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Effects of Acute Lying and Sleep Deprivation on the Behavior and Immune Function of Lactating Dairy cows

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I am submitting herewith a thesis written by Jessie Ann Kull entitled "Effects of Acute Lying and Sleep Deprivation on the Behavior and Immune Function of Lactating Dairy cows." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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Effects of Acute Lying and Sleep Deprivation on the Behavior and Immune Function of Lactating Dairy cows

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DEDICATION

I would like to dedicate all who offered support.

First, Brock for moving to Knoxville with me and continually supporting me through grad school.

Secondly, my mentors Drs. Krawczel and Pighetti for helping me grow as a person and believing in me.

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ABSTRACT

The objective of the study was to determine the effects of sleep or lying deprivation on the behavior, production, metabolism and immune function of dairy cows. Data were collected from 8 multi- and 4 primi-parous cows (DIM = 199 ± 44 (mean ± SD); days pregnant = 77 ± 30). Each cow experienced: 1) 24 h sleep deprivation implemented by noise or physical contact and 2) 24 h lying deprivation imposed by a wooden grid placed on the pen floor that prevented a recumbent position. An 11-d collection period (from 2 d before the first treatment (trt) to 8 d after trt) was followed by 12-d washout periods. Study days were organized from 2100 to 2059. During habituation (d -2 and -1 before trt), baseline (d 0), and trt (d 1), housing was individual stalls (mattress with no bedding). After trt, cows returned to sand-bedded freestalls for a 7-d recovery period (d 2 to 8). Lying behaviors were recorded by accelerometers attached to the hind leg. Milk yield was recorded 2× daily. NEFA and glucose concentrations were evaluated from serum sampled at 0300, 0900, 1500, and 2100 on d 1 and 2. Data were analyzed using a mixed model in SAS including fixed effects of trt, day, and their interaction with significant main effects separated using a PDIFF statement ($P \leq 0.05$). Lying time decreased during trt and increased on the first day of recovery for lying deprivation compared to sleep deprivation (d 1: 1.9 vs. 8.4 ± 0.7 h/d (mean ± pooled SE); $P < 0.001$; d 2: 16.8 vs. 13.6 ± 0.7 h/d; $P = 0.002$). Milk yield decreased during lying deprivation compared to sleep ($P = 0.002$). NEFA and glucose varied by time ($P \leq 0.03$). IL-1β and TNF-α were higher during trt, compared to baseline for both treatments (day: $P = 0.04$ and $P = 0.004$, respectively). Collectively, this suggests, lack of access to resting resources rather than the relative comfort of that resource, may have greater long-term effects on the welfare of dairy cows.

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INTRODUCTION

The term ‘welfare’ in farm animals, refers to three broad questions: (i) is the animal free from suffering and pain; (ii) is the animal healthy and productive; and (ii) is the animal free to express natural and normal behaviors (Fraser et al., 1997, Fraser and Duncan, 1998). In dairy cows, lying time can be used to measure welfare. For example, if cows are lying deprived, they are in some degree of discomfort (i), milk production can be decreased (iii), and they are prevented from expressing a natural behavior; lying (iii). However, as described in a dairy cow’s time budget, lying time is only one activity that she needs to achieve within a 24-h period. In a freestall setting, a cow spends 12 to 14 h/d lying down, 3 to 5 h/d feeding, 2.5 to 3.5 h/d outside the pen, 2 to 3 h/d socializing, and 30 min/d drinking (Grant, 2000, Gomez and Cook, 2010). However, a cow’s time budget can vary depending on the environment and management system. Cows on pasture will lie down between 8.3 and 9.8 ± 0.6 h/d, and graze between 8.3 and 9.0 ± 0.4 h/d (Tucker et al., 2007). Furthermore, in tie stalls, lying time ranged from 9.7 to11.3 ± 0.8 h/d, and cows spent 3.8 to 4.6 ± 0.2 h/d eating (Norring et al., 2012). Although time budgets may vary, they are relatively consistent across management systems. The most time consuming behavior in confined housing operations in a 24-h period is lying, suggesting, it is a high priority activity. In fact, other behaviors such as feeding and socializing have been given up to spend more time lying down (Metz, 1985, Munksgaard and Simonsen, 1996). Thus, any factors that diminish a cow’s ability to achieve her desired lying time, could result in negative welfare.

Sleep is defined as a behavioral state that is required for survival (Everson, 1995, Carskadon and Dement, 2005). However, it is often not accounted for in a dairy cow’s time budget. Sleep may provide a means to evaluate the quality of a cow’s lying time, rather than just gross quantity. In other species like humans and laboratory animals, sleep is considered

imperative for health and welfare (Everson et al., 1989, Everson and Crowley, 2004). Sleep serves a restorative function as it is a way for the body to conserve energy that could not otherwise be accomplished during wakefulness (Schmidt, 2014). Sleep is also important for clearing certain metabolites that build up during wakefulness, such as adenosine, which is a product of energy expenditure (Bjorness and Greene, 2009, Xie et al., 2013). Dairy cows sleep 3 to 4 h, in short 3 to 5 minute bouts throughout the day, which is only a quarter of the time she spends lying down (Ruckebusch, 1972, Ternman et al., 2012). Although some sleep can be accomplished while cows are standing, a recumbent position, due to skeletal muscle paralysis, is required for REM sleep (Aserinsky and Kleitman, 1953, Ruckebusch, 1972). Certain management factors have the potential to reduce a cow's overall lying time, and potentially sleep. This can alter her time budget, behavior, and welfare. For example, overstocking, which limits access to a stall, may affect her lying time (Wierenga, 1983, Winckler et al., 2015). However, even if stall space is available, depending on stall design, how cows utilize that stall may provide insight on quality of lying time, such as sleep (Fregonesi et al., 2009). Collectively, there is likely a difference between cows that have access to an uncomfortable stall, versus cows that do not have a stall available at all. Therefore, it is not only important to consider how much a cow is lying, but also what she is doing while she is lying, such as sleeping.

To engage in REM sleep, a lying position is required (Ruckebusch, 1974). Consequently, if cows are lying deprived, their overall sleep pattern is likely shifted as well. Therefore, any effects observed during lying deprivation may be due to the cumulative effect of sleep and lying deprivation. For example, restraint and transportation stress both alter reproduction when applied separately (Hayashi and Moberg, 1987). While restraint or transportation stress can be considered sole stressors the animal can cope with, the process of ovulation is seen as an

additional stressor which then causes distress. Moberg (2000) reported that ideally animals have the energy reserves to cope with one stressor, such as sleep deprivation, and maintain normal functioning. However, when another stressor is applied at the same time, such as lying deprivation, energy resources are diverted towards that stressor, and away from other physiological processes. Thus, she is left in a vulnerable state, and distress may occur. While there is a growing body of work on sleep and lying deprivation, a summary describing the potential cumulative effects of both stressors has not been well documented.

Lying Deprivation

Lying Deprivation

Lying is a highly desired behavior of dairy cows (Metz, 1985), which makes lying deprivation an interesting area of research. Cooper et al. (2007) evaluated the effects of a 2- or 4-h lying deprivation period. During the 2-h deprivation period, cows stomped their feet and repositioned themselves more relative to the control period. Similar results were observed during the 4-h deprivation period, however, head butting, and cows continually shifting their weight was also observed. These behaviors were consistently detected during lying deprivation periods of 22 h/d (Ruckebusch, 1974) and 3 h/d (Metz, 1985) as well. This indicates, cows are likely frustrated during times of lying deprivation, and welfare is reduced. Metz (1985) evaluated the effects of solely feed deprivation, versus feed and lying deprivation. When cows were deprived of food and lying for 3 h/d, cows chose to lie down rather than feed. This suggests, cows prioritize lying over feeding in a confinement system, indicating lying is a basic requirement for overall welfare.

While lying deprivation alters behavior, it is important to recognize that physiological and metabolic changes occur as well. When cows were deprived of lying for 14 h/d, they had a greater ACTH concentration at the beginning and end of treatment (Munksgaard and Simonsen,

1996), which could lead to excess cortisol secretion, and in turn, metabolic diseases such as hyperglycaemia (Forslund et al., 2010). Other physiological effects of lying deprivation include a reduction in milk yield. Grant (2004) concluded that with each additional hour of lying time, a 0.9 to 1.5 kilogram increase in milk per day per cow occurs. Conversely, as stall availability decreases, milk yield is reduced (Bach et al., 2008). This may be partly due to growth hormone concentration being reduced during lying deprivation (Munksgaard and Løvendahl, 1993), as growth hormone helps promote milk production. This suggests, lying time facilitates production, and any loss of lying time may reduce milk yield.

While various studies have been designed to evaluate the direct effects of lying deprivation, on farm factors may also indirectly reduce lying time. Factors such as, overstocking (Krawczel et al., 2012), heat stress (Cook et al., 2007) and bedding (Fregonesi et al., 2007a) can all indirectly affect lying time. When cows were stocked at 142 and 150%, lying time was reduced from 12.9 to 12.3 ± 0.2 h/d (Krawczel et al., 2012), and 11.2 ± .26 h/d (Fregonesi et al., 2007b), respectively. Furthermore, latency to lie down was 23 minutes less when stalls were stocked at 150% (Fregonesi et al., 2007b). This suggests, overstocking decreases lying time and the latency to lie, therefore, altering cow behavior. Heat stress is another factor that indirectly affects lying time. Cows lied down for 10.9 h/d during the coolest observation period, and for only 7.9 h/d during the hottest observation period (Cook et al., 2007). The increased standing time can partly be explained by cows trying to stand under the fans and soakers to try and dissipate heat. Therefore, during incidences of heat stress, cow's behavior changes and lying time is decreased. Lastly, even type and quality of bedding play a role in lying time. When not given a choice between dry (86.4 ± 2.1% DM), and wet bedding (26.5 ± 2.1%), cows lied for 13.8, and 8.8 ± 0.8 h/d, respectively (Fregonesi et al., 2007a). However, when given the choice

between dry and wet bedding, cows lied down for 12.3 on the dry bedding, and 0.9 ± 0.3 h/d on the wet bedding (Fregonesi et al., 2007a). Cows showed a clear preference for dry bedding, and their lying time was significantly reduced on wet bedding during the no-choice phase. While these factors may not directly induce lying deprivation, they do cause cows to decrease lying time and therefore, welfare. Research studies have been designed to evaluate the effects of lying deprivation in dairy cows. However, on farm management practices may indirectly diminish a cow's desired lying time depending on how she is able to cope. Therefore, research into areas of specific management practices allows us to better understand how a cow interacts with her environment under less than ideal conditions. Thus, the consequences she endures can then be better understood as we start to evaluate management factors that potentially decrease lying time.

Sleep Deprivation

Overview of sleep

Sleep, in general, can be defined as a non-vigilant state where consciousness is reduced, but can be quickly reversed back to wakefulness (Siegel, 2005, Lange et al., 2010). Non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep are considered the two main non-vigilant states of sleep (Irwin, 2015). However, in some animals, there is an intermediate state between wakefulness and NREM sleep, known as drowsing (Ruckebusch, 1972). Drowsing can be characterized by a mixture of low voltage, fast activity (LVFA) and high voltage, slow activity (HVSA) types of electrocorticographic (ECoG) signals. With drowsing, a small decrease in muscular tone and respiratory rate is observed (Ruckebusch, 1972). Once in a state of drowsing, the transition to NREM sleep is likely for dairy cows (Ternman et al., 2012). NREM sleep is broken into 4 different stages, with stages 3 and 4 being the deepest sleep (Irwin, 2015). NREM sleep, also known as slow-wave sleep (SWS), is characterized by high-amplitude, low

frequency components as observed by the electroencephalogram (EEG). Once in NREM sleep, the conversion to REM sleep or to wakefulness is usually observed in mammals (Carskadon and Dement, 2005, Ternman et al., 2012). In contrast to NREM sleep, brain activity is similar to waking in REM sleep, and is characterized with rapid eye movements and muscle paralysis (Motivala and Irwin, 2007). REM sleep is the vigilant state where dreams occur and has been observed to have a rapid, low-voltage EEG (Irwin, 2015). Typically, cows will transition through these non-vigilant states multiple times per night (Ternman et al., 2012).

Importance of various vigilant states

The importance and structure of sleep related to health has been widely studied in humans and laboratory animals. One essential function of sleep is to restore body and brain functions that undergo fatigue during wakefulness (Schmidt, 2014), and memory consolidation (Stickgold and Walker, 2007). NREM sleep is beneficial for energy conservation and recuperation of the nervous system (Siegel, 2005). It has been stated that NREM sleep and hibernation are related non-vigilant states where metabolic rate, body temperature, and respiration rate are all reduced, suggesting a common purpose; energy conservation (Berger and Phillips, 1995, Zepelin et al., 2005). This is important as energy expenditure is lower during sleep, so it reduces the amount of energy needed in a day (Jung et al., 2011). Thus, the energy saved during sleep is allocated to other physiological processes such as immune function (Everson, 1993, Jung et al., 2011). Furthermore, NREM sleep increases when an infection occurs, whereas REM sleep decreases (Imeri and Opp, 2009). The increase in NREM sleep is likely due to the reduced energy expenditure during NREM sleep, rather than when the animal is in REM sleep (Mignot, 2008). During an infection, NREM sleep is increased, however, it is more fragmented than normal NREM sleep and therefore, promotes shivering and helps reduce

heat loss (Parmeggiani, 2003, Olivadoti and Opp, 2008). This fragmented sleep helps promote fever, and is critical to recovering from an infection (Kluger et al., 1996). Furthermore, REM sleep is thought to be reduced during an infection because the animal can not engage in shivering, which is critical to maintaining a high body temperature (Imeri and Opp, 2009). Allowing cows enough time to sleep during may be critical to overcoming bacterial infections or diseases.

Although alertness is reduced during REM sleep, in mammals, brain metabolism and neuronal activity are higher, respiration and heart rate are increased, rapid eye movements, and body twitches occur (Aserinsky and Kleitman, 1953, Siegel et al., 1998). It is unknown why the body undergoes these physiological processes during REM sleep. However, research has hypothesized that waking up from REM sleep is more beneficial because animals have better sensory-motor function when awakened during REM sleep, than those awakened during NREM sleep (Horner et al., 1997). Awakening from a state that allows you to be more alert is especially beneficial for prey animals, such as cows. Furthermore, it is known that REM sleep helps establish crucial brain connections during development, indicating REM sleep and brain size are positively correlated (Siegel, 2005). Therefore, if REM sleep is prevented, especially at a young age, there could be developmental consequences.

Each non-vigilant state plays a key role in protecting the health of all animals. While many studies have evaluated the effects of sleep and sleep loss in other species, sleep loss in cattle is unknown. However, inferences can be made, and if one of these vigilant states is prevented in cows, there could be detrimental effects that she is unable to cope with. To better understand the behavior and welfare of cows, research should start focusing on sleep, rather than the gross amount of lying time.

