STRESS AND INFLAMMATION DURING THE PERIPARTURIENT PERIOD IN HOLSTEIN DAIRY COWS – ASSOCIATIONS WITH HEALTH AND PERFORMANCE AND THE INFLUENCE OF OVERSTOCKING

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STRESS AND INFLAMMATION DURING THE PERIPARTURIENT PERIOD IN HOLSTEIN DAIRY COWS – ASSOCIATIONS WITH HEALTH AND PERFORMANCE AND THE INFLUENCE OF OVERSTOCKING

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One of the most challenging periods for a dairy cow is during the 3-week period before and after calving. During this period cows undergo several changes in diet, social re-groupings, and dramatic metabolic and physiological adaptations to prepare for parturition and lactation; cows that cannot adapt to these challenges are at increased risk for disease and lower milk yield after calving. The objectives of this research were to: 1) evaluate how physiological parameters associated with stress (plasma cortisol and fecal cortisol metabolites), inflammation (haptoglobin; Hp), and energy metabolism (nonesterified fatty acids: NEFA) measured during the period around calving relate to health status, milk yield and reproductive performance after calving, and 2) identify specific management practices that increase prepartum stress-load and the mechanisms by which health is affected under these conditions. Increased concentrations of analytes related to stress and inflammation measured during the 3 weeks before calving were poor predictors of postpartum disease incidence when compared to increased concentrations of plasma NEFA, a measure of negative energy balance. However, increased concentrations of plasma Hp and fecal cortisol metabolites, particularly during the week after calving, were better predictors of milk yield and reproductive performance than NEFA. Despite it's widespread use in research as a measure of stress, concentrations of plasma cortisol are easily confounded by the stress associated with handling and sample collection; for use in field diagnostics to identify high

risk cattle or herds, plasma cortisol is a weak and inconsistent predictor of health and performance. In the second study, overstocking during the dry period was identified as a management practice capable of compromising physiological health. Cattle that were overstocked had greater concentrations of fecal cortisol metabolites and plasma NEFA, and altered energy metabolism as evidenced by reduced glucose clearance rates and an attenuated insulin response to a glucose challenge. Cattle that are the least successful at competing for access to the overstocked feed bunk were at the greatest risk for these metabolic disturbances. The knowledge gained from this research will be used to improve management of cattle around calving to promote health, productivity and overall animal well-being.

BIOGRAPHICAL SKETCH

Juliana (Julie) Mae Huzzey was born in Calgary, Alberta but grew up in the scenic Fraser Valley of British Columbia. Despite being a "city girl" Julie always knew that she would have a career working with animals. After high school, Julie attended the University of British Columbia (UBC) to pursue a Bachelors degree in Agroecology (2003) while completing her pre-veterinary requirements. During this program Julie developed a strong appreciation for agriculture and an interest in the field of Animal Welfare Science. Under the mentorship of her ruminant nutrition professor, Dr. Marina von Keyserlingk, who would later become her Masters supervisor Julie undertook an undergraduate research project at the UBC Dairy Education and Research Center, with the UBC Animal Welfare Program. This was Julie's first experience working with dairy cattle and her first introduction to research, however at the completion of this project she was convinced that research was *not* something she wanted to do! After a brief hiatus spent traveling in Australia, Julie returned to the UBC Dairy Education and Research Center as a research technician for the Animal Welfare Program and decided to give research a second chance. It was during this period, that Julie's interest in dairy cattle behavior and health firmly took hold and where she realized that research was going to be an important part of her future.

Julie's Masters research with Drs. Marina von Keyserlingk and Dan Weary at the UBC Animal Welfare Program focused on using feeding and social behavior as an early indicator of disease in dairy cattle around the calving period. After completing her Masters degree in the spring of 2007 she quickly transitioned to a Doctoral program that fall at Cornell University under the supervision of Dr. Tom Overton. Julie's professional goals are to obtain a faculty or research position that will allow her to develop an applied research program aimed at improving dairy cattle well-being.

Dedicated to Grandma Doris

~ Who always called me her Doctor Julie but wouldn't get to see me graduate ~

Love You!

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ART	Adrenal response test
AUC	Area under the curve
ADF	Acid detergent fiber
BCS	Body condition score
BHBA	β-hydroxybutyrate
BW	Body weight
CI	Competition index
CP	Crude protein
CR	Clearance rate
CRH	Corticotropin releasing hormone
CV	Coefficients of variation
DA	Displaced abomasum
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
DOA	Dead on arrival
FB	Feed bunk
FCORT	Fecal cortisol metabolites
FSH	Follicle stimulating hormone
GTT	Glucose tolerance test
HPA	Hypothalamic-pituitary-adrenal

HiHp	High Haptoglobin
Hp	Haptoglobin
HS	High success
LH	Luteinizing hormone
Ln	Natural logarithmic transformation
LS	Low success
MP	Multiparous
MS	Medium success
NDF	Neutral detergent fiber
NDI	No disorder of interest
NEFA	Nonesterified fatty acid
PCORT	Plasma cortisol
PP	Primiparous
RP	Retained placenta
SCK	Subclinical ketosis
TMR	Total mixed ration
305ME	Mature-equivalent 305 day milk yield
11,17-DOA	11,17-dioxoandrostane

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The Transition Cow Challenge

The transition period, which is typically defined as the period from 3 weeks before to 3 weeks after calving, is a critical period in a dairy cow's lactation cycle. Dairy cows undergo dramatic physiological changes to accommodate parturition and the commencement of lactation (Bauman and Currie, 1980; Bell, 1995). One of the main challenges for transition dairy cows is a sudden increase in nutrient requirements to support the onset of lactation at a time when dry matter intake (DMI), and thus nutrient supply, lags far behind (Drackley, 1999). Consequently, nearly all transition cows enter a period of negative energy balance, with this energy deficit being most pronounced during the first few weeks following calving (Grummer et al., 2004). Negative energy balance places transition cows at increased risk for a host of health complications including increased risk for disease (Goff, 2006), reduced milk yield (Duffield et al., 2009; Ospina et al., 2010b) and compromised reproductive performance (Butler, 2003).

In recent years scientists have made tremendous progress in understanding the nutritional management of transition cows and nutritionists have sufficient knowledge to formulate effective rations, particularly for the prepartum transition cow. Overton and Waldron (2004) provided a comprehensive review of many of these nutritional advancements. However, despite this progress, many dairy farms continue to have periodic or chronic trouble with transition cow health and performance. Approximately 75% of disease in dairy cows typically occurs within the

first month after calving (Ingvartsen, 2006; LeBlanc et al., 2006). Further, survey results collected by the USDA's National Animal Health Monitoring System that represent approximately 83 % of dairy cows in the U.S. indicated that most disorders affecting transition cows have increased in prevalence over the last 10 years (USDA 2008; Table 1.1). Regardless of whether this increase is due to a true increase in prevalence or improved disease detection, these results suggest that research must look for new ways to improve transition cow health and management.

Herd health and performance is not just a function of nutrition but also genetics, reproduction, environment, and management. Data from a recent study by Bach et al. (2008) suggests that over 50% of the observed variation in milk yield across dairy farms is not related to nutrition but rather management or environmental factors. In this study, 47 herds were fed the same ration and shared a similar genetic base yet the average milk yield per cow ranged from 20.6 to 33.8 kg/d. Age at first calving, presence or absence of feed refusals, number of free-stalls per cow, and frequency of feed push-ups were found to be among the most important management-related factors affecting milk production (Bach et al. 2008).

The ability of the dairy cow to successfully navigate the transition period by remaining healthy and productive after calving is almost certainly influenced by non-nutritional factors (Grant and Albright, 1995; Cook and Nordlund, 2004). In addition to the physiological and metabolic adaptations cows will undergo during the final stages of gestation, parturition, and then lactation, they must also adapt to a number of environmental and management changes during this time. The transition period is characterized by pen moves, social regroupings, commingling cows and heifers, and the introduction to novel environments like the milking parlor (for heifers). There may also be challenges with overstocking particularly during the dry period, exposure to heat or cold stress, or aspects of facility design that affect cow comfort

(Grant and Albright, 1995). These non-nutritional factors may represent potential stressors capable of negatively affecting behavior and physiology, and thus may contribute to increased risk for health or production related complications.

Opportunities to Improve Transition Cow Management

Presently, dairy producers lack objective tools to measure how much of an impact these potential non-nutritional management stressors have on overall health and performance. In most cases the effects (e.g., disease, reduced milk yield, or compromised reproductive performance) will not be observed until weeks or months after exposure to the stressor. The ability to identify the first signs of herd distress could lead to prompt intervention and thus benefit future herd health and productivity.

There are a multitude of hormones involved in the physiological stress response, including glucocorticoids (cortisol and corticosterone) and catecholamines (norepinephrine and epinephrine). Cortisol, commonly referred to as the "stress hormone," has been used extensively for the assessment of animals stress responses (Mormède et al., 2007); however, its use as a predictive measure of health and performance in transition dairy cattle has received limited attention. Measures of immune activation (e.g. induction of the acute phase response) during the transition period may also provide information about an animals' level of risk for developing subsequent health and production complications. There has been growing interest in using non-specific markers of inflammation or infection for the early identification of common infectious disorders after calving, including metritis (Huzzey et al., 2009) and mastitis (Hirvonen et al., 1996). The acute phase response may also be sensitive to environmental stressors (Lomborg et al., 2008) and therefore further investigation of their use in transition cow herd health programs is warranted.

By improving producers' ability to identify early on whether their animals are at increased risk for health complications or production loss, assessments can then be made on whether opportunities exist to improve overall herd performance. Understanding how environments and/or management systems like overstocking specifically moderate health and performance of transition cows will also be important for developing specific recommendations on how management programs should be modified to facilitate decreased risk for future complications.

Table 1.1. Prevalence (% of cows sampled) of disease reported by dairy herds in the United States. Data collected by the United States Department of Agriculture (USDA) National Animal Health Monitoring System in 1996, 2002 and 2007. This table was generated from data presented by USDA (2008).

	Percentage of cows					
	Dairy 1996		Dairy 2002		Dairy 2007	
Disease or Disorder	%	SE	%	SE	%	SE
Clinical Mastitis	13.4	(0.3)	14.7	(0.3)	16.5	(0.5)
Lameness	10.5	(0.3)	11.6	(0.3)	14.0	(0.4)
Retained Placenta	7.8	(0.2)	7.8	(0.2)	7.8	(0.2)
Reproductive Disease (e.g. dystocia, metritis)	N/A		3.7	(0.2)	4.6	(0.3)
Milk Fever	5.9	(0.1)	5.2	(0.1)	4.9	(0.1)
Displaced Abomasum	2.8	(0.1)	3.5	(0.1)	3.5	(0.1)
Not Pregnant at 150 DIM[1]	11.6	(0.3)	11.9	(0.3)	12.9	(0.3)
Abortions (Heifers and Cows)	3.5	(0.1)	4.0	(0.1)	4.5	(0.2)

[1] Days in Milk

STRESS AND THE TRANSITION COW

What is Stress?

To understand stress, one should first have an understanding of the concept of homeostasis. Walter Cannon (1929) provided the first definition of homeostasis, describing it as

a process of complex but coordinated physiological reactions that occur in order to maintain a state of internal consistency when changes in the surroundings produce internal disruptions of the system. Later, Hans Selye described a consistent pattern of physical and physiological responses to various "noxious agents" (Selye, 1936) and found that that these responses changed over time depending on the length of exposure to the agent. Selye determined these responses to be disruptions in the normal homeostatic mechanisms of the body, later coining the term "stress" to describe this pattern of response (Selye, 1955). Stress can be physical, psychological or physiological in nature, but often is a combination of all three of these components (von Borell, 1995). In dairy cattle, physical stressors could include heat or cold stress, psychological stressors could include social isolation or cow-calf separation, and physiological stressors could be related to nutritional imbalance and corresponding changes in energy balance.

Using the definitions described above there are clear challenges with understanding the term stress since nearly anything could be considered a stressor, particularly during the transition period. For this reason, the concept of homeorhesis as described by Bauman and Currie (1980) is important to consider when asking the question "*What is stress?"* during the transition period. Homeorhesis, as defined by Bauman and Currie (1980), is a coordination of metabolism by body tissues (i.e. partitioning of nutrients) to support the dominant physiological process (i.e. pregnancy or lactation).

For the purposes of this review and subsequent research chapters, it is important to differentiate between stress that is part of normal biological processes (such as the homeorhetic adaptations during pregnancy) and stress that threatens overall fitness (i.e. the ability to survive and reproduce). During the transition period, producers are concerned with stressors that threaten long-term survivability and productivity. Therefore, the following definitions of stress, stressors and the stress response will be used:

Stress: A state internal imbalance, during which an individuals overall fitness (ability to survive and reproduce; long-term viability) is challenged.

Stressor: The physical, physiological, or psychological event that threatens overall fitness.

Stress Response: The coordinated set of physiological or behavioral adaptations employed in order to respond to the stressor and alleviate the stress (regain internal balance).

The physiological and behavioral adaptations to stress are complex and depend on the nature of the stressor, the period of exposure to the stressor, and the individual coping strategy employed (Koolhaas et al., 1999; Mormède et al., 2007). These are important concepts to consider when evaluating the potential usefulness of measures of stress for the identification of cows at increased risk for health or production complications during the transition period.

Physiological Stress Response – Key Features

The typical stress response to an acute (sudden) stressor involves a series of endocrine changes involving the hypothamic-pitutitary-adrenal (**HPA**) axis (Sapolsky et al., 2000). Within seconds of initial exposure to a stressor there will be increased secretion of catecholamines (epinephrine and norepinephrine) via the sympathetic nervous system and release of corticotropin-releasing hormone (**CRH**) from the hypothalamus. Less than a minute later there will be increased secretion of adrenocorticotrophic hormone (**ACTH**), prolactin, and glucagon and decreased secretion of gonadotropins. After this first and rapid wave of endocrine changes, a second slower wave of change involves the steroid hormones. Within minutes glucocorticoid secretion (cortisol and corticosterone) from the adrenal gland is stimulated and following this

gonadal steroid secretion is reduced (Sapolsky et al., 2000).

Following activation of the initial stress response, the resulting biological effects will follow a timeline in accordance with the rate of secretion of hormones modifying the effect (Sapolsky et al., 2000). For example, catecholamines will quickly (within minutes) stimulate increased cardiovascular tone (e.g. increased heart rate, blood pressure, and blood flow), immune activation (cytokine secretion), energy metabolism (e.g. mobilization of energy reserves, increase nonesterified fatty acid), and decreased proceptive and receptive sexual behaviors (Sapolsky et al., 2000). These biological effects characterize the first of three stages (i.e. the *alarm* stage) of Seyles' general adaptation syndrome theory on stress (Selye, 1955). The purpose of this stage is to increase the individual's capacity for responding to the stressor; therefore biological effects during the alarm stage are characterized by an increase in energy availability and vascular tone to facilitate the fight or flight response. Further, behaviors that are not immediately necessary for survival (i.e. reproductive and foraging behaviors) are suppressed through hormonal regulation (Mormède et al., 2007). Therefore, acute stress responses are generally thought of as being adaptive and important for the maintenance of overall fitness.

The biological effects of glucocorticoids take longer to appear and have different effects depending on the length of exposure to the stressor. In the short-term, glucocorticoids facilitate the actions of catecholamines described above, helping to improve fitness by directly supporting energy mobilization (Raynaert et al., 1976) and enhancing the ability of catecholamines to exert their effects. For example, glucocorticoids prolong catecholamine actions in neuromuscular junctions by inhibiting catecholamine reuptake and decrease levels of peripheral enzymes that would inactivate catecholamines (Dailely and Westfall, 1978; Gibson, 1981). These responses describe the second stage of Selyes' general adaptation syndrome, the *resistance* stage.

During prolonged stressors, the effects of glucocorticoids eventually become suppressive

(Sapolsky et al., 2000) and this represents the final stage of Selyes' model of stress, *exhaustion*. Chronically high levels of glucocorticoids decrease overall fitness by contributing to immunosupression and muscular atrophy (Munck et al., 1984), reduced intake (Tempel and Leibowitz, 1994), and compromised reproductive performance (Johnson et al., 1992). The consequences of prolonged stress responses on overall health and performance will be outlined in more detail in the following sections.

Cortisol – Secretion and Metabolism

Cortisol and corticosterone are two important glucocorticoids produced by the adrenal cortex in response to stress; however, the relative importance of each differs by species (Mormède et al., 2007). In Holstein cattle, cortisol is the most important glucocorticoid, superceding corticosterone concentrations by 4:1 (Venkatasehu and Estergreen, 1970). Upon adrenal stimulation by ACTH, cortisol is released from adrenal cortex and moves within the circulatory system bound to corticosteroid binding globulin (**CBG**) or albumin. CBG is a specialized glycoprotein that binds cortisol with high affinity and regulates its bioavailability (Breuner and Orechinik, 2002). Under baseline conditions, approximately 80% of cortisol will be bound to CBG, 10% by albumin, and 10% of cortisol will be "free" and thus biologically active (Gayrard et al., 1996). Cortisol is lipophilic in nature and so when it is not bound to CBG or albumin, cortisol will readily cross cell membranes to exert its effects. Cortisol will interact both with glucocorticoid and mineralcorticoid receptors; however, mineralcorticoid receptors are generally protected from cortisol due to the presence of the enzyme 11β-hydroxysteroid deydrogenase (11β-HSD) that metabolizes cortisol to its inactive derivative cortisone (Stewart and Krozowski, 1999).

During the weeks before and after calving, basal plasma cortisol concentrations will

range from 2 to 5 ng/mL (Peter and Bosu, 1987; Patel et al., 1996); however, beginning approximately 2 d prior to parturition, concentrations of cortisol will increase exponentially and peak on the day of calving at a concentration 3 to 5 times that of basal levels (Patel et al., 1996). In cattle, normal daily cortisol secretion is episodic and subject to substantial individual variation with concentrations typically ranging from 0.4 to 10.0 ng/mL (Thun et al., 1981). Cortisol secretion from the adrenal gland is also characterized by a distinct circadian pattern; concentrations of cortisol are lowest during the afternoon and evening hours (1700h to 0100h) with episodic bursts not exceeding 3.5 ng/mL and highest (secretions > 8 ng/mL) at the onset of daylight and during the early morning hours (Thun et al., 1981). It has been reported that the half-life of exogenous cortisol (20 mg dose) administered to beef cows is approximately 30 min (Dunlap et al., 1981).

This variation in cortisol secretion has presented challenges in its use as a measure of stress in animals. Without repeated blood sampling, to account for variation associated with the pulsatile and diurnal patters of cortisol secretion, interpretation of plasma cortisol concentration can be a challenge. Further, restraint and handling, which are required during blood sampling, can activate the HPA axis and raise circulating cortisol concentrations quickly (Cook et al., 2000). Consequently, there has been increased interest in exploring other less-invasive methods of measuring cortisol production, namely from saliva, urine, milk, or feces.

Alternative Methods for Measuring Cortisol

Saliva

Glucocorticoids have been measured in the saliva of a variety of farm animal species including pigs (Cook et al., 1996), sheep (Fell et al., 1985), goats (Greenwood and Shutt, 1992) and cattle (Negrao et al., 2004). While saliva may be a relatively non-invasive sampling method,

concentrations of cortisol in saliva are highly correlated with the un-bound "free" cortisol fraction in blood (Aardal and Holm, 1995), entering saliva via passive diffusion (Vining et al., 1983); therefore, salivary cortisol is likely subject to the same high degree of variability and susceptibility to handling stressors as plasma cortisol, making it a challenging measure to interpret as an indicator of stress in animals.

Urine

Urine is the primary route of glucocorticoid elimination in ruminants with approximately 72% of metabolized cortisol being eliminated in this manner (Palme et al., 1996). The advantages of collecting urine for estimating cortisol production are that urine can be collected non-invasively and excreted cortisol metabolites in urine accumulate over a period of time before elimination and thus provide an integrated measure of cortisol production (Mormède et al., 2007). Urinary cortisol concentrations have been reported to demonstrate a strong linear correlation with plasma cortisol concentrations (Lindholm and Schultz-Möller, 1973). Hay et al. (2000) validated the used of urine to monitor changes in the activity of both the HPA axis and sympathetic nervous system during pregnancy in pigs. These researchers found that diurnal variations in urinary cortisol were comparable to plasma concentrations with concentrations being lowest during the evening hours and peaking in the morning just prior to feed delivery (Hay et al., 2000). For urine sampling in cattle, animals must still be restrained and made to urinate in order to collect a sample; therefore, cortisol determination from urine sampling also presents challenges.

Milk

Glucocorticoids are found in milk but concentrations are very low. Tucker and Schwalm

(1977) reported that glucocorticoid concentrations in milk range from 0.2 to 0.6 ng/mL; this represented only 4% of glucocorticoid levels that were detected in serum (7 to 13 ng/mL). These researchers also found that milk glucocorticoids decrease in concentration as lactation advances but are not affected by stage of the estrous cycle (Tucker and Schwalm, 1977). Researchers have used milk cortisol concentrations to evaluate stress associated with different types of milking systems (automated milking versus parlor milking); in one study, no differences in milk cortisol levels were detected between milking systems (Gygax et al., 2010) while in another study automated milking systems were associated with higher milk cortisol concentrations (Wenzel et al., 2003). In the latter study, while differences were observed the absolute value of milk cortisol estimates were still very low (0.4 to 0.9 ng/mL); therefore, the use of milk cortisol for the evaluation of stress responses will require highly sensitive laboratory assays able to detect low cortisol concentrations. Further, the use of this measure is limited to lactating dairy cattle and thus could not be used to assess stress responses during the dry period.

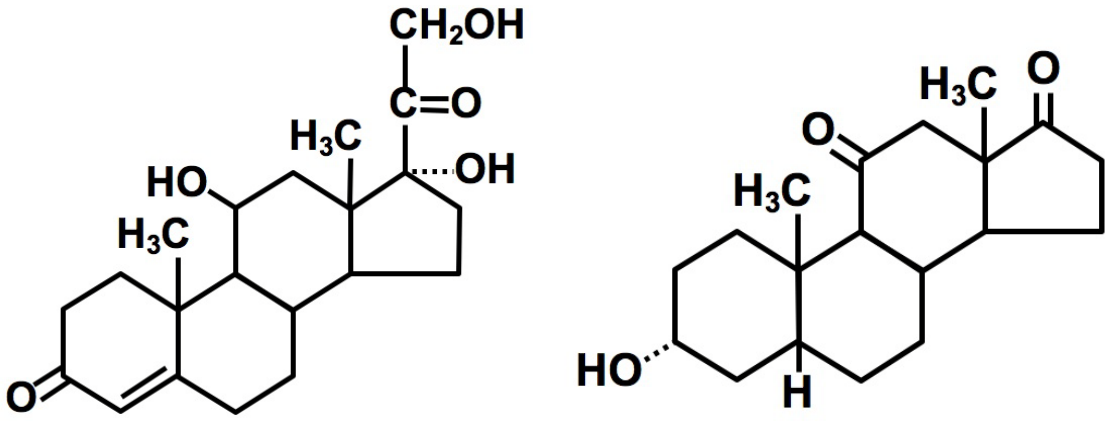
Feces

After cortisol is metabolized in the liver, its metabolites are excreted via bile into the intestine. In ruminants, approximately 28% of metabolized cortisol will be excreted via feces (Palme et al., 1996). There are at least 21 metabolites of cortisol found in the feces of cattle (Möstl et al., 2002). Palme et al. (1999) determined that 11,17-dioxoandrostane (**11,17-DOA**) is a common fecal cortisol metabolite and a good estimate of cortisol production in cattle. Figure 1.1. presents the chemical structure of cortisol and 11,17-DOA. Fecal cortisol metabolites may be an alternative to plasma cortisol as a measure of the stress response in cattle, due to the feedback-free nature of the sampling method (Palme et al., 1999). The correlation between 11,17-DOA and plasma cortisol has been validated in ruminants by ACTH and dexamethasone

tests (Palme et al., 1999); it was determined that concentration of 11,17-DOA paralleled that of cortisol in the blood with a delay time of 10 to 12 h. Although cortisol levels in feces have diurnal fluctuations, variations due to the secretory patterns of cortisol from the adrenal gland are attenuated in feces (Palme et al., 1999). The antibodies for the fecal cortisol metabolite assay described by Palme et al. (1999) have some cross-reactivity with gonadal steroids of placental origin; this can cause elevated levels of immunoreactive products in the feces of cattle particularly during the period just prior to parturition (Möstl et al., 2002); therefore, concentrations of fecal cortisol metabolites measured close to calving should be interpreted with caution.

Changes in DMI, which are common during the transition period, can alter manure output (Nennich et al., 2005); however, previous research suggests that level of intake does not meaningfully influence the concentration of steroid metabolites in the feces (Rabiee et al., 2001). Further, increased fecal cortisol metabolite concentrations in cattle have been reported in response to transport stress (Palme et al., 2000) suggesting that this measure has potential for describing natural fluctuations in cortisol secretion arising from environmental stressors.

Figure 1.1. The chemical structure of cortisol relative to its metabolite 11,17-dioxoandrostane found in feces.



Cortisol 11,17-dioxoandrostane

Effects of Stress on Animal Health and Performance

Stress and Immunity

Corticosteroids are well known for their anti-inflammatory properties. Studies have shown that when cattle or swine immune cells are exposed to physiologically relevant high concentrations of cortisol there is a decrease in the ability of lymphocytes to proliferate, cytokine production (e.g. IL-2) production drops, and neutrophil function is impaired (Westley and Kelley, 1984; Blecha and Baker, 1986; Salak et al., 1993). Other work, however, has shown that activation of the HPA axis does not negatively alter all aspects of immunity. For example, when exogenous cortisol was administered to pigs, causing plasma cortisol concentrations to double within 2 h of injection, cytotoxicity of natural killer (**NK**) cells was not affected (Salak-Johnson et al., 1996). In an earlier study it was shown that ACTH administration resulted in increased NK cell activity in pigs (McGlone et al., 1991). These results highlight the fact that stress responses moderate immunity in different ways and are not always immunosuppressive; many of these differences are likely attributed to whether the stressor is acute or chronic. During acute stress, such as an injury that challenges the integrity of the body, stress hormones are more likely to be associated with priming the immune system in a manner that prepares it for potentially invading pathogens and subsequent infection (Carroll and Forsberg, 2007). However, when the stressor is chronic the effects of stress hormones on the immune system shift and become less preparatory and more suppressive (Sapolsky et al., 2000).

Chronic stress and the corresponding sustained increase in glucocorticoids can also affect immune cell gene expression. Immune cells including lymphocytes, macrophages and granulocytes possess glucocorticoid receptors that are stimulated to a higher degree when glucocorticoid concentrations are high. When these glucocorticoid receptors are activated this can interfere with important transcription factors such as nuclear factor kappa B (NF-κB); this

transcription factor regulates cytokine production from both macrophages and T_H cells (Adcock and Caramori, 2001). Glucocorticoids can also influence overall immunity through their actions on the thymus. Within the thymus, developing thymocytes and stromal cells drive hematopoietic progenator cells to become mature T lymphocytes. High concentrations of glucocorticoids can interfere with the survival, proliferation and differentiation of thymocytes, thus altering T-cell production (Brommhardt et al., 2004).

Studies exploring environmental stressors in cattle have also demonstrated the relationship between increased glucocorticoid production and compromised immunity. In a study by Hopster et al. (1998), two groups of Holstein dairy cows were identified as either high or low plasma cortisol responders to a physiological stressor (a novel environment test). When later exposed to an endotoxin-induced mastitis challenge there was an increase in cortisol production in both groups which was also associated with a decrease in the number of circulating lymphocytes; however, this immunosupression was greatest in animals identified previously as being the greatest responders to the novel environment test (Hospter et al., 1998). Not only does this study provide supporting evidence that adrenal hormones alter lymphocyte numbers in circulation, but it also shows that other factors such as individual stress responses are also important mediators.

The immunosuppressive effects associated with chronic or persistent stress no doubt contribute to differences in disease susceptibility for stressed and non-stressed animals. For example, Peter and Bosu (1987) reported that cows that went on to develop retained placenta (**RP**) after calving had higher serum cortisol concentrations 6 d prior to parturition compared to cows without RP.

Stress and Reproduction

Stress can affect reproductive performance through a variety of pathways such as delays in the onset of puberty, behavioral alterations, failure or delay of ovulation, failure of embryo implantation, or spontaneous abortion (Johnson et al., 1992). The mechanisms underlying these dysfunctions are largely mediated through alterations in the reproductive endocrine system and interactions between the hypothalamic-pituitary-gonad (**HPG**) axis and the HPA axis. These mechanisms are complex and likely to differ depending on the type of stressor. The purpose of this review is to highlight some key examples of ways in which the physiological stress response may alter reproductive performance.

When the stress response has been activated, the corresponding rise in corticosteroids (i.e. cortisol, corticosterone, aldosterone) can directly interfere with the HPG axis. For example, during chronic stress in rats it was found that luteinizing hormone (**LH**) and follicle stimulating hormone (**FSH**) levels were decreased; this decrease, however, was not due to a loss of pituitary sensitivity to GnRH but rather lower hypothalamic gonadotropin releasing hormone (**GnRH**) concentrations (Lopez-Calderon et al., 1991). GnRH neurons have receptors for corticosteroids (Ahima and Harlan, 1992), which provide supporting evidence for a direct effect of corticosteroids on HPG axis activity at the level of the hypothalamus. However, corticosteroids have also been shown to affect the HPG axis at the level of the pituitary. For example when dexamethasone, an exogenous steroid that provides negative feedback to the pituitary to suppress the secretion of ACTH, was given to male rats there was a significant decline in GnRH-induced gonadotropin secretion from the pituitary (Rosen et al., 1988). Corticosteroids are not the only potential modifiers of the HPG axis. In adrenalectomized rhesus monkeys, injection of CRH resulted in decreased secretion of LH and FSH (Xiao et al., 1989).

Corticosteroids can also have direct effects at the level of the ovary. Pigs that were treated with ACTH during the luteal phase of the oestrus cycle had a reduction in viable

granulose cells and altered patterns of steroidogenesis; this can have down stream negative effects on subsequent follicular development (Viveiros and Liptrap, 1995). Embryo viability can also be compromised during periods of increased stress. Macedo et al. (2011) investigated the effects of stress on embryo production in superovulated Nelore (Bos indicus) cattle and found that among the cattle that had an increased physiological stress response (increased cortisol production) to human-animal interactions, the viability rate of their embryos was 19% lower than the viability rate of the embryos of less stressed cattle. However, as mentioned earlier these relationships are complex and increased cortisol production is not associated with all reproductive maladies. Spontaneous ovarian cyst disease in dairy cows is a disorder that has significant negative effects on reproductive performance. Probo et al. (2011) found no differences in plasma cortisol concentrations between cattle with and without cysts; however, this study utilized a relatively small sample size (n = 6 per group) and since cortisol secretion is known to vary greatly between individuals, this variability may have influenced their results.

