes associated with lactation

The lack of annotation of genes in the context of the mammary gland was a limitation inherent to the approach of this work. Specifically, with the bovine array, many of the differentially expressed probes either lacked annotation or annotation was not consistently available across analysis platforms (DAVID vs. IPA). To overcome this lack of annotation, comparisons were made between photoperiod-responsive genes and those identified in other microarray studies of lactation. Identifying those genes that had previously been associated with lactation and mammary function provided an additional layer of annotation. In each of the four studies presented here, genes associated with lactation have been identified. These share two important characteristics; they are photoperiod responsive in the mammary gland and may have a role in lactation. Therefore, these genes provide ideal targets for further study of the mechanisms underlying the response of the mammary gland to photoperiod manipulation.

Thyroid hormone signaling

Thyroid hormone-related gene expression was apparent in all three of the mouse studies, and although not explicitly mentioned in **Chapter 3**, was present in the bovine

interaction (23 genes). Thyroid signaling is now a well-established mechanism of seasonal and reproductive responses to photoperiod (Yoshimura, 2013; Dardente et al., 2014). The majority of the physiological effects of thyroid signaling are mediated through the pituitary gland and augmentation of hormone secretion (luteinizing hormone, follicle-stimulating hormone) (Yoshimura, 2013). In addition, thyroid hormone signaling is known to affect the mammary gland. Exogenous thyroid hormone increases milk production in dairy cows and *in vitro* exposure to 3,3'5-triiodothyronine (T3) affects the expression of both α-lactalbumin and casein proteins by increasing the effect of lactogenic hormones (Houdebine et al., 1978; Bhattacharjee and Vonderhaar, 1984). Among the genes representing thyroid-related signaling the mammary gland are thyroid stimulating hormone (TSH) receptor (*Tshr*), and tyrosine hydroxylase. In dairy cows, Capuco et al. (2008) reported expression of thyroid hormone receptor (TRβ1) and DIO2 is affected between late gestation and early lactation, thereby altering the responsiveness of the mammary gland to thyroid hormones. It is plausible that altered thyroid signaling could affect the mammary gland either by enhancing the secretion of prolactin or oxytocin from the pituitary gland or by altering the sensitivity of the mammary gland to thyroid signaling. Ultimately, our data support a role for thyroid hormone signaling in mediating the effects of photoperiod in the mammary gland and genes identified in our studies should serve as targets for continued investigation.

Circadian gene expression

The interconnectedness of photoperiod and circadian biology, although known, is not fully understood. Similarly, there is evidence of a relationship between circadian gene expression and mammary function; however, the nature of this relationship has not been fully described. Using human milk fat globules Maningat and coworkers showed that 7% of genes

in the lactating mammary gland undergo circadian variation in expression, including circadian clock genes (Maningat et al., 2009; Maningat et al., 2011). Disruption of circadian clock genes can affect a variety of normal cell functions like cell cycle progression and differentiation (Matsuo et al., 2003), two physiological changes vital to the onset of lactation. In addition, circadian gene expression changes with developmental stage in the mouse mammary gland (Metz et al., 2006). Using microarray analysis in rats, (Casey et al., 2009) identified differential expression of 15 core clock genes (including: *Clock*, *Nr1d1*, *Bhlhb2*, *Dbp*) in the mammary gland, liver, and adipose tissue between G20 and L1. Subsequently, Casey et al. (2009) proposed the circadian clock functions to coordinate the changes in physiology required at the onset of lactation.

Our findings in the mouse lactation study provide evidence that circadian genes may mediate the effects of photoperiod in the mammary gland, early in lactation. In general, SD photoperiod enhanced the expression of clock related genes relative to LD photoperiod, suggesting SD photoperiod promotes coordination of mammary function through clock gene expression. In addition, we also reported differential expression of serotonin receptors. Serotonin regulates tissue metabolism and functions in the entrainment of circadian rhythms (Weber et al., 1998; Morin, 1999). Serotonin has also been proposed regulator of lactation in the mouse, human, and bovine mammary gland (Hernandez et al., 2009; Collier et al., 2012). In conclusion, our findings provide additional evidence of a relationship among photoperiod, the circadian clock and lactation.