Sleep in cattle

Dairy cows spend 12 h/d lying down (Ito et al., 2009, Gomez and Cook, 2010), however, they also spend 3 to 4 h/d sleeping (Ruckebusch, 1972). Unlike humans, their sleep is very fragmented and spread out in short 3 to 5 minutes bouts throughout the day (Ruckebusch, 1972, Ternman et al., 2012). More specifically, they spend roughly 3 h/d in NREM sleep, 30 to 45 min/d in REM sleep, and 8 h/d drowsing (Ruckebusch, 1972, Nilsson, 2011). Although it is unclear the true value of drowsing, it is speculated that it is the bodies compromise between fully asleep and wakefulness so that ungulates, such as cattle, can more quickly react to predatory threats (Zepelin et al., 2005). For prey animals, it is advantageous to spend minimal time asleep to reduce the time they are vulnerable to predators (Allison and Cicchetti, 1976). While drowsing and NREM sleep can be accomplished when forced to stand (Ruckebusch, 1974), the cow must be in a recumbent position to engage in REM sleep. This suggests, if a cow is lying deprived, her overall sleep pattern may be altered.

Although a general structure of a cow's total time asleep is known, sleep patterns do change based on environment and stage of lactation (Ruckebusch, 1975, Nilsson, 2011). Cows housed indoors primarily sleep at night (Ruckebusch, 1975). However, when housed on pasture, cows lie down between 8.3 and 9.8 ± 0.6 h/d (Tucker et al., 2007), and sleep throughout the day and night (Ruckebusch, 1975). This suggests, although lying time is less on pasture, cows housed inside may be in a more disruptive environment throughout the day, which decreases the time she can sleep, relative to cows on pasture. Cows in early (2.5 ± 1.0 h/d), and peak lactation (3.5 ± 1.1 h/d) tend to sleep less in a 24-h period than cows in their dry period (4.5 ± 1.3 h/d) (Nilsson, 2011). One possible explanation for this is when food intake increases, there is less time for other activities, such as sleep. During the dry period, cows will increase NREM sleep from 2 to 2.5

h/d, to 3.9 h/d, while cows in peak lactation increase their REM sleep to 0.9 h ± 0.3 h/d (Nilsson, 2011). This is 0.3 or 0.5 h/d more than cows in the dry or early lactation period get, respectively. This suggests, cows in peak lactation could be compensating for the sleep loss during and after parturition due to the increase in energy requirements and food intake. Although total sleep time does not change, the general structure and time spent in each vigilant state is altered depending on environment and stage of lactation.

Measuring sleep in dairy cows

The gold standard for measuring sleep is to use electrophysiological equipment to score the vigilant state based on encephalography (EEG), electromyography (EMG) and electrooculography (EOG) as described by Rechtschaffen and Kales (1968). However, while previous work has used more of an invasive method in cattle, such as electrodes implanted on the brain surface (Ruckebusch, 1974), more recent work has validated the use of surface electrodes (Hänninen et al., 2008, Ternman et al., 2012). Cows can sleep in multiple different postures, depending on the vigilant state (Ruckebusch, 1974, Ternman et al., 2012). For example, since REM sleep is characterized by muscle paralysis, cows must be lying down with their head resting on their flank during REM sleep (Aserinsky and Kleitman, 1953, Ruckebusch, 1972, Ternman et al., 2012). For NREM sleep, they are typically lying on the ground with eyes closed, but head lifted off the ground (Ternman et al., 2012). Although, when forced to stand for a long duration of time, they can engage in NREM while standing (Ruckebusch, 1974). However, this is not typically observed, and may serve as a coping mechanism during lying deprivation. The problem with relying on behavioral postures to score sleep is that the same postures can be displayed for multiple vigilant states. Therefore, behavioral postures can not be used to accurately evaluate sleep in dairy cows (Ternman et al., 2014). For example, in calves and cows,

muscle paralysis is required for REM sleep, particularly in the neck muscles. This requires the head to be positioned on the ground, requiring that the animal assumes a recumbent position (Hänninen et al., 2008). However, these characteristics can also be representative of NREM or drowsing in cows (Ternman et al., 2014). Furthermore, drowsing and NREM sleep are sometimes hard to differentiate because they can both portray the same behavioral postures as well. During NREM sleep and drowsing, the cows eyelids are relaxed, but may be partially open, making it difficult to distinguish between the two (Ruckebusch, 1972). This further reinforces the importance of adequate lying time in cattle, especially for REM sleep, which requires a recumbent position.

The effects of sleep deprivation

The effects of sleep deprivation on the host defense system have been widely studied. On average, total sleep deprivation kills rats after 2 to 3 weeks (Everson et al., 1989, Obermeyer et al., 1991, Rechtschaffen and Bergmann, 1995). Food deprivation alone kills rats after 17 to 19 d (Dewasmes et al., 1989, Everson et al., 1989), and water deprivation a few days longer (Bivin et al., 1979). This suggests that sleep deprivation has similar effects to deprivations of basic needs.

While death occurs after a few weeks, signs of suffering occur earlier during deprivation. Signs of fatigue, an increase in whole body energy expenditure, and loss in body weight can occur earlier, indicative of signs of stress and poor welfare (Everson et al., 1989, Everson, 1995, Everson and Crowley, 2004). Rats also showed ulcerative and hyperkeratotic lesions on the tail and paw area, which are likely due to deprivation. Other symptoms of sleep deprivation include; decreased body temperature, high metabolic rate, and decreased host defense, suggesting sleep maintains vital bodily functions. Inflammatory cytokines have also been reported to increase during sleep deprivation which can alter the immune response (Altemus et al., 2001, Shearer et

al., 2001b). When people were sleep deprived for 40 (Moldofsky et al., 1989), and 24 h (Altemus et al., 2001), both IL1-β and TNF-α increased, which are inflammatory cytokines. Therefore, there is some degree of inflammation associated with sleep loss, which can lead to diabetes and other cardiovascular disorders (Chae et al., 2001, Thorand et al., 2003). Everson et al. (1989) reported bacterial invasions post sleep deprivation, and concluded that the rats may have died from septicemia. While inflammatory markers are increased during sleep deprivation, suggesting immune activation, it is insufficient to overcome microbial invasion (Everson, 2005)This suggests, sleep deprivation leads to the breakdown of the host defense system, and may be why total sleep loss is fatal.

With sleep deprivation affecting the immune response, it is not surprising that other physiological processes are also altered. For milk ejection to occur in rats, even if the pups are sucking, the mother must have a synchronized EEG, similar to that of NREM sleep (Lincoln et al., 1980). However, this is not the case in sows, where milk ejection can occur during a state of arousal (Poulain et al., 1981). Although comparisons across species may not be applicable in all cases, it is unknown if sleep is involved with milk ejection or production in dairy cows. Furthermore, it is unknown how pre calving management, or the time period prior to calving may affect sleep in dairy cows. However, there are reports that most women endure some degree of sleep deprivation during pregnancy (Osborn et al., 1990), which can lead to complications during birth. Women who slept less than 6 h/d a month prior to giving birth, had longer labors and more C-section births, relative to women who received more than 6 h/d of sleep (Lee and Gay, 2004). While this may be due to other stressors that occur during pregnancy, sleep could be considered a contributing factor. Lastly, women who worked over a 100 h week during their 1st trimester, and likely were sleep deprived, were 9.8% more likely to have a preterm birth than

women who worked less than a 100 h per week (Klebanoff et al., 1991). Indirectly, this indicates that sleep may play a role in milk production, labor duration, and timing of birth. While not directly evaluated in dairy cows, implications can be made as these effects may be observed if sleep deprivation occurs.

While many studies have evaluated the effects of sleep deprivation in humans and rodents (Moldofsky et al., 1989, Everson, 1995, Achie, 2015), few studies have looked at sleep deprivation in cattle. Ruckebusch (1974) recorded the effects of a 14, 20 and 22 h/d lying and food deprivation period on sleep in dairy cows for a total of 8 weeks. REM sleep was prevented, and NREM sleep was reduced during the deprivation periods. Interestingly, when lying deprivation was increased to 22 h/d, and when the free choice period (no deprivation) was limited to 2 h/d, cows chose to eat for that entire time rather than sleep. Contradictory to work from Metz (1985), who reported cows prioritized lying over feeding when deprived of both. This is likely due to the extreme 8 week deprivation implemented by Ruckebusch (1974), whereas, Metz (1985) only deprived the cows of lying and eating for 3 h/d for 2 weeks. Therefore, in extreme circumstances, such as a 22 h/d deprivation period, feeding is prioritized, likely for survival reasons. One limitation to Ruckebusch (1974)'s study is that he imposed sleep deprivation by lying depriving the cows. Therefore, the results he reported may be due to the cumulative effects of lying, feeding, and sleep deprivation. Nonetheless, when the free choice period was 10 h/d, cows ate and engaged in the same amount of REM sleep as they did during the baseline period, but NREM sleep was reduced (Ruckebusch, 1974). This suggests, although cows consumed the same amount of feed, and engaged in REM sleep, their circadian rhythm was altered (Ruckebusch, 1974). Following the deprivation period, continuing until 4 d later, a rebound effect occurred where both NREM and REM sleep nearly doubled their normal

duration. This suggests, the sleep loss experienced during deprivation must be compensated for at some point, or consequences to the cow's welfare may occur.

Previously, research has primarily focused on the effects of lying deprivation (Munksgaard et al., 1999, Cooper et al., 2007), or implementing sleep deprivation by lying depriving the cows (Ruckebusch, 1974), which serves as a confounding factor. While the impacts of sleep deprivation are less known in dairy cows, the various fatal effects of sleep deprivation has been demonstrated. This provides a clear path of where research needs to progress to understand how a cow's environment can affect not only her lying time, but also the time she spends sleeping.

Stress, Distress and Cumulative Stressors

Defining stress and distress

Stress, in general, alters biological function by shifting energy resources (Moberg, 1985). Moberg and Mench (2000) define stress as "the biological response elicited when an individual perceives a threat to its homeostasis." Stress challenges the body; but that stress can be overcome using coping mechanisms (Moberg, 2000). However, unlike stress, the term 'distress' is used when the stress response threatens animal well-being (Moberg, 2000, Council, 2010). Therefore, it is important to understand the differences between stress and distress, and recognize when stress becomes distress.

One way to differentiate stress and distress is to assess the biological cost of that stress (Moberg, 2000). When ewes were exposed to heat and nutritional stress, the effects on weight gain, feed intake, respiration, and various other physiological responses, were worse than either stressor alone (Sejian et al., 2010). Similarly, when rams were exposed to those same stressors simultaneously, growth and reproductive performance were reduced more than either stressor

alone (Maurya et al., 2016). This suggests, energy was redirected to cope with the multiple stressors, and less resources were allocated towards growth or other productive functions. While the biological cost may be low if only one of those stressors were applied, the cumulative effect of both stressors leads to distress. As long as the energy resources to cope with the stressor are sufficient, the stressor is likely not life threatening. In addition, if a stressor is only short term, such as escaping a predator, energy, such as glycogen, is quickly replenished, and the biological cost is small (Moberg, 2000). Overall, the biological cost of a stressor plays a key role in determining if stress may lead to distress.

Subclinical stress

Although not directly causing distress, subclinical stress can increase the risk of distress (Moberg, 1985, 1999). Subclinical stress will shift energy resources, however, not enough to affect normal functioning (NHMRC, 2008). This suggests, the biological cost associated with subclinical stress is low. However, some amount of energy is diverted elsewhere, potentially leaving the animal vulnerable if another stressor is encountered (Moberg, 2000). One primary consequence with subclinical stress is that altered behavior or other clinical signs may not be observed. Therefore, it is not obvious that the animal is coping with a stressor. When ducks consumed food with petroleum, there were no clinical signs of distress and weight was maintained (Holmes et al., 1979). However, when the ducks encountered a second stressor, such as cold temperatures, a higher mortality rate was observed relative to the ducks that consumed uncontaminated food. Furthermore, subclinical disorders make diagnosing ill dairy cows difficult, as current means of detection may be insufficient (Mordak and Stewart, 2015). While subclinical stress may not directly reduce welfare, it leaves the animal susceptible to distress by shifting energy resources away from other productive functions.

Cumulative stressors

The biological cost of subclinical stress is worse if a second stressor is encountered simultaneously, because the animal is forced to cope with the effects of multiple stressors (Schreck, 2000, NHMRC, 2008). When rats were restrained 4 h/d for 7 d, then injected with LPS, the combined exposure had greater effects on growth, energy deposition, plasma corticosterone concentration, and heat energy production, than either stressor (restraint or LPS) alone (Laugero and Moberg, 2000). This suggests, the combination of both stressors had a greater biological cost to the animal, than either stressor by itself. This is further supported by a similar study where Laugero and Moberg (1998) observed an initial decrease in body weight in restrained mice. However, towards the end of the experimental period, the mice reached a plateau, and were able to maintain body weight. This indicates, although weight was initially lost, the mice were able to shift enough resources to cope with the stress, as well as maintain body weight. It can be speculated that if another stressor was applied during that time, the mice would succumb to the effects of multiple stressors. Laying hens died after exposure to heat stress and injection with LPS, whereas no hens died when only exposed to one of the stressors (Star et al., 2008). This suggests, some hens could not overcome the combination of both stressors on thermoregulation and immunity. However, they were able to cope when only one stressors was applied. Lastly, when cows are heat stressed, they decrease their lying time (Cook et al., 2007, Herbut and Angrecka, 2017). While it is recognized that many physiological processes are likely altered during this time, the combination of multiple stressors may be why other productive functions, such as milk yield (Klinedinst et al., 1993, Ravagnolo et al., 2000), and fertility (Dash et al., 2016) are reduced. Overall, the cumulative effects of multiple stressors is likely worse than experiencing them separately.

Both lying and sleep deprivation have been studied in various species (Everson, 1993, Irwin et al., 1996, Munksgaard et al., 1999). Ruckebusch (1974) sleep deprived dairy cows by preventing them from lying. While the objective of this study was to observe the effects of sleep deprivation, he was lying depriving them as well. Thus, while aiming to evaluate sleep deprivation, it is unknown if lying deprivation was a confounding factor. On the other hand, other lying deprivation studies in dairy cows did not take into account sleep when implementing lying deprivation (Metz, 1985, Cooper et al., 2007). Since certain vigilant states can only be accomplished while lying (Ruckebusch, 1972, Hänninen et al., 2008), there is likely some degree of sleep deprivation occurring. Therefore, based on other animal models, it can be speculated that the cumulative effect of lying and sleep deprivation may exacerbate any symptoms.

Conclusions

Previously, research has focused on evaluating the effects of lying deprivation. While having sufficient space for cows to lie is important, it is also important to consider what she is doing while she is lying, such as sleeping. This concept of total time spent sleeping is likely critical to a cow's overall health and welfare. Additionally, the effects observed during lying deprivation may be due to the cumulative effects of sleep and lying deprivation, rather than solely lying deprivation. A cow may be coping with subclinical stress if she is sleep deprived because her stall is uncomfortable. Although no clinical signs are observed, this leaves her in a vulnerable state. Now, the pen is overstocked and she is to some degree, lying deprived. She now has no energy reserves left to manage that stress, and resources are being pulled from other productive functions. Therefore, while these physiological stressors may not have biological consequences alone, the cumulative effect of both stressors could be damaging.