INFLAMMATION AND THE TRANSITION COW

Parturition is a time associated with a high degree of inflammation (Norman et al., 2007; Bertoni et al., 2008). The process of parturition is extremely physically demanding and tissue damage and swelling, particularly around the vulva, is common during the delivery process. For these reasons the days around calving are associated with a period of immune activation and increased concentrations of plasma proteins associated with inflammation (Uchida et al., 1993). Increases in these inflammatory biomarkers at parturition occur regardless of the dairy cows' subsequent health status, although they appear to be more pronounced in cattle that go on to have serious health complications (Huzzey et al., 2009).

Over the last decade there has been growing interest in monitoring inflammatory

responses in animals for clinical or experimental purposes (Eckersall, 2000). Measures of immune activation (e.g. induction of the acute phase response) during the transition period may provide information about an animals' level of risk for developing subsequent health and production complications. Researchers are becoming increasingly interested in using markers of the acute phase response as a tool to identify disease in animals (Eckersall, 2000). The acute phase protein haptoglobin, a non-specific marker of inflammation, injury, or infection has been shown to be particularly useful for the early identification of common transition cow disorders including metritis (Huzzey et al., 2009) and mastitis (Hirvonen et al., 1996). The acute phase response may also be sensitive to environmental stressors (Lomborg et al., 2008) and therefore further investigation into their usefulness for monitoring transition cow health and performance is warranted.

The Acute Phase Response

During infection or injury, inflammatory cytokines including interleukin-1 (IL-1), IL-6 and tumor necrosis factor α (TNF-α) are released by macrophages and monocytes at the site of infection. These pro-inflammatory cytokines serve as messenger proteins to provide a variety of signals to different target tissues to respond to the infection (Parham, 2005). At the liver, these cytokines stimulate hepatocytes to increase or decrease production of key proteins associated with the immune response; this is termed the acute phase response and the liver derived proteins that change in response to immune system activation include positive acute phase proteins (those that increase in concentration) and negative acute phase proteins (those that decrease in concentration). In general the acute phase response begins a few hours after injury and subsides within 24 to 48 h (Parham, 2005). Acute phase proteins (**APP**) may include complement components, clotting factors, protease inhibitors and metal-binding proteins, all of which serve to

facilitate host defenses to an invading pathogen (Tizard, 2000).

There are a variety of APP of veterinary importance (Petersen et al., 2004); however, the relative importance of each APP differs depending on species. In cattle, haptoglobin (**Hp**) and serum amyloid A (**SAA**) are considered to be the most important positive APP of the inflammatory response because their concentrations increase markedly during the acute phase response (Skinner et al., 1991; Alsemgeest et al., 1994). C-reactive protein is a positive APP important in swine, dogs, humans, rats and rabbits (Petersen et al., 2004) but it is generally not considered an APP in cattle since it has very little or no response to the typical acute phase reaction (Nakajima et al., 1993). Fibrinogen is a positive APP that plays a key role in the formation of blood clots; however, during the acute phase response in cattle, the rise in fibrinogen is moderate compared to the rise observed in Hp or SAA (Petersen et al., 2004). Negative APP responses have received significantly less attention than positive APP responses; however, research has shown that lower paraoxonase levels may reflect inflammatory conditions in cattle during the periparturient period (Bionaz et al., 2007).

Paraoxonase

Paraoxonase is a glycoprotein produced in the liver during conditions of normal biological functioning. It is suggested to have antioxidant properties, as one of its functions is to protect low-density lipoproteins from oxidation (Mackness and Durrington, 1995). After calving paraoxonase activity is low; this may be due to liver oxidative damage caused by inflammation associated with parturition or fat accumulation in the liver due to excessive negative energy balance (Turk et al., 2005). Bademkiran et al. (2008) also reported lower paraoxonase activity after calving, but found that postpartum drops in this negative APP were not significantly different between cows with and without dystocia, an important modifier of inflammation status

at calving. Paraoxonase also tended to be influenced by calf birth weight and twinning (Bademkiran et al., 2008) which could complicate its use during the period around calving as an indicator of inflammation associated with long-term health or production complications. Research that has explored the relationships between inflammation and paraoxonase activity have used plasma concentrations of Hp (as an inflammatory reference) to validate these associations (Bionez et al., 2007); therefore, Hp is likely a more direct measure of inflammation at calving relative to paraoxonase.

Serum Amyloid A

Basal concentrations of SAA in the plasma of normal, healthy cattle are around 10 to 20 μg/dL (Heegaard et al., 2000; Ametaj et al., 2005) but can increase by factor of 1 to 10 times during infection (Petersen et al., 2004). The function of SAA is not as well described as the function of Hp, however several mechanisms by which SAA facilitate the immune response to infection or injury have been proposed (Petersen et al., 2004). SAA appears to have an inhibitory effect on fever (Shainkin-Kestenbaum et al., 1991) and also an inhibitory effect on the oxidative burst of neutrophilic granulocytes, which may help to limit oxidative tissue damage during infection (Linke et al., 1991). SAA may also facilitate chemotaxis of monocytes and polymorphonuclear leucocytes (Badolato et al., 1994). Concentrations of SAA were found to be useful for differentiating between acute and chronic inflammation (SAA is higher during acute inflammation; Horadagoda et al., 1999). SAA concentrations are also increased during fatty liver disease (Ametaj et al., 2005) and mastitis (Grönlund et al., 2005). A potential challenge for using SAA as a routine analyte in herd health monitoring programs is that laboratory testing is complicated and the availability of commercial assays for measuring SAA are limited (Eckersall, 2000).

Haptoglobin

Haptoglobin is regarded as the most sensitive APP in cattle. Conner et al. (1988) reported that Hp concentrations could increase by up to 100 fold within 2 hours of a localized inflammatory insult. In normal bovine serum Hp concentrations are very low or undetectable (Alsemgeest et al., 1994) and this is a major reason why it has been a key APP of interest in cattle. The primary function of Hp is to prevent the loss of iron through its formation of very stable complexes with free hemoglobin in the blood (Laurell and Nymann, 1957); in doing so Hp is thought to inhibit bacterial growth by limiting iron availability to pathogens (Bullen, 1981; Eaton et al., 1982). In cattle Hp has also been suggested to be an immunomodulator. In transported calves there was a significant correlation ($r = 0.57$) between Hp concentrations and suppression of the capacity of lymphocytes to proliferate (Murata and Miyamoto, 1993).

A benefit to using Hp over SAA for analyte testing programs on dairy farms is that it is a relatively easy protein to measure with laboratory assay. Assays designed to measure Hp are based on the binding of Hp to hemoglobin and then subsequently measuring the resulting change in peroxidase activity. Free hemoglobin exhibits peroxidase activity, which is inhibited at low pH. When Hp is present in the sample it combines with hemoglobin and at low pH preserves the peroxidase activity of the bound hemoglobin. Preservation of peroxidase activity of hemoglobin is directly proportional to the amount of Hp present in the specimen (Eckersall, 2000). There are also opportunities for semi-automatic or robotic methods of sampling Hp (Smith et al., 1998b) as this APP is also found in milk with concentrations elevated during mammary infection (Grönlund et al., 2005; Akerstdet et al., 2011). For these reasons and because of the highly sensitive nature of Hp in cattle to inflammatory and infectious conditions, this APP was the focus of the research conducted within this dissertation and therefore the following review will focus

on Hp.

Haptoglobin and Disease

Haptoglobin has been repeatedly shown to be associated with a variety of infectious or inflammatory disorders in cattle, including metritis, mastitis, dystocia, and RP. Increased concentrations have also been observed in response to injury and periods of excessive negative energy balance in cattle.

Reproductive Disease

Bacterial contamination of the uterus following calving in cattle is associated with increased Hp concentrations as well as delayed uterine involution (Sheldon et al., 2001). Skinner et al. (1991) reported increased Hp concentrations among cows with severe metritis and RP with average concentrations ranging from 0.76 to 1.06 g/L; these researchers, however, did not identify a significant increase in Hp in response to chronic endometritis. Hirvonen et al. (1999) reported that acute uterine infection associated with a high increase in Hp concentration was associated with a poor prognosis; in this study the animals with the most severe infections and highest Hp levels were eventually culled due to poor condition and reduced fertility. Bertoni et al. (2008) found that cows with a low liver activity index around calving had a more pronounced inflammatory status at calving as evidenced by high haptoglobin concentrations; this status was also associated with an increased frequency of calving related disorders including dystocia, milk fever, and retained placenta as well as a greater number of days open following calving, relative to cows with a high liver activity index status and low overall Hp concentration. Increased Hp concentrations have also been associated with cases of dystocia in sheep (Scott et al., 1992). While most of the above mentioned studies indicate that Hp concentrations are useful diagnostic

measures of uterine infection, Huzzey et al. (2009) reported that increased concentrations of Hp precede the onset of clinical signs of metritis by 3 d and thus Hp may also be useful as an early indicator of subsequent disease.

Mastitis

Hp concentrations > 1 g/L were reported by Skinner et al. (1991) in cows diagnosed with serious mastitis cases. Eckersall et al. (2001) compared serum and milk Hp concentration of cows identified with clinical signs of mild (clots in milk) and moderate (clots + swelling, redness, fever) mastitis to cows without these conditions. They found that the median (range) Hp concentration in serum of healthy cows was < 0.02 g/L (undetectable levels – 0.1 g/L), mild mastitis 0.47 g/L (0.02 – 1.36) and moderate mastitis 0.74 g/L (0.02 – 1.84). 12 of the 16 cows in the healthy group were found to be below the detection limit (0.2 g/L) of the Hp assay. To determine the diagnostic value of Hp these researchers used a reference level of > 0.05 g/L and found the Se and Sp for differentiating between healthy cows and cows with mild or moderate mastitis to be 82 and 94% respectively. Grönlund et al. (2005) found that quarter milk Hp concentrations were greater among animals with sub-clinical mastitis as identified by elevated SCC in the absence of clinical symptoms of infection. In contrast, Rezamand et al. (2007) did not find a relationship between Hp and cows with subclinical mastitis; however aspects of this study design and the lack of consideration for other conditions known to affect Hp status may have influenced their results.

Energy Balance

Changes in energy status and lipid metabolism during the transition period can lead to excessive NEFA infiltration of the liver and fatty liver syndrome (Bobe et al., 2004). Several

researchers have described an association between fatty liver disease and increased Hp concentrations (Uchida et al., 1993; Katoh, 2002; Ametaj et al., 2005). In one of these studies, the Hp concentration of cows with fatty liver were nearly 3 times that of cows without fatty liver and this increase lasted for a period of 2 wk after calving (Ametaj et al., 2005). Katoh (2002) suggests that this relationship is due in part to damaged liver cells. Similar to infection or trauma, fatty infiltration of the liver represents a harmful stimulus to liver parenchymal cells, and consequently these cells will increase the production of Hp (Katoh, 2002). This relationship, however, may only be present during periods of excessive negative energy balance; other research has found no association between ketosis and Hp concentrations (Skinner et al., 1991).

Injury

Hirvonen et al. (1997) examined Hp concentrations of dairy cattle that underwent emergency slaughter and found that the presence muscle trauma in the hind legs or sternal muscles, or arthritis in the stifle and tarsal joints were associated with increased Hp concentrations (range: 0.6 to 0.9 g/L) while minor injuries such as teat lesions were associated with low Hp concentrations (around 0.01 g/L). Haptoglobin has been shown to increase in response to the physical trauma associated with castration (Fisher et al., 2001). Lameness also induces an acute phase response in cattle. Kujala et al. (2010) reported that SAA concentrations were more responsive to hoof disease than concentrations of Hp; however, these results were based on a small sample of cows (15 lame and 15 control). In another study cattle 60 cattle with lameness but no other inflammatory problems such as pneumonia, mastitis, or uterine infection were compared to 10 healthy control cows (Smith et al., 2010); these research found that claw disorders (bacterial infections or non-infectious disorders of the claw) were associated with increased Hp concentrations (range: 0.3 g/L to > 1.0 g/L) while Hp concentrations of healthy

animals were consistently below the assay detection limit (Smith et al., 2010).

Haptoglobin and Environmental Stressors

While environmental stressors have been shown by several researchers to be associated with activation of the acute phase response in cattle, studies are divided on whether or not these stressors influence Hp concentrations specifically. For example, Alsemgeest et al. (1995) found that when calves were stressed by housing in a pen with very slippery flooring, SAA concentrations were significantly higher in the stressed calves compared to their non-stressed counterparts but Hp concentrations did not differ between groups. In another study, Arthington et al. (2003) reported no Hp response to transportation or commingling stress in newly weaned beef calves despite increased production of SAA. Hickey et al. (2003) studied the effects of abrupt weaning (inclusive of social group disruption and maternal separation) on the acute phase response in calves and found that while fibrinogen concentrations were greater in abruptly weaned calves, Hp was not affected by treatment. On the other hand, Lomborg et al. (2008) did report increased Hp concentrations among cattle exposed to a complex stressor. In this study cattle were sampled before and after exposure to transportation to a new facility in which they were then housed in solitary tie-stalls; the complex stressor for this study encompassed transportation stress, social isolation and a novel environment. Murata and Miyamoto (1993) reported a 5-fold increase in Hp concentration during the process of transportation and then found that there was a further 100% increase in Hp concentration on the day following transport. Differences in the response kinetics of SAA and Hp may explain some of the differences in APP responses to various environmental stressors. Hp is a slower reacting APP than SAA (Horadagoda et al. 1999; Jacobsen et al., 2004); therefore, studies should account for this lag time when assessing the effects of a potential environmental or management stressor on the acute

phase response.

The mechanisms behind stress-induced APP responses are unclear; however, they may be mediated by the actions of the HPA axis and the corresponding rise in plasma glucocorticoid concentrations. Higuchi et al. (1994) isolated parenchymal cells from the liver of male calves and then stimulated these cells with glucocorticoids. These researchers found that without dexamethasone or cortisol treatment, there was little to no Hp detected in culture media; however, cells that were treated with these glucocorticoids released considerable amounts of Hp into the medium suggesting a direct link between steroid hormones associated with the stress and liver production of Hp (Higuchi et al. 1994).

EARLY IDENTIFICATION OF HIGH RISK TRANSITION COWS

Most transition cow health disorders have a metabolic or physiological component that is associated with the onset or progression of the disorder. Metabolic changes associated with calving and the initiation of milk synthesis each contribute to increased energy demands during transition; however, cows in the transition period usually reduce their DMI by 2-4 kg per day (a 20 to 30% decrease) during the last week prior to calving (Bertrics et al., 1992). These events are important in the pathogenesis of negative energy balance during the transition period (Grummer, 1995) and contribute to other health problems including decreased body condition and body weight, increased disease incidence, and decrease reproductive performance (Butler et al., 2003; Goff, 2006). Excessive negative energy balance results in increased fat mobilization and subsequent elevations in ketone body concentrations that can be measured via concentrations of nonesterified fatty acid (**NEFA**) and beta-hydroxybutyrate (**BHBA**), respectively. Because of these relationships there has been increasing interest in using metabolite testing as a tool for monitoring transition cow health and performance. This work has focused heavily on NEFA and

BHBA testing; however, other analytes such as cortisol or Hp may also be useful in transition cow analyte testing programs.

Metabolite Testing

Several researchers (LeBlanc et al., 2005; Duffield et al., 2009; Ospina et al., 2010a; Chapinal et al., 2011) have explored testing of blood analytes related to energy balance (i.e. NEFA and BHBA) for identifying opportunities to improve dairy cow management. What differentiates these studies from others also exploring the relationship between negative energy balance and health or production outcomes, is in their identification of specific concentration thresholds of NEFA or BHBA that identify increased disease risk.

NEFA

Nonesterified fatty acids are elevated during periods of negative energy balance as a result of increased fat mobilization from adipose reserves to support energy demands (Grummer et al., 2004). Relationships between both prepartum and postpartum NEFA concentrations with postpartum health status and performance have been reported. Ospina et al. (2010a) determined that cows with circulating NEFA concentrations greater than 0.29 mEq/L during the 2-wk period before calving had twice the risk for postpartum disorders including displaced abomasum (**DA**), clinical ketosis (**CK**), metritis, and RP. Chapinal et al. (2011) found that cows with NEFA greater than 0.3 mEq/L during the week before calving had a 2-fold increase in risk for developing RP or metritis; however, these researchers found that a higher prepartum NEFA threshold (greater than 0.5 mEq/L) was required for identifying animals at increased risk for displaced abomasum. LeBlanc et al. (2004) also found that when serum NEFA concentrations were greater than 0.5 mEq/L during the week before calving, the risk of RP was increased by

80%. NEFA greater than 0.27 to 0.33 mEq/L during the prepartum period is also reported to be associated with a 19 % decreased rate of conception and lower 305-d milk yield, respectively (Ospina et al., 2010b).

Increased NEFA after calving is also associated with compromised health, milk yield, and reproductive performance. Cows with circulating NEFA concentrations greater than 0.57 mEq/L during the 2-wk period after calving had 4 times the risk for disorders including DA, CK, metritis and RP relative to cows with NEFA concentrations below this cutpoint (Ospina et al., 2010a). Chapinal et al. (2011) identified a postpartum NEFA threshold of greater than 1.0 mEq/L during the week after calving for the identification of cows at increased risk for DA. Ospina et al. (2010b) found that increased NEFA during the postpartum period (greater than 0.72 mEq/L) was associated with lower milk yield in multiparous cows only; first lactation heifers with postpartum NEFA greater than 0. 57 mEq/L had greater projected milk yields. NEFA greater than 0.72 mEq/L during the postpartum period was also found to be associated with a 16 % decreased rate of conception for both multiparous and primiparous cows (Ospina et al., 2010b).

BHBA

Beta-hydroxybutyrate concentration thresholds have also been associated with health, milk yield and reproductive performance. Ketone bodies (BHBA, acetone, and acetoacetate) are produced in the liver as intermediate metabolites of the oxidation of fatty acids. As the supply of NEFA to the liver exceeds the ability of the liver to completely oxidize fatty acids for energy, as is the case during periods of excessive negative energy balance, the liver will begin using acetyl-CoA derived from the β-oxidation of fatty acids for the biosynthesis of ketone bodies (Botham and Mayes, 2006). These ketone bodies can then be used as an alternate fuel source to glucose, thus sparing glucose for milk production (Herdt, 2000).

Unlike NEFA, BHBA sampling is generally restricted to the postpartum period since concentrations are typically low before calving. Ospina et al. (2010a) reported that cows with BHBA greater than 10 mg/dL during the 2-wk period after calving had 3 times the risk for developing DA, CK, or metritis; animals above this threshold also had a 13% decreased rate of conception and, among multiparous cows only, BHBA greater than 10 mg/dL was associated with a 393 kg drop in projected 305-d milk yield (Ospina et al., 2010b). Duffield et al. (2009) found that when BHBA was greater than 12.5 mg/dL during the week after calving cows were at increased risk for subsequent DA and metritis; this threshold however, was dependant on time relative to calving as BHBA greater than 18.8 mg/dL during the second week after calving was determined to be the best predictive threshold for determining subsequent DA risk (Duffield et al., 2009). Cows with BHBA greater than 14.6 mg/dL within the 2-wk period following calving had an increased risk of developing CK.

Herd-Level Risk Assessment and Interpretation

Metabolite testing programs aimed at evaluating herd-level risk for disease or reproductive problems involve first collecting samples from a subset of animals within a target group. It has been suggested that for NEFA and BHBA analyses, collecting samples from 15 animals within the pen of interest (regardless of the number of animals in that pen) is enough to achieve 90% confidence that estimates will represent herd-level prevalence of increased NEFA or BHBA (Ospina et al., 2010a; 2010b; 2010c). Oetzel (2004) has suggested that a minimum of 12 animals is required to give reasonable confidence (75% or more) that the classification of the test results from the 12 cows sampled will correctly represent the true classification of the entire group.

If an analyte is associated with disease when it is above a biological concentration

threshold (cutpoint) but not when it is below that threshold, then it should be evaluated as a proportional outcome (Oetzel, 2004). For example, if NEFA greater than 0.3 mEq/L is associated with an increased risk of DA than NEFA concentrations below this threshold are not associated with DA; therefore, there is little value in interpreting mean NEFA results from the sampled group. Interpreting herd-based tests for metabolic disease should focus on proportions of animals falling above (or below were appropriate) suggested cutpoints rather than overall group means. With this information specific herd alarm levels can then be set (i.e. the proportion of sampled animals with high NEFA or BHBA) that reflect detrimental herd-level effects. Ospina et al. (2010c) suggest that when more than 15 to 20 % of sampled animals on a herd have NEFA greater than 0.27 mEq/L within 2 wk prepartum, NEFA greater than 0.70 mEq/L within 2 wk postpartum or BHBA greater than 10 to 12 mg/dL within 2 wk postpartum, then the herd is at increased risk for disease, lower projected milk yields and a decreased rate of conception.

Alternative Analytes for Herd-Testing

The transition period encompasses both nutritional stressors (i.e. negative energy balance) as well as non-nutritional stressors (i.e. management or environment). The successful application of analytes associated with negative energy balance, including NEFA and BHBA, for herd testing programs suggest that there may be opportunities to include new measures that are also related to health and performance (i.e. analytes associated with stress and inflammation) for the improvement of existing testing programs.

Cortisol

To date there has been very little work characterizing cortisol concentrations around the transition period and no studies describing specific concentration thresholds of cortisol that may

be associated with an increased risk for disease, lower milk yield, or compromised reproductive performance. Activation of the physiological stress response, and the corresponding rise in concentrations of glucocorticoids, have been shown to be important moderators of health and reproductive status (refer to review above for references) particularly during periods of chronic stress. The transition period, defined by numerous changes to environment, management and physiology, may represent a period of chronic stress for some cattle and thus cortisol concentrations around calving may provide useful information about an animals risk for health or production complications after calving. Investigations into the use of cortisol for this purpose, however, should also focus on alternative methods of measuring cortisol secretion (e.g., fecal cortisol metabolites) since plasma cortisol concentrations are subject to a high degree of variability associated with the pulsatile and diurnal patterns of cortisol secretion from the adrenal gland and sensitivity to handling and sample collection stressors (Cook et al., 2000).

Haptoglobin

Studies exploring the associations between haptoglobin with health status have focused primarily on using haptoglobin as a diagnostic indicator of infection or inflammation (e.g. Skinner et al., 1991; Smith et al., 1998a; Sheldon et al., 2001; Grönlund et al., 2005). For example Skinner et al. (1991) concluded that Hp greater than 0.4 g/L indicated significant infection while a concentration greater than 0.2 g/L was a possible indictor of early or mild infection. Recently however, there has been interest in exploring Hp as an early indicator of disease. Huzzey et al. (2009) assessed a range of potential Hp concentration thresholds around calving for the prediction of postpartum metritis; these researchers found that cows with Hp greater than 1.0 g/L on the third day after calving were nearly 7 times more likely to develop severe or mild metritis and that this predictive threshold had a sensitivity of 50% and specificity

of 87%. Increased Hp concentrations around calving in the absence of observable signs of disease may provide information about subclinical infection of inflammation; in this manner Hp may provide useful information about disease risk.

OVERSTOCKING DAIRY CATTLE

Identifying cows at increased risk for health or production problems is only one half of the puzzle. Once these high-risk animals or herds have been identified, producers must know how to manage them in order to decrease risk. During the transition period cows may be exposed to a variety of environmental or management related stressors that have the potential to impact health and productivity; these include pen moves, social regroupings, commingling cows and heifers, exposure to novel environments, overstocking, exposure to heat or cold stress, or aspects of facility design that affect cow comfort (Grant and Albright, 1995; Cook and Nordlund, 2004). Each of these factors is important and has been the focus of many studies; however, the research described in this dissertation focused on overstocking as a potential modifier of disease risk.

Current industry recommendations are to provide cows with 0.6 m or 2 feet of linear feed bunk space per animal (or one headlock per cow) and 1 lying space per animal in the group (NFACC, 2009). Many farms however do not meet these guidelines. In a survey of over 300 dairy herds in Wisconsin, over 68% reported some level of overstocking on their farms with average stocking rates of 111% in four-row barns and 104% in six-row barns being reported (Bewley et al., 2001). Stocking rate is typically calculated by dividing the number of cows housed in the pen by the number of available free-stalls (lying spaces); therefore, estimates of stocking rate in the literature (as in the Bewley study) reflect stocking levels at the lying stalls but likely underestimate overstocking at the feed bunk. There are typically fewer feeding spaces per animal then there are free-stalls per animal (particularly in six-row barns); therefore, herds

that report stocking rates of 104% in a six-row barn based on free-stall availability, may actually have much higher stocking rates at the feed bunk. Presently, there are no good estimates of the true extent of overstocking on commercial dairy herds, particularly at different stages of lactation. Despite this, it is not unreasonable to assume that the dry period, when cows are not lactating, could be one of the most heavily overstocked stages of lactation. Cows that are overstocked during the dry period may face added challenges as they enter the transition period due to the interactions between stress and health. The behavioral consequences of overstocking have been well described; however, the physiological consequences of overstocking require further investigation.

Behavioral Consequences of Overstocking

There is mounting evidence to show that overcrowding can have dramatic effects on dairy cow behavior. Feeding time is decreased and inactive standing time is increased when cows are overstocked at the feed bunk, regardless of feed barrier type (Huzzey et al., 2006). Hill et al. (2009) reported that when cows are overstocked at the lying stalls and feed bunk, lying time decreases, total standing time increases (less standing in the stalls but more standing in the alley-ways) but feeding time does not change. Other research has shown that cows will sacrifice feeding time to gain additional resting time when access to both resources is limited (Metz, 1985). Aggressive competitive displacements at the feed bunk are increased at high stocking densities (Huzzey et al., 2006) likely because cows stand closer together while at an overstocked feed bunk (DeVries et al., 2004). Feeding rate is increased during overstocking and is correlated with displacement success; cows that displace other cows less frequently then they are displaced themselves have the highest feeding rates at an overstocked feed bunk (Proudfoot et al., 2009). Increased competition and reduced feeding times however do not necessarily translate into

reduced DMI. Proudfoot et al. (2009) observed a tendency for lower intake among overstocked multiparous cows but no change in the intake patterns of overstocked heifers. Olofsson (1999) found that a greater feeding rate during overstocking, despite lower daily feeding times, resulted in a tendency for slightly higher DMI during crowding. Huzzey et al. (2006) found that as overstocking increases at the feed bunk, the proportion of cows feeding during the hours following fresh feed delivery is substantially reduced but that cows do not compensate by feeding more during other periods of the day when bunk attendance is lower, such as during the overnight hours.

Consequences for Health and Performance

Research has shown that average daily gains of growing beef heifers are lower when space availability is also low (Fisher et al., 1997). Velasco et al. (2007) found that when free-stall availability was reduced but not access to the feed bunk during the entire dry period, milk yield was not compromised during the subsequent lactation. Bach et al. (2008) also observed no association between space availability at the feed bunk with subsequent milk yield; however, reduced lying stall availability was found to be an important risk factor for reduced milk yield. Decreased feed bunk space has also been associated with a decreased probability of pregnancy by 150 DIM (Caraviello et al., 2006) and an increased risk of health disorders such as DA (Cameron et al., 1998).

There is strong evidence to show that overcrowding has negative effects on dairy cow behavior such as reducing feeding or resting time and increasing competition. The assumption then is that these alterations in behavior could lead to down stream negative consequences for subsequent health and productivity in the herd; however, more research is required to understand

these potential relationships between overstocking and health.

Overstocking – A Chronic Stressor?

When cows are overstocked at the feed bunk, perhaps the most stressful time is the period immediately following fresh feed delivery; this is a period when cows are highly motivated to eat and when competition at the feed bunk is the greatest (DeVries et al., 2003; Huzzey et al., 2006). Overcrowding limits feed bunk access particularly for animals that do not engage in aggressive interactions at the bunk or for those animals that are not successful in displacing others to gain access to feed (Huzzey et al., 2006). Consequently, the period following feed delivery may be considered a chronic intermittent stressor particularly during periods of overcrowding.

The physiological consequence of overstocking has received little attention yet understanding this relationship is vital to understanding how overstocking affects overall health and performance. Previous work has shown that when cows are regrouped into a high stocking density pen (Friend et al., 1977) or subjected to overcrowding in the resting area (Friend et al., 1979) they have a greater cortisol response to ACTH challenge compared to cows that are not regrouped or overcrowded, respectively. Gonzalez et al. (2003) found that the proportion of time spent feeding during overstocking (2:1, lying stall to cow ratio) was negatively correlated with cortisol levels at 60 and 90 mins following ACTH challenge. This work suggests that there may be alterations in adrenal function in response to the stress of overstocking. Changes in stress physiology may be a reflection of the physiological adaptations that occur as cows try to cope with an overcrowded environment and an increase in plasma cortisol concentration may influence other physiological processes.

Glucocorticoids are important regulators of energy metabolism; they help to raise circulating glucose concentrations by increasing hepatic gluconeogenesis and inhibiting

peripheral tissue uptake of glucose; they also contribute to the regulation of lipolysis and lipogenesis, and facilitate increased plasma NEFA concentrations (Parker and Rainey, 2004). These actions essentially oppose the actions of insulin and excess glucocorticoid production has been linked to insulin resistance (Andrews and Walker, 1999). Glucocorticoids can decrease insulin receptor binding affinity without decreasing the number of insulin receptors (de Pirro et al., 1980) and decrease receptor number and affinity (Beck-Nielsen et al., 1980). The translocation of GLUT-4 glucose transporters to cell surfaces in response to insulin can be inhibited in the presence of glucocorticoids (Weinstein et al., 1995; Dimitriadis et al., 1997). Glucocorticoids also appear to play an important role in counter-acting insulin regulation of key rate limiting enzymes in the gluconeogenic pathway, such as phosphoenol-pyruvate carboxykinase (PEPCK) (Hanson and Reshef, 1997). In addition to their effects on insulin resistance glucocorticoids can directly inhibit insulin secretion from pancreatic β-cells (Delaunay et al., 1997).