IGF-1 and prolactin signaling

IGF-1 and prolactin have been principal targets of investigations into the mediators of the response of the mammary gland to photoperiod (Dahl et al., 1997; Dahl, 2008; Dahl et

al., 2012). In the bovine photoperiod study, IGF-1 signaling was identified as the top canonical pathway, and numerous genes were predicted to be regulated by IGF-1. In the mouse mammary gland, IGF-1 was identified as an *upstream regulator* of 15 genes in the comparison of SD_{ND} in the lactation study and 16 genes in the comparison of LD_{ND} in the gestation study on day 10 of lactation. In total, 52 genes predicted to have IGF-1 as an *upstream regulator* have been identified as responsive to photoperiod in our studies. These genes are ideal targets for the continued investigation of the role of IGF-1 as a mediator of the effects of photoperiod in the mammary gland during gestation and lactation.

Prolactin was also predicted to affect photoperiod-responsive genes, but to a lesser extent than IGF-1. In the bovine study, several genes were predicted to be regulated by prolactin in the main effect of photoperiod (AKR1C3, GPNMB, and SERPINA3) and 23 genes in the interaction. Genes downstream of prolactin in the interaction function in several mammary related *biofunctions* including cell differentiation, morphogenesis and branching (**Figure A1**). Secretion of prolactin is regulated by the hypothalamus by inhibitory actions of dopamine (Freeman et al., 2000). In the mouse gestation study on L10, we report differential expression of prolactin-related genes as well as dopamine decarboxylase (DDC), which was also differentially expressed in the mammary gland of cows on LD and SD (**Chapter 3**). In the mouse lactation study, 6 genes were predicted to have prolactin as an *upstream regulator*. There is considerable evidence prolactin may partly mediate the effects of photoperiod in dairy cows, and to a lesser extent in mice.

One potential explanation for the above discrepancy is that unlike the mice used in our studies, the cows were multiparous. Therefore the mammary gland of the cows was undergoing involution brought on by the cessation of milk removal and redevelopment in

preparation for the subsequent lactation (Hurley and Loor, 2011). This process is often characterized by gene expression associated with immune signatures (Stein et al., 2004), which prolactin is thought to mediate in dairy cows (Auchtung and Dahl, 2004; Dahl, 2008).

Limitations to gene expression data

Our data provide the first report of the effects of photoperiod on the mammary transcriptome in cows and mice. To date, few publications on the effects of environmental factors (temperature, photoperiod, etc.) on large-scale gene expression have been carried out. Therefore, the foundation of literature pertaining to photoperiod-responsive gene expression is minimal, making data interpretation more difficult. Annotation of differentially expressed probes, even in mice, was a considerable limiting factor since 11% of all differentially expressed probes were completely unmapped. Annotation of gene functions was an even more significant limitation to the interpretation of bovine microarray data. At the time of analysis, 33% of differentially expressed probes were not annotated; therefore, no interpretation of their function could be derived. Furthermore, many of the functional analysis tools available (DAVID, IPA) refer only to human, mouse and rat data. Incomplete annotation of genes creates significant challenges when attempting to interpret biological meaning. As more information is known about the gene function, further interpretation of our microarray findings may be possible.

One of the limitations of previous studies, including our own in dairy cows, is the use of only two photoperiods, LD and SD. This approach is used in part because of the cost of using large animals and the logistics of light exposure. Subsequently, the data from the cow study (**Chapter 2**) represents only the comparison of LD vs SD (LD_{SD}). Therefore, the microarray data represents the difference (LD – SD) rather than a comparison relative to a

common photoperiod, such as ND photoperiod. One limitation of the current work is that only the comparisons of LD_{ND} and SD_{ND} have been made in the mouse model. Based on the objectives of this research, the comparisons of LD_{ND} and SD_{ND} provided the most information and were the principle comparisons made to date. However, the results of the comparison of LD_{SD} in the mouse studies may more closely align with gene expression differences observed in the bovine photoperiod study. Future work may include analysis of the genes differentially expressed in the comparison of LD_{SD} followed by comparison of those genes to the genes identified here.

Another limitation of this work is the small sample size of both cows and mice. The sample size in cows was a result of the logistical difficulty of exposing cows to photoperiod for long periods, and the expense of working with large animals. In mice, the n = 6 was selected based power calculations using data from other published works (Wall et al., 2005a; Wall et al., 2005b; Ciarleglio et al., 2011), to establish expected variability of gene expression in the mammary gland in response to photoperiod

Future directions

The knowledge gleaned from this work has shed light on the genes and potential mechanisms underlying the effects of photoperiod in the mammary gland. There are still many unanswered questions regarding the effects of photoperiod on mammary function. These questions include how is photoperiodic information conveyed to mammary cells and how does differential expression of the genes we have identified ultimately mediate changes in mammary physiology and function? In dairy cows, the genes identified here should be assessed as potential markers for the enhancement of lactation performance in response to photoperiod manipulation. In mice, the effects of gestational photoperiod on mammary

development in offspring should be explored, to determine if pre-natal photoperiod has beneficial or detrimental effects on mammary development. In addition, it would be interesting to know if photoperiod has enduring effects on mammary gene expression and function in subsequent lactations. Lastly, in humans, investigation of the effects of photoperiod on mammary gene expression and function may provide insights into the long-term effects on lactation and the role of disturbed circadian rhythms on mammary carcinogenesis.

