University of Veterinary Medicine Hannover

Effects of Dexamethasone and Training on the Hypothalamic-Pituitary-Adrenal Response on Mild Stress Challenge in Dairy Cows

Thesis

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This work was dedicated to my parents and my friends

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List abbreviations

ACTH	Adrenocorticotropic hormone
AUC	Area under the curve
BW	Body weight
BL	Base line
CBG	Corticosteroid binding globulin
CRH	Corticotropin releasing hormone
CV	Coefficient of variation
CA	California
Cº	Grad celsius
DEXA	Dexamethasone
D	Day
e.g.	For example
EDTA	Ethylenediaminetetra-acetic acid
gm	Gram
HPA	Hypothalamic-pituitary adrenal axis
HF	Holstein Friesian
HSL	Hormone sensitive lipase
HR	Heart rate
I.U.	International unit
I.V	Intravenous
Kg	Kilogram
LR	Lateral recombancy
mg	Milligram
min.	Minute
ml	Millilitre
mmol	Millimol
µg	Microgram

µmol	Micro mol
NEFA	Non-esterified fatty acids
ng	Nanogram
n.s.	Not significant
NaCl	Sodium chloride
Plac	Placebo
p-value	Probability- Value
PG	Pico gram
P	Period
RR	Respiratory rate
Resp.	Respectively
SAS	Statistical Analysing System
SD	Standard deviation
SEM	Standard error of the mean
s.c	Subcutaneously
t	Time
Tr	Treatment
t-	before lateral recombancy
USA	United States of America
®	Trademark
-	Minus
%	Percent
*	Multiplication

1. Introduction

High yielding dairy cattle are exposed to several stressors resulting from environment, disease and animal-human interactions which may affect their well being. Such stressors and hassles related to the management and handling of cows can not be completely eliminated or avoided during the daily work operations.

Stress has been defined as a consequence of adverse effects of environment, physical, psychological or management systems which force changes in neuro-hormonal, haemato-chemical, or behavior to avoid physiological malfunctioning and to prepare animals for coping with stressful situations (CUNNINGHAM et al.; 2007, FAZIO et al:; 2003). The adrenal glands play a central role in hormonal reactions to stress as they are involved both in the hypothalamic–pituitary–adrenocortical axis (HPA) and the symphatho-adrenomedullary system (MOBERG, 2000, WILLIAM et al:, 2004). Adrenal release of cortisol and catecholamines during stress induce metabolic changes as well as cardiovascular and respiratory adaptation.

From other species it is known that exogenous glucocorticoids, such as dexamethasone, will suppress the HPA function and thereby adrenal cortisol release due to negative feed back (PERON et al 1960; SALMENPERAE et al 1976; RIJNBERK et al. 2010; VEISSIER et al. 2001). Dexamethasone is frequently used as a single treatment in dairy cows either in low doses (40µg/kg body weight; BW) for treatment of metabolic diseases or in higher doses (0.1 – 0.5 mg/kg BW) for anti-inflammatory treatment (KUSENDA 2010). To the best of the author's knowledge no information is available on the extent and duration of low dose dexamethasone effects on HPA function, although serum cortisol concentrations are commonly used to identify stress responses in cattle.

Commonly cows are restrained for claw trimming, a typical and repeatedly performed husbandry procedure, either in standing position in claw trimming chutes or in lateral recumbency on surgical tipping tables. Restraining is experienced as severe stress leading to the typical

hormonal, metabolic, and cardio-respiratory stress responses (PESENHOFER et al., 2006; RIZK et al., 2011a). Thus, restraining cows on surgical tipping tables appears as an adequate stress challenge model which is not painful for the restrained cow (RIZK et al 2011a). However, stress experiences depend largely on the individual stress responsiveness as well as previous experiences. Thus, the stress response may abate when cows are turned repeatedly into lateral recumbency under calm and reasonable handling conditions.

Animal handling and confinement by itself may be stressful and may confound results of blood cortisol measurements in studies on stress management (HOPSTER et al., 1999; COOK et al., 2000). Several authors have investigated non- or minimal invasive sampling procedures such as corticoid and metabolite determination in the urine (HAY et al., 1998), saliva (COOPER et al., 1989), milk (VERKERK 1998), faeces (KLEINSASSER et al 2010) or in blood using permanent indwelling venous catheters to minimize the stress effect (FELL and SHUTT, 1986; COOK et al., 1996; RIZK et al., 2011a)). Recently in human medicine measurement of cortisol concentration in lachrymal fluid was suggested (BANBURY 2009) which may have the advantage of being minimal invasive and therefore less stressful than blood collection. Also lachrymal cortisol concentrations display the "free" form of endogenous glucocorticoids, since lachrymal fluid is devoid of binding proteins (BANBURY 2009).

The aim of the present study was to investigate in cows restrained in lateral recumbency on a surgical tipping table as a minimal invasive stress challenge model

1. the extent and duration of a low dose dexamethasone treatment effect on HPA function,
2. the effect of training on hormonal stress response when cows are turned repeatedly into lateral recumbency, and
3. whether measurement of cortisol in lachrymal fluid may be a useful non invasive parameter to asses stress in cows.

2. Literature

2.1. Stress

Stress is defined as a consequence of adverse effects of the environment or management systems which force changes in an animal's physiology or behavior to avoid physiological malfunctioning (FAZIO et al., 2003). Adaptation or stress reactions represent modifications of ongoing physiological mechanisms and behavior that allow an animal to respond to stress stimuli with minimum alteration in homeostasis (STERLING and EYER, 1988; MUDRO et al 2005). Appleby (1999) defined animal welfare as the state of well-being brought about by meeting the physiological, environmental, nutritional, behavioral, mental and social needs of an animal. According to Broom (1986) well-being and welfare is impaired when adaptation mechanism fail to enable an animal to cope with its environment.

Dairy cows are exposed to a variety of stressors during their life such as climate stress, social stress among herd mates, stress due to inadequate environment, disease and pain, parturition as well as metabolic stress. Also unfamiliar husbandry or veterinary procedures, human-animal interaction and exposure to a new environment may be experienced as severe stress by cattle. The extent of stress experiences related to handling, environment and contact to humans or herdmates depends largely on the individual stress responsiveness and previous experiences of the animal (EFSA, 2009).

Handling stress is one of the main environmental effects leading to poor animal welfare and based on the animal's response to detectable or potential danger (BROOM, 1991; EFSA 2009). Handling for husbandry of veterinary procedures is associated with stress by social isolation of the animal from the herd, animal-human contact and exposure to new environments. Animals respond to such stressors by changes in behavior as freezing, avoidance or aggression. Furthermore, neuro-humoral responses occur and due to activation of the hypothalamic-pituitary-adrenal axis (HPA) and adrenal release of catecholamine cardio-respiratory and metabolic alterations are seen (ARCHER, 1979; BROOM, 1991). Recording hormonal, metabolic, cardio-

respiratory and behavioral changes is often used to identify and to quantify the magnitude of stressors in studies on stress and welfare. However, it is known that some animals are much better at coping with stress than others. While genetic factors are known to play a role in the susceptibility to stress, the prior experience of an individual with stressful events for instance handling are also known to exert a major influence on responses (REECE, 2004).

Measurement of serum concentrations of cortisol is frequently performed in studies on stress and welfare in cattle (BEERDA et al., 2004; FISHER et al., 2002; MORROW et al., 2002). Cows which are in stressful situations show higher levels of cortisol than cows in non-stressful settings (WHISNANT et al., 1985; MENCH et al., 1993; BOISSY et al 1997, GONZÁLEZ et al. 2003). Corticotropin releasing hormone (CRH) rise after exposure to stress which causes the pituitary gland to increase arenocorticotropin hormone (ACTH) release (Figure 1). ACTH binds to receptors in the adrenal cortex and stimulates in turn the secretion of cortisol (McDONALD et al., 1989, CUNNINGHAM et al., 2007). When serum concentrations of cortisol are high, the excess hormone will suppress the release of (ACTH) as a negative feedback regulator which in turn causes a decrease in serum cortisol concentration (PERON et al., 1960; SALMENPERAE et al., 1976; McDONALD et al., 1989).

After release cortisol is bound to one of the two main plasma protein carriers; corticosteroid binding globulin (CBG) and albumin. CBG has only one cortisol receptor and thus a low capacity for binding cortisol but in contrast to albumin, cortisol binds strongly with CBG. While 75 percent of cortisol binds to CBG, approximately 15 percent of cortisol binds to albumin, and the remaining 10-12 % remains undbound or free (JAMES et al., 2007).

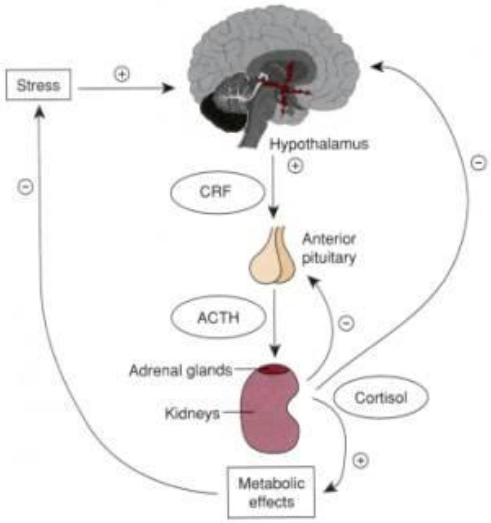


Figure 1. Hypothalamic-Pituitary-Adrenal Axis

(ENCEPHALOS ASSOCIATION, www.encephalos.gr/48-3-07e.htm)

Through the effect of stress on the HPA and the sympathetic autonomic nervous system the body will be flooded with catecholamines and cortisol, leading to increasing heart rate, blood pressure, myocardial activity with a simultaneous increase in myocardial oxygen consumption as well as peripheral vasoconstriction and increasing respiratory rate (MELLOR and STAFFORD, 1999; HUDSON et al., 2008; SCHUBERT et al, 2009; RIZK et al 2011a). Typical metabolic consequences are in turn high serum concentrations of glucose, non-esterified fatty acids (NEFA), and lactate as well as changes in water, acid-base and electrolyte balance and generally catabolic metabolism (MELLOR and STAFFORD, 1999; ANIL and DEEN 2002).

Stress can be divided into two types of stresses, acute and chronic stress. Acute stress results from a temporal exposure to an aversive situation such as spontaneous restraint, transport or a surgical intervention (BOISSY et al., 1997; KENT et al., 1983; MASON GJ et al., 2001, SYLVERTER et al., 1998) and leads to a stimulation of the HPA axis resulting in an immediate surge of cortisol

concentrations in the blood. The levels may remain elevated for up to one hour after exposure to the stressor (MORMÈDE et al., 1980; VEISSIER et al., 1988). However, blood cortisol concentrations do not reflect precisely the severity of the stressor. For example, cows which are only restrained had higher cortisol concentrations than cows which were first separated from their peers and then restrained in a crush, and no difference was observed between cows which were transported in addition to being separated and restrained (SANCHEZ et al, 1996).

Chronic stress involves a stressor that is continuously or repeatedly sustained for weeks, months, or even years and involves more complex mechanisms. In cattle (MORMÈDE et al., 2007) and rodents (LACHUER et al., 1994; DHABHAR et al., 1997; FERNANDEZ et al., 2002; ARMARIO et al., 2004) chronic exposure to the same stressor eventually reduces activity and reactivity of the HPA axis. For example, crowding results in an increased ACTH-induced cortisol response for 2–7 days (FRIEND et al., 1977; FRIEND et al., 1979), whereas longer-lasting spacial confinement suppresses adrenocortical responses (BENEKE et al., 1983; FISHER et al., 1997).

This process involving the return to pre-stress or baseline concentrations of ACTH and corticosteroids despite sustained exposure to the stressor has been termed habituation or adaptation (SAKELLARIS et al., 1975; LAY et al., 1996). The stressor is no longer perceived as novel or no longer feared, and thus the stimulatory inputs on ACTH secretion from the hypothalamus and higher brain centers are reduced (RIVIER et al., 1987).

Adaptations at the level of the HPA system to environmental challenges are flexible and dynamic over time (MORMÈDE et al., 2007). They involve both sensitization and desensitization, for example, due to reversible changes in the density and sensitivity of ACTH and cortisol receptors. Furthermore, depending upon the onset of the stressful condition and the nature of the stressor, the reactivity of the pituitary–adrenal system may be increased, unchanged or even depressed (MORMÈDE et al., 2007).

2.2. Stress evaluation

Stress can be evaluated by changes in physiological parameters or indirectly by interpreting animal behavior (WAIBLINGER et al., 2006). The evaluation of physiological parameters is based on changes in parameters of the sympathetic nervous system and the HPA (REECE 2004). For example, an isolated sheep or a newly branded dairy heifer will have an immediate, yet temporal surge in catecholamine concentrations in circulating blood, due to stimulation of the adrenal medulla by presynaptic sympathetic axons (REECE 2004).

Plasma cortisol concentration is the most widely used parameter to asses stress events. Cortisol concentrations are sensitive to environmental factors, such as handling and constraint (JAMES et al., 2007). To distinguish between effects on cortisol concentrations by the stress event and by the sampling procedure per se it is suggested to take blood sample within 2–3 min after catching the animal, before the adrenal cortex has been activated (MORMÈDE et al., 2007) or use of permanent indwelling catheters for sample collection (FELL and SHUTT 1986; COOK et al., 1996). Glucocorticoids can also be measured in obtained saliva samples in a variety of species such as pigs (SCHONREITER et al., 2000; COOK et al., 1996), cattle (NEGRAO et al., 2004; CHACON PEREZ et al., 2004), sheep (FELL et al., 1985) and goats (GREENWOOD and SHUTT 1992). Saliva can be collected for up to four minutes with out effect of handling being reflected in cortisol concentrations (REECE 2004). However, analytical sensitivity and specificity of assays are regarded as less accurate than plasma assays (BLACKSHAW et al., 1989; COOPER et al., 1989).

The main elimination route of glucocorticoids is through the urine; around one to two percent of the total cortisol secretion is excreted in this way (JAMES et al., 2007). Urinary cortisol displays a close linear correlation with unbound plasma cortisol (LINDHOLM and SCHULTZ-MÖLLER 1973). Although urine can be collected non-invasively and thus without stress for the animal, it is only available in unforeseen intervals (HAY and MORMÈDE 1997). Catecholamines and their metabolites can also be measured in urine (HAY and MORMÈDE 1997).

The measurement of fecal 11,17-dioxoandrostanes (11,17-DOA), a group of cortisol metabolites, has been proven useful to evaluate adrenocortical activity in a variety of species (PALME and MOSTL 1997, PALME 2005; MORMÈDE et al., 2007; KLEINSASSER et al., 2010). A careful validation for each species and sex should be taken into consideration as well as the dependency of 11,17-DOA concentrations on faecal temperature (TOUMA and PALME 2005). After collection within one h an increase by 136% of 11,17-DOA concentration was seen, thus immediate freezing of faecal samples and storage at −20 °C until analysis should be considered (MORMÈDE et al., 2007). Even after freezing some of the 11,17-DOA producing enzymes remain active and are only inactivated by heating the sample to 95 °C (PALME et al., 2000).

Milk concentrations are highly correlated to plasma concentrations (VERKERK et al., 1998). Collecting milk samples in dairy animals is very convenient and usually not particularly stressful for the animal. However, concentrations may depend on dilution effects according to varying daily milk yield.

In some studies beside cortisol also ACTH blood concentrations are determined to evaluate HPA responses to stress. FAZIO et al. (2008) showed a significant increase in ACTH after stress caused by transportation in stallions, whereas BEEKMAN (2004) described an elevation of ACTH in mice after exposure to psychological stressors. In cows, an increase of serum ACTH has been reported during transportation stress (KNIGHTS and SMITH 2007)
Stress induced hormonal response causes metabolic and cardiorespiratory changes which are frequently measured for assessment of stress.