To date, no researchers have explored the interactions between stress and energy metabolism during overstocking. These interactions may be an important mechanism by which overstocking during the dry period increases a cows' risk for health and production complications during the transition period.

CONCLUSIONS AND RESEARCH OBJECTIVES

There is no other stage in a dairy cow's lactation cycle that presents as many challenges as the transition period and the effects of an inability to cope are likely to be manifested by increased disease incidence, reduced milk yield, and poor reproductive performance. What is clear from the literature is that both stress and inflammation have the capacity to alter the health and productivity of dairy cattle. What has not been thoroughly investigated is whether measures of stress and inflammation can be used to improve the detection of cattle at increased risk for subsequent health and production complications. Increased concentrations of NEFA and BHBA have been used successfully as measures of disease risk and risk for reduced milk yield and compromised reproductive performance. The question is: Are there other physiological measures that may provide additional information about a cows' level of risk for subsequent health and production challenges?

By improving producers' ability to identify early on whether their animals are at increased risk for health or production complications, assessments can then be made on whether opportunities exist to improve overall herd performance. Understanding how environments and/or management systems like overstocking, specifically (i.e. behaviorally and physiologically) moderate health and performance of transition cows, will be important for developing specific recommendations on how management programs should be modified to facilitate decreased risk for future complications.

The objectives of this research were to:

1) Study physiological measures that may be related to non-nutritional management stressors during the transition period including those related to stress and inflammation
2) Determine whether physiological parameters associated with stress (plasma cortisol and

fecal cortisol metabolites) and inflammation (haptoglobin) measured during the period around calving are associated with health status, milk yield and reproductive performance after calving

3) Compare analytes associated with stress and inflammation to analytes associated with negative energy balance as potential predictive measures of disease, reduced milk yield or compromised reproductive performance

4) Describe the physiological effects of overstocking, including behavioral and physiological mechanisms by which health may be affected

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CHAPTER TWO

ASSOCIATIONS OF PREPARTUM PLASMA CORTISOL, HAPTOGLOBIN, FECAL CORTISOL METABOLITES AND NONESTERIFIED FATTY ACIDS WITH POSTPARTUM HEALTH STATUS IN HOLSTEIN DAIRY COWS

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ABSTRACT

The association between negative energy balance and health has led to the testing of blood analytes, such as nonesterified fatty acids (NEFA), to identify opportunities for improving management of transition dairy cows. The objective of this study was to evaluate whether prepartum analytes associated with stress (cortisol) or inflammation (haptoglobin) could also identify dairy cattle at increased risk for health complications after calving. Prepartum blood and fecal samples were collected once weekly from 412 Holstein dairy cows on two commercial dairy farms (wk -3, -2, and -1 relative to calving) and analyzed for concentrations of NEFA, haptoglobin (Hp), and cortisol in plasma and cortisol metabolites in feces. Retained placenta (RP), displaced abomasum (DA), subclinical ketosis (SCK), high Hp concentration (HiHp) and death were recorded up to 30 d after calving and animals were subsequently categorized into 3 health categories: 1) No disorder of interest (NDI); 2) One disorder (RP, DA, SCK, or HiHp); or 3) More than one disorder (RP, DA, SCK, HiHp) or death. With the exception of prepartum NEFA, there were no associations detected between prepartum concentrations of our analytes of interest and the occurrence of one disorder (RP, DA, SCK or HiHP) by 30 DIM. Haptoglobin concentration tended to be greater during wk -2 and -1 in cows that developed more than one disorder or that died by 30 DIM; however, when calving assistance was included as a covariate in the analysis prepartum Hp was no longer a significant risk factor for this postpartum health outcome. Primiparous cows with plasma cortisol concentrations > 22.2 nmol/L during wk -2 had reduced odds [odds ratio (OR) 0.41; 95 % confidence interval (CI) 0.17 – 0.98] of developing more than one disorder or death by 30 DIM while multiparous cows with plasma cortisol > 34.1 nmol/L during wk -2 tended to have greater odds (OR 2.53; 95 % CI 0.87 – 7.37) of developing more than one disorder or death by 30 DIM. Individual variation in daily cortisol secretion patterns and stress responses to the restraint and handling associated with sample collection

make prepartum plasma cortisol data and its relationship to postpartum health difficult to interpret. Among multiparous cows, for every 500 unit (ng/g fecal DM) increase in fecal cortisol metabolite concentration during wk -2, there were increased odds (OR 1.41; 95 % CI 1.12-1.79) of developing more than one disorder or dying after calving. For multiparous cows, every 0.15 mmol/L increase in plasma NEFA concentration during any of the 3 wk before calving was associated with approximately a 2 fold increase in the odds of developing more than one disorder or dying by 30 DIM. Fecal cortisol metabolite concentration during the prepartum period did not predict which cows would go on to develop more than one disorder or die within 30 DIM as well as prepartum NEFA concentration; therefore, this analyte is not a suitable substitute for NEFA for assessing opportunities to improve herd health.

Keywords: transition period, cortisol, haptoglobin, health

INTRODUCTION

Maintaining healthy dairy cows during the periparturient period continues to be a challenge. Several researchers (Kaneene et al., 1997; Oetzel 2004; LeBlanc et al., 2005; Ospina et al., 2010a) have explored testing of blood analytes to identify opportunities for improved dairy cow management. This work has primarily focused on the relationship between health and analytes related to energy status (e.g., NEFA and BHBA). For example, Ospina et al. (2010a) reported that cows with circulating NEFA concentrations > 0.29 mEq/L during the 2-wk period before calving had twice the risk for postpartum disorders (displaced abomasum, clinical ketosis, metritis, or retained placenta). Furthermore, cows with circulating NEFA concentrations > 0.57 mEq/L during the 2-wk period after calving, had 4 times the risk for these disorders compared to cows with NEFA concentrations below this cutpoint (Ospina et al., 2010a).

Although evidence for NEFA and BHBA as useful analytes for predicting disease

continues to mount, few studies have explored whether alternative physiological analytes may also be useful predictors of postpartum health status. During the transition period, environmental and management changes occur within a relatively short period of time including social re-groupings, mixing of heifers and cows, and changes in diet formulation (Grant and Albright, 1995). These situations represent potential stressors that may affect health and performance if individual coping strategies cannot effectively adapt to these challenges. Physiological indicators of stress, particularly during the period before calving, may reveal opportunities for improvements in transition cow management.

Changes in the activity and functioning of the hypothalamic-pituitary-adrenal (HPA) axis are often used to quantify an animal's response to a potential stressor (Mormède et al., 2007). Increased plasma cortisol concentrations have been associated with management and environmental factors thought to be stressful. Circulating cortisol concentrations in cattle increase in response to overstocking (Friend et al., 1979), transportation (Lay et al., 1996), and re-penning or re-grouping (Friend et al., 1977; Gupta et al., 2005). There is also evidence to show that changes in adrenal activity precede health disorders. Peter and Bosu (1987) reported that cows that went on to develop RP after calving had higher serum cortisol concentrations 6 d prior to parturition compared to cows without RP. Despite these relationships, plasma cortisol is likely a poor analyte for field diagnostics for several reasons. Restraint and handling, which are required during blood sampling, can activate the HPA axis and raise circulating cortisol concentrations quickly (Cook et al., 2000). Further, the release of cortisol from the adrenal gland throughout the day is pulsatile, has a diurnal cycle, and is subject to substantial individual variation (Thun et al., 1981); to obtain an accurate assessment of HPA axis activity for the day, as was done in the study by Peter and Bosu (1987), multiple plasma cortisol samples would have to be evaluated.

A practical analyte for field diagnostics or for the identification of herds or individuals at increase risk for disease is one that does not require repeated sampling over the course of a day or days. Fecal cortisol metabolites may be an alternative to plasma cortisol as a measure of the stress response in cattle, due to the feedback-free nature of the sampling method (Palme et al., 1999). The correlation between fecal cortisol metabolites (11,17-dioxoandrostanes) and plasma cortisol has been validated in ruminants by ACTH and dexamethasone tests (Palme et al., 1999); it was determined that concentration of fecal cortisol metabolites paralleled that of cortisol in the blood with a delay time of 10 to 12 h. Changes in DMI, which are common during the transition period, can alter manure output (Nennich et al., 2005); however, previous research suggests that level of intake does not meaningfully influence the concentration of steroid metabolites in the feces (Rabiee et al., 2001). Further, increased fecal cortisol metabolite concentrations in cattle have been reported in response to transport stress (Palme et al., 2000) suggesting that this measure has potential for describing natural fluctuations in cortisol secretion arising from environmental stressors.

Environmental stressors also induce an acute phase response in cattle. Transportation and re-grouping in cattle increase acute phase proteins such as haptoglobin (**Hp**) and serum amyloid A (Arthington et al., 2003; Lomborg et al., 2008). The acute phase response is activated during periods of inflammation, tissue damage, and infection. Although there are many acute phase proteins, Hp has been of particular interest for the detection of sick animals due to its high relative increase during the acute phase response from the low concentration threshold observed during normal or healthy conditions (Eckersall, 2000). Several researchers have demonstrated that Hp can be used as a diagnostic analyte (Skinner et al., 1991; Hirvonen et al., 1999; Sheldon et al., 2001). Huzzey et al. (2009) reported an increase in Hp 3 d prior to the onset of clinical signs of metritis; however, more research is needed to determine whether Hp can predict cows at

risk for disease when measured during the weeks leading up to calving. Greater prepartum Hp concentrations among cows that develop health complications after calving could indicate sub-clinical infections not yet detectable through visual symptoms, increased susceptibility to stressful environmental conditions, or even severe disruptions in energy balance. Changes in energy status and lipid metabolism during the transition period can lead to excessive NEFA infiltration of the liver and fatty liver syndrome. Similar to infection or trauma, fatty infiltration represents a harmful stimulus to liver parenchymal cells, and consequently these cells will increase the production of Hp (Katoh, 2002).

The objective of this study was to evaluate the association between prepartum concentrations of plasma cortisol, fecal cortisol metabolites, and plasma Hp with postpartum health status. The association between prepartum NEFA and postpartum health status was also evaluated so that our analytes of interest could be discussed relative to a well-described metabolic predictor of disease.

MATERIALS AND METHODS

Animals, Housing and Diet

The Cornell University Institutional Animal Care and Use Committee approved all procedures involving animals prior to the beginning of the study. This study was conducted between February 2008 and July 2008 on two commercial Holstein dairy herds in New York State. Herd size of the two farms ranged from approximately 1,300 to 1,500 milking cows. During this time, a convenience sample of 12 to 20 animals that were within 4 wk of calving was selected each week for enrollment. Any animals that were visibly sick or lame were not enrolled in the study. On Farm A, 202 cows were sampled [113 multiparous cows (**MP**) and 89 primiparous cows (**PP**)] and on Farm B 210 cows were sampled (117 MP and 93 PP) for a total

enrollment of 412 animals in the study. Primiparous cows were purposely targeted to increase the number of PP cows sampled to ensure that statistically valid comparisons between parities could be made. The average parity (mean ± SD) of all animals (including the calving event that occurred during the course of the study) was 2.3 ± 1.6 on Farm A and 2.1 ± 1.4 on Farm B with an overall average of 2.2 ± 1.5. Housing facilities on each farm included free-stalls for the close-up and fresh cow pens, and separate maternity pens consisting of straw-bedded packs. Cows were moved to the maternity pen when they showed physical signs of imminent calving (e.g., placenta or feet visible). After calving, cows were moved at the discretion of the farm staff into either a high-lactation fresh group or a transition fresh group if there were complications associated with calving (i.e. clinical disease). On Farm B, PP and MP cows were housed separately after calving; at all other periods, cows of all parities were commingled.

Cows in both herds were fed once daily in the morning, with feed push-ups occurring at regular intervals throughout the day. Weekly samples of the TMR were collected from the close-up and fresh cow groups. The weekly TMR samples for each farm were combined into 4-wk composite samples and submitted to a commercial laboratory for wet chemistry analysis (Dairy One Cooperative Inc., Ithaca, NY). The results of this analysis are provided in Table 1. Cows had ad libitum access to water and fresh cows were milked three times daily. Body condition score (Wildman et al., 1982) for each individual was recorded weekly beginning 4 wk prior to the expected calving date and once within the week following calving. Farm staff were responsible for collecting calving information including calving ease (assisted versus non-assisted), twinning, and calves born dead on arrival (**DOA**). Calving data and pre- and postpartum BCS were used as covariates in the statistical analysis.

Table 2.1. Component analysis (mean ± SD; % of DM unless otherwise noted) of 4-wk composite TMR samples collected between February and July 2008 from Farm A and B.

	Close-up Cow TMR		Fresh Cow TMR	
Compo nent	Farm A	Farm B	Farm A	Farm B
CP	13.8 ± 3.0	13.5 ± 2.0	18.2 ± 0.7	17.9 ± 0.8
ADF	31.3 ± 7.3	28.7 ± 4.2	18.8 ± 1.3	19.3 ± 0.9
NDF	48.9 ± 3.0	47.0 ± 5.6	31.3 ± 2.8	31.8 ± 2.2
NE_L (Mcal/kg)	1.55 ± 0.06	1.56 ± 0.04	1.68 ± 0.03	1.67 ± 0.02
Ca	1.31 ± 0.56	1.14 ± 0.42	0.99 ± 0.08	0.98 ± 0.10
P	0.33 ± 0.07	0.37 ± 0.03	0.45 ± 0.01	0.38 ± 0.02
Mg	0.38 ± 0.09	0.37 ± 0.09	0.37 ± 0.01	0.36 ± 0.03
K	1.12 ± 0.09	1.01 ± 0.18	1.27 ± 0.06	1.25 ± 0.08
Na	0.12 ± 0.04	0.11 ± 0.03	0.47 ± 0.05	0.51 ± 0.09

Blood and Fecal Sample Collection and Analysis

Blood and fecal samples were collected weekly from the 412 enrolled cows beginning approximately 4 wk prior to each individual's expected calving date and continued until parturition. For all experimental cows a postpartum blood sample was collected between 3 to 10 DIM. Blood was collected from the coccygeal vessel into 10-mL sterile tubes coated with sodium heparin (BD Vacutainer, Franklin Lakes, NJ, USA) then stored in coolers until they could be returned to the laboratory for processing. Plasma was harvested after centrifugation (2,800 x *g* for 15 min at 4˚C), and stored at -20˚C for later analyses. Plasma concentrations of NEFA and Hp were measured by enzymatic analysis (NEFA-C: Wako Pure Chemical Industries, Osaka, Japan; Phase Range Haptoglobin Assay: Kit no. TP801, Tridelta Diagnostics Ltd., NJ, USA). Intra- and interassay coefficients of variation (**CV**) for the NEFA assay were 2.2 and 4.7

%, respectively and for the Hp assay were 6.2 and 10.0 %, respectively. All spectrophotometric measurements were conducted using a $Versa_{max}$ tunable microplate reader (Molecular Devices, Sunnyvale, CA). A commercial solid-phase radioimmunoassay kit was used to determine plasma concentrations of cortisol (Coat-A-Count, Diagnostic Products, Los Angeles, CA). Intra- and interassay CV for the cortisol RIA were 1.7 and 2.9 %, respectively.

Fecal samples were collected fresh and stored on ice until they could be frozen at -20˚C for later processing. Steroids from the fecal samples were extracted using the wet extraction method described by Palme (2005). Briefly, 0.5 g of each raw fecal sample was weighed and vortexed with 5 ml of 80 % methanol for 30 min. Samples were then centrifuged for 15 min at 2,800 x *g* and the supernatant was divided into aliquots and stored at -20˚C until further analysis. The DM percentage of each fecal sample was obtained by weighing samples before and after drying in a hot oven (105˚C) for 24 h. Concentration of fecal cortisol metabolites (11,17-dioxoandrostanes) were measured using a competitive enzyme immunoassay developed by Palme and Möstl (1997) and validated for use in cattle (Palme et al., 1999). Intra- and interassay CV for the fecal cortisol metabolite assay were 3.9 and 6.5 %, respectively. To reduce the variation associated with differences in manure consistency, the concentration of fecal cortisol metabolites obtained from the EIA were adjusted for the DM content of the raw fecal sample and reported on a DM basis.

At the end of the study, blood and fecal samples were sorted by week relative to the actual calving date. Samples collected during d -21 to -15 relative to the actual calving date were used to represent wk -3, d -14 to -8 (wk -2), and d -7 to -2 (wk -1). Samples collected during d -1 were not included in the data set due to the increase in circulating cortisol and NEFA concentrations that occur during the day before parturition (Vazquez-Añon et al., 1994; Patel et al., 1996).

Determination of Health Outcomes

For the description of postpartum health status only disease cases that were recorded by both farms and that used similar case definitions were included. Cases of retained placenta (**RP**), displaced abomasum (**DA**), and death (not including voluntary culls) were collected from DairyCOMP 305 (**DC305**; Valley Agricultural Systems, Tulare, CA) up to 30 DIM. Case definitions for the disease outcomes were discussed with farm staff prior to the commencement of the study to ensure consistency of diagnosis between farms. RP was defined as failure to expel the placenta within 24 h after parturition. DA was diagnosed based on an auscultation of a "ping" during percussion of the left side of the abdomen and was generally confirmed during subsequent surgical correction. Cows were classified as sub-clinically ketotic (**SCK**) if plasma BHBA concentration from a postpartum blood sample collected between 3 to 10 DIM was greater than 0.96 mmol/L (10 mg/dl; Ospina et al., 2010a). Plasma concentrations of BHBA were measured by enzymatic analysis (BHBA dehydrogenase: Kit no. 310, Sigma Chemical). Intra- and interassay CV for the BHBA assay were 2.2 and 5.1 %, respectively. Cases of metritis and mastitis were not consistently recorded on the two herds and therefore could not be reliably measured. Researchers have shown that plasma Hp concentrations can be used as a diagnostic tool or predictive measure for cows at risk for metritis and mastitis has also been associated with elevated Hp concentrations in milk. Dubuc et al. (2010) reported that odds of metritis were 4 times greater when cows had Hp > 0.8 g/L between d 1-7 postpartum (Sensitivity (**Se**): 51% and Specificity (**Sp**): 79%) and Huzzey et al. (2009) found that cows with Hp > 1 g/L on d +3 were 6.7 times more likely to develop severe or mild metritis (Se: 50% and Sp: 87%). Wenz et al. (2010) reported Hp concentrations in the milk of severely infected mammary quarters was doubled that of quarters with only mild infection (0.50 g/L versus 1.01 g/L, respectively). Based on this literature a postpartum plasma Hp concentration > 1 g/L (**HiHp**) was considered a disease

outcome in this study. This cutpoint was associated with the higher Sp for the detection of metritis and this was preferred so that a positive result from the test would be associated with a higher probability of the presence of disease, thereby minimizing false positives.

Cows were divided retrospectively into three health categories for statistical analyses: 1) "No disorder of interest" (**NDI**) included cows that did not have RP, DA, SCK, HiHp or die by 30 DIM; 2) "One disorder" included cows that developed only one health disorder (RP, DA, SCK, or HiHp) by 30 DIM; and 3) "More than one disorder or death" included cows that developed 2 or more health disorders (RP, DA, SCK, HiHp) or that died by 30 DIM.

Statistical Analysis

The associations between categorical variables, parity and farm and the individual disease outcomes were analyzed using 2 x 2 contingency tables generated by the PROC FREQ statement in SAS (Version 9.2; SAS Institute, 2009). From these tables the Mantel-Haenszel Chi-Square test statistic was used to determine the type 1 error risk of the relationship. Correlations between NEFA, Hp, plasma cortisol, and fecal cortisol metabolites at each period prior to calving were determined using PROC CORR; for these analyses data for cows in each health category were pooled into one data set.

Statistical analyses were performed using SAS with cow as the experimental unit. NEFA, Hp, and fecal cortisol metabolites concentrations were analyzed as continuous outcomes using PROC MIXED. The differences in these analytes between NDI cows versus cows with one disorder, and between NDI cows versus cows with more than one disorder or death, were analyzed by period (wk -3, -2, and -1) using the contrast statement. These data were stratified by period rather than analyzed using a repeated measures model due to independent a priori hypotheses by time. Each period was considered an independent test because the concentrations of the analytes of interest will change relative to time from calving and it was predicted that the

level of association with the health outcome was likely to be influenced by the time lapse between the analyte being tested and the onset of the disease. The model included the fixed effects of farm (A vs. B), parity (PP vs. MP) and health category (NDI, one disorder, more than one disorder or death) and the relevant interaction terms (farm x health, parity x health). When an interaction term with $P<0.05$ was detected data were stratified by that factor.

After completing the plasma cortisol assay it was found that a large number of the samples (53%) fell below the lowest standard used in the assay (13.8 nmol/L). Consequently, plasma cortisol was analyzed as a categorical explanatory variable with logistic regression. Plasma cortisol concentrations marking the 95^{th}, 90^{th}, and 75^{th} percentiles (data from PP and MP cows combined) were used as the potential predictive cutpoints for the two postpartum health outcomes of interest.

When an association or a tendency for an association was detected ($P \leq 0.1$) between an analyte and a health outcome using PROC MIXED the data were evaluated further using multivariable logistic regression analysis. The LOGISTIC procedure in SAS was used to determine if the relationship between the analyte and the health outcome persisted once additional explanatory variables (covariates) were also accounted for. In addition to the analyte of interest (NEFA, Hp, plasma cortisol, or fecal cortisol metabolites) the following explanatory variables all of which preceded the diagnosis of disease, were considered for each model: farm (A vs. B), parity (PP vs. MP), calving ease (assisted vs. not assisted), twins (yes vs. no), calves born dead on arrival (DOA; yes vs. no), and prepartum BCS. Manual backwards-stepwise logistic regression was used to remove explanatory variables from the model when $P > 0.05$. Odds ratios (OR) were used to describe the level of association between the analyte of interest and the postpartum health outcome (NDI vs. One disorder or NDI vs. More than one disorder or death). The OR is the odds of the disease in the exposed group divided by the odds of disease in

the non-exposed group.

RESULTS

Descriptive Data

On farm A the median (range) BCS during the prepartum period was 3.5 (2.25 to 4.75) and during the postpartum period was 3.5 (2.0 to 4.5). On farm B, median prepartum BCS was 3.5 (1.75 to 4.25) and postpartum was 3.25 (1.75 to 4.0). The lactational incidence of death, DA and HiHp was not different between the two herds ($P > 0.20$); however, Farm A had more cases of SCK ($P < 0.01$) and tended to have more cases of RP ($P = 0.06$; Table 2.2.). Farm A had more sets of twins (19 vs. 6, $P < 0.01$), but farms did not differ in calves born DOA (12 vs. 14 for Farm A and B, respectively). Multiparous cows had more cases of RP and DA ($P < 0.001$ and $P = 0.04$, respectively), more twins (24 vs. 1, $P < 0.001$), and tended to have fewer calves born DOA (10 vs. 16, $P = 0.07$) than PP cows. Primiparous cows tended to have more cases of HiHp (P = 0.08). The mean (median) DIM of death for affected cows was 11.7 (12) and for DA was 12.6 (10). The number and proportion of cows that were allocated to the three experimentally defined health categories are provided in Table 2.2., stratified by parity and farm. Table 2.3. describes the distribution of health disorders and calving events by the 3 experimentally defined health categories.

During each period before calving, NEFA and fecal cortisol metabolites were positively correlated with each other (r = 0.19, 0.19, 0.29 for wk -3, -2 and -1, $P < 0.001$), as were NEFA and Hp (r = 0.22, 0.19, 0.41 for wk -3, -2 and -1, $P < 0.001$). NEFA was correlated with plasma cortisol during wk -2 and -1 (r = 0.17 and 0.16, respectively $P \leq 0.002$). Hp and fecal cortisol metabolites were positively correlated during all periods (r = 0.13, 0.29, 0.30 for wk -3, -2 and -1, $P \leq 0.01$) while Hp and plasma cortisol were only correlated during wk -2 and -1 (r = 0.17 and

0.16, $P \leq 0.003$). At no period prior to calving was there a correlation between plasma cortisol and fecal cortisol metabolites ($P > 0.48$).

Analytes by Postpartum Health Status

NEFA. Due significant parity x health status interactions at each period before calving ($P \leq 0.02$) the NEFA data were stratified by parity. Multiparous cows with more than one disorder or that died by 30 DIM had greater NEFA concentrations than NDI cows during each week prior to calving, while MP cows with only one disorder after calving had greater NEFA relative to the NDI group during wk -1 only. Primiparous cows with more than one disorder or that died by 30 DIM had greater NEFA during wk -2 and -1 relative to PP NDI cows. Prepartum NEFA concentrations were not different between PP cows with only one postpartum disorder by 30 DIM and those in the NDI category (Table 2.4.).

Haptoglobin. Haptoglobin concentration was greater in PP cows during wk -1 (0.33 vs. 0.23 g/L; P = 0.03) and tended to be greater during wk -2 (0.34 vs. 0.26 g/L) and wk -3 (0.27 vs. 0.21 g/L), relative to MP cows ($P \leq 0.09$). Relative to cows in the NDI category, Hp tended to be greater during wk -2 and wk -1 for cows that developed more than one disorder or that died by 30 DIM (P = 0.12 and 0.09, respectively; Table 2.4.). No other differences in prepartum Hp were detected between health categories.

Fecal Cortisol Metabolites. At all periods before calving PP cows had greater fecal cortisol metabolite concentrations than MP cows (wk -3, 1912 vs. 1441 ng/g fecal DM; wk -2, 2035 vs. 1560 ng/g fecal DM; and wk -1, 2332 vs. 1714 ng/g fecal DM; $P < 0.001$). Relative to cows in the NDI category, fecal cortisol metabolite concentrations tended to be greater during wk -3 and wk -2 for cows that developed more than one disorder or that died by 30 DIM (P = 0.07 and 0.10, respectively; Table 2.4.). No other differences in prepartum fecal cortisol metabolite concentrations were detected between health categories.

Plasma Cortisol. The concentrations of plasma cortisol that marked the 95th, 90th, and 75th percentile for all animals were: 48.6, 36.4, and 22.0 nmol/L respectively, during wk -3; 44.5, 34.1, and 22.2 nmol/L respectively, during wk -2; and 42.5, 36.1, and 24.6 nmol/L respectively, during wk -1. The relationships between these cutpoints and postpartum health status are described in the following section.

Multivariable Logistic Regression Analyses

Calving ease (assisted versus non-assisted) was the only additional covariate, other than parity, that was associated with the two health outcomes of interest in this study ($P \leq 0.007$). For statistical validity reasons, twinning and DOA calvings could not be included as covariates in these analyses due to the low frequency of these events occurring in the NDI category (Table 2.3.).

Prepartum NEFA was the only analyte associated with the development of one disorder by 30 DIM after accounting for parity and calving difficulty, however this association was detected during wk -1 only. Among MP cows, for every 0.15 mmol/L increase in plasma NEFA concentration during any 3 wk period before calving the odds of developing more than one disorder or death by 30 DIM were approximately 2 times greater than the odds of this health outcome without this increase in NEFA; however, among PP cows plasma NEFA was not associated with postpartum health status although calving assistance was (Table 2.5.).

Prepartum Hp was no longer associated with the development of more than one disorder or death by 30 DIM for either PP or MP cows after accounting for calving assistance as a covariate ($P > 0.15$).

During wk -2 only, the associations of plasma cortisol and fecal cortisol metabolites with postpartum health status (development of more than one disorder or death versus NDI) remained after accounting for calving assistance (Table 2.6.). For every 500 ng/g of fecal DM increase in

fecal cortisol metabolites among MP cows during wk -2 only, the odds of developing more than one health disorder or death increased 1.4 times. During wk -2, MP cows with plasma cortisol concentrations greater than 34.1 nmol/L (90^{th} percentile) tended to have greater odds (OR = 2.5) of developing more than one disorder or dying by 30 DIM while PP cows with plasma cortisol concentrations greater than 22.2 nmol/L (75^{th} percentile) tended to have lower odds (OR = 0.4) of developing this health outcome after calving.

Table 2.2. Number of health disorders during the first 30 DIM by farm (A, n = 202; B, n = 210) and parity [primiparous (PP), n = 182; multiparous (MP), n = 230] including overall lactational incidence (n = 412).

	Farm		Parity		Overall	
Disorder & Health Category [1]	A	B	PP	MP	n	%
Retained Placenta (RP)	28	17	9	36	45	10.9
Displaced Abomasum (DA)	11	14	6	19	25	6.1
Sub-clinical Ketosis (SCK) [2]	74	50	46	78	124	30.1
Haptoglobin > 1g/L (HiHp) [2]	66	80	73	73	146	35.4
Died	7	13	5	15	20	6.3
NDI	89	94	86	97	183	44.4
One Disorder	65	62	58	69	127	30.8
> One Disorder or Death	48	54	38	64	102	24.8

[1] NDI = cows that did not develop RP, DA, SCK, HiHp or die by 30 DIM; One Disorder = cows that developed only one disorder (RP, DA, SCK or HiHp) by 30 DIM; > One Disorder or Death = cows that developed 2 or more disorders (RP, DA, SCK, HiHp) or died by 30 DIM
[2] Measured post-partum (3 – 10 DIM)

Table 2.3. Frequency (no.) of health disorders and calving events and median (range) prepartum body condition score (BCS) in the three experimentally defined health categories[1].

Disorder & Calving Events [1]	Total no. Events	NDI (n = 183)	One Disorder (n = 127)	> One Disorder or Death (n = 102)
Retained Placenta (RP)	45	0	10	35
Displaced Abomasum (DA)	25	0	3	22
Sub-clinical Ketosis (SCK) [2]	124	0	48	76
Haptoglobin > 1g/L (HiHp)	146	0	66	80
Died	20	0	0	20
Twins	25	1	6	18
DOA	26	5	8	13
Assisted Calvings	106	32	35	36
Prepartum BCS	-	3.5 (2.0 - 4.25)	3.5 (2.5 - 4.75)	3.5 (1.75 - 4.50)

[1] NDI = cows that did not develop RP, DA, SCK, HiHp or die by 30 DIM; One Disorder = cows that developed only one disorder (RP, DA, SCK or HiHp) by 30 DIM; > One Disorder or Death = cows that developed 2 or more disorders (RP, DA, SCK, HiHp) or died by 30 DIM

[2] Measured post-partum (3 – 10 DIM)

Table 2.4. Least squares means (± SE) for plasma NEFA[1], plasma haptoglobin (Hp), and fecal cortisol metabolite (FCORT) concentrations for cows in three different postpartum health categories[2] during 3 wk before calving.