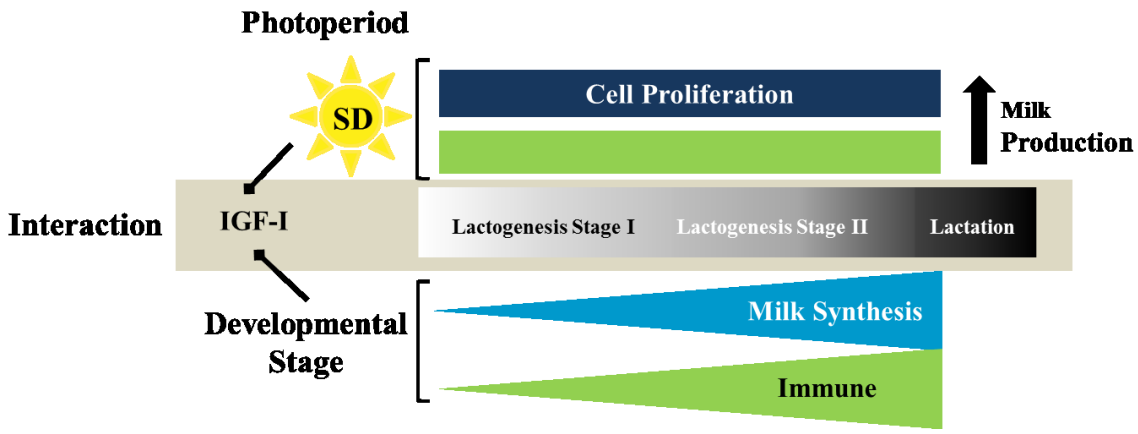


Figure 7.1. Schematic model of the effects of photoperiod, time and the interaction of photoperiod and developmental stage on mammary function during the dry period.

Analysis of the effects of photoperiod on the mammary transcriptome during the dry period demonstrates enrichment of cell proliferation and immune signatures between lactogenesis stage I and lactogenesis stage II. Cows exposed to short day (SD, 8 h light: 16 h dark) photoperiod subsequently produce more milk in the ensuing lactation compared to counterparts on long day (16 h light: 8 h dark) photoperiod. Developmental stage measured by time relative to partition affected genes associated with milk synthesis and immune function, with expression increasing as lactation is approached. Functional analysis of differentially expressed genes in the interaction of photoperiod and time indicated that the genes associated with the IGF-1 signaling pathway may mediate the effect of photoperiod on mammary function during the dry period.

Table 7.1. Common differential expression of genes in the mouse mammary gland in response to photoperiod

Chapter	Study	# of common genes	% of all differentially expressed genes
4	**Mouse Lactation : Day 10L**	14	1.5
5	**Mouse Gestation: Day 17G**	39	6.9
6	**Mouse Gestation: Day 10L**	111	8