The stress-induced release of glucocorticoides stimulates hepatic gluconeogenesis (DESBOROUGH, 2000; REECE, 2004). Reduced insulin sensitivity of peripheral tissues by glucocorticoides may also contribute to hyperglycemia (KUSENDA et al., 2011).

Activation of the hormone sensitive lipase (HSL) by catecholamines will stimulate release of nonesterified fatty acids (NEFA) from adipose tissues resulting in elevated NEFA blood levels

(MUDRON et al., 1994). Studies have also shown increased NEFA release from adipocytes in response to glucocorticoids (BAXTER and FORSHAM, 1972; MELLOR and STAFFORD 1999; WATERMAN-PEARSON 1999; CAMBRIDGE et al. 2000; OTTOSSON et al., 2000).

Catecholamine induced vasoconstriction and thereby reduced oxygenation in peripheral tissues may enhance anaerobic glycolysis resulting in increased lactate blood levels (RIZK et al 2011a).

Stress induces activation of the sympathetic autonomic nervous system. The response floods the body with catecholamines and leads to increasing heart rate, respiratory rate, blood pressure and myocardial oxygen consumption, (MELLOR and STAFFORD, 1999; HUDSON et al., 2008, Schubert et al., 2009).

Individual stress responsiveness can be assessed by the ACTH stimulation test. (DORIN et al., 2003), where the adrenal cortex is stimulated by intravenous injection of synthetic ACTH at a dose of 0.5–1 IU/kg $BW^{0.75}$ for young animals and 1–2 IU/kg $BW^{0.75}$ for adults (FRIEND et al., 1985; LADEWIG and SMITH, 1989, VEISSIER et al., 2001). Occasionally, CRH at a dose of 0.03 - 1 μg/kg is used (ZHANG et al., 1990, JANNSSENS et al., 1996, VEISSIER et al., 1999, DÈSAUTÈS et al., 1999, HAY et al., 2000, REENEN et al., 2005).

The cortisol response to ACTH and CRH challenges is rapid; after CRH injection the peak response will occur within 15 min and cause a 50% elevation in plasma cortisol level. Within 90 min a return to baseline is expected (REECE, 2004). ACTH injection causes a two- to three-fold plasma cortisol elevation within 60 to 120 minutes followed by a return to baseline levels in the following four hours (REECE, 2004). Collection of blood samples should be implemented at regular intervals after the injection of synthetic ACTH to determine ACTH and corticosteroid levels (ALAM et al., 1986, MORMÈDE et al., 2007). For integrated response evaluation, areas under the curves of corticosteroids or ACTH should be calculated (REECE 2004). It has been found that animals reared in poor conditions or subjected to repeated stressors exhibit an increased cortisol response to ACTH injection (FRIEND et al., 1985). In contrast, other authors

found a decreased response in cattle subjected to tethering or to crowding (LADEWIG and SMIDT, 1989; HASEGAWA et al., 1997). VEISSIER and Le NEINDRE (1988) showed that the intramuscular administration of ACTH induces higher, but more variable cortisol responses than intravenous injection. Other factors affecting the ACTH induced cortisol response are age (RIEGLE and NELLOR, 1967), high environmental temperatures (SHAYANFAR et al., 1975), time span after parturition (DUNLAP et al., 1981; GWAZDAUSKAS et al., 1986), and the presence of suckling calves (DUNLAP et al., 1981). Male sex hormones suppress ACTH induced cortisol responses, thus steers have higher responses than bulls (VERKERK and MACMILLAN, 1997).

The dexamethasone suppression test is widely used in human and veterinary medicine to verify hyperadrenocorticism (CAROLL et al., 1981). This test consists of an injection of dexamethasone (synthetic Glucocorticoids) and subsequent measurement of plasma cortisol concentrations and optionally ACTH level (RIJNBERK et al., 2010). In cattle the HPA response can be blocked by injecting 15–20 μg/kg BW dexamethasone i.m. Cortisol plasma concentrations will decrease by at least 50% within three hours and will return to baseline concentrations in the following 24 to 36 hours (VEISSIER et al., 2001).

3. Materials and methods

3.1 Study design

The study was approved by the Ethical Animal Care and Use Committee of the Federal State of Lower Saxony, Germany (research permit number 33.12-42502-04-10/0135).

In a blinded study in cross over design six healthy German HF cows were randomly divided into two groups of three cows each. In two subsequent study periods with an interim of four weeks each time all cows were restrained three times on day 1, day 3 and day 5 in lateral recumbency (LR) on a surgical tipping table without sedation. While cows of group one received on day 0 of period 1 dexamethasone in a dose of 40 µg/kg BW cows of group 2 were treated accordingly on day 0 of period 2 (table 1). During each session of lateral recumbency heart and respiratory rate as well as the hormonal and metabolic stress response were assessed.

Table 1: Scheme of study design and medical treatment

	Period 1		Period 2	
	Group 1	Group 2	Group 2	Group 1
d 0	DEXA	Placebo	DEXA	Placebo
LR d1	Cow 1, 2, and 3	Cow 4, 5, and 6	Cow 4, 5, and 6	Cow 1, 2, and 3
LR d3	Cow 1, 2, and 3	Cow 4, 5, and 6	Cow 4, 5, and 6	Cow 1, 2, and 3
LR d5	Cow 1, 2, and 3	Cow 4, 5, and 6	Cow 4, 5, and 6	Cow 1, 2, and 3

DEXA: Treatment with dexamethasone (40 µg/kg BW IV),
Placebo: Treatment with saline (equivalent dose IV)
LR: Restrain for 30 min in lateral recumbency as mild stress challenge
d: day
Interim time between study period 1 and 2: four weeks

3.2 Experimental animals, housing and feeding

Six healthy, non-pregnant, non-lactating, German Holstein Frisian cows (age: 4.37 ± 3.27 years; body weight (BW): 610 ± 87.9 kg) were included in this study. All cows were housed in tie stalls with straw bedding at the Clinic for Cattle of the University of Veterinary Medicine Hannover,

Germany. Cows were fed a diet based on hay and one kg of concentrate according to maintenance with free access of water.

3.3 Instrumentation

All cows were fitted with indwelling jugular vein catheters under aseptic conditions one day before the start of each study period (day: d-1). For implantation of venous catheters after surgical skin preparation local infiltration anesthesia was applied using 5 - 8 ml of procaine (procaine 2% ad. us. vet.; Selectavet. GmbH, Weyarm-Holzolling- Germany). The venous catheter (Stericlin®, AD 2,4mm, length 20cm with Teflon catheter (Walter, veterinar- instrumente e.k, Baruth/ Mark, Germany) was inserted into the jugular vein and fixed with two skin sutures using synthetic non absorbable suture material (Filovet Bengen ®, WDT, Garbsen- Germany). Additionally a skin fold was applied over the catheter at the insertion site using non absorbable suture material (USP 8, Silk Braided®, SMI, Belgium) to protect the catheter against mechanical irritation and bacterial contamination. The insertion site was covered by a bandage. After implantation catheters were flushed with heparinised 0.9% sterile saline (10,000 IU heparin\L, Heparin-Calcium Ratiopharm, Germany; sodium chloride solution, B.Braun Melsungen AG, Germany). All cows received 20,000 IU\kg BW of procaine penicillin (Procaine-Penicillin-G; AniMEDICA, Germany) subcutaneously for three subsequent days.

3.4 Lateral recumbency on a surgical table

Food was not withheld before cows were restrained. The last concentrate feeding (1 kg) was about two hours before restrain in lateral recumbency (LR). LR war performed as described by RIZK (2010) every day between 11 am and 2 pm. Briefly, all cows carried a halter with a 1.5 m long strong rope which was used to guide the cows. With the hydraulic tipping table in perpendicular position cows were led to the table with one person at the head and a second person

in the pelvic region. While the person at the head fixed the head with a neck belt to the head pad of the table, the second person pushed the cow where necessary at the tuber coxae in a position parallel and close to the table unless the cow took this position voluntarily. Then the abdominal and thoracic belts were tightened thoroughly but not so tight, that thoracic movements were significantly inhibited. Thereafter cows were turned into left LR in horizontal position and the two hind legs were fixed tightly on leg pads in middle of the metatarsus. After legs were fixed the abdominal and thoracic belts were loosened so that the animal remained safely fixed but the compression of the belts on the abdomen and thorax were reduced and the animals could breath freely. To let cows stand up, the procedure was performed inversely. Abdominal and thoracic belts were tightened, the legs released, the table was turned in perpendicular position and the belts (first thoracic, abdominal and then neck belt) removed when the feet had safe contact to the ground. When cows moved very slowly and reluctantly to the tipping table no more than gentle and patient pushing and encouragement by voice was used to direct cows into the correct position for fixation. The whole table was padded with a rubber mat and an extra soft rubber cushion was put under the whole left front limb in contact with the table to avoid muscular and nerve damage during LR.

3.5 Treatments

Cows of group I (cow 1, 2, and 3) were given on d0 of study period 1 40 µg\kg BW Dexamethasone (Dexamethason Injektionslösung®; CP-Pharma, Germany) intravenously, 24 hours before cows were restrained the first time in lateral recumbency (d1) on the tipping table. 72 hours (d3) and 120 hours (d5) after treatment animals were laid down again in LR on the tipping table. The same procedure (study period 2) was repeated 4 weeks after study period 1 except cows were treated with placebo (sodium chloride solution, B.Braun Melsungen AG, Germany) in to dexamethasone treatment corresponding volume and route of application.

Cows of group 2 (cow 4, 5, and 6) were treated as cows of group 1 except these cows received on d0 of study period 1 the placebo and on d0 of period 2 the dexamethasone.

3.6. Monitoring

3.6.1. Heart and respiratory rate

Heart and respiratory rate were monitored on each day when cows were restrained in LR (time = t0). Baseline values were recorded 30 and 15 minutes before LR (t-30min, t-15min), during LR (t10min, t20min, and t30min) and after LR (t45min, t60min, and t90min). Respiratory rate was assessed by counting thoracic excursions and heart rate by auscultation of heart beats.

3.6.2. Blood sampling and analysis

3.6.2.1 Blood sampling

Blood samples were withdrawn from the jugular vein catheter 30 and 15 minutes before LR (t-30min, t-15min), during LR (t10min, t20min, and t30min) and after LR (t45min, t60min, and t90min). Blood were collected in sodium fluoride tubes (Fa. Sarstedt, Sarstedt) for glucose analysis and in serum tubes (Fa. Sarstedt) for analysis of cortisol, nonesterified fatty acids (NEFA), and lactate. Blood samples for ACTH measurement were taken in EDTA tubes (Fa. Sarstedt) containing a protease inhibitor (Trasylol®, Bayer, Leverkusen, Germany). Fluoride and EDTA samples were kept on ice after collection. All samples were centrifuged for 10min at 1,500g and 4°C about 30 minutes after collection. Plasma and serum aliquots were stored at minus 80°C until analysis.

3.6.2.2 Lachrymal fluid sampling

Lachrymal fluid was collected 15 min before LR (t-15min), at the end of LR (t30min), and 60 min after LR (t90min) using 2ml syringes (Bd-discarded II, Company, Spain) connected to a blunt, soft tube (Senso connect HV, Meditrade Medicare, Medizinprodukte GmbH, Oberer Stadtplatz, Germany; AD 1mm) from the conjunctival sack. Lachrymal fluid was stored immediately after collection at -20 C until analysis.

3.6.2.3 Analytical procedures

Serum cortisol concentrations was measured by an automated competitive chemiluminescence immunoassay (LKCO1, Immulite® 1000 System, Siemens Diagnostics,USA). The intra-assay coefficient of variance (CV %) was 6.3-10%. The analytical sensitivity was 0.2 ng/ml. The assay has following cross reactivities: Prednisolone 49%,

Plasma ACTH concentration was measured using a automated chemiluminescence immunoassay based on a sandwich immunoassay principle (Immulite® System, Siemens Diagnostics, USA). The intra-assay CV % was 3.1-9.6%. The analytical sensitivity was < 5 pg/ml.

Concentrations of plasma glucose as well as serum NEFA and lactate were measured on an automated analyser (Cobas Mira®, Hofmann-La Roche) using commercial test kits.

Before measuring lacrimal cortisol concentrations the samples were centrifuged at 1,500g (10 min, 4C°) after thawing. Cortisol concentration was then measured using a commercial available Cortisol ELISA (Beckman Coulter, CA, USA) with following modifications. The supplied standard curve was adjusted to a lower measurement range of 0.125 ng/ml by dilution with buffer. An intra assay CV% was determined by measuring one sample of lachrymal fluid ten times within one test routine. The intra-assay CV% was 13.5. The analytical sensitivity was <0,03 µg/dl.

3.7. Calculations and statistical analyses

Data were analysed using the statistical analysis system (SAS version 9.1 for Windows, SAS institute Inc, Cary, NC, USA).

For all assessed parameters t-baseline was calculated as mean from results obtained on t-30min and t-15min. Thereafter, the area under the curve (AUC) was calculated from t-baseline, t10min, t20min, t30min, t45min, t60min, and t90min. The peak value during LR was assessed for each parameter.

AUC and Peak values were analysed by three factorial analysis of variance for repeated measurements (Proc GLM, Repeated statement). The statistical models included the factors treatment (placebo, DEXA) and period (period 1 and 2) and for the repeated statement the factor stress challenge by LR on d1, d3, and 5 (LR d1, LR d3, LR d5). The model included also interactions between treatment, period and LR d. Multiple comparisons of group means were performed by the LSMEANS statement (pdiff/tdiff option). Within groups results were tested for significant differences in group means on d1, d3, and d5 by means of paired t-test.

Results obtained at t-baseline were analysed by three factorial analysis of variance (PROC GLM). The factors treatment, period and LR day as well as their interactions were included into the statistical model. To test for differences between group means the LSMEANS statement (pdiff/tdiff option) was used.

Statistical analysis of curves of results obtained from t-baseline, t10min, t20min, t30min, t45min, and t60min was performed using the GLM procedure of SAS for repeated measurements. As factors treatment, period and LR day as well as their interactions were included into the model. For the repeated statement the time of sampling during LR was used.

Laboratory parameters (ACTH, cortisol, glucose, NEFA, lactate) deviated significantly from normal distribution (tested by PROC UNIVARIATE). Thus, for analysis of variance of these parameters the common logarithm was calculated and used.

The results of cortisol concentrations in lachrymal fluid were analysed by regression analysis (PROC Reg) comparing serum and lachrymal fluid cortisol concentrations at t-baseline, t30min (end of LR) and t90min and serum AUC cortisol with lachrymal fluid cortisol at t90min.

The level of significance was set at $P < 0.05$, a trend for significance was assumed for $P < 0.10$. Data were presented as mean ± SEM.

The program EXCEL (EXCEL® Office 2003, Microsoft Corp.) was used to create graphs.

4. Results

4.1. Baseline blood concentrations, heart and respiratory rate

4.1.1 ACTH

In control cows plasma baseline ACTH concentrations were significantly lower on day 3 and day 5 before stress challenge by restrain in LR compared to day 1 (table 2; figure 2 a-c). On day 1 and day 2 plasma ACTH level were significantly lower in dexamethasone treated cows than in controls (treatment effect: $p < 0.001$). While plasma ACTH baseline concentrations declined from day 1 to day 5 in controls in DEXA treated cows ACTH concentrations raised (treatment * LR day effect: $p = 0.009$). Baseline serum concentrations were numerically on day 1 and 3 and significantly higher on day 5 in period 2 than in period 1 no matter if cows were treated with DEXA or placebo (period effect: $p = 0.004$). In both periods cortisol concentrations in DEXA cows were lower than in controls.

Table 2: Results of statistical analysis regarding effects of treatment, study period, day of restrain in lateral recumbency (LR day) and their interactions for baseline plasma concentrations of ACTH.