		Period		
Analyte	N	wk -3	wk -2	wk -1
NEFA (mmol/L) - Primiparous Cows				
NDI	86	0.29 ± 0.02	0.34 ± 0.02	0.38 ± 0.02
One Disorder	58	0.31 ± 0.02	0.37 ± 0.02	0.41 ± 0.03
> One Disorder or Death	38	0.28 ± 0.03	0.41 ± 0.03 *	0.49 ± 0.04 **
NEFA (mmol/L) - Multiparous Cows				
NDI	97	0.17 ± 0.02	0.22 ± 0.02	0.29 ± 0.03
One Disorder	69	0.20 ± 0.02	0.28 ± 0.03 †	0.40 ± 0.04 *
> One Disorder or Death	64	0.29 ± 0.02 ***	0.42 ± 0.03 ***	0.60 ± 0.04 ***
Hp (g/L)				
NDI	183	0.24 ± 0.03	0.27 ± 0.03	0.23 ± 0.03
One Disorder	127	0.29 ± 0.03	0.29 ± 0.04	0.28 ± 0.04
> One Disorder or Death	102	0.18 ± 0.04	0.34 ± 0.04 †	0.33 ± 0.05 †
FCORT (ng/g fecal DM)				
NDI	183	1593.8 ± 58.4	1764.5 ± 66.7	1979.0 ± 98.7
One Disorder	127	1659.4 ± 69.9	1678.9 ± 79.9	1972.1 ± 119.5
> One Disorder or Death	102	1777.0 ± 84.1 †	1950.9 ± 92.5 †	2117.9 ± 139.4

Type I error risk for the differences in these analytes between NDI cows vs. cows with one disorder, and between NDI cows vs. cows with more than one disorder or that died: † $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

[1] Data were stratified by parity due to a significant parity × health status interaction during each wk prior to calving ($P \leq 0.02$)

[2] NDI = cows that did not develop RP, DA, SCK, HiHp or die by 30 DIM; One Disorder = cows that developed only one disorder (RP, DA, SCK or HiHp) by 30 DIM; > One Disorder or Death = cows that developed 2 or more disorders (RP, DA, SCK, HiHp) or died by 30 DIM

Table 2.5. Final logistic regression models describing the association between plasma NEFA at 3 periods before calving (accounting for additional covariates) and the risk for the experimental health outcomes of interest: 1) more than one disorder or death by 30 DIM versus NDI, and 2) one disorder by 30 DIM versus NDI[1].

Outcome: More than one disorder or death versus NDI						
	Models[2]	Level	OR	95% CI[3]		*P*
Primiparous						
wk -3	Ease	Assisted	2.85	1.22	6.68	0.02
	NEFA	Per 0.15 mmol/L increase	0.95	0.65	1.38	0.78
wk -2	Ease	Assisted	3.31	1.47	7.46	0.004
	NEFA	Per 0.15 mmol/L increase	1.30	0.88	1.91	0.19
wk -1	Ease	Assisted	5.66	2.27	14.09	<0.001
	NEFA	Per 0.15 mmol/L increase	1.23	0.94	1.62	0.14
Multiparous						
wk -3	Ease	Assisted	1.52	0.56	4.16	0.41
	NEFA	Per 0.15 mmol/L increase	1.87	1.31	2.67	<0.001
wk -2	Ease	Assisted	2.04	0.78	5.34	0.15
	NEFA	Per 0.15 mmol/L increase	1.94	1.41	2.67	<0.001
wk -1	Ease	Assisted	2.28	0.71	7.33	0.17
	NEFA	Per 0.15 mmol/L increase	2.04	1.50	2.79	<0.001
Outcome: One disorder versus NDI						
	Models	Level	OR	95% CI		*P*
wk -3	Parity	Primiparous	0.65	0.39	1.10	0.11
	Ease	Assisted	2.37	1.35	4.19	0.003
	NEFA	Per 0.15 mmol/L increase	1.18	0.94	1.49	0.16
wk -2	Parity	Primiparous	0.72	0.44	1.20	0.21
	Ease	Assisted	2.16	1.24	3.75	0.007
	NEFA	Per 0.15 mmol/L increase	1.17	0.95	1.45	0.14
wk -1	Parity	Primiparous	0.70	0.41	1.18	0.18
	Ease	Assisted	2.52	1.36	4.68	0.003
	NEFA	Per 0.15 mmol/L increase	1.33	1.09	1.61	0.004

[1] NDI = cows that did not develop RP, DA, SCK, HiHp or die by 30 DIM; One disorder = cows that developed only one disorder (RP, DA, SCK or HiHp) by 30 DIM; More than one disorder or death = cows that developed 2 or more disorders (RP, DA, SCK, HiHp) or died by 30 DIM

[2] Analyses were stratified by parity due to a significant NEFA × parity interaction during wk -3 and -1 ($P \leq 0.007$) and a tendency for an interaction during wk -2 ($P=0.11$).

[3] CI = Confidence Interval

Table 2.6. Final logistic regression models describing the association between fecal cortisol metabolite concentration (FCORT) and plasma cortisol (P. Cortisol) during wk -2 relative to calving (accounting for additional covariates) and the risk for more than one disorder or death by 30 DIM versus NDI[1].

	Models[2]	Level[3]	OR	95% CI [4]		*P*
Primiparous						
wk -2	Ease	Assisted	3.64	1.56	8.50	0.003
	FCORT	Per 500 ng/g fecal DM increase	0.90	0.74	1.10	0.31
wk -2	Ease	Assisted	2.89	1.28	6.56	0.01
	P. Cortisol	Above 22.2 nmol/L	0.41	0.17	0.98	0.05
Multiparous						
wk -2	Ease	Assisted	2.79	1.12	6.95	0.03
	FCORT	Per 500 ng/g fecal DM increase	1.41	1.12	1.79	0.004
wk -2	Ease	Assisted	2.66	1.10	6.44	0.03
	P. Cortisol	Above 34.1 nmol/L	2.53	0.87	7.37	0.09

[1] NDI = cows that did not develop RP, DA, SCK, HiHp or die by 30 DIM; More than one disorder or death = cows that developed 2 or more disorders (RP, DA, SCK, HiHp) or died by 30 DIM

[2] Analyses were stratified by parity during wk -2 due to a FCORT × parity interaction (P=0.004) and a plasma cortisol × parity interaction ($P \leq 0.05$).

[3] Plasma cortisol was modeled as a dichotomized predictor variable based on the 75th (22.2 nmol/L) or the 90th (34.1nmol/L) percentile of cortisol concentrations measured among all 412 experimental cows.

[4] CI = Confidence Interval

DISCUSSION

The results of this study support the conclusions of others (Kaneene et al., 1997; LeBlanc et al., 2005; Ospina et al., 2010a) that increased concentrations of NEFA, measured during the prepartum period, can be used to identify animals at increased risk for postpartum health disorders. In the present study, however, this relationship depended on the degree of illness after calving (one disorder vs. more than one disorder or death) and parity. A stronger association between prepartum NEFA and the development of multiple disorders (RP, DA, SCK or HiHp) after calving may suggest that negative energy balance plays a greater role in the pathogenesis of these disorders when they occur together. Including cows that died in this health category did not drive the strength of this relationship. The stronger association between elevated prepartum NEFA and multiple postpartum disorders compared to elevated NEFA and the development of only one disorder after calving remained even after removing cows that had died within 30 DIM.

The relationship between prepartum NEFA and postpartum health status appeared to be greater among MP cows than among PP cows of different health categories. Ospina et al. (2010a) did not observe an interaction between prepartum NEFA and parity in the prediction of cows at risk for DA, clinical ketosis, RP, metritis or a combination of these disorders after calving. In that study a larger sample size was used than in the present study, which may have allowed for more sensitivity in identifying differences within PP cows. There is, however, other evidence in the literature to suggest that PP and MP cows may require different prepartum predictors for postpartum performance. Ospina et al. (2010b) found that PP cows with NEFA concentrations $\geq$ 0.57 mEq/L during 3-14 DIM had greater projected milk yields while MP cows with NEFA concentrations $\geq$ 0.72 mEq/L during this period had lower projected milk yields. First calf heifers may undergo different homeorhetic adaptations to accommodate their first lactation and still support growth and maintenance requirements; this may explain the observed

discrepancies between risk factors. Understanding the interactions between parity and known risk factors for disease or production problems will be important for future herd health monitoring programs; failure to do so may lead to inaccurate assessments of risk and inappropriate managerial actions such as failing to identify the high risk animals and thus neglecting desirable management interventions or incorrectly identifying high risk animals and implementing unnecessary interventions.

Although prepartum NEFA concentrations were positively correlated with plasma haptoglobin, plasma cortisol, and fecal cortisol metabolite concentrations the correlation coefficients were low and therefore the biological significance of these relationships should not be over interpreted. Cortisol helps to facilitate NEFA mobilization from adipose tissue during periods of stress to increase energy availability (Sapolsky et al., 2000) however the biological functions of glucocorticoids are complex. For example, the actions of glucocorticoids are not always lipolytic; excess glucocorticoids are associated with fat accretion at truncal or abdominal areas (Rebuffé-scrive et al., 1992) and glucocorticoids can also stimulate appetite (Santana et al., 1995). Other researchers have described a strong correlation between NEFA and Hp ($r = 0.93$; Kovác et al., 2009) but these correlations incorporated data from the weeks following calving. Excessive negative energy balance, leading to fatty liver syndrome, may damage liver cells thus leading to increased Hp concentrations (Katoh, 2002). In the current study the correlation between NEFA and Hp was considered during the prepartum period only, when negative energy balance is typically less of a concern. Cortisol can also stimulate hepatic Hp production directly (Higuchi et al., 1994). Clearly NEFA, cortisol and Hp are interrelated but these relationships are complex and vary depending on the physiological status of the cow; this may explain why low correlation coefficients were observed in the current study while other researchers report strong correlations between these analytes.

Prepartum Hp concentration was not found to be a significant risk factor for the development of more than one disorder or death by 30 DIM when modeled along with calving assistance. Calving assistance is typically required during a prolonged or particularly difficult calving resulting from a very large or mal-positioned calf (Mee, 2008). Dystocia has been identified as a risk factor for retained placenta and metritis (Laven and Peters, 1996; Bruun et al., 2002; Dubuc et al., 2010), disorders that are also associated with elevated Hp concentrations during the week following calving (Huzzey et al., 2009; Dubuc et al., 2010). Among the cows that developed more than one disorder or that died by 30 DIM 78% of them had high Hp concentrations (> 1g/L) between d 3-10 post partum and 34% had a RP so it is not surprising that calving assistance was strongly associated with this health category and overwhelmed the tendency for an association between prepartum Hp concentration and this health outcome. These results also suggest that a sub-clinical infection or inflammatory condition during the prepartum period was probably not a major factor contributing to the development of the postpartum disorders measured in this study. Prepartum Hp concentrations do not seem to provide any additional information related to a cow's level of risk for postpartum health complications that calving assistance or dystocia couldn't already provide.

Higher variability in the plasma cortisol data likely explained the weaker association between wk -2 concentrations of this analyte with postpartum health status relative to fecal cortisol metabolites during wk -2; the chance of committing a type I error when using wk -2 fecal cortisol concentrations to predict disease in MP cows was much lower ($P = 0.004$ versus $P = 0.09$). Greater daily production of cortisol during the prepartum period, as reflected by greater fecal cortisol metabolite concentrations, could affect health of transition cows by altering immune function. For example glucocorticoids can inhibit the synthesis, release, and efficacy of cytokines such as IL-1 and TNF-α, important mediators of immune and inflammatory reactions

including the acute phase response and can alter the migration and function of peripheral cells of the immune system including lymphocytes, macrophages and monocytes (reviewed in Sapolsky et al., 2000).

Although there was no association between prepartum fecal cortisol metabolite concentration and postpartum health in PP cows, PP cows with greater plasma cortisol concentrations (75^{th} percentile) during wk -2 relative to calving had reduced risk of developing more than one disorder or dying by 30 DIM. These seemingly contradictory results could be explained by an exaggerated stress response during sampling in PP cows that are less familiar with handling and the process of sample collection than MP cows. Previous work has shown that average plasma cortisol concentrations of heifers increase by about 20 ng/mL within 10 min of being introduced to a novel environment (Veissier and LeNeindre, 1988). It is possible that by the time heifers were sorted and restrained in headlocks many would have had elevated plasma cortisol concentrations prior to the collection of the blood sample.

The confounding effects of the sample collection procedure also likely explain the lack of correlation between plasma cortisol and fecal cortisol metabolites during the prepartum period. Fecal cortisol metabolite concentrations reflect the amounts of excreted, and thus produced, cortisol 10 to 12 hours prior to sample collection (Palme et al., 1999); in the current study this period would have reflected the overnight hours (approximately midnight – 3 am) and so there would have been little to no human interaction with the cattle. Researchers that validated the use of fecal cortisol metabolites as a measure of cortisol production by demonstrating the paralleling concentrations of plasma cortisol and its fecal metabolites following ACTH administration and also by reporting the correlation ($r = 0.77$; $P = 0.006$) between ACTH dose and the % increase in fecal cortisol metabolites, minimized the sampling effect by collecting blood through jugular catheters of animals well accustomed to handling (Palme et al., 1999).

The natural increase in maternal cortisol production during the days just prior to parturition (Patel et al., 1996) may have prevented the detection of a relationship between prepartum cortisol and postpartum health during wk -1. Further, the antibodies used in the fecal cortisol metabolite assay have some cross-reactivity with gonadal steroids of placental origin; this can cause elevated levels of immunoreactive products in the feces of cattle particularly during the period just prior to parturition (Möstl et al., 2002). While data from d -1 was eliminated from the analyses in order to limit these confounding effects, the results could suggest that samples collected at any time during the week prior to calving may be influenced by maternal or placental changes in steroid production that are associated with the onset of parturition.

Although there was an association between wk -2 fecal cortisol metabolite concentrations and postpartum health status among multiparous cows, this analyte is probably not a suitable measure for identifying cows at increased risk for postpartum health complications, when compared with prepartum NEFA. A major challenge for prepartum blood testing programs is the inability to know exactly when parturition will occur; actual calving dates can vary from the predicted dates by ± 5 d. It would be very difficult for producers to accurately select cows for sampling that were exactly in their second-to-last week prepartum, and thus very difficult for producers to accurately interpret the results of the fecal cortisol metabolite assay without knowing precisely when the sample was collected prior to parturition. Because prepartum NEFA is associated with health status during each of the 3 wk before calving in MP cows, interpretation of the data would still be possible without knowing the exact proximity to parturition.

CONCLUSION

In general the relationships between prepartum analytes and postpartum health status

were stronger among MP cows than among PP cow and, with the exception of prepartum NEFA, there were no associations detected between prepartum concentrations of our analytes of interest and the occurrence of one disorder (RP, DA, SCK or HiHP) by 30 DIM. After accounting for important covariates including parity and calving assistance, prepartum Hp was not a significant predictor of cows that would go on to develop more than one disorder or die by 30 DIM and prepartum plasma cortisol and fecal cortisol metabolites were only associated with this health when measured during wk -2 relative to calving. Prepartum NEFA concentrations were associated with the development of more than one disorder or death by 30 DIM during all three weeks prior to calving and the strength of these associations were greater than the strength of the associations between plasma cortisol or fecal cortisol metabolites during wk -2 and this health outcome (lower type I error risk). Using a sampling protocol that mimics field-level herd testing (i.e., cross-sectional sampling with an appropriate sample size), NEFA is a more suitable analyte to evaluate, relative to fecal cortisol metabolites, plasma Hp, or plasma cortisol for identifying opportunities to improve herd health.

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CHAPTER THREE

ASSOCIATIONS OF PERIPARTUM PLASMA CORTISOL, HAPTOGLOBIN, FECAL CORTISOL METABOLITES AND NONESTERIFIED FATTY ACIDS WITH REPRODUCTIVE PERFORMANCE AND MILK YIELD IN HOLSTEIN DAIRY COWS

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ABSTRACT

The objectives of this study were to evaluate the relationship between peripartum concentrations of nonesterified fatty acids (**NEFA**), plasma cortisol, fecal cortisol metabolites (11,17-dioxoandrostane; **11,17-DOA**), and plasma haptoglobin (**Hp**) with milk yield and reproductive performance. Blood and fecal samples were collected weekly from 412 Holstein dairy cows from wk -3 through wk +1 (d 3 to 10 postpartum) relative to calving. Reproduction and milk production were the 2 outcomes of interest and measured as conception within 150 DIM and projected 305ME milk yield at 102 DIM, respectively. The associations between plasma NEFA, Hp and 11,17-DOA with reproductive performance were strongest when analytes were measured during the week before or after calving and were stronger among primiparous (**PP**) cows than multiparous (**MP**) cows. Primiparous cows with Hp > 1.3 g/L or NEFA > 0.45 mEq/L after calving (3 to 10 DIM) had a 41% or 39% decreased risk of conception, respectively, compared to primiparous cows below these concentration thresholds. Increased plasma cortisol concentrations among MP cows during wk -2 (> 31.1 nmol/L) and wk -1 (> 38.6 nmol/L) were associated with an increased risk of conception, while PP cows with increased 11-17-DOA during wk -2 and wk -1 (> 2300 ng/g fecal DM) had a 36 to 42% lower risk of conception. The strongest associations between Hp, plasma cortisol, and 11,17-DOA with 305ME milk yield were observed when these analytes were measured during the postpartum period. Postpartum Hp > 1.1 g/L was associated with 947 kg lower 305ME milk yield and plasma cortisol > 19.3 nmol/L was associated with 666 kg increase in 305ME milk yield among all sampled animals. Among MP cows only increased 11,17-DOA concentrations after calving (> 400 ng/g fecal DM) were associated with a 663 kg lower 305ME milk yield. During wk -2 and -1 relative to calving increased NEFA concentrations were better predictors of postpartum milk performance; PP and MP cows with NEFA > 0.55 mEq/L during wk -1 had a 1360 kg lower projected 305ME milk

yield. In each model tested, Hp and 11,17-DOA remained the most significant predictor (lowest risk of committing a type I error) of milk yield and risk of conception, when NEFA was included as a covariate. Additionally, in particularly during the period after calving, increased Hp and 11,17-DOA concentrations are associated with greater estimates of lower 305ME milk yield and lower conception rates; therefore, analyte testing programs on dairy herds aimed at identifying opportunities to improve milk yield or reproductive performance may benefit by measuring 11,17-DOA or Hp in addition to NEFA.

Key words: milk production, reproduction, cortisol, haptoglobin

INTRODUCTION

Improving the early detection of animals or herds at increased risk for reduced milk yield or compromised reproductive performance is important component of herd health programs. The period around calving (transition period) is characterized by nutritional and metabolic stressors as cows must adapt to the increased energy demands of lactation. Although there are significant physiological adaptations that occur to prioritize nutrient use in order to facilitate lactation (Bauman and Currie, 1980), dry matter intake (**DMI**) during the first few weeks following calving is insufficient to support energy expenditure and so the transition period is characterized by a period of negative energy balance (Grummer et al., 2004). Elevated concentrations of plasma nonesterified fatty acid **(NEFA**) and β-hyrdoxybutyrate (**BHBA**), both indicators of negative energy balance, have repeatedly been shown to be associated with reduced milk yield and increased time to conception (Reist et al., 2000; Duffield et al., 2009; Walsh et al., 2007; Ospina et al., 2010). For example, Ospina et al. (2010) reported that the risk of pregnancy within 70-d post-voluntary waiting period was 19% lower for cattle with a NEFA concentration ≥ 0.27 mEq/L during the 2-wk period before calving and that projected 305-d mature equivalent milk

yield was decreased by 683 kg when NEFA was ≥ 0.33 mEq/L during this prepartum period.

In addition to nutritional stressors during the transition period cattle also face numerous non-nutritional stressors during this time. The transition period is characterized by social regroupings, pen-moves, cow-calf separation, and for primiparous animals exposures to novel environments such as the milking parlor. Compared to the work done on physiological measures of energy balance, there has been far less research done to explore physiological measures of stress during transition and their association with milk yield and reproductive success.

Despite its widespread use as a physiological measure of stress in animals, plasma cortisol concentrations are easily confounded by the stress associated with handling and sampling collection which makes interpretation of concentration levels difficult. Fecal cortisol metabolites may be an alternative to plasma cortisol as a measure of the stress response in cattle because concentrations of these metabolites are not subject to the acute feedback mechanisms that plasma cortisol is subject to (Palme et al., 1999). Recent work has shown that increased concentrations of fecal cortisol metabolites during wk -2 relative to calving can be used to identify cows at increased risk for disease following after calving (Huzzey et al., 2011). The relationship of peripartum concentrations of cortisol with reproductive performance and milk yield, including the identification of cortisol concentration thresholds above which predict compromised performance, have yet to be investigated.

Non-nutritional stressors experienced during the transition period may also be associated with immune or inflammatory responses in cattle. There has been substantial work done to show that the transition period is a time of compromised immune status in dairy cattle (reviewed in Mallard et al., 1998). Increased concentrations of analytes associated with immune system activation may also provide information about subsequent risk for health or production complications. There has been increasing interest in using Haptoglobin (**Hp**) concentrations as

an early indicator of disease due to its high relative increase during immune stimulation from the low concentration thresholds observed in healthy animals (Eckersall, 2000). Previous work has shown that Hp is elevated prior to the observation of clinical metritis (Huzzey et al., 2009) and is increased during environmental stressors such as transportation and social re-grouping in cattle (Arthington et al., 2003). Recently Chan et al. (2010) described an association between increased Hp concentrations (Hp concentrations > 0.13 g/L between 2 – 6 mo. postpartum) with longer days to conception; however, this study used a small population of animals and did not identify predictive Hp concentrations thresholds of reproductive performance, during the transition period. Increased concentrations of Hp may be associated with lower milk production due to its positive relationship with subclinical and clinical mastitis (Grönlund et al., 2004).

The objectives of this study were to: 1) evaluate the associations of peripartum concentrations of plasma cortisol, fecal cortisol metabolites (11,17-dioxoandrostane), plasma Hp and plasma NEFA as biomarkers of stress, inflammation, and energy balance, with milk yield and reproductive performance, 2) establish cutpoints for these analytes that could serve as predictive thresholds for identifying animals at increased risk for reduced milk yield or reproductive performance, and 3) determine whether analytes associated with stress and inflammation provide additional information relative to NEFA, pertaining to level of risk for compromised milk yield and reproductive performance.

MATERIALS AND METHODS

Study Population and Study Design

The Cornell University Institutional Animal Care and Use Committee approved all procedures involving animals before the beginning of the study. This study was conducted

between February 2008 and July 2008 on two commercial herds in New York State. During this period, a convenience sample of 12 to 20 animals that were within 4 wk of calving were selected each week for enrollment into the study. Any animals that were visibly sick or lame were excluded from enrollment. On farm A, 202 cows were sampled [113 multiparous cows (**MP**) and 89 primiparous cows (**PP**)] and on Farm B, 210 cows were sampled (117 MP and 93 PP) for a total enrollment of 412 animals in the study. Primiparous cows were purposefully targeted to increase the number of PP cows sampled so that statistically valid comparisons between parities could be made. The average parity (mean ± SD) of all animals (including the calving event that occurred during the course of the study) was 2.3 ± 1.6 on Farm A and 2.1 ± 1.4 on Farm B, with an overall average of 2.2 ± 1.5. All cows were housed in free stall pens before and after calving, and were moved to bedded pack maternity pens for the calving event. After calving, at the discretion of farm staff, cows were moved into either a high lactation fresh group or a transition fresh group if there were complications associated with calving (i.e. clinical disease). On Farm B PP and MP cows were housed separately after calving; however, at all other periods during the study all parities were co-mingled.

Cows on both herds were fed once daily in the morning, with feed push ups occurring at regular intervals throughout the day. Weekly samples of the total mixed ration (**TMR**) were collected from the close-up and fresh cow groups, combined into 4-wk composite samples and submitted to a commercial laboratory for wet chemistry analysis (Forage Testing Laboratory, Dairy One Cooperative Inc., Ithaca, NY; Table 3.1.). Cows had ad libitum access to water.

Table 3.1. Component analysis (mean ± SD; % of dry matter unless otherwise noted) of 4-wk composite TMR samples collected between February and July 2008 from Farm A and B.

	Close-up Cow TMR		Fresh Cow TMR	
Component	Farm A	Farm B	Farm A	Farm B
CP	13.8 ± 3.0	13.5 ± 2.0	18.2 ± 0.7	17.9 ± 0.8
ADF	31.3 ± 7.3	28.7 ± 4.2	18.8 ± 1.3	19.3 ± 0.9
NDF	48.9 ± 3.0	47.0 ± 5.6	31.3 ± 2.8	31.8 ± 2.2
NE_L (Mcal/kg)	1.55 ± 0.06	1.56 ± 0.04	1.68 ± 0.03	1.67 ± 0.02
Ca	1.31 ± 0.56	1.14 ± 0.42	0.99 ± 0.08	0.98 ± 0.10
P	0.33 ± 0.07	0.37 ± 0.03	0.45 ± 0.01	0.38 ± 0.02
Mg	0.38 ± 0.09	0.37 ± 0.09	0.37 ± 0.01	0.36 ± 0.03
K	1.12 ± 0.09	1.01 ± 0.18	1.27 ± 0.06	1.25 ± 0.08
Na	0.12 ± 0.04	0.11 ± 0.03	0.47 ± 0.05	0.51 ± 0.09

Blood and fecal samples were collected weekly from the 412 enrolled cows beginning approximately 4 wk before each individual's expected calving date and continued until parturition. A postpartum blood sample was collected between 3 and 10 DIM from all study animals. Blood was collected from the coccygeal vessels into 10-mL sterile tubes coated with sodium heparin (BD Vacutainer, Franklin Lakes, NJ, USA) then stored in coolers until they could be returned to the lab for processing. Plasma was harvested after centrifugation (2,800 × *g* for 15 min at 4˚C) and stored at -20˚C for later analyses. Plasma concentrations of NEFA and haptoglobin (Hp) were analyzed by enzymatic analysis (NEFA-C: Wako Pure Chemical Industries, Osaka, Japan; Phase Range Haptoglobin Assay: Kit no. TP801, Tridelta Diagnostics Ltd., NJ, USA). Intra- and interassay CV for the NEFA assay were 2.2 and 4.7%, respectively and for the Hp assay were 6.2 and 10.0%, respectively. All spectrophotometric measurements

were conducted using a $Versa_{max}$ tunable microplate reader (Molecular Devices, Sunnyvale, CA). A commercial solid-phase radioimmunoassay kit was used to determine plasma concentrations of cortisol (Coat-A-Count Cortisol RIA, Diagnostic Products, Los Angeles, CA). Intra- and interassay CV for the cortisol assay were 1.7 and 2.9%, respectively. Fecal samples were collected fresh and stored on ice, until they could be frozen at -20˚C for later processing. Steroids from the fecal samples were extracted using the wet extraction method described by Palme and Möstl (1997). The percentage DM of each fecal sample was obtained by weighing samples before and after drying in a hot oven (105˚C) for 24 hrs. Fecal cortisol metabolite concentrations (11,17-dioxoandrostanes, **11,17-DOA**) were measured using a competitive enzyme immunoassay developed by Palme and Möstl (1997) and validated for use in cattle (Palme et al. 1999). Intra- and interassay CV for the 11-17-DOA assay were 3.9 and 6.5%, respectively. To reduce the variation associated with differences in manure consistency, the concentration of 11,17-DOA obtained from the assay were adjusted for the DM content of the raw fecal sample and reported on a DM matter basis.

At the end of the study blood and fecal samples were sorted by week relative to actual calving. Samples collected during d -21 to d -15 relative to the actual calving date were used to represent wk -3, d -14 to d -8 (wk -2), d -7 to d -2 (wk -1), and d +3 to d +10 (postpartum). Samples collected during d -1 were not included in the data set due to the rapid increase in circulating cortisol concentration that occurs during the day before parturition (Patel et al. 1996) and samples were not collected during the first 2 d following calving due to the natural elevation in plasma Hp associated with parturition (Uchida et al., 1993; Huzzey et al., 2009).

The reproductive information collected consisted of DIM at first breeding, calving date, DIM at conception, and (when applicable) the date the cow was no longer in herd (due to culling or death) and the pregnancy status at removal from herd. Both herds utilized an ovulation

synchronization program; cows displaying visible estrus during the pre-synchronization stage were bred and the rest of the cows were bred on timed AI. The voluntary waiting period for both herds was 50 DIM. Pregnancy diagnosis was performed by the herd veterinarian by rectal palpation or ultrasound and recorded by herd personnel. Reproductive success was measured as pregnancy within 150 DIM. Parity was dichotomized into 2 groups: parity 1 (PP) or ≥ 2 (MP). Body condition score was assessed at the time of the postpartum sample collection and was dichotomized into 2 groups: < 3.75 or ≥ 3.75.

Milk production information was collected as predicted 305-Mature Equivalent (**305ME**) milk yield measured at the 3rd test date, which occurred on average (± SD) at 102 ± 17 DIM. The DIM at the 3rd test date was recorded for each cow and log linear somatic cell count (SCC) scores from the 2nd and 3rd test dates were averaged to provide an estimate of overall log linear SCC score for each cow. This information was retrieved from each herd's on-farm Dairy Comp 305 records.

Statistical Analyses

All data were stratified based on time of sample collection (i.e. wk -3, wk -2, wk -1, and postpartum) for analyses. Reproduction and milk production were the 2 outcomes of interest and measured as conception within 150 DIM and projected 305ME milk yield at 102 DIM, respectively. For both of these outcomes, plasma NEFA, plasma cortisol, Hp, and 11,17-DOA were each evaluated independently as categorical predictors in 4 models based on the sampling period relative to calving (wk -3, wk -2, wk -1, and postpartum).

Analyte concentrations were categorized by first identifying a concentration threshold (cutpoint) to dichotomize the analyte by, then assigning a 0 to cows below the cutpoint and a 1 to cows above the cutpoint. Appropriate analyte concentration thresholds for evaluating subsequent milk yield and reproductive performance were determined first by creating incremental cutpoints

for each analyte of interest within a biologically relevant concentration range and then examining the threshold that had the greatest impact on milk yield or reproductive outcome with the smallest type I error risk.