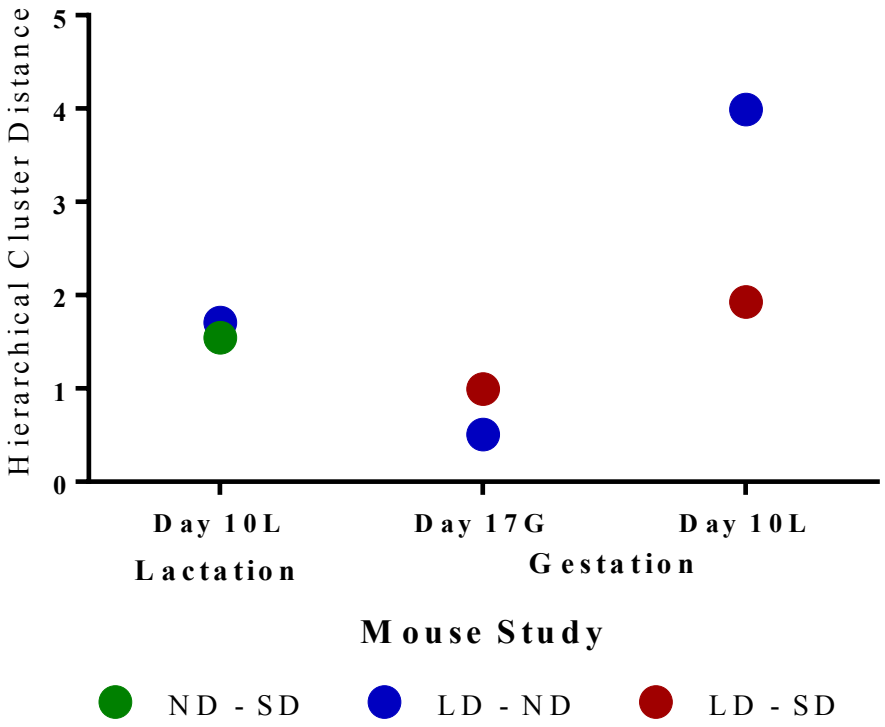


Figure 7.2. Distances between photoperiod clusters identified using two-way cluster analysis of commonly differentially expressed genes.

Hierarchical clustering was conducted in Jmp® Pro 10 using the Ward method. The larger the value of distance the more distant the relationship between photoperiod clusters.

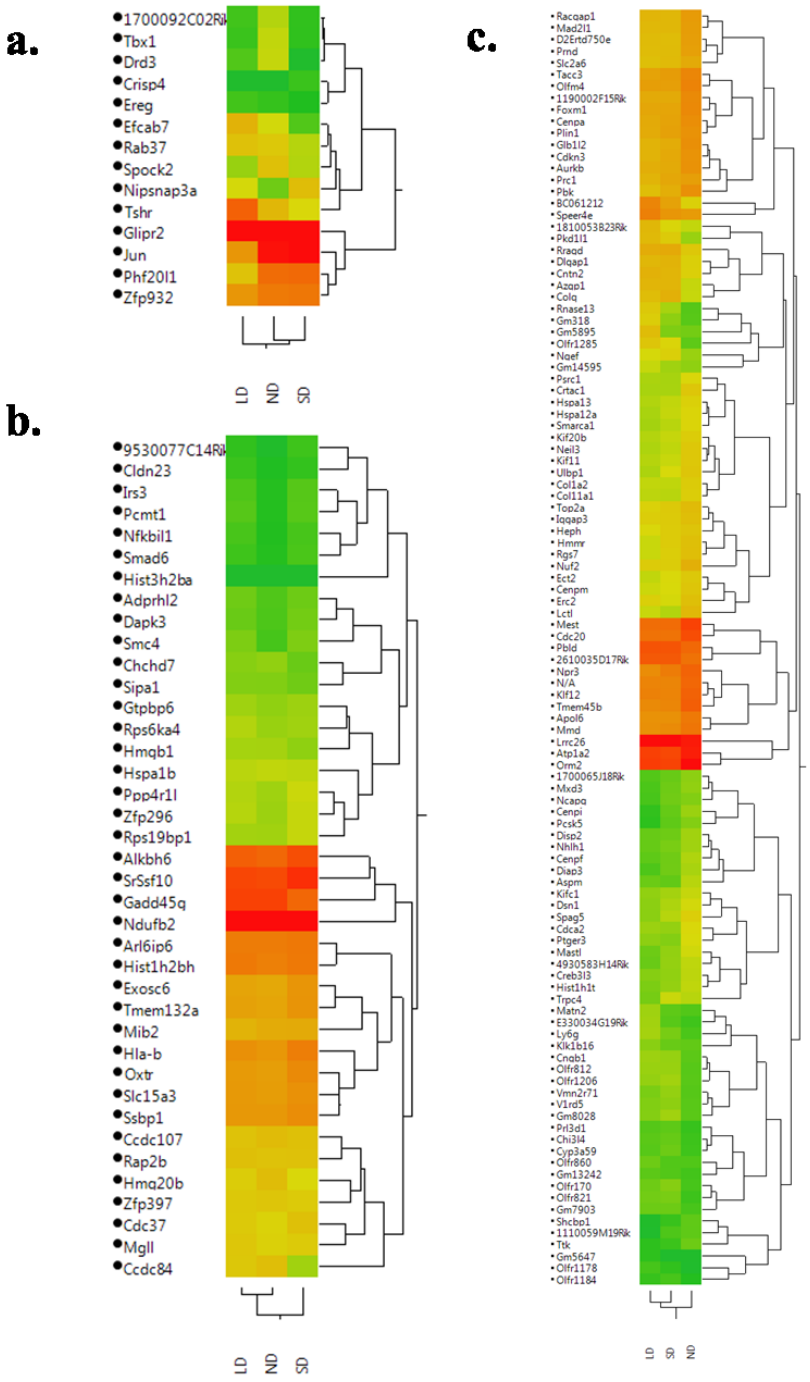


Figure 7.3. Two-way hierarchical clustering of genes commonly differentially expressed in the comparisons of LD_{ND} and SD_{ND}.

a. Lactation day L10, **b.** gestation day G17, and **c.** gestation day L10.

