Lehrstuhl für Physiologie
Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt
Technische Universität München

Importance of light and of the serotonin-melatonin-system on neurophysiology of milk synthesis and ejection in dairy cows

Maria Theresia Kollmann

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Agrarwissenschaften

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. L. Dempfle
Prüfer der Dissertation:	1. Univ.-Prof. Dr. H.H.D. Meyer
	2. Univ.-Prof. Dr. R.M. Bruckmaier, (Universität Bern, Schweiz)

Die Dissertation wurde am 03.07.2007 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 30.07.2007 angenommen.

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ABBREVIATIONS

AFR	average flow rate
AUC	area under the curve
bST	bovine somatotropin
BW	body weight
C	control treatment, performed without additional lighting
cAMP	cyclic adenosine mono phosphate
CLA	conjugated linolic acid
D-form	dextro-form
CREB	cAMP response element binding protein
d 0	day before tryptophan supplementation
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immuno sorbent assay
5-HT	serotonin (5-hydroxytryptamine)
5-HTP	5-hydroxytryptophan
GH	growth hormone
IGF-I	insulin-like growth factor I
L-form	levo-form
MJ	megajoule
NEFA	nonesterified fatty acid
NEL	netto energy lactation
NOEL	non observable effect level
NPN	non protein nitrogen
nXP	utilizable crude protein
PFR	peak flow rate
RIA	radioimmunoassay
REM	rapid eye movement
SE	standard error
tPFR	time to reach peak flow rate
UDP	undegraded protein
UV	ultraviolet light treatment, performed 10 min before milking and during milking
UVIR	ultraviolet and infrared light treatment, performed 10 min before milking and during milking

UVMO	ultraviolet light treatment, performed only during milking, not before
vs.	versus

1 ABSTRACT

The serotonin-melatonin-system regulates many functions in the vertebrate body. Mood, anxiety, sleep-wake-rhythm, body temperature, immune-response and many more functions are under its control. Milk synthesis and ejection related hormones like oxytocin and prolactin were also reported to be affected by the system in rats. Which component of the system is the mediator of the effect and if it operates inhibitory or stimulatory is not completely clear. A deviation of the effect in cows from those in rats is supposable because of their different activity period (diurnal vs. nocturnal).

The aim of the study was to influence the serotonin-melatonin-system and thus to investigate its function in the regulation of milk synthesis and ejection in dairy cows. Furthermore the influence of blood melatonin concentration on the melatonin concentration in milk and its transport mechanism was determined.

With the supplementation of rumen-protected tryptophan the serotonin-melatonin-system in heifers and dairy cows was tried to be modified. Tryptophan plasma concentration increased in heifers and dairy cows as an effect of supplementation. In heifers melatonin concentration increased, too, but not in dairy cows, what could derive from the high demand of tryptophan for milk protein synthesis in dairy cows.

With additional artificial sunlight at milking time, a faster decrease of the nightly elevated melatonin was observed in its extent dependent on the light spectrum and the duration of illumination. The suppression of melatonin was faster with the wider light spectrum, and higher after the longer duration of illumination.

The only method that influenced the concentration of plasma prolactin was the change of the seasonal photoperiod; the others had no effect, neither during milking nor during continuous measuring for 24 h.

Oxytocin did neither increase with tryptophan supplementation nor a difference between the seasons (summer and winter) was observed, but increased after illumination with artificial sunlight, whereas it was only a short-term effect. With longer illumination oxytocin already started to decrease, thus it seems to be mediated by the light off-on sequence.

Cortisol was not affected by a variation of photoperiod and illumination with artificial sunlight. Different cortisol levels were observed at morning and evening

milking under natural conditions, but did not exist when lighting with artificial sunlight around milking was performed.

With no treatment total milk yield could be increased. Most likely, the milking conditions were optimal in our experiments and oxytocin was not limiting. Under suboptimal conditions in stressed cows with reduced oxytocin release, illumination with artificial sunlight during milking could possibly act as a management tool to increase oxytocin level and attenuate milk ejection inhibition and increase the reduced milk yield. With a variation of the photoperiod or supplementation of rumen-protected tryptophan this seems not to be possible.

In bovine milk melatonin was also detected and showed a diurnal rhythm similar to that in blood. The correlations, the regression and the amphiphilic character of melatonin let us conclude that melatonin diffuses from bloodstream into milk. The amount of melatonin in milk was low, i.e. in the range of 10^{-12} g/ml. With the used methods melatonin in milk of twice daily milking could not be influenced. Fractionized milk sampling (foremilk, main milk fraction and residual milk) did also not influence the melatonin concentration in milk.

2 ZUSAMMENFASSUNG

Das Serotonin-Melatonin-System regelt viele Funktionen im Körper von Wirbeltieren. Die Stimmung, die Angst, der Schlaf-Wach Rhythmus, die Körpertemperatur, die Immunantwort und viele andere Funktionen sind unter dessen Kontrolle. Es wurde bei Ratten berichtet, dass milchsynthese- und milchejektionsabhängige Hormone wie Ocytocin und Prolaktin von diesem System ebenso beeinflusst werden. Welcher Bestandteil des Systems der Vermittler des Effekts ist, und ob er hemmend oder stimulierend wirkt, ist bis jetzt nicht vollständig geklärt. Eine Abweichung des Effekts in Ratten von dem in Kühen ist zudem denkbar wegen ihrer unterschiedlichen Aktivitätsphasen (nachtaktiv vs. tagaktiv).

Das Ziel dieser Untersuchung war, das Serotonin-Melatonin-System zu beeinflussen und seinen Effekt auf die Milchsynthese und Milchejektion bei Milchkühen zu erforschen. Weiterhin wurde der Einfluss der Melatoninkonzentration im Blut auf die Melatoninkonzentration in der Milch und sein Transportmechanismus untersucht.

Mit der Ergänzung von pansengeschütztem Tryptophan wurde versucht das Serotonin-Melatonin-System bei Färsen und Milchkühen zu beeinflussen. Die Plasmatryptophankonzentration stieg bei den Färsen und bei Milchkühen als Folge der Ergänzung an. Bei den Färsen stieg die Melatoninkonzentration ebenso an, aber nicht bei den Milchkühen, was auf den hohen Bedarf an Tryptophan für die Milchproteinsynthese bei den Milchkühen zurückzuführen sein könnte.

Mit zusätzlichem künstlichem Sonnenlicht rund um den Melkvorgang wurde ein schnellerer Abfall des während der Nacht erhöhten Melatonins beobachtet, der in seinem Ausmaß vom Lichtspektrum und von der Dauer der Beleuchtung abhängig ist. Die Unterdrückung des Melatonins war schneller mit einem breiteren Lichtspektrum und stärker bei längerer Beleuchtungsdauer.

Die einzige Methode, die die Plasmakonzentrationen von Prolaktin beeinflusste, war die Veränderung der saisonalen Photoperiode; die anderen zeigten keinen Effekt, weder während des Melkens noch während wiederholter Messungen über 24 h.

Ocytocin stieg weder mit der Methode der Tryptophanergänzung an, noch zeigte sich ein Unterschied zwischen den Jahreszeiten (Sommer und Winter), aber es stieg nach einer Beleuchtung mit künstlichem Sonnenlicht an, wobei dies lediglich

ein Kurzzeiteffekt war. Mit verlängerter Beleuchtungsdauer sank Ocytocin bereits wieder, folglich scheint es durch den Licht Aus-Ein-Wechsel vermittelt zu werden.
Cortisol wurde nicht durch eine Veränderung der Tageslichtlänge oder durch Beleuchtung mit künstlichem Sonnenlicht beeinflusst. Unterschiedliche Cortisol Niveaus wurden während des Morgen- und des Abendgemelks unter natürlichen Bedingungen beobachtet, bestanden aber nicht, während der Beleuchtung mit künstlichem Sonnenlicht.
Mit keiner Methode konnte die absolute Milchmenge beeinflusst werden. Möglicherweise waren die Melkbedingungen in unserem Experiment optimal und Ocytocin war nicht begrenzend. Unter suboptimalen Bedingungen wie in Stresssituationen, die mit reduzierter Ocytocinausschüttung einhergehen, könnte eine Beleuchtung mit künstlichem Sonnenlicht während des Melkens als Managementwerkzeug dienen, um das Ocytocinniveau anzuheben und die Milchejektionshemmung abzuschwächen und die reduzierte Milchmenge zu erhöhen. Mit einer Veränderung der Tageslichtlänge oder einer Ergänzung mit pansengeschütztem Tryptophan scheint dies nicht möglich zu sein.
In Kuhmilch wurde Melatonin ebenfalls nachgewiesen und zeigte einen Tagesrhythmus ähnlich dem im Blut. Die Korrelationen, die Regression und der amphiphile Charakter von Melatonin lassen den Schluss zu, dass Melatonin vom Blutstrom in die Milch diffundiert. Die Menge des Melatonins in der Milch war niedrig in der Grössenordnung von 10^{-12} g/ml. Mit den benutzen Methoden war Melatonin in der Milch von zweimal täglichem Melken nicht beeinflussbar. Fraktionierte Milchprobennahme (Vormilch, Hauptmilch und Residualmilch) beeinflusste die Konzentration in der Milch nicht.

3 INTRODUCTION

3.1 AMINO ACIDS AND PROTEIN METABOLISM

3.1.1 Amino acid structure and classification

The monomer components of proteins are amino acids. They are connected with peptide bonds to proteins. Amino acids have two functional groups, the carboxy group (-COOH) and the amino group (-NH2). Only 21 of these compounds, called proteinogenic amino acids, appear in proteins. In proteinogenic amino acids the amino group is in α-position to the carboxy group (Kirchgessner, 1997; Jeroch et al., 1999). The form of amino acids is L(levo)-form or D(dextro)-form. A mixture of both which results from technical production, is called racemic mixture. In proteins appear only L-amino acids, because only this form can be used in protein synthesis (Kirchgessner, 1997). The amino acids differ in the side chain which chemical structure is the basis for the classification of amino acids by Koolman and Ropstad (1996). There the amino acids are divided in aliphatic amino acids (glycin, alanin, valin, leucin and isoleucin), acidic amino acids (aspartic acid, glutamic acid), basic amino acids (lysine, arginine), sulfur-containing amino acids (cysteine, cystine, methionine), aromatic amino acids (phenylalanine, tyrosine, tryptophan, histidine), neutral amino acids (serin, threonin, asparagine, glutamine) and in prolin, the imino acid, a special type.

Furthermore amino acids can be classified by its role in nutrition, but this classification is not valid for all species and age groups. The amino acids are divided into essential amino acids, non-essential amino acids and semi-essential amino acids. Essential amino acids are amino acids, which cannot be produced in metabolism, they must be supplied with nutrition. Non-essential amino acids can be produced in metabolism. With transaminating of 2-oxo-acid many amino acids can be synthesized (Kirchgessner, 1997). Semi-essential amino acids can be produced, but not in adequate amounts. They can be limiting for a short period of growth, for high production or permanently. A semi-essential amino acid is arginin in the growing pig and also cysteine and methionine in poultry (Kirchgessner, 1997). In high yielding dairy cows lysine and methionin (Socha et al., 2005; Schwab et al., 1976) or histidine (Vanhatalo et al., 1999) especially in grass silage based diets can be semi-essential for milk protein synthesis (Korhonen et al., 2002).

3.1.2 Protein metabolism in monogastric animals

In monogastric animals proteins are degraded by hydrolytic enzymes, which can be divided in endopeptidases (pepsin, trypsin, chymotrypsin) and exopetidases (carboxypeptidases, aminopeptidases, dipeptidases).

The degradation of proteins begins in the stomach with pepsin and goes further in the intestine with trypsin and chymotrypsin. Endopeptidases split proteins and peptides in the middle of the peptide chain. Where they affect the peptid chain exactly is dependent on the side chain of the peptide and of the endopeptidase. Exopeptidases, which are secreted from the pancreas and the intestine split the peptides from the end of the chain. They act after the endopeptidases and finish the degradation with primarily free amino acids and partially small peptides. The free amino acids are resorbed with an active transport system, that consist mostly of a natrium dependent carrier. The resorbed amino acids are transported along the portal vein to the liver. They are used for synthesis of body protein, for modification to other compounds (creatin, purine, hormones, etc.), for synthesis of specific products (milk, wool, eggs, etc.) for energy, or they are rebuilt by transaminating, desaminating or decarboxylation and excreted. The protein metabolism is in steady turnover. In growing animals protein synthesis is higher as proteolysis whereas in fully-grown animals proteolysis and protein synthesis are balanced.

3.1.3 Protein metabolism in ruminants

Ruminants have in contrast to monogastric animals four stomachs and thus the protein metabolism differs from the one of monogastric animals. In ruminants the protein degradation starts in the rumen. The first step is the degradation of proteins with proteolytic enzymes of the microorganism. The proteins are splitted in peptides and afterwards in amino acids, and mostly they are splitted into ammonia. Microorganism can also use NPN-(non protein nitrogen) compounds for protein turnover. These are for example free amino acids, alkaloids, amides, ammonia salts, betain, cholin, nitrates and purins. Some proteins are leaving the rumen undegraded (undegraded protein=UDP). The degradation of proteins in the intestine is the same as in monogastric animals. The microbal protein together with UDP which enters the small intestine is called utilizable crude protein (nXP).

Because of protein metabolism in rumen, ruminants are not addicted to protein quality of nutrition. Rumen bacteria can synthesize essential amino acids, thus the mixture of amino acids in nutrition are not as important as in monogastric animals. Ammonia, that is not used for synthesis, is absorbed and goes into the rumino-hepatic-cycle.

3.1.4 Amino acid supplementation

In modern monogastric animal nutrition, amino acids are important. To enhance the quality of proteins and reduce the excretion of ammonia, single amino acids are supplemented. In growing pigs a supplementation with lysine, the first-limiting amino acid, can increase the rate of protein synthesis and reduce the ammonia excretion (Salter et al., 1990).

In cattle methionine and lysine are discussed to be first limiting for milk protein synthesis (Socha et al., 2005; Schwab et al., 1976). To supply additional amino acids to cows, two approaches can be used: 1) inclusion of protein sources in the diet that are not degraded in the rumen and that pass to the small intestine or 2) optimization of ruminal fermentation to make extensive use of the microbial protein, thereby increasing the available amino acids for absorption (Overton et al., 1996). For maximal production of milk and milk protein in dairy cows microbial protein synthesis is not sufficient (Piepenbrink et al., 1996) thus dietary escape protein is important. Supplemented amino acids like in monogastric animals would be degraded in the rumen of ruminants but they have to bypass the rumen. With different methods amino acids can be made rumen persistent. One possibility is to coat the amino acid with an insoluble substance, for example with a special “protected” fat coating or with formaldehyde substances. Another possibility is to link the amino acid with less soluble substances, for example to make amino acid-trace element-chelates.

3.2. THE SEROTONIN-MELATONIN SYSTEM

3.2.1 Components of the serotonin-melatonin system

Tryptophan is an essential precursor in the synthesis of serotonin and melatonin. It is an amino acid which is essential for monogastric animals. This approach can

not be used in ruminants because of the extensively metabolisation of amino acids by rumen microbes, they do not have a direct requirement of an amino acid.

Tryptophan circulates in blood as free amino acid by 10-20 % while the remainder is bound to serum albumine. Tryptophan is the only amino acid that binds to a plasma protein (Wurtman and Fernstrom, 1975). The fraction of free tryptophan is dependent on the concentration of nonesterified fatty acids (NEFA), because NEFA also bind to albumin. Therefore with increasing concentration of NEFA the fraction of albumine bound tryptophan decreases and the fraction of free tryptophan increases (Wurtman and Fernstrom, 1975), because NEFA have a higher affinity to albumine than tryptophan (Davis et al., 2000). The transportation of tryptophan into the brain is coupled to a carrier. Thus tryptophan has to share this carrier with six other large neutral amino acids (tyrosine, phenylalanine, leucine, isoleucine, valine and methionine) and its transfer rate into the brain trough the blood brain barrier depends on the concentration of these six amino acids (Fernstrom and Wurtman, 1972; Fernstrom J.D. et al., 1973; Fernstrom and Faller, 1978). Although only free tryptophan can bind to the carrier into brain and pass blood brain barrier, binding to serum albumine is not limiting, indeed it is the opposite, because it acts as storage. After insulin secretion the concentration of amino acids in blood declines, because they are absorbed, whereas tryptophan levels can remain elevated because it is mostly bound to albumin and not affected by insulin (Wurtman and Fernstrom, 1975).

It was reported, that tryptophan concentration in plasma varies during the day. In humans lowest levels were observed at 0200-0400 h and rose by 50-80 % to a plateau in late morning or early afternoon (Wurtman and Fernstrom, 1975). In rats a diurnal rhythm with low levels at 1200 h and high levels at 0000 h was reported (Gutierrez et al., 2003). The variation in tryptophan concentration is not a real circadian rhythm; it rather depends on the eating behaviour of the subjects (Wurtman and Fernstrom, 1975) that was confirmed with a study in fasting man (Marliss et al., 1970) whereas no circadian rhythm was observed after 4 weeks of fasting.

In cows, too much tryptophan supply can cause pulmonary emphysema or fog fever (Kerr and Linnabary, 1989). High amounts of tryptophan are converted in rumen by microorganism to the pneumotoxic 3-methylindole, which is responsible

for the disease. It occurs primarily in late summer or fall when too much of lush pasture grasses are supplied, which have a high tryptophan content.

Serotonin (5-hydroxytryptamine), produced by hydroxylation and decarboxylation of tryptophan, is an indoleamine neurotransmitter. Furthermore serotonin is a precursor for melatonin. The brain serotonin synthesis is influenced by the uptake rate of tryptophan into the brain. Serotonin in brain shows a diurnal rhythm like tryptophan, whereas the rhythm of serotonin may result partly from the daily rhythm in plasma tryptophan (Fernstrom and Wurtman, 1971).

However, less than 2 % of serotonin are in brain (Llambias et al., 2003). Peripheral serotonin is derived from the enterochromaffin cells in the grastrointestinal tract, in the upper small intestine (Llambias et al., 2003) and stored in platelets, where it is involved in vasoconstriction, homeostasis, and the control of immune responses (Walther and Bader, 2003). It is suggested that serotonin cannot pass the blood brain barrier (Heine, 1999), which implicates that the serotonin used in brain must be produced in brain. Ohtsuki (2004) found that there are serotonin transporter located at the luminal and abluminal membranes. The abluminal transporter functions as an inactivation system for neurotransmitter, but the function of the luminal transporter is still unclear (Ohtsuki, 2004). Moreover, the serotonin transporter plays a minor role, thus brain serotonin must be produced in brain. Serotonin in brain is particularly produced in the pinealocyte where it is an intermediate product for melatonin synthesis. Furthermore it is synthesized by the raphe nuclei in the midbrain, pons and medulla oblongata (Walther and Bader, 2003). The presynaptic serotonin release is under control of the firing frequency of the raphe-neurons (Huether et al., 1992). This is rather stable during the wake period and completely stopped during REM-sleep. Serotonin is released from free ending axon terminals, too. Nevertheless a physiological increase of neuronal serotonin can be associated with an increase of the secretion rate into synapses (Wurtman and Fernstrom, 1975).

Serotonin is known to play an important role in a wide variety of functions including, mood, anxiety, aggression, sleep, appetite, sexual function (Bell et al., 2001) and body temperature (Fernstrom and Wurtman, 1971). Because of its functions, serotonin is suggested to be pivotal in psychiatric disorders. To study serotonin effects in psychiatry, the method of tryptophan depletion to lower brain serotonin, is often used. Thereby serotonin synthesis is limited by limiting

tryptophan with a tryptophan-free diet. Furthermore serotonin plays a role in the physiology of stress. Different forms of stress influence the release and synthesis of serotonin (Jorgensen et al., 2002). Chronically stressed animals show an increased serotonergic activity in the hippocampus (Gamaro et al., 2003) and stress induced depression can be attenuated by selective serotonin re-uptake inhibitors (Hashimoto et al., 1999; Gamaro et al., 2003).

Melatonin is a neurohormone derived from serotonin in absence of light. It is produced particularly in the pineal gland, but also in the retina of vertebrates (Tosini and Fukuhara, 2003). In mammals retinal melatonin has no influence to the circulating melatonin levels and its function is assumed to have some regulatory effects in the retinal physiology (Tosini and Fukuhara, 2003). Melatonin is suggested to be produced in the mucosa of the gastrointestinal tract, too, and released into the portal vein. Tissue concentration of the gastrointestinal tract can surpass blood levels, particularly during daytime. Blood melatonin levels during day can be influenced by gastrointestinally produced melatonin. But the melatonin produced there is not under photoperiodic control and is of no relevance for the nocturnal melatonin peak and the circadian rhythm of melatonin. Its function is the synchronization of sequential digestive processes as a luminal hormone (Bubenik, 2001). Pineal melatonin, the main source, is released without storage directly into the blood and cerebrospinal fluid (Delagrange et al., 2003). It can pass morphophysiological barriers like blood-brain barrier and placenta and move into all body cells (Bubenik, 2001).

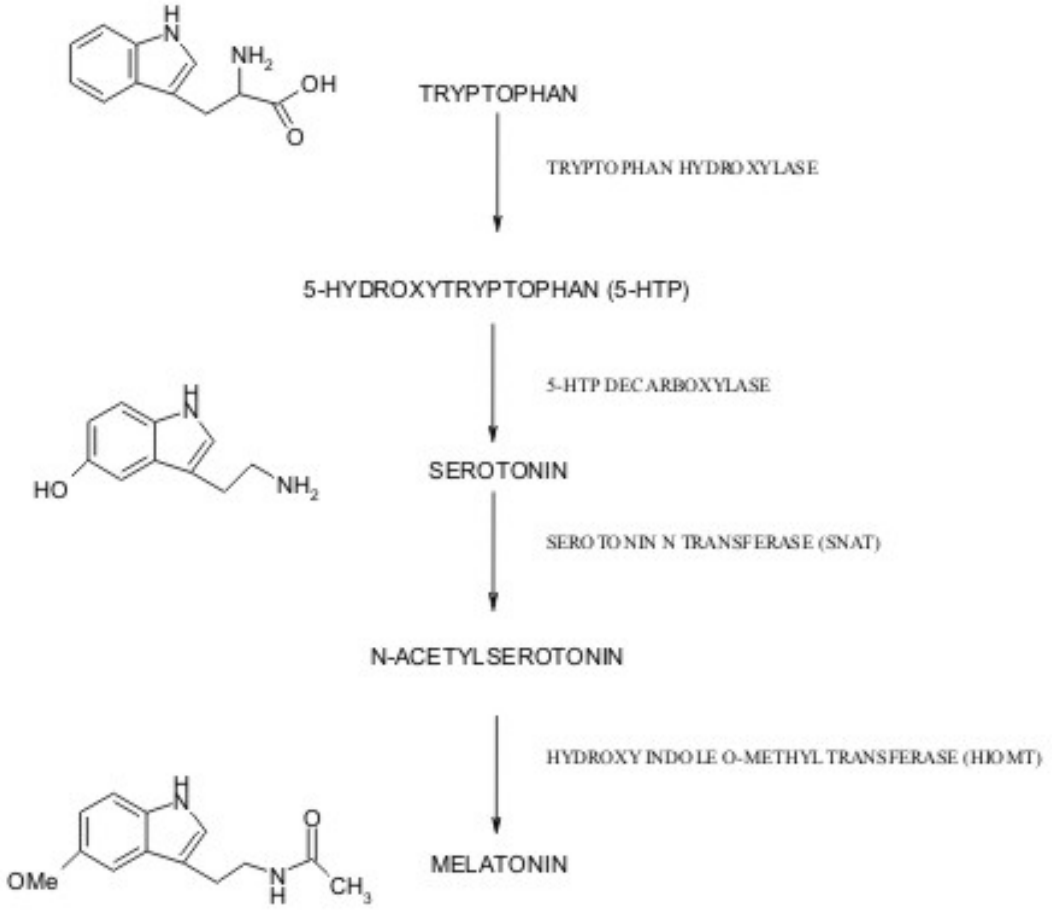
Melatonin mediates physiological, endocrinological and behavioural processes including the regulation of circadian rhythm, sleep, mood, reproduction, immune response and aging in the vertebrate body (Harumi and Matsushima, 2000).