Plasma NEFA concentration thresholds were explored within a prepartum range of 0.25 to 0.75 mEq/L and a postpartum range of 0.40 to 0.85 mEq/L (0.05 mEq/L increments); this range was established around the NEFA cutpoints identified by Ospina et al. (2010) for describing risk of conception and subsequent milk yield. For the analysis of haptoglobin, incremental cutpoints of 0.1 g/L within the range of 0.2 to 1.4 g/L were evaluated; this range was based on the work of Huzzey et al. (2009) that evaluated a range of Hp concentrations for the evaluation of subsequent risk for metritis. For the analysis of 11,17-DOA, incremental cutpoints of 50 to 100 ng/g fecal DM within the range of 1800 to 3000 ng/g fecal DM prepartum and 300 to 1000 ng/g fecal DM postpartum were evaluated. Plasma cortisol thresholds were explored within a range of 0.4 to 1.4 µg/dL (11.0 to 38.6 nmol/L) in 0.1 µg/dL increments. The concentration ranges for both 11,17-DOA and plasma cortisol were based on a univariate analysis of the analyte distributions at each week relative to calving; the concentration range that was explored fell within the 50^{th} and 95^{th} analyte quantiles.

Because previous research has shown NEFA to be a useful predictor of reproductive performance and milk yield (Ospina et al., 2010), NEFA was included as a covariate in the models testing our main predictive analytes of interest (Hp, Cortisol, and 11,17-DOA). This was done to determine whether these alternative analytes could explain additional variation in the outcomes of interest (milk yield and reproductive performance) that NEFA could not explain.

Reproduction. Time to conception was modeled using a semiparametric proportional hazards model (Cox, 1972) with Prog Phreg (SAS Institute, 2009). In addition to the main effects of NEFA, Hp, plasma cortisol, and 11,17-DOA, the covariates farm, BCS, parity, 305ME milk

and NEFA (where appropriate) were evaluated. Initially, PP and MP cows were evaluated separately, but if the association between the covariates and the outcome of interest was similar they were grouped together for the final analysis. Projected 305ME milk yield was dichotomized based on the median production of all 412 experimental cows for inclusion as a covariate in this analysis. The effect of an elevated analyte concentration on time to conception was evaluated by assessing a range of critical thresholds for the analyte of interest and dichotomizing the concentrations based on these thresholds. The dichotomized NEFA, Hp, plasma cortisol, and 11,17-DOA concentration that resulted in the smallest chance of committing a type I error and largest estimate (hazard ratio for time to conception) was kept in the final model. Kaplan-Meier estimator graphs (Proc Lifetest) of the time to conception were created for animals that were above and below the critical threshold found to be the most strongly associated with reproductive performance (SAS Institute, 2009).

Milk Production. Predicted 305ME milk yield was modeled using Proc Mixed in SAS (2009). In addition to the main predictors (NEFA, Hp, plasma cortisol, and 11,17-DOA), the covariates farm, parity, BCS, average log linear SCC, DIM at the 3rd test day, sample period NEFA concentration (where appropriate), and the interaction between parity and the analyte of interest were evaluated. If an interaction was detected between parity and the predictive analyte ($P \leq 0.05$), the data were stratified by parity. The effects of an elevated analyte concentration on projected 305ME milk yield were evaluated by assessing a range of critical thresholds for the analyte of interest and dichotomizing the concentrations based on these thresholds. The dichotomized NEFA, Hp, plasma cortisol, and 11,17-DOA concentration that resulted in the smallest chance of committing a type I error and largest 305ME estimate was kept in the final model.

RESULTS

Descriptive Data

The average (± SD) days to first breeding was 67 ± 11 d on Farm A and 68 ± 19 d on Farm B. In total 180 animals (69 PP and 111 MP cows) were right-censored for the reproductive performance analysis from the original 412 animals enrolled in the study. Among the censored PP cattle 6 died, 14 were sold, and 49 were not pregnant by 150 DIM. Among the censored MP cattle 24 died, 22 were sold, and 65 were not pregnant by 150 DIM. For PP cattle that did become pregnant within 150 DIM (n = 113) the conception rate at first AI was 50% and for the MP cattle that became pregnant by 150 DIM (n = 119) the conception rate at first AI was 53%.

In total 18 PP cows and 39 MP cows were excluded from the milk production analyses due to early culling (sold or died) prior to the 3rd DHI test day. For the remaining 164 PP cows the median (range) 305ME milk yield assessed at 102 DIM was 12,986 (5,382 – 19,077) kg and for the remaining 191 MP cows the median (range) 305ME milk yield was 12,586 (5,895 – 17,150) kg.

Compared to the other periods of analysis, there were very few associations detected between concentrations of the analytes of interest measured during wk -3 and the outcomes of interest. When associations were detected they were always weaker (increased risk of committing a type 1 error) than the associations observed during the other periods relative to calving; therefore, the following results will highlight only the associations observed for samples collected during wk -2 and wk -1 before calving and between 3 to 10 d after calving.

Analytes to Predict Reproductive Performance

NEFA. Dichotomized concentrations of plasma NEFA during wk -2 within a range of critical thresholds (0.25 to 0.75 mEq/L) were not associated with reproductive performance in either PP or MP cattle ($P > 0.21$). Plasma NEFA concentrations were not associated with

reproductive performance in MP cows across a range of potential thresholds during wk -1 (0.25 to 0.75 mEq/L; $P \geq 0.18$) or the postpartum period (0.45 to 0.85 mEq/L; $P \geq 0.19$). For PP cows, a NEFA concentration > 0.40 mEq/L during wk -1 was associated with a 42% decreased risk of conception (hazard ratio = 0.58; $P = 0.02$; Table 3.2) while a NEFA concentration > 0.45 mEq/L during the postpartum period was associated with a 39% decreased risk of conception (hazard ratio = 0.61; $P = 0.02$; Table 3.2); 44% and 40% of sampled PP cows were above these thresholds during the week before and after calving, respectively. Figure 3.1A presents the Kaplan-Meier graph of time to conception for PP cows with NEFA concentrations above or below the postpartum critical threshold of 0.45 mEq/L.

Haptoglobin. Among MP cows, a Hp concentration > 0.70 g/L during wk -2 was associated with a 102% increase in the risk of conception (hazard ratio = 2.02; $P = 0.02$; Table 3.3); 9% of the sampled MP cows had Hp concentrations above this threshold. Plasma Hp concentrations were not associated with reproductive performance in MP cows across a range of potential thresholds (0.2 to 1.4 g/L) during wk -1 ($P \geq 0.50$) or the postpartum period ($P \geq 0.40$). Among PP cows, a Hp concentration > 0.4 g/L during the wk before calving was associated with a 41% decreased risk of conception (hazard ratio = 0.59; $P = 0.05$; Table 3.3) and a Hp concentration > 1.3 g/L during the postpartum period (3 to 10 DIM) was also associated with a 41% decreased risk of conception (hazard ratio = 0.59; $P = 0.02$; Table 3.3); 22% and 31% of sampled PP cows were above these thresholds during the wk before and after calving, respectively. Figure 3.1B presents the Kaplan-Meier graph of time to conception for PP cows with Hp concentrations above or below the postpartum critical threshold of 1.3 g/L.

Plasma Cortisol and Fecal Cortisol Metabolites. Dichotomized concentrations of plasma cortisol (between 11.0 to 38.6 nmol/L) and 11,17-DOA (between 300 to 1000 ng/g fecal DM),

measured during the postpartum period were not associated with reproductive performance ($P \geq$ 0. 27 and $P \geq$ 0. 19, respectively).

Increased plasma cortisol concentrations during wk -2 and -1 were associated with an increased risk of conception among MP cows only (Table 3.4); there was no association between this analyte and reproductive performance in PP cows. During wk -2, MP cows with a plasma cortisol concentration > 33.1 nmol/L tended to have a 58% increased risk of conception (hazard ratio = 1.58; $P = 0.06$; Table 3.4); 15% of the sampled MP cows were above this threshold. During wk -1, MP cows with plasma cortisol concentrations > 38.6 nmol/L during wk -1 had a 127% increased risk of conception (hazard ratio = 2.27; $P = 0.02$; Table 3.4); 9% of the sampled MP cows were above this threshold during the wk before calving.

Increased 11,17-DOA concentrations during wk -2 and -1 were associated with a lower risk of conception among PP cows only (Table 3.5); there was no association between this analyte and reproductive performance in MP cows. During wk -2 and wk -1, 11,17-DOA concentrations > 2300 ng/g fecal DM were associated with a 36% and 42% decreased risk of conception, respectively (wk -2 hazard ratio = 0.64, $P = 0.05$; wk -1 hazard ratio = 0.58, $P =$ 0.02; Table 3.5); 30% and 42% of the sampled PP cows had 11,17-DOA concentrations above this threshold during wk -2 and -1 relative to calving, respectively.

Biomarkers to Predict Milk Yield

NEFA. Concentrations of plasma NEFA were associated with milk production during wk -2 and -1 relative to calving and during the postpartum period (Tables 3.6, 3.7, and 3.8 respectively). During wk -2, for MP cows only, those with NEFA > 0.45 mEq/L had 1464 kg lower projected 305ME milk yield ($P = 0.001$); 16% of sampled MP cows were above this threshold. During wk -1, both PP and MP cows with NEFA > 0.55 mEq/L had 1360 kg lower 305ME milk yield ($P = 0.002$); 19% of the sampled animals were above this threshold.

Increased NEFA during the postpartum period (> 0.70 mEq/L) among PP cows was associated with a 1049 kg increase in 305ME milk yield ($P = 0.02$) while MP cows above this threshold tended to have 592 kg lower 305ME milk yield projections ($P = 0.08$); 15% of PP cows and 25% of MP cows were above this NEFA threshold after calving.

Haptoglobin. Concentrations of plasma Hp were associated with milk yield during wk -1 relative to calving and during the postpartum period (Tables 3.7 and 3.8). When NEFA was included as a covariate in each of the models exploring the relationship between Hp concentration and milk yield, NEFA was found to be either the least significant predictive analyte (increased type I error risk) or was not a significant predictor relative to Hp. Among MP cows only sampled during wk -1, Hp > 0.20 g/L was associated with a 943 kg lower 305ME milk yield projection ($P = 0.01$); 23% of MP cows were above this Hp threshold. After calving, MP and PP cows with a Hp concentration > 1.1 g/L had 947 kg lower 305ME milk yield projections ($P = 0.001$); 33% of the animals sampled postpartum were above this Hp threshold.

Plasma Cortisol and Fecal Cortisol Metabolites. Concentrations of plasma cortisol and 11,17-DOA were associated with milk yield when measured during the postpartum period only (Table 3.8). Among MP cows only and after accounting for postpartum NEFA concentrations as a covariate, 11,17-DOA concentrations > 400 ng/g fecal DM were associated with a 663 kg lower 305ME milk yield projection ($P = 0.03$); 32% of the sampled MP cows were above this 11,17-DOA threshold. Increased plasma cortisol concentration (> 19.3 nmol/L) during the postpartum period was associated with a 666 kg increase in 305ME milk yield for both MP and PP cows ($P = 0.009$); in total 25% of the sampled animals were above this threshold.

Table 3.2. Cox proportional hazards models by study period for the effect of nonesterified fatty acid (NEFA) and covariates on days to conception within 150 DIM (n = 412).

		Primiparous Cows				Multiparous Cows			
Model (Period)	Variable	Parameter Estimate	SE	Hazard Ratio	*P*	Parameter Estimate	SE	Hazard Ratio	*P*
1 (wk -1)	NEFA (> 0.40 mEq/L)	-0.55	0.23	0.58	0.02	0.09	0.23	1.09	0.70
	Farm[1]	-0.47	0.23	0.63	0.04	0.90	0.23	2.46	0.001
	BCS[2]	0.39	0.22	1.47	0.08	0.39	0.33	1.48	0.23
	305ME[3]	-0.27	0.21	0.77	0.22	0.30	0.21	1.35	0.15
2 (Postpartum)	NEFA (> 0.45 mEq/L)	-0.49	0.21	0.61	0.02	-0.26	0.20	0.77	0.19
	Farm[1]	-0.25	0.19	0.78	0.20	0.77	0.21	2.16	0.001
	BCS[2]	0.14	0.21	1.16	0.48	0.39	0.32	1.48	0.22
	305ME[3]	-0.15	0.19	0.86	0.43	0.28	0.19	1.33	0.14

[1]Farm dichotomized as B vs. A.

[2]BCS dichotomized as ≥ 3.75 vs. < 3.75.

[3]305 Mature Equivalent milk yield dichotomized as ≥ vs. < than median 305ME projected at 102 DIM for all animals.

Table 3.3. Cox proportional hazards models by study period for the effect of Haptoglobin (Hp) and covariates on days to conception within 150 DIM (n = 412).

		Primiparous Cows				Multiparous Cows			
Model (Period)	Variable	Parameter Estimate	SE	Hazard Ratio	*P*	Parameter Estimate	SE	Hazard Ratio	*P*
1 (wk -2)	Hp (> 0.70 g/L)	-0.06	0.26	0.94	0.81	0.70	0.31	2.02	0.02
	Farm[1]	-0.43	0.21	0.65	0.05	0.87	0.21	2.39	0.001
	BCS[2]	0.24	0.20	1.28	0.23	0.39	0.31	1.47	0.21
	305ME[3]	-0.21	0.20	0.81	0.28	0.39	0.20	1.47	0.05
	NEFA[4]	1.12×10^{-3}	6.65×10^{-4}	1.00	0.08	4.37×10^{-4}	4.32×10^{-4}	1.00	0.31
2 (wk -1)	Hp (> 0.40 g/L)	-0.53	0.27	0.59	0.05	-0.16	0.34	0.85	0.63
	Farm[1]	-0.18	0.21	0.84	0.39	0.99	0.23	2.70	0.001
	BCS[2]	0.43	0.22	1.53	0.05	0.37	0.33	1.45	0.25
	305ME[3]	-0.27	0.21	0.77	0.20	0.29	0.21	1.34	0.17
	NEFA[4]	-	-	-	-	5.36×10^{-4}	4.18×10^{-4}	1.00	0.20
3 (Postpartum)	Hp (> 1.3 g/L)	-0.52	0.23	0.59	0.02	-0.18	0.25	0.84	0.48
	Farm[1]	-0.23	0.19	0.80	0.24	0.80	0.22	2.23	0.001
	BCS[2]	0.22	0.20	1.25	0.27	0.32	0.32	1.38	0.31
	305ME[3]	-0.21	0.20	0.81	0.29	0.27	0.19	1.32	0.16
	NEFA[4]	3.04×10^{-4}	3.74×10^{-4}	1.00	0.42	9.97×10^{-5}	3.79×10^{-4}	1.00	0.79

[1]Farm dichotomized as B vs. A.

[2]BCS dichotomized as ≥ 3.75 vs. < 3.75.

[3]305 Mature Equivalent milk yield dichotomized as ≥ vs. < than median 305ME projected at 102 DIM for all animals.

[4]NEFA modeled as a continuous predictor. During wk -1 when NEFA was included in the primiparous cow model it was not associated with the reproduction outcome ($P = 0.99$); however, its inclusion in the model significantly altered the association between Hp and reproductive performance (type I error risk); therefore, NEFA was removed from the final model.

Table 3.4. Cox proportional hazards models by study period for the effect of plasma cortisol (PCORT) and covariates on days to conception within 150 DIM.

		Primiparous Cows				Multiparous Cows			
Model (Period)	Variable	Parameter Estimate	SE	Hazard Ratio	*P*	Parameter Estimate	SE	Hazard Ratio	*P*
1 (wk -2)	PCORT (> 33.1 nmol/L)	-0.45	0.46	0.64	0.34	0.45	0.24	1.58	0.06
	Farm[1]	-0.40	0.21	0.67	0.05	0.81	0.21	2.25	0.001
	BCS[2]	0.22	0.20	1.25	0.27	0.35	0.31	1.41	0.26
	305ME[3]	-0.20	0.20	0.82	0.30	0.26	0.19	1.30	0.18
	NEFA[4]	-1.16×10^{-3}	6.62×10^{-4}	1.00	0.08	-	-	-	-
2 (wk -1)	PCORT (> 38.6 nmol/L)	-0.27	0.52	0.77	0.61	0.82	0.35	2.27	0.02
	Farm[1]	-0.46	0.23	0.63	0.05	0.98	0.23	2.67	0.001
	BCS[2]	0.40	0.22	1.49	0.07	0.46	0.32	1.58	0.16
	305ME[3]	-0.28	0.21	0.76	0.19	0.34	0.21	1.40	0.11
	NEFA[4]	-1.14×10^{-3}	6.56×10^{-4}	1.00	0.08	3.89×10^{-4}	3.84×10^{-4}	1.00	0.31

[1]Farm dichotomized as B vs. A.

[2]BCS dichotomized as ≥ 3.75 vs. < 3.75.

[3]305 Mature Equivalent milk yield dichotomized as ≥ vs. < than median 305ME projected at 102 DIM for all animals.

[4]NEFA modeled as a continuous predictor. During wk -2 when NEFA was included in the multiparous cow model it was not associated with the reproduction outcome ($P = 0.23$); however, its inclusion in the model significantly altered the association between PCORT and reproductive performance (type I error risk); therefore, NEFA was removed from the final model.

Table 3.5. Cox proportional hazards models by study period for the effect of fecal cortisol metabolites (11,17-DOA) and covariates on days to conception within 150 DIM.

		Primiparous Cows				Multiparous Cows			
Model (Period)	Variable	Parameter Estimate	SE	Hazard Ratio	*P*	Parameter Estimate	SE	Hazard Ratio	*P*
1 (wk -2)	11,17-DOA (> 2300 ng/g fecal DM)	-0.44	0.23	0.64	0.05	-0.06	0.31	0.95	0.85
	Farm[1]	-0.24	0.19	0.79	0.22	0.82	0.21	2.26	0.001
	BCS[2]	0.30	0.20	1.35	0.14	0.31	0.31	1.37	0.31
	305ME[3]	-0.24	0.20	0.79	0.23	0.34	0.19	1.40	0.08
	NEFA[4]	-	-	-	-	6.91×10^{-4}	4.14×10^{-4}	1.00	0.10
2 (wk -1)	11,17-DOA (> 2300 ng/g fecal DM)	-0.54	0.23	0.58	0.02	-0.18	0.30	0.84	0.55
	Farm[1]	-0.43	0.23	0.65	0.06	1.00	0.23	2.72	0.001
	BCS[2]	0.42	0.22	1.52	0.06	0.43	0.33	1.53	0.20
	305ME[3]	-0.27	0.21	0.76	0.20	0.31	0.21	1.37	0.14
	NEFA[4]	-7.26×10^{-4}	6.61×10^{-4}	1.00	0.27	5.53×10^{-4}	4.18×10^{-4}	1.00	0.19

[1]Farm dichotomized as B vs. A.

[2]BCS dichotomized as ≥ 3.75 vs. < 3.75.

[3]305 Mature Equivalent milk yield dichotomized as ≥ vs. < than median 305ME projected at 102 DIM for all animals.

[4]NEFA modeled as a continuous predictor. During wk -2 when NEFA was included in the primiparous cow model it was not associated with the reproduction outcome ($P = 0.15$); however, its inclusion in the model significantly altered the association between 11,17-DOA and reproductive performance (type I error risk); therefore, NEFA was removed from the final model.

Table 3.6. Mixed model for the effect of NEFA during wk -2 and covariates on milk production measured by 102 DIM mature-equivalent 305-d milk yield (305ME). Data were stratified by parity due to a significant NEFA x Parity interaction (P = 0.04).

		Multiparous Cows			Primiparous Cows		
Variables	Reference	Difference in 305ME (kg)	SE	P	Difference in 305ME (kg)	SE	P
SCC Linear Score	per unit increase	-216	83	0.01	-286	109	0.01
DIM at test day	per unit increase	35	12	0.01	11	13	0.39
NEFA	> vs. < 0.45 mEq/L	-1464	411	0.001	-197	374	0.60

***** Farm and BCS were not associated with 305ME milk yield ($P \geq 0.56$); therefore, the 305ME estimates for these covariates are not shown.

Table 3.7. Mixed model for the effect of NEFA and Hp during wk -1 and covariates on milk production measured by 102 DIM mature-equivalent 305-d milk yield (305ME). Data for the Hp model were stratified by parity due to a significant wk -1 Hp x Parity interaction (P = 0.04).

		All Animals			Multiparous Cows (MP)			Primiparous Cows (PP)		
Variables	Reference	Difference in 305ME	SE	*P*	Difference in 305ME	SE	*P*	Difference in 305ME	SE	*P*
Model 1: NEFA (wk -1)										
Parity	PP vs. MP	889	557	0.11	-	-	-	-	-	-
SCC Linear Score	per unit increase	-266	72	0.001	-	-	-	-	-	-
DIM at test day	per unit increase	23	10	0.02	-	-	-	-	-	-
NEFA	> vs. < 0.55	-1360	394	0.002	-	-	-	-	-	-
NEFA x Parity	-	-	-	0.19	-	-	-	-	-	-
Model 2: Hp (wk -1)										
SCC Linear Score	per unit increase	-	-	-	-232	92	0.01	-283	134	0.04
DIM at test day	per unit increase	-	-	-	35	14	0.01	10	14	0.50
NEFA (wk -1; mEq/L)	per unit increase	-	-	-	-0.79	0.60	0.19	-0.39	0.85	0.65
Hp	> vs. < 0.20 g/L	-	-	-	-943	375	0.01	1	405	0.99

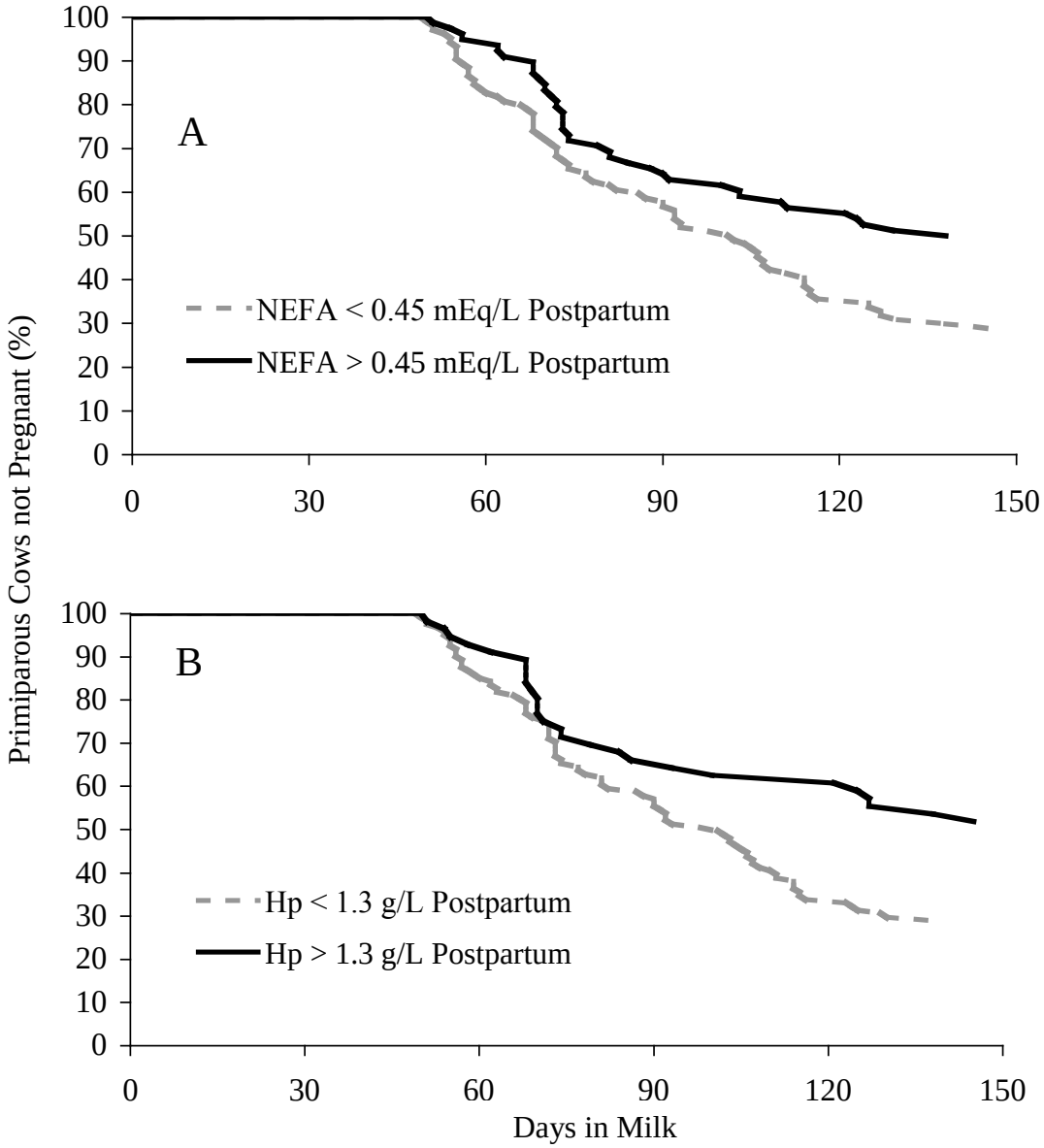
***** Farm and BCS were not associated with 305ME milk yield ($P \geq 0.41$); therefore, the 305ME estimates for these covariates are not shown.

Table 3.8. Mixed model for the effect of NEFA, haptoglobin (Hp), plasma cortisol (PCORT), and fecal cortisol metabolite (11,17-DOA; units = ng/g fecal DM) concentrations measured during the postpartum period (3 to 10 DIM) and covariates on milk production measured by 102 DIM mature-equivalent 305-d milk yield (305ME). Data for the NEFA and 11,17-DOA models were stratified by parity due to a significant NEFA x Parity (P = 0.005) and 11,17-DOA x Parity (P = 0.05) interactions.

		All Animals			Multiparous Cows (MP)			Primiparous Cows (PP)		
Variables	Reference	Difference in 305ME (kg)	SE	P	Difference in 305ME (kg)	SE	P	Difference in 305ME (kg)	SE	P
Model 1: NEFA (Postpartum)		-	-	-						
SCC Linear Score	per unit increase	-	-	-	-250	85	0.004	-266	107	0.01
DIM at test day	per unit increase	-	-	-	33	13	0.01	6	13	0.62
NEFA	> vs. < 0.70 mEq/L	-	-	-	-592	333	0.08	1049	428	0.02
Model 2: Hp (Postpartum)										
Parity	PP vs. MP	-327	561	0.36	-	-	-	-	-	-
SCC Linear Score	per unit increase	-200	65	0.002	-	-	-	-	-	-
DIM at test day	per unit increase	20	9	0.02	-	-	-	-	-	-
NEFA (wk -1; mEq/L)	per unit increase	-0.02	0.51	0.02	-	-	-	-	-	-
Hp	> vs. < 1.1 g/L	-947	318	0.001	-	-	-	-	-	-
Parity x Hp	-	-	-	0.71	-	-	-	-	-	-
Model 3: 11,17-DOA (Postpartum)										
SCC Linear Score	per unit increase	-	-	-	-220	85	0.01	-271	107	0.01
DIM at test day	per unit increase	-	-	-	34	13	0.003	9	13	0.50
NEFA (wk -1; mEq/L)	per unit increase	-	-	-	-0.39	0.52	0.45	1.31	0.54	0.01
11,17-DOA	> vs. < 400 units	-	-	-	-663	308	0.03	122	353	0.73
Model 4: PCORT (Postpartum)		-	-	-	-	-	-	-	-	-
Parity	PP vs. MP	-766	608	0.11	-	-	-	-	-	-
SCC Linear Score	per unit increase	-215	66	0.001	-	-	-	-	-	-
DIM at test day	per unit increase	18	9	0.04	-	-	-	-	-	-
NEFA (wk -1; mEq/L)	per unit increase	-0.61	0.53	0.39	-	-	-	-	-	-
PCORT	> vs. < 19.3 nmol/L	666	357	0.009	-	-	-	-	-	-
Parity x PCORT	-	-	-	0.95	-	-	-	-	-	-

***** Farm and BCS were not associated with 305ME milk yield ($P \geq 0.24$) thus 305ME estimates for these covariates are not shown.

Figure 3.1. Graph of Kaplan-Meier estimator of days to conception for primiparous cows with postpartum (3 to 10 DIM) nonesterified fatty acid (NEFA; A) concentrations > or ≤ 0.45 mEq/L (P = 0.02) and postpartum haptoglobin (Hp; B) concentrations > or ≤ 1.3 g/L (P = 0.02 with NEFA included as a covariate in analysis).



DISCUSSION

Consistent with other studies (Walsh et al., 2007; Ospina et al., 2010), results from this study have shown that NEFA concentrations around calving are associated with milk yield and reproductive performance. The critical NEFA thresholds identified in the present study are different than the thresholds reported by others; however, the general relationships persist. For example Ospina et al. (2010) reported that when NEFA was ≥ 0.33 mEq/L (among both PP and MP cows) during the 2 wk period before calving 305-d milk yield predicted at 120 DIM was reduced by 683 kg. In the present study the prepartum NEFA thresholds were higher (wk -2: > 0.45 mEq/L and wk -1: > 0.55 mEq/L) and were associated with greater estimates of milk yield loss than in the Ospina study (e.g. -1360 kg for PP and MP cows based on wk -1 sample); these differences may have been associated with sample size (larger in the Ospina Study), the time relative to calving when the samples were collected, or the concentration threshold used. All transition cows will go through a period of increasing negative energy balance as they approach parturition, with the height of this imbalance occurring during the weeks following calving (Grummer et al., 2004); NEFA concentrations will reflect these changes in energy balance and thus predictive thresholds for compromised milk yield or reproductive performance will also vary depending on when samples are collected relative to calving. In the present study, the greater differences in 305ME milk yield estimates based on prepartum NEFA concentrations may have been a consequence of greater negative energy balance since a higher predictive NEFA threshold was used compared to that used in the Ospina study.