Effect by	P - value
Treatment	< .001
Period	.004
LR day	.24
Treatment * LR day	.009
Treatment * Period	.14
Period * LR day	.40
Treatment * Period * LR day	.96

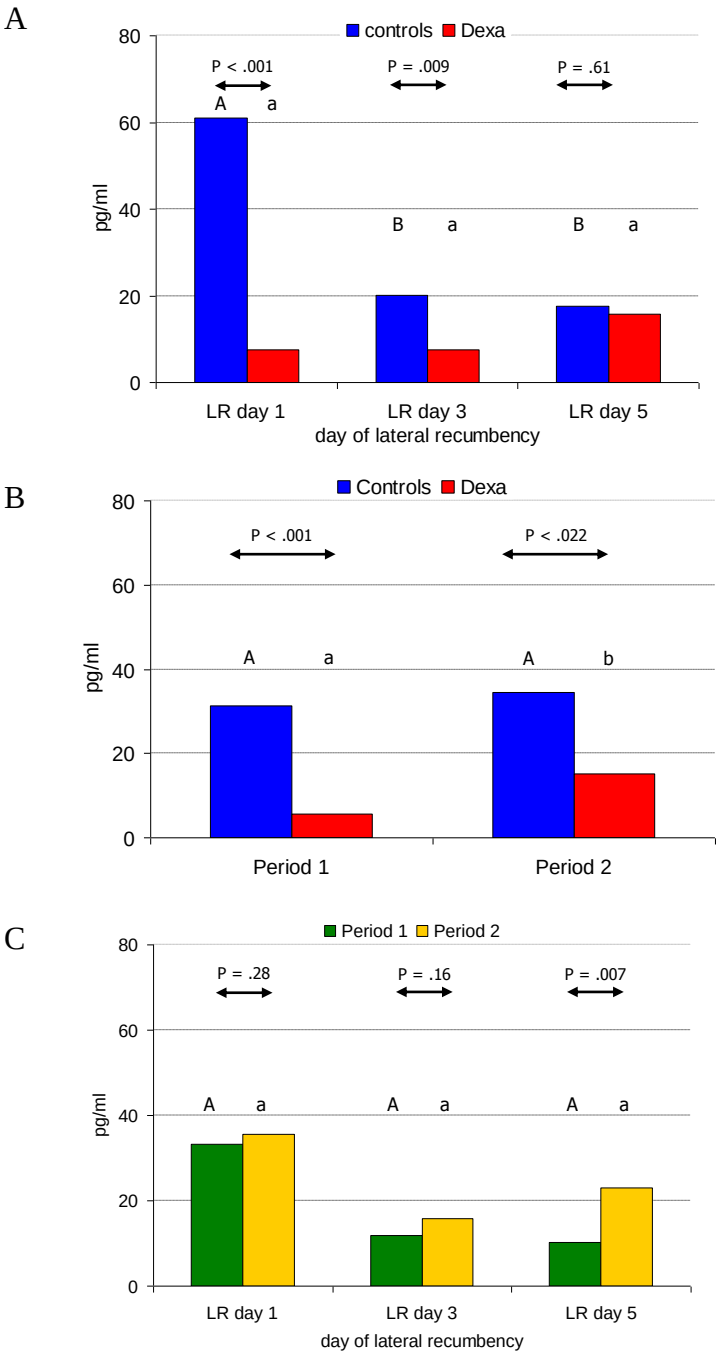


Figure 2 A-C: Mean baseline plasma ACTH concentrations of six dairy cows either treated with dexamethasone (dexa; 40µg/kg BW IV) or placebo before mild stress challenge by lateral recumbency (LR) on day 1, 3 and 5 (A), in period 1 and 2 (B), and in period 1 and period 2 related to the day of LR (C). Significantly different means within groups are marked by different letters. Significantly different means between groups are marked by arrows and differences are given by p-values above arrows.

4.1.2 Cortisol

After dexamethasone treatment mean serum baseline cortisol concentrations were significantly lower than in controls on all study days and in both study periods (treatment effect: $p < 0.001$; Table 3, Figure 3 A-C). While in control cows from day 1 to day 5 baseline cortisol serum concentrations decreased, in DEXA treated cows serum cortisol increased (treatment * LR day effect: $p = 0.007$). In controls baseline cortisol serum concentrations decreased compared to period 2 but in DEXA treated cows mean cortisol concentrations were almost same (treatment * period effect: $p = 0.025$).

Table 3: Results of statistical analysis regarding effects of treatment, study period, day of restrain in lateral recumbency (LR day) and their interactions for baseline serum cortisol concentrations.

Effect by	P - value
Treatment	< .001
Periode	.89
LR day	.44
Treatment * LR day	.007
Treatment * Period	.025
Period * LR day	.11
Treatment * Period * LR day	.10

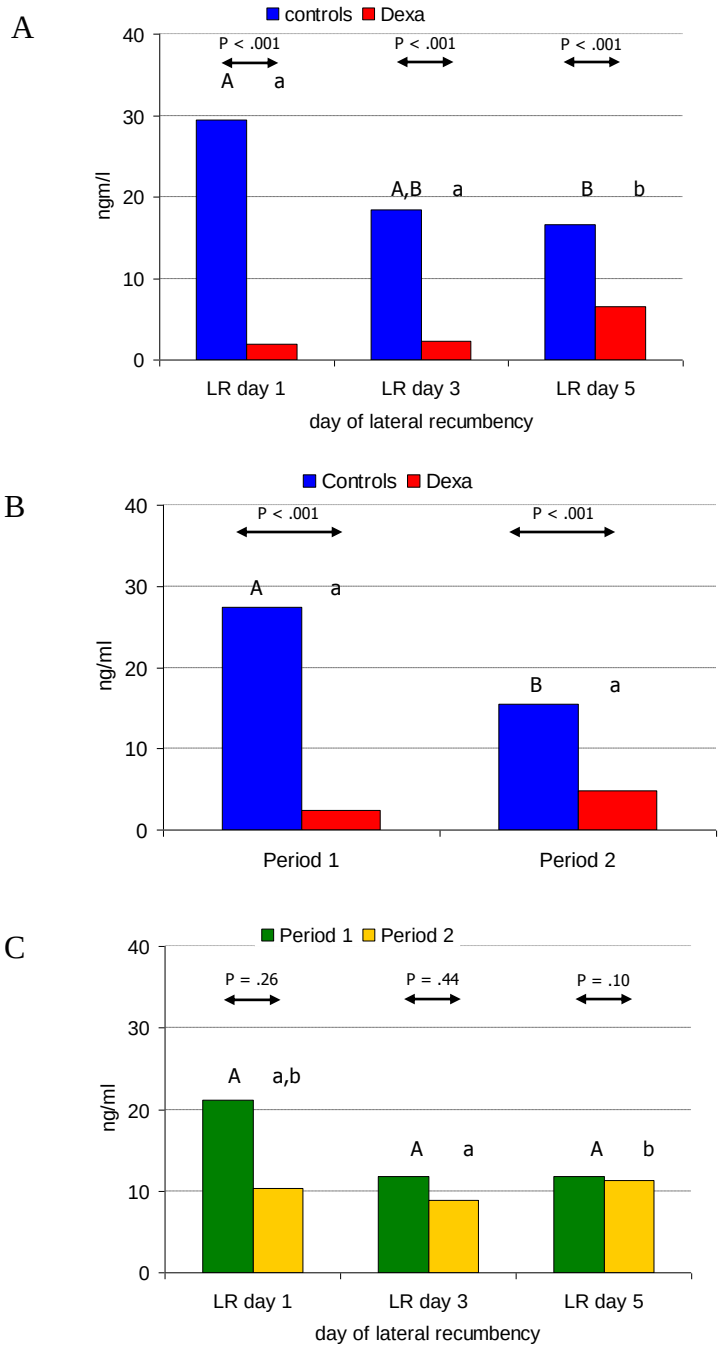


Figure 3 A-C: Mean baseline serum cortisol concentrations of six dairy cows either treated with dexamethasone (dexa; 40µg/kg BW IV) or placebo before mild stress challenge by lateral recumbency (LR) on day 1, 3 and 5 (A), in period 1 and 2 (B), and in period 1 and period 2 related to the day of LR (C). Significantly different means within groups are marked by different letters. Significantly different means between groups are marked by arrows and differences are given by p-values above arrows.

4.1.3 Glucose

On LR day 1 DEXA treatment resulted in significantly higher plasma glucose baseline concentrations than in controls, which this was not seen on day 3 and day 5 before stress challenge(day 1: $p < 0.001$; treatment * LR day: $p < 0.001$; Table 4, Figure 4 A-C). In control cows there were no significant differences in plasma baseline glucose level between day 1, day 2 and day 3 of stress challenge by LR where as in dexamethasone treated cows the plasma glucose level were significantly lower on day 3 and day 5 compared to day 1 ($p <0.05$). Baseline plasma glucose level were significantly lower in the control group and significantly higher in DEXA treated cows in period 2 than in period 1 (treatment * period effect: $p = 0.010$). Plasma glucose levels before stress challenge were in both periods lower on day 3 and 5 compared to day 1 (LR day effect: $p < 0.001$).

Table 4: Results of statistical analysis regarding effects of treatment, study period, day of restrain in lateral recumbency (LR day) and their interactions for baseline serum glucose concentrations.

Effect by	P - value
Treatment	< .001
Periode	.17
LR day	< .001
Treatment * LR day	< .001
Treatment * Period	.010
Period * LR day	.68
Treatment * Period * LR day	.55

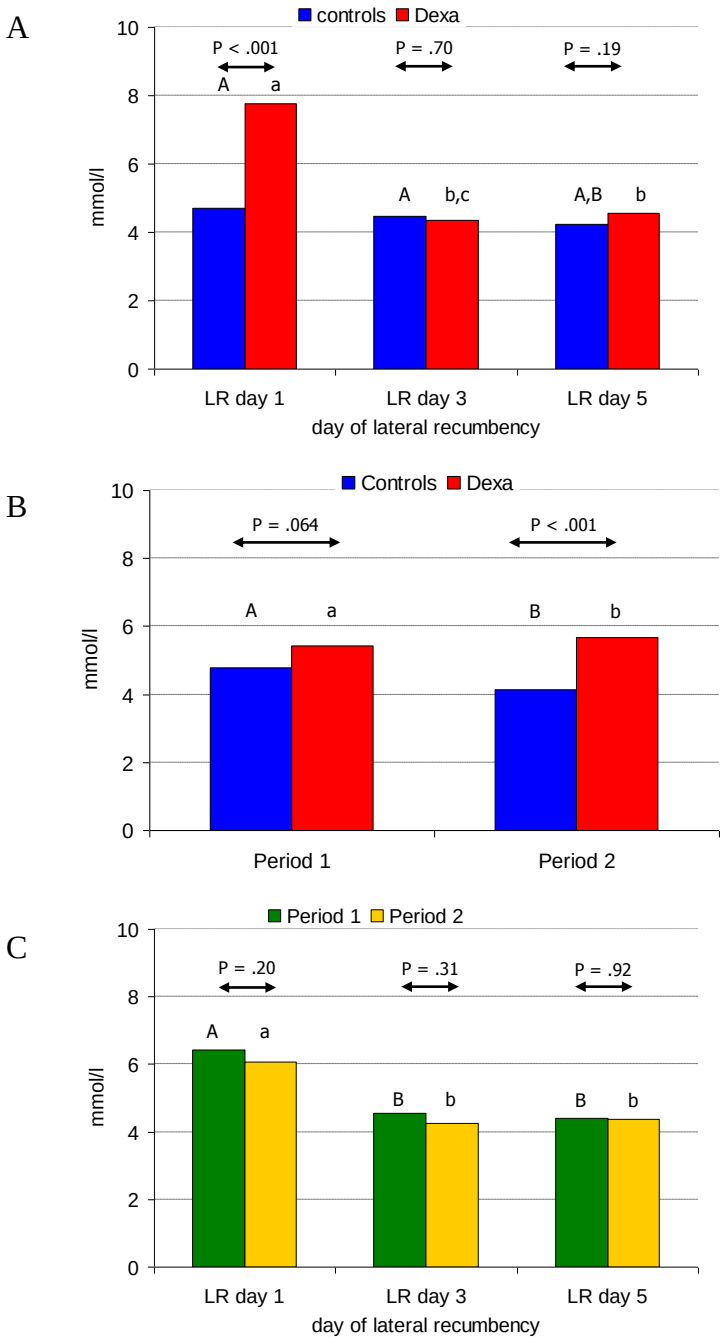


Figure 4 A-C: Mean baseline serum glucose concentrations of six dairy cows either treated with dexamethasone (dexa; 40µg/kg BW IV) or placebo before mild stress challenge by lateral recumbency (LR) on day 1, 3 and 5 (A), in period 1 and 2 (B), and in period 1 and period 2 related to the day of LR (C). Significantly different means within groups are marked by different letters. Significantly different means between groups are marked by arrows and differences are given by p-values above arrows.

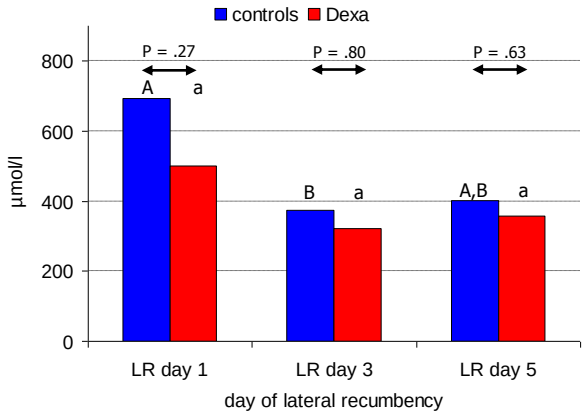
4.1.4 NEFA

Treatment, day of LR and period had no significant effects on baseline serum NEFA concentrations in this study (Table 5 A-C; Figure 5 a-c). However, mean baseline plasma NEFA concentrations were significantly lower in DEXA treated cows compared to controls in period 1 while NEFA concentrations were almost same in period 2 (treatment * period effect: $p = 0.020$).

Table 5: Results of statistical analysis regarding effects of treatment, study period, day of restrain in lateral recumbency (LR day) and their interactions for baseline serum NEFA concentrations.

Effect by	P - value
Treatment	.32
Periode	.12
LR day	.10
Treatment * LR day	.76
Treatment * Period	.020
Period * LR day	.77
Treatment * Period * LR day	.71

A



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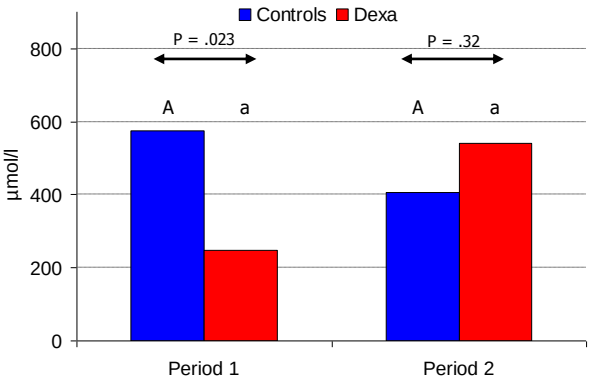

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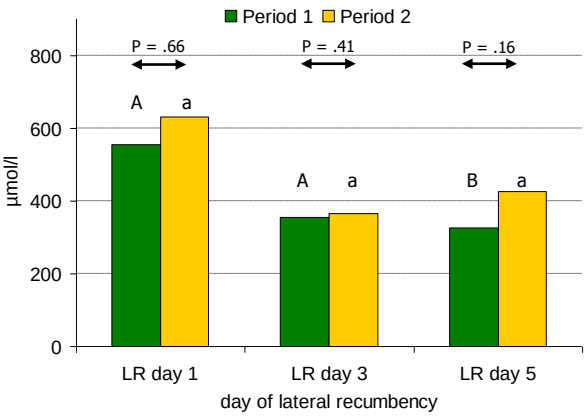


Figure 5 A-C: Mean baseline serum NEFA concentrations of six dairy cows either treated with dexamethasone (dexa; 40µg/kg BW IV) or placebo before mild stress challenge by lateral recumbency (LR) on day 1, 3 and 5 (A), in period 1 and 2 (B), and in period 1 and period 2 related to the day of LR (C). Significantly different means within groups are marked by different letters. Significantly different means between groups are marked by arrows and differences are given by p-values above arrows.