With the concept of multiple stressors having worse effects than solely one stressor, the effects observed during lying deprivation in dairy cows, may be the cumulative effect of sleep and lying deprivation. Thus, it is important to understand not only lying time, but to also consider sleep. To fully understand the effects of a stressor, understanding the effects of implementation must be considered to fully appreciate that stressor.

CHAPTER ONE

Effects of Acute Lying and Sleep Deprivation on the Behavior and Production of Lactating Dairy Cows

Abstract

The objective was to determine the effects of sleep or lying deprivation on the behavior and production of dairy cows. Data were collected from 8 multi- and 4 primiparous cows (DIM = 199 ± 44 (mean ± SD); days pregnant = 77 ± 30). Using a crossover design, each cow experienced: 1) sleep deprivation implemented by noise or physical contact when their posture suggested sleep, and 2) lying deprivation imposed by a grid placed on the pen floor. One day before treatment (baseline), and treatment day (trt) were followed by a 12-d washout period. Study days were organized from 2100 to 2059. During habituation (d -3 and -2 before trt), baseline (d -1), and trt (d 0), housing was individual boxstalls (mattress with no bedding). After trt, cows returned to sand-bedded freestalls for a 7-d recovery period (d 1 to 7) where data on lying behaviors were collected. Lying time, lying bouts, bout duration, and steps were recorded by dataloggers attached to the hind leg for 25 d. Milk production was collected automatically 2x daily. Data were analyzed using a mixed model in SAS including fixed effects of trt, day, and their interaction with significant main effects separated using a PDIFF statement ($P \leq 0.05$). Interactions between trt and day were evident for lying time and bouts. Lying time was reduced for both trts during the trt period relative to baseline. Lying time increased during the recovery period for both lying and sleep deprived cows. However, it took 4 d for the lying deprived cows to fully recover their lying time after trt, whereas it only took the sleep deprived cows 2 d for their lying time to return to baseline levels. The lying deprived cows produced less milk on d 1 and 2 ($P \leq 0.02$).This data suggests that while lying deprivation altered behavior and reduced production more, both sleep and lying deprivation can have detrimental effects on cow behavior and welfare. Management factors that limit freestall access likely reduce lying time and sleep, causing negative welfare implications for dairy cows.

Introduction

Lying time is critical for biological function; however, there are various factors on farm that diminish a cow's ability to lie or influence how she utilizes that lying space once she has occupied it. Management factors such as, overstocking (Krawczel et al., 2012) or heat stress (Cook et al., 2007) may decrease lying time, either by reduced access or altered motivation. Additionally, facility factors such as bedding type (Fregonesi et al., 2007a) and stall design (Fregonesi et al., 2009) can influence how she utilizes her time in a stall, even if stalls were accessible or a cow's motivation to lie down remained the same. Thus, there is likely a difference between lack of access to a stall, versus change in utilization of that lying surface.

Within their time budget, dairy cows lie down between 11 and 13 h/d in confinement (Tucker and Weary, 2004, Jensen et al., 2005, Ito et al., 2009). However, some of that lying time is spent sleeping. Time budgets, and specifically lying time, have the potential to be redefined with the inclusion of sleep. Sleep is divided into two main vigilant states; non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep (Irwin, 2015). However, drowsing in some animals is observed, and described as an intermediate state between wake and NREM sleep (Ruckebusch, 1972). Dairy cows sleep for about 4 h/d, in short 3 to 5 minute bouts throughout the day (Ternman et al., 2012). Specifically, cows spend 3 h/d in NREM sleep, 30 to 45 min/d in REM sleep, and 8 h/d drowsing (Ruckebusch, 1972). Furthermore, cows can drowse and engage in some NREM sleep when forced to stand, though, this is not normally observed (Ruckebusch, 1972). All vigilant states cannot be achieved while standing. A recumbent position must be assumed for cows to engage in REM sleep (Ruckebusch, 1972) Therefore, any loss of lying time has the potential to alter the time cows spend in each vigilant state.

Negative effects are associated with lying and sleep deprivation on the productivity of dairy cows and other species. Bach et al. (2008) found a relationship between stalls per cow and milk production. As stall access decreased and the potential for lying as well, milk production was reduced, suggesting lying time plays a critical role in milk yield. With each additional hour of lying time, the cow produces 2 to 3.5 extra lbs of milk per day (Grant, 2004). Similar to lying deprivation, sleep deprivation has various effects on the productivity of animals. Growth hormone and prolactin, which are key hormones associated with milk production, are decreased during sleep deprivation in people. This suggests, milk production may be affected in cattle as well (Davidson et al., 1991).

In dairy cows, because of the difficulty in evaluating sleep, research has primarily focused on studying lying time, rather than what she is doing during that time, such as engaging in sleep. The importance of sleep related to welfare has been widely studied in other species (Everson et al., 1989, Irwin, 2015). Previous research concluded that rats died after two to three weeks of complete sleep deprivation (Rechtschaffen and Bergmann, 1995). Signs of fatigue, an increase in whole body energy expenditure, and loss of body weight can occur earlier, indicative of signs of stress (Everson et al., 1989, Everson, 1995, Everson and Crowley, 2004). Furthermore, rats developed lesions on their paws and tail as early as d 2 after sleep deprivation (Kushida et al., 1989). This can be attributed to malnutrition and indicate poor welfare. This suggests, sleep deprivation is stressful to animals, and therefore, decreases their overall health and welfare.

Lying time is an important behavior in dairy cows, suggesting, if lying time is restricted, welfare is reduced. When given the choice, cows relinquish other activities such as feeding and socializing to spend more time lying down (Munksgaard et al., 2005). Many studies have

evaluated the effects of lying deprivation because it is such a high priority behavior. During a 2 or 4-h lying deprivation period, cows stomped their feet, shifted their weight, and head butted neighboring cows (Cooper et al., 2007). These behaviors were consistently observed during lying deprivation periods of 22 h/d for two weeks (Ruckebusch, 1974), and 3 h/d for one week (Metz, 1985). Collectively, this suggests cows are likely expressing frustration, restlessness and lack of comfort during this time. While lying time was reduced in these studies, some degree of sleep deprivation was likely imposed as well, because cows cannot engage in REM sleep while standing (Ruckebusch, 1974). Therefore, it is not known if the effects observed during lying deprivation are solely a result of lying deprivation, or the cumulative effects of lying and sleep deprivation.

The concept of dual stressors having cumulative effects was originally proposed by Moberg (2000) in his theory on subclinical stress. Rats that were restrained for 4 h/d for 7 d, and then injected with LPS as a second stressor, had greater detrimental effects than rats that only experienced one stressor (Laugero and Moberg, 2000). More relative to cows, when cows experienced heat stress, lying time was decreased (Cook et al., 2007, Herbut and Angrecka, 2017). While many physiological processes are altered during this time, the combination of multiple stressors may contribute to the negative effects observed during heat stress (Ravagnolo et al., 2000, Dash et al., 2016). Thus, the cumulative effects of multiple stressors may be worse than experiencing one stressor. Within the current study, cows may have the energy reserves to cope with sleep deprivation, and maintain productivity, but when another stressor is applied, such as lying deprivation, the animal becomes distressed and is unable to function normally. Although the effects of lying deprivation have been evaluated, less is known about the potential cumulative effects of lying and sleep deprivation.

To date, research has evaluated the effects of lying deprivation, but did not account for sleep deprivation as an additional stressor. Little is known about the cumulative effects of lying and sleep deprivation. Although inferences can be made from sleep deprivation studies focused on humans and laboratory rodents, the effects of sleep loss in cattle are unknown. With this concept of cumulative stressors, the idea of lying time has the potential to be redefined with the addition of sleep. Thus, the primary objective of this study was to determine the effect of sleep and/or lying deprivation on the behavior of dairy cows. The second objective was to compare the behavioral response during baseline and treatment period, and to quantify the behavior throughout the recovery period.

Materials and Methods

Animals, Housing and Management

This study was conducted at the University of Tennessee's Little River Animal and Environmental Unit (Walland, TN) during April and May 2016. Mid to late-lactation Holstein dairy cows (n = 12) were enrolled based on DIM (DIM = 199 ± 44) and days pregnant (77 ± 30 d). Cows were milked twice daily starting at 0700 and 1730 h in a double-8 herringbone milking parlor (BouMatic, Madison, WI).). Cows are normally housed in deep-bedded sand freestall pens. During the 4-d observation period, cows were housed individually in a 4.11 × 3.32 m pen with a mattress. Visual and olfactory contact was possible for enrolled cows throughout the duration of the treatment phase. Individually housing in this manner facilitated the use of electrophysiological equipment to assess vigilant state and lying deprivation. Pens were thoroughly scrubbed with chlorhexidine solution (Durvet Inc., Blue Springs, MO) every morning at 0700 h when cows were being milked. Fecal matter was removed manually throughout the day to maintain pen and cow hygiene. Fresh water and a TMR were available ad libitum. The TMR

was comprised of 60% corn silage, 25% pelleted premix grain concentrate, 12% small grain silage, and 3% dry hay. All procedures described were approved by the University of Tennessee Institutional Animal Care and Use Committee.

Enrollment Criteria

From the cows meeting the selection criteria for DIM and pregnancy, a final group of 12 cows were selected using white blood cell count (WBC ≤ 12.6), and temperament. Blood samples from the target population of cows, were taken via the coccygeal vein and WBCs were analyzed to ensure cows were below the accepted threshold of 12.6 cell/mL as described by Schalm (1961), indicating the cows were not experiencing any prior illness. Thus, cows enrolled in the study were considered healthy. Temperament was evaluated using an approachability and brush test. For the approachability test, a researcher slowly approached the cow with one arm extended, and observed the cow's reaction (Lensink et al., 2003). Cows were scored based on the 1 to 4 scale described by Lensink et al. (2003), with 1 being defined as the cow allowing physical contact, and 4, the cow strongly withdrew from the researcher (Table 1). If the cow remained still and allowed physical contact or approached the researcher (a score of 1 or 2), the cow was considered suitable for the study. The brush test used was slightly modified from the brush test described by Ternman et al. (2014), where cows were restrained in pen headlocks, instead of free roaming. Cows were scored based on a 1 to 4 scale, similar to the scale defined by Lensink et al. (2003) (Table 1). For this test, the cow's head and neck area were brushed, particularly where the EEG equipment would be placed (Lensink et al., 2003, Ternman et al., 2014). If the cow did not pull away, or only slightly withdrew when brushing occurred (a score of 1 or 2), she was considered an acceptable candidate for the study. In total, 14 cows met the criteria for the

temperament tests, however, 2 cows were removed because their WBC count exceeded the accepted 12.6 cell/mL threshold (Schalm, 1961).

Treatments

Treatments were implemented using a crossover design with rolling enrollment. The study design progressed from a habituation (-3 d, -2 d), baseline (-1 d), treatment (0 d) and recovery (1 – 7 d) period, with a 12-d washout period between treatments. Because cows were moved to an unfamiliar pen, a 2-d habituation period was provided to allow cows to adapt to their new environment. When cow were regrouped into a novel pen (von Keyserlingk et al., 2008), or trained to use a robotic milking system (Jacobs and Siegford, 2012), it only took the cows 2 d to habituate, suggesting our 2-d habituation period was sufficient. Additionally, the mattress bedded pens the cows were placed in were only 8 m away from their home pen, so visual, and olfactory contact were maintained. During the study, cows experienced two treatments; a 24-h lying deprivation period, and a 24-h sleep deprivation period starting at 2100 h. After treatment, cows returned to their home deep-bedded sand freestall pen for a 12-d washout period before returning to an individual pen for their second treatment (whichever treatment they did not experience first). Observations were recorded every 30 minutes for each cow over the 48-h baseline and treatment period starting at 2100 h. The 7 d immediately after treatment were defined as the recovery period.

Lying Deprivation. The 24-h lying deprivation period was implemented using a wooden girl placed on the pen floor, preventing cows from assuming a recumbent position. The wooden grid was based on a design by Schütz et al. (2008) which prevented cows from lying during times of heat stress. If cows attempted to lie during treatment, a researcher would encourage her to stand up. If the researcher was unsuccessful, the cow would be returned to her home pen, and

removed from the study. Ultimately, no cows had to be removed, and only 2 cows attempted to lie down during treatment.

Sleep Deprivation. During the 24-h sleep deprivation period, cows were allowed to lie down, but were continuously monitored to ensure cows remained awake and alert. If a cow's posture suggested the onset of sleep, the cow would be touched to keep her awake as described by Ledoux et al. (1996) who used this method in cats. Gentle handling or touching was used because it implemented deprivation, but likely did not induce a stress response that would be caused by the method of deprivation (Graves et al., 2003).

Behavioral Data

Lying Behaviors. IceTag dataloggers (IceRobotics Ltd., Edinburgh, Scotland) were attached to the hind leg during milking two days prior to the start of the study to allow for habituation (MacKay et al., 2012). A total of 18 d worth of data were collected and analyzed from the IceTags for each cow. The IceTags collected daily lying times (h/d), lying bout frequency (number/d), lying bout length (min/bout), and total steps (number/d) (McGowan et al., 2007).

Electrophysiological equipment. During the baseline period, cows were fitted with the electrophysiological equipment that collected electroencephalographic (EEG), electrooculography (EOG), and electromyography (EMG) data (EEG; BioRadio, Great Lakes Neurotechnologies, Cleveland, OH). Cows were restrained in the headlock of the experimental pen for placement of the electrophysiological equipment. Hair at the location of each electrode was shaved using 40 blade clippers (Andis, Sturtevant, WI) and wiped clean with alcohol to ensure sufficient contact. Non-invasive electrodes were plugged into the EEG device and then placed on the cow using Durapore Surgical Tape (3m Healthcare, St. Paul, MN) and adhesive

glue (Gorilla Glue Inc., Cincinnati, OH) to secure the electrodes in place. Ten20 EEG conductive paste (Weaver and Company, Aurora, CO) was placed on both sides of the electrodes to help conduct the signal. In total, there were ten electrodes on the cow. Electrode configuration was placed on the head and neck area, and can be further illustrated in Figure 1 (Ternman et al., 2012). During the entire 48 h EEG recordings from the baseline and treatment periods, a researcher was present to monitor the cow and ensure the EEG device, and the electrodes remained in place. This data is being analyzed in conjunction with other collaborators and due to time constraints, the EEG data will not be prepared in time to be presented in this thesis.

Production Data

Milk sampling. Cows were milked twice daily starting at approximately 0730 and 1700 h. Milk weights were recorded at each milking on d -2, 2, 3, 4 and 5 automatically. The collars that register cows in the parlor were removed during the baseline and treatment period because they interfered with the EEG device. Therefore, milk weights were not recorded during this time. Data from the day prior to baseline was used to represent the baseline period. Milk weights were combined from morning and evening milking to obtain total daily production.