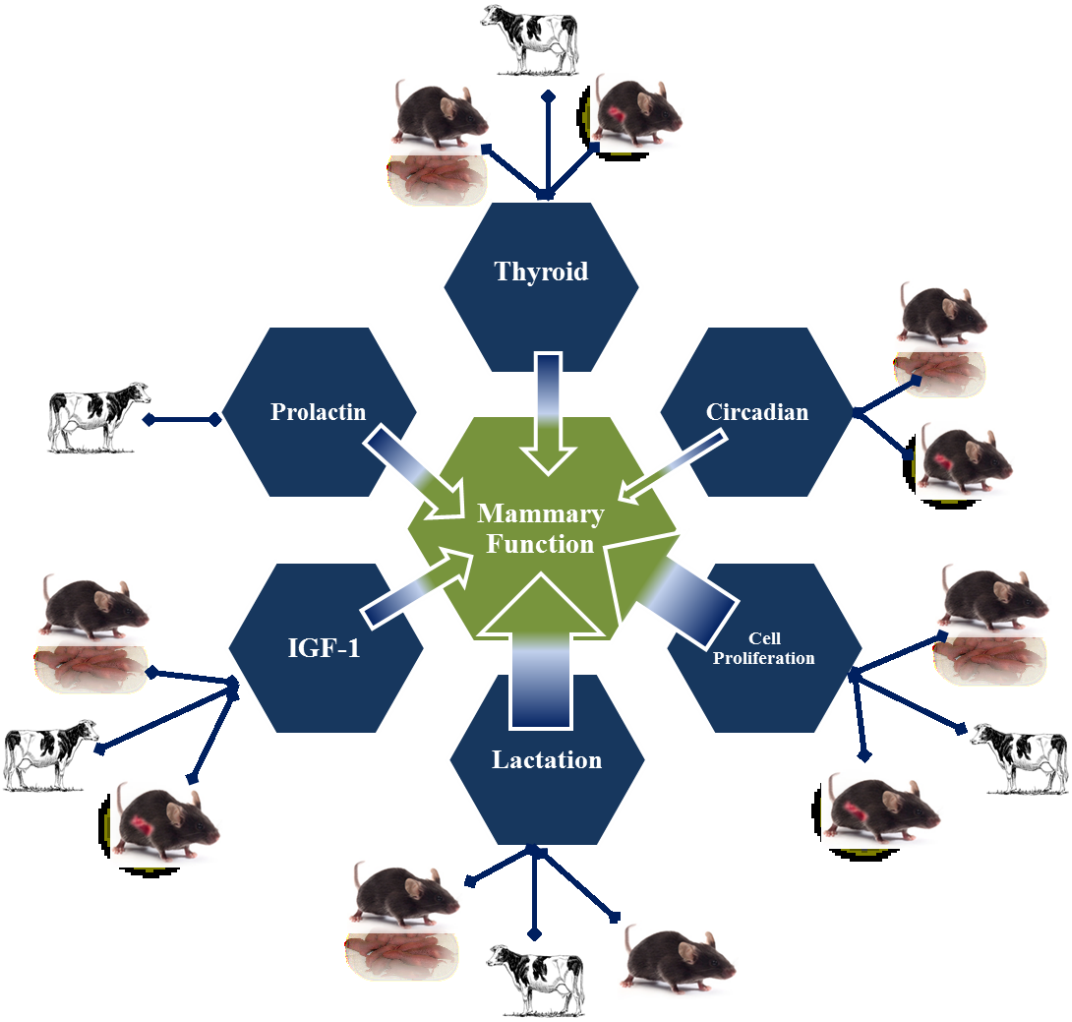


Figure 7.4. Model of the relationship of study findings to 6 potential mechanisms and their relative potential effects on mammary function.

REFERENCES

Auchtung, T. L. and G. E. Dahl. 2004. Prolactin mediates photoperiodic immune enhancement: Effects of administration of exogenous prolactin on circulating concentrations, receptor expression, and immune function in steers. Biol Reprod 71:1913.