4.1.5 Lactate

Compared to controls DEXA treatment resulted in significantly higher mean serum baseline lactate concentrations on day 1 ($p < 0.001$) and in period 2 ($p = 0.001$) (treatment effect: $p = 0.002$; Table 6 A_C, Figure 6 a-c). Mean lactate concentrations were on day 1 about two-fold and five-fold higher than on day 5 in controls and DEXA treated cows, resp. (LR day effect: $p < 0.001$; trend treatment * LR day effect: $p = 0.085$). In control cows baseline plasma concentrations were significantly lower in period 2 than in period 1 while no difference was seen in DEXA treated cows (trend period effect: $p = 0.062$).

Table 6: Results of statistical analysis regarding effects of treatment, study period, day of restrain in lateral recumbency (LR day) and their interactions for baseline serum lactate concentrations.

Effect by	P - value
Treatment	.002
Periode	.062
LR day	< .001
Treatment * LR day	.085
Treatment * Period	.11
Period * LR day	.90
Treatment * Period * LR day	.96

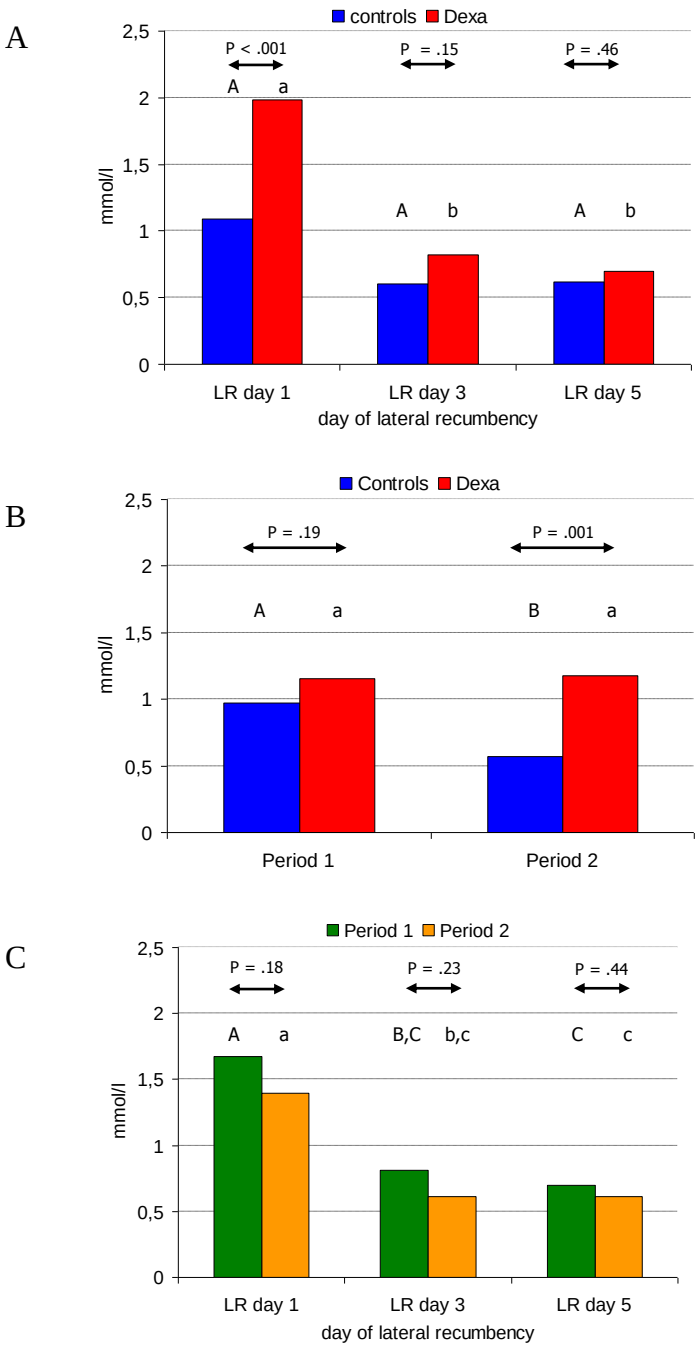


Figure 6: Mean baseline serum lactate concentrations of six dairy cows either treated with dexamethasone (dexa; 40µg/kg BW IV) or placebo before mild stress challenge by lateral recumbency (LR) on day 1, 3 and 5 (A), in period 1 and 2 (B), and in period 1 and period 2 related to the day of LR (C). Significantly different means within groups are marked by different letters. Significantly different means between groups are marked by arrows and differences are given by p-values above arrows.

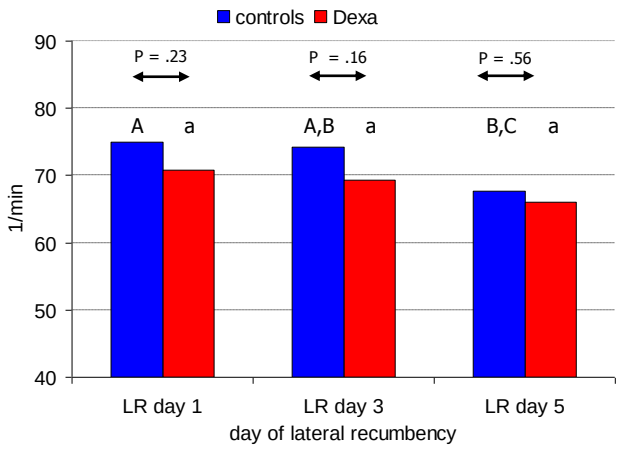
4.1.6 Heart rate

Mean baseline heart rates (HR) tended to be lower after DEXA treatment compared to controls (trend treatment effect: $p = 0.073$; Table 7 A-C, Figure 7 a-c). The day of LR revealed a significant effect ($p = 0.044$) on mean baseline heart rates which was predominantly seen in control cows (day 1 vs. day 5: $p < 0.05$). Period had no obvious effect on heart rate and evaluation of results revealed no further interactions of factors.

Table 7: Results of statistical analysis regarding effects of treatment, study period, day of restrain in lateral recumbency (LR day) and their interactions for baseline heart rate.

Effect by	P - value
Treatment	.073
Periode	.88
LR day	.044
Treatment * LR day	.82
Treatment * Period	.14
Period * LR day	.97
Treatment * Period * LR day	.97

A



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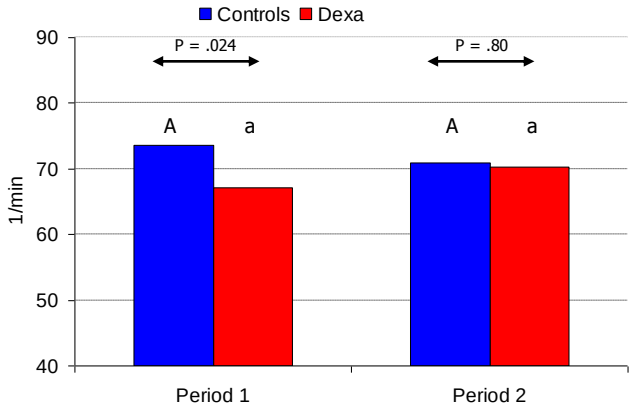

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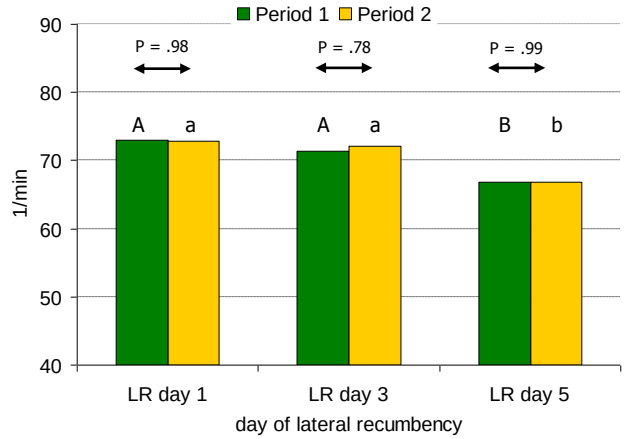


Figure 7 A-C: Mean baseline heart rates of six dairy cows either treated with dexamethasone (dexa; 40µg/kg BW IV) or placebo before mild stress challenge by lateral recumbency (LR) on day 1, 3 and 5 (A), in period 1 and 2 (B), and in period 1 and period 2 related to the day of LR (C). Significantly different means within groups are marked by different letters. Significantly different means between groups are marked by arrows and differences are given by p-values above arrows.

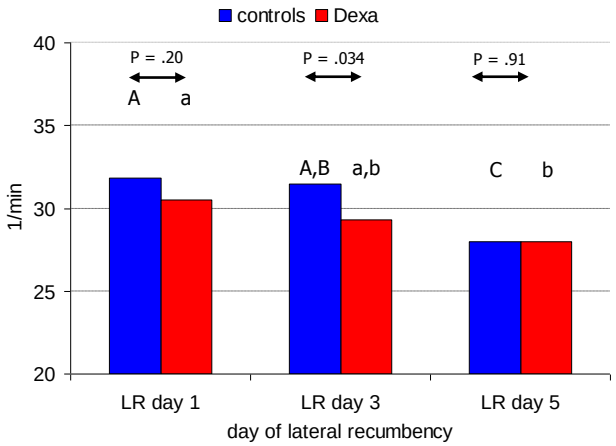
4.1.6 Respiratory rate

The factors treatment ($p = 0.045$), LR day ($p < 0.001$) and period ($p = 0.043$) had significant effects on baseline mean respiratory rates in study cows. Mean baseline respiratory rates were significantly lower in controls and DEXA treated cows on day 5 compared to day 1 of LR ($p < 0.05$) in DEXA treated cows compared to controls. After DEXA treatment evaluation of results revealed no differences in respiratory rates in period 1 but a significant treatment effect in period 2 ($p = 0.006$; treatment * period effect: $p = 0.046$). In both periods baseline mean respiratory rates were significantly lower on day 5 compared to day 1 (Table 8 A-C, Figure 8 a-c).

Table 8: Results of statistical analysis regarding effects of treatment, study period, day of restrain in lateral recumbency (LR day) and their interactions for baseline respiratory rate.

Effect by	P - value
Treatment	.045
Periode	.043
LR day	< .001
Treatment * LR day	.33
Treatment * Period	.046
Period * LR day	.80
Treatment * Period * LR day	.94

A



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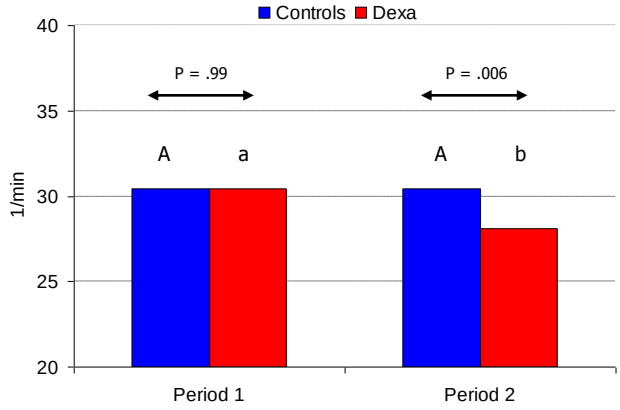

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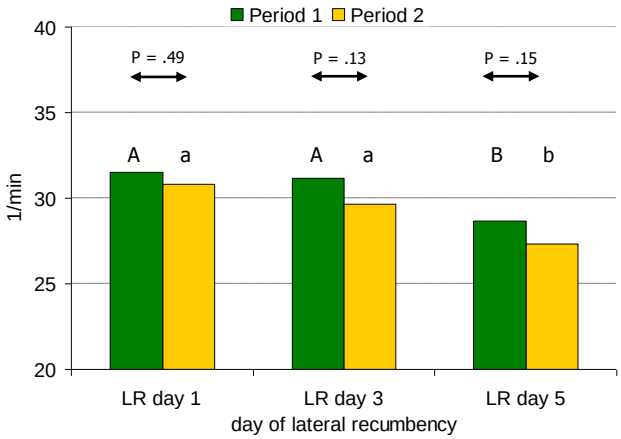

Figure 8: Mean baseline respiratory rates of six dairy cows either treated with dexamethasone (dexa; 40µg/kg BW IV) or placebo before mild stress challenge by lateral recumbency (LR) on day 1, 3 and 5 (A), in period 1 and 2 (B), and in period 1 and period 2 related to the day of LR (C). Significantly different means within groups are marked by different letters. Significantly different means between groups are marked by arrows and differences are given by p-values above arrows.

4.2. Stress response during lateral recumbency

4.2.1 Hormonal stress response

4.2.1.1 ACTH

The factors treatment ($p < 0.001$; $p = 0.002$) and the interactions LR day * treatment ($p = 0.020$; $p = 0.016$) and period *treatment ($p = 0.004$; $p < 0.001$) were found to affect significantly peak ACTH plasma concentrations and the area under the curve (AUC) of ACTH concentrations, resp., during the mild stress challenge by LR in this study (Table 9). DEXA treatment resulted in lower peak ACTH concentrations and AUC values compared to controls. While from day 1 to day 5 in DEXA treated cows ACTH peak and AUC increased, the opposite was seen in control cows. In control cows but not in DEXA treated cows peak and AUC ACTH were significantly lower on day 1 in period 2 compared to day 1 in period 1.

The evaluation of plasma ACTH concentrations over time during the LR revealed generally the same results as for peak and AUC ACTH values. In control cows ACTH levels increased significantly (time effect: $p < 0.001$) during LR, which was not seen after DEXA treatment (time * treatment effect: $p < 0.001$). In control cows the rise in ACTH concentrations seen in period 1 was almost absent in period 2 (time * period effect: $p = 0.003$). While in controls the increase of ACTH plasma concentrations in period 1 was less pronounced on day 3 and day 5 compared to day 1 in period 2 the rise in ACTH plasma concentrations in period 2 was almost same and absent on day 1, 3 and 5. After DEXA treatment neither in period 1 nor in period 2 on day 1, 3 and 5 ACTH concentrations increased noticeably (time * treatment * LRday effect: $p = 0.018$; time * treatment * period effect: $p = 0.008$; table 10, Figure 9 a-c).

Table 9: Mean peak concentrations and area under the curve (AUC) of ACTH plasma concentrations revealed from six dairy cows during mild stress challenge by restrain in lateral recumbency (LR) on three subsequent days (day 1, 3, and 5) in two study periods after either placebo or dexamethasone treatment (40µg/kg BW IV) on day 0.

		Treatment Controls		DEXA			P - value							
		Period 1	2	1	2									
Parameter	Day	A	B	C	D	SEM	A:B	A:C	A:D	B:C	B:D	C:D	Overall	p-value
ACTH AUC	1	11736 [a]	3565 [a]	152 [a]	366 [a]	1353	.005	< .001	< .001	.004	.005	.083	Treatment (T)	< .001
	3	9669 [a]	2724 [a]	260 [a]	516 [a]	3639	.28	.003	.013	.014	.079	.29	Period (P)	.43
	5	8780 [a]	1168 [a]	1321 [a]	2445 [a]	3198	.20	.26	.50	.84	.50	.62	T * P	.020
													Day (D)	.23
													D * T	.004
													D * P	.72
													D * T * P	.66
Peak (pg/ml)	1	944 [a]	175 [a]	5 [a]	13 [a]	134	.001	< .001	< .001	< .001	< .001	< .033	Treatment (T)	.002
	3	517 [a]	131 [a]	17 [a]	30 [a]	184	.31	.003	.012	.017	.066	.39	Period (P)	.45
	5	542 [a]	53 [a]	66 [a]	195 [a]	195	.13	.13	.42	.97	.43	.42	T * P	.016
													Day (D)	.21
													D * T	< .001
													D * P	.84
													D * T * P	.72

a)Differences in corresponding means of day 3 and day 5 compared to day 1 within one column are indicated by different superscript letters ($p < 0.05$)

Table 10: Results of statistical analysis regarding the treatment (placebo or dexamethasone: 40µg/kg BW), period and day of lateral recumbency (LR) effect and their interactions on plasma ACTH concentrations during mild stress challenge by LR in six dairy cows.