A composite milk sample was collected into a 15mL collection vial during milking on baseline, treatment and d 2, to monitor fat, protein and somatic cell count (SCC). Morning and evening milk composite data were combined daily for all study days. Milk composite samples were taken automatically via an inline sampler without additional handling of the cow. Samples were stored at room temperature for no more than 48 h before analysis. Milk fat, protein, and somatic cell counts (SCC) were analyzed by the Tennessee Dairy Herd Improvement Laboratory (Knoxville, TN).

Statistical Analysis

All data were analyzed in SAS 9.4 (SAS Institute, Inc., Carry, NC) using the cow as the experimental unit. Data were analyzed using the mixed model ANOVA with significance declared a $P \leq 0.05$ and a trend declared at $P = 0.05 – 0.1$. Behavioral and production data for the current study were analyzed using a crossover design with repeated measures. For the production data, fixed effects were period, day, treatment, and time of sampling. To facilitate the recording of the EEG data, animals were housed in two different environments. Therefore, comparisons of behavior are presented within the same environment. Fixed effects were study days, period and treatments. When there were significant interactions, treatment means within a day were separated using the PDIFF option of the LSMEANS statement of SAS. The random effect was cow within treatment and sequence of events. For data that was not normally distributed, a log transformation was used to normalize all data, and data were reported as back transformed means.

Results

Baseline to Treatment Period Comparison

The baseline and treatment periods were both occurred while cows were housed in the individual, mattress bedded pens and therefore, cows experienced the same environment during this time.

Lying Behaviors. All lying behaviors differed between baseline and treatment for the lying deprived cows ($P \leq 0.05$; Table 2). A tendency for reduced lying bouts and increased bout duration occured for sleep deprived cows relative to baseline ($P \leq 0.1$; Table 2).

Treatment to Recovery Period Comparison

The last day of the recovery period, d 7, be used as the comparison for lying behaviors as it better reflects a cow's typical daily lying time on sand bedding. Thus, using d 7 compares cow behavior on sand bedded freestalls, which is their normal housing environment.

Lying Behaviors. Lying behaviors were similar on d 7 for both sleep and lying deprivation ($P > 0.05$). The mean lying time was increased on d 1 relative to d 7 for both lying and sleep deprived cows ($P \leq 0.006$; Figure 2). Lying deprived cows took 4 d to completely recover their lying time after deprivation ($P = 0.62$; Figure 2). However, sleep deprived cows only took 2 d to recover their lying time ($P = 0.24$; Figure 2).

Lying bouts did not differ for either treatment on d 1 through 6, relative to d 7 ($P \geq 0.05$; Table 3). However, there was a tendency for cows to have more lying bouts on d 2, relative to d 7 for the lying deprived cows ($P = 0.07$; Table 3). Bout duration was greater on d 1, relative to d 7 for the lying deprived cows ($P < 0.0001$; Table 3). On d 2 through 7, bout duration did not differ relative to d 7 for the lying deprived cows ($P \geq 0.05$; Table 3). No differences in bout duration on any day were evident for the sleep deprived cows relative to d 7 ($P \geq 0.05$; Table 3). However, there was a tendency for bout duration to be longer on d 1, relative to d 7 for the sleep deprived cows ($P = 0.08$; Table 3). Steps did not differ between d 1 and 6, relative to d 7 for either treatment ($P \geq 0.05$; Table 3).

Production

Milk Yield. Milk production was similar during baseline for both treatments ($P = 0.44$). However, the lying deprived cows produced less milk on d 1 and 2 than during baseline (Table 4). Milk production tended to be lower on d 2 relative to baseline for the sleep deprived cows, but did not differ on any other day relative to the baseline period (Table 4).

Milk Composite. For lying deprived cows fat content was lower during baseline, relative to treatment, or d 1 ($P \leq 0.001$; Table 4). No other days differed relative to baseline for fat content ($P \geq 0.05$; Table 4). Protein content for the lying deprived cows was elevated on d 1, and 2 relative to baseline ($P \leq 0.04$; Table 4). Overall, the sleep-deprived cows had a lower protein content than the lying deprived cows ($P = 0.01$; Table 4). Protein content did not differ on any days for the sleep-deprived cows ($P \geq 0.05$). For SCC, there was a tendency for a period and treatment effect to occur ($P = 0.08$ and $P = 0.09$, respectively). Lying deprived cows tended to have a higher SCC, than the sleep deprived cows. However, there was no effect of day or treatment × day interaction ($P = 0.64$ and $P = 0.15$, respectively).

Discussion

Evaluating the effects of sleep and lying deprivation separately on behavior and production, has yet to be determined in dairy cows. Prior research has focused on lying deprivation and most likely reflects current difficulties in evaluating sleep, but has failed to consider the cumulative effect of lying and sleep deprivation during this time. Assessing the effects of sleep and lying deprivation separately is inherent to understanding the difference between gross quantity of lying time, and what she is doing while she is lying. Within the current study, both deprivations altered lying time after treatment, suggesting either deprivation alters cow behavior and welfare. Furthermore, although sleep deprivation had no effect on milk production, lying deprivation reduced milk yield. However, this could be due to the cumulative effect of lying and sleep deprivation during this time.

As expected all lying behaviors were reduced from the baseline to the treatment period for the lying deprived cows, similar to previous studies that implemented lying deprivation (Metz, 1985, Munksgaard et al., 1999). While a small amount of lying time was recorded during

lying deprivation, it is likely due to cows shifting their weight to alleviate pressure on their hooves. While we did not record these observations, these behaviors have been observed as a sign of frustration and discomfort in other lying deprivation studies (Ruckebusch, 1975, Metz, 1985, Cooper et al., 2007). In addition, the researchers who were present during the entire treatment period, recording direct observations every 30 minutes, did not observe any lying time. Furthermore, research has reported that accelerometers can record false lying behaviors due to horizontal leg movements (Kok et al., 2015). Overall, the recorded lying time during lying deprivation, was likely not real lying time.

While lying deprivation altered lying time, sleep deprivation did not reduce lying time relative to the baseline period. This suggests, while sleep deprivation kept the cows awake, it did not change their lying time. However, lying time during baseline for both treatments, was less than previous reports for mattress bedding (Manninen et al., 2002, Tucker and Weary, 2004, Ito et al., 2009). Tucker and Weary (2004) reported a mean lying time of 12.3 ± 0.53 h/d on a mattress surface with no bedding. This may be due to the transition from the cow's typical sand bedded freestall pen to an individual, mattress bedded pen, as cows change lying behaviors depending on bedding type (Tucker et al., 2003). Nonetheless, lying time the day after sleep deprivation was increased relative to treatment, suggesting, some amount of lying time may be lost during sleep deprivation as well.

Lying bouts (4.9 ± 0.82 bouts/d) and bout duration (58.9 ± 7.31 min/bout) during the baseline period were similar to previous literature, suggesting, researcher presence did not disrupt all lying behaviors. Previously, a mean of 8.5 ± 0.6 bouts/d (Tucker and Weary, 2004), and 10.7 ± 0.7 bouts/d (Manninen et al., 2002), were reported for dairy cows on mattress bedding. Although bout durations are shorter relative to reports by Tucker and Weary (2004) (90

± 6.0 min/bout), data within the current study is similar to Van Gastelen et al. (2011), and Manninen et al. (2002), who reported 71.7 ± 10.2, and 70.4 ± 4.5 min/bout, respectively. This suggests, lying bouts and bout duration were not greatly altered during baseline for either treatment. Lying bouts and bout duration had a tendency to differ between baseline and sleep deprivation, where during treatment, cows had a tendency to have less lying bouts and longer bout duration. Although, lying bouts only differed by 1.4 bouts/d and bout duration only differed by 11.09 min/d. Therefore, there may not be any biological relevance to the tendency, due to the minimal differences observed. Overall, lying deprivation altered lying bouts and bout duration more than sleep deprivation. Thus, cows can likely be sleep deprived without being fully lying deprived. However, the quality of lying time during sleep deprivation is like reduced due to the inability to engage in sleep.

The number of steps taken within the current study differed depending on the study days. During the baseline period, cows took more steps than what was reported previously for this herd. On sand bedding, cows took a mean of 1,611 ± 120.7 steps/d depending on the season (Kull et al., 2017). Although this is lower than steps taken within the current study, cow's activity varies across environment and bedding type (Manninen et al., 2002, Tucker et al., 2003). Thus, cows were more active on the mattress bedding, relative to their normal sand bedded freestalls. Furthermore, number of steps taken during baseline for sleep and lying deprivation did not differ. This suggests, that even though steps were higher than previously reported, baselines for both treatments were similar, indicating accurate comparisons can be made between treatments. The number of steps taken was less during baseline relative to lying deprivation, but did not differ between baseline and sleep deprivation. This implies that lying deprivation has a greater overall impact of a cow's daily activity than sleep deprivation.

Lying deprivation reduced milk production by 3.1 and 2.1 kg from the baseline period to d 1, and 2, respectively. Other studies either did not measure milk yield during lying deprivation (Ruckebusch, 1974, Metz, 1985, Munksgaard and Simonsen, 1996), or milk production was not affected (Munksgaard and Løvendahl, 1993, Cooper et al., 2007). However, when cows were lying deprived for 14 h/d for 23 d, growth hormone (GH) was reduced (Munksgaard and Løvendahl, 1993). GH in dairy cows is involved with the partitioning of energy resources in favor of milk production, as increased GH concentration is positively correlated with milk yield (Hart et al., 1978). While GH was not measured in the current study, it can be speculated that GH was a contributing factor to the reduction in milk yield for the lying deprived cows. Furthermore, GH hormone is also strongly tied to the sleep-wake cycle (Kim et al., 2011). GH secretion is typically increased during sleep and suppressed during sleep deprivation (Brandenberger et al., 2000, Everson and Crowley, 2004). While, sleep deprivation did not have an effect on milk yield in the current study, it may be the cumulative effect of lying and sleep deprivation that reduced milk yield during lying deprivation.

Milk composition was altered during the experimental period. However, all components fell within the normal range. Within the current study, milk fat and protein were similar to other studies that reported a range from 2.0 to 6.1, and 2.5 to 2.8%, respectively (Kelsey et al., 2003). Furthermore, results are consistent with Åkerlind et al. (1999), and Bouraoui et al. (2002), who reported milk fat and protein similar to the results presented in the current study. Although milk fat was elevated during the treatment period relative to baseline, milk fat is the most variable of all components (Woodford et al., 1986) and changes based on lactation (Council, 1988), milking duration (Wheelock, 1980), and season (Jenness, 1985). Thus, milk fat changing slightly across days is not alarming, and may not be biologically relevant. Although feed intake was not

measured in the current study, cows during lying deprivation do increase their feed intake (Cooper et al., 2007), which can increase milk fat percentage in dairy cows (Macmillan et al., 2017). This may be why fat content is higher during treatment and d 1, relative to baseline. SCC was increased for the lying deprived cows, relative to the sleep deprived cow. This may suggest that cows were stressed during this time, as SCC increased in cows during transportation (Yagi et al., 2004), and when mixed in groups (Kay et al., 1977), which can both be deemed as stressful events. However, SCC within this study were well below the 200,000 cell/mL threshold (Schepers et al., 1997, Bradley and Green, 2005), indicating the increase in SCC may not be biologically relevant. Collectively, the deprivation period may not have been long enough to alter milk composition significantly.

Overall, results were consistent among all lying behaviors for both treatments. The lack of differences between the baseline and treatment period for sleep deprivation suggests, while cows were sleep deprived, they were not lying deprived, indicating the successful separation of sleep and lying deprivation. While sleep deprivation alone did not reduce milk yield, there was likely a cumulative effect of lying and sleep deprivation during lying deprivation, and this may be why milk yield was reduced during lying deprivation. Furthermore, lying deprivation had a greater overall impact on cow activity and production.

Treatment to Recovery Period Comparison

Lying time increased for both deprivations after the treatment period. However, on d 7, the last day of recovery, lying time was similar to previous research that observed a lying time of 9.5 to 12.9 h/d in freestalls (Ito et al., 2009), and 12.0 h/d on sand bedded freestalls (Cook et al., 2004). This suggests, d 7 may be more reflective of a cow's typical lying time on sand bedding and will be used to evaluate post treatment responses. Lying time was higher on d 1 after lying

deprivation, suggesting, lying deprivation strongly raises the need for lying (Metz, 1985, Munksgaard et al., 1999). The lying deprived cows lied down for longer on d 1, relative to the sleep deprived cows, indicating their need for lying may be stronger. Furthermore, it took the lying deprived cows 4 d to fully recover their lying time, whereas it only took the sleep deprived cows 1 d. This is likely due to the lying deprived cows losing more lying time during treatment, than the sleep-deprived cows. This speculation is further supported by cows who were lying deprived for 4 h/d, and lied down for longer during the post deprivation period, than cows who were deprived of 2 h/d (Cooper et al., 2007). This suggests, long lying deprivation periods result in higher lying times the subsequent days post deprivation. Overall, both sleep and lying deprivation increased lying time after deprivation, and therefore, altered a cow's time budget and behavior. Thus, if her lying time is reduced due to lying or sleep deprivation, it could lead to poor welfare.

Lying bouts within the current study did not differ for either treatment, suggesting, cows did not have to recover any lying bouts after treatment. Additionally, results within the current study were consistent with prior data, who reported a range of 8.8 to 11.0 (Kull et al., 2017), and 10.2 to 10.3 bouts/d on sand bedding (Gomez and Cook, 2010). This indicated, even though lying time and bout duration were affected by treatment, the number of times cows lied down did not differ. To compensate for the loss in lying time, cows increased their bout duration rather than altering how many times they got up and down throughout the day.

Bout duration for the lying deprived cows was increased on d 1, relative to d 7, suggesting, cows lied down for longer before getting up the day after deprivation. However, bout duration during the rest of the recovery period was consistent with other studies who observed a mean of 88 (Ito et al., 2009), and 77 min/bout in a freestall environment. This increase in bout

duration is likely driven by an increase in the motivation to lie from the lack of lying during treatment. However, since bout duration recovered after 24 h for the lying deprived cows, it is more easily recovered than overall lying time. Bout duration only had a tendency to differ on d 1 for the sleep deprived cows, suggesting, their lying time or bout duration was not as affect by sleep deprivation. Thus, bout duration for the lying deprived cows was altered more, relative to the sleep deprived cows.

Consistent with bout duration, steps followed a very similar pattern. Steps were consistent with the data presented by Kull et al. (2017), indicating, cows within the current study behaved similarly to other cows on sand bedding. However, steps did not differ for either treatment, the entire recovery period. This suggests, even though lying behaviors were altered, cows were likely taking the same number of steps/d, but spent less time standing idle, and more time lying, post deprivation. Typically, cows spend between 2.1 (Gomez and Cook, 2010) and 2.4 h/d standing idle (Cook, 2008), thus, this time was likely consumed by lying rather than standing.