Melatonin acts as a transducer of photoperiodic information in photoperiodic species (Berthelot et al., 1993). Plasma melatonin concentrations are high during night, both in diurnal and nocturnal species, although it is in one during sleep and in the other during activity period. Thus melatonin rhythm is an endocrine marker for night (Vanecek, 1998). The duration of melatonin increase is short on long day photoperiods and long on short day photoperiods (Vanecek, 1998).

In many mammals seasonal reproduction is common. The breeding season is driven by annual changes in photoperiod (Notter and Chemineau, 2001). Photoperiodic animals use the information of melatonin to ensure the correct

timing of seasonally variable functions such as reproduction, coat growth, and the duration and organization of sleep (Arendt et al., 1999). Seasonal breeding in sheep is controlled by circadian variation in circulating melatonin and circannual variation in timing and duration of the nighttime rise (Notter and Chemineau, 2001; Malpaux et al., 1996). Cows are no seasonal breeders, but the onset of puberty is considered to be under control of melatonin (Tortonese and Inskeep, 1992).
Furthermore, melatonin has the ability to scavenge reactive molecules and regulate the gene transcription of antioxidative enzymes, thus it is a potent antioxidant (Lena and Subramanian, 2003; Rodriguez et al., 2004). Because of this ability, melatonin is regarded as anti-aging hormone.

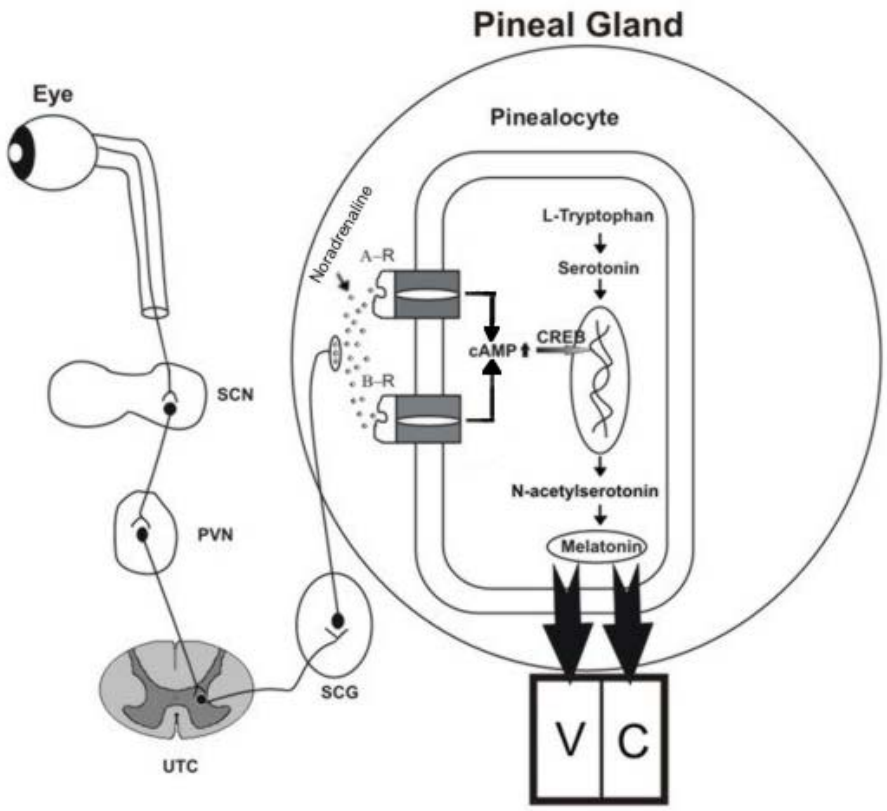
3.2.2 Melatonin biosynthesis



http://www.angelfire.com/yt/yas709neuroscience/melatonin1.htm

Figure 1: Biosynthesis of melatonin: pathway from the amino acid tryptophan to the hormone melatonin

The amino acid tryptophan is the substrate for the synthesis of serotonin and melatonin (Figure 1). Free plasma tryptophan penetrates the blood-brain-barrier and is afterwards converted in the pinealocyte by tryptophan hydroxylase to 5-hydroxytryptophan. Then the aromatic amino acid decarboxylase converts 5-hydroxytryptophan to serotonin (5-hydroxytryptamine). Serotonin is, in absence of light, converted by serotonin-N-transferase to N-acetlyserotonin and then finally to melatonin (N-aectly-5-methoxytryptamine). The serotonin synthesis is potentially limited by several factors: the total amount of free plasma tryptophan, the transfer rate of tryptophan through the blood-brain barrier, and the activity of the tryptophan hydroxylase enzyme (Bell et al., 2001). Under normal physiological conditions the enzyme is not the rate limiting step, because the Michaelis-Menten constant for tryptophan hydroxylase is several times higher than that of tryptophan concentration in the brain and thus it is not saturated with its substrate (Fadda, 2000; Fernstrom and Wurtman, 1972). Thus normally the availability of tryptophan may be rate limiting for brain serotonin synthesis (Fernstrom and Wurtman, 1972). The regulation of the melatonin synthesis is under the control of light. With light, the biosynthesis of melatonin is suppressed. Light is mediated via the retina. If light impinges the retina, the signal is transduced via the retinohypothalamic tract to the suprachiasmatic nucleus. From there, the signal passes through the paraventricular nucleus and attains via the medial forebrain bundle the intermediolateral cell column of the upper thoracic spinal cord. Then a projection to the superior cervical ganglion exists, from which sympathetic neurons (nervii conarii) innervate the pineal (Moller and Baeres, 2002). During night, noradrenalin is released from the pineal. This causes an α-adrenergic and β-adrenergic receptor activation which leads to an increase of cAMP. This activates the CREB (cAMP response element binding protein) which induces the transcription of serotonin-N-acetyltransferase (Figure 2). With light noradrenalin release is suppressed and a rapid inactivation of serotonin-N-acetyltransferase follows that implicates in cessation of melatonin release (Klein and Weller, 1972).



http://www.angelfire.com/yt/yas709neuroscience/melatonin1.htm

Figure 2: Melatonin biosynthesis: schematic representation of the role of light. Pathway from the eye to the pinealocyte.
SCN: suprachiasmatic nucleus; **PVN**: paraventricular nucleus; **UTC**: upper thoracic spinal cord; **SCG:** superior cervical ganglion; **A-R**: alpha adrenergic receptor; **B-R**: beta adrenergic receptor; **CREB**: cAMP response element binding protein; **V**: Ventricles of the Brain; **C:** Capillaries

3.2.3 Exogenous melatonin

Melatonin is used in animals to mimic short day photoperiod (Lincoln and Ebling, 1985). Administration of exogenous melatonin is a possibility to induce breeding season in short-day breeders. Melatonin alone in physiological quantities induced an early onset of the breeding season in ewes (Arendt et al., 1983) and sheep (Malpaux et al., 1996).

In humans, melatonin is used for therapeutic purpose. Exogenous melatonin can only phase shift the circadian rhythm when the production of the hormone is

inhibited (Dubocovich et al., 1996). Thus an effect of melatonin can be expected mainly in infants, children, old and mentally retarded people, and after phase shifts. The administration of exogenous melatonin can help in circadian rhythm sleep disorders (Dagan and Borodkin, 2005) or lessen the effects of jet lag (Smucny, 2002) or attenuate shift work sleep disorders (Skene et al., 1999). Administration of 5 mg exogenous melatonin to phase shifted volunteers resulted in improved subjective sleep, alertness, and performance (Deacon and Arendt, 1996). But even lower doses of melatonin (0.1 and 0.3mg) improved sleep in mentally retarded people with poor sleep efficiency (Niederhofer et al., 2003).

3.3 LIGHT AND PHOTOPERIOD

Photoperiod is the time period of daily exposure that an organism receives from daylight or artificial light. Many physiological and behavioural factors are influenced by photoperiod. The mediator of photoperiod is the hormone melatonin which gives information about the changing length of the night in the course of the year (Arendt et al., 1999). The influence of photoperiod to seasonal breeders is reported by several authors (O'Callaghan et al., 1991; Barrell et al., 2000).

In several species photoperiod is used as a management tool. Laying hens were exposed to extended photoperiod to increase the laying performance (Tucker and Ringer, 1982).

In dairy cows an increase of milk yield up to 10 % with extended photoperiod to 16-18 h light was reported (Peters et al., 1978; Peters et al., 1981; Marcek and Swanson, 1984; Stanisiewski et al., 1985; Bilodeau et al., 1989; Evans and Hacker, 1989; Dahl et al., 1997; Miller et al., 1999; Reksen et al., 1999).

Less illumination can cause seasonal affective disorders (Joffe et al., 1993). Especially in countries with long winter times the low illumination can act depressive. Light therapies with artificial light can have an antidepressant effect in humans (Joffe et al., 1993). Sunlight was also used in several species to boost general condition. Especially in horses a positive effect in erythrocytes and haemoglobin concentration was found (Stendel W., 1980).

The quality of light is also important for its influence on physiology. Differences in melatonin suppression in dependence of the wave length of light were reported. In rats the suppression was highest with green light (~485-585 nm) and could not be

detected by red light (~640-680 nm) (Cardinali et al., 1972). It was shown in humans that the circadian pacemaker is more sensitive to short (460 nm) versus long (555 nm) wave lengths of visible light (Lockley et al., 2003; Thapan et al., 2001). But regardless of the wave length, melatonin suppression increases with increasing irradiance (Thapan et al., 2001).

3.4 ENDOCRINE CONTROL OF LACTATION

For onset and maintenance of lactation the interaction of a couple of hormones is necessary. Metabolic hormones, growth factors and prolactin are necessary for normal development of the mammary gland (Svennersten-Sjaunja and Olsson, 2005) with some special importance for the sex steroids (Lamote et al., 2004). Lactogenesis and galactopoiesis are primarily controlled by GH and prolactin, whereas its role and importance is different in rodents and in ruminants (Flint and Knight, 1997). Milk ejection is primarily induced by oxytocin (Bruckmaier, 2005; Bruckmaier and Blum, 1998) and its release is necessary during the whole milking for undisturbed milk release (Bruckmaier and Blum, 1998).

3.4.1 Hormones related to milk synthesis, secretion and ejection

Growth hormone (GH) respectively somatotropin (ST) is a 191 amino acid peptide hormone, synthesized in somatotrope cells in the anterior pituitary gland (McMahon et al., 2001). Its primarily physiological role is the control of growth and metabolism. It is especially important for lactation.

GH is essential for growth of ducts in the pubertal phase of mammary development and for lobulo-alveolar growth during pregnancy (Sejrsen et al., 1999). Furthermore GH together with prolactin are important for the transition from a proliferative to a lactating mammary gland, whereas GH dominates in ruminants (Svennersten-Sjaunja and Olsson, 2005). The galactopoietic action of GH is suggested to be due to a greater utilization of available nutrients for milk synthesis (Bauman, 1992). More energy from fat, especially during early lactation, is available as effect of GH (Tucker, 2000). Because of the galactopoietic effect, exogenous bovine ST is used as management tool in some countries. With injection of bST milk yield can be increased by 10-15 % (Etherton and Bauman,

1998) i.e. 6-30 % (Svennersten-Sjaunja and Olsson, 2005). Its effect in lactating tissues is discussed to be only partly direct or furthermore indirectly mediated by IGF-I (Svennersten-Sjaunja and Olsson, 2005). Possibly the effect is direct and indirect. GH binding to ruminant mammary tissue was not detected, but the receptor is expressed during all stages of lactation (Röpke et al., 1994), thus the direct effect cannot be excluded (Svennersten-Sjaunja and Olsson, 2005). GH binds to hepatocytes in the liver which stimulates the release of IGF-I (Tucker, 2000). As IGF-I receptors are present in lactating mammary cells (Tucker, 2000) and a binding was detected (Cohick, 1998) the indirect effect was suggested.

Prolactin is a polypeptide hormone that is synthesized in the anterior pituitary gland. In addition mammary epithelial cells are capable to synthesize prolactin (Freeman et al., 2000). Prolactin is important for development of mammary gland and the induction of milk synthesis. In monogastric species it is additionally necessary for maintenance of milk synthesis. With the ergot alkaloid bromocryptine, prolactin was suppressed in rats and resulted in a suppression of milk secretion (Tucker, 2000). In cattle only a slight suppression of milk secretion after suppression of prolactin was observed (Karg and Schams, 1974; Koprowski and Tucker, 1973). Thus prolactin is rather more essential for mammogenesis and lactogenesis as for the maintenance of lactation in cow. Various factors influence the plasma concentration of prolactin. It is influenced by oestrus cycle (Madej et al., 1985) and increases during gravidity towards parturition (Schams and Karg, 1970). Season or photoperiod is reported to influence prolactin, too. Prolactin level is increased during summer or long day photoperiod and decreased during winter or short day photoperiod (Lincoln et al., 2003; Schams and Reinhardt, 1974; Miller et al., 2000; Newbold et al., 1991). Furthermore a circadian rhythm of prolactin was found (Zinn et al., 1986; Mollett and Malven, 1982; Madej et al., 1985; Lefcourt et al., 1994; Koprowski et al., 1972; Bines et al., 1983), but could not be confirmed by Fulkerson et al. (1980).

During milking prolactin increases (Schams and Karg, 1970; Forsling et al., 1974). It was shown that oxytocin initiates the release o prolactin (Bryant and Greenwood, 1968). The increase of prolactin during milking was reported to be 3- to 15-fold (Forsling et al., 1974) but the importance is still unclear.

During milking also cortisol increases continuously (Bruckmaier et al., 1993; Gorewit et al., 1992) but the physiological significance of milking-induced secretion or release of cortisol in ruminants is also not known.

Cortisol is a glucocorticoide, produced in the adrenal cortex. Its release shows a strong ultradian rhythm in lactating cows with a period around 100-120 min and a weak circadian rhythm (Lefcourt et al., 1994; Fulkerson et al., 1980). Cortisol concentrations are high from mid-night to mid-morning and lowest during afternoon (Fulkerson et al., 1980).

Stress activates the hypothalamo-pituitary-adrenal-axis and resulted in a release of corticosterone in rats, and cortisol in man (Young et al., 2004) and cows (Doecke, 1994). The cortisol release in response to stress can overlap the ultradian and circadian rhythm.

The cortisol release in response to milking does not seem regulated by the hyptophalamo-pituitary-adrenal axis, because ACTH did not increase as in stress response (Tancin et al., 2000). Furthermore, acute stress can induce a inhibition of milk ejection (Bruckmaier et al., 1993), but with cortisol a depression of milk ejection was not possible (Mayer and Lefcourt, 1987).

During mammogenesis cortisol, the predominat glucocorticoid in cattle, causes differentiation of the lobule-alveolar system (Tucker, 2000). In the onset of lactation glucocorticoids are involved, too. The onset of lactation can be induced in nonlactating cows with well-developed lobule-alveolar systems by injection of glucocorticoids, whereas the milk quantity is greater when prolactin secretion is also increased (Tucker, 2000).

For milk removal milk ejection is important. Less than 20 % of the milk can be removed without milk ejection, because only this is stored in the cistern (Bruckmaier and Blum, 1998). The major part of milk is fixed in the alveoli by capillary forces and can be released only with milk ejection. A contraction of the myoephitelial cells, caused by the hormone oxytocin, resulted in milk let down into the cistern. Thus for complete milk removal an increase of oxytocin concentration during milking is necessary.

Oxytocin is a hormone consisting of nine amino acids (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly). It is synthesized in the hypothalamic supraoptic nucleus and paraventricular nucleus, transported via the neurons to its storage in the posterior pituary gland (Swaab et al., 1975; Vandesande and Dierickx, 1975; Vandesande

et al., 1975). Its release is initiated by a neuroendocrine reflex arc, that responds to tactile stimulation (Bruckmaier and Blum, 1998). The neuroendocrine reflex arc is not under conscious control of the animal. The impulse is carried from the receptors, located primarily in the tip of the teat, to the anterior and posterior inguinal nerves, via the inguinal canal, to the lumbar nerves. The dorsal roots of the lumbar nerves terminate in the spinal column, and from there the signal is transported into the brain, the supraoptic and paraventricular nuclei (Bruckmaier and Blum, 1998). The oxytocin released stimulates the receptors on the myoepithelial cells and causes a contraction of these cells. The contraction of the myoepithelial cells surrounding the alveoli, respond in a squeeze of the milk ducts, whereas the alveoli milk finally is shifted into the cisternal space (Bruckmaier and Blum, 1998). To cause myoepithelial contraction, oxytocin has to pass a threshold level (Schams et al., 1984). Oxytocin concentration must be continuously elevated during the whole milking for complete milk removal (Bruckmaier et al., 1994). If the threshold level is passed the maximum intramammary pressure and consequently the maximum milk ejection succeeds. Additionally intramammary pressure and milk ejection are only achievable with supraphysiological amounts of oxytocin, which make the residual milk available and can respond in additional milk up to 30 % (Bruckmaier et al., 1994).

Milk ejection can be disturbed under various conditions whereat it can be a peripheral or a central inhibition of milk ejection. Peripheral milk ejection is due to a α-adrenergic receptor stimulation or an oxytocin blocking reagent (Bruckmaier et al., 1996; Bruckmaier, 2005). This inhibition is limited for experimental approach, whereat the central inhibition is common in dairy practice. Central inhibition expresses itself in an inhibition of oxytocin release from the pituitary gland. The exact physiology of central inhibition is not clear, but it is suggested that endogenous opioids play a role (Bruckmaier, 2005).

3.4.2 Importance of the serotonin-melatonin system for lactation

There is growing evidence that the serotonin-melatonin system influences milk removal. In rats a correlation of melatonin or serotonin to milk disposal seems possible, although the results are controversial. With a visual stimulus during sucking milk ejection could be inhibited in rats (Prilusky and Deis, 1982). Due to

the fact that rats kept in total darkness or continuously light are not influenced, the inhibition of milk ejection is therefore produced by light on-off sequence.

It was assumed that 5-HT increases peripheral release of oxytocin whereat the effect of serotonin on oxytocin is mediated via activation of 5-HT_{1a}, 5-HT_{2a} (Van de Kar et al., 2001) and 5-HT_{2c} receptors (Jorgensen et al., 2003). Alike it was reported that 5-HT inhibits milk ejection (Mizuno et al., 1967) and the suggested inhibitory effect of melatonin was rejected (Mizuno and Sensui, 1970). Serotonin seems to have different effects in conscious and anesthetisized rats: serotonin did not affect milk ejection reflex in conscious rats, even at the highest doses, however it had an inhibitory effect under urethane anesthesia (Moos and Richard, 1983). This let suppose that serotonergic neurons are involved in the afferent pathway of the milk ejection reflex (Moos and Richard, 1983).

An effect of melatonin on oxytocin was assumed in other studies. Melatonin inhibits oxytocin release in rats (Yasin et al., 1996; Yasin and Forsling, 1998) and also in hamsters (Juszczak et al., 1995). The sensitivity to melatonin depend on the time of day, because exposure to high melatonin levels during the dark period may downregulate the receptor binding sites, whereas during the day at low melatonin levels the number of receptor sites may increase (Yasin et al., 1996). Melatonin inhibits basal oxytocin secretion from hypothalami during light hours (Evans et al., 1999). Melatonin possibly affects afferent pathways mediated by acetylcholine, dopamine and/or prostaglandine and influences that way the neurohypophysial hormone release (Yasin and Forsling, 1998). The effect of melatonin to oxytocin seems to be dose dependent (Yasin et al., 1993). Low doses of melatonin inhibit oxytocin while higher doses stimulate the release in rat (Juszczak and Stempniak, 1997). In ewes melatonin infusion resulted in an increase of plasma oxytocin concentration in nonpregnant ewes, but had no effect in pregnant ewes (Ross et al., 1985). This let suggest that its effect could be suppressed by other hormones and is dependent on the reproductive phase (Ross et al., 1985). Furthermore it was reported that melatonin inhibits prolactin (Juszczak and Stempniak, 1997).

It seems that the effect of serotonin and melatonin on milk ejection is possibly dose-dependent and species dependent.

As these results are found in nocturnal rats which have their activity period and suckle primarily during night, the results can be different in diurnal cows, which

have their activity period during day. Thus rats have high melatonin concentration during their active period and cows during sleep.

3.5 MILK COMPOSITION

Milk is originally produced for the supply of nutrients to the newborn. The composition of milk varies between the species and is adapted to the demand of the newborn. Because of the composition of cow milk, it is used in human nutrition.

3.5.1 Major constituents

Milk is synthesized continuously in the alveolar cells of the udder. The substrates for milk synthesis are carried to the udder via the bloodstream. Most milk components are synthesized in the alveolar cells, but some are only transferred from blood into milk. For the production of 1 l of milk around 540 l of blood must pass the udder (Gravert, 1983). To improve the absorption of substrates from bloodstream in udder vasodilation is used. The main components of bovine milk are: water (87 %), fat (4.2 %), lactose (4.7 %), proteins (3.4 %) and salts (0.8 %) (Schlimme and Buchheim, 1999).

Milk composition can be altered by natural genetic variation. Especially fat content varies between the breeds. For example Jersey cows with around 5.5 % fat have a higher content than Holstein-Friesian with around 4.0 %. But the target of genetic selection until now was more milk production than milk composition.

Feeding can also influence the composition of milk. A deficiency in energy supply results in a reduced protein content of milk. Milk fat can be influenced by its content and its composition. Feed ration with low levels of crude fibre can decrease the fat content. The consistency of milk fat, that is important for further processing of milk, can be influenced by the dietary source, too. It is dependent on the percentage of unsaturated fatty acids in the feed.

3.5.2 Hormones and growth factors in milk

Beside the main components some other substrates i.e. drugs and hormones are in milk (Gravert, 1983). Most hormones are transferred from the bloodstream into milk by diffusion, whereas the profile in milk is similar to that in plasma (Schams

and Karg, 1986). An active transport system exists for some hormones, too, i.e. for prolactin (Schams and Karg, 1986). Several hormones and growth factors appear to be synthesized within the mammary gland as well as transported from the maternal circulation (Grosvenor et al., 1993). The function of bioactive substances in milk is not completely known. But it is suggested that several substances are important for regulation of growth and secretory functions of maternal mammary tissue and the regulation of growth, development and maturation of the gut, immune system and several endocrine systems in the neonate (Grosvenor et al., 1993). However, the concentrations of hormones in cow milk are very low and are assumed to be of little significance in human nutrition, because the endogenous production is manyfold higher.