Auchtung, T. L., A. G. Rius, P. E. Kendall, T. B. McFadden, and G. E. Dahl. 2005. Effects of photoperiod during the dry period on prolactin, prolactin receptor, and milk production of dairy cows. J Dairy Sci 88:121.

Bhattacharjee, M. and B. K. Vonderhaar. 1984. Thyroid hormones enhance the synthesis and secretion of alpha-lactalbumin by mouse mammary tissue in vitro. Endocrinology 115:1070.

Brooks, E., D. Patel, and M. M. Canal. 2014. Programming of mice circadian photic responses by postnatal light environment. PLoS ONE 9:e97160.

Buckles, K. S. and D. M. Hungerman. 2013. Season of birth and later outcomes: Old questions, new answers. Rev Econ Stat 95:711.

Capuco, A. V., E. E. Connor, and D. L. Wood. 2008. Regulation of mammary gland sensitivity to thyroid hormones during the transition from pregnancy to lactation. Exp Biol Med (Maywood) 233:1309.

Casey, T., O. Patel, K. Dykema, H. Dover, K. Furge, and K. Plaut. 2009. Molecular signatures reveal circadian clocks may orchestrate the homeorhetic response to lactation. PLoS ONE 4:e7395.

Ciarleglio, C. M., J. C. Axley, B. R. Strauss, K. L. Gamble, and D. G. McMahon. 2011. Perinatal photoperiod imprints the circadian clock. Nat Neurosci 14:25.

Collier, R. J., L. L. Hernandez, and N. D. Horseman. 2012. Serotonin as a homeostatic regulator of lactation. Domest Anim Endocrinol 43:161.

Dahl, G. E. 2008. Effects of short day photoperiod on prolactin signaling in dry cows: A common mechanism among tissues and environments? J Anim Sci 86:10.

Dahl, G. E., T. H. Elsasser, A. V. Capuco, R. A. Erdman, and R. R. Peters. 1997. Effects of a long daily photoperiod on milk yield and circulating concentrations of insulin-like growth factor-1. J Dairy Sci 80:2784.

Dahl, G. E., S. Tao, and I. M. Thompson. 2012. Effects of photoperiod on mammary gland development and lactation. J Anim Sci.

Dardente, H., D. G. Hazlerigg, and F. J. Ebling. 2014. Thyroid hormone and seasonal rhythmicity. Front Endocrinol (Lausanne) 5:19.

Freeman, M. E., B. Kanyicska, A. Lerant, and G. Nagy. 2000. Prolactin: Structure, function, and regulation of secretion. Physiol Rev 80:1523.

Goldman, B. D. 2003. Pattern of melatonin secretion mediates transfer of photoperiod information from mother to fetus in mammals. Sci STKE 2003:PE29.

Hernandez, L. L., S. W. Limesand, J. L. Collier, N. D. Horseman, and R. J. Collier. 2009. The bovine mammary gland expresses multiple functional isoforms of serotonin receptors. J Endocrinol 203:123.

Horton, T. H., S. A. Stachecki, and M. H. Stetson. 1990. Maternal transfer of photoperiodic information in siberian hamsters. Iv. Peripubertal reproductive development in the absence of maternal photoperiodic signals during gestation. Biol Reprod 42:441.

Horton, T. H. and M. H. Stetson. 1990. Maternal programming of the fetal brain dictates the response of juvenile siberian hamsters to photoperiod: Dissecting the information transfer system. J Exp Zool Suppl 4:200.

Houdebine, L. M., C. Delouis, and E. Devinoy. 1978. Post-transcriptional stimulation of casein synthesis by thyroid hormone. Biochimie 60:809.

Hurley, W. L. and J. J. Loor. 2011. Mammary gland: Growth, development and involution. Pages 338 in Encyclopedia of dairy sciences (second edition). J. W. Fuquay, ed. Academic Press, San Diego.

Jackson, C. R., M. Capozzi, H. Dai, and D. G. McMahon. 2014. Circadian perinatal photoperiod has enduring effects on retinal dopamine and visual function. J Neurosci 34:4627.

Maningat, P. D., P. Sen, M. Rijnkels, D. L. Hadsell, M. S. Bray, and M. W. Haymond. 2011. Short-term administration of rhgh increases markers of cellular proliferation but not milk protein gene expression in normal lactating women. Physiol Genomics 43:381.

Maningat, P. D., P. Sen, M. Rijnkels, A. L. Sunehag, D. L. Hadsell, M. Bray, and M. W. Haymond. 2009. Gene expression in the human mammary epithelium during lactation: The milk fat globule transcriptome. Physiol Genomics 37:12.

