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Uppsala 2012

Degree project / Swedish University of Agricultural Sciences, Department of Animal Nutrition and Management, **401**

Examensarbete, 30 hp
Masterarbete
Husdjursvetenskap
Degree project, 30 hp
Master thesis
Animal Science

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Examinator:
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Omfattning:
Extent: 30 hp
Kurstitel:
Course title: Degree project
Kurskod:
Course code: EX0551
Program:
Programme: Animal Science, Master thesis
Nivå:
Level: Advanced A2E
Utgivningsort:
Place of publication: Uppsala
Utgivningsår:
Year of publication: 2013
Serienamn, delnr:
Series name, part No: Degree project / Swedish University of Agricultural Sciences, Department of Animal Nutrition and Management, 401
On-line publicering:
On-line published: http://epsilon.slu.se
Nyckelord:
Key words: Dairy cows, light program, IGF-1, prolactin, milk yield

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Abstract

The aim of this study was to investigate the relationship between sleep, photoperiod, milk yield and hormones connected to milk formation. Variations of prolactin (PRL) and Insulin-Like growth factor-1 (IGF-1) in plasma were measured during two different light programs. The light programs consisted of either 24 hours of light (L24:D0) or four hours of light and 20 hours of darkness (L4:D20). Five cows of the Swedish Red breed were used in a cross-over experiment and each light program consisted of five days. Milk yield was measured at every milking and milk samples were analyzed with regard to fat, lactose and protein. The difference between morning and evening milking in a 12-hour milking interval was also examined. Blood samples were collected via a permanent catheter during milking and after periods of sleep during the last 36 hours of each light program.

There were no significant differences in milk yield or milk composition between the light programs, neither did morning or evening milk yield vary. There were small variations in amounts of sleep between the two light programs. Plasma PRL did not differ significantly between the light programs besides at 1 min and 11 min after start of milking. Levels of IGF-1 were significantly higher during L4:D20. There was no significant effect of sleep on IGF-1. Sleep, light and milk formation needs to be further studied in order to investigate possible connections and underlying mechanisms.

Sammanfattning

Syftet med studien var att undersöka hur sömn, ljus, mörker och hormoner kopplade till mjölkbildning, är relaterade. Variationer i nivåer av prolaktin (PRL) och insulin-like growth factor-1 (IGF-1) mättes i två olika ljusprogram. Ljusprogrammen bestod aningen av 24 timmars ljus (L24:D0) eller fyra timmars ljus och 20 timmars mörker (L4:D20). Fem kor av rasen Svensk rödbrokig boskap användes i en crossover-studie där varje ljusprogram bestod av fem dagar. Avkastning registrerades vid varje mjölkning och mjölkprover togs för analys av fett, laktos och protein. Skillnaden i avkastning mellan morgon- och kvällsmjölkning i ett 12-timmars mjölkningsintervall studerades även. Blodprov togs med hjälp av en permanentkateter under mjölkningen samt efter perioder av sömn under de sista 36 timmarna av varje ljusprogram.

Varken avkastning eller mjölksammansättning skiljde sig signifikant åt mellan ljusprogrammen, det fanns inte heller någon skillnad i avkastning mellan morgon- eller kvällsmjölkning. Det fanns inga stora skillnader i mängden av sömn i de två ljusprogrammen. Nivåer av PRL skiljde sig inte åt mellan ljusprogrammen med undantag för +1 min och +11 min efter starten av mjölkningen. Mängden IGF-1 var signifikant högre under L4:D20, men sömn hade ingen effekt på nivån av IGF-1. Sömn, ljus och mjölkbildning behöver undersökas mer för att förstå grundläggande underliggande mekanismer och samband.

Introduction

Dairy cows have been bred for a long time to produce high amounts of milk. Modern dairy farming often involves robotic milking with production running 24 hours a day. How the constant production and light in the barn is affecting sleep and milk production is unknown. Researchers showed early that when milking intervals are equally long (12 hours), morning milk yield has been found to be 0.10-0.65 kg higher than evening milk yield in dairy cows (Everett & Wadell, 1970; Putnam & Gilmore, 1970; Gilbert *et al*., 1973). Higher milk production in the morning could be due to different patterns of hormone release associated with the night or with sleep. A widespread perception is that sleeping might increase the production of hormones that are related to milk formation. There are few studies made regarding sleep in dairy cows, Ruckebusch (1972) estimated that dairy cows sleep about four hours per day and Nilsson (2011) concluded that cows spend more time sleeping at night compared to daytime.

Different photoperiods, the daily period of light and dark, affects reproduction, lactation and health in cattle (Dahl *et al*., 2012). Lactating dairy cows exposed to long day photoperiod (LDPP, approximately 16 hours of light and 8 hour of darkness) have an increased milk production with 10-15 % (Peters *et al*., 1978) or with 2.5 kg/cow per day (Dahl *et al*., 2000) compared to cows held in short day photo period (SDPP, approximately 8 hours of light and 16 hours of darkness). The pattern is reported to be reversed during the dry period as dry cows exposed to SDPP produce more milk in the subsequent lactation compared to cows held in LDPP during the dry period (Miller *et al*., 2000). The exposure of continuous lighting does not seem to have a positive effect on milk production (Marcek & Swanson, 1984). Using photoperiod as a management tool is gaining greater acceptance among farmers looking for ways to increase productivity in dairy production. The effect of photoperiod is well documented but the full underlying galactopoietic mechanism, and why continuous lighting does not elicit the same effect, is still unknown (Dahl *et al*., 2000; Kendall *et al*., 2003).

Initially prolactin (PRL) was believed to be the mediating factor of the response of LDPP but several arguments have been raised that there are other underlying factors that require consideration (Dahl *et al.,* 2000). One explanation of the galactopoietic response to LDPP is the interaction between PRL and insulin growth factor-1 (IFG-1) and their receptors (Flint *et al*., 2001; Dahl & Petitclerc, 2003). Increased levels of IGF-I was found to be the mediator of LDPP in lactating cows (Dahl *et al*., 1997) but the effect of SDPP in dry cows is believed to be mediated by variations in PRL sensitivity in the mammary tissue (Auchtung *et al*., 2005).

Development of new strategies and management tools are dependent on studies that examine biochemical mechanisms in the dairy cow. Only in that way efficiency, milk production and animal welfare can be improved (Casey & Plaut, 2012). To our knowledge, no studies have been made to investigate the relationship between photoperiod and sleep in dairy cows. The relationship between sleep and hormones related to milk formation is another field of knowledge that needs to be further

investigated. A comparative study with plasma samples from sleeping and awake cows has never been performed before, and therefore it might not be possible to reach any conclusions regarding the hormone secretion during sleep and the impact it has on milk formation during these analyses. The results could however give a deeper knowledge about basic physiological functions of the link between sleep, light and milk formation.

The aim of this project was to evaluate variations of PRL and IGF-1 during different light programs and during different stages of sleep. Furthermore, the aim was to investigate possible correlations between light treatment and milk yield.