Nowadays there is ambition to increase special components, particularly bioactive components in milk which should enhance health of humans. The term “functional food” describes food or food components that have beneficial effects on human health beyond the nutritive value (Bauman et al., 2006). Especially the composition of fatty acids in milk is tried to advance. Conjugated linolic acid (CLA) was found to have anticarcinogenic effects, and thus it was tried to be increased. Feeding fresh pasture or the supplementation with plant or marine oils to dairy rations are the most common method to increase rumenic acid (*c9,t11*-CLA) (Bauman et al., 2006).

For some factors management practices can be determinative. To increase the hormone melatonin in milk, the time and the illumination during milking were suggested to be important.

4 OBJECTIVE OF THE STUDY

The objective of this study is to test whether the serotonin-melatonin-system can be influenced by different lighting, by different photoperiodical conditions or by additional substrate supply, and thereafter determine the effect of the influenced serotonin-melatonin-system to milk synthesis and ejection in dairy cows.

Thus the study will try to influence the serotonin-melatonin-system with different methods and evaluate its effect to milk synthesis and ejection in cattle.

We hypothesize that the serotonin-melatonin-system can be regulated by influencing the amount of substrate availability. With supplementation of rumen-protected tryptophan in heifers and dairy cows, an increase of plasma tryptophan was aimed because the amount of plasma tryptophan is a limiting factor for serotonin synthesis (Bell et al., 2001), and thus with increasing plasma tryptophan a higher concentration of plasma melatonin was assumed.

As second method, the regulation of illumination time, illuminance and light spectra around milking will be tested, because melatonin is suppressed by light, depending on the wave length.

The influence of photoperiod to the serotonin-melatonin-system will be investigated by utilization of the natural season (summer vs. winter).

With the use of these methods further influence of the serotonin-melatonin-system to prolactin, cortisol, oxytocin, milk yield and milkability factors may be tested.

Additionally, the influence of plasma melatonin level to melatonin level in milk will be ascertained, to get information about the transport of melatonin into milk and its levels.

5 MATERIAL AND METHODS

5.1 ANIMALS AND HUSBANDRY

The first two studies were performed on the experimental station Veitshof of the Physiology Weihenstephan, Technical University Munich, Germany. For all experiments, conducted in Weihenstephan, Brown Swiss cows or heifers were used. The dairy cows were housed in a free stall barn. They had free access to a mixed ration providing energy and other nutrients to cover the demand for maintenance and milk production (138 MJ NEL/d, 3225 g nXP/d and 25 % UDP, (Kollmann et al., 2006). The heifers were housed in a tie stall barn and fed with corn silage and hey ad libitum.

Study III was performed on experimental station Agroscope Liebefeld-Posieux in cooperation with the University Bern, Switzerland. The cows were of different breeds (1 Holstein Friesian, 5 Brown Swiss, 6 Red Holstein). They were housed in a tie stall barn during the experiment and fed with grass silage, corn silage and hey.

5.2 EXPERIMENTAL DESIGN

The following studies were performed:

Study I (see appendix I)

The objective of the study was to test if a supplementation with rumen-protected tryptophan can increase plasma tryptophan and hence increase serotonin and melatonin and by this way influence hormones related to milk synthesis (prolactin) and milk ejection (oxytocin). 62,5 g rumen protected tryptophan (250 g of 25% tryptophan, Nutreco, Bussolengo, Italy) was administered to 6 heifers and 6 lactating cows twice daily. Blood sampling was performed in 3-h intervals between 0800 h and 0500 h on the day before tryptophan supplementation (d 0) and on day 2, 5 and 7 of tryptophan supplementation, and in heifers additionally on d 21, i.e. 2 weeks after tryptophan supplementation was ceased. All samples were analysed for melatonin and prolactin and in samples taken at 1100 h and 0200 h for tryptophan and serotonin. Additionally proportional milk samples of the whole milk during twice daily milking at 0415 h and 1545 h were taken for analysis of melatonin in milk.

To avoid light effects on the pineal activity, all blood samples during night were taken only with a small head lamp and direct illumination of the animals´ eyes was carefully avoided.

In cows additionally blood samples at the pre-treatment day and at day 7 were taken in 1 min intervals during milking and analysed for prolactin and oxytocin.

Study II (see appendix II)

To investigate the effect of photoperiodical differences and artificial sunlight to milk removal in dairy cows two experiments were designed.

Four different treatments, existing of illumination with different light spectra and different duration of lighting were tested during four consecutive days at morning and evening milking in every cow. For illumination an animal solarium with 18 infrared and 7 ultraviolet lamps was used that mimic the natural sunlight (Turnier II, Weinsberger International, Weinsberg, Germany) and it was employed 10 min before and during milking.

The treatments were peformed after the cows were moved to the milking parlor. They were exposed to additional lighting with ultraviolet and infrared light (UVIR) or only ultraviolet light (UV) 10 min before milking, then milking was started with continuing light, or they were moved to the parlor and milked at once with additional ultraviolet light but without additional lighting before milking (UVMO). As control treatment, the cows were moved to the parlor, where they had to stay for 10 min without additional illumination; neither before milking nor during milking lighting was performed (C).

Blood samples were taken after entering the parlor, immediately before milking and in 1 min intervals during milking also as 10 min and 30 min after milking. The samples were analysed for oxytocin, melatonin (only in the second sample) and prolactin and cortisol (in the basal sample, the sample taken 4 min after cluster attachment , in the sample taken during the last minute of milking, i.e. 9.7 ± 0.4 min after cluster attachment and the sample taken 30 min after milking).

The second experiment was performed during June/July, when daylength was maximal and during December, when daylength was minimal. Blood samples were taken during morning and evening milking at three consecutive days in 1 min intervals and analysed for oxytocin in all and for cortisol in the basal sample, the sample taken 4 min after cluster attachment and in the sample taken during the last minute of milking, i.e. 8.8 ± 0.1 min after cluster attachment.

Study III (see appendix III)

The correlation of plasma melatonin concentration and milk melatonin concentration was tested. Blood and proportional milk samples of the total milk were taken in 1 h intervals over 24 h starting at 0700h and analysed for melatonin. All samples during night were taken only with a small head lamp as in study I. Additionally milk samples taken during normal twice daily milking in June/July and December at Veitshof in Weihenstephan were compared. Furthermore milk fraction samples (fore milk, main milk and residual milk) were taken by hand milking and analysed for melatonin to see differences in melatonin concentration between the fractions.

5.3 MILKING SYSTEM

Cows in study I and II were milked twice daily at 0415 h and 1545 h in a 2 × 2 tandem milking parlor equipped with Stimopuls clusters (WestfaliaSurge GmbH, Oelde, Germany). Milk yield and milk flow was measured with a strain gauge system as described previously (Bruckmaier et al., 1992) (study I) or with LactoCorder® (study II).

Cows in study III were milked with a bucket milker (Lemmer-Fullwood AG, Meierskappel, Switzerland) every hour. Milk yield was measured with a digital scale.

5.4 SAMPLE COLLECTION

For blood sampling the animals were catheterized at least at the day before the experiments with a permanent catheter inserted into a jugular vein (Cavafix Certo Spilttocan 335, length 32 cm, diameter 1.8 × 2.35 mm Braun. Melsungen, Germany). Blood samples were anticoagulated with EDTA and cooled on ice until centrifugation at 2000 × g for 15 min. at 4°C (1000 × g for 20 min. at 4°C in study III) and plasma was stored at -20°C until analysed in assays.

Proportional milk samples were taken during milking of the total milk. They were stored at –20°C until further analysis.

5.5 HORMONE AND TRYPTOPHAN ANALYSIS

5.5.1 Tryptophan

Plasma samples were prepared similar to the method of Teerlink et al. (1994) except of the usage of EDTA instead of heparin for anticoagulation. 400 µL of plasma were transferred into cups with 100 µL 5-sulfosalycilic acid, frozen in liquid nitrogen and stored at -80°C until analysing. Tryptophan concentration were analysed with a HPLC-method according to Schuster (1988) using reagents as described previously (Teerlink et al., 1994).

5.5.2 Serotonin

Plasma serotonin concentrations were determined with a commercial ELISA kit (Beckman Coulter, Krefeld, Germany, REF: 1749).

5.5.3 Melatonin

Plasma melatonin concentration were measured by using a commercial ELISA kit (IBL Hamburg, Germany , Kat.-Nr. RE 540 21).
Milk melatonin concentrations were measured with the same ELISA kit after an optimized sample preparation (Kollmann et al., 2006). Reliable results and highest recovery level were achievable with skimmed milk. Skimming was performed by centrifugation at 3300 × g for 15 min (4°C). Afterwards skimmed milk was extracted as plasma samples (500µl in study I and 1 ml in study III).

5.5.4 Oxytocin

Oxytocin plasma concentrations were determined by radioimmunoassay as described previously (Schams, 1983).

5.5.5 Prolactin

Prolactin plasma concentrations in study I were measured with an ELISA as described (Kollmann et al., 2006). The intra-assay variation was 11 % and the inter-assay variation was 20 %. The sensitivity of the test was 0.7 ng/ml.

Prolactin concentration in study II were measured by RIA as previously (Schams and Karg, 1970).

5.5.6 Cortisol

Cortisol in plasma was measured by using a competitive ELISA as previously described (Sauerwein et al., 1991).

5.6 LIGHT MEASUREMENT

In study I illuminance (in lux) was measured with a luxmeter (digital luxmeter Peak Tech® 5020, Ahrensburg, Germany).
In study II illuminance was measured (in lux) with the datalogger LiCor Li-1000 (LiCor Biosciences. Lincoln Nebraska, USA)
Spectral measurement was performed with a spectroradiometer LiCor Li-1800 (LiCor Biosciences. Lincoln Nebraska, USA)

5.7 DATA HANDLING AND STATISTICAL ANALYSES

Results are presented as means ± SE. All data were processed by the SAS system (version 9.1). For analysis of variance the MIXED procedure was used. The repeated subject was always the animal. Treatment effects were tested for significance ($P<0.05$) using Bonferroni´s t-test based on least square means. To calculate Pearson´s coefficient of correlation the CORR procedure and for calculation of linear regressions the REG procedure was used. For hormone profiles often the area under the curve was calculated and for melatonin (study I) a linear correction with pre-treatment means was done afterwards. Differences were indicated as statistically significant in case of $P < 0.05$, unless stated otherwise. A detail description of the statistical models is shown in appendix (Kollmann et al., 2006; Kollmann et al., 2007b; Kollmann et al., 2007a).

6 RESULTS AND DISCUSSION

The serotonin-melatonin-system could be partially influenced by using the method of substrate supply regulation and light control.

6.1 EFFECT OF TRYPTOPHAN SUPPLEMENTATION TO THE SEROTONIN-MELATONIN-SYSTEM

It is generally assumed that amino acids in ruminants are not deficient because they are synthesized by ruminal microbes, and thus plasma levels can not be increased. A sufficient energy supply which was assured in the ration (138 MJ NEL/d, 3225 g nXP/d and 25 % UDP, (Kollmann et al., 2006) is important for the *de novo* synthesis of protein by ruminal microbes. In contrast Fenderson and Bergen (1975) reported, that with very low protein ration amino acids can become limiting in cattle. Microbial protein synthesis can be insufficient in high yielding cows for maximal production of milk and milk protein (Piepenbrink et al., 1996), thus dietary escape protein is important. The additional supply of the limiting methionine for milk fat synthesis as rumen-protected product was able to increase milk fat in cows (Overton et al., 1996). Thus we hypothesized that by tryptophan supplementation serotonin and melatonin can be increased in cattle.

In heifers and dairy cows a supplementation with rumen-protected tryptophan increased plasma tryptophan levels, whereas the increase was higher in heifers than in dairy cows (Kollmann et al., 2006), what could derive from the demand of the animals. The demand in non pregnant heifers (BW 536 ± 13 kg) resulted primarily for maintenance whereas in high yielding dairy cows nutrients are primarily used for milk production. After 14 days without tryptophan supplementation, the plasma concentration of tryptophan reached nearly the level of day 0 what confirms the effect of treatment. Tryptophan plasma levels in bovine species displayed a circadian rhythm as reported in rats (Gutierrez et al., 2003) and humans (Wurtman and Fernstrom, 1975) whereas the peaks occur at different times in different species. In cattle the tryptophan plasma concentration was higher during nighttime than during daytime (Kollmann et al., 2006). The circadian rhythm of tryptophan plasma levels is suggested to depend on the feeding behaviour of the species (Gutierrez et al., 2003; Wurtman and Fernstrom, 1975).

Thus the different lag of blood sampling to the prior feeding of the product caused the difference in the samples. The interval between feeding and blood sampling differed between 3-4 h and 8-9 h (daytime and nighttime sampling respectively).

Serotonin synthesis and consequently melatonin level is regulated by the amount of free plasma tryptophan (Bell et al., 2001). Thus an increase of both was expected after an increased tryptophan level. Because of the fact that serotonin can not pass the blood-brain barrier (Heine, 1999) in adequate amounts for melatonin synthesis, it must be produced in the brain. In newest research luminal and abluminal serotonin transporter are identified, but their function is still unclear (Ohtsuki, 2004) and suggested of marginal importance. The amount of serotonin in brain is less than 2 % of the total serotonin, whereas virtually all serotonin in blood derives from the gastrointestinal tract (Llambias et al., 2003). In dairy cows and in heifers plasma serotonin was not influenced by tryptophan supplementation (Kollmann et al., 2006), but serotonin measured in plasma does not represent the brain serotonin content. In rats a significant increase in serotonin content in diencephalic regions when tryptophan was administered by day, could be shown (Esteban et al., 2004). Rats fed with tryptophan supplemented feed, presented an increase in brain serotonin (Sarwar and Botting, 1999).

The increase in tryptophan level resulted in a significant increase of plasma melatonin concentration in heifers, but could not be detected in dairy cows (Figure 3) (Kollmann et al., 2006). As a consequence of the results in rats and the increase of melatonin in heifers, an increase of brain serotonin in cattle can be assumed.

Melatonin concentration could only be increased with tryptophan supplementation in heifers but not in cows. This could be due to the lower tryptophan level in heifers and the consumption of tryptophan for milk protein synthesis in dairy cows. Furthermore the high variety in melatonin concentration between the animals could be a reason. The variation possibly derived from a strong genetic influence like it is reported in ewes. Ewes have a heritability of melatonin of h^2=0.45 (June and December) (Zarazaga et al., 1998). That the increase of melatonin concentration in heifers derives from the tryptophan supplementation was shown with the results of the day 21, 14 days after tryptophan supplementation was ceased. The concentration of melatonin at day 21 was nearly as low as at day 0,

i.e., the effect was reversible and reached the basal level after finishing the treatment.

Melatonin is light suppressed (Vanecek, 1998). The inverse correlation of light and melatonin could be seen with study I. Melatonin began to increase in the experiment when light decreased to less than 20 lux as reported previously (Berthelot et al., 1990).

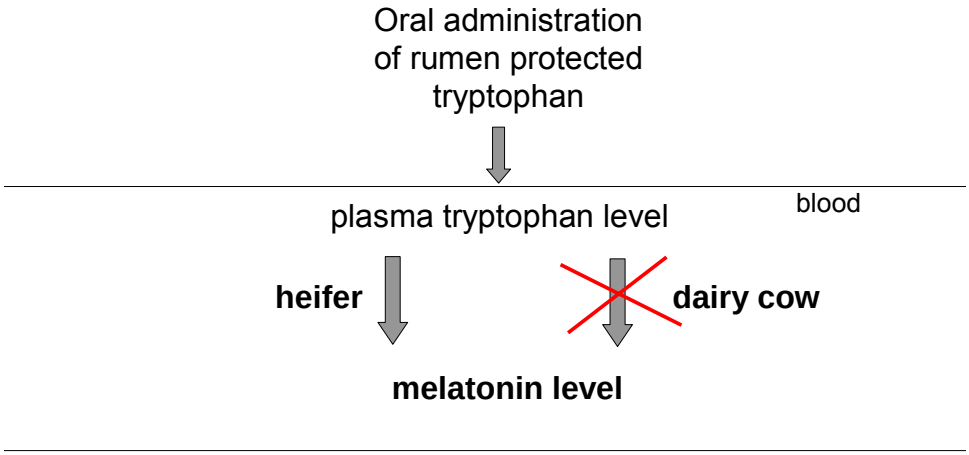


Figure 3: Effect of oral administration of rumen protected tryptophan to heifers and dairy cows

6.2 EFFECT OF LIGHT TO THE SEROTONIN-MELATONIN-SYSTEM

Photoperiodical differences show different blood melatonin levels. The blood pattern of melatonin in dependence of photoperiod was reported several times (Arendt, 2006; Berthelot et al., 1990; Kollmann et al., 2006) and melatonin was accepted as the active mediator of photoperiodic response (Dahl et al., 2000).

Because of the different lighting times in different seasons, melatonin pattern was not measured in study II in dependence of season but light intensity and spectra (study II) were measured and thus melatonin pattern can be assumed. Melatonin is produced only in absence of light (Berthelot et al., 1990; Kollmann et al., 2006) and melatonin suppression increases with increasing irradiance (Thapan et al.,

2001). During long day photoperiod the duration of the melatonin increase is shorter than in short day photoperiod (Vanecek, 1998).

The illumination time was longer and illuminance (lux) was higher in summer compared to winter (Kollmann et al., 2007b). Light spectra switched from summer to winter to a different spectral composition. In summer a higher percentage of UV-light during 1230 h was observed whereas in winter UV-light was hardly present. The light spectra in winter switched to a higher percentage of blue light (420-500 nm) and a lower percentage of infrared light (>750 nm).

The importance of wave length to melatonin suppression was found in rats and humans whereas short wave lengths showed a higher suppression as long wave lengths (Cardinali et al., 1972; Lockley et al., 2003). The inhibition was highest in rats with only green light (76%) (Cardinali et al., 1972). In our experiment in cows melatonin was measured during illumination with ultraviolet and infrared light and it was observed that light spectra show significant influence to melatonin concentration in bovine. The suppression of melatonin was dependent on the time and on the spectrum of light. Melatonin was lower after illumination 10 min before milking and during milking (UV) than in control treatment (C) without additional illumination and in the treatment with illumination only during milking (UVMO) (Kollmann et al., 2007b). Furthermore, with additionally infrared light (UVIR), melatonin concentration was lower than only with ultraviolet light (UV) (Kollmann et al., 2007b). In rats lighting with only ultraviolet light showed a suppression of only 16 % whereas the suppression with a broad-spectrum light (cool-white) suppressed melatonin by 57 % (Cardinali et al., 1972).

Thus lighting around milking showed an effect to the serotonin-melatonin-system in cattle (Kollmann et al., 2007b), whereas the extent of the suppression depended on the duration of illumination and the light spectra. A wider spectrum of light and longer duration of illumination showed a faster suppression of melatonin in plasma than illumination with the tight spectrum and the short illumination time.

6.3 EFFECT OF CONTROLLED SEROTONIN-MELATONIN SYSTEM TO MILK SYNTHESIS AND EJECTION

Influencing the serotonin-melatonin-system by tryptophan supplementation (Kollmann et al., 2006) or by additional supply of sunlight around milking

(Kollmann et al., 2007b), prolactin did not show any regulation, neither during milking nor during sample taking every 3 h. Only seasonal changes in photoperiod showed a variation in plasma prolactin level (Figure 4) (Kollmann et al., 2007b).
By using the method of tryptophan supplementation, melatonin could be increased in heifers but not significantly in dairy cows. Neither in heifers nor in cows a regulation of prolactin as effect of tryptophan supplementation was observed. In humans and horses an increase of plasma prolactin concentrations was induced by infusion of tryptophan (Farris et al., 1998; Charney et al., 1982; Huether et al., 1992), because increased tryptophan availability stimulates brain serotonin synthesis (Chaouloff et al., 1989) and the increased brain serotonin is connected with a prolactin release (Farris et al., 1998; Jorgensen et al., 1992; Kahn and Wetzler, 1991; Van de Kar, 1991). Although plasma tryptophan concentration could be increased with the method of tryptophan supplementation (Kollmann et al., 2006), plasma prolactin was not affected in bovine species. With the sampling interval of 3 h a circadian rhythm could not be confirmed as found by others (Zinn et al., 1986; Madej et al., 1985; Mollett and Malven, 1982; Koprowski et al., 1972; Bines et al., 1983; Lefcourt et al., 1994). But a circadian rhythm was also not found by Fulkerson et al. (1980) although the sampling interval was shorter than in our study. Prolactin increased towards the end of milking in our studies (Kollmann et al., 2006; Kollmann et al., 2007b) same as others (Forsling et al., 1974; Schams and Karg, 1970) and decreased again after milking (Kollmann et al., 2007b). Prolactin was also reported to be under control of photoperiod (Schams and Reinhardt, 1974; Auchtung et al., 2005) that could be confirmed with study II. During summer prolactin concentration was significantly higher than during winter at morning and evening milking. In our experiment prolactin release during milking was influenced by photoperiod in contrast to Peters et al. (1981). But artificial sunlight before and during milking (UVMO, UV and UVIR) did not influence prolactin concentrations. During the last minute of milking, prolactin was significantly higher in UVIR treatment than in the others, but in all an effect of treatment to the level of prolactin could not be proven. Possibly prolactin can only be influenced by changing photoperiod or by pharmacological doses of melatonin (Auldist et al., 2006). Furthermore the prolactin level was during the treatment with artificial sunlight relatively high because of the season of experiment. The study was performed in April/May, whereat the days in our latitude are increasing from

21.12 to 21.6 of the year. Possibly an additional increase in prolactin concentration is not possible. With our studies, we could not show an inhibitory effect of melatonin on prolactin synthesis as others (Juszczak and Stempniak, 1997). An effect of illumination to prolactin independent of melatonin was also not observed.
Oxytocin was partly influenced by the modified serotonin-melatonin-system (Kollmann et al., 2006; Kollmann et al., 2007b). By the method of light control an effect was observed, but not by the method of tryptophan supplementation. Melatonin was not significantly increased with tryptophan supplementation in cows (Kollmann et al., 2006), thus possibly the effect to oxytocin cannot be seen. But an increase of oxytocin release during milking (Bruckmaier et al., 1992; Bruckmaier et al., 1993; Bruckmaier, 2005; Bruckmaier and Blum, 1998) could be stated with both studies (Kollmann et al., 2006; Kollmann et al., 2007b). Furthermore, different photoperiods did not show any effect in oxytocin pattern during milking (Kollmann et al., 2007b) which let to the conclusion that oxytocin was not regulated by season. With specific light supply a modification of oxytocin was observed (Figure 4). Additional ultraviolet light during milking (UVMO) increased oxytocin concentration (AUC/min) significantly. This effect seems to be a short-term effect, because with longer lasting illumination, i.e. additional illumination 10 min before milking (UV), oxytocin blood pattern was nearly as low as with control (C) treatment. The effect was also not observed, when additional infrared light was used 10 min before milking and during milking (UVIR). This AUC was similar to control treatment (Kollmann et al., 2007b). Oxytocin concentration is supposed to be under control of light, because in rats oxytocin increases during daylight and decrease during the night (Windle et al., 1992). In cows it was also reported that more oxytocin is released during light period than during night in well illuminated barns (Macuhova and Bruckmaier, 2004). Furthermore a visual stimulus during sucking inhibits milk ejection in rats (Prilusky and Deis, 1982). This was not observed in rats kept under complete darkness or in which the visual stimulus shone continuously, what let suppose that the inhibition is produced by the light on-off sequence (Prilusky and Deis, 1982). Because of the fact, that cows in contrast to rats are diurnal species which have their active period during daytime and suckle primarily during day, whereas rats are nocturnal species which are active and suckle primarily during night, the visual stimulus resulted in the opposite effect. It stimulated oxytocin release, that was shown with the increased AUC of

oxytocin after ultraviolet light treatment during milking (Kollmann et al., 2007b). The concentration of oxytocin with the treatment including lighting 10 min before milking, possibly acted as the continuous lighting; the animals were used to, and thus the concentration converged to the level as without treatment. Melatonin is not able to explain the light effect, because its effect occurred only for a short term after lighting as observed in rats (Prilusky and Deis, 1982). It seems to be independent of melatonin. Light could be mediated via the suprachisamatic nucleus. Possibly it is projected via the suprachiasmatic nucleus, the major oscillator, to the supraoptic nucleus, which synthesises oxytocin (Saeb-Parsy and Dyball, 2004). Oxytocin neurons in rats were excited by stimulation of the suprachiasmatic nucleus, partly inhibitory, partly stimulatory and partly in a complex manner (Saeb-Parsy and Dyball, 2004). Because of the oppositional effect of oxytocin to light stimulus in rats, the reaction in cows could deviate from this in rats. For evaluation of the exact mechanism, how light affects oxytocin release and thus milk ejection especially in cattle, more studies are necessary.