While both deprivations altered behavior, lying deprivation may be the cumulative effect of both, lying and sleep deprivation. This theory was first proposed by Moberg (2000), who believed the effects of multiple stressors being applied simultaneously, is biologically worse than experiencing one stressor. For example, when cows are heat stressed, their lying time is reduced as well (Cook et al., 2007, Herbut and Angrecka, 2017). While it is recognized that other physiological processes are altered during heat stress, the combination of both heat stress and lying deprivation, could be why other productive functions are also affected (Ravagnolo et al., 2000, Dash et al., 2016). Furthermore, when rats were restrained for a period of time, then injected with endotoxin, there were worse effects biologically, than the rats who only

experienced only one of these stressors (Laugero and Moberg, 2000). This may be due to energy resources being shifted towards the stressor(s), and away from other productive functions such as growth (Moberg and Mench, 2000). Thus, the effects that occur during lying deprivation could be the cumulative effect of both lying, and sleep deprivation. This may be why worse effects on behavior are observed during lying deprivation, relative sleep deprivation.

In conclusion, both deprivations altered behavior after treatment. Thus, depriving cows of either sleep or lying long term may have worse effects than what was observed within the current study. Overall, lying deprivation had a greater impact on a cow's lying time and milk production relative to sleep deprivation alone. Therefore, it may still be better for a cow to have access to an uncomfortable stall, where she can lie, but not necessarily engage in sleep, rather than not having a stall available at all. However, there is potential for cows to be experiencing both lying and sleep deprivation when lying time is reduced. Therefore, it could be the cumulative effect of both stressors occurring simultaneously, and why there are stronger changes in behavior during lying deprivation.

Appendix

Table 1. Approachability and brush test scoring guide using a 1 through 4 scale modified from Lensink et al. (2003). Cows were deemed acceptable for the study if they were scored a 1 or 2.

Score	Approach Test	Brush Test
1	No withdrawal and cow allows physical contact	No withdrawal and cow allowed brushing of the head and neck area
2	Cow steps away after being touched	Cows slightly withdrew when physical contact was applied
3	Slight withdrawal when arm is extended & touched	Slight withdrawal when arm was extended
4	Strong withdrawal when arm is extended (does not allow physical contact)	Strong withdrawal when cow was approached

Table 2. Mean and standard error of lying time, number of lying bouts, lying bout length, and total steps taken for cows during the baseline and treatment period on mattress bedding in individual box stalls.

Variable	Baseline	Treatment	SE	*P*-value
Lying Deprivation				
Lying time, h/d	8.78	1.88	0.77	<.0001
Number of lying bouts, d	9.58	4.10	0.82	<.0001
Lying bout length, min/d	58.85	15.30	7.31	<.0001
Total steps	2422.8	3318.3	260.7	<.0001
Sleep Deprivation				
Lying time, h/d	8.63	8.37	0.66	0.71
Number of lying bouts, d	9.00	7.58	0.75	0.07
Lying bout length, min/d	61.77	72.86	7.02	0.09
Total steps	2623.3	2537.8	260.7	0.58

Table 3. Mean and standard errors of lying bouts (LB; bout/d), bout duration (BD; min/bout), and steps (number/d), are presented below for both lying and sleep deprivation during the recovery period (d 1 – 7). All comparisons are made relative to d 7 (last day of the recovery period). Means with ^{*A}superscript differed from means on d 7 ($^{A}P \leq 0.05$ and, $^{*}P > 0.05$ but $\leq$ 0.10).

Day	Lying Deprivation			Sleep Deprivation		
	LB (bout/d)	BD (min/bout)	Steps/d	LB (bout/d)	BD(min/bout)	Steps/d
1	9.7 ± 0.7	110.0 ± 6.6^{A}	1,618.8 ± 260	9.8 ± 0.7	89.9 ± 7.0*	2,010.3 ± 260
2	10.9 ± 0.7*	82.1 ± 6.6	1,618.0 ± 260	9.3 ± 0.7	85.0 ± 7.0	1,828.3 ± 260
3	10.3 ± 0.7	80.6 ± 6.6	1,686.0 ± 260	10.0 ± 0.7	79.3 ± 7.0	1,756.8 ± 260
4	9.8 ± 0.7	76.5 ± 6.6	2,012.8 ± 260	9.7 ± 0.7	72.8 ± 7.0	1,924.5 ± 260
5	10.5 ± 0.7	73.1 ± 6.6	1,788.8 ± 260	9.9 ± 0.7	72.2 ± 7.0	1,819.1 ± 260
6	10.4 ± 0.7	74.0 ± 6.6	1,805.1 ± 260	9.8 ± 0.7	76.3 ± 7.0	1,784.0 ± 260
7	9.5 ± 0.7	76.4 ± 6.6	1,728.6 ± 260	10.0 ± 0.7	68.7 ± 7.0	1,807.6 ± 260

Table 4. Mean and standard error of milk production, and milk components are reported for lying and sleep deprived cows during baseline, and the sequential days (excluding treatment day for milk production). The *P*-value listed are all compared to the baseline period. N/A represents data not collected on that day.

Variable	Milk Yield (kg)	*P*-value	Protein (%)	*P*-value	Fat (%)	*P*-value	SCC (cell/mL)	*P*-value
Lying Deprivation								
Baseline	34.9 ± 2.48		2.89 ± 0.1		2.90 ± 0.2		53,596	
Treatment	n/a		2.97 ± 0.1	0.04	3.60 ± 0.2	0.001	64,800	0.2
Day 1	31.8 ± 2.48	0.001	3.01 ± 0.1	0.004	3.60 ± 0.2	0.001	61,464	0.4
Day 2	32.8 ± 2.48	0.02	n/a		n/a		n/a	
Day 3	34.6 ± 2.48	0.75	n/a		n/a		n/a	
Day 4	36.2 ± 2.48	0.17	n/a		n/a		n/a	
Sleep Deprivation								
Baseline	35.8 ± 2.48		2.86 ± 0.1		3.06 ± 0.2		53,873	
Treatment			2.85 ± 0.1	0.6	3.55 ± 0.2	0.01	48,012	0.5
Day 1	35.3 ± 2.48	0.61	2.85 ± 0.1	0.8	3.33 ± 0.2	0.14	41,159	0.08
Day 2	34.1 ± 2.48	0.07	n/a		n/a		n/a	
Day 3	36.0 ± 2.48	0.82	n/a		n/a		n/a	
Day 4	35.2 ± 2.48	0.48	n/a		n/a		n/a	

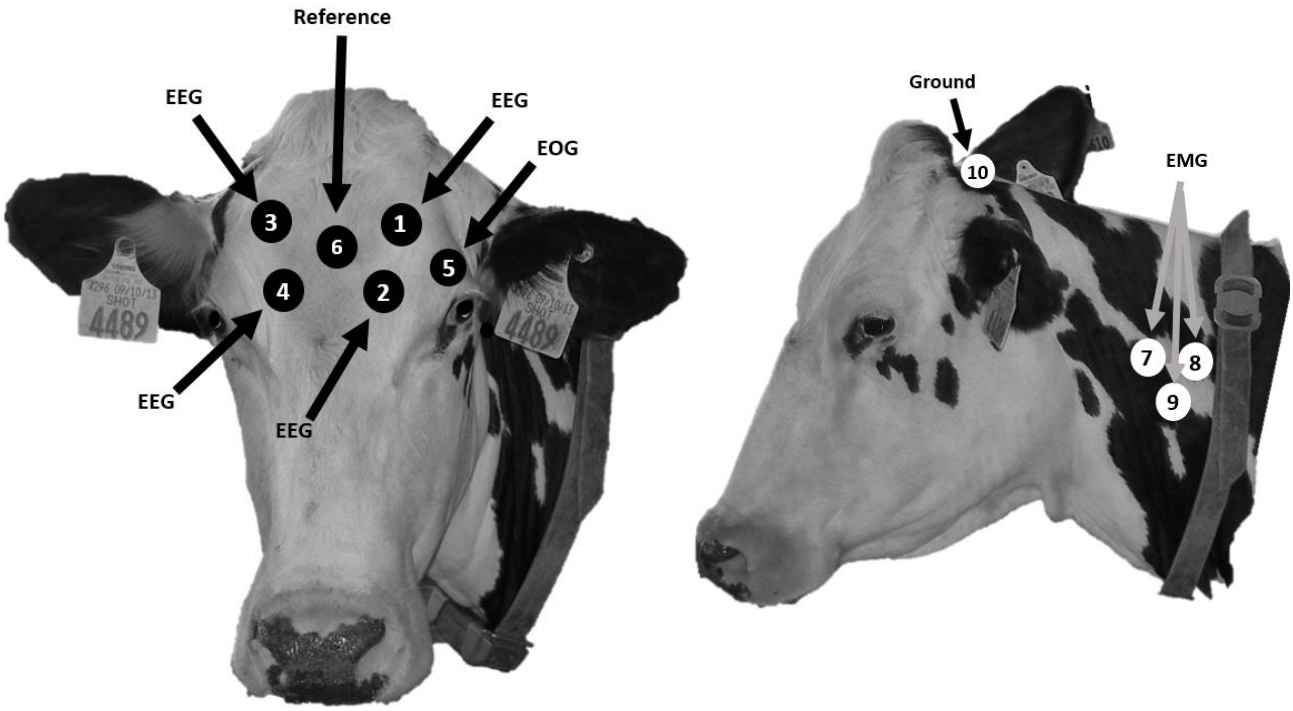

Figure 1. Placement of electrodes as outlined by Ternman et al. (2012)

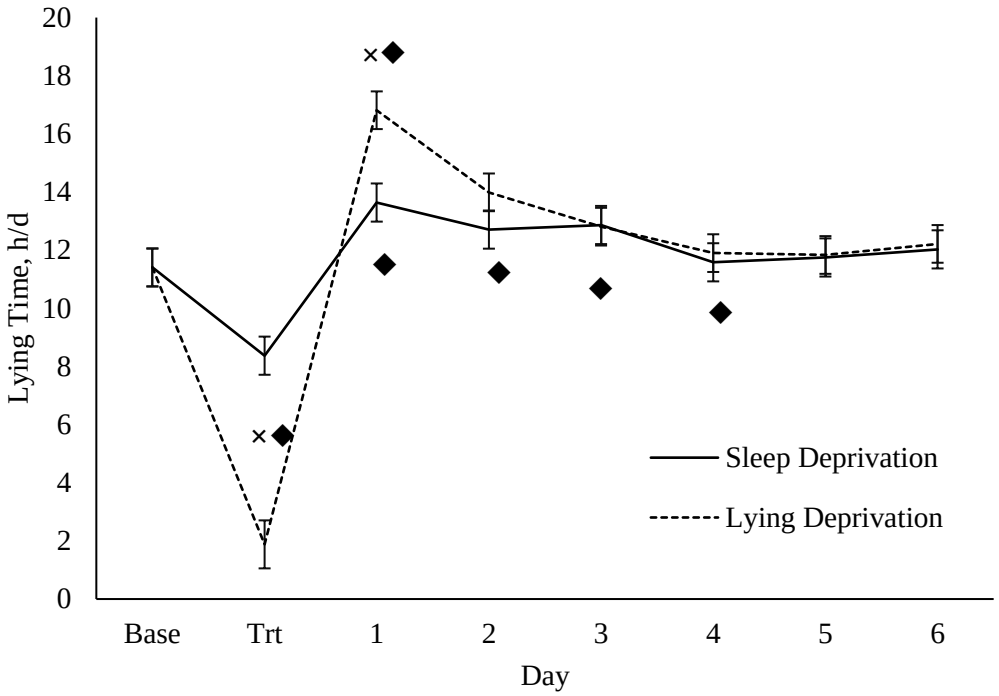

Figure 2. For lying time d 7 is used as the baseline period (Base) and d 1 through 6 illustrate the recovery period when cows were returned to their home sand bedded freestall pen. Lying time increased on d 1 for both treatments (trt) ($P \leq 0.0003$). Lying time did not return to baseline levels until d 5 for the lying deprived cows, and d 2 for the sleep deprived cows ($P \geq 0.05$). ˣ◆Values with a different superscript differ ($P < 0.05$). ˣIndicates across treatment differences, and ◆indicates within treatment differences relative to baseline.

CHAPTER TWO

Effects of Acute Lying and Sleep Deprivation on the Metabolism and Immune Response of Lactating Dairy Cows

Abstract

The objective of the study was to determine the effects of sleep and lying deprivation on metabolism and immunity of dairy cows. Data were collected from 8 multi- and 4 primiparous cows (DIM = 199 ± 44 (mean ± SD); days pregnant = 77 ± 30). Each cow was exposed to two 24 h baseline periods (d -1) followed by two 24 h treatment periods (d 0) using a crossover design: 1) sleep deprivation achieved by noise or physical contact and 2) lying deprivation imposed by a wooden grid placed on the pen floor. A 2 d acclimation period occurred before each baseline period, with a 12 d washout period between treatments. Baseline and treatment periods were imposed from 2100 to 2059 h. Cows were housed in individual boxstalls during the acclimation period, d -1 and d 0. NEFA and glucose concentrations were measured at 0300, 0900, 1500, and 2100 h on d -1 and 0. Functional activity of blood leukocytes was assessed at 2100 h on d -1 and 0. Blood samples were separated into two aliquots (5 mL each); one sample was stimulated with LPS (5 mg/mL), and one was not stimulated (Dulbecco's modified Eagle Medium added). From both samples, the expression of TNF-α, IL-1β and IL-6 mRNA generation was measured via quantitative RT-qPCR. Data were analyzed using a mixed model in SAS including fixed effects of treatment (sleep and lying deprivation), day (d -1 and 0), sampling time and their interaction with significant main effects separated using a PDIFF statement ($P \leq 0.05$). NEFA and glucose varied by time of day ($P \leq 0.03$), but were not affected by treatment or day ($P \geq 0.05$). Stimulated IL-1β and TNF-α were higher on d 0, compared to d -1 for both treatments (day: $P = 0.04$ and $P = 0.004$, respectively). When not stimulated, lying deprived cows tended to naturally produce more IL-1β on d 0, compared to sleep deprived cows (day: $P = 0.24$ and trt: $P = 0.08$). IL-6 concentration did not differ on any day ($P > 0.05$). To conclude, we found no effect of day or treatment on NEFA or glucose, suggesting shifts in energy balance did not occur when cows

are sleep or lying deprived for a short period. However, regardless of stimulation, both sleep and lying deprivation elicited an inflammatory response and may pose health and reproductive risks long term.

Introduction

Lying time is a highly prioritized behavior in dairy cows (Metz, 1985, Munksgaard and Simonsen, 1996). Cows will give up other activities such as feeding and socializing to spend more time lying (Metz, 1985). Lying is the most time consuming behavior, as cows prefer to lie for 12 to 14 h/d in a confinement system (Grant, 2000, Gomez and Cook, 2010). A reduction in lying time may indicate impaired welfare. The effects of lying deprivation on behavior in dairy cows have been evaluated (Ruckebusch, 1974, Munksgaard et al., 1999, Bach et al., 2008). One finding was that when cows were deprived of lying for 4 h/d for 1 d, they stomped their feet, repositioned themselves, and shifted their weight more, relative to the control cows. This suggests, cows were likely uncomfortable during this time, and lying time is important for the welfare of dairy cows.