Literature review

Photoperiod

Cattle are not strictly seasonal breeders (Hansen *et al*., 1983) but respond to different photoperiods (Dahl *et al*., 2012). The definition of photoperiod is the duration of light within a 24-hour period. A short-day photoperiod (SDPP) usually consists of 8 h of light and 16 h of darkness while long-day photoperiod (LDPP) is 16 h of light and 8 h of darkness (Dahl *et al*., 2012). Peters *et al.* (1978) made the initial report on the galactopoietic effect of LDPP in comparison with normal day length (9 to 12 h of light per day). The increase in yield associated with LDPP is consistently reported in different studies (Peters *et al*., 1978; Marcek & Swanson, 1984; Dahl *et al*., 1997; Reksen *et al*., 1999). Reksen *et al.* (1999) concluded that cows exposed to more than 12 h of light per day produce more than cows held in less than 12 h of light per day. There are few doubts that photoperiod has great impact not only on lactation but also on health, as dry cows held in SDPP has been reported to have an improved immune status at calving (Dahl *et al*., 2012).

Melatonin mediates regulation of sleep, immune response and circadian rhythm (Harumi & Matsushima, 2000) and is the mediator of the response to different photoperiods (Dahl *et al*., 2000). The response to different photoperiods starts with stimulation of photoreceptors in the eye, the signal is then transferred to the pineal gland (Stanisiewski *et al*., 1988) and secretion of melatonin changes (Collier *et al.,* 2006). Melatonin levels are low during light conditions, darkness raises the levels several fold (Stanisiewski *et al*., 1988). The response occurs independently of age or stage of lactation (Dahl *et al*., 2012). Melatonin response is not dependent on natural day light, artificial lightning influences the secretory pattern in a similar way (Dahl & Petitclerc, 2003).

Manipulation of the daily photoperiod is a management tool used to increase milk yield during the whole cycle of lactation by dairy farmers (Dahl *et al*., 2000). Dahl and Petitclerc (2003) presents a compilation with 10 studies published between 1978 and 2002, all supporting the positive effect of an increased day length compared to a natural daily photoperiod on milk yield in lactating cows.

The responsiveness to LDPP ranges across any stage of lactation and production level, the acclimatization and response develops gradually over 3-4 weeks (Dahl & Petitclerc, 2003). The composition of milk is generally unaffected of photoperiod, although decreased percentages of fat have been reported at LDPP (Dahl *et al*., 2000).

During the prepubertal period, manipulation of the photoperiod can be an effective tool for improving reproduction (Dahl *et al*., 2012) since heifers held in systems with LDPP reach puberty at a younger age (Hansen *et al*., 1983). As the late pregnant heifer approaches calving, SDPP increase milk production in the subsequent lactation (Dahl *et al*., 2012). There are consistent results that SDPP during the dry period followed by LDPP during lactation, increases milk production (Dahl & Petitclerc, 2003). There can be clear increases in production when the use of different light programs is a part of daily management (Dahl *et al*., 2012) but the effect of different photoperiods and its effect on among dairy cows sleep has not yet been investigated.

Dairy cows kept in continuous lighting are not associated with greater milk yield (Marcek & Swanson, 1984). A dark phase appears to be necessary to maintain the photoperiodic response, as animals held in continuous lightning seem to lose ability to keep track of day length (Buchanan *et al*., 1992). The length and light intensity of the required dark phase is not fully understood. Bal *et al.* (2008) concludes that intensities of 40-60 lux have no effect on concentrations of melatonin and can be considered as night time for dairy cows while Muthuramalingam *et al.* (2006) consider light intensities below 10 lux as night time for dairy cows.

PRL

During development of the mammary gland and later on during onset and control of lactation, PRL is an essential hormone (Flint & Gardner, 1994; Tucker *et al*., 2000). This peptide hormone is first and foremost synthesized in the anterior pituitary but some other tissues, such as the epithelial cells of the mammary gland, are also able to synthesize PRL (Freeman *et al*., 2000). PRL has been reported to have anti apoptotic effects on the mammary gland (Accorsi *et al*., 2002). *In vitro* studies clearly show that PRL is involved in survival of epithelial cells in the mammary gland and stimulate synthesis of milk components such as casein (Goodman *et al*., 1983), PRL also affects epithelial cell proliferation and differentiation (Olazabal *et al*., 2000). Among most mammals, suppression of PRL inhibits milk production (Flint & Gardner, 1994).

Evidence regarding the PRL involvement and relationship with the effects of LDPP are conflicting and of discussion as contradictory results have been reported (Lacasse *et al*., 2011). Several studies have been conducted with attempts to measure the effect on milk production by administration of exogenous PRL or by suppressing PRL. In a study by Plaut *et al.* (1987), 8 high producing dairy cows were injected with PRL postpartum, but no effect on established lactation could be detected prior to, or post peak lactation. In a study by Lacasse *et al*. (2011) 5 dairy cows in early lactation were injected with the PRL-releasing inhibitor quinagolide. Quinagolide decreased milk production but only milking-induced PRL was affected, not basal PRL levels, indicating the reduction in yield

can appear even in the absence of effects on basal PRL (Lacasse *et al*., 2011). In addition, administration postpartum of another PRL releasing inhibitor, bromocripine, reduced milk production in the subsequent lactation (Akers, 2002). Milking induced release of PRL could be a mediator of persistency of the ongoing lactation by stimulating maintained cell differentiation and limiting loss of secretory cells (Lacasse *et al*., 2011). Bernier-Dodier *et al.* (2010) milked two quarters of the same udder of dairy cows one time and the other two quarters three times per day and saw elevated gene expression of PRL receptors in the udder quarters milked more frequent. As PRL appears to have direct effects on the mammary gland, and milking frequency has been shown to affect both the number and isoforms of PRL receptors, PRL is stated to be galactopoietic in dairy cows (Lacasse *et al*., 2012).

PRL has been suggested to be the endocrine mechanism behind the effects on milk yield seen in LDPP (Dahl *et al*., 2012). The photoperiod influences the release of PRL (Freeman *et al*., 2000), increasing in response to LDPP and decreasing during SDPP (Miller *et al*., 2000). Both milking induced and basal levels of PRL increase as a response to decreased levels of melatonin (Dahl *et al*., 2012). PRL receptors expressed in mammary tissue have a reversed relationship to PRL concentrations in cows exposed to various photoperiods during the dry period (Auchtung *et al*., 2005). SDPP during the dry period in combination with LDPP during the subsequent lactation increases not only milk yield but also the expression of PRL receptors (Auchtung *et al*., 2005). The elevated milk yield observed, might be a result of the increased sensitivity to PRL during establishment of lactation (Auchtung *et al*., 2005). The ability to respond to circulating PRL, the PRL sensitivity, may be associated with the ability to increase milk yield, especially during the transition to lactation (Auchtung *et al*., 2005). Dahl *et al.* (2012) concludes that PRL is the factor mediating endocrine effects with regard to photoperiod.