While this study confirmed the relationship of peripartum NEFA concentrations with milk yield and reproductive success, the results also suggest that increased concentrations of both Hp and 11,17-DOA, in particular during the postpartum period can predict lower milk yield or compromised reproductive performance better than NEFA concentrations. This conclusion is

based on several observations. NEFA was included as a covariate in each of the models exploring the relationship between increased concentrations of the analytes of interest (Hp, plasma cortisol and 11,17-DOA) with milk yield and reproductive performance, and in each model where an association was detected between the analyte of interest and respective outcome, NEFA was always a less significant predictor of the outcome or not significant (increased type I error risk). Further, compared to NEFA modeled as an independent predictor, the estimates derived from the Hp and 11,17-DOA models relative to change in milk yield or time to conception were generally higher. For example, during the postpartum period, PP cows with Hp > than 1.3 g/L had a 41% decreased risk of conception while PP cows with NEFA > 0.47 mEq/L had a 39% decreased risk of conception. Similarly for milk yield, when Hp concentrations were > 1.1 g/L during the postpartum period, both PP and MP cows had a 947 kg lower projected 305ME while NEFA concentrations > 0.70 mEq/L only tend (P = 0.08) to be associated with a 592 kg lower 305 milk yield in MP cows only. After calving, increased 11,17-DOA concentrations in MP cows (>400 ng/g fecal DM) were associated with a 663 kg lower projected 305ME milk yield. Before calving, wk -1 NEFA concentrations were a better predictor of milk production than Hp (higher 305 ME milk yield estimates and association persisted for MP and PP cows); however, for reasons that will be described later, postpartum relationships may provide more meaningful information when considering how these results may be used for on-farm analyte testing. When considering the results derived from the postpartum sampling, there were also higher proportion of animals being identified above the concentration thresholds to predict milk yield for Hp (33% of all animals sampled) and 11,17-DOA (32% of MP cows sampled), relative to NEFA (25% of MP cow sampled).

Interestingly, the associations between plasma cortisol concentration and milk and reproductive outcomes generally contradicted the associations that were observed with 11,17-

DOA concentration. For example, during the postpartum period MP cows with 11,17-DOA concentrations > 400 ng/g fecal DM had 663 kg lower 305ME milk yield projections while increased concentrations of plasma cortisol (> 19.3 nmol/L) were associated with 666 kg greater 305ME milk yield projections among both PP and MP cows. Similarly when considering time to pregnancy as the outcome of interest, MP cows that had 11,17-DOA >2300 ng/g fecal DM during the 2 wk period before calving had on average a 39% decreased risk of conception while PP cows with plasma cortisol concentrations > 33.1 to 38.6 nmol/L during wk -2 or -1, respectively, averaged a 92% increased risk of conception. When considering these results a few important points need to be highlighted. First, while both plasma cortisol and fecal cortisol metabolites are estimates of circulating cortisol concentrations, these estimates reflect different periods of time relative to sample collection. The criticism of plasma cortisol as a measure of stress in animals is that concentration values can be difficult to interpret due to the confounding effect of the increased stress associated with handling, restraint, and the sample collection procedure (Cook et al., 2000). Cortisol secretion from the adrenal gland throughout the day is also pulsatile and follows a diurnal cycle that is subject to substantial individual variation (Thun et al., 1981). Therefore, for herd analyte-testing programs, that aim to generate meaningful information from a single blood sample, plasma cortisol measurements collected in this fashion are likely to yield highly variable and inconsistent results that are difficult to interpret. The physiological stress response in many situations (acute stressors) is an adaptive response, meaning the response improves the animals' capacity to address the threat (e.g. increased alertness, mobilization of energetic substrates towards tissues that will facilitate fight or flight responses) (Sapolsky et al., 2000). In the current study, the positive relationship between higher plasma cortisol and milk yield and reproductive performance may have reflected this adaptive physiological response to the acute stress of sample collection which presumably would be

greatest in healthy normal cattle. Fecal cortisol metabolites reflect cortisol production approximately 10 to 12 h prior to sample collection; therefore, concentrations of 11,17-DOA are not subject to the feedback mechanisms of the acute stress of handling (Palme et al., 1999). Fecal cortisol metabolite concentrations also a reflect an integration metabolized cortisol over a period of time; therefore the variation attributed to the pulsatile nature of cortisol secretion and the peaks and troughs in cortisol levels relative to time of day are reduced when considering fecal cortisol metabolites. For these reasons, herd-testing programs aimed at assessing overall stress-load in their animals would benefit from sampling fecal cortisol metabolite concentrations rather than plasma cortisol.

The mechanisms by which increased concentrations of Hp or 11,17-DOA relate to reduced milk yield and reproductive performance can be speculated on based on previous research. There are numerous studies providing evidence that increased glucocorticoid production compromises reproductive performance; these relationships are largely mediated through the negative interactions between glucocorticoids and hormones of the hypothalamic-pituitary-gonadal axis. These interactions can lead to a variety of negative downstream effects including impaired follicular growth and reduced embryo viability (reviewed in Dobson and Smith, 2000). Increased stress-load during the transition period may also directly compromise milk yield. Daniels et al. (2007) showed that by reducing postpartum stress in heifers associated with novel exposure to the milk parlor through the implementation of a prepartum milking program, increased milk yields were observed. However, another study found that increased stress associated with random exposure to stray voltage applied to the water trough, did not correlate with reduced milk yield (Rigalma et al., 2010). The mechanisms underlying these relationships will be complex and likely dependent upon a variety of interacting factors including behavior and physiological processes. Increased Hp concentrations during the postpartum period

have been shown to be a good diagnostic indicators of infectious disease including uterine infection and metritis (Hirvonen et al., 1999; Dubuc et al., 2010) and mastitis (Grönlund et al., 2004), disorders that have been associated with reduced reproductive performance (LeBlanc et al., 2002) and lower milk yield (Deluyker et al., 1991), respectively.

In general, the relationship between increased NEFA, Hp or 11,17-DOA concentrations with reduced reproductive performance was found to be limited to PP cows. On the other hand the relationship between increased concentrations of these analytes with reduced milk yield was stronger in MP cows than PP; however, in some cases this relationship was consistent across both parities (i.e. wk -1 NEFA and postpartum Hp). Similar to results reported by Ospina et al. (2010) increased concentrations of NEFA among PP cows after calving were associated with increased 305ME milk yield projections (NEFA > 0.70mEq/L = +1049 kg 305ME milk yield). While the mechanisms behind these seemingly contradictory finding are still unclear, differences in pathways regulating energy balance in support of continuing body growth in PP cows compared to MP cows that only have maintenance requirements may contribute to these discrepancies in the observed relationships between peripartum analyte concentrations with milk and reproduction outcomes.

When considering the practical application of this information for on-farm testing programs, it is important to consider at what period relative to calving is the best period to sample. Sampling during the postpartum period may be preferred. In most cases the estimates of change in milk yield or reproductive performance based on NEFA, Hp, or 11,17-DOA concentration thresholds were higher when samples were collected during the post partum period. In fact, the results of the current study highlight the point that the associations between these analytes (including NEFA) with milk yield and reproductive performance are highly influenced based on when each sample was collected relative to calving. The challenge for

prepartum analyte testing programs is that it is impossible to determine when the sample is being collected relative to calving because actual calving dates can vary from predicted calving dates by ± 5 d. Postpartum sampling programs offer the advantage that producers will know the appropriate time for sample collection and these collections can easily be incorporated into existing fresh-cow herd health checks.

CONCLUSIONS

This research provides evidence that increased concentrations of analytes associated with stress (i.e. 11,17-DOA) and inflammation (i.e. Hp) during the periparturient period, are associated with lower milk yield and compromised reproductive performance. These relationships were influenced by parity and time of sample collection relative to calving. Herd analyte testing programs focused on postpartum sampling may result in more reliable estimates of risk for reduced milk yield or lower conception rates because knowledge of sampling time relative to calving is assured.

When sampled during the postpartum period (3 to 10 DIM) both Hp and 11,17-DOA appear to be better measures of risk for lower milk yield and reduced reproductive performance than NEFA. Compared to NEFA modeled as an independent predictor, the estimates derived from the Hp and 11,17-DOA models relative to change in milk yield or time to conception were generally higher. For example, cattle with a postpartum Hp concentration greater than 1.1 g/L had 947 kg lower projected 305-d milk yield and heifers with postpartum Hp greater than 1.3 g/L had a 41% lower risk of conception. On the other hand, increased NEFA during the postpartum period was associated with higher milk yield in heifers, only tended to be associated with lower milk yield (592 kg) in multiparous cows, and was associated with a 39% lower risk of conception for heifers. Further, the proportion of animals identified above the postpartum Hp

and 11,17-DOA critical thresholds were higher than the proportion of animals above the postpartum NEFA critical threshold; this suggests that both concentrations of Hp and 11,17-DOA can identify more animals at risk for milk production losses or compromised reproductive performance than concentrations of NEFA. These results suggest that herd-based analyte testing programs aimed at identifying opportunities to improve milk yield or reproductive performance, may benefit by adding Hp or 11,7-DOA to the testing program.

Increased concentrations of plasma cortisol during the postpartum period were associated with greater projected 305ME milk yield in both MP and PP cows but was not related to reproductive performance; before calving high plasma cortisol was associated with an improved risk of conception. The discrepancies between plasma cortisol and 11,17-DOA results may be attributable to the confounding effects of handling, restraint, and sample collection stress; plasma cortisol concentrations can rise quickly due to these stressors and thus concentrations levels can be difficult to interpret.

Future work in this area will need to focus on characterizing the inter- and intra-herd variability in the concentrations of these alternative analytes of interest (i.e. Hp and 11,17-DOA). By understanding this variability accurate herd alarm levels for identified concentration thresholds (i.e. the proportion of sampled animals above the concentration threshold which indicates a herd-level opportunities for improvement) will be able to be identified.

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CHAPTER FOUR

THE EFFECTS OF OVERSTOCKING HOLSTEIN DAIRY CATTLE DURING THE DRY PERIOD ON CORTISOL SECRETION AND ENERGY METABOLISM

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ABSTRACT

The objective was to determine whether overstocking during the dry period could alter physiological parameters in dairy cattle associated with cortisol secretion and energy metabolism. Four groups of 10 late gestation, non-lactating Holstein cows (6 multiparous cows and 4 heifers per group) were exposed to both a control [1 lying stall/cow and 0.67 m linear feed bunk (FB) space/cow] and overstocked (1 stall/2 cows and 0.34 m FB space/cow) stocking density treatment in a replicated crossover design with 14-d treatment periods. On d 1, 3, 5, 7, 9 and 11 of each 14-d treatment period, blood and fecal samples were collected from each cow for the determination of plasma NEFA, glucose, insulin, and fecal cortisol metabolite (11,17-dioxoandrostane; 11,17-DOA) concentrations. Glucose and ACTH challenges were conducted on d 13 and 14, respectively, of each treatment period. Dry matter intake per cow was greater during the overstocked period than during the control period (15.9 vs. 14.9 ± 0.5 kg/d). During the overstocked period plasma NEFA and glucose concentrations were greater (0.11 vs. 0.09 ± 0.006 mEq/L and 65.3 vs. 64.2 ± 1.1 mg/dL, respectively) and 11,17-DOA concentration tended to be greater (891 vs. 792 ± 86 ng/g fecal DM) than during the control period. Insulin concentration was the same during the overstocked (29.0 ± 2.1 µIU/mL) and control period (31.2 ± 2.1 µIU/mL). Overstocking was associated with a slightly slower glucose clearance from circulation as evidenced by a greater area under the curve (AUC) estimate for the glucose response curves (2882 vs. 2657 ± 165 mg/dL x 180 min) but a more attenuated insulin response (Insulin AUC = 5258 vs. 6692 ± 1104 µIU/mL x 180 min for the overstocked and control periods, respectively). Changes in tissue glucose uptake may be mediated by changes in pancreatic insulin secretion or peripheral tissue responses to insulin. The role of glucocorticoids in mediating these changes in energy metabolism is still unclear as stocking density treatment was not associated with changes in adrenal secretion of cortisol following ACTH stimulation.

Keywords: overstocking, energy metabolism, cortisol

INTRODUCTION

Industry recommended best practice with regard to space allowance in dairy barns is to provide one lying stall for every cow and provide 60 cm (24 inches) of linear feed bunk space per animal (NFACC, 2009). Despite these recommendations overstocking is still common; survey data of free-stall farms in the United States show that 58 % of farms have less than the recommended 60 cm of feeding space and 43 % have less than the recommended lying stall availability based on average cow numbers on the farm during the year (USDA, 2010).

The behavioral consequences of overstocking are well documented. Feeding time is decreased and time spent standing idly (i.e. standing not feeding) is increased when cows are overstocked at the feed bunk (Huzzey et al., 2006). Increased aggressive displacements are often observed at the overstocked feed bunk or free-stalls (Huzzey et al., 2006; Fregonesi et al., 2007); these competitive environments can make it difficult for some cows to gain access to these resources particularly when motivation to eat or rest is high, such as after fresh feed delivery (DeVries et al., 2004) or during the overnight hours (Fregonesi et al., 2007), respectively. The relationship between overstocking and DMI is less clear and is likely influenced by feeding rate. Some researchers have reported no effects of overstocking on DMI (Proudfoot et al., 2009) while others have reported a slight increase in DMI (Olofsson, 1999) in response to overstocking.

The physiological consequences of overstocking have still not been thoroughly investigated. Previous work has shown that when cows are regrouped into a high stocking density group (Friend et al., 1977) or subjected to overcrowding in the resting area (Friend et al., 1979) they have a greater cortisol response to ACTH challenge relative to cows that are not regrouped or overcrowded, respectively. This work suggests that there may be alterations in

adrenal function in response to the stress of overstocking. Changes in stress physiology may be a reflection of the physiological adaptations that occur as cows try to cope with an overcrowded environment and an increase in plasma cortisol concentration may influence other physiological processes. Glucocorticoids are important regulators of energy metabolism; they help to raise circulating glucose concentrations by increasing hepatic gluconeogenesis and inhibiting peripheral tissue uptake of glucose; they also contribute to the regulation of lipolysis and lipogenesis, and facilitate increased plasma nonesterified fatty acid concentrations (reviewed in Parker and Rainey, 2004). Excess glucocorticoid production has also been associated with insulin resistance (reviewed in Andrews and Walker, 1999). To date, no work has explored changes in adrenal activity and energy metabolism in response to the potential stress associated with overstocking dairy cattle.

The hypothesis for this study was that if overstocking can lead to changes in glucocorticoid secretion there may also be observable changes in energy metabolism. The objective of this study was to measure physiological responses to overcrowding during the dry period. The effect of overcrowding on energy metabolism was evaluated by 1) measuring daily NEFA, insulin and glucose concentrations and 2) the response of these analytes to an intravenous glucose tolerance test. Changes in stress physiology (adrenal activity) were evaluated by 1) measuring daily fecal cortisol metabolite concentrations and 2) the plasma cortisol response to an ACTH challenge.

MATERIALS AND METHODS

Animals, Housing and Diet

This study was conducted between the months of January and April 2010 at the Cornell Teaching and Research Dairy Center. The Cornell University Institutional Animal Care and Use

Committee approved all procedures involving animals prior to the beginning of the study. Forty pregnant, non-lactating Holstein dairy cows [16 heifers and 24 multiparous cows (mean parity ± SD; 1.38 ± 0.65)] were used in this study. Cows were housed in a 2-row free-stall barn in groups of 10. Groups were balanced based on parity (4 heifers and 6 multiparous cows per group) and previous 305ME among multiparous cows. Each pen had 10 free-stalls that were arranged in a 2-row formation and bedded with a mattress and layer of sawdust. A post-and-rail feed barrier was used at the feed bunk. During both treatments cows were fed the same TMR once daily (approximately 0800 h) with feed push-ups occurring at regular intervals throughout the day. The diet was composed of wheat straw (24.6 % of DM), corn silage (41.0% of DM), and dry cow grain (34.4% of DM). Weekly samples of the TMR were collected and combined into a 4-wk composite sample that was sent to a commercial laboratory for wet chemistry analysis (Dairy One Cooperative Inc., Ithaca, NY). The TMR analysis consisted of (% of DM ± SD): CP = 14.5 ± 0.6; ADF = 31.4 ± 1.5; NDF = 49.8 ± 2.8; Starch = 17.7 ± 0.7; Ca = 0.76 ± 0.07; P = 0.29 ± 0.01; Mg = 0.23 ± 0.01; K = 1.06 ± 0.04; and Na = 0.19 ± 0.03. Group as-fed intake (kg/d) was measured daily using a mixer wagon equipped with FeedWatch (Valley Agricultural Software, Tulare, CA); group intakes were determined by subtracting the weight of the orts from the total weight fed during the previous day. As-fed group intake was corrected for the DM % of the TMR and reported on a per cow basis (group DMI / 10 cows).

Treatments and Experimental Design

In sets of two, all 4 groups were exposed to two stocking density treatments using a crossover experimental design (i.e. replicated crossover). The stocking density treatments were defined as follows: 1) Control: 1 lying stall per cow and 0.67 m linear feed bunk (**FB**) space per cow, and 2) Overstocked: 1 lying stall per 2 cows and 0.34 m linear FB space per cow. To simulate conditions of overstocking access to the 4 free-stalls facing (nearest) the FB and one

additional free-stall along the back wall of the pen were roped off to restrict resting space and access to the FB was restricted using plywood which was bolted across the feeding area. The first set of groups were formed between d –74 to –61 relative to the cows' expected calving dates and the second set of groups were formed between d –81 to –67 relative to the cows' expected calving dates. Cows were given 10 d to adapt to their respective groups before the first experimental treatment period began. Cows averaged 214 d in gestation at the beginning of the first experimental period. Each of the two treatment periods lasted 14 d and these periods were separated by a 3-d washout period during which time both groups were housed at the control stocking density. After the first set of two groups had been exposed to both treatments the second set of two groups were formed and the crossover design was repeated.

Behavior Data Collection

Behavior at the FB was monitored using video cameras (Sony CCD Digital ULTRA Pro Series, Hi-Resolution BW CCD Camera with Auto-Iris) connected to digital recording system (DiGiCam H.264, 120 & 240 FPS, DVR PC Version; Central Alarms Systems Inc., Littleton, CO). A camera was positioned directly above the feeding area to continuously record behavior at the FB. Hair dye was used to create unique alphanumeric symbols on the backs of the cows so that individuals could be identified on the video recordings. Daily feeding time, time to the FB post fresh feed delivery, and proportion of total daily feeding time during the 3 h period after fresh feed delivery was estimated from 10-min time scans of the video recordings over 4 consecutive days (d 7 to 10 of the 14-d overstocked period). A cow was considered to be feeding when its neck collar was visible beyond the top rail of the feed barrier on the feed alley side of the pen. To assess competitive behavior, 3 d of continuous (24-h; d 7 to 9) video recordings were reviewed and each competitive displacement that occurred at the FB was recorded. A displacement was recorded when a cow's head (actor) came in contact with a cow that was

feeding (reactor), resulting in the reactor withdrawing its head from the FB.

Blood and Fecal Collection and Analysis

On d 1, 3, 5, 7, 9 and 11 of each 14-d treatment period blood and fecal samples were collected from each cow. Blood was collected from the coccygeal vessel into 10-mL sterile tubes coated with sodium heparin (BD Vacutainer, Franklin Lakes, NJ, USA) and plasma was harvested after centrifugation (2,800 x *g* for 15 min at 4˚C). Plasma samples were stored at -20˚C for later laboratory analysis. Plasma concentrations of glucose and NEFA were measured by enzymatic analysis (glucose oxidase, P7119, Sigma Chemical; NEFA-C: Wako Pure Chemical Industries, Osaka, Japan). Spectrophotometric measurements were conducted using a $Versa_{max}$ tunable microplate reader (Molecular Devices, Sunnyvale, CA). The intra- and interassay coefficients of variation (**CV**) for the NEFA assay were 3.7 and 4.4 %, respectively, and for the glucose assay were 2.9 and 6.1 %, respectively. Plasma insulin concentration was measured by radioimmunoassay (Porcine Insulin RIA Kit #PI-12K, Millipore Corp., Billerica, MA). The intra- and interassay CV for the insulin assay were 3.3 and 3.2 %, respectively.

Fecal samples were collected fresh, sealed within plastic bags and placed immediately under ice. Within 2 h of sample collection, steroids from the fecal samples were extracted using the wet extraction method described by Palme and Möstl (1997). Briefly, 0.5 g of each raw fecal sample was weighed and vortexed with 5 ml of 80 % methanol for 30 min. Samples were then centrifuged for 15 min at 2,800 x *g* and the supernatant was divided into aliquots and stored at -20˚C until further analysis. The DM percentage of each fecal sample was obtained by weighing samples before and after drying in a hot oven (105˚C) for 24 h. Concentrations of fecal cortisol metabolites (11,17-dioxoandrostanes; **11,17-DOA**) were measured using a competitive enzyme immunoassay developed by Palme and Möstl (1997) and validated for use in cattle (Palme et al., 1999). The intra- and interassay CV for the 11,17-DOA assay were 3.2 and 3.4%, respectively.

Glucose Tolerance Test and ACTH Challenge

Between d 11 and 12 of each treatment period each cow was fitted with an indwelling sterilized jugular catheter (30 cm x 1.78 mm o.d., Tygon S-54-HL Medical tubing, Saint-Gobain Performance Plastics, Akron, OH). The catheter was secured within a fabric pouch that was attached to the neck of the cow using a topical adhesive approved for use in animals. The entire neck was wrapped with elastic bandages to prevent the cow from dislodging the catheter while in the group pen. Body weights of all animals were measured on their catheterization day to determine glucose and ACTH doses for the glucose tolerance test (**GTT**) and ACTH challenge, respectively. The GTT was performed on all cows on d 13 of each treatment period and the ACTH challenge was performed on all cows on d 14 of each period. Approximately 1 to 1.5 h before the start of the both the GTT and ACTH challenge cows were moved a short distance to a tie-stall barn where each could be tethered in an individual stall to facilitate sample collection.

On d 13, the GTT was administered to one group of 10 cows in the morning (0900 to 1200 h) and to the second group during the afternoon (1300 to 1600 h). Feed was removed from the cows 2 h before the start of the GTT. This test involved administering 0.25 g/kg of BW of glucose i.v. (dextrose 50% wt/vol., Butler Animal Health Supply, Dublin, OH) and then collecting jugular blood samples at -15, -5, 0, 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 120, 150, and 180 min relative to glucose administration. After the 180-min sample was collected, catheters were flushed with 5 mL of heparinized saline (500 IU/L), stored in the fabric pouch protected by the neck wrap and then the cows were returned to their free-stall group pen. Feed was not withheld from cows prior to the start of the ACTH challenge and the test was administered to all 20 cows during the morning of d 14 (0900 to 1300 h). The ACTH challenge involved administering 0.125 IU/kg of BW of ACTH i.v. (Porcine, A6303, Sigma Chemical, St. Louis, MO) and then collecting jugular blood samples at -60, -15, 0, 15, 30, 45, 60, 90, 120, 150, 180,

and 240 min relative to the administration of ACTH. During both the GTT and ACTH challenge samples were immediately centrifuged after collection and the plasma was harvested and stored at -20 ºC. At the end of the ACTH challenge catheters were removed, cows were returned to their respective group pens and the stocking density barriers were taken down to signal either the beginning of the 3-d washout period or the end of the experiment. Plasma cortisol concentration was determined for samples collected during the ACTH challenge using a radioimmunoassay (Coat-A-Count Cortisol RIA Kit, Siemens Medical Solutions Diagnostics, Los Angeles, CA). The intra- and interassay CV for the cortisol assay were 3.2 and 1.9 %, respectively.

Calculations and Statistical Analyses

All statistical analyses were performed using SAS version 9.1 (SAS Institute, 2009). Pen was considered the experimental unit for all analyses with measures from all cows in the group (10 cows / group) averaged to create one overall observation (by time where appropriate) per pen and treatment. In order to explore how primiparous cows responded to the stocking density treatments, measures from only the primiparous cows in each group (4 cows / group) were averaged to create one primiparous observation per pen and treatment; multiparous cow data was excluded for the calculation of primiparous group means. To explore how multiparous cows responded to the stocking density treatments, measures from only the multiparous cows in each group (6 cows / group) were averaged to create one multiparous observation per pen and treatment; primiparous data was excluded for the calculation of multiparous group means.

Differences in plasma NEFA, glucose, insulin, 11,17-DOA, and DMI measured every 2 d (daily for DMI) and the behavioral measures between stocking density treatments were analyzed as per a replicated crossover design using the MIXED procedure in SAS. The statistical model included the fixed effects of treatment, sequence, day, and the treatment x day interaction, and the random effects of period and pen within sequence. Day was identified as a repeated measure

and an autoregressive covariance structure was used in each model based on best fit using the Bayesian Information Criteria. The DMI on d 13 and 14 of each treatment period were excluded from this analysis because feed was withheld for approximately 5 h per day during the GTT and ACTH challenge. Logarithmic transformation was required for the 11,17-DOA data to comply with the model assumptions and improve fit. Least squares means and SEM for the 11,17-DOA data were estimated from untransformed values, whereas *P*-values reflect statistical analysis of transformed data.

Area under the curve (**AUC**) for the glucose, NEFA and insulin responses to the GTT and the cortisol response to the ACTH challenge were calculated using the trapezoidal method and actual concentration values after discounting the basal values. Positive and Negative AUC were calculated separately. Positive AUC (**+AUC**) included only the periods when the actual metabolite concentration was greater then the basal concentration, and negative AUC (**-AUC**) included only those periods when the actual metabolite concentration was lower then the basal concentration. This differentiation in AUC was done to better describe the differences in the metabolite curves when they dropped below the basal concentrations, as treatment differences were lost when including all the data together.

The NLIN procedure of SAS was used to fit exponential curves for glucose concentration during the first 60 min of GTT, for NEFA during the first 30 min of the GTT, for insulin between 15 and 75 min of the GTT (period following peak insulin concentration), and for cortisol between 60 and 180 min of the ACTH challenge using the following equation: $F(t) = A \times e^{(-k \times t)}$, where F(t) is the analyte concentration at time t; A is the maximum value of the analyte (estimated by model); t is the time (min); and k is the regression coefficient. Using this equation, calculated for each cow and treatment period, the following parameters were calculated: clearance rate of glucose, insulin and cortisol ($\mathbf{CR}_{ta\text{-}tb}$; %/min) = $\{(\ln[t_a] - \ln[t_b])/(t_b - t_a)\} \times 100$;

rate of NEFA decline from circulation (**Slope$_{ta-tb}$**; %/min) = {(ln[t_a] - ln[t_b])/(t_b - t_a)} × 100; time to reach half maximal glucose concentration (**$T_{1/2}$**; min) = {[ln(2)]/CR} × 100; and time to reach basal glucose concentration (**T_{basal}**; min) = ln([basal]/A)/(-k), where [t_a] and [t_b] are the concentrations of the analyte at time a and b respectively and [basal] is the basal analyte concentration, calculated by averaging the analyte concentration at t = -15 and -5 min for the GTT or t = -60 and -5 min for the ACTH challenge. The lowest NEFA concentration (NEFA nadir), highest insulin and cortisol concentration (peak insulin, peak cortisol) of each cow were identified from samples collected following the administration of dextrose or ACTH. These parameters were averaged by group and treatment for statistical analysis. Data were analyzed as per a replicated crossover design using the MIXED procedure in SAS. The statistical model included the fixed effects of treatment, and sequence, and the random effects of period and pen within sequence.

RESULTS

Overall Results

During the overstocked period cows took longer to approach the FB following fresh feed delivery (68 vs. 37 ± 9 min; $P = 0.02$), spent a smaller proportion of their total daily feeding time at the FB during the 3 h period following fresh feed delivery (22 vs. 28 ± 3 %; $P = 0.001$), and engaged in more competitive displacements at the FB over a 24-h period (50 vs. 28 ± 3 displacements/d; $P = 0.003$). Stocking density treatment did not affect average daily feeding time (241 vs. 242 ± 12 min/d for the control and overstocked periods, respectively; $P = 0.91$).

The average DMI across all experimental days was 15.9 ± 0.5 kg/d per cow during the overstocked treatment and 14.9 ± 0.5 kg/d per cow during the control treatment ($P < 0.001$). The average DMI of cows in each of the 4 groups during the control and overstocked period is

presented in Figure 4.1 by experimental day.

Plasma NEFA and glucose concentrations were greater during the overstocked period compared to the control period [0.11 vs. 0.09 ± 0.006 mEq/L (P = 0.002) and 65.3 vs. 64.2 ± 1.1 mg/dL (P = 0.05), respectively] and overall 11,17-DOA concentration tended to be higher during the overstocked period compared to the control period (891 vs. 792 ± 86 ng/g fecal DM; P = 0.10). There were no differences in the insulin concentration of cows between the overstocked and control periods (29.0 vs. 31.2 ± 2.1 µIU/mL, respectively; P = 0.20).

Glucose clearance from circulation was slower during the overstocked treatment as evidenced by a longer time to reach half maximal glucose concentration and to reach basal glucose levels, a greater +AUC throughout the 180 min of sampling, and a tendency for a lower glucose CR between 0 and 60 min of the GTT (Table 4.1; Figure 4.2 A; $P \leq 0.05$). There was an attenuated insulin response to the GTT during the overstocked treatment period relative to the control period; this was evidenced by lower peak insulin concentration following glucose administration, a lower +AUC value for the 240-min sampling period, and a lower insulin clearance rate between 15 and 75 min of the ACTH challenge (Table 4.1; Figure 4.2 B; $P \leq$ 0.02). The rate of NEFA decline from circulation was slower during the first 30 min of the GTT during the overstocked treatment (P = 0.04) and there was also a tendency (P = 0.09) for NEFA concentration to drop lower (lower nadir) following glucose infusion during the control treatment (Table 4.1; Figure 4.2 C).

During the ACTH challenge, cows did not differ in basal cortisol (11.2 vs. 11.2 ± 2.7 nmol/L; P = 0.97), peak cortisol (176.2 vs. 177.7 ± 8.8 nmol/L; P = 0.62), $CR_{60\text{-}180}$ (1.30 vs. 1.36 ± 0.06 %/min; P = 0.13), $+AUC_{240}$ (18824 vs. 18467 ± 859 nmol/L x 240 min; P = 0.48), or $-AUC_{240}$ (-52 vs. -64 ± 43 nmol/L x 240 min; P = 0.80) between the control and overstocked periods, respectively (Figure 4.3).

Multiparous Cows Only

During the overstocked period, multiparous cows spent a smaller proportion of their total daily feeding time at the FB during the 3 h period following fresh feed delivery (25 vs. 29 ± 2 %; $P = 0.05$). Average daily feeding time during the control and overstocked periods (263 vs. 265 ± 20 min/d) and the time to approach the FB following fresh feed delivery (40 vs. 59 ± 15 min) was not different in multiparous cows ($P = 0.85$ and $P = 0.20$, respectively).

Average glucose, insulin and 11,17-DOA concentrations were not different among multiparous cows during the overstocked or the control treatment periods. A treatment by day interaction for plasma NEFA ($P = 0.05$) indicated that multiparous cows had greater NEFA concentrations during the overstocked period relative to the control period, but on d 5 of the treatment period only (Figure 4.4).