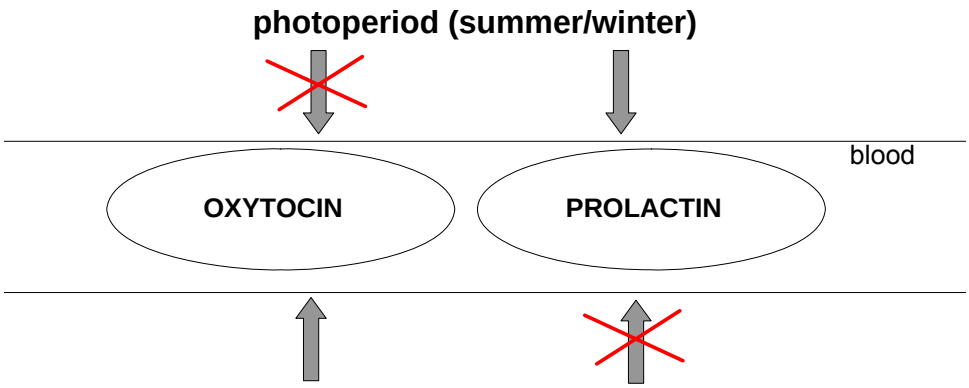


Figure 4: Effect of artificial sunlight during milking and photoperiod to oxytocin and prolactin in dairy cows

Altough oxytocin could be increased at least with lighting, with no method total milk yield and milkability factors (PFR, AFR, tPFR) could be increased (Kollmann et al., 2006; Kollmann et al., 2007b). With the method of tryptophan supplementation a slight increase of morning milk yield was observed (Kollmann et al., 2006). Because of higher melatonin levels during night compared to daytime, possibly the

effect in morning milking was higher as in evening milking and thus milk yield increased during the morning. An effect of increased oxytocin release occurred possibly during morning milking. That the increased oxytocin level in study II did not show any effect in total milk yield, could be due to the fact that the milking took place under optimal conditions. Oxytocin has to increase for complete milk ejection only over a threshold level (Bruckmaier et al., 1994; Bruckmaier et al., 1996; Schams et al., 1984) and is not limiting under good milking conditions. Oxytocin can be limiting in some situations as in milking stressed cows in unfamiliar surrounding (Bruckmaier et al., 1993). This can depress oxytocin release and thus inhibit milk ejection and reduce milk yield. Possibly additional lighting during milking in cows with suppressed milk ejection would attenuate the disturbance of milk ejection.

Cortisol is reported to be unaffected by season (Auchtung et al., 2005; Peters et al., 1981; Zinn et al., 1986) that we observed, too. Photoperiod did not affect cortisol release during milking and neither did illumination with artificial sunlight during milking. The UVMO, UV and UVIR treatment showed no significant difference compared to the C treatment. The course of cortisol during milking was increasing (Kollmann et al., 2007b) same as at others (Gorewit et al., 1992; Bruckmaier et al., 1993). By studying the influence of photoperiod, cortisol release was higher during morning than during evening milking. This could derive from the circadian rhythm of cortisol. Its concentrations are high from mid-night to mid morning and lowest during afternoon (Fulkerson et al., 1980). Possibly the circadian rhythm is affected and under control of light. By illumination of the cows with artificial sunlight during milking a difference in cortisol concentration between morning and evening milking could not be shown. The illumination with the same treatment during morning and evening milking possibly suppresses the difference, because the illuminance and the wave length were not different during the daytimes than by utilization of the natural photoperiod.

6.4 EFFECT IN MILK

Melatonin was detected in bovine milk (Kollmann et al., 2006; Kollmann et al., 2007a) as reported in human (Illnerova et al., 1993) and rat milk (Rowe and Kennaway, 2002). During daytime no melatonin was measurable in milk in

accordance with others (Illnerova et al., 1993; Rowe and Kennaway, 2002). Only in milk samples of twice daily milking melatonin was detected during day (Kollmann et al., 2006; Kollmann et al., 2007a). Milk melatonin concentration showed a diurnal rhythm in cattle (Kollmann et al., 2007a) and other species (Rowe and Kennaway, 2002; Illnerova et al., 1993). The rhythm was similar to that in blood. Melatonin concentration in blood and milk increased when illuminance decreased, and decreased rapidly after the onset of daylight. A regression ($P<0.01$) of blood and milk melatonin in dependence of the time could be calculated with $y=0.796x-2.879$ and $y=0.123x-0.680$ (blood and milk concentration respectively). Milk melatonin concentrations were influenced by blood concentrations. A correlation of milk and blood melatonin concentration could be calculated ($r=0.256$, $P<0.001$). An even higher correlation with $r=0.288$ ($P<0.01$) was found between blood melatonin concentration and total amount of melatonin in milk. Although blood melatonin reflects the situation only during blood sampling, and milk melatonin reflects the situation of the whole time since the last milking, at hourly sampling the correlation was high. A linear regression for milk melatonin concentration in dependence of blood concentration was found ($y=0.047x+0.606$, $P<0.01$) like for total amount of melatonin in milk in dependence of blood concentration ($y=55.9x+586.3$, $P<0.01$). The correlations together with the regression lines let suggest that melatonin is migrating from the bloodstream into the milk. This indicates that the transfer is not active, but is rather migrating by passive diffusion from the bloodstream. This is supported by the amphiphilic character of melatonin. Melatonin can freely diffuse through biological membranes (Vanecek, 1998).

At twice daily milking beginning at 0415 h and 1545 h none of our used methods did influence the milk melatonin concentration (Kollmann et al., 2006; Kollmann et al., 2007a). Neither with tryptophan supplementation nor with variation of the photoperiod (summer vs. winter) milk melatonin concentration could be increased. Tryptophan supplementation did not increase melatonin plasma concentration in cows, thus it did not influence melatonin milk levels significantly in twice daily milking. Nevertheless, after 7 days of supplementation, the concentration of milk melatonin was significantly higher in morning milking of supplemented cows than in control cows (Kollmann et al., 2006).

The season, i.e. the photoperiod, did also not influence melatonin level in the milk during normal milking. There was no significant difference between melatonin milk concentration in June/July and in December in twice daily milking at 0415 h and 1545 h. The means of melatonin milk concentration were 5.3 ± 0.6 and 2.4 ± 0.4 pg/ml in June and 4.2 ± 0.6 and 2.0 ± 0.5 pg/ml in December (morning and evening milking respectively). In other species in plasma the duration of the nightly melatonin peak was longer (Vanecek, 1998) and the amplitude was higher (Zarazaga et al., 1998; Brainard et al., 1982; Garidou et al., 2003; Garcia et al., 2003) in short day photoperiod than in long day photoperiod. Thus in winter a higher amount of melatonin should be produced. In milk, only a small amount of blood melatonin is found. About 35 % of blood melatonin are observed in human milk (Illnerova et al., 1993) and about 40 % were observed in our study (Kollmann et al., 2007a) in bovine milk, but with a high variation. Thus possibly the differences in milk are marginal and were overlapped by the individual variation, which was high and possibly derives from the high heritability (h^2=0.45) of melatonin (Zarazaga et al., 1998).

Milk composition is reported to be different in different fractions (Ontsouka et al., 2003; Sarikaya et al., 2005). Fat content increases continuously towards the end of milking and also a variation of protein content was found in different milk fractions (Sarikaya et al., 2005; Ontsouka et al., 2003). Melatonin concentration showed no variation in different milk fractions of fore milk, main milk fraction and residual milk. The melatonin concentration stayed constant during the milking with means of 6.9 ± 1.8, 7.7 ± 2.1 and 6.1 ± 1.9 pg/ml (fore milk, main milk fraction and residual milk respectively). The stability in concentration of melatonin in milk fractions possibly derives from the fact that 80 % of melatonin remained in the water phase (Illnerova et al., 1993).

Melatonin levels in milk of all experiments were lower than usual pharmacological doses. The NOEL in human was found to be 0.04 mg/kg body weight (European Agency for the Evaluation of Medicinal Products, 2007) – an amount far below any possible uptake from milk consumption. Thus an effect to human seems unlikely.

7 CONCLUSIONS

The serotonin-melatonin-system can be influenced by the different methods.

All tested methods could at least partially influence the serotonin-melatonin-system, but not all the methods were appropriate to evaluate the effect of the serotonin-melatonin-system to milk ejection and synthesis in dairy cows. Thus the method of additional lighting around milking seems to be the best method to influence the serotonin-melatonin-system for this purpose, because tryptophan supplementation did not influence melatonin concentration in dairy cows, only in heifers, and the variation in photoperiod by utilization of the natural season differences was not able to influence oxytocin and cortisol, possibly because of marginal differences of the serotonin-melatonin-system under the given conditions. Nevertheless, with no method an increase of total milk yield was achievable. Possibly the milking conditions in our experiment were optimal and oxytocin passes the threshold level anyway and is not limiting. But under suboptimal milking conditions or in stressed cows as after moving to unfamiliar surrounding oxytocin release can be limiting. An inhibition of milk ejection can occur and limit milk ejection and consequentially reduce milk yield. Possibly lighting with artificial sunlight can be used as management tool to attenuate the milk ejection inhibition by increasing the amount of oxytocin release and pass the threshold level. The other methods, variation in photoperiod or supplemental tryptophan supply have no effect on oxytocin and thus on milk ejection.

Melatonin could be also detected in bovine milk and showed a diurnal rhythm in milk similar to that in plasma. The correlation and the regression of milk melatonin in dependence of melatonin in plasma together with is amphiphilic character let conclude a passive transfer of melatonin from bloodstream into milk by diffusion.

Compared to pharmacological doses of melatonin, and to the NOEL in human, the concentration in milk is much lower. Thus an effect to human seems unlikely.

Milk melatonin concentrations are not influenceable at twice daily milking by any of these methods and not different in the various milk fractions. Thus the best method to influence the melatonin level in milk would be the optimization of the milking time.

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9 CURRICULUM VITAE

Name:	Maria Theresia Kollmann
Date of Birth:	05.10.1979
Place of Birth:	Krumbach (Schwaben)
Family Status:	unmarried

School education

1986-1990	Elementary School Ichenhausen
1990-1999	Gymnasium Krumbach (Schwaben)

Vocational training

1999-2004	study of agricultural science, Technical University Munich
1/2004	Graduation with Diploma (Dipl.-Ing. agr)
Since 3/2004	PhD student at Physiology Weihenstephan, Technical University Munich

Practical work

7/99-9/99	Agricultural Practice: Gut Unterwaldbach/Jettingen-Scheppach
03/01-04/01	Practice: Besamungsverein Nordschwaben e.V., Höchstädt
04/02-09/02	Agricultural Practice: Kratzhof, Harburg

10 SCIENTIFIC COMMUNICATIONS

Original publications printed or in „press“

M. Rovai, M. T. Kollmann, and R. M. Bruckmaier. 2007, Incontinentia lactis: physiology and anatomy conducive to milk leakage in dairy cows. Journal of Dairy Science. Feb; 90 (2): 682-90

M.T. Kollmann, M. Locher, F. Hirche, K. Eder, H.H.D. Meyer, R.M. Bruckmaier. 2006, Effects of tryptophan supplementation on plasma tryptophan and related hormone levels in heifers and dairy cows, Domestic Animal Endocrinology, Article in press

M.T. Kollmann, H.H.D. Meyer R.M. Bruckmaier. 2007, Short term and long term light effects on the release of oxytocin, prolactin and cortisol in dairy cows, submitted to Journal of Dairy Research

Original publications in preparation

M.T. Kollmann, V. Lollivier, S. Richter, O. Wellnitz, R.M. Bruckmaier. 2007, Twenty four hour pattern of melatonin in blood and milk of dairy cattle: ready for submission

M.T Kollmann., M. Rovai, R.M. Bruckmaier: 2006, Incidence of cows leaking milk in German dairy farms. in preparation for submission to Journal of Veterinary Medicine Series A-Physiology Pathology Clinical Medicine

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11 APPENDIX

Appendix I

M.T. Kollmann, M. Locher, F. Hirche, K. Eder, H.H.D. Meyer, R.M. Bruckmaier. 2006, Effects of tryptophan supplementation on plasma tryptophan and related hormone levels in heifers and dairy cows, Domestic Animal Endocrinology, Article in press

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Domestic Animal Endocrinology xxx (2006) xxx–xxx

DOMESTIC ANIMAL ENDOCRINOLOGY

www.journals.elsevierhealth.com/periodicals/dae

Effects of tryptophan supplementation on plasma tryptophan and related hormone levels in heifers and dairy cows

M.T. Kollmann[a], M. Locher[a], F. Hirche[b], K. Eder[b], H.H.D. Meyer[a], R.M. Bruckmaier[c,*]

[a] *Physiology Weihenstephan, Technical University Munich, Weihenstephaner Berg 3, D-85350 Freising, Germany*
[b] *Institute of Nutritional Sciences, Martin-Luther-University Halle-Wittenberg, Emil-Abderhalden-Strasse 26, D-06108 Halle/Saale, Germany*
[c] *Veterinary Physiology, Vetsuisse Faculty, University of Bern, Bremgartenstrasse 109a, CH-3001 Bern, Switzerland*

Received 6 July 2006; received in revised form 25 September 2006; accepted 27 September 2006

Abstract

This study was conducted to investigate the effects of rumen-protected tryptophan (125 g tryptophan per day) in heifers and dairy cows. Blood samples from dairy cows and heifers were collected for 24 h in 3-h intervals on the day before tryptophan supplementation, on day 2, 5 and 7 of tryptophan supplementation, and in heifers additionally on d 14 after tryptophan supplementation was ceased. Plasma tryptophan, melatonin, serotonin, and prolactin concentrations were determined. Tryptophan plasma concentrations on d 5 were augmented at day (11:00 h) and nighttime (02:00 h), ($P < 0.05$) in response to tryptophan supplementation in heifers by 119% and in dairy cows by 47%, respectively, as compared with d 0. Melatonin increased ($P < 0.05$) in response to tryptophan supplementation in heifers, but not in cows. The effect of tryptophan supplementation on plasma tryptophan and melatonin was reversible as demonstrated in heifers on d 14 after cessation of tryptophan supplementation. Serotonin and prolactin in plasma did not respond to tryptophan supplementation. However, milk yield during morning milking increased significantly in tryptophan supplemented cows on d 1, 3 and 4 as compared to the day before tryptophan supplementation.

Additional blood samples were taken during afternoon milking in cows at 1-min intervals for the analyses of oxytocin and prolactin on the day before the start and on d 7 of tryptophan supplementation. Milk flow curves were recorded during milking. No effect of tryptophan supplementation on the milking related release of oxytocin and prolactin and on any characteristic of milk flow was observed. In conclusion, tryptophan supplementation caused increased plasma tryptophan in cows and heifers and plasma melatonin in heifers. However, plasma serotonin, prolactin and oxytocin release in cows remained unchanged by tryptophan supplementation. Milk yield at morning milking increased slightly and transiently in response to tryptophan supplementation.

Keywords: Melatonin; Tryptophan; Serotonin; Prolactin; Dairy cow

Abbreviations: TRP, tryptophan; MEL, melatonin; 5-HT, serotonin; PRL, prolactin; OT, oxytocin; AUC, area under the curve; TMY, total milk yield; AFR, average flow rate; PFR, peak flow rate; d 0, day before tryptophan supplementation

* Corresponding author. Tel.: +41 31 631 2324; fax: +41 31 631 2640.
E-mail address: rupert.bruckmaier@physio.unibe.ch (R.M. Bruckmaier).

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doi:10.1016/j.domaniend.2006.09.005

Please cite this article in press as: Kollmann MT et al., Effects of tryptophan supplementation on plasma tryptophan and related hormone levels in heifers and dairy cows, Domest Anim Endocrinol (2006), doi:10.1016/j.domaniend.2006.09.005

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1. Introduction

The amino acid tryptophan (TRP) is the substrate for the biosynthesis to serotonin (5-hydroxytryptamine, 5-HT) and melatonin (MEL). Free plasma TRP penetrates through the blood-brain barrier into the brain and is enzymatically converted in the pinealocytes via 5-hydroxytryptophan to 5-hydroxytryptamine. 5-HT is converted by *N*-acetlytransferase via *N*-acteylserotonin to melatonin (*N*-acetyl-5-methoxytryptamine, MEL). Several factors in this pathway are potentially limiting for the synthesis of 5-HT and MEL. 5-HT synthesis is influenced by three factors: the plasma concentration of free plasma TRP, the transfer rate of free TRP through the blood-brain barrier, and the activity of the TRP hydroxylase [1]. Under normal physiological conditions the Michaelis-Menten constant for TRP hydroxylase is several times higher than the TRP concentration in the brain; hence the activity of this enzyme is not saturated in vivo [2]. The efficiency of TRP transfer from plasma into the brain depends also on the concentration of six other large neutral amino acids (tyrosine, phenylalanine, leucine, isoleucine, valine, and methionine) [3,4] because TRP competes with these amino acids for the neutral amino acid-carrier. It is commonly assumed that most amino acids including tryptophan are not deficient in ruminants, because they are synthesized by ruminal microbes. The major part of protein, entering the small intestine, has been synthesized de novo in the rumen [5]. However, a very low protein ration can cause a limitation of amino acid output from the rumen [6]. For maximum production of milk and milk protein in dairy cows microbial protein synthesis is not sufficient [7] and dietary ruminal escape protein is important. In cattle methionine and lysine are considered to be limiting for milk protein and fat synthesis at high milk production levels [8,9]. Their supplementation increases milk fat [8,10] and milk protein synthesis [8]. Thus, we hypothesized that TRP could potentially be limiting, if not for milk protein synthesis then at least for the synthesis of 5-HT and MEL in the brain. Because corn contains very little tryptophan [11,12], especially rations with a high corn content, as used in the present study, may cause a tryptophan deficiency. The rationale of this study was to investigate if a supplementation with rumen-protected TRP induces an elevation of plasma TRP and hence the plasma concentration of MEL and/or 5-HT. Because of the influence of MEL on many endocrine systems TRP supplementation might also influence the concentration of hormones related to milk synthesis (prolactin (PRL)) and milk removal (oxytocin (OT)). MEL was reported to inhibit PRL synthesis [13]. An effect on OT concentrations was reported in rats, either inhibitory [14,15] or stimulatory [13,16] possibly depending on the concentration level of MEL.

2. Materials and methods

2.1. Animals

Twelve nonpregnant Brown Swiss heifers (body weight: 536 ± 13 kg, age: 22 ± 3 mo) and 12 lactating Brown Swiss primiparous ($n = 3$; treated = 1, control = 2) and multiparous ($n = 9$; treated = 5, control = 4) cows were used in the experiments. The heifers were kept in a tie stall barn and fed corn silage and hay ad libitum. The cows were kept in loose housing and were 154 ± 28 days in milk with a daily milk yield of 35 ± 2 kg at the start of the experiments. They had free access to a mixed ration providing energy and other nutrients to cover the demands for maintenance and milk production. The diet for cows included 40% corn silage, 14% grass silage, 10% hay and 36% concentrate (dry matter basis). The ration had an energy content of 138 MJ net energy lactation (NEL)/d, 3225 g nXP (utilizable crude protein)/d and the content of undegraded protein was 25%.

Cows were milked twice daily at 0415 and 1545 h in a 2 × 2 tandem milking parlor equipped with Stimopuls clusters (WestfaliaSurge GmbH, Oelde, Germany). Milk yield was measured via a strain gauge system, as previously described [17]. Proportional milk samples were taken during morning and evening milkings on all experimental days. Samples were immediately stored at −20 °C for further analyses.

2.2. Experimental design

Rumen-protected TRP (with 25% TRP, Nutreco, Bussolengo, Italy) was administered by gavage to six heifers and six lactating cows twice daily in portions of 250 g each at 07:00 and 18:00 h for 7 d. Thus, the animals received 125 g additional TRP per day. The remaining six cows and six heifers served as controls. Control animals received water via the same procedure as for TRP administration in the treated animals. The experiment was carried out in June and July in heifers and in August and September in cows. Blood samples were taken in 3-h intervals between 08:00 and 05:00 h on the day before TRP supplementation (d 0) and on d 2, 5 and 7 of TRP supplementation. In heifers, blood samples were also taken on d 21, i.e. 2 weeks after TRP supplementation was ceased.

Blood samples (10 ml) were taken via a permanent catheter in the jugular vein (Cavafix Certo Splittocan

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335, length 32 cm, diameter 1.8 × 2.35 mm, Braun, Melsungen, Germany) which was inserted on the day before the start of experiments. Blood was anticoagulated with EDTA and cooled on ice until centrifugation at 2000 × *g* for 15 min at 4 °C. Plasma was aliquoted and stored at −20 °C until used in MEL, 5-HT and PRL assays. Blood samples for TRP analysis were taken at 11:00 h during daytime and at 02:00 h during the night. These samples were processed similar to the method of Teerlink et al. [18] except for the usage of EDTA instead of heparin as an anticoagulant. 400 μl of plasma were transferred into cups with 100 μl 5-sulfosalicylic acid (10%), frozen in liquid nitrogen and thereafter stored at −80 °C.

To prevent effects of lighting on the pineal activity, all blood samples during night were taken only with a small head lamp and direct illumination of the animals eyes was avoided.

Illuminance in the barn was measured before each blood sampling by using a luxmeter (digital Luxmeter, Peak Tech® 5020, Ahrensburg, Germany) at different positions in the barn (3 points for cows; 2 points for heifers) that represented the sojourn of the animals during the day adequately.

To test the effect of TRP supplementation on milking related OT and PRL release, the six TRP supplemented and six control cows were taken blood samples during afternoon milkings in 1-min intervals on d 0 and d 7 of TRP supplementation. The first blood sample was removed immediately before the first manual contact with the udder, followed by a sample at cluster attachment and start of a 1 min mechanical stimulation (Stimopuls, WestfaliaSurge GmbH, Germany) and thereafter at 1-min intervals until the end of milking.

2.3. Assay procedures

Plasma TRP levels were measured with a HPLC-method according to Schuster [19] using reagents as described previously [18].

Plasma MEL concentrations were measured by using a commercial ELISA-Kit (IBL Hamburg, Germany, Kat.-Nr. RE 540 21). The detection limit was 3.0 pg/ml and the intra-assay-variation was 7.5% and the inter-assay-variation was 11.3%.