IGF-1

Growth hormone (GH) is in control of growth and metabolism in the dairy cow (Sjaastad *et al*., 2003), mRNA for GH receptors is found in all stages of lactation but GH binding to mammary tissue has not been identified (Plath-Gabler *et al*., 2001). The effect of GH on lactation is discussed to be only partly direct and instead mediated by IGF-1 (Abribat *et al*., 1990; Svennersten-Sjaunja & Olsson, 2005). Receptors for IGF-1 can be found in all mammary cells and release of IGF-1 is stimulated by binding of GH to hepatocytes in the liver (Tucker, 2000), which is the main source of circulating IGF-1 (Akers, R. M, 2002). As there are some arguments against PRL being the regulating mechanism behind the galactopoietic effect of photoperiod, IGF-1 has been pointed out as another possible candidate (Dahl *et al*., 2000). The concentration of IGF-1 can be seen as an indicator of the physiological state of the dairy cow (Taylor *et al.,* 2004). Levels of IGF-1 are lower at the start of lactation and increase during the whole lactation, inversely related to milk yield (Abribat *et al*., 1990) which can be seen as contradictory if IGF-1 is the mediating factor behind higher milk yield.

The effect of LDPP on lactation is suggested to be mediated by increased levels of IGF-I as observed increases in circulating IGF-I seems to act independently to changes in

levels of circulating GH or GH-receptors (Dahl *et al*., 1997; Kendall *et al*., 2003). Spicer *et al.* (2007) held prepubertal heifers during 4 months in different photoperiods and saw that long days increased circulating IGF-I. The heifers that were held in 16 hours of light and 8 hours of darkness had higher circulating concentrations of IGF-I than those held in 8 hours of light and 16 hours of darkness (Spicer *et al*., 2007). Dahl *et al.* (1997) investigated the hypothesis that the increased levels of IGF-I, occurring from long days, were connected to the galactopoietic effect of long days and used 39 lactating cows that were exposed to either 18 h of light and 6 h of darkness (LDPP) or a natural photoperiod (approx. 13 h of light per day). Cows exposed to LDPP had higher levels of circulating IGF-I and produced more milk than those held in the natural photoperiod. The galactopoietic action of IGF-I on mammary tissue has nevertheless been reported inconsistent (Tucker, 2000) but there are evidence supporting the fact that increasing levels of IGF-I is the mechanism behind the response to long days (Dahl *et al*., 2000).

Flint & Knight (1997) argues that the former explanations are too simplified when trying to explain the role of GH and IGF-I. Results from studies on goats and rodents propose that the answer to the galactopoietic effect on long days is due to an interaction between the IGF-I system and PRL (Flint & Knight, 1997; Flint *et al*., 2001). IGF-binding protein-5 (IGFBP-5) is known to have apoptotic effects with regards to the mammary gland and there is an inverse relationship between PRL and IGFBP-5 (Dahl *et al*., 1997). Studies regarding dairy cows and IGFBP-5 are needed (Dahl & Petitclerc, 2003) as long days and increased levels of PRL are believed to result in lower expressions of IGFBP-5 and less effect on mammary cell reduction. The outcome would explain the greater persistency and higher milk production observed (Dahl *et al*., 1997; Dahl & Petitclerc, 2003).

12-hour milking interval

Variations in dairy milk production has been examined in cows milked twice daily in a milking interval of 12 hours (Quist *et al*., 2008). Putnam & Gilmore (1970) found milk yield to be greater at the morning milking compared to the evening milking, similar results has been reported by Gilbert *et al.* (1973). In the study by Gilbert *et al*. (1973), 51 dairy cows were milked with a 12 hour interval during 15 days. Average milk yield obtained at the morning milking was 0.65 kg ± 0.05 greater compared to evening milking, 73.8 percent of the cows had a higher milk yield at the morning milking. There was not a strict 12 hour milking interval in a study by Quist *et al*. (2008) but all of the 14 farms that had twice daily milking had higher milk production at the morning milking. Milking interval was not accounted for in the statistical model in the study by Quist *et al*. (2008). Natural diurnal variation has been discussed as the underlying mechanism behind higher milk yield at the morning milking when milking intervals are equal (Gilbert *et al*., 1973). In studies where a 12-hour milking intervals has been examined, sleep has not been a factor that has been considered when evaluating the results.

Sleep

Sleep may be divided in to two sleep stages, rapid eye movement (REM) and non-rapid eye movement (NREM) sleep (Staunton, 2005). Sleeping is a state of changed metabolism, the characteristics of NREM sleep is decreased muscle- and metabolic

activity (Åkerstedt & Nilsson, 2003) and sleep in the NREM state is providing rest for the physiological functions of the whole system of the body (Siegel, 2005). Deprivation of sleep affects the immune response and the relationship between health and immunity is proven (Bryant *et al*., 2004). REM sleep can be defined as lack of muscle activity and a basically awake brain (Siegel, 2005). The awake state of the dairy cow can be divided into alert wakefulness and drowsiness where drowsiness is a state in between awake and asleep. One third of the awake time is estimated to be drowsiness, cows can ruminate both awake and when drowsing (Ruckebusch, 1972).

Ruckebusch (1972) quantified that cows spent most of the day and night-time awake and that the awake state was dominated by drowsiness, especially during night-time. According to Ruckebusch (1972), the dairy cow enters a number of REM sleep bouts, at different periods during a 24 hour cycle and the total amount of sleep is approximately four hours during that cycle. Nilsson (2011) found the overall amount of sleep to be 3.5 hours per 24 hours and concluded that cows sleep more during the night. Research concerning total sleeping time per day among dairy cows has been performed by Ruckebusch (1972) and recently by Nilsson (2011), but intensity of light was not specified in any of those studies. How different intensities and how amount of light affect sleeping among dairy cows is not understood and needs to be investigated further.

Material and Method

Animals and experimental design

The trial was carried out at the Swedish Livestock Research Centre in Uppsala during February and March 2012. The experimental design and the handling of all animals were approved by Uppsala Ethical Committee. Five dairy cows of the Swedish Red breed were used in the trial. The cows were in their first lactation and from previous milking systems of VMS (Voluntary Milking System) and AMR (Automatic Milking Rotary). The AMR had a milking interval of 12 hours. The trial was four weeks long divided into two different light programs (Table 1). The cows were divided in two groups, group 1 and group 2, with two cows in group 1 (Cow ID 1557, 1558) and three cows in group 2 (Cow ID 1545, 1563, 1565). During the start of the trial at week 1, the cows were 191 ± 3.6 DIM. The experiment was designed as a cross-over design, both groups of cows were in the experiment at two separated periods of five days, where each period involved one of the light programs L24:D0 or L4:D20.