Matsuo, T., S. Yamaguchi, S. Mitsui, A. Emi, F. Shimoda, and H. Okamura. 2003. Control mechanism of the circadian clock for timing of cell division in vivo. Science 302:255.

Metz, R. P., X. Qu, B. Laffin, D. Earnest, and W. W. Porter. 2006. Circadian clock and cell cycle gene expression in mouse mammary epithelial cells and in the developing mouse mammary gland. Dev Dyn 235:263.

Morin, L. P. 1999. Serotonin and the regulation of mammalian circadian rhythmicity. Ann Med 31:12.

Pantazatos, S. P. 2013. Prediction of individual season of birth using mri. Neuroimage 88C:61.

Stein, T., J. S. Morris, C. R. Davies, S. J. Weber-Hall, M. A. Duffy, et al. 2004. Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving lbp, cd14 and stat3. Breast Cancer Res 6:R75.

Wall, E. H., T. L. Auchtung-Montgomery, G. E. Dahl, and T. B. McFadden. 2005a. Short communication: Short-day photoperiod during the dry period decreases expression of suppressors of cytokine signaling in mammary gland of dairy cows. J Dairy Sci 88:3145.

Wall, E. H., T. L. Auchtung, G. E. Dahl, S. E. Ellis, and T. B. McFadden. 2005b. Exposure to short day photoperiod during the dry period enhances mammary growth in dairy cows. J Dairy Sci 88:1994.

Weber, E. T., R. L. Gannon, and M. A. Rea. 1998. Local administration of serotonin agonists blocks light-induced phase advances of the circadian activity rhythm in the hamster. J Biol Rhythms 13:209.

Wei, J., P. Ramanathan, I. C. Martin, C. Moran, R. M. Taylor, and P. Williamson. 2013. Identification of gene sets and pathways associated with lactation performance in mice. Physiol Genomics 45:171.

Yoshimura, T. 2013. Thyroid hormone and seasonal regulation of reproduction. Front Neuroendocrinol 34:157.

APPENDIX A

Table A1. Exposure to photoperiod[1] during lactation does not alter mouse thymus weight[2].

		Long Day		Normal Day		Short Day			p-value[3]		
	Day of Lactation	**Weight (mg) ± sd**		**Weight (mg) ± sd**		**Weight (mg) ± sd**		**Means by Time**	**Time**	**PP**	**Time *PP**
Thymus	**5**	0.89	±0.32	0.98	±0.48	1.30	±0.23	1.08	0.7	0.9	0.1
	10	1.25	±0.30	1.05	±0.22	1.08	±0.31	1.13			
	15	1.19	±0.35	1.11	±0.28	0.82	±0.50	1.05			
Means by Photoperiod[4]		**1.14**		**1.05**		**1.08**					

[1.] Photoperiods: long day (16 h light: 8 h dark), normal day (12 h light: 12 h dark) and short day (8 h light: 16 h dark).

[2.] The effects of time, photoperiod, and the interaction of photoperiod and time were determined using the ANOVA procedure in JMP® Pro 10.

[3.] Separation of means by photoperiod or time were conducted using Tukey-Kramer HSD test, differences were characterized as significant at $p \leq 0.05$ and are indicated by differing superscript letters (a, b).

Table A2. Parturition data for mice exposed to different photoperiods[1] throughout gestation

		LD		ND		SD		p-value[2,3]
	Day	Weight (g)	±sd	Weight (g)	±sd	Weight (g)	±sd	
Gestation								
Terminal Dam	17	32.9	±2.42	33.7	±2.82	32.4	±4.68	0.821
	19	38.8[a]	±1.54	38.4[ab]	±0.70	34.0[b]	±4.82	0.031*
Conceptus	17	8.14	±1.75	7.67	±2.26	7.21	±4.26	0.902
	19	12.2	±1.03	10.5	±5.02	9.50	±4.46	0.509
Total wt of pups	17	5.03	±1.07	4.74	±1.48	4.28	±2.48	0.822
	19	9.20	±0.93	9.15	±3.14	7.04	±3.37	0.319
Number of pups	17	7.75	±2.06	7.00	±2.45	7.00	±4.43	0.930
	19	8.00	±0.89	6.86	±3.67	6.33	±3.20	0.607
Lactation								
Litter wt at birth	1	11.2	±2.08	10.5	±1.98	11.0	±2.41	0.784
# of pups at birth	1	8.71	±1.77	8.40	±1.58	8.67	±1.99	0.908
Pup wt. at birth	1	1.29	±0.12	1.26	±0.07	1.27	±0.05	0.676

[1.] Photoperiods: long day (LD, 16 h light: 8 h dark), normal day (ND, 12 h light: 12 h dark) and short day (SD, 8 h light: 16 h dark).