During the GTT, multiparous cows had a slower insulin clearance rate during the overstocked period relative to the control period ($CR_{15\text{-}75}$: 3.3 vs. 3.8 ± 0.3 %/min; $P = 0.02$). All other measured responses during the GTT were found to be either trends or not significant (Table 4.2). During the ACTH challenge, multiparous cows did not differ in basal cortisol (12.1 vs. 11.7 ± 3.8 nmol/L; $P = 0.81$), peak cortisol (186.5 vs. 186.8 ± 9.5 nmol/L; $P = 0.93$), $CR_{60\text{-}180}$ (1.28 vs. 1.35 ± 0.07 %/min; $P = 0.17$), $+AUC_{240}$ (20051 vs. 19431 ± 757 nmol/L x 240 min; $P = 0.44$), or $–AUC_{240}$ (-51 vs. -86 ± 57 nmol/L x 240 min; $P = 0.57$) between the control and overstocked periods, respectively.

Primiparous Cows Only

During the overstocked period, primiparous cows took longer to approach the FB following fresh feed delivery (81 vs. 32 ± 18 min; $P = 0.04$) and spent a smaller proportion of their total daily feeding time at the FB during the 3 h following fresh feed delivery (18 vs. 27 ± 3 %; $P = 0.001$). Stocking density treatment did not affect the average daily feeding time of

primiparous cows (208 vs. 208 ± 10 min/d for the control and overstocked periods, respectively; $P = 0.97$).

Primiparous cows had greater NEFA, glucose, and 11,17-DOA concentrations during the overstocked period relative to the control period ($P \leq 0.04$) but average daily plasma insulin was not affected by stocking density treatment (Figure 4.5).

During the GTT, primiparous cows had a lower rate of NEFA decline from circulation during the overstocked period relative to the control period ($Slope_{30}$: 1.92 vs. 2.63 ± 0.17 %/min, respectively; $P = 0.02$). All other measured responses during the GTT were found to be either trends or not significant (Table 4.3). During the ACTH challenge, primiparous cows did not differ in basal cortisol (9.9 vs. 10.4 ± 2.2 nmol/L; $P = 0.90$), peak cortisol (160.7 vs. 163.9 ± 8.8 nmol/L; $P = 0.73$), $CR_{60\text{-}180}$ (1.31 vs. 1.36 ± 0.08 %/min; $P = 0.20$), $+AUC_{240}$ (16983 vs. 17021 ± 1105 nmol/L x 240 min; $P = 0.62$), or $–AUC_{240}$ (-53 vs. -31 ± 33 nmol/L x 240 min; $P = 0.67$) between the control and overstocked periods, respectively.

Table 4.1. Effect of stocking density treatment (control vs. overstocked) on the glucose, insulin, and nonesterified fatty acid (NEFA) responses (LSMEANS ± SEM) to an intravenous glucose tolerance test (GTT) for 4 groups of cows (4 primiparous cows and 6 multiparous cows per group).

	All Cows (n = 10 per group)					
Item[1]	Control	Overstocked	SEM	Difference[2]	SED[3]	*P*
GLUCOSE (mg/dL)						
Basal	74.2	74.8	0.9	-0.6	0.6	0.40
$CR_{0\text{-}60}$ (%/min)	1.93	1.78	0.11	0.15	0.05	0.12
$T_{1/2}$ (min)	37.0	40.3	2.2	-3.3	0.7	0.04
T_{basal} (min)	51.5	55.1	2.9	-3.6	0.8	0.05
+AUC180	2657	2882	165	-225	61	0.03
–AUC180	-665	-513	113	-152	81	0.20
INSULIN (μIU/mL)						
Basal	21.0	22.9	1.6	-1.9	0.6	0.08
Peak	259.8	199.1	38.6	60.8	9.1	0.02
$CR_{15\text{-}75}$ (%/min)	4.3	3.6	0.4	0.7	0.1	0.02
+AUC180	6692	5258	1104	1434	130	0.008
–AUC180	-592	-609	83	17	53	0.78
NEFA (mEq/L)						
Basal	0.17	0.17	0.01	0.001	0.013	0.96
Nadir	0.07	0.08	0.004	-0.008	0.003	0.09
$Slope_{0\text{-}30}$ (%/min)	1.93	1.42	0.15	0.51	0.15	0.04
+AUC180	2907	2090	703	817	994	0.45
–AUC180	-7684	-7285	1285	-398	1694	0.83

[1] Basal = mean analyte concentration at t = -15 and -5 min of GTT; Peak = highest insulin concentration; Nadir = lowest NEFA concentration; $CR_{t1\text{-}t2}$ = clearance rate between t1 and t2 GTT; $Slope_{0\text{-}30}$ = rate of NEFA decline from circulation during the first 30 min of GTT; $T_{1/2}$ = time to reach half maximal glucose concentration; AUC180 = area under the curve during the 180 min of the GTT (+AUC refers to AUC for sampled analyte concentrations above basal and –AUC refers to AUC for sampled analyte concentrations below basal).
[2] Difference between the treatment LSMEANS.
[3] Standard error of the difference between LSMEANS.

Table 4.2. Effect of stocking density treatment (control vs. overstocked) on the glucose, insulin, and nonesterified fatty acid (NEFA) responses (LSMEANS ± SEM) to an intravenous glucose tolerance test (GTT) for 4 groups of multiparous cows.

	Multiparous Cows (n = 6 per group)					
Item[1]	Control	Overstocked	SEM	Difference[2]	SED[3]	*P*
GLUCOSE (mg/dL)						
Basal	72.8	73.3	0.8	-0.5	0.4	0.37
CR_{0-60} (%/min)	1.86	1.77	0.09	0.09	0.05	0.24
$T_{1/2}$ (min)	38.2	40.6	1.9	-2.4	0.6	0.06
T_{basal} (min)	52.8	55.0	0.9	-2.2	1.0	0.12
+AUC180	2656	2777	151	-121	51	0.10
–AUC180	-643	-591	84	-52	29	0.22
INSULIN (μIU/mL)						
Basal	19.6	20.3	2.3	-0.7	1.3	0.61
Peak	203.1	149.3	38.3	53.8	13.2	0.06
CR_{15-75} (%/min)	3.8	3.3	0.3	0.6	0.1	0.02
+AUC180	5354	4067	1116	1287	564	0.15
–AUC180	-590	-544	148	-46	145	0.78
NEFA (mEq/L)						
Basal	0.12	0.12	0.01	0.01	0.02	0.73
Nadir	0.06	0.06	0.002	-0.002	0.002	0.45
$Slope_{0-30}$ (%/min)	1.46	1.08	0.19	0.38	0.27	0.21
+AUC180	2215	1578	624	637	867	0.50
–AUC180	-4696	-3997	1707	-699	2165	0.76

[1] Basal = mean analyte concentration at t = -15 and -5 min of GTT; Peak = highest insulin concentration; Nadir = lowest NEFA concentration; CR_{t1-t2} = clearance rate between t1 and t2 GTT; $Slope_{0-30}$ = rate of NEFA decline from circulation during the first 30 min of GTT; $T_{1/2}$ = time to reach half maximal glucose concentration; AUC180 = area under the curve during the 180 min of the GTT (+AUC refers to AUC for sampled analyte concentrations above basal and –AUC refers to AUC for sampled analyte concentrations below basal).
[2] Difference between the treatment LSMEANS.
[3] Standard error of the difference between LSMEANS.

Table 4.3. Effect of stocking density treatment (control vs. overstocked) on the glucose, insulin, and nonesterified fatty acid (NEFA) response (LSMEANS ± SEM) to an intravenous glucose tolerance test (GTT) for 4 groups of primiparous cows.

	Primiparous Cows (n = 4 per group)					
Item[1]	Control	Overstocked	SEM	Difference[2]	SED[3]	*P*
GLUCOSE (mg/dL)						
Basal	76.3	77.2	1.0	-0.9	1.1	0.47
$CR_{0\text{-}60}$ (%/min)	2.03	1.80	0.15	0.23	0.10	0.14
$T_{1/2}$ (min)	35.2	39.8	2.7	-4.6	1.7	0.11
T_{basal} (min)	49.5	55.2	3.6	-5.7	2.5	0.15
+AUC180	2660	3040	204	-380	172	0.11
–AUC180	-697	-396	165	-301	229	0.26
INSULIN (μIU/mL)						
Basal	22.8	26.7	2.9	-3.9	3.1	0.28
Peak	339.8	269.1	48.8	70.7	27.2	0.12
$CR_{15\text{-}75}$ (%/min)	5.0	4.2	0.4	0.8	0.3	0.10
+AUC180	8533	6931	1539	1602	650	0.13
–AUC180	-585	-718	66	133	94	0.22
NEFA (mEq/L)						
Basal	0.24	0.25	0.03	-0.01	0.01	0.56
Nadir	0.08	0.10	0.01	-0.02	0.01	0.22
$Slope_{0\text{-}30}$ (%/min)	2.63	1.92	0.17	0.71	0.09	0.02
+AUC180	3946	2857	903	1088	1277	0.43
–AUC180	-12166	-12218	1676	53	1842	0.98

[1] Basal = mean analyte concentration at t = -15 and -5 min of GTT; Peak = highest insulin concentration; Nadir = lowest NEFA concentration; $CR_{t1\text{-}t2}$ = clearance rate between t1 and t2 GTT; $Slope_{0\text{-}30}$ = rate of NEFA decline from circulation during the first 30 min of GTT; $T_{1/2}$ = time to reach half maximal glucose concentration; AUC180 = area under the curve during the 180 min of the GTT (+AUC refers to AUC for sampled analyte concentrations above basal and –AUC refers to AUC for sampled analyte concentrations below basal).
[2] Difference between the treatment LSMEANS.
[3] Standard error of the difference between LSMEANS.

Figure 4.1. Least squares means (± SE) DMI of 4 groups (expressed on a per cow basis) exposed to both a control and an overstocked stocking density treatment.

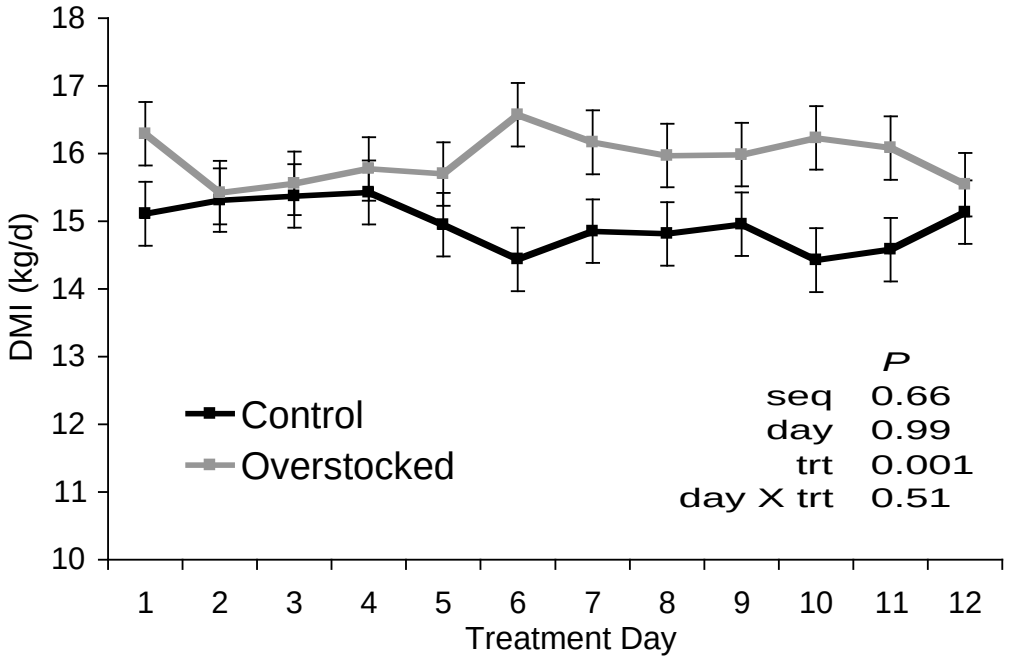


Figure 4.2. Effects of overcrowding on glucose (A), insulin (B), and nonesterified fatty acid (C) (arithmetic mean ± SE) response to an intravenous glucose tolerance test. Data presented is the average of 4 groups (n = 10 cows per group).

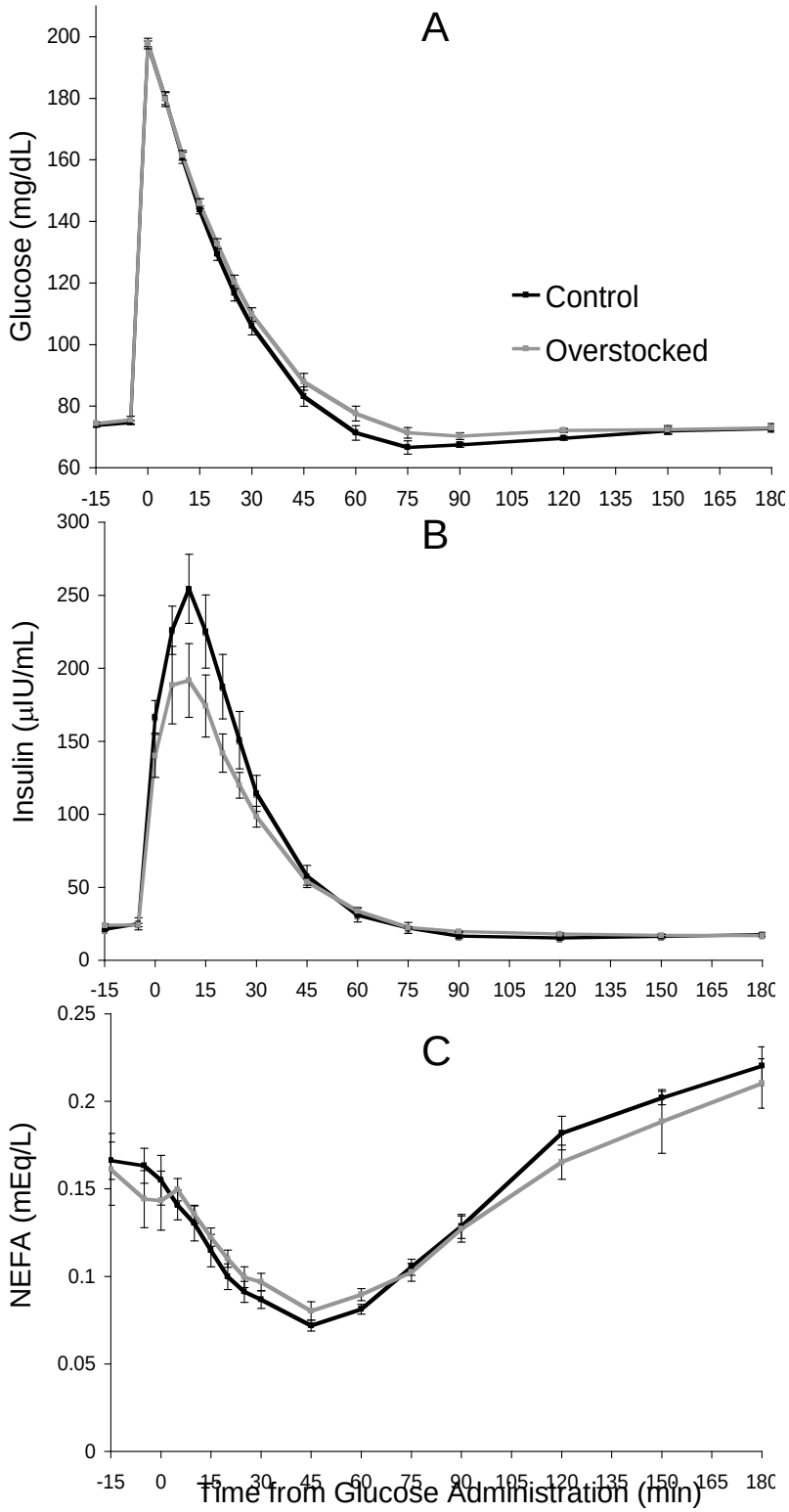


Figure 4.3. Effects of overcrowding on plasma cortisol (arithmetic mean ± SE) response to an intravenous ACTH challenge. Data presented is the average of 4 groups (n = 10 cows per group).

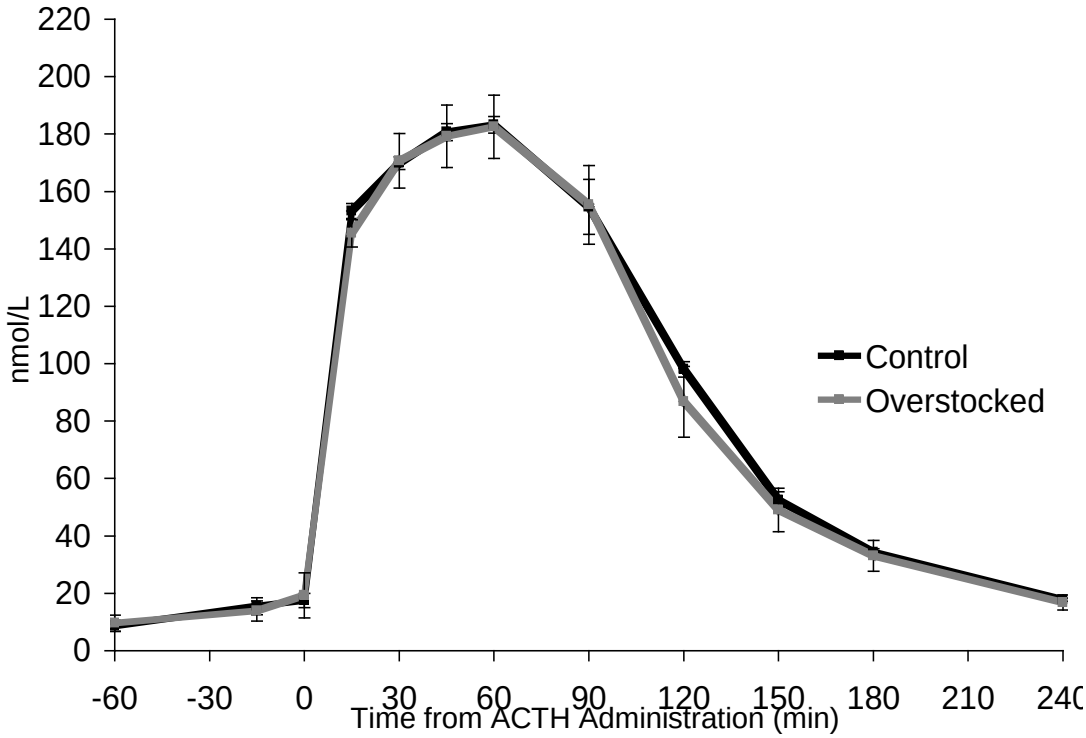


Figure 4.4. Least squares means (± SE) plasma glucose, nonesterified fatty acid (NEFA), insulin, and 11,17-dioxoandrostane (11,17-DOA) of multiparous cows in 4 groups during a control (solid line) and overstocked (dashed line) stocking density treatment.

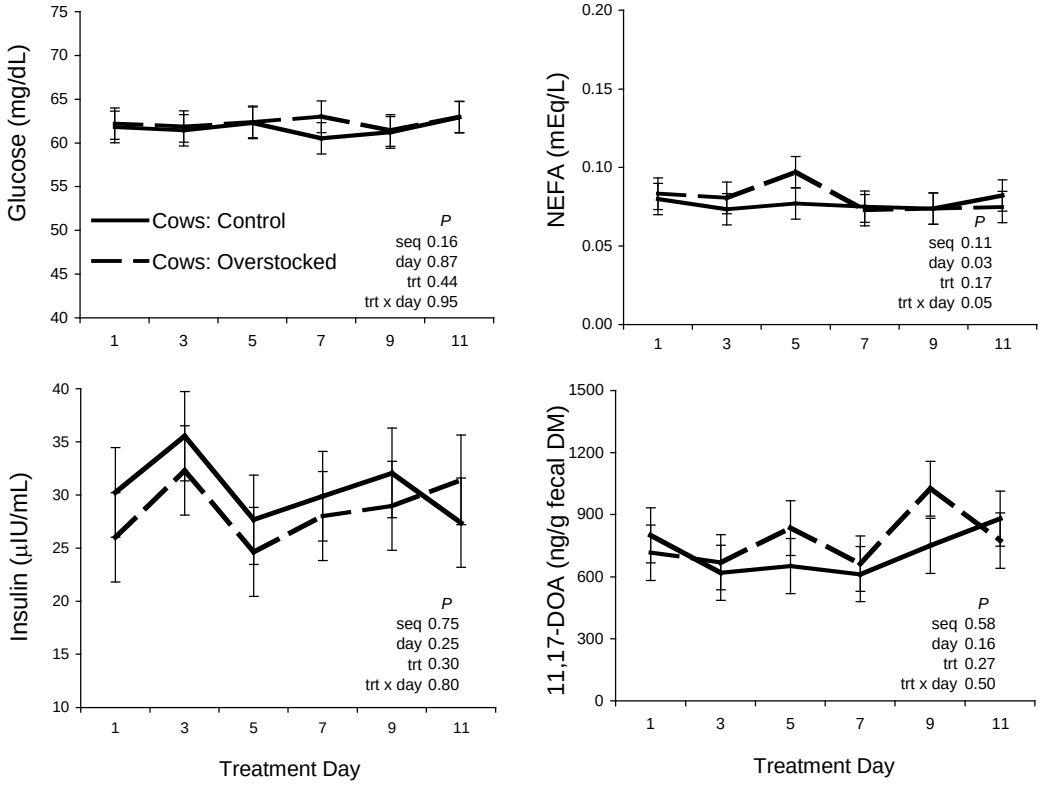
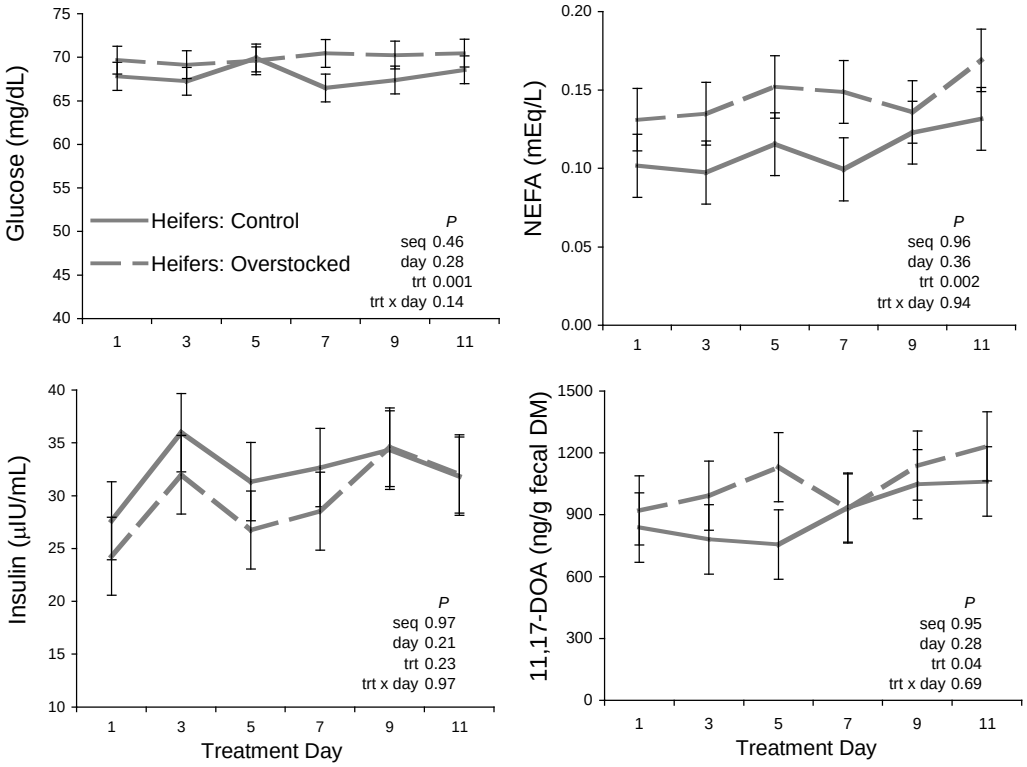


Figure 4.5. Least squares means (± SE) plasma glucose, nonesterified fatty acid (NEFA), insulin, and 11,17-dioxoandrostane (11,17-DOA) of primiparous cows in 4 groups during a control (solid line) and overstocked (dashed line) stocking density treatment.



DISCUSSION

The results of this study suggest that overstocking may alter physiological parameters associated with energy metabolism. While overall DMI increased during the overstocked treatment period relative to the control period, overall NEFA, averaged across the entire treatment period, was also higher during the overstocked period. In dairy cattle, elevated concentrations in plasma NEFA are typically observed when intake cannot support energy requirements thus requiring the mobilization of NEFA from adipose tissue to support energy demand (Bauman and Currie, 1980). The results of this study suggest that there may be factors other than intake regulating NEFA balance in the dairy cow during periods of overstocking; for example, changes in the concentrations of circulating hormones (e.g. insulin or glucocorticoids) that are important regulators of lipolysis and lipogenesis or changes to the sensitivity or responsiveness of tissues to these hormones may alter plasma NEFA concentrations (Bauman and Currie, 1980; Andrews and Walker, 1999).

Increased NEFA concentration during the weeks around calving (e.g. $\geq$ 0.3 mEq/L during the 2-wk period before calving) have been associated with an increased risk of disease, reduced milk yield, and compromised reproductive performance (Ospina et al., 2010a; Ospina et al., 2010b); however, this study was conducted during the early dry period (before 3 wk prepartum) and so it is unclear whether the increased NEFA concentrations observed during overstocked period could contribute to an increase risk for health or production complications after calving. Further, the NEFA concentrations observed in the present study during both treatment periods were low (0.9 to 0.11 mEq/L) compared to the prepartum NEFA concentration thresholds that Ospina et al. identified as being predictive of postpartum health and performance outcomes (e.g. $\geq$ 0.3 mEq/L); therefore, the observed increase in NEFA during the overstocked period may not be of biological significance. Future research will need to investigate whether

changes in NEFA concentrations during the early dry period could have downstream consequences on health and performance.

The results of the GTT provided additional evidence that overstocking is associated with changes in energy metabolism. Overall glucose AUC estimates, time to half maximal glucose concentration and time to basal glucose concentration were increased during the overstocked treatment period but the magnitudes of these differences were small and may not be of biological significance. For example, the time to reach basal glucose concentration following the GTT differed by only 4 min between stocking density treatments. On the other hand, stocking density treatment had a much greater effect on insulin response to glucose during the GTT. During the GTT, overall peak insulin secretion during the overstocked treatment was 61 μIU/L lower than the peak insulin secretion during the control treatment (199 vs. 260 μIU/L), a concentration difference 3 times that of basal insulin concentrations. Based on the insulin response, the glucose response curves to the GTT could be interpreted in two ways. First, decreased insulin secretion from the pancreas might explain the slightly reduced glucose clearance as there would be less endocrine signaling to up-regulate glucose transporters for cellular glucose uptake; alternatively, the overstocking treatment may have had an insulin-sensitizing effect, because less insulin was required to produce similar glucose clearance rates (Leney and Tavaré, 2009).

The physiological responses to overstocking appear to have commonalities with the responses observed during compromised nutritional status. For example, previous work has shown that plane of nutrition can influence insulin secretion. Hove (1978) reported an attenuated insulin response following an GTT in ketonaemic cows, while Holtenius et al. (2003) found that cattle that were fed below their metabolizable energy requirements had lower glucose induced insulin secretion from the pancreas. Despite these similarities, overall group intake was greater during the overstocked period; however, group DMI in response to overcrowding should be

interpreted with caution because it can mask individual differences in intake. It is likely that not all cows within a group have the same level of success at competing for access to the FB in order to achieve higher intake during overstocking. Future work will need to test this hypothesis by exploring how success at competitive interactions at the feed bunk during overstocking is related to feeding behavior and analytes associated with energy metabolism.

Glucocorticoids are also important moderators of energy metabolism; they increase the supply of glucose by promoting hepatic gluconeogenesis and also decrease the utilization of glucose by cells elsewhere in the body, possibly by altering these cells responsiveness to insulin (Andrews and Walker, 1999). These steroids can also directly inhibit insulin secretion from the pancreas (Lambillotte et al., 1997) and increase rates of lipolysis (Andrews and Walker, 1999). In the present study, no differences were observed in the plasma cortisol response to ACTH challenge between the two stocking density treatments. This was in contrast to the results of Friend et al. (1979) who reported a greater cortisol response to an ACTH challenge when cattle were exposed for 7 d to the same level of crowding in the lying stalls as used in the present study (1 stall/2 cows). Prolonged overstocking may be considered a chronically stressful situation and the physiological responses to prolonged stressors are not necessarily constant over time (Mormède et al., 2007). In the present study the ACTH challenge was administered after a 14 d treatment period and thus the lack of a cortisol response during the ACTH challenge could have reflected a physiological desensitization to stressors associated with crowding. Plasma cortisol response to ACTH challenge can also be influenced by ACTH dose level; as the dose of ACTH increases, maximum cortisol concentrations do not change but the cortisol response is prolonged over time (Lay et al., 1996) which may make treatment differences more difficult to detect The dose of ACTH used in the present study was adjusted for each cow's body weight and was lower than the dose used by Friend et al. (1979); therefore, it is unlikely that the current study utilized a

ACTH dose that was too high and masked treatment differences.

Concentrations 11,17-DOA tended to be greater during the overstocked period suggesting overall daily cortisol secretion might have been higher during overstocking. Overstocking is characterized by increased social interactions (Huzzey et al., 2006; Fregonesi et al., 2007) many of which can be aggressive and thus potentially experienced by the animal as acute stressors capable of inducing a physiological stress response. In the present study there was a greater frequency of competitive displacements from the FB during the overstocked period relative to the control period (50 vs. 28 displacements / d) and evidence of increased feeding rate (no difference in feeding time despite greater DMI during overstocking). Even with no difference in the amount of cortisol secreted from the adrenal gland upon stimulation, higher average 11,17-DOA concentrations could have been achieved if the adrenal gland was stimulated to secrete cortisol more frequently (e.g. during periods when cows were engaged in competitive displacements at the FB). There was no effect of day or a treatment by day interaction on 11,17-DOA concentrations, suggesting that concentrations of 11,17-DOA were constant overtime during each treatment period; this observation was not consistent with the hypothesis that there was physiological adaptation or desensitization at the level of the adrenal gland to the stress of overstocking. Although it is clear that cortisol has the capacity to affect energy metabolism through a variety of pathways, it is unclear whether the observed trends for greater 11,17-DOA concentration during overstocking reflect a raise in circulating cortisol sufficient enough to influence daily glucose and NEFA concentrations or the insulin response to the GTT. Although not measured in this study other moderators of the stress response such as epinephrine or norepinephrine may play a role in moderating these observed differences in energy metabolism (Malaisse et al., 1967).