Table 1. Week schedule for each group of cows and light program, L24:D0 and L4:D20. The length of the pause was one week

	L24:D0	L4:D20
Week 1	Group 1	
Week 2		Group 2
Pause		
Week 3		Group 1
Week 4	Group 2	

The light program of L24:D0 consisted of 24 hours of continuous lighting, the light program of L4:D20 consisted of four hours of light and 20 hours of darkness. The four hour period of light was in L4:D20 mostly due to practical reasons, for example the catheterization. Each group of cows had a two week pause between the different treatment periods. One trial week consisted of five days (approximately 100 hours) divided into an acclimatization period (approximately 60 hours) and the actual trial period of approximately 40 hours. Milk samples were collected at each milking (twice daily) during the five day period of each light treatment. During the 40-hour trial period, recordings of sleep were performed and blood samples collected.

Housing

The cows were kept in single pens with rubber mat and bedding consisting of peat and short chopped straw. To get the cows adapted to the environment and the light programs, the cows were moved to the pens the night before the start of the acclimatization period. The halter and udder holder for later attachments of the EEG device was put on when the cows were moved to the pen at the start of the acclimatization period. The cows were fed silage *ad libitum,* and concentrate according

to Swedish recommendations (Spörndly, 2003) four times per day at approximately 05:00, 11:00, 17:00 and 23:00. The rectal temperature of the cows was measured after every feeding. Water and salt lick were accessible at all times during the trial. The pens were cleaned prior to every milking as well as when the cows were fed. The first days after each trial period, the cows were housed in single pens and skin zones previously exposed to electrodes were carefully examined and temperature was checked for.

Data recording

Milking

Bucket milking was performed at quarter level by using a custom designed milking machine (provided by DeLaval International AB, Tumba, Sweden) with monovac, pulsation ratio 70/30, pulsation rate 60 cycles per minute and system vacuum 39 kPa. Milking day 1 and 2 the first week was, however, done by ordinary bucket milking. The cows were milked twice daily with 12-hour intervals, in total nine milkings per trial week. Morning milking started at 05:15 and evening milking at 17:15, milking and blood sampling took a total of 60 minutes per cow. In order to maintain a 12 hour milking interval cow number two was milked at 06:15 and 18:15 etc. To obtain a good milk ejection (Bruckmaier & Blum, 1998), concentrate was given to the cows during pre-stimulation. Pre-stimulation consisted of cleaning and stripping of fore-milk according to a set schedule.

Light treatments

The hours of light in the light program L4:D20 took place between 9:30 and 13:30. The light intensity at cow eye level during the dark time was less than 3 lux in all pens. However, during the light time in both programs light intensity differed between the pens; pen number 1 was exposed to 210 lux, pen number 2 to 410 lux and pen number 3 to 70 lux. Two red LED lamp lightchains (Konstsmide, DE-60-24W), 10 meters, were placed on the wall above the pens and above the area outside the pens to be able to work without additional light around the cows during the L4:D20 treatment.

Sleep

Non-invasive electrophysiological recordings were performed by electroencephalogram (EEG), electro-ocularography (EOG) and electromyography (EMG). The recordings were collected via a portable recording device (Embla titanium, Embla Systems, Broomfield, USA). Areas for electrode attachments were trimmed and shaved. Ten surface electrodes (Unilect, Unomedical Ltd, Stonehouse, Great Britain) were placed on the head of the cow with tissue adhesive (3M Vetbond, 3M Animal Care Products, St. Paul, USA) prior to the evening milking on day 3.

Blood samples

To collect the blood samples without disturbing the cows during periods of sleep, a permanent catheter was placed in the jugular vein of the cows after the morning milking on the second day in each trial week. Prior to the catheter surgery, areas for tube stitches on the neck of the cow were trimmed and shaved. The shaved areas were washed with soap and lukewarm water. Before the cows were moved to the fixation stall

the shaved areas were soaked with Jodopax. Placed in the fixation stall, the cows were sedated with Narcoxyl and the shaved areas prepared with 70 % ethanol. Moreover, at the site of catheterization, Lidokain was subcutaneously administrated before the incision was made to insert the catheter. The silicone extension tube (120-150 cm, volume 2-3 ml) of the permanent catheter was secured with stitches on the site of insertion and on two places on the neck. Stitches and extension tube was covered with bandaging tape (Vetrap, 3M, USA) during the whole trial. The cows were monitored 24 hours per day after the catheterization. The catheter was removed after the morning milking on the last day of the trial week, the total time as catheterized was approximately 70 hours per trial period.

Data collection

Milk yield on quarter level was recorded at every milking and representative samples of composite milk from each cow was preserved and refrigerated at 4°C in tubes prepared with 10 % bronopol, 2-bromo-2-nitropropane-1.3-diol (VWR International AB, Stockholm, Sweden). Evaluation of milk lactose, total protein and fat content was performed using mid-infrared spectroscopy (Fourier Transform instrument, FT 120, Foss, Hillerød, Denmark), SCC was analysed using fluorescence-based cell counting (Fossomatic 5000, Foss, Hillerød, Denmark) at Kungsängen Research Center, SLU, Uppsala. All milkings that occurred during the trial period were performed by the same person and a standardized milking procedure was utilized. One minute of cleaning and pre stimulation was followed by 30 seconds of hand milking before first teatcup was attached. Each teat had an individual milk flow meter and each teat cup was removed individually when the milk flow had decreased to 0.3 kg/min. Teat disinfectant was sprayed on the teats directly after completed milking.

The sleep recording started at day 3 prior to the start of milking at 17:15 and the technical device was connected to a lap top equipped with software for sleep registration (RemLogic 2.0.1, Embla, Systems, Broomfield, USA). The recording devices and the laptops were connected wireless by Bluetooth, enabling online sleep scoring. All raw data from the sleep recordings was saved after the recording sessions. The sleep registrations were ended after the morning milking on day 5 and all equipment was taken off the cows immediately.

During milking, blood samples was collected at -15, -5, -2, 0, 1, 2, 3, 5, 7, 9, 11, 13, 15, 20 and 30 minutes before and after the start of milking at time 0. Blood samples were also collected once every hour and after a period of three minutes of REM- or NREM-sleep. Samples collected in the context of sleep did not exceed more than ten samples per cow and trial period. Blood samples were collected with vacuum S-Monovette System® technique, into 9 ml Li-Heparin tubes. Heparinized saline, 10 ml, was injected afterwards to prevent clogging of blood in the extension tube. If the time in-between blood samples was three minutes or less, only saline was injected. Each blood sample was immediately placed on ice and then centrifuged (4500 rpm for 5 minutes in 4 °C) within 30 minutes of collection. Plasma was collected and stored at -20 °C until analyzed. A total of 5-10 ml of fluid from the extension tube was discarded before the blood sample was collected in

order to get a representative sample. The plasma samples were accidently thawed once and then refrozen, at -20 °C, before the analysis of IGF-1 and PRL started.