[2.] The effects of time, photoperiod determined using the ANOVA procedure in JMP® Pro 10.

[3.] Separation of means by photoperiod was conducted using Tukey-Kramer HSD test, differences were characterized as significant at $p \leq 0.05$ and are indicated by differing superscript letters (a, b).

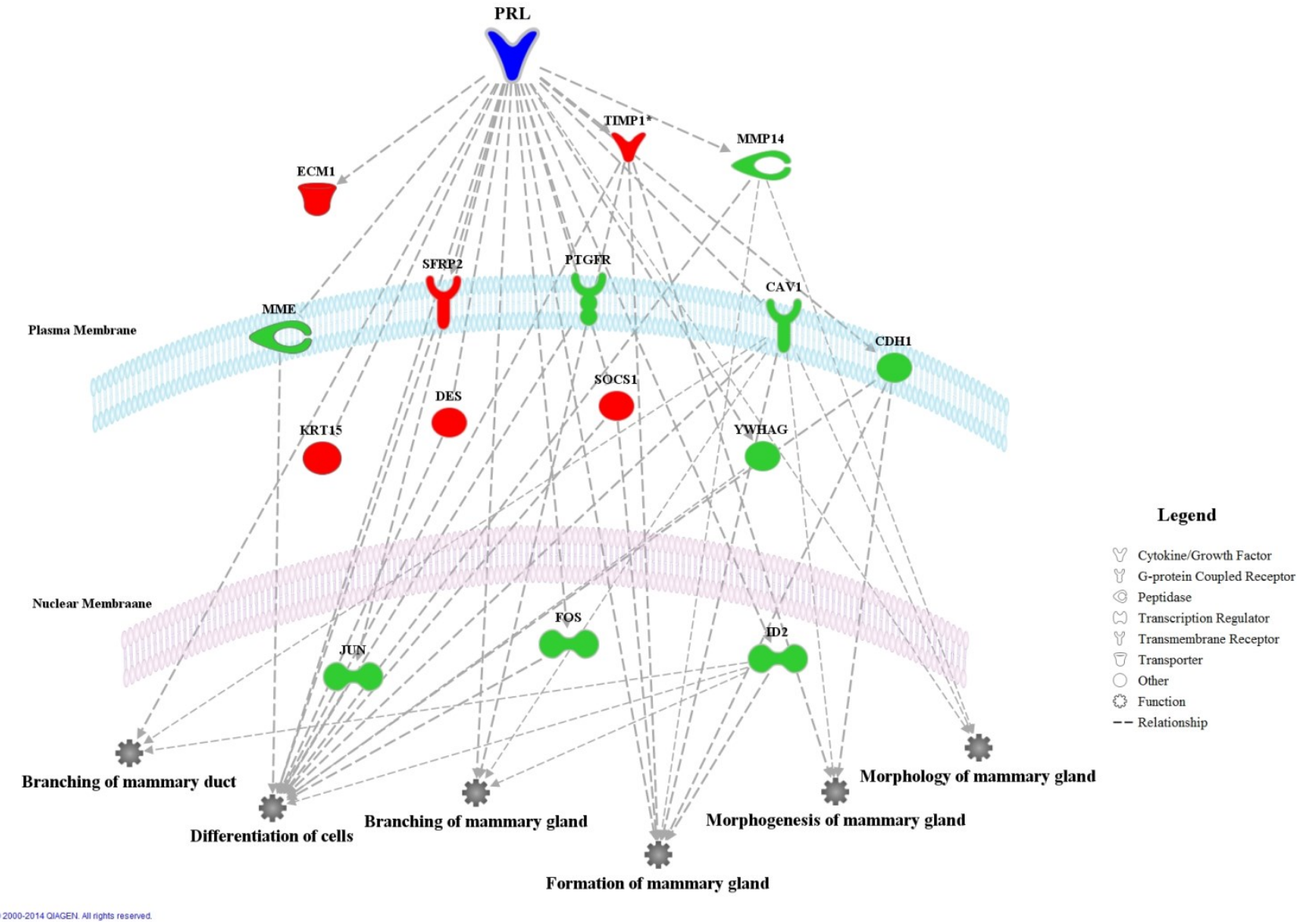


Figure A1. Prolactin signaling in the bovine mammary gland from genes identified in the interaction.

SUPPLEMENTAL TABLES

Provided in digital format