Effect by	P - value	Effect by	P - value
Treatment (Tr)	< .001	Time	< .001
Period (P)	.77	Time * Tr	< .001
LR day (D)	.15	Time * P	.003
Tr * D	.002	Time * D	.15
Tr * P	.002	Time * Tr * D	.018
P * D	.70	Time * Tr * P	.008
		Time * P * D	.60

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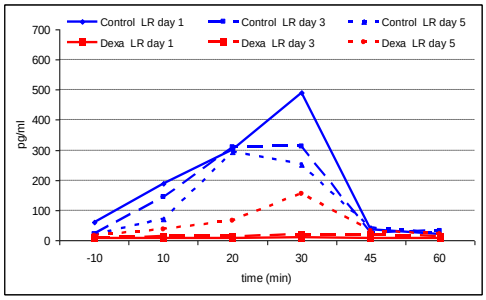

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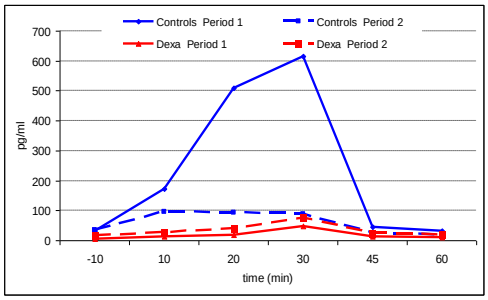

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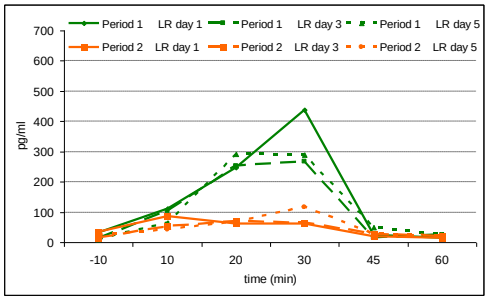


Figure 9 a - c: Mean plasma ACTH concentrations of six dairy cows during mild stress challenge by restrain in lateral recumbency (LR; from time 0 to time 30 min) after placebo or dexamethasone treatment (40 µg/kg BW) 24 h before challenge: A) treatment effect related to day of LR, B) treatment effect related to study period, and C) Effect of study period related to day of LR.

4.2.1.2 Cortisol

Peak and AUC of serum cortisol concentrations during stress challenge by LR were significantly affected by treatment ($p < 0.001$, $p < 0.001$, resp.), LR day ($p = 0.010$, $p = 0.005$, resp.) and the interaction of LR day * treatment ($p < 0.001$, $p < 0.001$, resp.; Table 11). Cortisol peak and AUC values were generally lower after DEXA treatment compared to controls. While after DEXA treatment AUC and peak cortisol values increased from day 1 to day 3 the opposite was seen in control cows.

During LR generally serum cortisol concentrations increased and dropped right after LR to baseline values again (time effect: $p < 0.001$; Table 12, Figure 10 a-c). Results showed that from day 1 to day 3 the increase in serum cortisol concentrations decreased while in DEXA treated cows the opposite was found (treatment * LR day effect: $p = 0.006$, time * treatment effect: $p = 0.004$, treatment * LR day effect: $p = 0.018$, treatment * LR day * treatment effect: $p = 0.026$).

Table 11: Mean peak concentrations and area under the curve (AUC) of cortisol serum concentrations revealed from six dairy cows during mild stress challenge by restrain in lateral recumbency (LR) on three subsequent days (day 1, 3, and 5) in two study periods after either placebo or dexamethasone treatment (40µg/kg BW IV) on day 0.

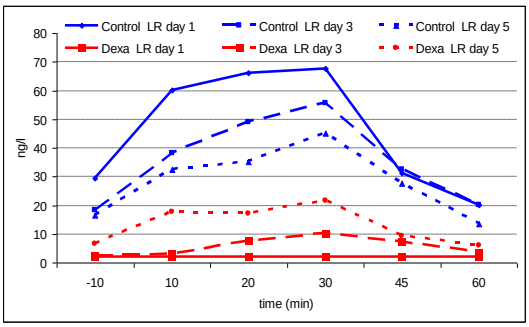
		Treatment					P - value							
		Controls		DEXA										
		Period												
		1	2	1	2									
Parameter	Day	A	B	C	D	SEM	A:B	A:C	A:D	B:C	B:D	C:D	Overall	p-value
Cortisol														
AUC	1	4703[a]	3247[a]	149[a]	151[a]	717	.13	< .001	< .001	< .001	< .001	.094	Treatment (T)	< .001
	3	3693[b]	2759[a]	612[a]	518[a]	772	.89	.028	.023	.035	.029	.90	Period (P)	.52
	5	3717[a]	1534[a]	1036[a]	1342[b]	563	.085	.012	.030	.24	.59	.55	T * P	.41
													Day (D)	< .010
													D * T	< .001
													D * P	.91
													D * T * P	.45
Peak	1	81.7[a]	61.4[a]	1.9[a]	1.9[a]	11.6	.19	< .001	< .001	< .001	< .001	.98	Treatment (T)	< .001
(ng/ml)	3	62.4[a]	52.4[a]	14.2[a]	9.3[a]	12.1	.93	.046	.024	.052	.028	.69	Period (P)	.49
	5	67.2[a]	29.1[a]	20.3[a]	26.1[b]	8.9	.080	.012	.028	.26	.51	.61	T * P	.59
													Day (D)	.005
													D * T	< .001
													D * P	.93
													D * T * P	.36

a)Differences in corresponding means of day 3 and day 5 compared to day 1 within one column are indicated by different superscript letters ($p < 0.05$)

Table 12: Results of statistical analysis regarding the treatment (placebo or dexamethasone: 40µg/kg BW), period and day of lateral recumbency (LR) effect and their interactions on plasma serum cortisol concentrations during mild stress challenge by LR in six dairy cows.

Effect by	P - value	Effect by	P - value
Treatment (Tr)	< .001	Time	< .001
Period (P)	.27	Time * Tr	.004
LR day (D)	.086	Time * P	.075
Tr * D	.006	Time * D	.018
Tr * P	.093	Time * Tr * D	.026
P * D	.96	Time * Tr * P	.10
		Time * P * D	.039

a



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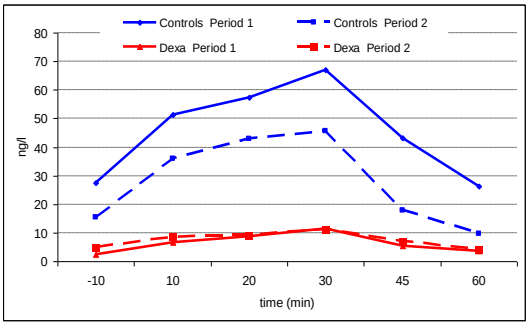

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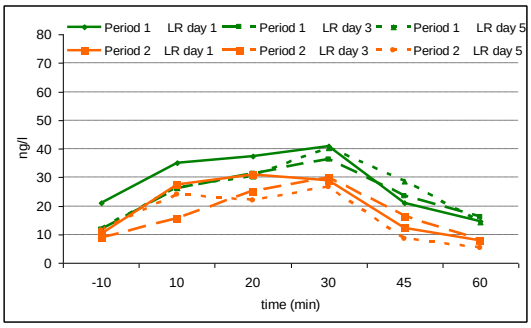


Figure 10 a - c: Mean serum cortisol concentrations of six dairy cows during mild stress challenge by restrain in lateral recumbency (LR; from time 0 to time 30 min) after placebo or dexamethasone treatment (40 µg/kg BW) 24 h before challenge: A) treatment effect related to day of LR, B) treatment effect related to study period, and C) Effect of study period related to day of LR.

4.2.2 Metabolic stress response

4.2.2.1 Glucose

Treatment with DEXA revealed a significant effect ($p = 0.022$) on AUC plasma glucose concentrations, which were generally higher after DEXA treatment than in controls, but not on peak concentrations (Table 13). For both AUC and peak of plasma glucose concentration significant effects were seen for effects of LR day ($p < 0.001$, $p < 0.001$, resp.) and interaction effects of treatment * LR day ($p < 0.001$, $p < 0.001$, resp.). In controls and DEXA treated cows AUC and peak plasma glucose decreased from day 1 to day 5. In controls AUC and peak were significantly lower in period 2 than in period 1 in control cows but not in DEXA treated cows (treatment * period effect: $p = 0.025$, $p = 0.009$, resp.).

During lateral recumbency generally plasma glucose concentrations increased (time effect: $p < 0.001$). The rise in plasma glucose in controls was less pronounced during LR on day 3 and day 5 compared to day 1 (LR day effect: $p < 0.001$). In DEXA treated cows highest plasma glucose concentrations were seen on day 1 of both periods, which were then significantly reduced on day 3 and 5 compared to day 1 (treatment * LR day effect: $p < 0.001$, time*treatment effect: p<0..01). While in controls plasma glucose concentrations during LR were lower in period 2 than in period 1, the opposite was seen in DEXA treated cows (treatment * period effect: $p < 0.001$; time * period effect: $p < 0.001$, time * treatment * period effect: $p < 0.001$). (Table 14. Figure 11)

Table 13: Mean peak concentrations and area under the curve (AUC) of glucose plasma concentrations revealed from six dairy cows during mild stress challenge by restrain in lateral recumbency (LR) on three subsequent days (day 1, 3, and 5) in two study periods after either placebo or dexamethasone treatment (40µg/kg BW IV) on day 0.

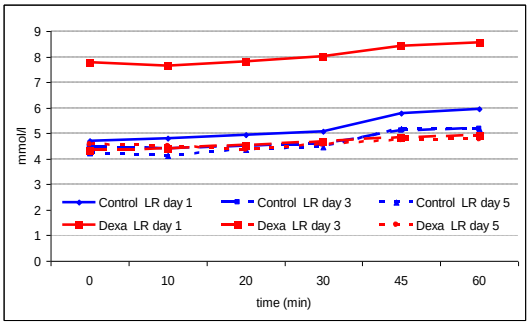
		Treatment					P - value							
		Controls		DEXA										
		Period												
		1	2	1	2									
Parameter	Day	A	B	C	D	SEM	A:B	A:C	A:D	B:C	B:D	C:D	Overall	p-value
glucose AUC	1	453 [a]	326 [a]	583 [a]	623 [a]	35	.037	.034	.010	.001	< .001	.45	Treatment (T)	.022
	3	390 [a]	313 [a]	335 [b]	360 [b]	20	.033	.10	.34	.49	.15	.41	Period (P)	.29
	5	384 [b]	305 [a]	316 [b]	369 [b]	21	.033	.058	.62	.73	.075	.12	T * P	.025
													Day (D)	< .001
													D * T	< .001
													D * P	.36
													D * T * P	.32
Peak (mmol/l)	1	7.41 [a]	4.88 [a]	8.15 [a]	9.09 [a]	0.53	.002	.33	.064	< .001	< .001	.30	Treatment (T)	.26
	3	6.09 [b]	4.52 [a]	4.82 [b]	5.22 [b]	0.45	.044	.099	.27	.60	.26	.51	Period (P)	.10
	5	6.30 [a]	4.44 [a]	4.56 [b]	5.18 [b]	0.38	.005	.008	.066	.78	.13	.20	T * P	.009
													Day (D)	< .001
													D * T	< .001
													D * P	.51
													D * T * P	.27

a)Differences in corresponding means of day 3 and day 5 compared to day 1 within one column are indicated by different superscript letters ($p < 0.05$)

Table 14: Results of statistical analysis regarding the treatment (placebo or dexamethasone: 40µg/kg BW), period and day of lateral recumbency (LR) effect and their interactions on plasma serum glucose concentrations during mild stress challenge by LR in six dairy cows.

Effect by	P - value	Effect by	P - value
Treatment (Tr)	< .001	Time	< .001
Period (P)	.046	Time * Tr	< .001
LR day (D)	< .001	Time * P	< .001
Tr * D	< .001	Time * D	.93
Tr * P	< .001	Time * Tr * D	.15
P * D	.61	Time * Tr * P	< .001
		Time * P * D	.63

a



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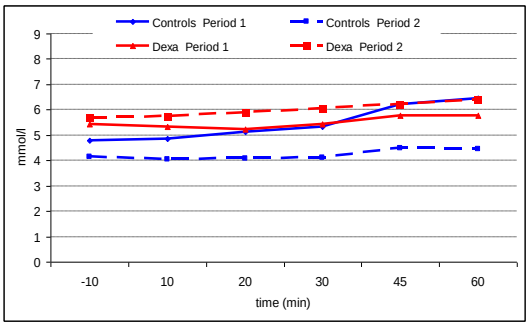

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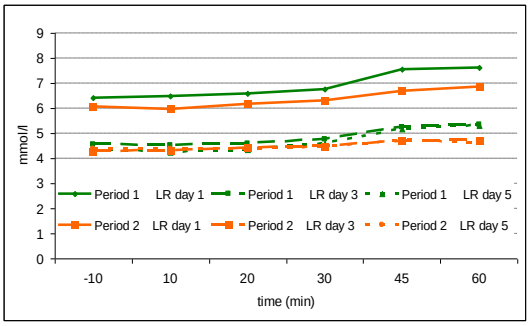


Figure 11 a - c: Mean serum glucose concentrations of six dairy cows during mild stress challenge by restrain in lateral recumbency (LR; from time 0 to time 30 min) after placebo or dexamethasone treatment (40 µg/kg BW) 24 h before challenge: A) treatment effect related to day of LR, B) treatment effect related to study period, and C) Effect of study period related to day of LR.

4.2.2.2 NEFA

In this study neither treatment, nor LR day and period as well as their interactions revealed statistically significant effects on peak and AUC NEFA serum concentrations during the stress challenge by LR (Table 15). However, during LR a slight increase of serum NEFA concentrations were seen (time effect: $p<0.001$, Table 16, Figure 12 a-c). The highest NEFA levels were found on day 1 compared to day 3 and day 5 (time * LRday effect: $p = 0.010$).

Table 15: Mean peak concentrations and area under the curve (AUC) of NEFA serum concentrations revealed from six dairy cows during mild stress challenge by restrain in lateral recumbency (LR) on three subsequent days (day 1, 3, and 5) in two study periods after either placebo or dexamethasone treatment (40µg/kg BW IV) on day 0.

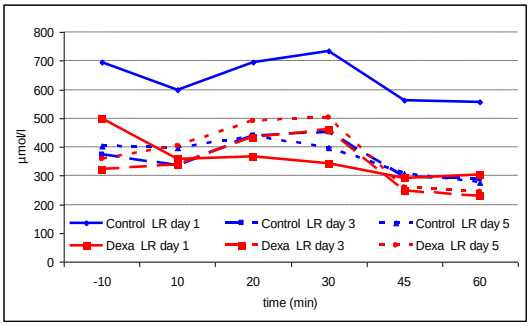
		Treatment												
		Controls		DEXA			P - value							
		Period												
		1	2	1	2									
Parameter	Day	A	B	C	D	SEM	A:B	A:C	A:D	B:C	B:D	C:D	Overall	p-value
NEFA														
AUC	1	49039 [a]	49363 [a]	23698[a]	27792 [a]	5777	.87	.070	.21	.089	.27	.47	Treatment (T)	.28
	3	26798 [a]	30375 [a]	21164[a]	33803 [a]	5864	.71	.61	.49	.39	.74	.24	Period (P)	.23
	5	29797 [a]	26596 [b]	21156[a]	39581 [b]	6207	.97	.23	.54	.24	.52	.088	T * P	.27
													Day (D)	.28
													D * T	.21
													D * P	.57
													D * T * P	.60
Peak	1	886 [a]	794 [a]	390 [a]	779 [a]	172	.68	.065	.63	.12	.94	.14	Treatment (T)	.35
(µmol/l)	3	488 [a]	527 [a]	331 [a]	609 [a]	140	.80	.40	.51	.29	.67	.15	Period (P)	.17
	5	551 [a]	460 [a]	352 [a]	700 [a]	164	.89	.19	.61	.23	.52	.087	T * P	.13
													Day (D)	.12
													D * T	.59
													D * P	.80
													D * T * P	.75

a) Differences in corresponding means of day 3 and day 5 compared to day 1 within one column are indicated by different superscript letters ($p < 0.05$)

Table 16: Results of statistical analysis regarding the treatment (placebo or dexamethasone: 40µg/kg BW), period and day of lateral recumbency (LR) effect and their interactions on plasma serum NEFA concentrations during mild stress challenge by LR in six dairy cows.