Each pen had camera surveillance from day 2 every trial week, as the cows were catheterized. During L4:D20, additional infrared lamps were used to support the cameras with clear pictures for monitoring. The cameras were linked to laptops, with the software MSH Video Client, in a nearby room enabling surveillance without disturbing the cows. The cows were placed in the same pen during both light treatments. The light intensity varied between the different pens during L24:D0, the intensity of the artificial light ranged from 70 to 210 lux due to uneven placement of light sources. There was no difference during L4:D20 as all pens received the same amount of light, <3 lux.

Data analysis

Scoring and evaluation of different vigilance states was performed by Emma Ternman and Lisa Andersson, for more detailed explanation see Andersson (2012). Analysis of blood samples for IGF-1 and PRL were conducted at Universitat Autònoma de Barcelona in May 2012. A solid-phase, enzyme-labeled chemiluminescence immunometric assay of the plasma samples of IGF-1 was performed by IMMULITE® (Siemens Healthcare Diagnostics, Los Angeles, USA). The plasma samples were pretreated with acidic solution to release IGF-1 from the binding proteins. Analysis of plasma PRL samples was made with an ELISA Kit (MyBioSource, San Diego, California, USA). The statistical analysis was performed by using PROC MIXED model in SAS (SAS, 2003). A level of 95 % of significance was used in the model of analysis and non-significant posts were removed from the model. The fixed effects of total resting time (TRT; REM, NREM and drowsing), feed, pen and week were included in all models. A random effect of cow was also included. In the model for milk composition, milk yield was included.

Results

There was a change in roughage during the study, but the feed intake among the cows appeared unaffected.

One cow (1563) was excluded from the data set when PRL was analyzed because of plasma values out of range.

Milk yield

The difference in milk yield was greater in-between cows, independently of light program, rather than in-between the different light programs for each cow (Figure 1). No significant differences in milk yield were found between the different light programs (Figure 2). The fixed effect of total resting time (TRT) was significant in the statistical model for milk yield (P=0.026). There were no significant differences in milk yield between evening and morning milkings in any of the light programs (Figure 3).

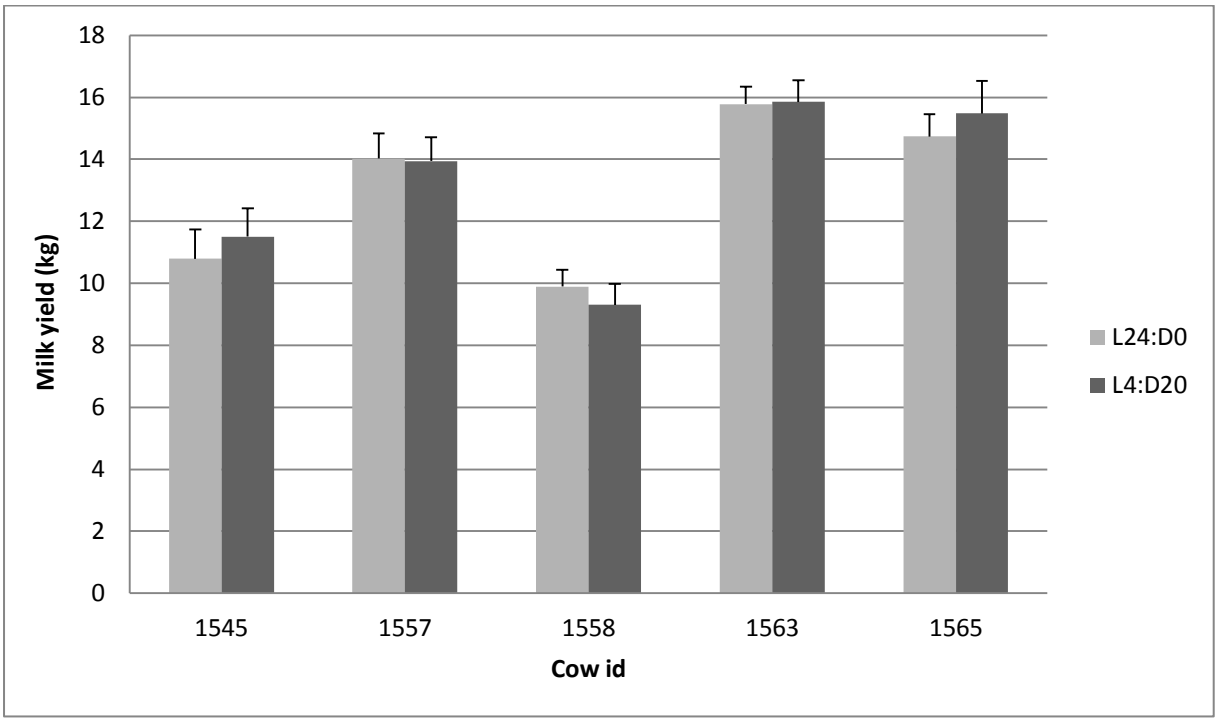

Figure 1. Milk yield from five cows during two different light programs, 24 hours of light (L24:D0), four hours of light and 20 hours of darkness (L4:D20). Error bars display standard error of the mean.

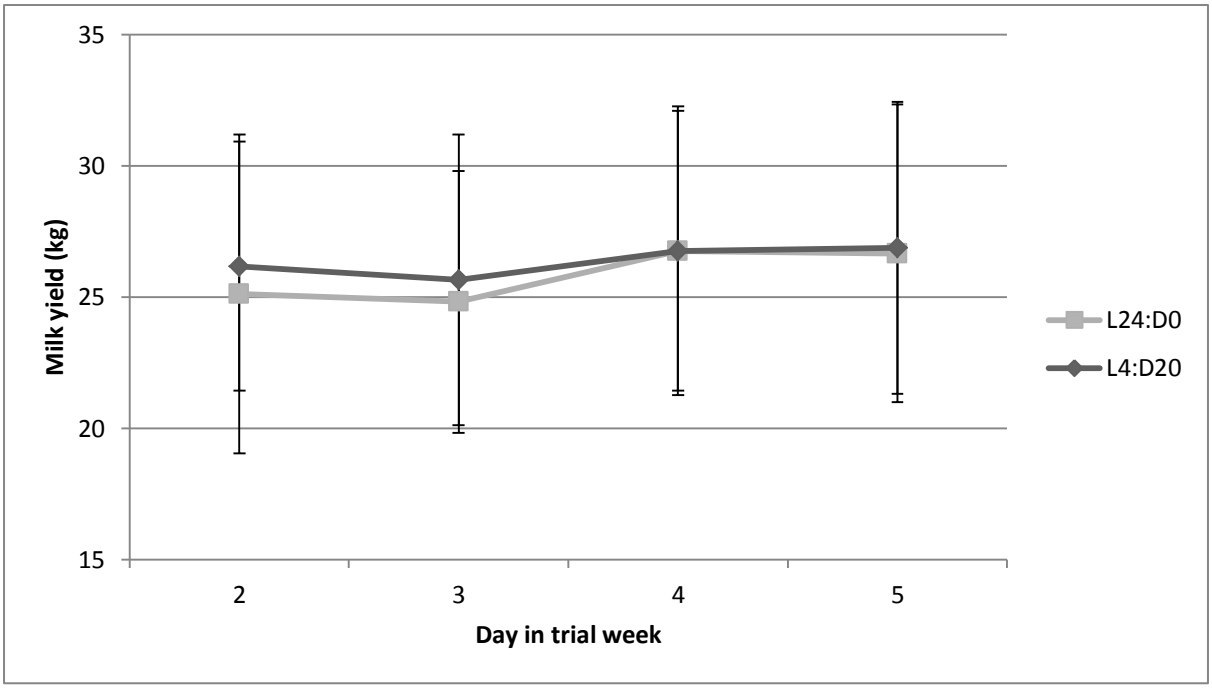

Figure 2. Mean values of milk yield from five cows during two light treatments, 24 hours of light (L24:D0), four hours of light and 20 hours of darkness (L4:D20) displayed per day of the trial week of five days. Day one is removed from the data set because of only one evening milking. Error bars display standard error of the mean.