Milk MEL levels were measured with the same ELISA-Kit after an optimized sample preparation. Repeatable results and highest recovery levels were achieved with skimmed milk. Skimming was performed by centrifugation at 3300 × *g* for 15 min (4 °C). Skimmed milk underwent the same extraction as the plasma samples. A repeatability coefficient of 99.88% was achieved.

OT concentrations were determined by radioimmunoassay as previously described [20].

Plasma PRL concentrations were measured by using an indirect competitive ELISA. Plates were coated with 1 μg sheep anti-rabbit IgG dissolved in ELISA buffer (with 0.75% $Na_2HPO_4 \cdot xH_2O$, 0.11% KH_2PO_4, 0.12% NaCl, 0.18% EDTA, pH 7.4) and incubated at 4 °C overnight. Then plates were washed and afterwards blocked with 250 μl blocking buffer (ELISA buffer with 0.1% BSA) for 30 min at ambient temperature. The used rabbit-anti-prolactin antibody was previously characterized and used in a radioimmunoassay by Schams and Karg [21]. The same standard solution NIH-P-B2, ranging from 0.5 to 128 ng/ml, as described previously [21,22] was used and the samples were diluted 1:10 before application in the assay. Fifty microlitres diluted samples, standards and 100 μl antibody (diluted 1:320,000) were pipetted into wells and incubated overnight at ambient temperature. Thereafter, 100 μl of the prolactin-biotin-conjugate, described previously [23], diluted 1:6000, were added and incubated for 30 min at ambient temperature. Afterwards 20 ng streptavidinperoxidase (Sigma) diluted in 100 μl ELISA buffer were added and another incubation for 30 min at ambient temperature followed. Thereafter, substrate (with tetramethylbenzidine) [23] was added and the reaction stopped after 40 min with 50 μl 4N H_2SO_4. The absorbance was read at 450 nm. The intra-assay-variability was 11% and the inter-assay-variabilty was 20%. The sensitivity of the assay was 0.7 ng/ml. The additivity was 109% on average and the very good parallelism of diluted samples is shown in Fig. 1.

5-HT plasma levels were determined by using an ELISA-Kit (Beckman-Coulter, Krefeld, Germany, REF:

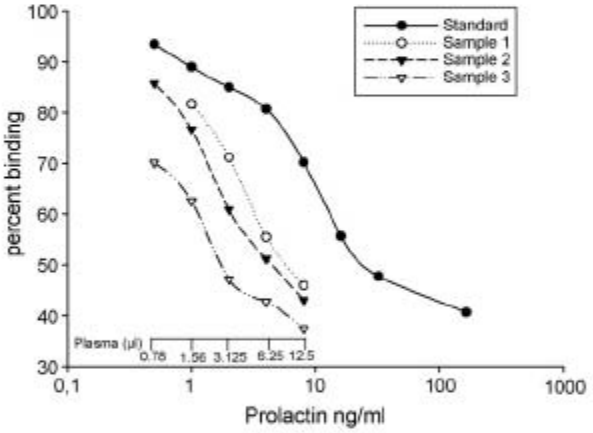

Fig. 1. Test of parallelism for bovine prolactin standards with three serially diluted bovine plasma samples containing different amounts of prolactin in the competitive ELISA for prolactin.

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1749). The samples were diluted 1:100 to be in the range of the standard curve. 5-HT was measured in samples taken at 11:00 and 02:00 h only.

2.4. Mathematical and statistical evaluations

Data in text, figures and tables are presented as means ± S.E. For statistical analysis of MEL and PRL plasma concentrations the results of each test day were divided in two areas under the curve (AUC). AUC 1 included the area between 08:00 and 17:00 h and AUC 2 included the area between 17:00 and 05:00 h. A linear normalization of the AUC was performed by using the means of d 0.

For statistical analysis of OT and PRL during milking AUC/min was calculated. A repeated measures analysis of variance was performed by using the MIXED procedure of the SAS program package (SAS Institute, version 9.1). The repeated subject was the animal. Significant differences ($P<0.05$) between means, also between heifers and cows, were localized by using Bonferroni's t-test based on least square means.

Regression analyses (PROC REG) were used to develop models that could explain the influence of TRP and 5-HT to MEL plasma level or of plasma MEL to the MEL level in milk.

3. Results

Pretreatment TRP plasma concentrations were higher in cows than in heifers ($P<0.01$) (Figs. 2 and 3). They did not significantly differ between day (11:00 h) and night (02:00 h) neither in heifers nor in cows.

TRP supplementation caused an increase of plasma TRP levels in heifers (Fig. 2b) and in cows (Fig. 3b) ($P<0.05$) both at daytime and nighttime. In heifers a significant increase was first observed at 02:00 h on d 2. On d 5 and 7, the increase compared with d 0 was higher during night than at daytime. The TRP concentrations on d 21, i.e. 14 d after cessation of TRP supplementation, were not different as compared to d 0, i.e. the effect of TRP supplementation was reversible. In control heifers TRP concentrations remained on a similar level on all experimental days at daytime as well as at nighttime (Fig. 2a).

In cows TRP supplementation induced an augmentation of plasma TRP by 43% which was first observed at 11:00 h on d 2. On d 5 and d 7, the TRP concentration during daytime was by 35 and 19% and during nighttime by 47 and 39% ($P<0.05$) higher than on d 0, respectively. Similar as in heifers, the TRP levels in control

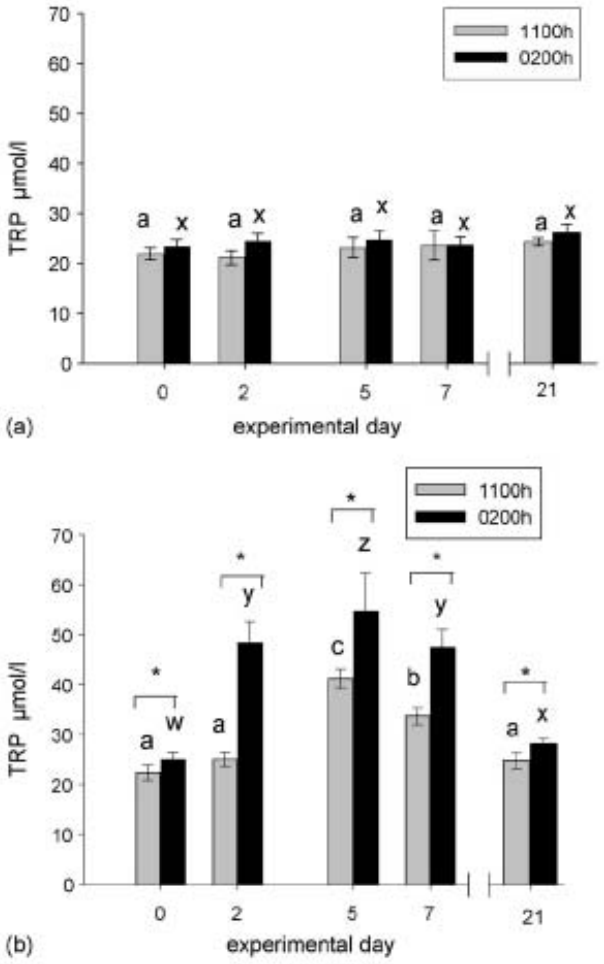

Fig. 2. Tryptophan (TRP) concentration in plasma (µmol/l) of control heifers (a) and tryptophan supplemented heifers (b) at 11:00 h (gray) and 02:00 h (black) on the day before tryptophan supplementation (day 0), day 2, 5 and 7 of tryptophan supplementation and on day 14 after cessation of tryptophan supplementation. Data are presented as means ± S.E. Means within a time point without common superscripts are significantly different ($P<0.05$). Asterisks indicate differences between the time points within the experimental day ($P<0.05$).

cows remained unchanged during the entire experiment (Fig. 3a).

On all experimental days, plasma levels of TRP of the treated animals were higher ($P<0.05$) at night than at daytime except in cows on d 2.

During the experiment light intensity decreased after sampling at 17:00 h and during sampling at 20:00 h there were always less than 20 lux. Light intensity started to increase at around 05:00 h and values were around 60 lux at this time. With decreasing light MEL increased and vice versa.

The MEL plasma levels in control heifers were numerically lower than in TRP supplemented heifers on d 0 during daytime (AUC 08:00–17:00 h) (Fig. 4a) and

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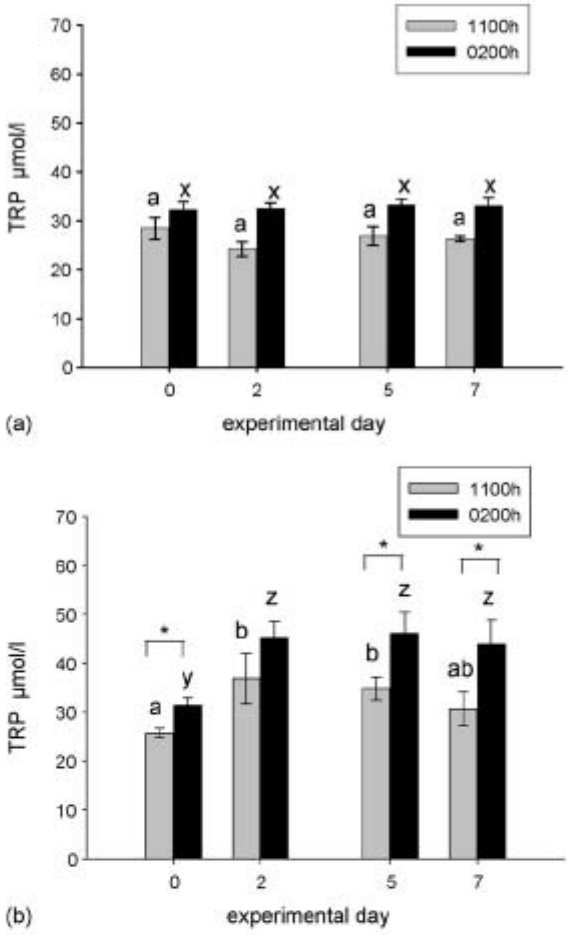

Fig. 3. Tryptophan (TRP) concentration in plasma (µmol/l) of control cows (a) and tryptophan supplemented cows (b) at 11:00 h (gray) and 02:00 h (black) on the day before tryptophan supplementation (day 0), day 2, 5 and 7 of tryptophan supplementation. Data are presented as means ± S.E. Means within a time point without common superscript are significantly different ($P < 0.05$). Asterisks indicate differences between the time points within the experimental day ($P < 0.05$).

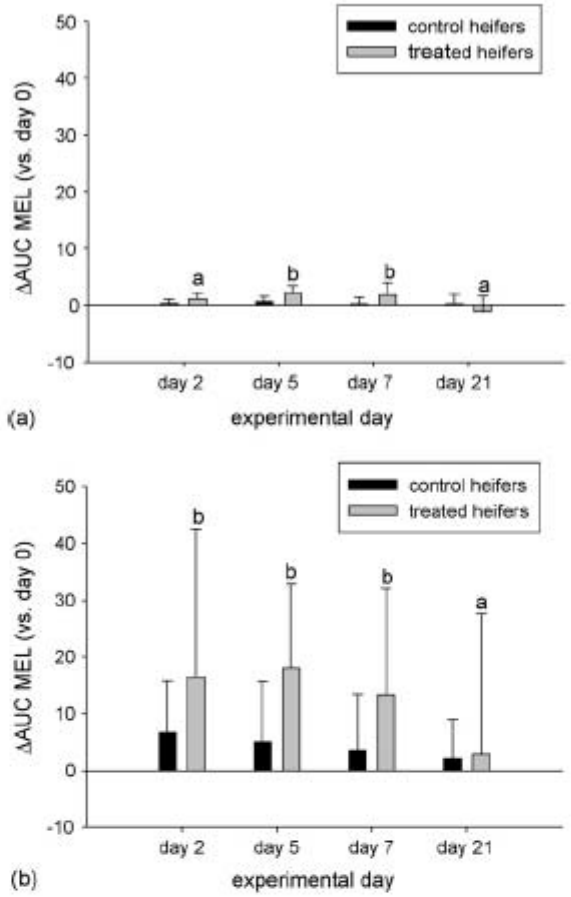

Fig. 4. Melatonin concentration (AUC per 3 h in pg/ml × 3 h) in plasma of control heifers (black) and tryptophan supplemented heifers (gray) during day time (a) and nighttime (b) on day 2, 5 and 7 of tryptophan supplementation and day 14 after tryptophan supplementation was ceased expressed relative (linear corrected) to the day before supplementation (Δ AUC MEL). Means within the treatment group without common superscripts are significantly different ($P < 0.05$).

during night (AUC 17:00–05:00 h) (Fig. 4b). MEL levels varied considerably between animals, most obvious during the night. In heifers the AUC of MEL on d 0 during night ranged from 2.4 to 36.5 pg/ml × 3 h.

In heifers, MEL concentration increased during the course of experiment in response to TRP supplementation. During the night, plasma MEL concentrations on d 2, 5 and 7 were higher ($P < 0.05$) than on d 0. The MEL level on d 21 (14 d after cessation of the TRP supplementation) was lower than during the period of TRP supplementation and did not significantly differ from d 0. Also during daytime a weak but significant ($P < 0.01$) increase of MEL in response to TRP supplementation was observed on d 5 and 7. In control heifers there was no change in MEL concentration during daytime or during nighttime during the entire experimental period.

In cows, no significant changes of MEL concentration occurred in response to the TRP supplementation neither during day (Fig. 5a) nor during night (Fig. 5b). MEL was also measured in milk samples (Table 1). With TRP supplementation it was not possible to generally increase milk MEL levels. There were no overall differences between the experimental days and the treatments with respect to milk MEL levels. In controls and TRP treated cows the level of MEL in milk did not change. However, on d 7 of experiment MEL in milk was significantly higher in treated cows as compared to controls. MEL levels were significantly higher in the milk obtained at morning milking (start at 04:15 h) as compared to evening milking (start at 15:45 h). Across all treatments

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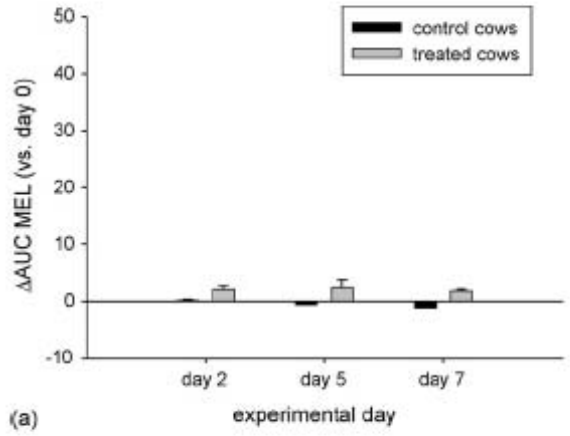

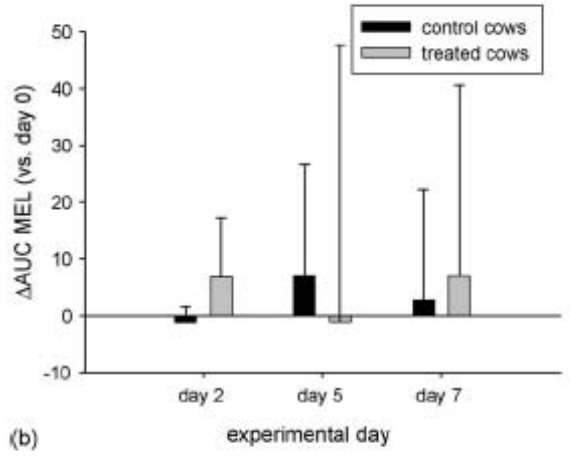

Fig. 5. Melatonin concentration (AUC per 3 h in pg/ml × 3 h) in plasma of control cows (black) and TRP supplemented cows (gray) during day time (a) and nighttime (b) on day 2, 5 and 7 of tryptophan supplementation expressed relative (linear corrected) to the day before supplementation (Δ AUC MEL). Means within the treatment group without common superscripts are significantly different ($P<0.05$).

and days, milk MEL levels showed a significant correlation of $r=0.39$ ($P<0.001$) to MEL plasma concentration (AUC). This correlation was closer in cows with more than 200 days in milk (0.58, $P<0.001$) than in cows with less than 200 days in milk (0.32, $P<0.01$).

Levels of 5-HT varied considerably between individuals and groups, both before and during the treatment

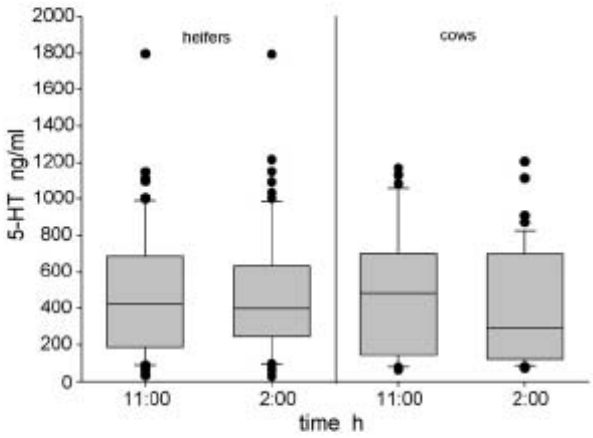

Fig. 6. 5-HT plasma concentration (ng/ml) at 11:00 and 02:00 h across all treatment days and treatments in heifers and in dairy cows. Mean concentration within animals category and time is given by the horizontal line in the grey boxes.

period (Fig. 6). At 11:00 h on d 0 the average 5-HT concentrations were 241 ± 71, 799 ± 267, 334 ± 81 and 352 ± 126 ng/ml in control heifers, treated heifers, control cows and treated cows, respectively, and no effect of TRP supplementation on 5-HT plasma levels was detected. As expected, there was also no significant correlation between 5-HT and TRP or between 5-HT and MEL, respectively.

Concentrations of PRL were lower in heifers than in cows (Table 2), but did not change in response to the TRP treatment neither in heifers nor in cows. However, PRL varied significantly between experimental days in heifers and cows and in treated and control animals (Table 2). In heifers, the PRL levels were significantly higher during nighttime as compared to daytime in treated and in control animals. This difference was also observed in control cows ($P<0.05$), but not in TRP treated cows.

Milking characteristics (average flow rate (AFR), peak flow rate (PFR) and the time of plateau) did not differ between treatment groups on any of the experimental days (data not shown). Daily milk yield was not significantly affected by TRP supplementation. However, total

Table 1
Melatonin (MEL) concentration in milk during morning and evening milking in tryptophan (TRP) supplemented cows and control cows on day 0, 2, 5 and 7 of experiment. No statistical differences were found

Melatonin (MEL) (milk) pg/ml	Tryptophan (TRP) supplemented group ($n=6$)				Control group ($n=6$)			
Experimental day	0	2	5	7	0	2	5	7
Milking								
Morning	8.3 ± 2.0	9.4 ± 2.8	8.9 ± 2.2	10.5 ± 2.5	6.3 ± 1.0	6.4 ± 1.2	6.7 ± 0.9	3.9 ± 1.2
Evening	3.1 ± 0.7	3.4 ± 0.7	3.6 ± 0.3	4.0 ± 0.5	3.0 ± 1.1	4.4 ± 0.7	1.8 ± 0.7	2.8 ± 0.9

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Table 2
Prolactin (PRL) concentrations in plasma (AUC per 3 h) during daytime (08:00–17:00 h) and during nighttime (17:00–05:00 h) in tryptophan (TRP) supplemented and control heifers and cows on day 0, 2, 5, 7 and 14 days after tryptophan supplementation was ceased (d 21, only heifers)

Prolactin (PRL)		Tryptophan (TRP) supplemented group					Control group				
Experimental day		0	2	5	7	21	0	2	5	7	21
Cows											
AUC day	ng/ml × 3 h	127 ± 30^{a}	113 ± 26^{ab}	103 ± 28^{ab}	103 ± 26^{b}	–	108 ± 33^{a}	108 ± 38^{a}	101 ± 38^{a}	85 ± 37^{a}	–
AUC night	ng/ml × 3 h	101 ± 23^{a}	112 ± 25^{a}	107 ± 29^{a}	103 ± 23^{a}	–	146 ± 46^{ab}	161 ± 62^{b}	159 ± 61^{b}	99 ± 34^{a}	–
Heifers											
AUC day	ng/ml × 3 h	64 ± 11^{ab}	60 ± 6^{ab}	53 ± 3^{b}	75 ± 16^{a}	75 ± 11^{a}	57 ± 7^{a}	61 ± 8^{a}	63 ± 10^{a}	75 ± 9^{a}	62 ± 12^{a}
AUC night	ng/ml × 3 h	72 ± 10^{b}	81 ± 8^{ab}	71 ± 5^{b}	97 ± 14^{a}	90 ± 23^{ab}	59 ± 8^{a}	76 ± 12^{ab}	79 ± 11^{ab}	91 ± 11^{b}	94 ± 15^{b}

Means in the same row with different superscripts (a, b) differ significantly ($P \leq 0.05$).

Table 3
Total milk yield during the whole experiment in tryptophan (TRP) supplemented cows and control cows

Experimental day			0	1	2	3	4	5	6	7
Total milk yield (kg)	Morning	Tryptophan supplemented	16.9 ± 2.0^{ac}	18.4 ± 1.8^{bc}	17.8 ± 1.8^{abc}	18.9 ± 2.2^{bc}	18.3 ± 2.5^{bc}	18.7 ± 2.1^{abc}	18.0 ± 2.0^{ab}	17.9 ± 2.0^{ab}
		Control	17.5 ± 1.4^{a}	16.6 ± 1.1^{ab}	16.3 ± 1.2^{ab}	16.3 ± 1.3^{ab}	17.8 ± 1.4^{ab}	16.4 ± 1.6^{ab}	16.1 ± 0.8^{b}	16.7 ± 1.0^{ab}
	Evening	Tryptophan supplemented	16.5 ± 1.5^{a}	15.7 ± 1.9^{a}	15.4 ± 1.6^{a}	15.4 ± 1.5^{a}	15.1 ± 2.0^{a}	17.8 ± 2.6^{a}	16.3 ± 2.2^{a}	16.1 ± 1.9^{a}
		Control	14.5 ± 1.3^{ab}	14.4 ± 1.2^{ab}	13.7 ± 1.4^{ab}	12.9 ± 0.9^{a}	15.3 ± 1.1^{ab}	14.8 ± 1.2^{b}	15.2 ± 1.3^{b}	14.5 ± 1.6^{ab}

Means in the same row with different superscripts (a, b) differ significantly ($P \leq 0.05$).

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milk yield (TMY) at morning milking increased transiently in TRP treated animals (Table 3). TMY on d 0 was significantly lower than on d 1, 3 and 4 of experiment at morning milking. TMY on d 5, 6 and 7 was not different from that on d 0. However, on d 5 and d 6 the morning TMY in control cows was 2.3 and 3.2% lower than on d 0 ($P = 0.08$ and 0.04, respectively). The release of PRL during milking was not affected by the TRP treatment. On d 0 the milking related PRL release was similar in control and TRP treated animals (112 ± 19; 113 ± 22 ng/ml × min, respectively). On d 7, the concentration of PRL was 91 ± 28 and 103 ± 24 ng/ml × min in control and treated cows, respectively. The PRL level increased during milking and at the end of milking PRL concentration was significantly higher ($P < 0.05$) than at the start of milking.

Milking-related OT release was not affected by TRP supplementation. However, OT during milking increased significantly in all animals ($P < 0.001$). AUC of OT for treated cows was 23.2 pg/ml × min on d 0 and 18.0 pg/ml × min on d 7. In control cows, the mean was 21.5 pg/ml × min on d 0 and 20.9 pg/ml × min on d 7.