Effect by	P - value	Effect by	P - value
Treatment (Tr)	.14	Time	< .001
Period (P)	.12	Time * Tr	.22
LR day (D)	.29	Time * P	.074
Tr * D	.49	Time * D	.010
Tr * P	.12	Time * Tr * D	.68
P * D	.80	Time * Tr * P	.088
		Time * P * D	.85

a



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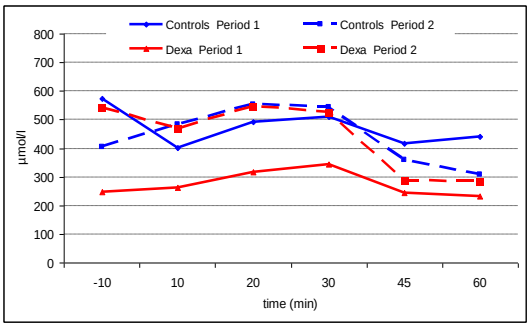

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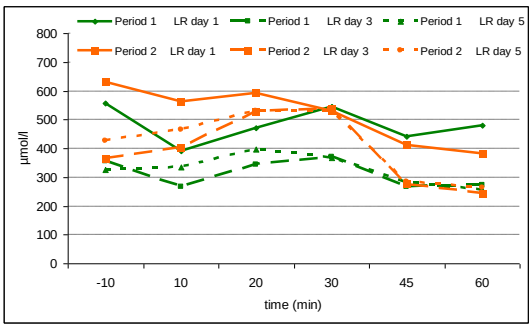


Figure 12 a - c: Mean serum NEFA concentrations of six dairy cows during mild stress challenge by restrain in lateral recumbency (LR; from time 0 to time 30 min) after placebo or dexamethasone treatment (40 µg/kg BW) 24 h before challenge: A) treatment effect related to day of LR, B) treatment effect related to study period, and C) Effect of study period related to day of LR.

4.2.2.3 Lactate

Mean AUC and peak of serum lactate concentrations during LR were significantly affected by treatment ($p = 0.017$, $p = 0.037$, resp.), LR day ($p < 0.001$, $p < 0.001$, resp.) as well as interactions of treatment * period ($p < 0.001$, $p < 0.001$, resp.), and treatment * LR day ($p < 0.001$, $p < 0.001$, resp.). After DEXA treatment AUC and peak serum lactate were generally higher than in control cows. While AUC and peak lactate were lower in period 2 than in period 1 in controls, this was the opposite in DEXA treated cows. In period 1 and period 2 on day 3 and day 5 AUC and peak lactate were significantly lower in DEXA treated cows compared to day 1. In contrast, in control cows AUC and peak lactate were almost same on day 1, 3, and 5 in both periods.

During the stress challenge by LR mean serum lactate increased over time (time effect: $p < 0.01$; Table 18, Figure 13 a-c). Highest lactate concentrations were found in cows on day 1 after DEXA treatment (treatment effect: $p < 0.001$). These concentrations were significantly higher than on day 3 and day 5 (LR day effect: $p < 0.001$). In contrast in control cows mean lactate serum concentrations presented an almost same pattern on day 1, 3 and 5 (treatment * LR day effect: $p < 0.001$). In period 2 serum lactate level in DEXA treated cows were found to be higher than in period 1, while in control cows lactate concentrations were lower in period 2 than in period 1 (treatment period effect: $p < 0.001$).

Table 17: Mean peak concentrations and area under the curve (AUC) of lactate serum concentrations revealed from six dairy cows during mild stress challenge by restrain in lateral recumbency (LR) on three subsequent days (day 1, 3, and 5) in two study periods after either placebo or dexamethasone treatment (40µg/kg BW IV) on day 0.

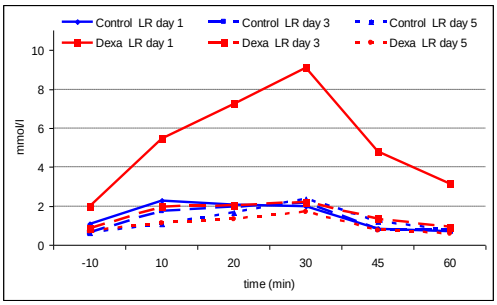
		Treatment												
		Controls		DEXA										
		Period					P -	value						
		1	2	1	2									
Parameter	Day	A	B	C	D	SEM	A:B	A:C	A:D	B:C	B:D	C:D	Overall	p-value
Lactate AUC	1	195 [a]	48 [a]	360 [a]	630 [a]	64	.004	.078	.006	< .001	< .001	.14	Treatment (T)	.017
	3	179 [a]	52 [a]	109 [b]	158 [b]	46	.026	.41	.71	.10	.048	.63	Period (P)	.074
	5	202 [a]	41 [a]	69 [b]	114 [b]	48	.007	.052	.26	.22	.044	.32	T * P	.005
													Day (D)	< .001
													D * T	<.001
													D * P	.93
													D * T * P	.55
Peak (mmol/l)	1	3.96 [a]	0.78 [a]	6.50 [a]	10.1 [a]	1.29	.003	.15	.024	< .001	< .001	.027	Treatment (T)	.037
	3	3.43 [a]	0.86 [a]	1.92 [b]	2.75 [b]	0.88	.022	.34	.56	.11	.056	.69	Period (P)	.066
	5	4.17 [a]	0.66 [a]	1.12 [c]	2.39 [c]	1.07	.006	.036	.32	.27	.030	.18	T * P	.005
													Day (D)	< .001
													D * T	< .001
													D * P	.97
													D * T * P	.35

a) Differences in corresponding means of day 3 and day 5 compared to day 1 within one column are indicated by different superscript letters ($p < 0.05$)

Table 18: Results of statistical analysis regarding the treatment (placebo or dexamethasone: 40µg/kg BW), period and day of lateral recumbency (LR) effect and their interactions on plasma serum lactate concentrations during mild stress challenge by LR in six dairy cows.

Effect by	P - value	Effect by	P - value
Treatment (Tr)	< .001	Time	< .001
Period (P)	.011	Time * Tr	.13
LR day (D)	< .001	Time * P	.091
Tr * D	< .001	Time * D	.43
Tr * P	< .001	Time * Tr * D	.001
P * D	.94	Time * Tr * P	< .001
		Time * P * D	.073

a



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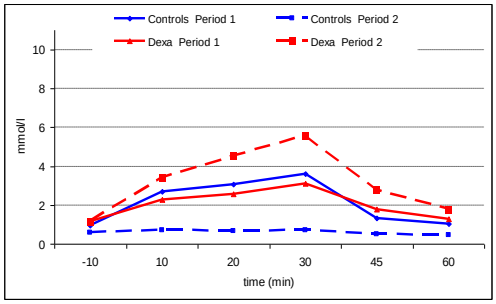

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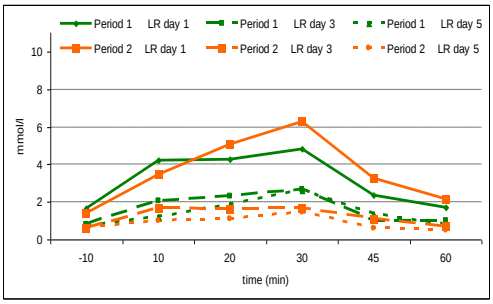


Figure 13 a - c: Mean serum lactate concentrations of six dairy cows during mild stress challenge by restrain in lateral recumbency (LR; from time 0 to time 30 min) after placebo or dexamethasone treatment (40 µg/kg BW) 24 h before challenge: A) treatment effect related to day of LR, B) treatment effect related to study period, and C) Effect of study period related to day of LR.

4.2.3 Cardio-respiratory stress response

4.2.3.1 Heart rate

Mean AUC and peak values of heart rate during LR were generally reduced by DEXA treatment (treatment effect: $p = 0.023$, Table 19)). In both periods on day 5 AUC heart rate was significantly lower than on day 1 and day 3 in controls and DEXA treated cows (LR day effect: $p < 0.001$), while this pattern was found significant for peak heart rate only in control cows (LR day effect $p < 0.001$). The reduction from day 1 to day 5 in AUC heart rate was more pronounced in control cows than in DEXA treated cows (treatment*LR day effect: $p = 0.032$)

As response to the mild stress challenge by LR generally mean heart rate increased (time effect: $p < 0.001$, Table 20, Figure 14 a-c). In control cows the rise in mean heart rate was more distinct than in DEXA treated cows (treatment effect: $p = 0.003$, time * treatment effect: $p<0.001$). In controls and DEXA treated cows a steady decline in the increase of mean heart rates were seen during LR (LR day effect $p = 0.036$). The behaviour of mean heart rates was in both periods and both groups almost same.

Table 19: Mean peak level and area under the curve (AUC) of heart rate revealed from six dairy cows during mild stress challenge by restrain in lateral recumbency (LR) on three subsequent days (day 1, 3, and 5) in two study periods after either placebo or dexamethasone treatment (40µg/kg BW IV) on day 0.

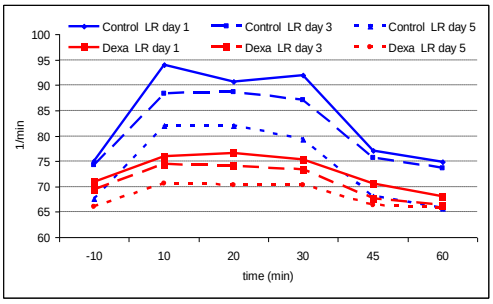
		Treatment												
		Controls		DEXA			P - value							
		Period												
		1	2	1	2									
Parameter	Day	A	B	C	D	SEM	A:B	A:C	A:D	B:C	B:D	C:D	Overall	p-value
Heart rate														
AUC	1	6570 [a]	6398 [a]	5458 [a]	5626 [a]	252	.68	.015	.032	.029	.063	.63	Treatment (T)	.023
	3	6321 [b]	6150 [b]	5146 [a,b]	5591 [a,b]	303	.73	.024	.13	.043	.22	.31	Period (P)	.75
	5	5705 [c]	5675 [b]	5051 [b,c]	5281 [b,c]	298	.94	.15	.33	.16	.37	.58	T * P	.48
													Day (D)	< .001
													D * T	.032
													D * P	.50
													D * T * P	.36
Peak	1	94 [a]	93 [a]	75 [a]	79 [a]	4.0	.84	.009	.028	.012	.039	.47	Treatment (T)	.013
(1/min)	3	90 [b]	88 [b]	72 [a]	78 [a]	4.6	.74	.021	.098	.036	.16	.35	Period (P)	.70
	5	82 [c]	82 [c]	70 [a]	73 [a]	4.4	.99	.084	.16	.084	.17	.66	T * P	.52
													Day (D)	< .001
													D * T	.089
													D * P	.93
													D * T * P	.34

a) Differences in corresponding means of day 3 and day 5 compared to day 1 within one column are indicated by different superscript letters ($p < 0.05$)

Table 20: Results of statistical analysis regarding the treatment (placebo or dexamethasone: 40µg/kg BW), period and day of lateral recumbency (LR) effect and their interactions on heart rate during mild stress challenge by LR in six dairy cows.

Effect by	P - value	Effect by	P - value
Treatment (Tr)	.003	Time	< .001
Period (P)	.62	Time * Tr	< .001
LR day (D)	.036	Time * P	.80
Tr * D	.68	Time * D	.89
Tr * P	.22	Time * Tr * D	.63
P * D	.96	Time * Tr * P	.91
		Time * P * D	.97

a



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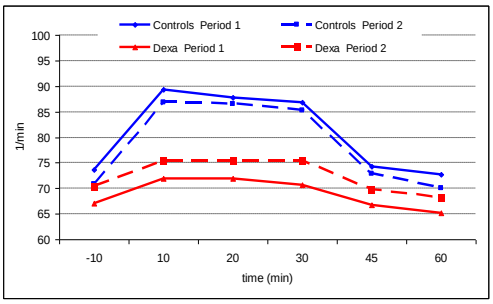

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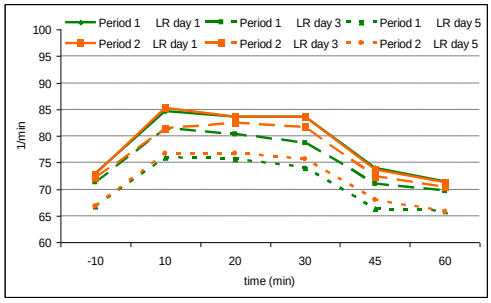

Figure 14 a - c: Mean heart rate of six dairy cows during mild stress challenge by restrain in lateral recumbency (LR; from time 0 to time 30 min) after placebo or dexamethasone treatment (40 µg/kg BW) 24 h before challenge: A) treatment effect related to day of LR, B) treatment effect related to study period, and C) Effect of study period related to day of LR.

4.2.3.2 Respiratory rate

For mean respiratory AUC and peak values statistically significant effects were revealed for the factors treatment ($p = 0.002$, $p = 0.001$, resp.) and LR day ($p < 0.001$, $p<0.001$, resp., Table 21). After DEXA treatment AUC and peak values were generally lower than in controls. Highest AUC values were found in control cows during period 1 (treatment * LR day * period effect: $p = 0.002$).

Mean respiratory rates increased during LR stress challenge ($p < 0.001$, Table 22, Figure 15 a-c), but to less extent in DEXA treated cows compared to controls (treatment effect: $p < 0.001$, time*treatment effect: $p < 0.001$). From day 1 to day 5 the rise in respiratory rates was less pronounced in both controls and DEXA treated cows (LR day effect: $p < 0.001$, time * LR day effect: $p = 0.043$).

Table 21: Mean peak level and area under the curve (AUC) of respiratory rate revealed from six dairy cows during mild stress challenge by restrain in lateral recumbency (LR) on three subsequent days (day 1, 3, and 5) in two study periods after either placebo or dexamethasone treatment (40µg/kg BW IV) on day 0.

		Treatment												
		Controls		DEXA										
		Period					P - value							
		1	2	1	2									
Parameter	Day	A	B	C	D	SEM	A:B	A:C	A:D	B:C	B:D	C:D	Overall	p-value
Respiratory rate														
AUC	1	3101 [a]	2956 [a]	2580 [a]	2595 [a]	102	.39	.007	.008	.027	.035	.90	Treatment (T)	.002
	3	2941 [b]	2828 [b]	2510 [a,b]	2403 [a,b]	101	.49	.018	.005	.055	.015	.42	Period (P)	.33
	5	2731 [c]	2688 [c]	2411 [b,c]	2210 [b,c]	92	.79	.044	.004	.068	.005	.13	T * P	.90
													Day (D)	< .001
													D * T	.97
													D * P	.13
													D * T * P	.002
Peak	1	49 [a]	46 [a]	37 [a]	37 [a]	2	.43	.002	.002	.008	.009	.99	Treatment (T)	.001
(1/min)	3	45 [a,b]	43 [b]	37 [a,b]	35 [a,b]	2	.57	.021	.009	.051	.023	.62	Period (P)	.37
	5	43 [b]	41 [b]	34 [b]	31 [b]	2	.49	.007	.001	.022	.003	.22	T * P	.94
													Day (D)	< .001
													D * T	.053
													D * P	.13
													D * T * P	.058

a) Significant differences in corresponding means within one column are indicated by different superscript letters ($p < 0.05$)

Table 22: Results of statistical analysis regarding the treatment (placebo or dexamethasone: 40µg/kg BW), period and day of lateral recumbency (LR) effect and their interactions on respiratory rate during mild stress challenge by LR in six dairy cows.