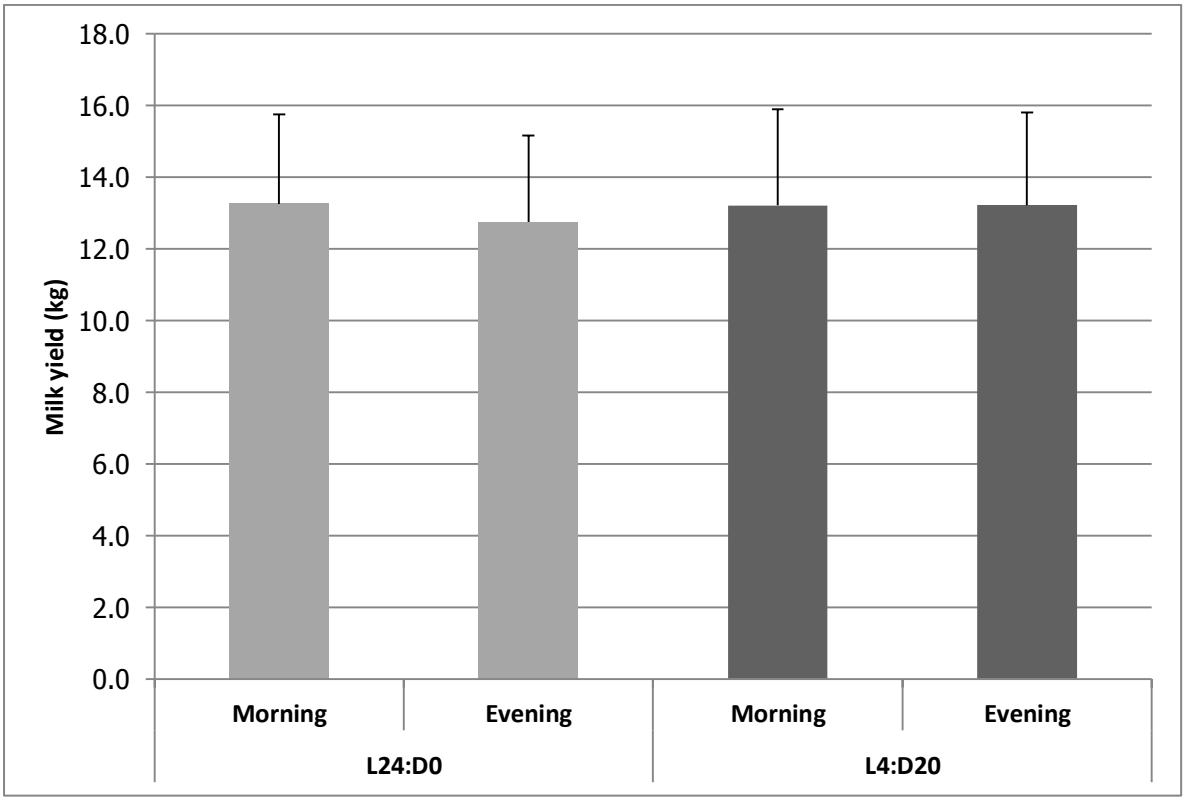

Figure 3. Milk yield from five cows during five days in two different light programs, 24 hours of light (L24:D0), four hours of light and 20 hours of darkness (L4:D20), divided into morning and evening yield. Error bars display standard error of the mean.

Milk composition

No significant differences in milk composition (fat, lactose and protein) were found between light programs (Figure 4).

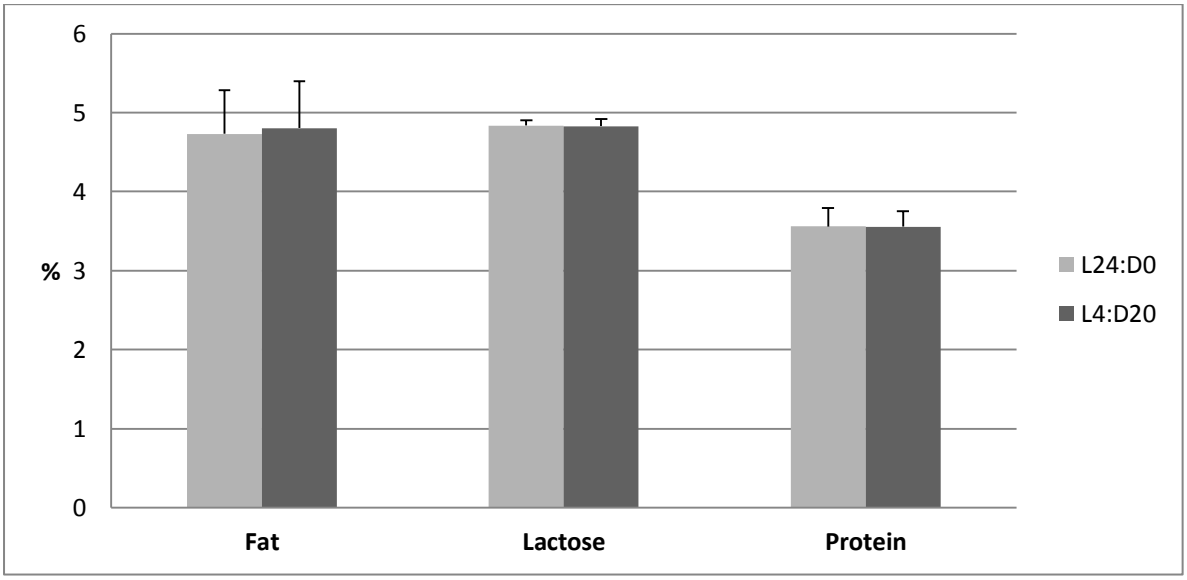

Figure 4. Mean values of fat, lactose, protein content in milk from five cows in two different light programs, 24 hours of light (L24:D0), four hours of light and 20 hours of darkness (L4:D20). Error bars display standard error of the mean.