4. Discussion

It is generally assumed, although not tested in dairy cows, that the amino acid TRP is not deficient in ruminants. Amino acids are synthesized by ruminal microbes and primarily a sufficient energy supply is important for the extent of microbial protein synthesis. The ration fed to the experimental animals used in this study contained sufficient energy to fully cover the energy requirement in the respective lactational stages. The demand of amino acids is different in heifers than in cows because non-pregnant heifers need nutrients only for maintenance and growth. Because the heifers used in this study were in a late stage of development (BW 536 ± 13 kg), their demand was primarily for maintenance, whereas the major demand of nutrients in the high yielding dairy cows was used for milk production. Thus, much of the supplemented TRP was potentially used for milk production in dairy cows, causing a less pronounced effect of TRP supplementation on plasma TRP in cows than in heifers. In this study, the effect of TRP supplementation was investigated in heifers and dairy cows fed a high proportion of corn silage in the ration. It was possible to show that plasma TRP can be increased by supplementation with rumen-protected TRP. The TRP level in plasma of supplemented animals was significantly higher during night as compared to the level during day. It was previously shown in rats that the concentration of TRP in the serum and in the pineal gland undergoes a circadian rhythm with much lower values at noon than at midnight [24]. A circadian rhythm of TRP was observed in human too, with peak concentrations occurring 8–10 h earlier than in the rat [25]. The circadian rhythm is suggested to depend on the feeding behaviour of the respective species [24,25]. In our study the time of blood sampling was set during daytime around 3–4 h after feeding and during nighttime around 8–9 h after feeding. The difference of this lag time may have influenced the difference between nighttime and daytime concentrations. The TRP plasma levels of cows showed nearly the same range as in rats [24] whereas TRP plasma levels were higher in heifers. Similar TRP levels as seen in heifers and cows in the present study were oberved in humans [26], depending on the protein content of the food and the time of day.

MEL plasma levels were affected by TRP supplementation in heifers. In lactating cows this effect of TRP supplementation could not be demonstrated. The difference could in part be due to the high variance in MEL levels, but also to the lower TRP plasma levels observed in dairy cows. Possibly the precursors TRP or 5-HT or other precursors of MEL biosynthesis are less available in cows than in heifers because of the use of amino acids for milk protein synthesis.

Pretreatment MEL plasma levels of cows during the photoperiod were in accordance with Berthelot et al. [27], whereas the maximum values during scotoperiod were higher in some cows in this study than in the study of Berthelot et al. [27]. In fact MEL showed large individual differences between the animals, some remaining low also during night and some increasing to levels higher than 150 pg/ml during night even without TRP supplementation. Possibly the MEL blood level is under strong genetic influence like in ewes, where seasonal MEL concentrations (June and December) were shown to have a heritability of $h^2 = 0.45$ [28].

MEL synthesis is most active during night and is suppressed by illumination. In the experiment with heifers and with cows, an inverse correlation between light intensity and MEL was obvious. The average intensity of light had different levels during day but nevertheless, at 20:00 h the average intensity of all measure points was always lower than 20 lux and as a consequence at 20:00 h MEL plasma levels started to increase [27].

MEL levels in milk and in plasma exhibited a clear circadian rhythm in cows, although great individual variation in the profile was apparent [29]. MEL levels in milk were higher in the milk obtained during morning milking as compared to evening milking. Milk MEL concentrations reflected MEL blood levels with a short time delay [29]. A correlation of AUC of MEL in plasma and milk was found in our experiment, too. Cows in a late stage of

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lactation showed a more effective accumulation of MEL in milk than cows in early stage of lactation [29]. Accordingly we found a closer correlation between blood and milk MEL in cows with more than 200 DIM than in cows with less than 200 DIM.

A circadian regulation of 5-HT was not obvious. In addition, 5-HT levels in plasma did not significantly increase after TRP supplementation. 5-HT which is also a metabolite in MEL biosynthesis is produced in the brain, because 5-HT cannot pass the blood-brain barrier [30]. However, because less than 2% of the total 5-HT is present in the brain, virtually all 5-HT in peripheral blood is derived from the gastrointestinal tract [31]. New studies assume 5-HT transporters at luminal and abluminal membranes, but their functions and the physiological importance are still unclear [32]. Possibly, the plasma concentration of 5-HT is not reflecting the 5-HT produced in the brain and thus may be not suitable to show a possible effect of TRP supplementation on 5-HT in brain. On the other hand a significant increase of 5-HT content in diencephalic regions was demonstrated in response to oral TRP administration in rats [33,34]. No significant correlation of plasma 5-HT with MEL and TRP was observed possibly again because only the brain 5-HT concentration is expected to correlate with peripheral MEL and TRP which may be not reflected by plasma 5-HT which is possibly more influenced by other factors than by TRP uptake.

Plasma PRL concentrations are affected by many factors, such as seasonal influences [35]. The present study was conducted during summer, when PRL levels should be higher than in winter [35]. Nevertheless, PRL seems to have a circadian rhythm [36–41]. In our study with a sampling interval of 3 h, a clear circadian rhythm was not obvious and was also not observed by others although the sampling intervals were shorter than 3 h [42]. PRL concentrations in our study were lower in heifers than in dairy cows. During the course of milking, PRL increased in accordance with previous studies [22,43]. It was reported that TRP infusion in human and horse induces an increase in plasma PRL concentrations [44–46]. Increased TRP availability stimulates brain 5-HT synthesis [47] and a rise in brain 5-HT is associated with PRL release [44,48–50]. In contrast, TRP supplementation did not alter PRL concentrations to a significant degree in this study neither in heifers nor in cows.

Oxytocin concentration during milking increased as expected from previous results [17,51–53]. Differences of OT concentrations between the experimental days were however not observed. In previous studies MEL was reported to affect OT release, either inhibitory or stimulatory depending on the dosage [13,16] and possibly on the species. Because MEL was not increased significantly in response to TRP supplementation in cows, an influence of TRP on the milking related OT release was very unlikely and also not demonstrated. However, milk yield at morning milkings on d 1, 3, 4, and 6 was elevated in response to TRP supplementation. Possibly a stimulatory effect of TRP on milk secretion occurred only during morning milking because the MEL levels are high during the night when most of the milk is secreted which is removed at morning milking. It is very unlikely that the increased milk yield was due to an improved OT release milk ejection because changes of OT release during the course of the experiment could not be shown although low doses of MEL was shown to inhibit oxytocin release while higher doses had a stimulatory effect in the rat [54]. Furthermore, the influence of TRP supplementation is not only mediated by MEL. As mentioned earlier it was impossible to detect changes of 5-HT in the CNS. However, changes of central concentrations of 5-HT cannot be excluded. An effect of 5-HT on milk ejection has been discussed to be inhibitory [55] or stimulatory [56,57] in rats. It remains unclear and needs to be further investigated if similar types of mechanisms can also occur in dairy cows.

5. Conclusion

Plasma TRP concentrations are increased by supplementation of rumen-protected TRP in ruminants and hence MEL plasma levels in animals with a low TRP requirement for production. TRP supplementation can slightly increase milk yield at morning milking. Peripheral PRL, 5-HT and OT levels are not affected by TRP supplementation.

Acknowledgments

The authors thank Mrs. T. Dicker and Mrs. W. Schmidt for their excellent technical assistance during hormone assays. The help of Mrs. C. Fochtmann and Mr. J. Riederer during animal experiments is gratefully appreciated. This study was supported by the H. Wilhelm Schaumann Foundation, Hamburg, Germany.

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Please cite this article in press as: Kollmann MT et al., Effects of tryptophan supplementation on plasma tryptophan and related hormone levels in heifers and dairy cows, Domest Anim Endocrinol (2006), doi:10.1016/j.domaniend.2006.09.005

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Appendix II

M.T. Kollmann, H.H.D. Meyer, R.M. Bruckmaier. 2007, Short term and long term light effects on the release of oxytocin, prolactin and cortisol in dairy cows, submitted to Journal of Dairy Research

Short term and long term light effects on the release of oxytocin, prolactin and cortisol in dairy cows

Maria T. Kollmann[1], Heinrich H.D. Meyer[1], Rupert M. Bruckmaier[2*]

[1]Physiology Weihenstephan, Technical University Munich, Weihenstephaner Berg 3, D-85350 Freising, Germany

[2]Veterinary Physiology, Vetsuisse Faculty, University of Bern, Bremgartenstrasse 109a, CH-3012 Bern, Switzerland

[*]Corresponding author:

Tel.: +41-31-631-2324

Fax: +41-31-631-2631

E-mail address: rupert.bruckmaier@physio.unibe.ch

ABSTRACT

The effect of artificial sunlight during milking and of the seasonally different photoperiod on the milking-related release of oxytocin, prolactin and cortisol in dairy cows was investigated. In experiment I, 7 Brown Swiss dairy cows were exposed to 3 different light treatments and a control treatment (without additional illumination) at four consecutive days at morning and evening milking in a randomized sequence. The light treatments consisted of different light spectra (only UV light or UV light plus infrared light) at different duration of illumination (10 min before milking plus during milking or during milking only). They resulted in an immediate decrease of the nightly elevated plasma melatonin levels at morning milking. The decrease was more pronounced if infrared light was used in addition to ultraviolet light than with ultraviolet light only. Furthermore, melatonin was suppressed more efficiently if the illumination was started at 10 min before milking than if the illumination was performed during milking only. Prolactin and cortisol baseline concentrations and their milking-related release were not affected by any treatment. However, milking related oxytocin release was higher during ultraviolet light treatment than in control if the illumination was switched on during milking only, but not at the other light treatments. In experiment II, 10 Brown Swiss cows were exposed to the environmental light conditions in June/July (summer) and in December (winter). Oxytocin concentrations during milking did not differ between the seasons and neither did cortisol, however prolactin concentrations were higher in summer than in winter ($P<0.05$). In conclusion, light-induced changes of the release of milking-related hormone differs between short term illumination and long term seasonal changes of the photoperiod.

INTRODUCTION

Changes of the photoperiod play a key role in the regulation of many physiological complexes which are linked to seasonal and circadian rhythms. Based on that, artificial illumination is used as a management tool in various livestock species. In dairy cows an increase of milk yield up to 10 % with extended photoperiod to 16–18 h light was repeatedly described. Changes of IGF-I and prolactin **(PRL)** and the interaction with their receptors (Dahl *et al.* 2000) are likely responsible for the increased milk yield (Dahl *et al.* 2000, Miller *et al.* 2000, Dahl & Petitclerc 2003). It is widely accepted that melatonin **(MEL)** and serotonin are involved in the regulation of PRL release and milk production because MEL is suppressed by light.

It can be hypothesized that there exists also a positive effect of the photoperiod on oxytocin **(OT)** release and milk ejection. A previous study in dairy cows showed a greater milking-related OT release during daytime than during night milkings in an automatic milking system, however only in a well illuminated barn (Macuhova & Bruckmaier 2004). The aim of the current study was to investigate the effects of photoperiodical differences and of artificial sunlight on the milking-related hormone release and milk removal in dairy cows and their possible dependency on melatonin concentrations.

MATERIALS AND METHODS

Animal husbandry: Experimental cows were kept in loose housing and had free access to a total mixed ration consisting of 40% corn silage, 14% grass silage, 10% hay and 36% concentrate (dry matter basis), providing enough nutrients for the production of 30 kg of milk/d.

The blood samples from each cow were collected through an indwelling catheter (Cavafix Certo Splittocan 335, Braun Melsungen, Germany) which was inserted in a jugular vein 2 d before the start of experiments. To prevent coagulation, blood samples were treated with EDTA and cooled on ice until centrifugation at 3000 × g for 15 min at 4 °C. Plasma was aliquoted and stored at –20 °C until analyses.

Milking Routine: Milking experiments were conducted during routine milking time twice daily from 0415 h until 0615 h and 1545 h until 1745 h in a 2 × 2 tandem milking parlour. Milking routine consisted of a short manual udder preparation including forestripping and cleaning of the teats with wet towels for 10 to 15 s, and an 1 min vibration stimulation (Stimopuls, WestfaliaSurge GmbH, Oelde, Germany). When milk flow decreased below 0.2 kg/min at the end of milking, machine stripping was performed and subsequently clusters were manually removed. Milk yield and milk flow were recorded by a well established mobile device (LactoCorder®, WMB Balgach, Switzerland).

Experiment I: Experiment I was designed to investigate the effect of artificial sunlight. The experiment was performed in April/May and 7 multiparous Brown Swiss cows in week 25 ± 2 of lactation with a daily milk yield of 28.9 ± 0.9 kg were used.

The experiment was performed as a completely balanced crossover design with randomizing the sequence of the treatments for each individual cow during morning and evening milkings for four consecutive days. The experiments were preceded by two days of adaptation to the catheters and 14 d to the light treatments. Three different treatments

and a control treatment were tested during four days at morning and evening milking in each cow. The treatments consisted of different light spectra, and different illumination times. For illumination an animal solarium (Turnier II, Weinsberger international, Weinsberg, Germany) with 18 infrared and 7 ultraviolet lamps was used inside the milking parlour. The ultraviolet lamps emitted UV-A and UV-B light at 280-380 nm and partly visible light at 400-750 nm; the infrared lamps emitted infrared light at more than 780 nm, but no long wavelength infrared. The cows were moved to the milking parlour and milking was started 10 min later in all treatments. In treatment 1 **(C)**, cows were milked without additional illumination. In treatment 2 **(UVMO)**, cows were exposed to ultraviolet illumination during the milking procedure only. In treatment 3 **(UV),** illumination with ultraviolet light started 10 min before milking and was maintained until the end of milking. In treatment 4 **(UVIR),** the ultraviolet illumination from 10 min before until the end of milking was supplemented by infrared light. For each treatment, blood samples (10 ml) were collected after reaching the milking parlour, immediately before the start of milking, then during milking in 1-min intervals and, in addition at 10 min and 30 min after the end of milking.

Experiment II: The experiment II was performed during summer in June/July during maximum day length (18 h photoperiod) and during winter in December during shortest day length (9 h photoperiod). The illuminance was measured during the whole experiment at 10-min intervals with LiCor Li-1000 (LiCor Biosciences, Lincoln Nebraska, USA) and light spectra were recorded at 1230 h and 1815 h (with LiCor Li-1800; LiCor Biosciences, Lincoln Nebraska, USA) during every experimental day. During both seasons each, 10 Brown Swiss cows in their first to sixth lactation were used. Lactational stages varied between weeks 11 and 52, and milk yield was 31.9 ± 2.0 kg/d during the period one of experiment in summer (June/July). Cows were between week 2 and 57 of lactation with a milk yield of 28.4 ± 2.1 kg/d in period two of the experiment in winter (December). Two cows were used in two successive lactations in periods one and two, respectively. Blood samples were collected through an indwelling catheter which was inserted into one jugular vein, a day before the start of experiments. Blood samples (10 ml) were collected during morning and evening milking, at three consecutive days and at 1-min intervals from 1 min before milking until the end of milking.

Hormone Analyses: Plasma OT and PRL concentrations were determined by radioimmunoassay (RIA) (Schams & Karg 1970, Schams 1983). MEL concentration was measured in the sample removed during cluster attachment by a commercial enzyme

immunoassay kit (IBL Hamburg, Germany, Kat.-Nr. RE 540 21). The cortisol was assayed via an enzyme immunoassay as previously described (Sauerwein *et al.* 1991). Cortisol and PRL were only analysed in the basal sample i.e., before touching the udder, at 4 min after cluster attachment, at the end of milking (9.7 ± 0.4 and 8.8 ± 0.1 min after cluster attachment in experiment I and II respectively), and in experiment I, additionally at 30 min after milking.

Mathematical and Statistical Evaluations: Results are presented as means ± SE. To analyse OT results the area under the curve (AUC/min) of the entire milking process was calculated. For MEL, cortisol and PRL means were calculated within point in time. For statistical analysis a repeated measures analysis of variance (MIXED procedure of the SAS program package, SAS Institute, 2005) was used. In experiment I and II, the cow was the repeated subject. Treatment effects were tested for significance ($P<0.05$) by using Bonferroni´s t-test based on least square means.

RESULTS

Experiment I: Plasma concentrations of MEL before milking (Figure 1) were higher at morning milking than at evening milking ($P<0.05$). Furthermore, MEL levels did not differ among treatments at evening milking. At morning milking, the illumination from 10 min before milking with UV caused a slight but non-significant decrease of MEL levels as compared to C. In response to UVIR the MEL levels at the start of milking were significantly ($P<0.05$) decreased. Because treatments C and UVMO did not differ until the start of milking, the MEL concentration did not differ between these treatments at this time.

Premilking concentrations of PRL did not differ among treatments. During the course of milking PRL increased, and at 30 min after the end of milking, PRL levels had decreased again and were significantly lower than those during the last minute of milking. PRL levels did not differ between morning and evening milking (Figure 2). PRL concentration was higher ($P<0.05$) at the end of evening than at morning milking in treatments UV and UVMO. Cortisol concentrations did not differ between treatments or between morning and evening milkings. However, the levels of cortisol increased significantly ($P<0.05$) during milking and decreased thereafter to concentrations partially lower than pre-milking cortisol at 30 min after the end of milking.

Pre-milking OT concentrations were low and similar in all treatments (6.6±1.7, 5.7±1.6; 5.5±1.1, and 5.8±1.1 pg/ml in the morning and 7.6±1.1; 5.5±1.0, 5.6±1.0, and 6.4±1.9 in

treatments C, UVMO, UV, and UVIR, respectively). The concentration of OT increased during milking in all treatments. Based on AUC, concentrations of OT did not differ between morning and evening milkings. However, OT release was greater in UVMO than in C during morning and evening milking ($P<0.05$; Figure 3).

Milk yield, average and peak milk flow rates did not differ among the treatments.

Experiment II: The intensity of light was higher and the duration of lighting was longer in summer as compared to winter experiments. In July there were around 17 h of light with a peak intensity around 4050 lux and in December the light period was around 10.5 h with peak intensity not higher than 350 lux. Light composition differed between winter and summer. Thus, the proportion of UV light at 1230 h was higher in summer than in winter, where it was barely detectable. In summer, the light spectrum contained a small proportion of blue light and a higher proportion of infrared light as compared to winter.

The pattern of plasma OT during milking did not differ between seasons, neither before milking nor during the course of milking. The mean concentration of OT during milking (calculated from AUC) was 12.8 ± 1.9 and 16.7 ± 3.0 pg/ml×min in summer, and 14.5 ±1.2 and 15.0 ± 1.9 pg/ml×min in winter, at evening and morning milkings, respectively.

Concentrations of PRL during both, morning and evening milkings were significantly higher in summer than in winter. An increase of PRL concentration during the course of milking was observed in summer (morning and evening) ($P<0.01$), but in winter only during evening milkings ($P<0.05$; Figure 4). At morning milking in winter, PRL was elevated before the start of milking as compared to the evening milking ($P<0.05$) but did not show a further increase during milking.

In summer, the concentrations of cortisol increased significantly ($P<0.05$) during morning and evening milkings (Figure 5). In winter, the concentration of cortisol increased significantly ($P<0.05$) only during evening milkings. Levels of cortisol during morning milkings were higher than during evening milkings in summer ($P=0.0056$) and winter ($P=0.0394$), however only significant ($P<0.05$) at the basal concentration in winter, and at 4 min after cluster attachment and in the last min of milking (8.8 ± 0.1 min after cluster attachment) in summer.

DISCUSSION

The concentrations of MEL in this study were in the range of previously published values (Berthelot *et al.* 1990). In the present study we could show that artificial sunlight suppresses MEL within a period as short as 10 min. However, the inhibition of MEL

release was only obvious at morning milking, because shortly after 0400 h pre-milking MEL showed still the nightly elevation. It could be demonstrated that the spectrum of light is crucial for its suppressive effect on MEL. With infrared lamps in addition to UV the decrease was more pronounced than without. An influence of the light spectrum on MEL suppression was previously reported in rats (Cardinali *et al.* 1972) but was to our knowledge shown for the first time in cattle.

In the present study PRL increased towards the end of milking as reported by others (Schams & Karg 1970, Forsling *et al.* 1974), and decreased again after the end of milking. Thus, at 30 min after milking, the level of PRL was already significantly lower than at the end of milking. In addition we could confirm the finding of previous studies (Schams & Reinhardt 1974, Newbold *et al.* 1991, Auchtung *et al.* 2005) that PRL in dairy cows follows a seasonal pattern with higher values during long day photoperiod. A short illumination with artificial sunlight, however, did not affect PRL concentration in the present study. Although during morning milking PRL was higher ($P<0.05$) during the last minute of milking in UVIR treatment than in the other treatments; any regulation that could be caused by the treatments was not obvious. The effect of light on PRL which is mediated by MEL is obviously a long-term effect (Auldist *et al.* 2007). Therefore, the duration of lighting in experiment I of only some minutes before and during milking was too short to affect PRL.

In the present study, lighting during milking could increase the OT level in cows. It was shown in rats that OT plasma concentration increase during daylight and decrease during the night (Windle *et al.* 1992). A higher amount of OT was released in cows milked during the daylight period as compared to those milked during night in an automatic milking system in a well illuminated barn (Macuhova & Bruckmaier 2004). The greatest and significant release during milking was evident in our study if artificial sunlight was administered during milking only. The illumination already 10 min before milking caused only a slight but no significant increase of OT values. Possibly, the illumination of the cow resulted only in a short-term effect with respect to the readiness to release OT in response to milking-related stimuli.

Possibly the treatment with artificial sunlight during milking can reduce the problem of too low OT release and thus reduce the incidence of disturbed milk ejection.

In the present study, the concentration of cortisol increased during milking as shown in previous studies (Gorewit *et al.* 1992, Bruckmaier *et al.* 1993), and decreased again after milking. In addition, the level of cortisol was higher during morning than during evening

milking in experiment II. The diurnal pattern of cortisol usually shows high levels from midnight until the morning while lowest concentrations occur during the afternoon (Fulkerson *et al.* 1980). Seasonal changes of the photoperiod did not affect the cortisol concentration in our study as it was already observed in previous investigations (Peters *et al.* 1981, Zinn *et al.* 1986, Auchtung *et al.* 2005), and neither did the short-term treatment with artificial sunlight.

In conclusion, short term lighting at milking time increases the milking-related release of OT but does not alter PRL and cortisol. In contrast long term seasonal changes of the photoperiod affect PRL but do not affect OT and cortisol release during milking. Cows with disturbed milk ejection, based on an insufficient OT release (Bruckmaier *et al.* 1993), are an obviously increasing problem in practical dairy farming. Artificial sunlight in the milking environment is possibly a tool to reduce the frequency of these disturbances.

We thank the H. Wilhelm Schaumann Foundation, Hamburg, Germany for supporting this study.

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Figure 1:

Melatonin plasma levels (pg/ml) in dairy cows at cluster attachment, during morning and evening milking, with different lighting (C, UVMO, UV, UVIR). The means within the same time without common superscript are significantly different (P≤0.05). Asterisks indicate differences between the times (P≤0.05).

Figure 2:

Prolactin plasma concentrations (ng/ml) in dairy cows during milking with different light treatments **a)** C, **b)** UVMO, **c)** UV, **d)** UVIR. Samples are: basal samples taken 1 min before and samples taken 4 min after cluster attachment, samples taken during the last minute of milking (9.7 ± 0.4 min after cluster attachment) and 30 min after the end of milking. Means within the course of either morning or evening milking without common superscripts are significantly different (P≤0.05). Asterisks indicate differences between respective means of morning and evening milkings (P≤0.05).