Lying deprivation also can affect other aspects of a dairy cow, such as metabolism. When cows were overcrowded at the freestalls, indicating some degree of lying deprivation, NEFA was elevated (Huzzey et al., 2012). Although intake increased, NEFA was still mobilized from the tissues to support the energy demands required during overstocking. Previously, increased NEFA concentrations in transition cows also has been associated with an increased risk of disease, reduced milk yield, and fertility (Ospina et al., 2010). While glucose has not been measured during lying deprivation, it is altered during other stressful events such as hoof trimming (Trevisi et al., 2007), transportation (Tarrant et al., 1992, Early and O'riordan, 2006), and heat stress (Wheelock et al., 2010). Sleep deprivation, another stressor dairy cows may experience during

lying deprivation, also plays a role in metabolism (Spiegel et al., 1999, Broussard et al., 2015). NEFA increased during 4 d of 4.5 h of sleep deprivation in men (Broussard et al., 2015). Immediately after partial sleep deprivation, glucose had a slower rate of clearance, suggesting some degree of insulin resistance in people (Spiegel et al., 1999). Increased NEFA and glucose have both been found to alter immune function in people (Esposito et al., 2002, Lacetera et al., 2004). Therefore, both sleep and lying deprivation may impact how cows utilize energy stores and ultimately, metabolism.

While not measured in dairy cows, the impact of sleep deprivation on the immune system has been studied (Motivala and Irwin, 2007, Lange et al., 2010, Besedovsky et al., 2012). During sleep deprivation, a pro-inflammatory state can occur, which is evident by an increase in IL-1β, IL-6 and TNF-α, which are pro-inflammatory cytokines. (Everson, 2005, van Leeuwen et al., 2009, Chennaoui et al., 2011). While an increase in these inflammatory cytokines are critical to fighting off diseases, over activation of these systems can lead to chronic inflammation, autoimmune disorders, and immune system impairments (McPherson, 2001). Although these cytokines increase during endometritis in cows (Brodzki et al., 2015), and during weaning stress in calves (Kim et al., 2011), the concentrations of these cytokines during sleep or lying deprivation has yet to be determined in dairy cows. However, other than the inflammatory response, sleep deprivation has other effects. Sleep is critical to health, because when sleep is prevented, rats die within 2 to 3 weeks (Everson et al., 1989, Obermeyer et al., 1991, Rechtschaffen and Bergmann, 1995). Before death occurred, WBC concentrations are altered, energy expenditure increased, body weight was lost, and lesions formed on the tail and paws (Everson et al., 1989, Everson and Crowley, 2004, Everson, 2005). This suggests the host defense system was compromised and sleep maintains vital bodily functions. Although the

effects of sleep deprivation have not been evaluated in dairy cows, implications can be made that similar effects may occur across species.

While the effects of lying deprivation have been studied in dairy cows, the effects of sleep deprivation are unknown. However, implications can be made using the data from human and rodent models. Furthermore, it is not known if the effects observed during lying deprivation are solely due to lying deprivation, or the cumulative effect of lying and sleep deprivation. Thus, the objective of this study was to separate these two deprivations, and determine the effect of sleep and/or lying deprivation on metabolism and immunity.

Materials and Methods

Animals, Housing and Management

This study was conducted at the University of Tennessee's Little River Animal and Environmental Unit (Walland, TN) during April and May 2016. Mid to late-lactation Holstein dairy cows (n = 12) were enrolled based on DIM (DIM = 199 ± 44), and days pregnant (77 ± 30 d). Cows were milked twice daily starting at 0700 and 1730 h in a double-8 herringbone milking parlor (BouMatic, Madison, WI). Cows are normally housed in deep-bedded sand freestall pens. During the 4-d observation period, cows were housed individually in a 4.11 × 3.32 m pen with a mattress. Visual and olfactory contact was possible for enrolled cows throughout the duration of the treatment phase. Individually housing in this manner facilitated the use of electrophysiological equipment to assess vigilant state and lying deprivation. Pens were hosed and cleaned with chlorhexidine solution (Durvet Inc., Blue Springs, MO) every morning at 0700 h when cows were in a double-8 herringbone milking parlor (BouMatic, Madison, WI). Fecal matter was removed manually throughout the day to maintain pen and cow hygiene. Fresh water and a TMR were available ad libitum. The TMR was comprised of 60% corn silage, 25%

pelleted premix grain concentrate, 12% small grain silage, and 3% dry hay. All procedures described were approved by the University of Tennessee Institutional Animal Care and Use Committee.

Enrollment Criteria

From the cows meeting the selection criteria for DIM and pregnancy, a final group of 12 cows were selected using white blood cell count (WBC ≤ 12.6), and temperament. Blood samples from the target population of cows, were taken via the coccygeal vein and WBCs were analyzed to ensure cows were below the accepted threshold of 12.6 cell/mL as described by Schalm (1961), indicating the cows were not experiencing any prior illness. Thus, cows enrolled in the study were considered healthy. Temperament was evaluated using an approachability and brush test. For the approachability test, a researcher slowly approached the cow with one arm extended, and observed the cow's reaction (Lensink et al., 2003). Cows were scored based on the 1 to 4 scale described by Lensink et al. (2003), with 1 being defined as the cow allowing physical contact, and 4, the cow strongly withdrew from the researcher (Table 5). If the cow remained still and allowed physical contact or approached the researcher (a score of 1 or 2), the cow was considered suitable for the study. The brush test used was slightly modified from the brush test described by Ternman et al. (2014), where cows were restrained in pen headlocks, instead of free roaming. Cows were scored based on a 1 to 4 scale, similar to the scale defined by Lensink et al. (2003) (Table 5). For this test, the cow's head and neck area were brushed, particularly where the EEG equipment would be placed (Lensink et al., 2003, Ternman et al., 2014). If the cow did not pull away, or only slightly withdrew when brushing occurred (a score of 1 or 2), she was considered an acceptable candidate for the study. In total, 14 cows met the criteria for the

temperament tests, however, 2 cows did not enter the study because their WBC count exceeded the accepted 12.6 cell/mL threshold (Schalm, 1961).

Treatments

Treatments were implemented using a crossover design with rolling enrollment. The study design progressed from a habituation (-3 d, -2 d), baseline (-1 d), treatment (0 d) and recovery (1 – 7 d) period, with a 12-d washout period between treatments. Because cows were moved to an unfamiliar pen, a 2-d habituation period was provided to allow cows to adapt to their new environment. When cow were regrouped into a novel pen (von Keyserlingk et al., 2008), or trained to use a robotic milking system (Jacobs and Siegford, 2012), it only took the cows 2 d to habituate, suggesting, our 2-d habituation period was sufficient. Additionally, the mattress bedded pens the cows were placed in were only 8 m away from their home pen, so visual, and olfactory contact were maintained. During the study, cows experienced two treatments; a 24-h lying deprivation period, and a 24-h sleep deprivation period that both started at 2100 h. After the cow's first treatment, they returned to their home deep-bedded sand freestall pen for a 12-d washout period before returning to an individual pen for their second treatment (whichever treatment they did not experience first). Visual observations were recorded every 30 minutes for each cow over the 48-h baseline and treatment period, starting at 2100 h. The 7 d immediately after treatment were defined as the recovery period.

Lying Deprivation. The 24-h lying deprivation period was implemented using a wooden grid placed on the pen floor, preventing cows from assuming a recumbent position. The wooden grid was based on a design by Schütz et al. (2008) which prevented cows from lying during times of heat stress. If cows attempted to lie during treatment, a researcher would encourage her to stand up. If the researcher was unsuccessful, the cow would be returned to her home pen, and

removed from the study. Ultimately, no cows had to be removed, and only 2 cows attempted to lie down during treatment.

Sleep Deprivation. During the 24-h sleep deprivation period, cows were allowed to lie down, but were continuously monitored to ensure cows remained awake and alert. If a cow's posture suggested the onset of sleep, the cow would be touched to keep her awake as described by Ledoux et al. (1996) who used this method in cats. Gentle handling or touching was used because it implemented deprivation, but likely did not induce a stress response that would be caused by the method of deprivation (Graves et al., 2003).

Electrophysiological equipment

During the baseline period, cows were fitted with the electrophysiological equipment that collected electroencephalographic (EEG), electrooculography (EOG), and electromyography (EMG) data (EEG; BioRadio, Great Lakes Neurotechnologies, Cleveland, OH). Cows were restrained in the headlock of the experimental pen for placement of monitoring devices. Hair at the location of each electrode was shaved using 40 blade clippers (Andis, Sturtevant, WI) and wiped clean with alcohol to ensure sufficient contact. Non-invasive electrodes were plugged into the EEG device and then placed on the cow by creating a pocket with Durapore Surgical Tape (3m Healthcare, St. Paul, MN) and adhesive glue (Gorilla Glue Inc., Cincinnati, OH) to secure the electrodes in place. Ten20 EEG conductive paste (Weaver and Company, Aurora, CO) was placed on both sides of the electrodes to help conduct the signal. In total, there were ten electrodes on the cow. Electrode configuration was placed on the head and neck area and can be further illustrated in Figure 3 (Ternman et al., 2012). During the entire 48h EEG recordings from the baseline and treatment periods, a researcher was present to monitor the cow and ensure the EEG device, and the electrodes remained in place. This data is being analyzed in conjunction

with other collaborators and due to time constraints, the EEG data will not be prepared in time to be presented in this thesis.

Physiological Measures

Blood sampling. Blood samples were taken during the baseline and treatment period for each treatment, every 6 hours (± 1 h) once baseline started at 2100 h (8 samples total; 4 during baseline, 4 during treatment). By locating the ventral midline of the tail, blood was collected via the coccygeal vein using a sterile needle (1-1.5" x 16G) while cows were restrained in a headlock. Blood was centrifuged at a speed of 3000 x *g* at 4 °C for 10 minutes; serum was harvested, and stored in -80 °F freezer for later analysis Alhussien et al. (2015).

WBC differential. Estimation of WBC differential was used using the Wright-Giemsa method with the Fisher HealthCare Protocol HEMA 3 Fixative and solutions kit following manufacturer's instructions (ThermoFisher Scientific, Waltham, MA). Microscope slides (ThermoFisher Scientific, Waltham, MA) were read following the procedure by Levkut et al. (2002).

Assess cytokine production by whole blood leukocytes via real-time quantitative polymerase chain reaction (RT-qPCR). To measure the functional activity of peripheral blood leukocytes, whole blood from the 2100 h sampling time for d 0 (end of baseline/start of treatment) and 1 (24 h after treatment started), was incubated with Dulbecco's modified Eagle Medium added or LPS (final concentration: 5 µg/µl; *Escherichia coli 0111:B4* lipopolysaccharide, Sigma L4391*)* for 3.5 h at 37C (Røntved et al. (2005). Changes of TNF-α, IL-1 and IL-6 mRNA expression was measured via RT-qPCR. RNA was initially isolated and stabilized using a LeukoLOCK kit (Thermo Fisher Scientific, Pittsburgh, PA). RNA was purified following manufacturer's instructions (Thermo Fisher Scientific, Pittsburgh, PA). Later, RNA

quality and quantity was assessed using an Experion– capillary electrophoresis station (Bio-Rad, Hercules, CA) with Experion StdSens RNA chips and reagents (Bio-Rad, 7007154). Total RNA (1.0 µg) was heat denatured at 70°C for 2 minutes prior to reverse transcriptase. RNA was reverse transcribed into cDNA using GoScript reverse transcriptase (Promega, a5003) following the manufacturer's instructions with RNasin ribonuclease inhibitor. The adapters used to prime the reverse transcription were oligo-dT (15-mers) and random hexamers at a final concentration of 12.5ng/µl each. RNasin was used at a concentration of 1u/µl and the enzyme used had a final concentration of 8u/µl. Samples were incubated in the thermocycler (iCycler, Bio-Rad) at 20ºC for 5 min, 42ºC for 1 hr, 85ºC for 5 mins and then held at 4ºC. Lastly, real-time quantitative PCR was performed on a QuantStudio6 (Applied Biosystems, Foster City, CA) using Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The PCR for each gene was run in triplicate in a 384 well plate and included 2 µl of cDNA. It also included, 100 nM of each specific forward and reverse primers (Primer sequences are located in Table 6) and 1x Sybr Green master mix in a final volume of 5 µl. Conditions of the PCR reaction included an initial 2 min at 50°C, then 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. A melt curve was run to assess specificity of reaction. Cytokine gene expression was relative to the expression of 2 reference genes that were selected from a pool of nine genes that were most consistent with the sample type (*YWHAZ* and *S24*). An inter-run calibrator was created by pooling an equal volume of all samples. Reference genes and an inter-run calibrator were included on all plates. The primers were designed using Primer3 (Untergasser et al., 2012) and ordered from IDT (Coralville, IA; Table 6). The mean value for each triplicate sample was normalized to the geometric mean of the reference genes as outlined previously by Vandesompele et al. (2002) using the formula ΔCq=Cq target – Cq reference (Livak and

Schmittgen, 2001). ΔCq values within a plate also were normalized to the ΔCq values of the inter-run calibrator to remove technical variability between plates, resulting in ΔΔCq. By using the formula $X=2^{(-\Delta\Delta Cq)}$ were linearized into a value representing expression of the target gene relative to the two reference genes (Livak and Schmittgen, 2001)

Metabolic factors. Non-esterified fatty acids (NEFA) in sera were analyzed using the NEFA Wako commercial kit (Wako Chemicals, Richmond, VA) following manufacturer's instructions except for modification of the volume of reagents A and B to 130 µl and 65 µl, respectively. Similarly, glucose samples were run in duplicates using the Glucose Hexokinase Reagent Kit (Thermo Electron Corp., Waltham, MA) following the manufacturer's instructions. The intra- and inter-assay CV were, respectively, 1.61% and 1.99% for NEFA and 4.05% and 4.13% for glucose.

Statistical Analysis

All data were analyzed in SAS 9.4 (SAS Institute, Inc., Carry, NC) using the cow as the experimental unit. Data were analyzed using a mixed model ANOVA with significance declared a $P \leq 0.05$ and a trend declared at $P = 0.05 – 0.1$. A crossover design was used with split-split plot treatments and repeated measures. The base model included the fixed effects of day, treatment, period (e.g. treatment was received first or second). The random effect was cow within treatment. For the cytokine data, the model also included stimulation, e.g., the unstimulated Dulbecco's modified Eagle Medium added versus stimulated with LPS. For the metabolite data, time of sampling was also included in the model. When significant interactions occurred, treatment means within a day were separated using the PDIFF option of the LSMEANS statement of SAS. For data that was not normally distributed, a log transformation

was used to normalize all data and data were reported as back transformed means (milk components and cytokine data).

Results

Metabolism

NEFA and Glucose. NEFA and glucose concentrations did not differ for period, day, treatment or any interactions ($P > 0.05$). However, both NEFA and glucose differed depending on the time of collection ($P \leq 0.03$; Figure 4 and 5, respectively).

Immune System

WBC Differential. Lymphocytes differed depending on the time of sampling (P = 0.03). The percentage of lymphocytes were higher at 0300 (54.2 ± 2.0%) relative to 0900, 1500, or 2100 (50.3, 51.4, 50.3 ± 1.9%, respectively; P ≤ 0.05). However, neutrophils, monocytes and eosinophils did not differ the entire experimental period. Mean percentage is as followed for lymphocytes (52.5 ± 9.2%), neutrophils (38.2 ± 8.8%), monocytes (5.2 ±3.5%), and eosinophils (3.6 ± 3.2%).