Effect by	P - value	Effect by	P - value
Treatment (Tr)	< .001	Time	< .001
Period (P)	.070	Time * Tr	< .001
LR day (D)	< .001	Time * P	.33
Tr * D	.96	Time * D	.043
Tr * P	.32	Time * Tr * D	.89
P * D	.67	Time * Tr * P	.094
		Time * P * D	.52

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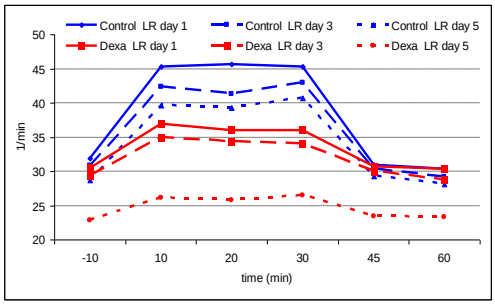

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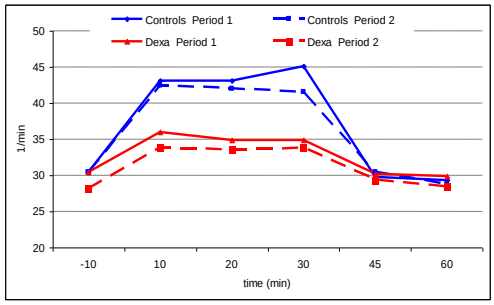

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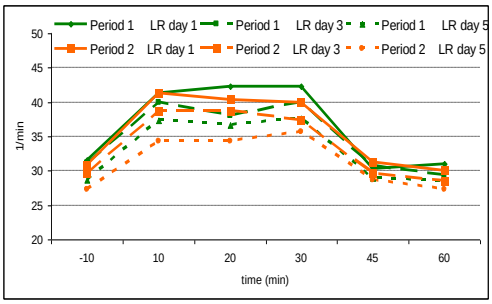
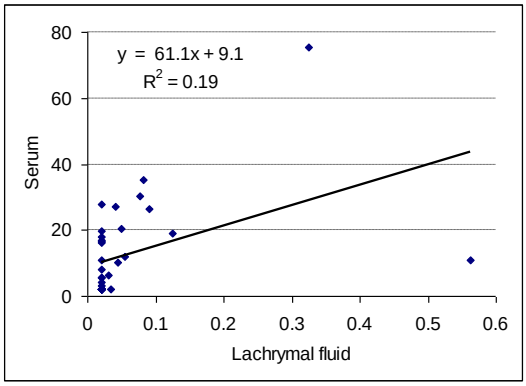


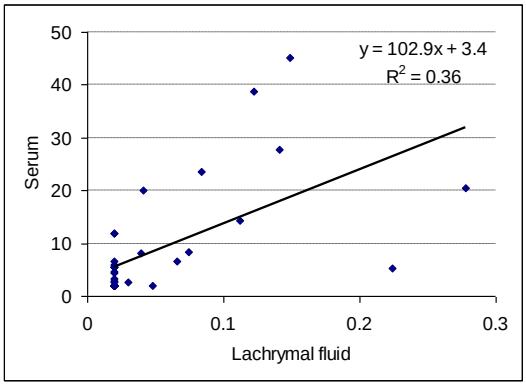
Figure 15 a - c: Mean respiratory rate of six dairy cows during mild stress challenge by restrain in lateral recumbency (LR; from time 0 to time 30 min) after placebo or dexamethasone treatment (40 µg/kg BW) 24 h before challenge: A) treatment effect related to day of LR, B) treatment effect related to study period, and C) Effect of study period related to day of LR.

4.3 Cortisol concentrations in serum and lachrymal fluid

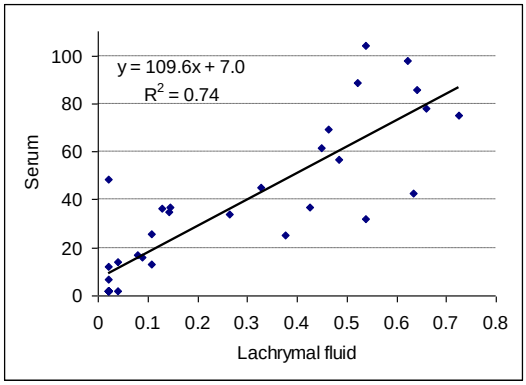
Significant correlations were found by regression analysis between serum cortisol concentrations and cortisol concentrations in lachrymal fluid 15 minutes before (Figure y a; $R^2 = 0.18$, $p < 0.05$), during LR (Figure x b, $R^2 = 0.74$, $p < 0.001$), and 30 minutes after LR (Figure 16 c, $R^2 = 0.36$, $p < 0.001$) as well as between AUC of serum cortisol during LR with cortisol levels in lachrymal fluid during LR (Figure x d, $R^2 = 0.75$, $p < 0.001$).

a - b





c - d



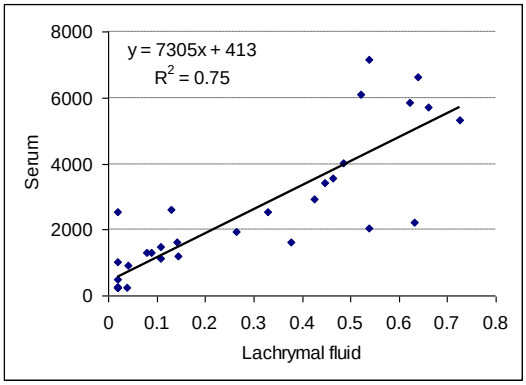


Figure 16 a-d: Results of regression analysis between cortisol concentrations (µg/dl) in lachrymal fluid and serum revealed from six dairy cows during repeated stress challenges by restrain in lateral recumbency (LR; N = 72). Serum vs. lachrymal fluid cortisol concentrations a) 15 minutes before LR, b) 30 min after LR, c) during LR (peak), and d) the area under the curve (AUC from 15 min before to 60 min after LR) of serum cortisol vs. lachrymal fluid cortisol concentrations during LR. R^2: coefficient of determination.

5. Discussion

5.1. Lateral recumbency as stress model

Though cows are living in dairy herds under the protection and care of humans they are exposed to numerous stressors such as social stress among herd mates, heat stress during summer time, pain stress resulting from diseases or trauma, calving stress, metabolic stress, exposure to new environments or unfamiliar husbandry procedures for instance claw trimming and dehorning (HEMSWORTH et al., 1989; MUNSGAARD et al., 1997, HEMSWORTH et al., 2000; HERSKIN and MUNSGAARD 2000, HERSKIN et al., 2003a, HERSKIN et al., 2003b, MUNSKGAARD et al., 2001, HEMSWORTH 2003, NIGEL and NOORDLUND 2009; ROUHA-MULLEDER et al., 2010). Also human-animal contact with inappropriate pushing, yelling, chasing or beating is experienced as severe stress by cows (de PASSILLÈ et al., 1996; RUSHEN et al., 1999). Stress impairs performance of cows and interferes with animal well-being and welfare (KNIERIM and WARAN 1994, RUSHEN et al., 1999; HEMSWORTH et al., 2000; WAIBLINGER et al., 2002, EFSA 2009; Ivemeyer et al., 2011) and is therefore in recent years increasingly subject of research.

As in previous studies restraining cows in lateral recumbency (LR) on a surgical tipping table appears as an adequate stress model (RIZK et al., 2011a, RIZK et al., 2011b). Animals are taken out of their familiar stall environment and are exposed to a new environment and restrain, which is a frequently performed painless but stressful husbandry procedure (WAIBLINGER et al., 2004). Cows of this study were familiar with frequent human contacts as they are used for student training, e.g. clinical examination procedures, rectal palpation and blood sampling. They were always guided by a halter to the tipping table in the surgery hall by same two experienced animal keepers using only a calm, friendly low voice, gentle pushing in the pelvic region and the animals was given time to explore the new environment. Thus, the procedure of turning cows into LR was

performed as stressless as it can be, under common farming conditions for the animals. Though cows were used to close human contact they showed the typical hormonal, metabolic and cardio-respiratory stress responses during LR as reported before by PESENHOFER et al., (2006) and RIZK et al., (2011a).

Stress induced activation of the HPA axis (FLECKNELL 2000, FISHER et al., 2002, MORROW et al., 2002; BEERDA et al., 2004; REECE 2004; RIJNBERK et al., 2010) led to a significant rise in plasma ACTH and serum cortisol concentrations during LR as reported before (ABILAY et al., 1975; ALAM et al., 1986; BOANDL et al., 1989; BREARLEY et al., 1990; TAGAWA et al., 1994; RIZK et al., 2011a) which fell quickly to baseline levels after the cows were turned back into a standing position. The sympatho-adrenomedullary system is activated as response to stressful stimuli resulting in elevation of plasma catecholamine levels and increasing heart rate, blood pressure and myocardial activity as well as respiratory activity (ABILAY et al., 1975; ALAM et al., 1986; BOANDL et al. 1989; BREARLEY et al. 1990; TAGAWA et al. 1994, MELLOR and STAFFORD 1999; HUDSON et al., 2008, SCHUBERT et al., 2009). Thus, the increase in heart rate (HR) and respiratory rate (RR) of controls in this study is probably mostly due to adrenal release of catecholamines. Also positioning cows in LR may mildly impair respiration (KLEIN and FISHER1988, WAGNER et al., 1990, RIZK et al., 2011a), which may have also contributed to the rise in RR. The observed metabolic responses in blood glucose, NEFA and lactate concentrations were reported before (PESENHOFER et al., 2006; RIZK et al., 2011a) and resulted predominantly from hormonal responses. Cortisol is assumed to increase gluconeogenesis (DESBOROUGH 2000, Reece 2004) and to reduce glucose utilization by peripheral tissues (KUSENDA et al., 2009, KUSENDA et al., 2010), thereby leading to hyperglycemia. Despite cortisol promoted NEFA utilization in peripheral tissues (Tappy et al., 1994), serum NEFA level increased which was probably mainly caused by catecholamine stimulated lipolysis in adipose tissue (SUMNER and McNAMARA 2007) as reported under several stressful conditions (BAXTER & FORSHAM 1972, MUDRON et al., 1994; MELLOR and STAFFORD 1999;

WATERMAN-PEARSON 1999; CAMBRIDGE et al. 2000). Cortisol has also been shown to amplify the induction of lipolysis (OTTOSSON et al. 2000). Catecholamines increase arterial blood pressure by induction of vasoconstriction in peripheral tissues which in turn will reduce oxygen supply. In consequence glucose in peripheral tissues will be metabolized more anaerobically to lactate (EL-GHOUL and HOFFMANN 2002; CHACON et al., 2005; MUDRON et al., 2005, OFFINGER et al., 2011).

In conclusion, restraining cows in a standing position in a crush (PESENHOFER et al 2006; GIBBONS et al., 2011) or in LR on a tipping table (PESENHOFER et al., 2006; RIZK et al., 2011a) is a stress model with typical stress responses and without harm for the animal. Since restraining itself is pain free, stress responses are caused mainly by the human-animal contact, the new environment and the husbandry procedure of LR (GIBBONS et al., 2009).

5.2. Training effects on stress response

Cows in this study learned quickly that restrain in LR is pain free and harmless which in turn reduced anxiety and fear. Accordingly, a steady decline in hormonal, metabolic and cardio-respiratory stress responses was seen from d1 to d5. This was not only seen during restrain in LR but also in recorded baseline data. Cows taken out from their familiar stall environment are stressed by human-animal contact, social isolation from herd mates and novelty due to the new environment (WAIBLINGER et al., 2006; SCHMIED et al., 2008), which is already reflected in this study in recorded baseline data. However, cows quickly got used to the procedure which was demonstrated in the reduction of ACTH and cortisol baseline blood concentrations from d1 to d5. Baseline serum NEFA concentrations as well as heart and respiratory rate were significantly lower on d5 compared to d1. Whether and how quickly cows learn to get used to new situations depends mainly on how they are handled and in particular the experience of

pain during the new situation (RUSHEN et al., 1999, WAIBLINGER et al., 2006; WINDSCHNURER et al., 2008). A friendly, calm and low voice as well as giving time to explore new situations will inspire cows with trust. Additionally, tactile contact between humans and animals will help to provide confidence. In particular regular stroking in the ventral neck region, the region which is commonly licked during social grooming between cows is effective to reduce fear to humans (SCHMIED et al., 2008a and 2008b, WINDSCHNURER et al., 2009; SCHMIED et al., 2010). However, in this study cows did not receive any extra attention by stroking, since cows need to be stroked on several days and each time for several minutes to receive a significant and lasting effect on behavior. In this study contacts were limited to voice and common tactile contacts during the leading of cows by a halter to simulate typical farming conditions.

Subsequent fixation of cows in the new environment at the tipping table and turning cows into LR provided additional stress by anxiety and fear as indicated by a surge of ACTH, cortisol, glucose, NEFA and lactate blood concentrations as well as heart and respiratory rates. However, during the stress challenge by restrain in LR, the short term memory learning of dairy cows became obvious by the fact, that the increase in serum cortisol as well as heart and respiratory rates was significantly less pronounced on d5 than on d1.

According to the study design the three stress challenges by LR in period 1 were repeated four weeks later again in period 2. Data of this study provided some indications that stress challenges from period 1 were transferred into the long term memory as harmless experience and LR in period 2 appeared less stressful to the cows than in period 1. In controls of this study baseline plasma glucose, serum cortisol and lactate concentrations were reduced in period 2 compared to period 1. During the stress challenge of LR a significant reduction in plasma ACTH and serum glucose and lactate concentrations was found in period 2 compared to period 1. These results may indicate that the stress during LR was less pronounced in period 2 than in period 1. However, no

effect of the study period was seen in heart and respiratory rates. In other studies clear evidence was found that positive experiences with humans made e.g. by stroking animals in the ventral region will be memorized for at least four to eight weeks resulting in reduced avoidance behavior and changes in heart rates when animals are approached (WAIBLINGER et al., 2004, SCHMIED et al., 2008a and 2008b; WINDSCHNURER et al., 2009; SCHMIED et al., 2010). There are some indications that positive experiences are memorized differently than negative, fear inducing experiences (HEMSWORTH et al., 2000, SCHMIED et al., 2008a and 2008b). Thus, a fear inducing husbandry procedure like restrain in LR might be less lasting memorized as harmless by dairy cows compared to positively experiences like stroking.
However, according to the study design, control cows of period 2 were treated with DEXA in period 1. Although the interim period between period 1 and 2 was four weeks it may be possible that the reduction of ACTH, cortisol, glucose and lactate was still a consequence of the HPA suppression by DEXA treatment in period 1 (see 5.3.).

In conclusion, results revealed that cows quickly learn to cope with unfamiliar husbandry procedures when handled appropriately and giving them time to get used to new environments and handling. However, due to the study design there is no clear evidence that this experience will be transferred into the long term memory and will last for longer periods when cows are not continuously exposed to such situations.

5.3. Dexamethasone effect on stress response

Low dose treatment with the glucocorticoid dexamethasone suppressed the HPA response (FERGUSON and HOEING, 2001) to stress almost completely for more than 5 days. Both baseline concentrations of ACTH and cortisol as well as concentrations during LR were close to the analytical detection limit of the hormones on d1 and d3. During LR almost no rise of ACTH and cortisol blood concentrations was seen in DEXA treated cows. The slight increase in blood ACTH and cortisol concentrations on d5 did

not reach the level of control cows on the corresponding day. Also WETTEMANN et al.,(1981) showed that treatment of stressed heifers with synthetic glucocorticoids will decrease serum concentrations of endogenous glucocorticoids by decreasing ACTH secretion.