Figure 3:

Oxytocin plasma levels (AUC/min) in dairy cows during morning and evening milking, with different lighting (C, UVMO, UV, UVIR). Means within the same time without common superscript are significantly different (P≤0.05). Asterisks indicate differences between the times (P≤0.05).

Figure 4:

Prolactin plasma concentrations (ng/ml) in dairy cows during milking at different seasons (summer **(a)** and winter **(b)**) before milking, 4 min after cluster attachment, during the last minute of milking (8.8 ± 0.1 min after cluster attachment) and 30 min after the end of milking. Means within the same treatment without common superscripts are significantly different (P≤0.05). Asterisks indicate differences between the milking times (P≤0.05).

Figure 5:

Cortisol plasma concentrations (ng/ml) in dairy cows during milking at different seasons (summer **(a)** and winter **(b)**) before milking, 4 min after cluster attachment, during the last minute of milking (8.8 ± 0.1 min after cluster attachment) and 30 min after the end of milking. Means within the same treatment without common superscripts are significantly different (P≤0.05). Asterisks indicate differences between the milking times (P≤0.05).

Figure 1:

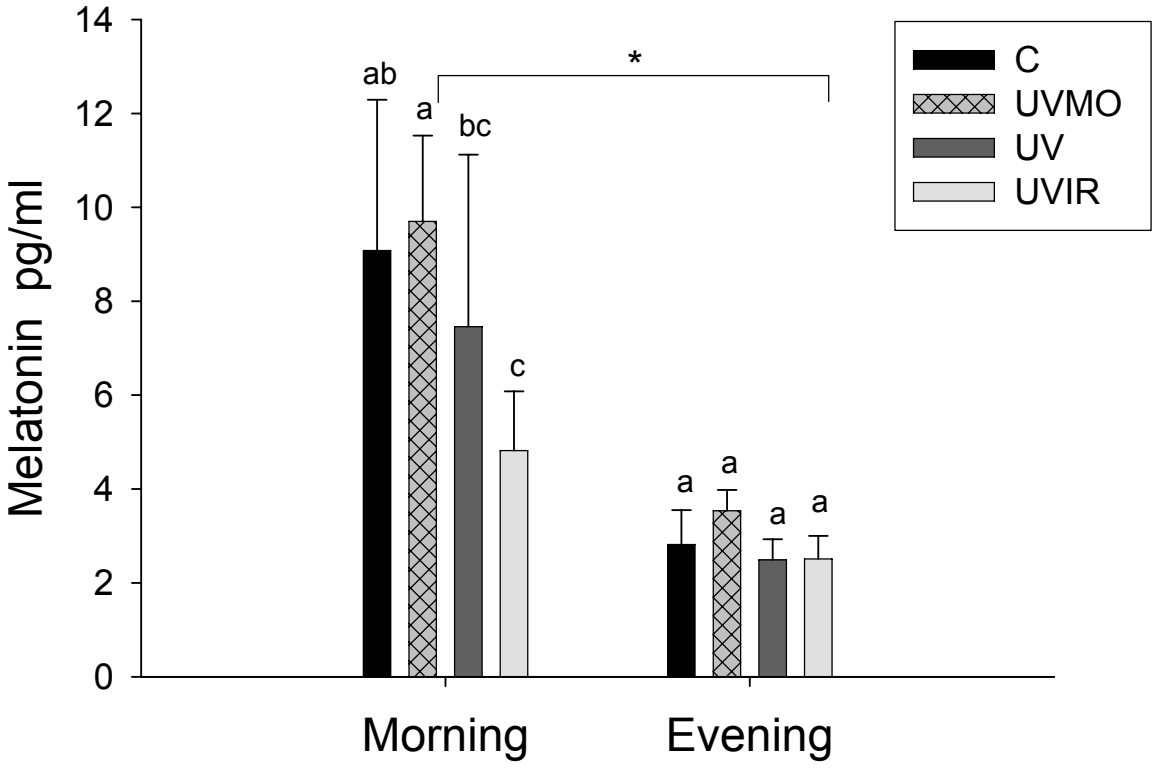
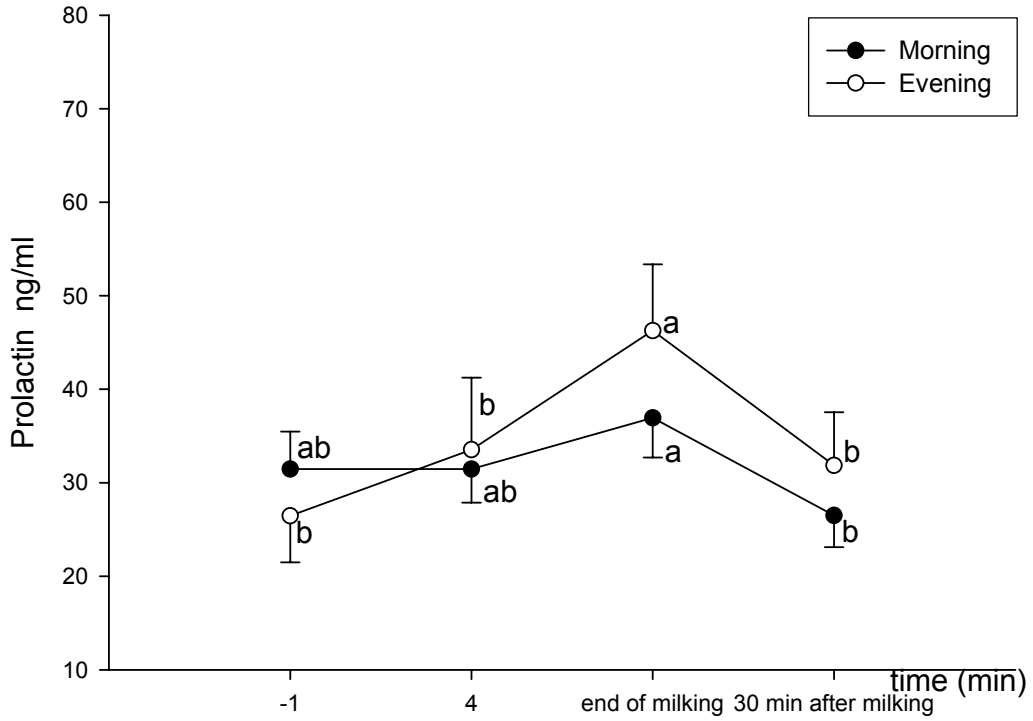
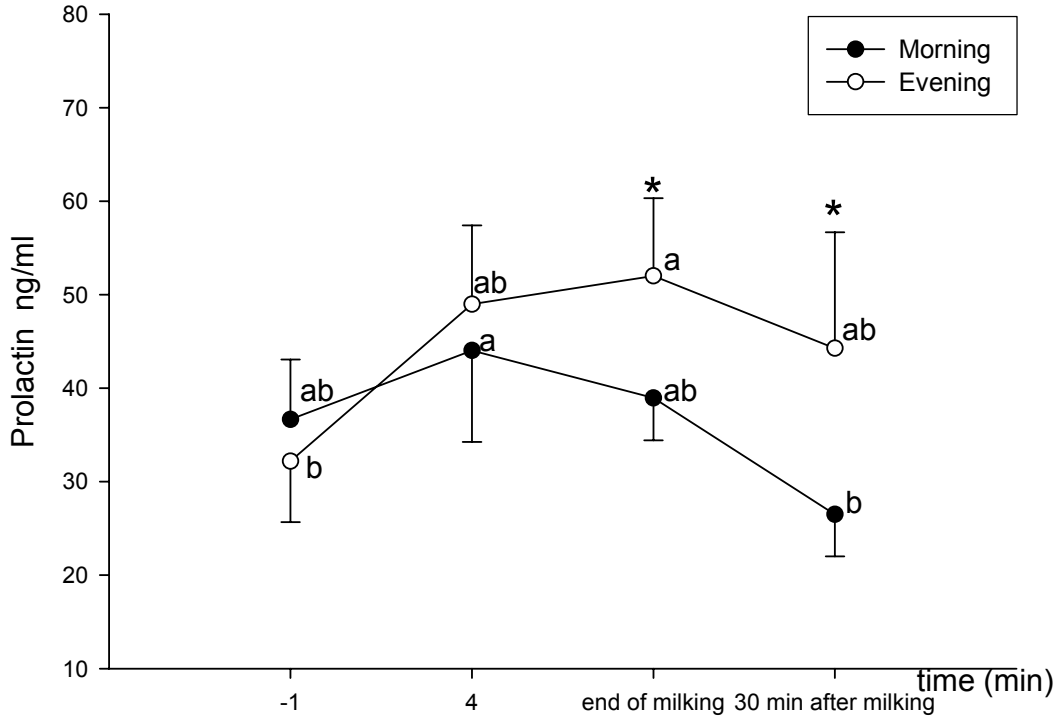

Melatonin pg/ml
14
12
10
8
6
4
2
0
ab
a
bc
c
a
a
a
a
*
C
UVMO
UV
UVIR
Morning
Evening

Figure 2:

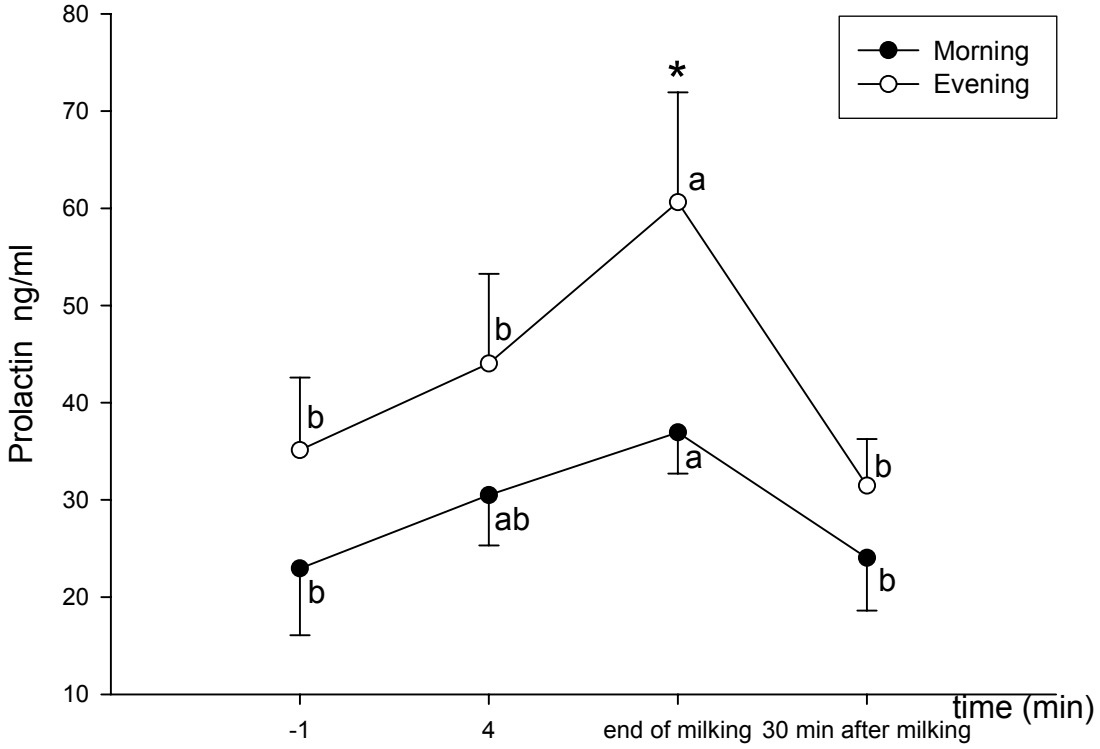
a)


Morning
Evening
Prolactin ng/ml
80
70
60
50
40
30
20
10
ab
b
ab
a
a
b
b
b
time (min)
-1
4
end of milking
30 min after milking

b)


Morning
Evening
Prolactin ng/ml
80
70
60
50
40
30
20
10
*
*
ab
a
ab
a
ab
ab
b
b
time (min)
-1
4
end of milking
30 min after milking

c)


Morning
Evening
Prolactin ng/ml
80
70
60
50
40
30
20
10
*
a
b
b
a
b
ab
b
b
time (min)
-1
4
end of milking
30 min after milking

d)

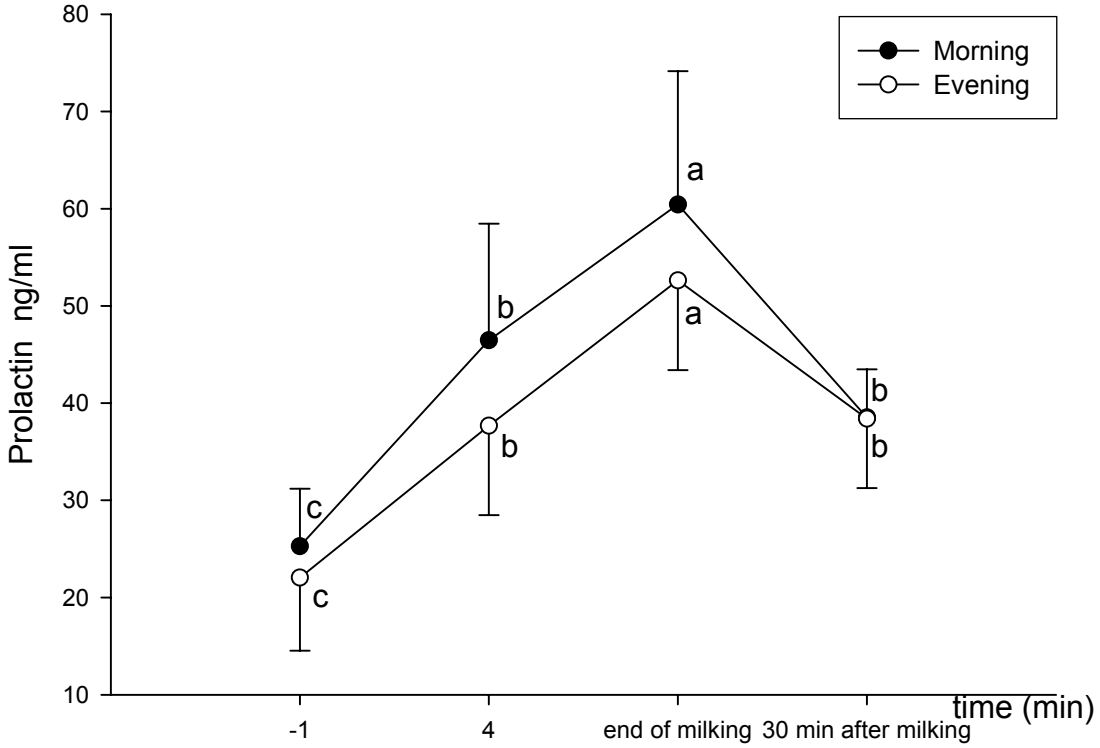

Morning
Evening
Prolactin ng/ml
80
70
60
50
40
30
20
10
a
b
a
b
b
c
b
c
time (min)
-1
4
end of milking
30 min after milking

Figure 3:

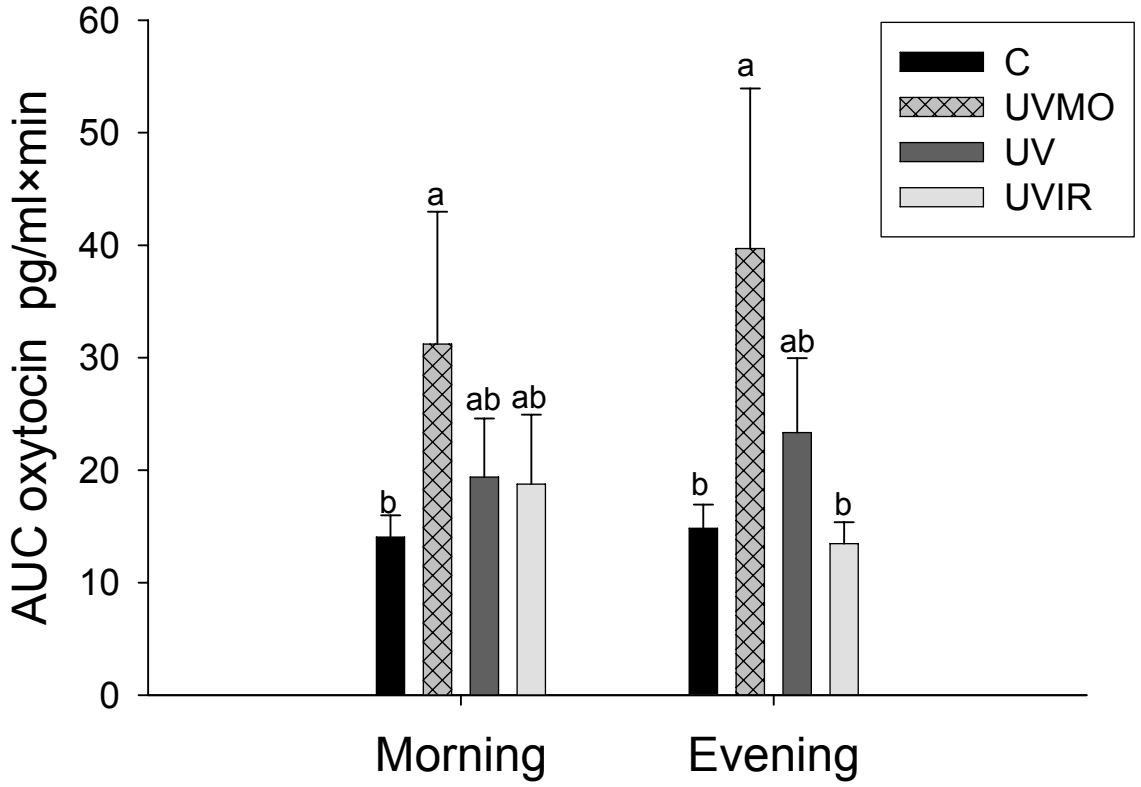
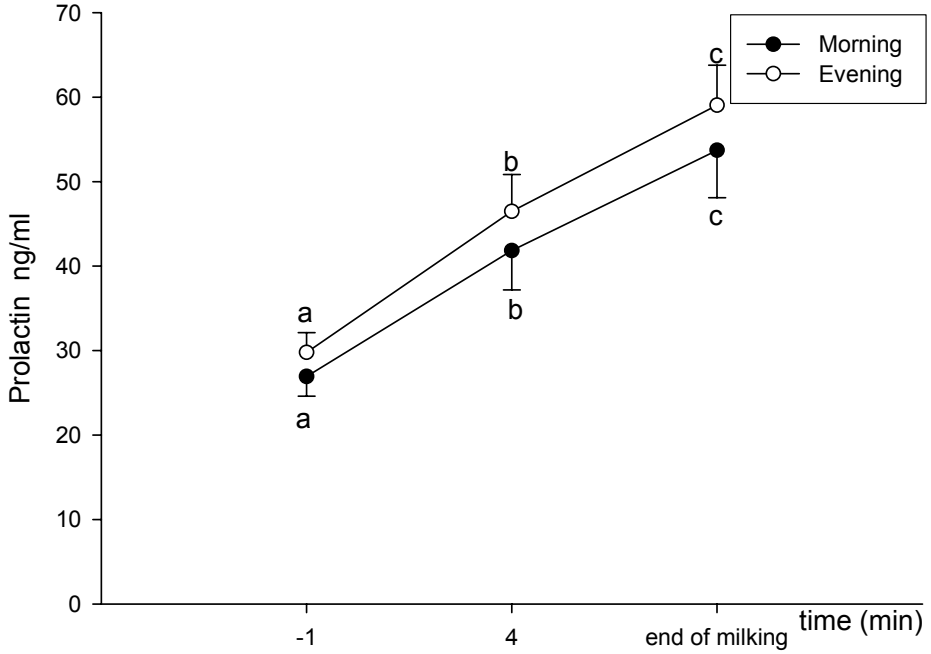

AUC oxytocin pg/ml×min
60
50
40
30
20
10
0
C
UVMO
UV
UVIR
a
ab
ab
b
a
ab
b
b
Morning
Evening

Figure 4:

a)


Morning
Evening
Prolactin ng/ml
time (min)
-1
4
end of milking
0
10
20
30
40
50
60
70
a
a
b
b
c
c

b)

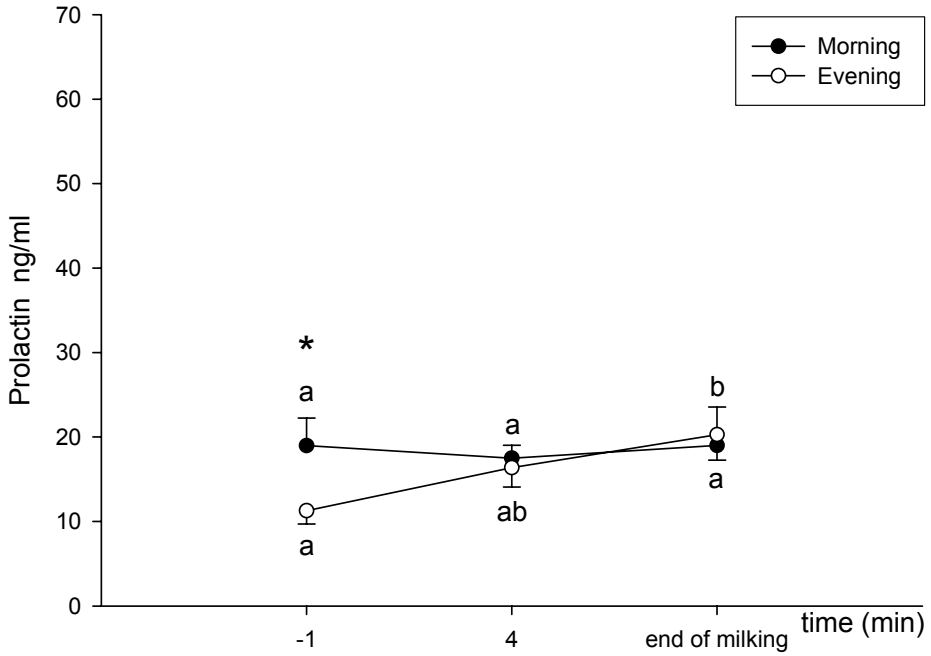
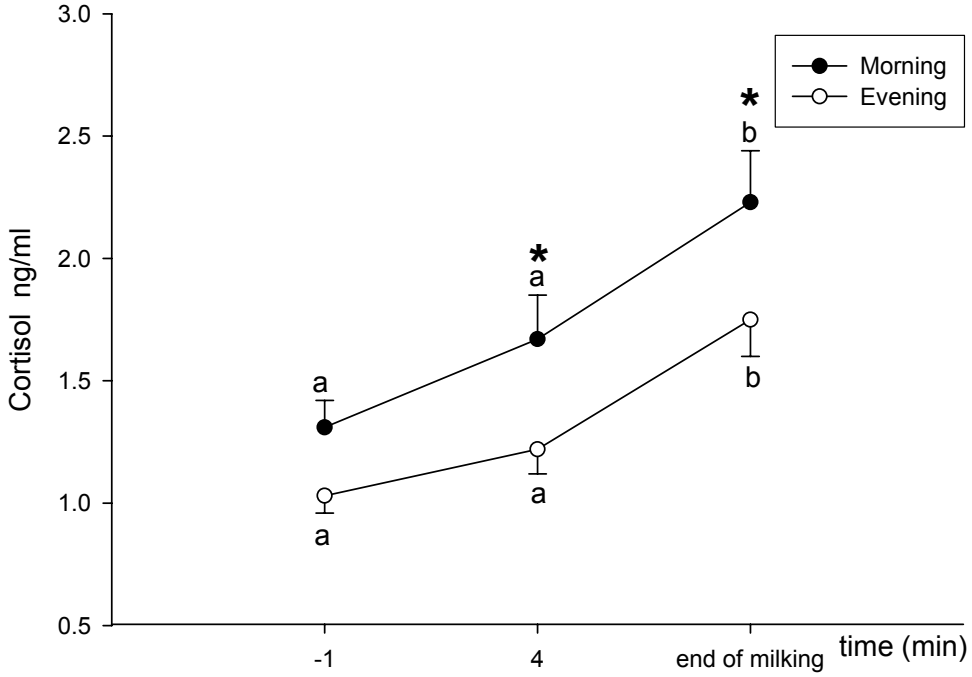
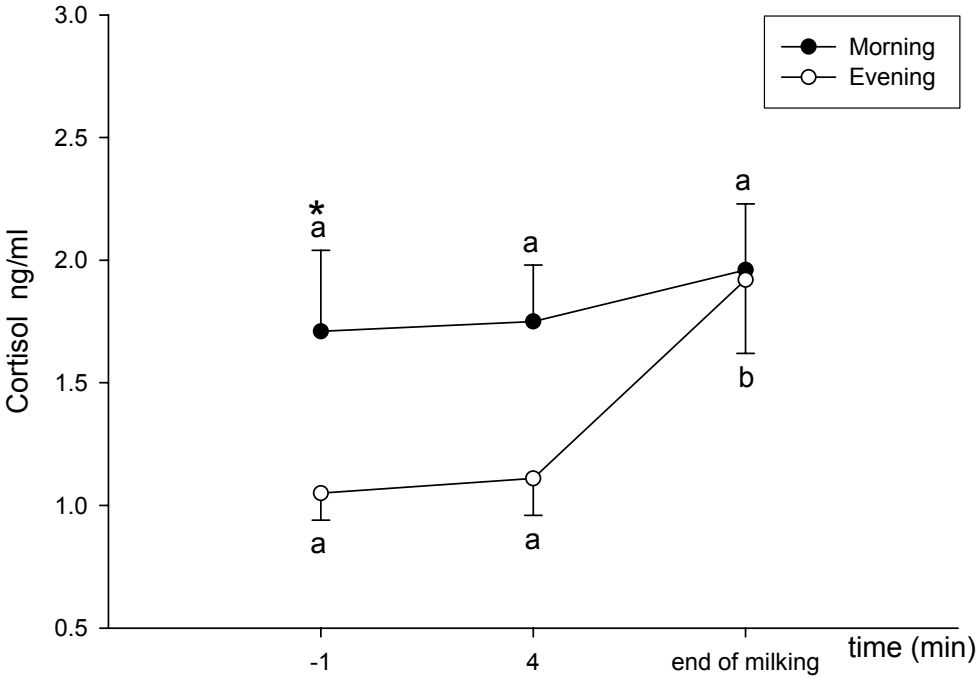