Inflammatory Cytokines. The unstimulated sample (only Dulbecco's modified Eagle Medium added) and the stimulated sample (LPS added) always differed regardless of the cytokine evaluated ($P < 0.05$). This suggests that stimulation with LPS was capable of inducing cytokine generation.

Overall, TNF-α was greater during the treatment period (d0) versus the baseline period (d-1) (Baseline: 1.96 ± 0.21 versus Treatment: 2.44 ± 0.27; $P = 0.004$). TNF-α had a period effect ($P = 0.0002$), but no other difference for were observed ($P > 0.05$; Figure 6). When only the non-stimulated samples were analyzed, TNF-α had a tendency to increase during treatment, relative to the baseline period (P = 0.07; Figure 7). Similar to TNF-α, a period effect was evident

for IL-1β ($P = 0.002$). A day effect was evident where cows produced more IL-1β during the treatment period than during baseline (Baseline: 0.61 ± 0.07 versus Treatment: 0.77 ± 0.09; $P = 0.04$; Figure 8). A tendency for a treatment × day interaction also occurred ($P = 0.06$). Cows in during lying deprivation produced more IL-1β than during the baseline period ($P = 0.006$; Figure 8). However, IL-1β did not differ between the baseline periods or during sleep deprivation ($P \geq 0.05$), which may explain why there is only a tendency. There was a tendency for treatments to differ where cows during lying deprivation spontaneously produced more IL-1β (0.32 ± 1.9), relative to cows during sleep deprivation (0.21 ± 1.9; $P = 0.08$; Figure 8). Lastly, IL-6 concentration did not differ at any time ($P > 0.05$). However, a period effect did occur ($P = 0.002$; Figure 9). When assessing only the non-stimulated samples, IL-6 had a tendency for a treatment × day interaction where the lying deprived cows spontaneously produced more (0.76 ± 1.4) IL-6, relative to the baseline period (0.43 ± 1.4; $P = 0.08$;).

Discussion

The effects of lying and sleep deprivation on the metabolism and immune response of dairy cows was evaluated. Measuring various physiological parameters is critical to understanding the effects of lying and sleep deprivation related to the biological functioning of dairy cows. The present results suggest, lying and sleep deprivation modified the immune response, however, lying deprivation produced a stronger reaction. However, this may be due to other things occurring during lying deprivation such as poor circulation or increased energy expenditure during that time. Furthermore, NEFA and glucose were not altered other than over sampling times, which followed the typical diurnal pattern of those metabolites (Nielsen et al., 2003, Rottman et al., 2014). While lying deprivation had a greater impact on measured

parameters than sleep deprivation, there may be a cumulative effect of both lying and sleep deprivation during lying deprivation.

Metabolite concentrations can be used to assess the health of dairy cows (Adewuyi et al., 2005, González et al., 2011). Within the current study, NEFA only differed with time of sampling. However, this is not surprising as NEFA has a diurnal pattern (Thomson et al., 2003) and can change based on feeding frequency (Sutton et al., 1988), sampling time, and housing type (Kolver and MacMillan, 1993, Blum et al., 2000). Consistent with other studies, NEFA peaked at 0900, and decreased around 0300 and 1500 for dairy cows (Nielsen et al., 2003, Thomson et al., 2003). NEFA concentrations were ≤ 0.2 mM but greater than 0, which is what Hammon et al. (2006), and Drackley (2000), reported as a positive energy balance. Collectively, NEFA followed a normal diurnal pattern and was below the accepted threshold suggesting this short term treatment had no effect on NEFA concentrations. Glucose, another metabolite correlated with dairy cow health, also changed with sampling time and followed the diurnal pattern reported by Rottman et al. (2014). Oddly though, glucose concentrations for the current study appeared to be elevated during the baseline and treatment period. Results were above the normal 55 to 70 mg/dl range previously reported for lactating dairy cows (Ametaj et al., 2009, Rottman et al., 2014). However, glucose can increase during times of stress, such as hoof trimming (Trevisi et al., 2007), transportation (Tarrant et al., 1992, Early and O'riordan, 2006), heat stress (Wheelock et al., 2010), and sleep deprivation (Donga et al., 2010). Glucose concentrations were similar to those of cows that just experienced abdominal surgery, who had a mean glucose concentration of 107.6 ± 32.4 mg/dl 2 h after surgery (Mudroň et al., 2005). In our study cows were potentially stressed during the baseline period because of the attached electrophysiological equipment which required frequent attention and the blood sampling that

occurred every 6 hr. However, since NEFA has been reported to increase during times of stress due to alterations in other circulating hormones (Collier et al., 1982, Andrews and Walker, 1999, Drackley, 2000) and it was not increased, it cannot be concluded that glucose was elevated solely due to stress during the baseline period.

Various immune cells, such as WBC are altered during sleep deprivation (Irwin et al., 1996, Everson, 2005). During 22 d of total sleep deprivation in rats, neutrophil and monocyte concentration drastically increased, suggesting leukocytosis and inflammation (Everson, 2005). Furthermore, when men were only allowed to sleep 4 h a night for 3 nights, neutrophil concentrations increased as well as an overall increase in total WBC (Boudjeltia et al., 2008). These differences did not occur until halfway through the deprivation period (Everson, 2005) or after the 3rd night of sleep restriction (Boudjeltia et al., 2008). This suggests, it may take longer than 24 h for WBC populations to be altered, and may be why for the current study, there were no differences among WBC populations. However, lymphocytes were consistently higher during the 0300 sample relative to the other sampling times. This is consistent with results from Fox and Laird (1970) and Melillo (2007) who reported lymphocytes being highest during the early morning hours, and lowest during the evening in rabbits. Furthermore, concentration of all WBC populations within the current study were similar to the results reported by Tvedten and Korcal (1996) for bovine. Thus, the difference in lymphocytes across time is likely normal, and not biologically relevant.

Pro-inflammatory cytokines can increase during sleep deprivation in various species, and alter immune function (Altemus et al., 2001, Shearer et al., 2001a, Chennaoui et al., 2015). Regardless of deprivation type, both lying and sleep deprivation produced greater IL-1β and TNF-α following LPS stimulation, relative to the baseline period, suggesting a pro-

inflammatory state. However, the lying deprived cows spontaneously produced more IL-1β than the sleep deprived cows. These cytokines may be more exaggerated during lying deprivation because of the cumulative effects of lying and sleep deprivation during that time. This theory is supported by Cooper et al. (2007), and Laugero and Moberg (2000), who reported worse effects when multiple stressors were applied simultaneously. For example, when rats were restrained 4 h/d for 7 d, then injected with LPS, the combined effects of both stressors had greater effects on health and productivity, than either stressor alone (Laugero and Moberg, 2000). This is likely due to the increase in basal heat production, which partitions more energy into heat as opposed to growth or other productive functions. Specifically, sleep deprivation may exacerbate symptoms when the health of an animal is already compromised, further suggesting the detrimental effects of multiple stressors (Everson, 1993).

However, other factors may be occurring during lying deprivation that may contribute to this stronger response. Tomei et al. (1999) reported professions that require more than 50% of the shift standing, had greater incidences of chronic venous disorders, suggesting increased swelling in the feet and legs. Furthermore, increased standing in humans increases energy expenditure (Buckley et al., 2013). This may indicate that when cows are lying deprived, decreased circulation or swelling in the feet and legs may occur, as well as increased energy expenditure that occurs during standing. Thus, sleep deprivation may not be the only negative impact of lying deprivation. Moreover, since a whole blood assay was used in the current study, metabolites such as hormones were still in the blood when assessing cytokine production. Therefore, hormones such as cortisol or ACTH may have altered cytokine generation (Smits et al., 1998). Although cortisol was not measured, Smits et al. (1998) who evaluated the effects of cortisol on inflammatory cytokines through a whole blood assay, found a decrease in these

cytokines. This suggests, hormones can play a role in cytokine production and whole blood assays accurately reflect real life scenarios because all components are included.

During sleep deprivation, IL-1β would be expected to stimulate innate immunity, and humoral responses, which could impair immune function if gone uncontrolled (Everson, 2005). Other studies reported an increase in IL-1β, and TNF-α after 40 h (Moldofsky et al., 1989), 36 h (Hu et al., 2003), and 1 night of sleep deprivation (Born et al., 1997). Although IL-6 concentrations did not change, this is similar to Ruiz et al. (2012) who reported no changes in IL-6 concentrations after 2 nights of total sleep deprivation, or 4 nights of REM deprivation. Furthermore, similar to our study design, Frey et al. (2007) did not observe an increase in IL-6 after 1 night of sleep deprivation. However, results have been contradictory, as IL-6 increased after 1 night of partial sleep deprivation (from 0300 to 0700) (Irwin et al., 1996), and 4 nights of total sleep deprivation (Rosa Neto et al., 2010). However, IL-6 only had a tendency to differ between baseline and lying deprivation, suggesting the deprivation period may not have been long enough to increase IL-6. Collectively, an increase in these proinflammatory cytokines can lead to chronic inflammation (Hu et al., 2003) and cardiovascular diseases (Yndestad et al., 2007). Furthermore, chronic inflammation can cause autoimmune diseases and impair the host defense (Everson, 1993, Deon et al., 2001), leaving dairy cows more susceptible to disease. More relative to dairy cows, within the current study, it is important to recognize that the results observed were based on mid to late lactation cows, who likely were not experiencing any inflammation prior to the study. However, it can be speculated that cows in a more vulnerable state, such as fresh cows, may have a stronger immune response during either deprivation since they are already experiencing some degree of inflammation (Humblet et al., 2006).

In conclusion, the effects of lying and sleep deprivation elicit some degree of an inflammatory response in dairy cows. However, within the currently study, it was necessary to select the most calm and tame cows for this project due to the frequent reattachment of the EEG equipment. If less tame cows were used, the results observed may have been more exaggerated based on the individual's temperament, and how she handles stressful events such as sleep or lying deprivation. Thus, the results observed in the current study may to some degree be less reflective of cows who are naturally more anxious or nervous. Overall, while the effects of lying deprivation were worse than solely sleep deprivation, there is likely a cumulative effect of sleep and lying deprivation during lying deprivation. This is evident by lying deprivation eliciting a stronger immune response relative to sleep deprivation. However, other factors may be occurring during lying deprivation that may contribute to this stronger response. Therefore, many benefits come from lying and if lying is prevented, there is potential for sleep to be prevented as well.

Appendix

Table 5. Approachability and brush test scoring guide using a 1 through 4 scale modified from Lensink et al. (2003). Cows were deemed acceptable for the study if they were scored a 1 or 2.

Score	Approach Test	Brush Test
1	No withdrawal and cow allows physical contact	No withdrawal and cow allowed brushing of the head and neck area
2	Cow steps away after being touched	Cows slightly withdrew when physical contact was applied
3	Slight withdrawal when arm is extended & touched	Slight withdrawal when arm was extended
4	Strong withdrawal when arm is extended (does not allow physical contact)	Strong withdrawal when cow was approached

Table 6. Reference and target genes and primer sequences used in the current study.

Target genes	Genebank ID		Primer sequence (5' – 3')	Primers originally published
IL6	NM_173923.2	For	CACCCCAGGCAGACTACTTC	13
		Rev	CCAGAAGACCAGCAGTGGTT	
IL1β	NM_174093.1	For	CAACCGTACCTGAACCCATCA	15
		Rev	GCTGGTTGTCTTCCAGCTTCA	
TNF-α		For	CGGTGGTGGGACTCGTATG	
		Rev	GCTGGTTGTCTTCCAGCTTCA	
Reference genes				
YWHAZ	GU817014.1	For	GCATCCCACAGACTATTTCC	17
		Rev	GCAAAGACAATGACAGACCA	
RPS24	XM_005226403.2	For	TTTGCCAGCACCAACGTTG	11
		Rev	AAGGAACGCAAGAACAGAATGAA	

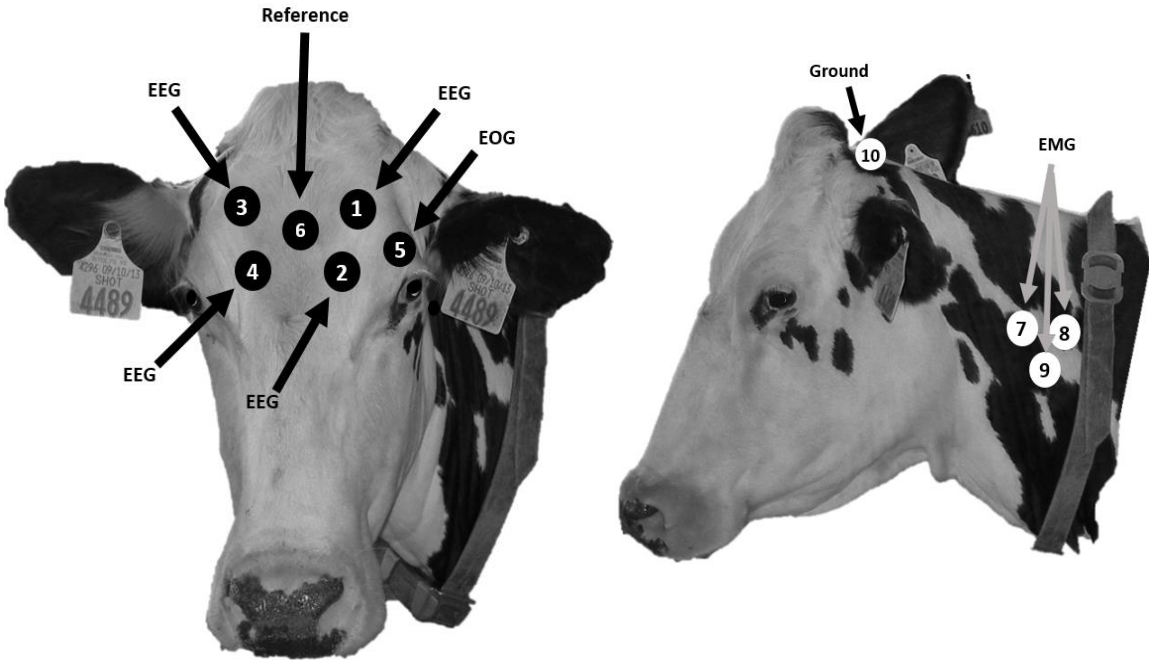

Figure 3. Placement of electrodes as outlined by Ternman et al. (2012).