Sleep

The amount of sleep during the last 36 hours for the cows in the study was less than 5 % REM and less than 10 % NREM in both light treatments (Figure 5). There were great individual differences in amount of sleep among the cows.

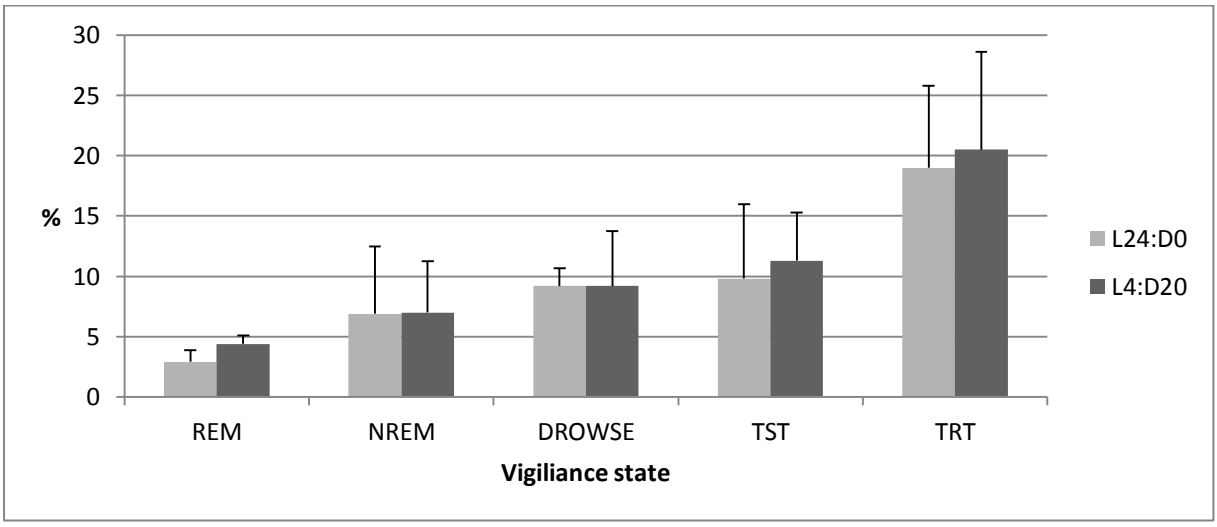

Figure 5. Percentage of different states of vigilance during the last 36 hours of each light program, 24 hours of light (L24:D0), four hours of light and 20 hours of darkness (L4:D20). TST=REM+NREM. TRT=REM+NREM+DROWSE. Error bars display standard error of the mean.

PRL

Over all, there were no significant differences in plasma levels of PRL between the two light programs (Figure 6). After the start of milking, significant differences were found at time +1 min (P=0.016), at +11 min (P=0.049) and there was a tendency for difference at +30 min (P=0.053).

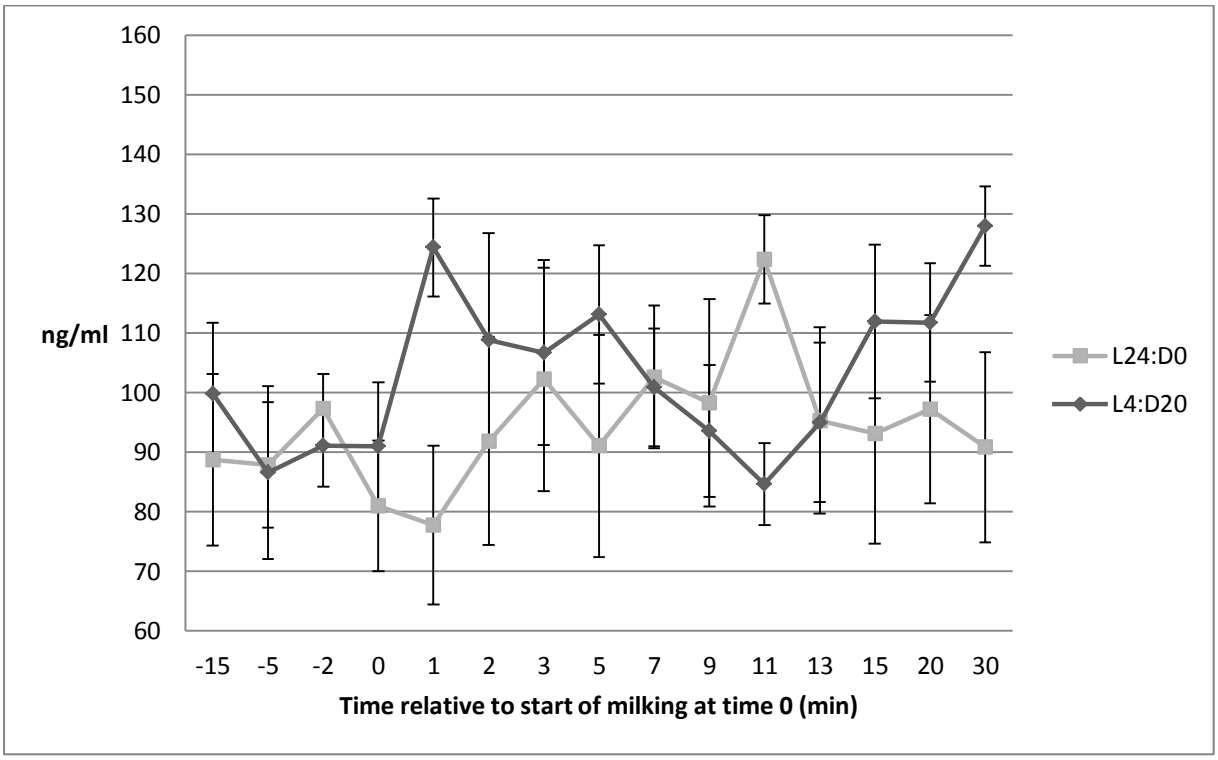

Figure 6. Mean values of plasma PRL from four cows during two milkings during the last 36 hours of a trial week during two light program, 24 hours of light (L24:D0), four hours of light and 20 hours of darkness (L4:D20). Error bars display standard error of the mean.

IGF-1

There was a significant difference (P = 0.024) in plasma levels of IGF-1 between the two light programs as IGF-1 levels were higher during L4:D20 than during L24:d0 (133±11.6 ng/ml and 124±19.5 ng/ml respectively). Numbers of observations were slightly higher for L4:D20 (n=70) compared to L24:D0 (n=51). Similar levels of IGF-1 were found during the different vigilance states.

Discussion

Milk yield and milk composition

Manipulation of the daily photoperiod is associated with possibilities to increase milk yield and is frequently used by modern dairy producers worldwide (Dahl *et al*., 2000). There were no significant differences in milk yield between L24:D0 and L4:D20 in this study, some cows had small numerical differences in milk yield between the two photoperiods. Continuous lighting has not been reported to be associated with an increase in milk yield (Marcek & Swanson, 1984) and the results of this study are in agreement with their findings.