According to the study design control cows of period 2 were treated with DEXA in period 1. As mentioned above after an interim period of four weeks the significantly lower ACTH concentrations of control cows on d1 of period 2 may still reflect ongoing depression of the HPA response. However, it may also be an effect of a combination of dexamethasone treatment and training. As known from experiments with animals and humans, glucocorticoids affect memorization of events in the long term memory. Glucocorticoids have shown to enhance memory consolidation of emotionally arousing experiences but impair memory retrieval and working memory during emotionally arousing experiences (QUERVAIN et al., 2009). Thus, it is also possible, that experiences during the stressful events of LR in period 1 under dexamethasone treatment were more strongly memorized. In consequence the stress response after the interim period of four weeks may therefore turned out less pronounced than in cows which did not receive DEXA as pretreatment before the repeated LR procedures. A final conclusion can not been drawn from this study, but for the author it appears more likely that ACTH depression on d1 in period 2 were mainly a consequence of DEXA induced suppression of the HPA, since no learning effect became obvious in catecholamine induced changes of heart and respiratory rate.

A significant reduction of heart and respiratory rate was induced by DEXA treatment compared to control cows. It has been shown before in rats (BROWN and FISHER 1986), sheep (KOMESAROFF and FUNDER 1994) and humans (SHARARA et al., (2009) LITERATUR) that administration of exogenous glucocorticoids attenuate or suppress stress induced elevation of plasma nor-epinephrine and epinephrine. The control mechanism of adrenal steroids on catecholamine response to stress is not clear

(Munck, 1984). Thus, a dexamethasone induced depression of adrenal catecholamine release is supposed to be the cause for reduced heart and respiratory rates in studied cows. However, DRUCE et al.(2011) found no significant changes in plasma catecholamines at the end of the low-dose dexamethasone suppression test compared to baseline under standardised conditions.

It is possible that glucocorticoids have the potential of reducing retrieval of aversive memories and enhance fear extinction (QUERVAIN et al. 2009). Thus, reduced heart and respiratory rates may be also a result of central effects of dexamethasone which in consequence led to less fearful perception of LR and thereby reduced adrenal response to the procedure.

DEXA treatment on d0 in the used dosage induced a surge in plasma baseline glucose concentrations on d1 which was not seen anymore on d3 and d5 of the study. The short hyperglycemia after DEXA treatment is in line with previous reports and is attributed mostly to reduced insulin sensitivity of peripheral tissues and thereby reduced glucose utilization (Kusenda et al., 2011) and less to increased hepatic gluconeogenesis (Starke et al submitted). During LR on d1 in DEXA treated cows only a slight increase was seen in glucose plasma concentrations compared to baseline while this was more pronounced in control cows. On d3 and d5 glucose concentrations during LR followed almost the same pattern. This data shows that during the hyperglycemic phase after DEXA treatment, serum glucose concentrations are not a reliable indicator of stress.

Highest serum lactate concentrations in baseline and peak values during the stress challenge by LR were seen on d1 of DEXA treated cows. Commonly the surge in serum lactate concentrations during stress is explained by catecholamine induced vasoconstriction and in consequence anaerobic glycolysis from pyruvate in peripheral tissues. Thus, due to high serum glucose concentrations after DEXA treatment, more substrate may have been available for glycolysis. Otherwise DEXA treatment reduces

glucose uptake by peripheral tissues. Another explanation may be that dexamethasone has a direct influence on the cardio-vascular function (STARKE et al 2011) with a positive inotrope (ANDREWS and WALKER 1999) and vasoconstrictive effect (DODT et al 2009). Thus, vasoconstriction during the stress challenge may have been more pronounced leading to enhanced anaerobic glycolysis than in control cows. However, the effect of dexamethasone treatment on serum lactate concentrations disappeared almost completely on d3 and d5.

5.4. Lachrymal fluid cortisol concentrations for assessment of stress response

Cortisol release is stimulated by both mental and pain stress. Blood collection by venipuncture makes serious restrain necessary and the procedure is by itself associated with mild pain. Both may therefore affect results of cortisol measurements. Thus, indwelling vein catheters are necessary to reduce effects of sample collection on cortisol results. In this study lachrymal fluid could be collected easily without stimulation of lacrimation in all cases. The collection procedure was painless for the animals and was possible just by gentle holding of the animal by a trained keeper while animals were standing and without additional fixation during LR. Therefore the technique appears to bear less risk of influencing analytical results of cortisol. In accordance with a report in humans (Banbury 2009) cortisol concentrations in lachrymal fluid are only about 1% of serum cortisol. However, results of samples taken at the end of LR showed a close correlation between lachrymal fluid cortisol concentrations and serum concentrations (R^2 = 0.76) and with AUC of the serum cortisol concentration curve during LR (R^2 = 0.76). In the view of the author, assessment of cortisol concentrations in a single lachrymal fluid sample at the end of stress challenges reflect the adrenal cortisol response to the stress challenge sufficiently. The technique could therefore be suitable to replace frequent blood sampling during the procedure for serum cortisol determination.

5.5. Conclusion

According to the results in blood concentrations of ACTH, cortisol, glucose, NEFA and lactate as well as heart and respiratory rate, restrain in lateral recumbency is experienced as stress by dairy cows, although the procedure was performed reasonably in a gentle, calm, and painless manner. Dexamethasone treatment almost completely suppressed the HPA responses, as indicated by blood ACTH and cortisol concentrations. This effect lasted for more than five days after treatment, possibly even for several weeks. Stress responses are substantially reduced after repeatedly performed LR, indicating that experiences such as harmless LR are transferred efficiently into the short term memory. This study provided only some evidence that experiences with fear-inducing but painless procedures are transferred into the long term memory and affect stress responses at future dates. Pretreatments with glucocorticoids and training effects need to be considered in studies on stress in dairy cows, since both affect hormonal, metabolic and cardio-respiratory stress responses significantly.

6. Abstract

Effects of dexamethasone and training on the hypothalamic-pituitary-adrenal response on mild stress challenge in dairy cows

Nimer Khraim, Nablus, West Bank

Assessment of hormonal, metabolic and cardio-respiratory changes is frequently used in studies on stress in cattle. In Cattle the glucocorticoid dexamethasone (DEXA) is frequently administered for treatment of metabolic disorders or as anti-inflammatory drug. From other species it is known that DEXA treatment suppresses the function of the hypothalamic-pituitary-adrenal axis (HPA). However, no information is available in cattle about the extent and duration of HPA suppression after dexamethasone treatment. Cattle adapt to repeated stress situations. This results in a reduced HPA response on such situations. A frequently performed stressful setting for cattle is restrain in lateral recumbency (LR) for husbandry procedures. Cows are stress by social isolation from herd mates, close animal-human contact and new environment and procedures. Thus, the aim of the study was to investigate in cows during restrain in LR the effect of DEXA treatment and training on hormonal, metabolic and cardio-respiratory stress response. Additionally it was studied, if cortisol measurement in a single sample of lachrymal fluid, which can be collected non-invasively and taken at the end of a stress challenge, sufficiently reflects the hormonal stress response.

The randomized and blinded study was conducted in six non-lactating, non-pregnant dairy cows in cross over design. In two different periods with an interim period of four weeks all cows were turned into LR for thirty minutes on day 1, 3 and 5. On day 0 cows were once either treated with DEXA (40µg/kg BW IV) or an equivalent dose of saline. In short intervals blood samples were collected from indwelling jugular vein catheters for analysis of ACTH, cortisol, glucose, nonesterified fatty acids (NEFA) as well as lactate

and heart and respiratory rate were recorded. Before, at the end and after the stress challenge lachrymal fluid was collected.

Compared to controls DEXA treated cows revealed significantly reduced blood levels of ACTH and cortisol as well as heart and respiratory rates in baseline data and during the stress challenge by LR (peak values and area under the curve (AUC)). The effect lasted for more than five days. On d1 and d3 almost no HPA response was detectable. On d1 blood glucose and lactate concentrations were significantly higher in baseline samples and during LR (peak and AUC) in DEXA compared to control cows. DEXA treatment revealed no effect on NEFA serum levels. The repeated LR resulted in adaptation of control cows leading to a steady reduction from d1 to d5 of blood levels of ACTH, cortisol, glucose and lactate as well as heart and respiratory rate. Controls in period 2 which were treated with DEXA in period 1 revealed significantly lower ACTH, cortisol, glucose and lactate blood level than controls in period 1.

In conclusion, DEXA treatment revealed a very strong effect on hormonal, metabolic as well as cardio-respiratory stress response lasting for more than five days. The effect needs to be considered in studies on stress management in dairy cows. Three repetitions of LR result in clear adaptation of dairy cows to stressful settings, when cows are handled carefully, with friendly and low voice and giving them reasonable time to explore the new and unfamiliar situation. From this study it remained unclear if the reduced stress response seen in controls in period 2 was a result of adaptation due to experiences memorized from period 1 or if this was a four weeks lasting effect of DEXA treatment.

Key words: ACTH, cortisol, dexamethasone, stress, adaptation, learning, animal-human contact

7. Zusammenfassung

Effekte von Dexamethason und Training der Hypothalamus-Hypophysen-Nebennieren Reaktion auf leichten Stress als Herausforderung bei Milchkühen

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Die Beurteilung der hormonellen, metabolischen und kardio-respiratorischen Veränderungen, wird häufig in Studien zum Thema Stress bei Rindern verwendet. Für gewöhnlich wird Kühen Glukokortikoid Dexamethason (DEXA) zur Behandlung von Stoffwechselerkrankungen oder als entzündungshemmendes Medikament verabreicht. Von anderen Tierarten ist bekannt, dass die DEXA Behandlung die Funktion der Hypothalamus-Hypophysen-Nebennieren-Achse (HPA) unterdrückt. Es sind jedoch keine Informationen zu Rindern über das Ausmaß und die Dauer der HPA Unterdrückung nach einer Dexamethason-Behandlung verfügbar. Kühe haben sich an wiederholte Stress-Situationen gewöhnen, was zu einer verringerten HPA Reaktion auf solche Situationen führt. Eine regelmässig herbeigeführte stressige Umgebung für Kühe ist die erzwungene Seitenlage (LR) durch landwirtschaftliche Prozesse. Kühe stehen unter Stress, durch soziale Isolation von der Herde, durch einen nahen Tier-Mensch-Kontakt und durch neue Umgebungen und Verfahren. Somit war das Ziel der Studie, bei Kühen während der erzwungenen LR die Wirkung von DEXA Behandlung und die Auswirkung auf den Hormonhaushalt, Stoffwechsel-und Herz-Lungen-Stress-Reaktion zu untersuchen. Zusätzlich wurde untersucht, ob eine Cortisol Messung in einer einzigen Probe von Tränenflüssigkeit, die nicht-invasiv, am Ende einer Stressherausforderung entnommen werden kann, ausreichend die hormonelle Stress-Auswirkung reflektiert.

Die zufällig ausgewählte Studie, wurde an sechs nicht-laktierenden, nicht-schwangeren Kühen im "Cross over-Design" durchgeführt. In zwei verschiedenen Perioden, mit einer Übergangszeit von vier Wochen, wurden alle Kühe in die LR für 30 Minuten am Tag 1, 3 und 5 gedreht. Am Tag 0 wurden die Kühe entweder mit DEXA (40µg/kg BW IV) oder

mit einer äquivalenten Dosis von Kochsalzlösung behandelt. In kurzen Abständen wurden Blutproben durch einen innen befindlichen Jugularis-Katheter für die Analyse von ACTH, Cortisol, Glukose, nicht veresterten Fettsäuren (NEFA) sowie Laktat-, Herz- und Atemfrequenz aufgezeichnet. Zuvor wurden, am Ende und nach der Stress Herausforderung, Tränenflüssigkeit gesammelt.

Im Vergleich zu den Kontrollen, zeigen DEXA behandelten Kühen deutlich reduziert Blutspiegel von ACTH und Cortisol sowie die Veränderung der Herz-und Atemfrequenz in den Basisdaten und während der Stress Herausforderung durch LR (Spitzenwerte und die Fläche unter der Kurve (AUC)). Die Wirkung dauerte mehr als fünf Tage. Auf d1 und d3 war fast keine HPA Reaktion nachweisbar. Die auf d1 im Blut enthaltene Glukose und Laktat-Konzentrationen waren signifikant höher in den Baseline Proben und während der LR (peak und AUC) in DEXA, als im Vergleich zur Kontrollgruppe. Die DEXA Behandlung zeigte keinen Einfluss auf den NEFA-Serumspiegel. Die wiederholte LR, resultierend aus der Anpassung der Kontrolle von Kühe, führt zu einem stetigen Rückgang von d1 bis d5 der Blutspiegel von ACTH, Cortisol, Glukose und Laktat sowie Herz-und Atemfrequenz. Kontrollen in Periode 2, die mit DEXA in Periode 1 behandelt wurden, zeigten deutlich geringere ACTH, Cortisol, Glukose und Laktat im Blut, als bei den Kontrollen in Periode 1.

Im Ergebnis zeigte die DEXA Behandlung eine sehr starke Wirkung auf den Hormonhaushalt, Stoffwechsel-sowie Herz-und Atemwegserkrankungen, bei Stress-Situationen, die länger als fünf Tage anhielten. Diese Wirkung muss in Studien über Stress-Management bei Milchkühen in Betracht gezogen werden. Drei Wiederholungen von LR Situationen, führten zu einer Anpassung der Milchkühe auf stressige Situationen, wenn die Kühe vorsichtig behandelt werden, wenn sie mit freundlicher und leiser Stimme angesprochen werden und wenn ihnen angemessene Zeit gewehrt wird, um die neue und unfamiliäre Situation zu erkunden. Aus dieser Studie blieb unklar, ob die reduzierte Reaktion auf Stress, die bei den Kontrollen in der Periode 2 zu sehen

waren, ein Ergebnis der Anpassung aufgrund von Erfahrungen, gespeichert aus Periode 1 war oder ob dies eine 4 Wochen anhaltende Wirkung der DEXA Behandlung war.

Key words: ACTH, Cortisol, Dexamethason, Stress, Anpassung, Lernen, Tier-Mensch-Kontakt

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Appendix

Table 23: Means of baseline serum concentrations of ACTH, cortisol, glucose, nonesterified fatty acids (NEFA), and lactate as well as heart (HR) and respiratory rate (RR) before three subsequent mild stress challenges on day 1, 3 and 5 by restrain in lateral recumbency (LR) in study period 0 or 1 in six dairy cows after placebo or dexamethasone treatment (40 µg/kg BW IV) on day 0.

		Treatment												SEM
		Placebo						Dexa						
		Period												
		0			1			0			1			
		LR on day												
Parameter		1	3	5	1	3	5	1	3	5	1	3	5	
ACTH	pg/ml	61.5	18.7	13.8	60.7	21.4	21.5	4.99	4.99	6.81	10.2	10.3	24.6	15.8
Cortisol	ng/ml	40.2	20.9	21.3	18.6	15.8	12.0	1.99	2.71	2.37	1.99	1.99	10.6	6.36
Glucose	mmol/l	5.12	4.72	4.54	4.31	4.19	3.90	7.71	4.36	4.24	7.84	4.32	4.86	0.30
Nefa	µmol/l	816	427	478	571	318	326	310	246	183	691	398	530	154
Lactate	mmol/l	1.52*	0.64*	0.74*	0.65*	0.55*	0.49*	1.82*	0.98*	0.66*	2.14*	0.66*	0.72*	0.22*
HR	1/min	76.3	75.3	69.3	73.6	73.0	66.0	69.6	67.3	64.3	72.0	71.3	67.6	3.47
RR	1/min	31.6	31.6	28.0	32.0	31.3	28.0	31.3	30.6	29.3	29.6	28.0	26.6	0.96