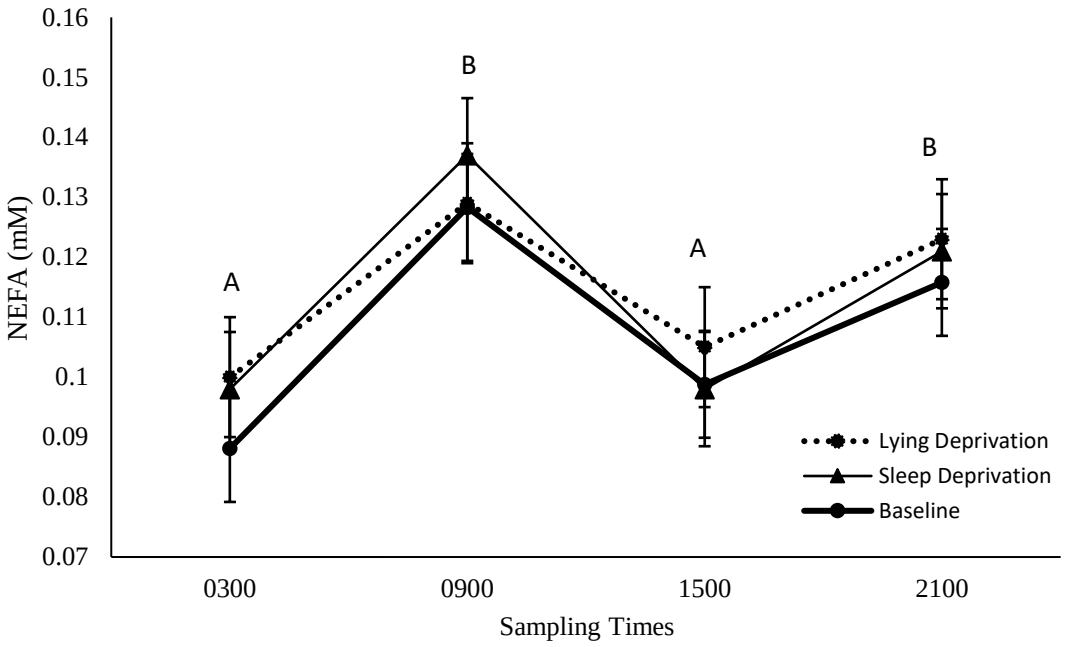

Figure 4. Means and SE are presented in mM for NEFA. [A,B]Values with a different superscript differ relative to other sampling times ($P \leq 0.05$). Specific days or treatments did not differ, so superscripts are only representative of sampling times. NEFA concentration varied depending on the time of sampling ($P \leq 0.05$).

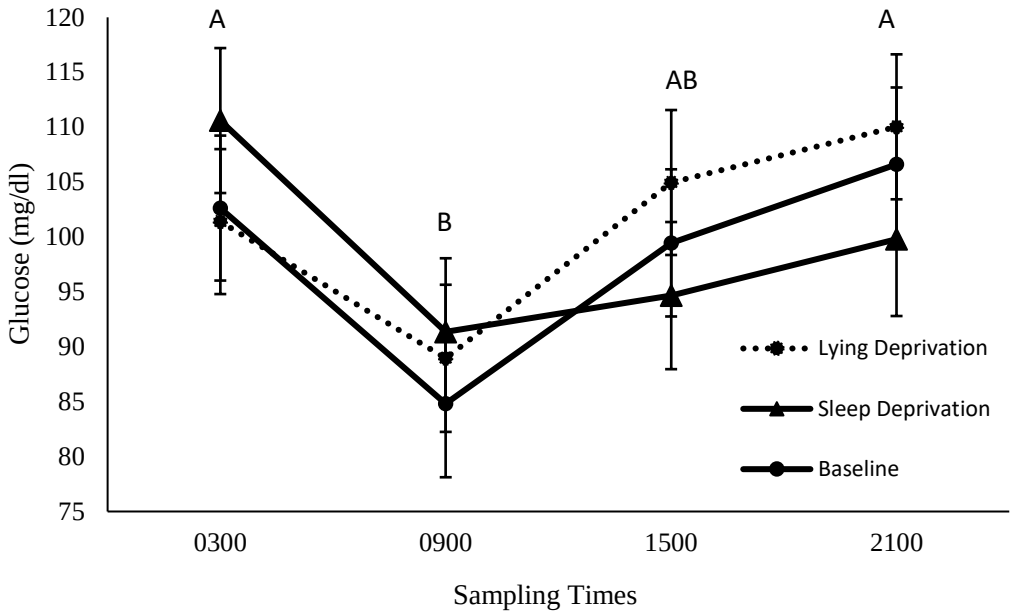

Figure 5. Means and SE are presented in mg/dl for glucose. [a,b]Values with a different superscript differ relative to other sampling times ($P \leq 0.05$). Specific days or treatments did not differ, so superscripts are only representative of sampling times. Glucose concentration varied depending on the time of sampling ($P \leq 0.05$).

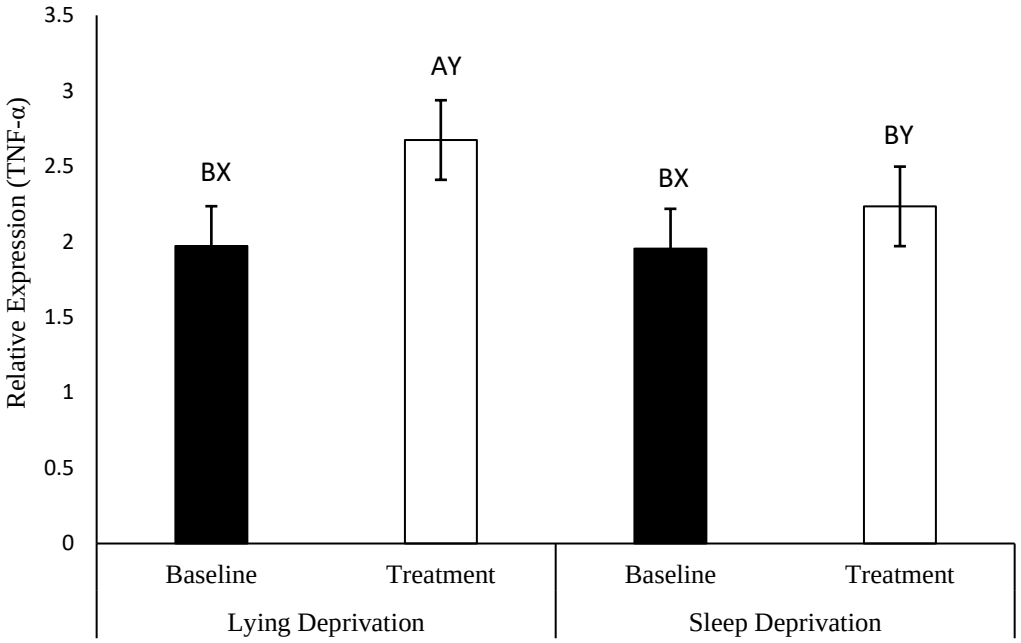

Figure 6. TNF-α generated by peripheral blood leukocytes after LPS stimulation. Means and SE are presented as relative gene expression values. ^{A,B,X,Y}Values with a different superscript, within a treatment, or across treatments differ ($P \leq 0.05$). Cows in the baseline period produced less TNF-α, than they did during lying deprivation ($P \leq 0.05$; Trt: $P = 0.52$, Day: $P = 0.004$, Trt*Day: $P = 0.23$, Period: $P \leq 0.01$).

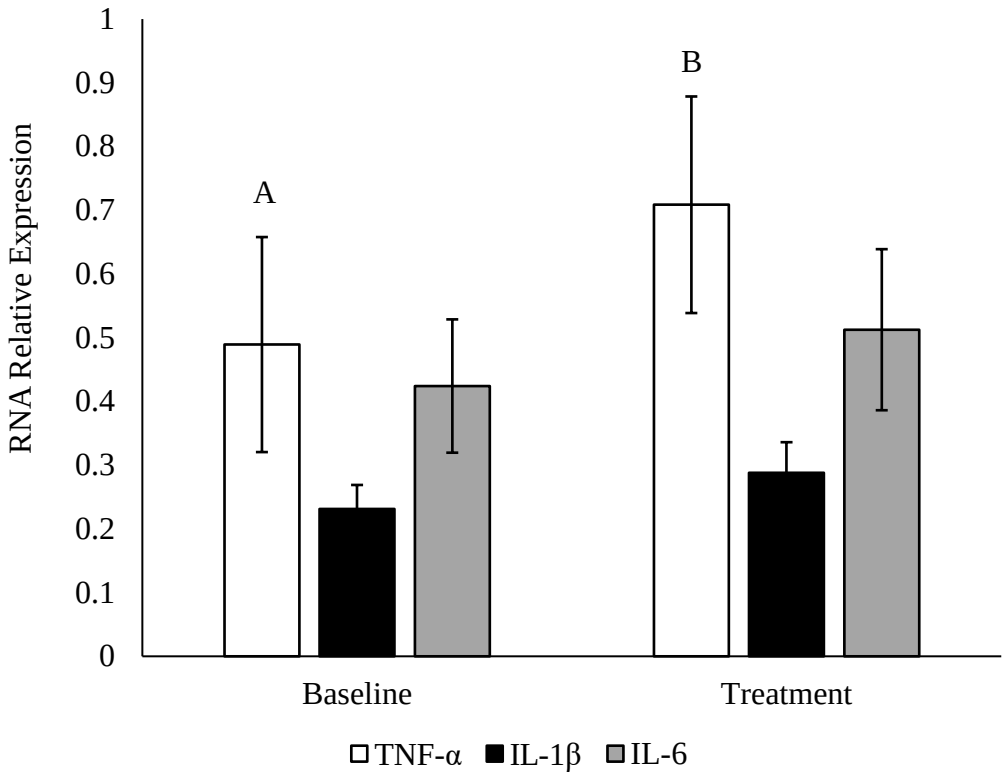

Figure 7. Cytokine generation by non-stimulated peripheral blood leuckocytes. Means and SE are presented as relative gene expression values. [A,B]Values with a different superscript, within a treatment have a tendency to differ ($P < 0.10$). Cows in the baseline period produced less TNF-α, than during the treatment period ($P = 0.06$).

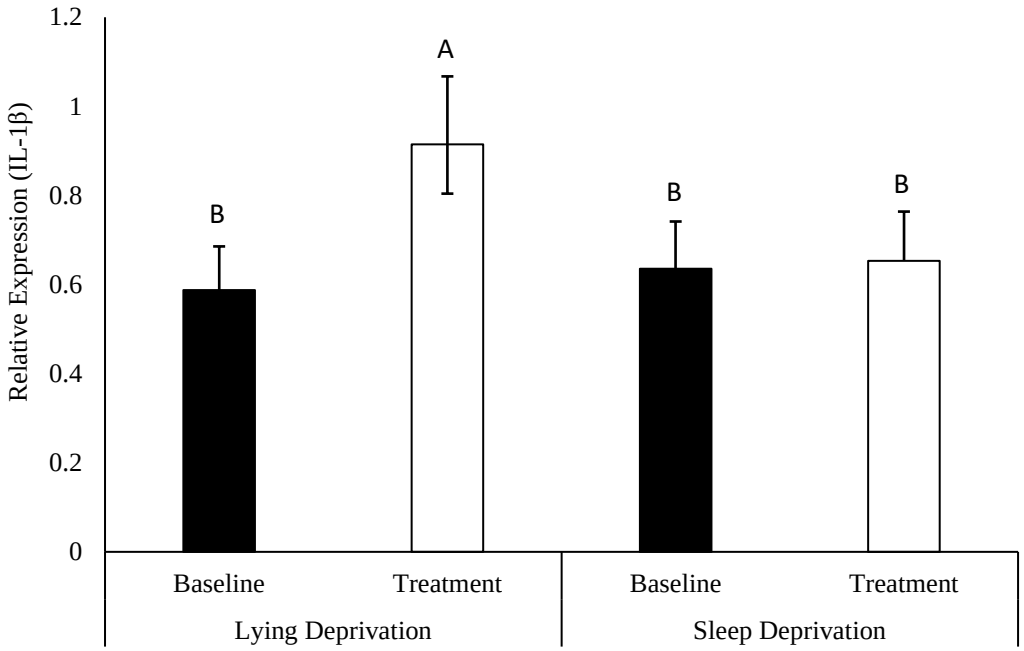

Figure 8. IL-1β generated by peripheral blood leukocytes after LPS stimulation. Means and SE are presented as relative gene expression values. [A,B]Values with a different superscript, within a treatment differ ($P \leq 0.05$). Cows in the baseline period produced less IL-1β, than they did during lying deprivation ($P \leq 0.05$; Trt: $P = 0.54$, Day: $P = 0.04$, Trt*Day: $P = 0.06$, Period: $P = 0.002$).

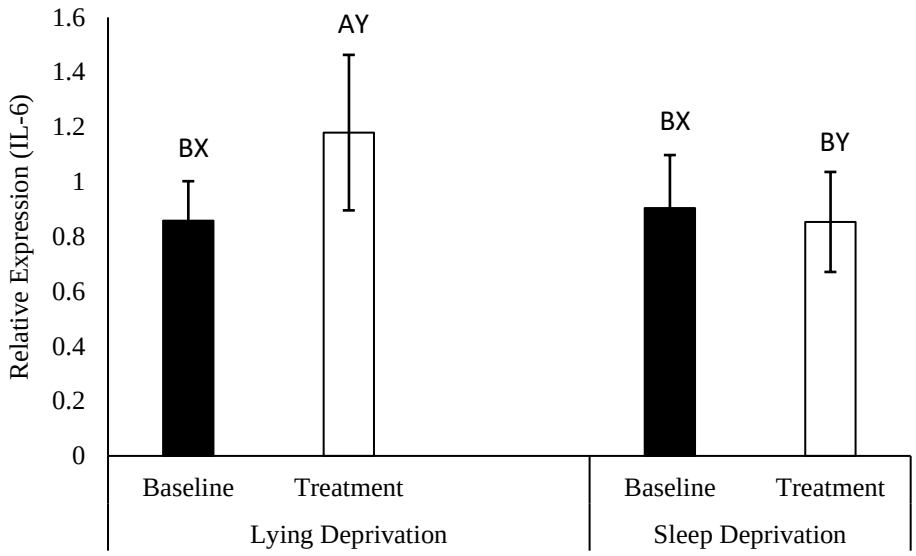

Figure 9. IL-6 generated by peripheral blood leukocytes after LPS stimulation. Means and SE are presented as relative gene expression values. ^{A,B,X,Y}Values with a different superscript, within a treatment have a tendency to differ ($P < 0.10$). IL-6 concentrations did not differ on any occasion ($P \geq 0.05$; Trt: $P = 0.99$, Day: $P = 0.18$, Trt*Day: $P = 0.18$, Period: $P = 0.002$)).

CONCLUSIONS

In conclusion, the effects of lying and sleep deprivation have detrimental effects on the behavior, production, and immune response of dairy cows. Lying deprivation produced worse outcomes relative to solely sleep deprivation, however, there is likely a cumulative effect of sleep and lying deprivation during lying deprivation. This is evident by the stronger response in immunity, production, and behavior. Therefore, it may still be better for a cow to have access to an uncomfortable stall, where she can lie, but not necessarily engage in sleep, rather than not having a stall available at all. However, cows are likely to some degree sleep deprived when they are lying deprived. Therefore, while dairy cows spend over half their day lying, they are also engaging in sleep. Thus, depriving cows of either sleep or lying long term may have worse effects than what was observed within the current study.

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