The small negative trend in milk yield between day two and three might be an effect of the catheterization, the cows were sedated and the event can be assumed to be somewhat stressful for the cows (Figure 2). Also, the changed environment and housing for the cows might have affected their milk yield. The development of the milk yield curve of each light program would be interesting to follow during a longer period as the curve had an overall slight positive slope during the trial week, overlooking the drop at day three. Day one was excluded from the figure as only one milking was performed.

The results of this study, concerning milk yield and milk composition, are based on milk collected from all five days during a trial week in each photoperiod. The acclimatization period to each of the photoperiods (L24:D0 or L4:D20) was only three days long and might have been too short to give significant differences. According to Dahl & Petitclerc (2003), responsiveness to light programs might take 3-4 weeks. This would indicate that the results of this study might not be the effects of the different photoperiods the cows where held in.

Several studies have shown that when milking intervals are equal, morning milking tends to result in higher yields (Putnam & Gilmore, 1970; Gilbert *et al*., 1973). The low number of cows (n=5) along with individual differences in milk yield (Figure 1) might have affected the result as no significant difference was found in milk yield between morning- and evening milking (Figure 3). The underlying mechanism behind differences in milk yield between morning- and evening milkings has been hypothesized to derive from natural diurnal variations (Quist *et al*, 2008). Above the diurnal variations, there might be an effect of sleep that is not understood yet as no studies have measured sleep associated with milk yield among dairy cows. In the statistical model for milk yield, TRT was included as a fixed effect and was found significant (P= 0.026)which might indicate the importance of sleep for milk production.

There were no significant differences in milk composition (Figure 4) between L24:D0 and L4:D20 which is in agreement with Dahl *et al*. (2000). Decreased fat content has been reported during LDPP studies (Dahl *et al*., 2000) but was not found in this study.

Sleep

There was a difficulty in keeping the recording equipment in place since the cows in periods were frustrated and scratched off the electrodes. The frustration might have reduced the amount of time spent lying down which in its turn might have affected the amount of sleep achieved among the cows. The plasma samples obtained after periods of sleep might also have reduced the overall sleep time among the cows in the study. This since the general impression was that they were disturbed by the measurements and from activity in the pens next to them.

There were minor variations between the two light programs in time spent in the different vigilance stages (Figure 5). Cows tend to sleep more during the night compared to day time (Nilsson, 2011) which might imply that darkness is a factor associated with sleep among dairy cows. L24:D0 did not provide a dark phase which according to Buchanan *et al*. (1992) cows might need, as a period of darkness keeps track of day length.

PRL

Photoperiod influences the release of PRL in dairy cows (Freeman *et al*., 2000) which has, along with IGF-1, been suggested to be the regulating mechanism behind the galactopoietic response of LDPP (Dahl *et al*., 2012). In this study, PRL plasma levels differed during milking with fluctuating curves (Figure 6). There were apparent differences during certain times but levels of plasma PRL did not differ significantly between the two light programs. The result might have been affected by the short time in each light program or by the limited time period for measurements, and the low number of plasma samples (n=60) per cow. A more uniform and describing pattern for a period of 24 hours would be preferable when discussing what role PRL might have during different photoperiods.

From -15 min until the start of milking, both light programs followed each other in a similar pattern and values did not differ greatly. At the start of milking, the curves diverged with a peak value in L4:D20 whereas L24:D0 reached the lowest value. This might indicate a difference in response of the milking-induced released of PRL at the start of the milking at time 0 and that basal levels of PRL probably did not differ between light programs.

At +1 min after the start of milking, PRL levels were significantly different with a high value in L4:D20 and with a low value for L24:D0. The milking induced response might have been a bit delayed in L24:D0 as there was a peak but it came after 11 min of milking. These differences in response might possibly derive from differences within each individual cow and cannot be seen as an effect of photoperiod. These results are in agreement with Peters *et al*. (1981) who found that milking induced release of PRL was not affected by photoperiod.

At time +11 min after the start of milking, values differ significantly again but in a reversed pattern and when the sampling was ended at +30 min there was a tendency

(P=0.053) for a significant difference again. The significant differences between the two light programs at certain points during the milking are most likely not reflections of parameters associated with photoperiod. The differences might be associated with other events around the milking or individual differences in response among the cows. The three measurements before onset of milking could give an indication of the basal PRL levels for the cows in this study which indicate similar basal levels in both light programs. Absence of darkness leads to decreasing melatonin levels (Stanisiewski *et al*., 1988) and both milking induced and basal levels of PRL increase as a response to the changed melatonin level (Dahl *et al*., 2012). In order to look into this further, longer duration of measurements needs to be performed and not only be done around milking.

IGF-1

IGF-1 has been suggested to be the regulatory mechanism behind the galactopoietic effects seen in photoperiod manipulation (Dahl *et al*., 2000) and cows held in LDPP has been associated to elevated levels of plasma IGF-1. The literature is however inconsistent (Tucker, 2000) and our results did not follow the expected pattern as levels of IGF-1 in L24:D0 were significantly lower (P=0.024) compared to L4:D20. The exposure of continuous lighting, lacking a dark period, might have had a negative effect on levels of IGF-1 in this study. The optimal time period for adaptation might be longer than the three days of acclimatization that was used in this trial.

The immune system activity increases during sleep (Åkerstedt & Nilsson, 2003) but no differences in plasma levels of IGF-1 could be detected between any of the sleep stages in this study (Figure 5). Standard deviations were large among all vigilance states and with this large variation it is difficult to comment if any of the vigilance states are connected to IGF-1. Studies with longer duration and equal numbers of samples in each vigilance state would provide more information needed for stating the importance of sleep for the dairy cow with regard to IGF-1.

There were large variations in number of observations in each vigilance state, ranging from 9 observations of drowsing to 63 observations of awake. The large range might have affected the result and as no previous study has investigated how sleep and IGF-1 is connected there is no literature that can support the results. Due to the small number of individuals and the relatively short duration of time of each light program, the results should be interpreted carefully.

Conclusions

The different photoperiods of this study (L24:D0/L4:D20) did not have an effect on milk yield in a 12-hour milking interval. In addition, there were no differences between morning- and evening milk yield or any differences in milk composition. Plasma PRL did not differ between photoperiods in this study (L24:D0/L4:D20). There was a significant difference in plasma levels of IGF-1 where cows had higher levels in L4:D20. There were no significant variations in plasma IGF-1 between the different vigilance states. The link

between sleep, light and milk formation needs to be further investigated as the physiological functions still are poorly understood.

Acknowledgement

During this study, milking was performed by use of a custom-built milking machine (the Octupus) from DeLaval, great thanks for assistance and service during the weeks of milking.

I would like to thank my supervisor Sabine Ferneborg for all her help and for the wise answers to all of my questions, I also want to thank my examiner Kjell Holtenius.

In addition, I want to thank the staff and people working at Universitat Autònoma de Barcelona, and Vilhelm Ektander for great support and endless patience.

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