Morning
Evening
Prolactin ng/ml
time (min)
-1
4
end of milking
0
10
20
30
40
50
60
70
*
a
a
a
ab
b
a

Figure 5:

a)


3.0
2.5
2.0
1.5
1.0
0.5
Cortisol ng/ml
Morning
Evening
*
b
*
a
a
b
a
a
-1
4
end of milking
time (min)

b)


3.0
2.5
2.0
1.5
1.0
0.5
Cortisol ng/ml
Morning
Evening
*
a
a
a
b
a
a
-1
4
end of milking
time (min)

Appendix III

M.T. Kollmann, V. Lollivier, S. Richter, O. Wellnitz, R.M. Bruckmaier. 2007, Twenty four hour pattern of melatonin in blood and milk of dairy cattle: ready for submission

Twenty four hour pattern of melatonin in blood and milk of dairy cattle

M.T. Kollmann[1], V. Lollivier[2], S. Richter[3], O. Wellnitz[3], H.H.D. Meyer[1], R.M. Bruckmaier[3*]

[1]Physiology Weihenstephan, Technical University Munich, Weihenstephaner Berg 3, D-85350 Freising, Germany

[2]UMR INRA-Agrocampus Production du Lait, 35000 Rennes, France

[3] Veterinary Physiology, Vetsuisse Faculty, University of Bern, Bremgartenstrasse 109a, 3012 Bern, Switzerland

[*]Corresponding author:

Tel.: +41-31-631-2324

Fax: +41-31-631-2631

E-mail address: rupert.bruckmaier@physio.unibe.ch

ABSTRACT

This study was conducted to investigate the diurnal pattern and the concentration of melatonin in bovine blood and milk. Blood and fractionized milk samples were taken every hour for 24 hours from 12 dairy cows which were housed under natural light conditions in June (approx. 16 h daylight). Melatonin blood and milk concentrations showed a diurnal rhythm with high levels during night and low levels during day. A positive correlation between milk and blood melatonin concentration was found ($r=0.256$, $P<0.001$). The regression line ($y=0.047x+0.606$) of milk melatonin in dependence of blood melatonin indicates that melatonin is migrating by passive diffusion from the bloodstream into milk.

With further experiments it was possible to show that the season did not influence the milk melatonin concentration during twice daily milking. Milk samples taken during July and December did not show different melatonin concentrations but samples varied significantly between morning and evening milking.

The melatonin concentration did not differ between milk samples of different milk fractions (fore milk, main milk fraction and residual milk). In conclusion it could be demonstrated that melatonin in bovine milk shows a diurnal rhythm reflecting the blood melatonin concentration thus indicating that the water soluble melatonin reflects directly the blood plasma melatonin concentration via diffusion and is most likely not actively transported from blood into milk.

KEY WORDS:

Melatonin, diurnal rhythm, blood, milk, milk fraction

INTRODUCTION

The neurohormone melatonin is produced in the absence of light and is suppressed by illumination. A circadian rhythm with high levels during nighttime and low levels during daytime can be observed in blood. Thus melatonin acts as the key transducer of photoperiodic information (Arendt, 1988; Berthelot et al., 1993) and is an endocrine marker for darkness (Vanecek, 1998; Schomerus et al., 2002).

Several hormones pass from the bloodstream into milk by diffusion, while the profile in milk is similar to that in plasma (Schams and Karg, 1986). The presence of melatonin in milk was reported in rats (Rowe and Kennaway, 2002), humans (Illnerova et al., 1993) and also in bovine milk (Kollmann et al., 2006). The passage mechanism of melatonin into milk is not clear until now. Possibly the circadian rhythm in blood can be seen in milk, too, but this needs to be tested. This study was designed to determine whether the levels in bovine milk exhibit a similar diurnal rhythm as plasma levels and whether a correlation exists between concentrations in plasma and milk.

The concentration of hormones in mature milk under normal conditions is very low and of no physiological significance in human, because their normal production is several orders of magnitude higher. The objective of the study was to determine the course and concentration of melatonin in milk and by the correlation of blood and milk melatonin to get some information about the passage mechanism of the hormone into milk.

MATERIALS AND METHODS

Experiment I

12 primiparous (n=3) and multiparous (n=9) dairy cows were used for experiment I. The cows were of different breeds (5 Brown Swiss, 1 Holstein Friesian, 6 Red Holstein). They were housed in a tie stall barn during the experiment where they were fed with hay, grass and maize silage and concentrate according to their individual production levels.

The cows were between day 92 and 188 of lactation and had a milk yield around 25 kg/day before the start of the experiment. They were milked with a bucket milker (Fullwood AG, Meierskappel, Switzerland) every hour. The experimental animals were kept under natural light conditions without using artificial light during the experiment. The experiment was conducted in June with a photoperiod of about 16 h of daylight. Consecutive samples of blood and milk were taken at 1-h intervals during 24 h starting at 7:00 h in the morning. The cows were catheterized on the day before the experiment with a permanent catheter (Cavafix Certo Splittocan 335, length 32 cm, diameter 1.8 × 2.35 mm, Braun, Melsungen, Germany) inserted in one jugular vein.

At each hourly sampling, a blood sample (10 ml) was taken via the catheter, then 1 I.U. synthetic oxytocin (diluted in 5 ml 0.9 % NaCl) was injected into the catheter and flushed with 10 ml saline solution (0.9 %) with 5000 I.U./l heparin.The cows were milked immediately. Blood samples were anticoagulated with EDTA and cooled on ice until centrifugation at 1000 × g for 20 min at 4°C. Plasma was aliquoted and stored at -20°C until used in the melatonin assay. Milk samples were taken from the total milk every hour and stored at -20°C until preparation for the melatonin assay. Milk yield was determined by digital scales.
To avoid illumination of the animals´ eyes samples during night were taken only with a small head lamp and care was taken to avoid direct illumination of the eyes.

Experiment II

Milk samples proportional of the total milk yield were taken from 9 Brown Swiss cows in two different seasons. In June (long days) and also in December (short days) milk samples were taken during the normal twice daily milking starting at 0415 and 1545h. The cows were housed in loose housing under natural light conditions. Milk samples were processed and analysed for melatonin as described for experiment I.

Experiment III

6 Brown Swiss cows were used for the experiment. Milk samples of the different fractions were taken during normal twice daily milking under natural light conditions. One sample of the foremilk (foremilk fraction), one in the middle of milking (main milk fraction) and one after finishing of milking (residual milk fraction) was taken by hand milking. Milk samples were processed and analysed for melatonin as described for experiment I.

Assay procedure

Melatonin in blood was measured by using a commercial ELISA-kit (IBL Hamburg, Germany, Kat.-Nr. RE 540 21). The concentration of melatonin in milk was measured with the same kit after a special preparation of the milk as described previously (Kollmann et al., 2006). For the extraction 1 ml of the skimmed milk was used and extracted similarly as plasma.

Mathematical and statistical evaluations

Results are presented as means ± SE. Concentrations below the detection limit of 2.4 pg/ml were defined as 0. A repeated measure of analysis was performed by using the MIXED procedure of the SAS program package (SAS Institute, version 9.1). The repeated subject was the cow. Significant differences were localized by using Bonferroni´s t-test based on least square means. Correlations were calculated by using the CORR procedure and linear regressions by using the REG procedure.

RESULTS

Experiment I

A diurnal rhythm of melatonin was observed in blood and in milk. After the onset of darkness (around 2200 h) melatonin started to increase in blood and in milk and decreased again when illuminance exceeded 20 lux (around 0600h). During the experimental phase the days were long with around 16 h of light and around 8 h of darkness. A significant regression of blood and milk melatonin concentration in dependence of the time could be calculated (Figure 1). The course of melatonin concentration was increasing in blood and in milk. Highest melatonin levels in blood and in milk were observed in the middle or towards the end of the dark phase. The highest melatonin concentration in blood was reached at 0100 h with 25.36 ± 5.60 pg/ml and with 2.99 ± 0.74 pg/ml in milk at 0400 h (Figure 2). Melatonin levels varied between the cows (Figure 1). In some animals plasma melatonin increased to more than 50 pg/ml during night and in others it did not exceed 6 pg/ml. Milk melatonin was only measurable during night, during day it did not exceed the detection limit. During night it reached at most 9.30 pg/ml.

The concentration of milk melatonin correlated ($r=0.256$, $P<0.001$) with plasma melatonin. A correlation of blood melatonin concentration with the total amount of melatonin in milk was observed, too. This was even higher with $r=0.288$ ($P<0.01$). A regression ($P<0.001$) of melatonin milk concentration in dependence of melatonin blood concentration could be calculated: $y=0.047x+0.606$. The regression of total amount of melatonin with blood concentration was also significant ($P<0.001$): $y=55.9x+586.3$.

Experiment II

Melatonin concentration in milk during twice daily milking was significantly different at morning milking from evening milking ($P<0.01$). An effect of photoperiod was not obvious in samples during normal twice daily milking. The average concentrations of melatonin in milk were during evening milking 2.4 ± 0.4 pg/ml and 2.0 ± 0.5 pg/ml and during morning milking 5.3 ± 0.6 pg/ml and 4.2 ± 0.6 pg/ml (June and December respectively).

Experiment III

Melatonin concentration in milk did not show significant differences in different milk fractions and was 6.9 ± 1.8, 7.7 ± 2.1 and 6.1 ± 1.9 pg/ml in foremilk, main milk fraction and residual milk respectively.

DISCUSSION

Melatonin was detected in bovine milk as previously described (Kollmann et al., 2006) and demonstrated a diurnal rhythm as reported in rats (Rowe and Kennaway, 2002) and in human (Illnerova et al., 1993). In our study during the day no melatonin was detectable in bovine milk unlike the observations for rat and human milk (Illnerova et al., 1993; Rowe and Kennaway, 2002). A similar rhythm of melatonin in milk like in blood was found. The average concentration of melatonin in human milk was about 35 % (Illnerova et al., 1993), whereas in cows we reached about 40 %, but the variation was high. The correlation of melatonin concentration in milk and blood suggest that melatonin is continuously transferred from blood into milk. The pattern of melatonin concentrations let us suppose that its transport is not active, more probably it is a diffusion of melatonin. Furthermore it was reported that melatonin freely diffuses through biological membranes (Vanecek, 1998). The correlation of blood and milk concentration was high although blood concentration showed only the situation of the moment of blood sampling and milk concentration showed always the situation of one hour, because milk is secreted continuously and milked only every hour in the respective experiment. Thus melatonin concentration of the obtained milk represents the transfer of melatonin during the past production period. The variation in melatonin plasma concentration was high as reported in earlier studies (Kollmann et al., 2006) and in ewes (Zarazaga et al., 1998).

The nighttime rise in blood melatonin was lower as found in other studies (Berthelot et al., 1990; Kollmann et al., 2006). This could have a genetical reason, because it was reported for ewes that melatonin blood level has a heritability of h^2=0.45 - measured in June and December- (Zarazaga et al., 1998). In hamster (Brainard et al., 1982; Garidou et al., 2003), ewe (Zarazaga et al., 1998) and red deer (Garcia et al., 2003) the melatonin peak is higher in short day photoperiod as in long day photoperiod and its duration is longer (Vanecek, 1998). This could partly explain the low level of melatonin in our study, but to evaluate the level of melatonin it must be measured at another season, too. However, no difference of melatonin concentration in milk of twice daily milking between the seasons was found. This, possibly derives from the fact, that only a small amount of blood melatonin diffuses into milk. Possibly the effect is marginal in milk, so that the individual differences between the animals overlap these effect.

The higher melatonin level in morning milking as compared to evening milking was expected because of the higher levels of melatonin in blood and milk measured every hour during night compared to these during day. The morning milking showed an accumulation of the milk produced during night and thus an accumulation of melatonin produced during this time whereas evening milking showed an accumulation of milk and melatonin produced during the day.

Milk fat increases towards the end of milking (Ontsouka et al., 2003; Sarikaya et al., 2005) and protein increased first and decreased towards the end of milking again (Sarikaya et al., 2005). Milk melatonin concentration was not influenced during milking. Melatonin in human milk remained at about 80 % in the water phase (Illnerova et al., 1993), what could explain the consistency in concentration in milk. Possibly it is secreted with the water. Thus specific selection of a milk fraction would not increase milk melatonin concentration.

In milk, removed during night, more melatonin was found as in those, milked during daylight.

Conclusion

Melatonin concentrations in milk show a diurnal rhythm similar to that in blood. A high regression between concentrations in blood and milk exists what let suppose that melatonin is migrating into milk by diffusion from the bloodstream. Seasonal

differences of milk melatonin concentration are not obvious at twice daily milking and also no difference between the milk fractions exists.

Acknowledgement

This study was supported by H. Wilhelm Schaumann Foundation, Hamburg, Germany

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Figure 1:

Scatter plot of blood and milk melatonin concentration of 12 cows for 24 h with regression line for blood and milk concentration in dependence of the time.

Figure 2:

Average blood and milk melatonin concentration of 12 cows for 24 h, beginning at 0700h. Results are represented as means ± SE.

Figure 1:

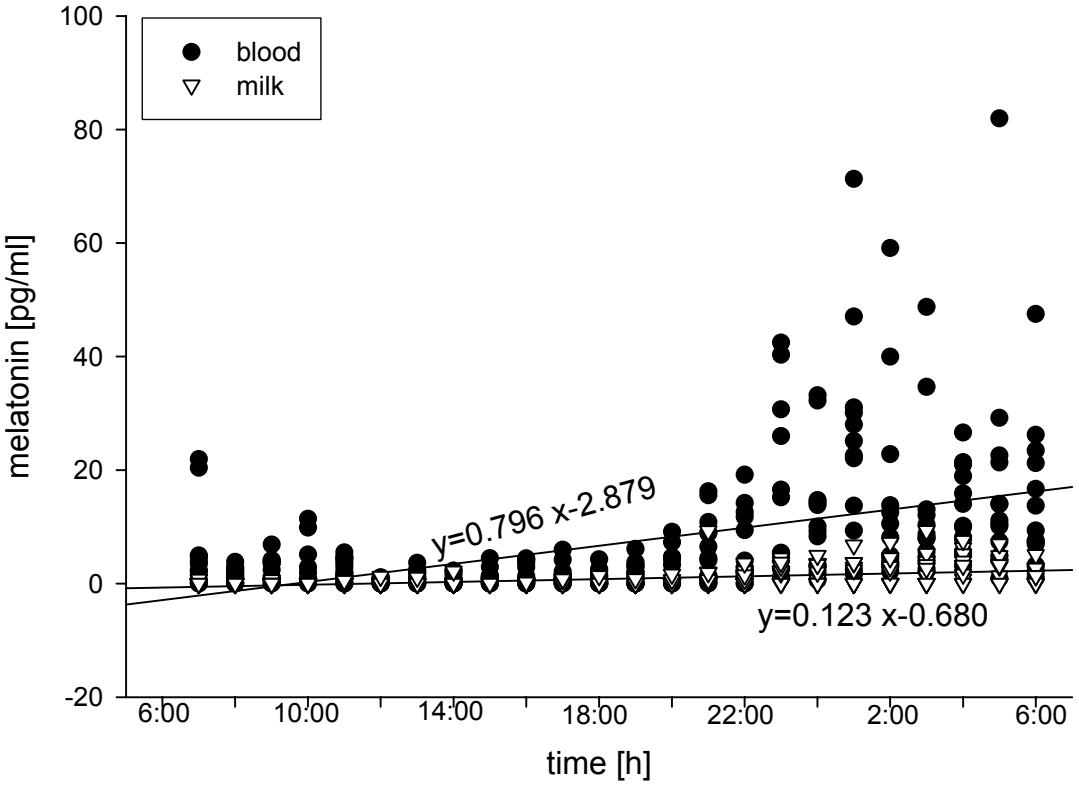
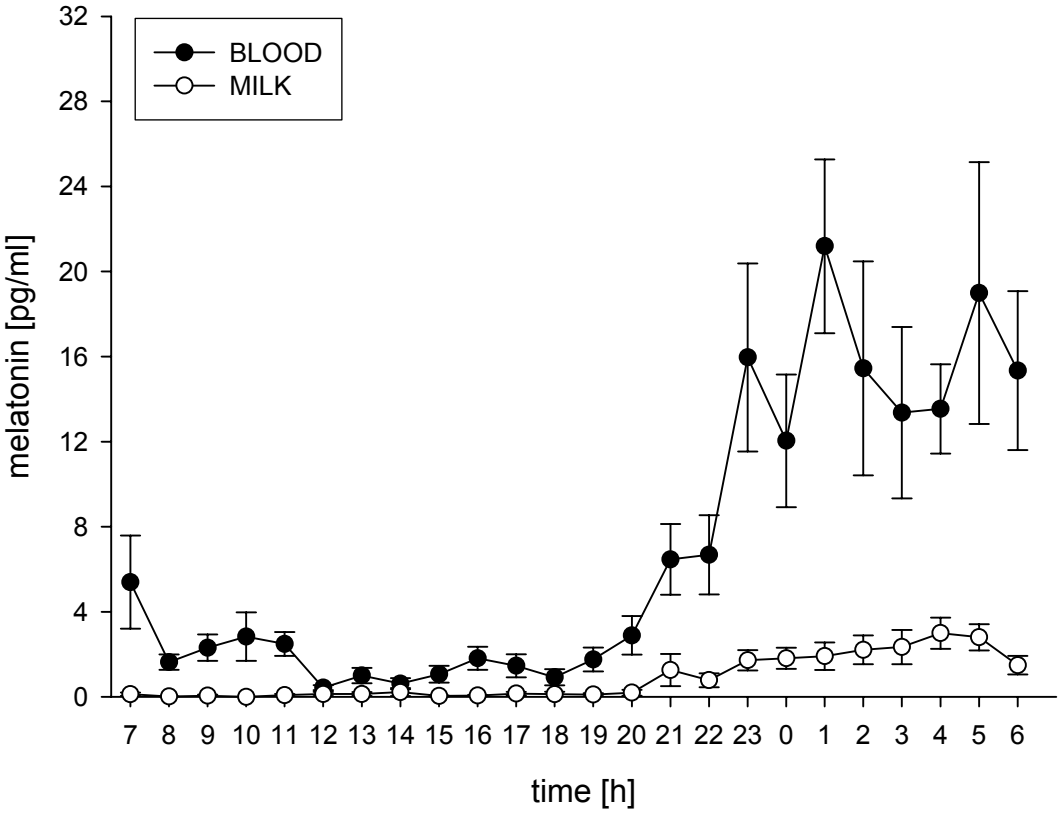

blood
milk
melatonin [pg/ml]
100
80
60
40
20
0
-20
y=0.796 x-2.879
y=0.123 x-0.680
6:00
10:00
14:00
18:00
22:00
2:00
6:00
time [h]

Figure 2:


BLOOD
MILK
melatonin [pg/ml]
32
28
24
20
16
12
8
4
0
7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 0 1 2 3 4 5 6
time [h]

12 ACKNOWLEDGEMENT

First of all I would like to thank the H. Wilhelm Schaumann foundation for their financial support. Without their help, this work would not exist.

My gratefully thanks I would like to convey to everybody who helped weather with comments, discussion, editing or helpful ideas to realize this work:

To Prof. Dr. R.M. Bruckmaier, who encouraged me to begin this thesis, for friendly and competent supervision as well as for editing and correcting of the publications.

To Prof. Dr. H.H.D. Meyer who enabled me to work at the Institute Physiology Weihenstephan and for helpful discussion.

To Prof Dr. L. Dempfle for the chairmanship.

To T. Dicker for her help during animal experiment and never ending analyses in the lab.

To W. Schmid and G. Schwentker for their excellent technical assistance.

To C. Fochtmann, O. Wellnitz and S. Richter for help during animal experiments

To Mr. Agr. Ing M. Schmölz for very good working conditions at Veitshof during experiment.

To Agroscope Liebefeld- Posieux for provision of the location and the animals for experiment.

To J. Riederer, P. Wametsberger and the milkers at Agroscope Liebefeld- Posieux for their friendly and necessary help and the good ambience during work.

To Dr V. Lollivier for very good collaboration with a friendly atmosphere.

To the diploma student M. Locher contributed very well to the continuing work.

To F. Hirche and K. Eder for good collaboration.

To E. Merkus for correcting proofs.

To Weinsberger Solargesellschaft W. Stendel GmbH, Weinsberg, Germany for providing of the animal solarium.

To my family and my boy-friend Bernhard for patience and support in hard times, DANKE.