In the present study cows were overstocked both at the lying stalls and at the FB;

overstocking both of these resources likely influenced overall treatment responses. Previous work has shown that cows will sacrifice feeding time in order to gain additional resting time when access to both resources is limited (Metz, 1985). Behavior at the lying stalls was not measured in the current study; however, during the overstocked period cows took longer to approach the FB following fresh feed delivery and spent a smaller proportion of their total daily feeding time within the 3 h period following fresh feed delivery. This observation may be evidence of some cows displaying a preference for resting during a time that would otherwise be considered a peak feeding period; cows are highly motivated to eat during the period following fresh feed delivery (DeVries and von Keyserlingk, 2005). These altered feeding patterns during the overstocked period suggest that stocking pressure (cow numbers) at the FB during peak feeding time was lower than the treatment defined stocking rate of 200%. If overstocking the lying stalls reduced stocking pressure (and thus competition level) at the FB it is possible that the physiological response to overstocking may differ depending upon which resource is overstocked and the magnitude of the stocking rate. For example, while there was a greater frequency of competitive displacements at the FB during the overstock treatment relative to the control treatment, it is possible that this level of competition may have been greater had the stalls not also been overstocked, thus resulting in a different physiological profile for the cows in the group. These hypotheses require further investigation.

Stocking density treatment had few effects on the measured physiological and behavioral parameters of multiparous cows (e.g. Figure 2); this may be evidence of multiparous cows being effective in adapting to a competitive feeding and resting environment. The glucose, insulin and NEFA responses of multiparous cows to the GTT were similar to the overall group responses (e.g. multiparous cows had lower peak insulin secretion during the overstocked treatment relative to the control treatment) but nearly all these associations were trends. This was likely due to

fewer animals being used to generate group averages (6 vs. 10 cows per group) leading to more variation between groups and thus a higher type I error risk for the GTT parameters.

When considering the responses of only the primiparous cows in each of the 4 groups, overstocking was associated with higher glucose, NEFA and 11,17-DOA concentrations relative to the control period (Figure 3). Primiparous cows also took longer to approach the FB following fresh feed delivery and had a smaller proportion of their total daily feeding time within the 3 h period following fresh feed delivery during the overstocked period, relative to the control period. Glucose, insulin and NEFA responses to the GTT were also similar to the overall group responses but were associated with a higher type I error risk, likely due to the lower number of primiparous animals used to generate group means (4 primiparous cows / group).

This study was not designed to make direct comparisons between the primiparous and multiparous cows housed together as this was a pen study and the cows within a pen were not independent units of analysis. However, after evaluating group responses to overstocking based on summarized data from either primiparous cows only or multiparous cows only, the results suggest that the physiological responses to overstocking may be influenced by parity. Because primiparous and multiparous cows were commingled in the present study, the exact manner by which parity may moderate physiological responses to overstocking is unclear. Heifers have been reported to spend less time feeding, have lower DMI, spend less time lying down, and be involved in more aggressive interactions when grouped with multiparous cows (Phillips and Rind, 2001); therefore it seems reasonable to speculate that when commingled together in an overstocked environment primiparous cows may experience more adverse effects relative to multiparous cows. This hypothesis, however, requires further testing using experimental designs appropriate for comparing responses between parities. Future work in this area could begin by exploring whether overstocking can alter aspects of physiology in cows housed within groups of

the same parity.

CONCLUSION

It is important to understand how overstocking influences dairy cow physiology in addition to behavior because this knowledge contributes to a better understanding of the ways in which overstocking can affect overall dairy cattle health and well-being. The overall results of this study show that overstocking during the dry period is associated with changes in physiology. These changes were related to energy metabolism and may be moderated by altered pancreatic insulin secretion or peripheral tissue responses to insulin. The role of cortisol in influencing these effects is still unclear. Additional research is required in order to determine whether these physiological changes are of a magnitude significant enough to affect subsequent health and performance.

Future work in this area should also explore whether physiological changes vary depending on the magnitude of overstocking, whether these changes differ depending on the resource being overstocked (i.e. lying stalls vs. feed bunk), and the effects of parity on the behavioral and physiological responses to overstocking. Finally, understanding how individual behavioral strategies to overstocking correlate with physiological outcomes may help to identify management interventions that can minimize both the negative behavioral and physiological consequences of overstocking.

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CHAPTER FIVE

Short Communication

RELATIONSHIP BETWEEN COMPETITIVE SUCCESS DURING DISPLACEMENTS AT AN OVERSTOCKED FEED BUNK AND MEASURES OF PHYSIOLOGY AND BEHAVIOR IN HOLSTEIN DAIRY CATTLE

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ABSTRACT

The objective of this study was to evaluate how behavioral and physiological parameters are affected based on a cow's level of success at displacing others at an overstocked feed bunk. Forty Holstein non-lactating, late gestation dairy cattle were housed in an overstocked pen (5 stalls/10 cows and 0.34 m linear feed bunk (**FB**) space/cow) in groups of 10 (4 heifers and 6 multiparous cows) for 14 d. Plasma NEFA, glucose and fecal cortisol metabolites (11,17-dioxoandrostanes**, 11,17-DOA**) were measured in blood and feces sampled every 2 d. A glucose tolerance test (**GTT)** and an ACTH challenge were conducted on all cows on d 13 and 14, respectively to further explore the effects of competitive success on energy metabolism and stress physiology. Feeding behavior and displacements at the FB were recorded between d 7 to 10 of the observation period. A competition index (**CI**) was calculated for each cow by dividing the number of time the cow displaced another at the FB by the total number of displacements the cow was involved in, either as an actor or reactor. Cows were then divided into 3 sub-groups based on their CI: High-success (**HS**: $CI \geq 0.6$), Medium-success (**MS**: $0.4 \leq CI < 0.6$), and Low-success (**LS**: $CI < 0.4$). Heifers accounted for 7%, 36% and 79% of the total number of animals in the HS (n=15), MS (n=11), and LS (n=14) groups, respectively. There were no differences in daily feeding time, total number of displacements, and time to approach the FB following fresh feed delivery between the 3 CI groups; however, cows in the LS group had greater daily NEFA and 11,17-DOA concentrations relative to cows in the HS group. There were no differences in cortisol response to an ACTH stimulation test between CI categories. During the GTT, glucose response curves were the same between all 3 CI categories; however, the peak insulin response of LS cows was 130 μIU/mL greater than the peak HS response indicating LS cows may have reduced tissue responses to insulin or increased pancreatic responses to glucose. In an overstocked environment, dairy cattle physiology is associated with a cow's level of success at

displacing other individuals at the feed bunk.

Keywords: overstocking, competition index, physiology, behavior

INTRODUCTION

When cows are crowded at the feed bunk (**FB**) aggressive displacements increase as cows vie to gain access to feed (Huzzey et al., 2006); it is likely that some cattle are more successful than others during these interactions. Previous work has shown that level of success in agonistic interactions may be an important determinant of an animal's ability to cope with an aversive environment. A compromised ability to cope may be evidenced by a greater physiological stress response or increased risk for health disorders. For example, Mendl et al. (1992) showed that pigs who were aggressive but also displaced frequently during agonistic interactions (Low Success) had greater salivary cortisol concentrations, a greater cortisol response to an ACTH challenge test, and lower weight gains than individuals that were aggressive but successful at displacing others (High Success). Galindo and Broom (2000) reported that cattle that were the least successful in agonistic interactions were at greater risk for lameness because they spent less time lying down and more time standing with two feet in the cubicle.

Competitive success during overstocking is associated with behavior in Holstein cattle. Cows that are displaced by a greater number of individuals at the feed bunk than they can displace themselves (Low success) eat faster (Proudfoot et al., 2009) and have a greater increase in feeding activity when more feed bunk space is provided compared to cows with higher competitive success (DeVries et al., 2004). Val-Laillet et al. (2008) found that cattle with low success in agonistic interactions at the feed bunk spent a smaller percentage of their time at the feeder compared to high success cattle. These behavioral differences may be associated with

differences in physiological parameters; however, this has never been explored. The objective of this study was to evaluate how stress physiology and energy metabolism are affected based on a cow's level of success during competitive interactions at the feed bunk.

MATERIALS AND METHODS

Forty non-lactating, late gestation Holstein dairy cows were housed in 4 groups of 10 cows (4 heifers and 6 multiparous cows per group) in a 2-row free-stall barn and managed according to the guidelines set by the Cornell University Institutional Animal Care and Use Committee. Groups were formed when cows were between 61 to 81 d from their expected calving date and cows were allowed 10 d to adapt to their respective groups before the feeding and resting space in the pens was modified to simulate conditions of overstocking. Access to the 4 free-stalls facing (nearest) the feed bunk and one additional free-stall along the back wall of the pen were roped off to restrict resting space and access to the feed bunk was restricted using plywood which was bolted across the feeding area. During the overstocked period, which lasted 14 d, each group of 10 animals had access to 5 free stalls and 0.34 m of linear post-and-rail feed bunk space per animal; this represented a stocking rate 200% that of industry recommendations (NFACC, 2009).

Cows were fed a TMR once daily at approximately 0800 h, feed-push ups occurred at regular intervals throughout the day, and all cows had ad libitum access to water. The TMR consisted of wheat straw (24.6 % of DM), corn silage (41.0% of DM), and dry cow grain (34.4% of DM) and a wet chemistry analysis (Dairy One Cooperative Inc., Ithaca, NY) of a composite of weekly feed samples revealed the following TMR composition (% DM ± SD): CP = 14.5 ± 0.6; ADF = 31.4 ± 1.5; NDF = 49.8 ± 2.8; Starch = 17.7 ± 0.7; Ca = 0.76 ± 0.07; P = 0.29 ± 0.01; Mg = 0.23 ± 0.01; K = 1.06 ± 0.04; and Na = 0.19 ± 0.03.

Behaviors were monitored using video cameras (Sony CCD Digital ULTRA Pro Series, Hi-Resolution BW CCD Camera with Auto-Iris) connected to digital recording system (DiGiCam H.264, 120 & 240 FPS, DVR PC Version; Central Alarms Systems Inc., Littleton, CO). A camera was positioned directly above the feeding area to continuously record behavior at the feed bunk. Hair dye was used to create unique alphanumeric symbols on the backs of the cows so that individuals could be identified on the video recordings. Daily feeding time and time to the feed bunk post fresh feed delivery was estimated from 10-min time scans of the video recordings over 4 consecutive days (d 7 to 10 of the 14-d overstocked period). A cow was considered to be feeding when its neck collar was visible beyond the top rail of the feed barrier on the feed alley side of the pen. To assess competitive behavior, 3 d of continuous (24-h; d 7 to 9) video recordings were reviewed and each competitive displacement that occurred at the feed bunk was recorded. A displacement was recorded when a cow's head (actor) came in contact with a cow that was feeding (reactor), resulting in the reactor withdrawing its head from the feed bunk. These observations were used to calculate a competition index (**CI**) for each cow. This index has previously been used in cattle (Val-Laillet et al., 2008; Galindo and Broom, 2000) and was calculated as follows:

$$CI = \frac{\text{No. of times cow is the ACTOR}}{\text{No. of times cow is the ACTOR} + \text{No. of times cow is the REACTOR}}$$

For each cow the CI score could vary from 0 to 1; an index value of 0 would indicate that a cow was never successful at displacing another individual but was displaced themselves, whereas an index value of 1 would indicated that a cow could displace others and but never be displaced themselves. These index values were used to categorize cows into 3 subgroups according to their level of success during competitive interactions at the feed bunk (Val-Laillet et al., 2008;

Galindo and Broom, 2000): Low Success (**LS**: CI < 0.40), Medium Success (**MS**: 0.40 < CI ≤ 0.60), and High Success (**HS**: CI > 0.60).

Blood and fecal samples were collected on d 1, 3, 5, 7, 9 and 11 of the 14-d overstocked period. Plasma concentrations of glucose and NEFA were measured by enzymatic analysis (glucose oxidase, P7119, Sigma Chemical; NEFA-C: Wako Pure Chemical Industries, Osaka, Japan). The intra- and interassay coefficients of variation (**CV**) for the NEFA assay were 3.7 and 4.4%, respectively, and for the glucose assay were 2.9 and 6.1%, respectively. Fecal samples were collected fresh, sealed within plastic bags and placed immediately under ice. Steroids from the fecal samples were extracted using the wet extraction method described by Palme and Möstl (1997). Concentrations of fecal cortisol metabolites (11,17-dioxoandrostanes; **11,17-DOA**) were measured using a competitive enzyme immunoassay developed by Palme and Möstl (1997) and validated for use in cattle (Palme et al., 1999). The intra- and interassay CV for the 11,17-DOA assay were 3.2 and 3.4%, respectively. The 11,17-DOA concentrations determined by the assay were corrected for the DM content of the raw fecal samples.

A glucose tolerance test (**GTT**) and adrenocorticotropic hormone (**ACTH**) challenge were administered to each cow on d 13 and 14, respectively, of the overstocked period. Prior to these tests (d 11 and 12) all cows were fitted with a jugular catheter that was secured to the neck within a fabric pouch using a topical adhesive approved for use on animals. The cow's neck was wrapped with elastic bandages to prevent the cow from dislodging the catheter while residing in the group pen. Body weights (**BW**) were measured on the day of catheterization to determine glucose and ACTH doses for the GTT and ACTH challenge, respectively. Approximately 1 to 1.5 h before the start of the GTT and ACTH challenge all cows were moved a short distance from their group pen to individual stanchions; this was done to facilitate sample collection during the procedures. Feed was removed from the cows 2 h prior to the start of the GTT. The GTT

involved administering 0.25 g/kg of BW of glucose i.v. (dextrose 50% wt/vol.; #002460, Butler Animal Health Supply, Dublin, OH) and then collecting blood samples at -15, -5, 0, 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 120, 150, and 180 min relative to the administration of the glucose. The ACTH challenge was performed by administering 0.125 IU/kg of BW of ACTH i.v. (Porcine, A6303, Sigma Chemical, St. Louis, MO). Blood samples were collected at -60, -15, 0, 15, 30, 45, 60, 90, 120, 150, 180, and 240 min relative to the administration of ACTH. During both procedures blood samples were immediately centrifuged, plasma harvested and stored at -20ºC for later determination of plasma glucose, NEFA and insulin concentration (GTT) or plasma cortisol concentration (ACTH challenge). Plasma insulin and cortisol were measured by radioimmunoassay (Porcine Insulin RIA Kit #PI-12K, Millipore Corp., Billerica, MA; Coat-A-Count Cortisol RIA Kit, Siemens Medical Solutions Diagnostics, Los Angeles, CA). The intra- and interassay CV for the insulin assay were 3.3 and 3.2%, respectively and for the cortisol assay were 3.2 and 1.9%, respectively.

All statistical analyses were performed using SAS version 9.2 (SAS Institute, 2009). Cow was considered the experiment unit for all analyses. Measures that were sampled over multiple days during the observation period (e.g. plasma NEFA, glucose, 11,17-DOA, displacements, feeding time, and time to feed bunk post fresh feed delivery) were averaged to generate one overall estimate of each measure per cow. These overall estimates were modeled as dependent variables using proc MIXED with the following included as fixed effects: group, parity, CI category, and the parity x CI category interaction. In each of these models the interaction term was not significant ($P \geq 0.23$) and so was removed from the models. The contrast statement in proc MIXED was used to describe the differences in the dependent variable between each of the 3 CI categories. To analyze the NEFA, insulin and glucose response to the GTT and the cortisol response to the ACTH challenge, area under the curve (**AUC**) was estimated for each cow's

response curves. AUC was calculated using the trapezoidal method and sampled concentrations after discounting basal values. For the glucose, insulin, and cortisol response curves only sampled concentrations that were above basal concentration were included in the AUC calculation while for the NEFA response only sampled concentrations that were below basal concentration were included in the AUC calculation (a negative estimate for NEFA). Natural logarithmic transformation (**Ln**) was required for all AUC data to comply with MIXED model assumptions and improve model fit. The absolute value of the negative NEFA AUC estimate for each cow was used for the log transformation. The Ln AUC estimates for each response curve were modeled as dependant variables using the same MIXED model set-up as described above.

RESULTS AND DISCUSSION

The distribution of CI scores for the 40 experimental cows is presented in Figure 5.1. The number of heifers, 1st lactation, 2nd lactation and 3rd lactation cows by CI category were: LS: 11, 2, 1, 0; MS: 4, 5, 1, 1; and HS: 1, 10, 3, 1, respectively. The average (± SD) body weight of cows in the LS, MS, and HS groups were 603 ± 62 kg, 680 ± 67 kg, and 713 ± 89 kg, respectively.

Average daily feeding time per cow and time to approach the feed bunk following fresh feed delivery was not different between CI categories ($P \geq 0.28$; Figure 5.2). There was no difference in the total number of displacements that cows in the LS, MS and HS groups engaged in per day, either as an actor or reactor (28, 32, and 32 ± 3 displacements per day, respectively; $P \geq 0.41$).

The LS group had greater plasma NEFA and fecal 11,17-DOA concentrations during the overstocked observation period relative to the MS and HS groups ($P \leq 0.05$; Figure 5.2) while average (± SE) glucose concentration did not differ between CI categories (LS: 65.9 ± 0.8, MS:

67.2 ± 0.9, HS: 65.4 ± 0.9 mg/dL; $P \geq 0.14$).

There were no differences in the plasma cortisol response to an ACTH challenge between cows in the 3 CI categories (Ln AUC Cortisol; LS: 9.78 ± 0.06, MS: 9.74 ± 0.06, HS: 9.83 ± 0.06 nmol/L x 240 min ACTH challenge; $P \geq 0.26$). Mean (± SD) cortisol concentration for cows in all CI categories before the ACTH challenge was 13.8 ± 2.8 nmol/L with cortisol concentrations peaking at t = 60 min following ACTH administration at 173.8 ± 13.8 nmol/L. Glucose response curves did not differ between CI categories following the GTT (Ln AUC Glucose; LS: 8.02 ± 0.06, MS: 7.90 ± 0.06, HS: 7.91 ± 0.06 mEq/L x 180 min GTT; $P \geq 0.21$) suggesting that glucose clearance rate was the same for all animals. The LS group had a greater insulin response to the GTT than the HS group (Ln AUC Insulin; LS: 8.73 ± 0.14 vs. HS: 8.14 ± 0.15 μIU/L x 180 min GTT; $P = 0.01$) and tended to have a greater NEFA response than the HS group (Ln |AUC| NEFA; LS: 8.82 ± 0.25 vs. HS: 8.02 ± 0.27 μEq/L x 180 min GTT; $P = 0.06$). The Ln transformed AUC estimates for the insulin (8.42 ± 0.14 μIU/L x 180 min GTT; $P > 0.14$) and NEFA (8.35 ± 0.27 μEq/L x 180 min GTT; $P > 0.23$) response curves of the MS group did not differ from the other two CI Categories. Figure 5.3 presents the non-transformed glucose, insulin and NEFA response curves to the GTT for the 3 CI categories.

These results suggest that there is an association between CI and physiological status. While no differences in the glucose response curves during the GTT between CI categories were observed, the insulin response to the GTT among LS cows was greater than for HS cows; this may suggest that among LS cows there is decreased tissue sensitivity to insulin (more insulin was required to yield the same glucose response) or greater pancreatic sensitivity to glucose, both of which may be indicators of insulin resistance. Insulin resistance can lead to a cascade of health problems that are analogous to Type 2 diabetes in humans; most notably rates of lipolysis are increased and plasma NEFA concentrations are high (Schinner et al., 2005). In the present

study, greater plasma NEFA concentrations were observed among the LS cattle throughout the observation period and at the start of the GTT, which may support the hypothesis that LS cows are more insulin resistant than HS cows. While increased NEFA concentrations during the 2 wk period before calving have been shown to be a risk factor postpartum health disorders including displaced abomasum, ketosis, retained placenta and metritis (e.g. LeBlanc et al., 2005; Ospina et al., 2010), it is unclear whether higher NEFA concentrations during the far-off dry period (before 3 wk prepartum) also are associated with increased disease risk. In the present study, average daily NEFA concentrations observed across all CI groups were relatively low (0.97 to 0.13 mEq/L) compared to NEFA thresholds that previous researchers have identified as being a risk factor for disease (e.g. greater than 0.3 during the 2-wk period before calving; Ospina et al., 2010). Overstocking during the far-off period and the corresponding changes in energy metabolism related to insulin resistance may set cows up for additional physiological challenges as they approach calving; this hypothesis however requires further investigation to explore its validity.

Insulin resistance can arise from a number of different factors including plane of nutrition and stress. Increased plasma NEFA concentrations have been shown to reduce insulin sensitivity through direct free fatty acid interactions with insulin receptor proteins; these interactions lead to dysfunctional insulin signaling and thus impaired translocation of insulin-dependant glucose transporters to cellular membranes (Schinner et al. 2005). Individual DMI was not able to be determined in the present study and so it's uncertain whether increased NEFA concentrations in LS were a function of reduced nutrient intake. No differences were detected in average daily feeding time between cows in the 3 CI groups or in the time it took them to approach the feed bunk following fresh feed delivery, suggesting that all animals had equal opportunity to consume a feed that was not over sorted and thus of high quality. Differences in feeding rate between

animals can influence total DMI when feeding time is held constant; however, previous research showed no relationship between DMI and success at displacing others in an overstocked feeding environment (Proudfoot et al., 2009).

Increased stress, leading to a rise in plasma glucocorticoid concentration, has also been linked to insulin resistance (reviewed in Andrews and Walker, 1999). A stressor can be any situation or event that threatens or is perceived by the animal to threaten overall fitness. Glucocorticoids, including cortisol, oppose the actions of insulin so that there can be increased substrate (NEFA) for oxidative energy metabolism and this in turn helps the animal respond to the stressor. A variety of pathways by which glucocorticoids can contribute to insulin resistance have been suggested such as by reducing the translocation of GLUT4 transporters to cell surface, increasing NEFA concentrations by promoting lipolysis, or up-regulating enzymes such as Glucose-6-Phosphate and PEPCK to increase hepatic gluconeogenesis (Andrews and Walker, 1999). In the present study, no differences in the plasma cortisol response to the ACTH challenge between CI groups were detected suggesting that adrenal capacity for cortisol secretion following acute stimulation was unaltered. This observation could be evidence that after a 14-d period of overstocking there is a level of desensitization in the physiological stress response to overcrowding at the level of the adrenal gland. Friend et al. (1979) observed a greater cortisol response to an ACTH challenge when cattle were exposed to the same level of crowding at the lying stalls as used in the present study (1 stall per 2 cows); however, this challenge was performed after only 7 d of exposure to the crowded environment.

Although there were no differences between CI groups in the cortisol response to the ACTH challenge, higher concentrations of fecal cortisol metabolites (11,17-DOA) in the LS group suggest there was still higher cumulative cortisol secretion in this group relative to the other two CI categories. Even with no difference in the amount of cortisol secreted from the

adrenal gland upon stimulation, higher average 11,17-DOA concentrations could have been achieved if the adrenal gland was stimulated to secrete cortisol more frequently. Fecal 11,17-DOA concentrations are an integrated reflection of cumulative cortisol secretion about 10 to 12 h prior to the fecal sample collection and therefore are also not confounded by the stress of sample collection (Palme et al., 1999). The results of the current study suggest that it is not necessarily the act of participating in displacements at the feed bunk that determines stress response but rather the frequency of success during those interactions. All cows in the present study were involved in competitive displacements at the feed bunk and the average number of displacements per day was not different between the 3 categories. The observation that the MS and HS groups did not differ in physiological responses to overstocking suggests that to some degree cattle can cope with some failure during competitive displacements at the feed bunk; however, for those animals that are the least successful during displacements at an overstocked feed bunk, their 11,17-DOA profile is indicative of a greater physiological stress response.

Previous work has shown that cows will sacrifice feeding time in order to gain additional resting time when access to both resources is limited (Metz, 1985). Behavior at the lying stalls was not measured in the current study; however, if cows did maximize their occupancy of the lying stalls throughout the day this may have reduced stocking pressure (cow numbers) at the feed bunk and thus decreased the level of competition for feed. Previous work has shown that cows are highly motivated to feed during the period following fresh feed delivery (DeVries and von Keyserlingk, 2005). While there was no difference in the average time cows in each CI category took to approach the feed bunk following fresh feed delivery these times averaged between 45 to 90 min (range approximately 0 min to 3 hours); these lag times may be a reflection of some cows showing a preference for lying rather than feeding when these resources are both limited. If overstocking the lying stalls reduced competition pressure at the feed bunk it

is possible that the physiological response to overstocking may differ depending on which resource is overstocked and the magnitude of the stocking rate. These hypotheses require further investigation.

The proportion of heifers in the LS group was much higher (79% of group) than in the HS group (7% of the group) and this may have also been a confounding effect in the present study. During the weeks leading up to calving heifers have greater NEFA concentrations than multiparous cows and appear to be able to withstand higher NEFA concentrations without developing health conditions such as fatty liver relative to multiparous cows (Vandehaar et al., 1999); however, it is unclear whether these relationships also exist during the far-off period. The extent to which innate physiological differences between heifers and cows explain the observed differences in NEFA profiles of LS and HS cows is unclear; however, insulin responses during the GTT likely cannot be explained by parity alone. The average BW of cows in the LS group was over 100 kg lower than the average BW of cows in the HS group; this is almost certainly explained by the higher proportion of growing heifers in the LS group. Insulin is an important regulator of skeletal muscle protein synthesis and insulin sensitivity is reported to be higher in growing animals (Davis and Fiorotto, 2005). If the observed differences in insulin response to the GTT were attributable to only an innate difference between heifers and older cows, higher tissue sensitivity to insulin would be expected in the younger animals; this was not observed in the current study.

CONCLUSION

In conclusion, this study shows that LS cows, which are displaced frequently from the feed bunk and are not successful at displacing others, have a different physiological profile than HS cows. There is evidence of a greater physiological stress response as evidenced by greater

average daily fecal cortisol metabolite concentrations as well as altered energy metabolism that is suggestive of insulin resistance. This study also found that cattle could cope with some failure during competitive displacements at the feed bunk, as it was found that there were no physiological differences between MS and HS cows. Finding ways to reduce displacement frequency, such as by using a headlock feed barrier (Huzzey et al. 2006) or feeding partitions that extend from the feed bunk into the pen (DeVries et al. 2006), might be a strategy producers can use to improve the overall well-being of LS cows, possibly by making it more difficult for others to displace them.

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Figure 5.1. Competition index (CI) of 40 experimental cows that describe their level of success during competitive interactions at the feed bunk; 3 groups are distinguished: a High Success group (CI ≥ 0.6), Medium Success group (0.4 ≥ CI < 0.06), and Low Success group (CI < 0.4).

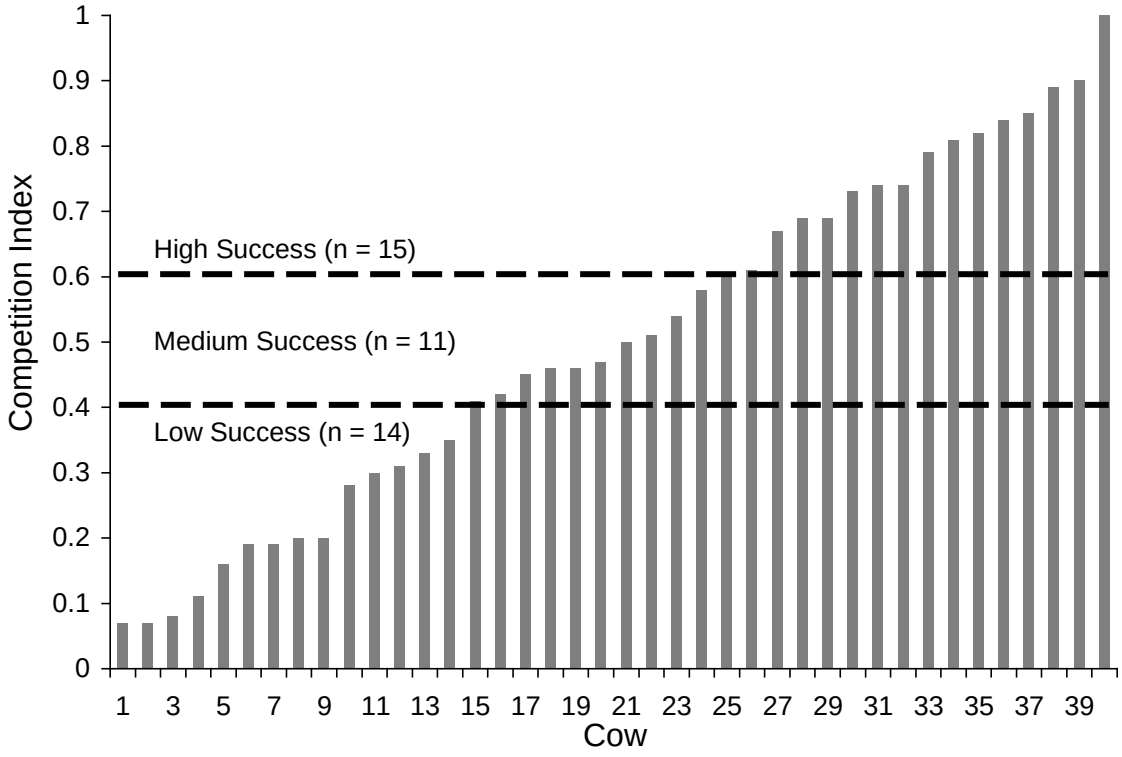


Figure 5.2. Least squares means (± SE) plasma NEFA, fecal cortisol metabolite (11,17-DOA), daily feeding time and time to approach the feed bunk (FB) following fresh feed (FF) delivery of cows grouped into 3 categories based on their competition index (CI) score: High Success group (**HS**: CI ≥ 0.6), Medium Success group (**MS:** 0.4 ≥ CI < 0.06), and Low Success group (**LS:** CI < 0.4).

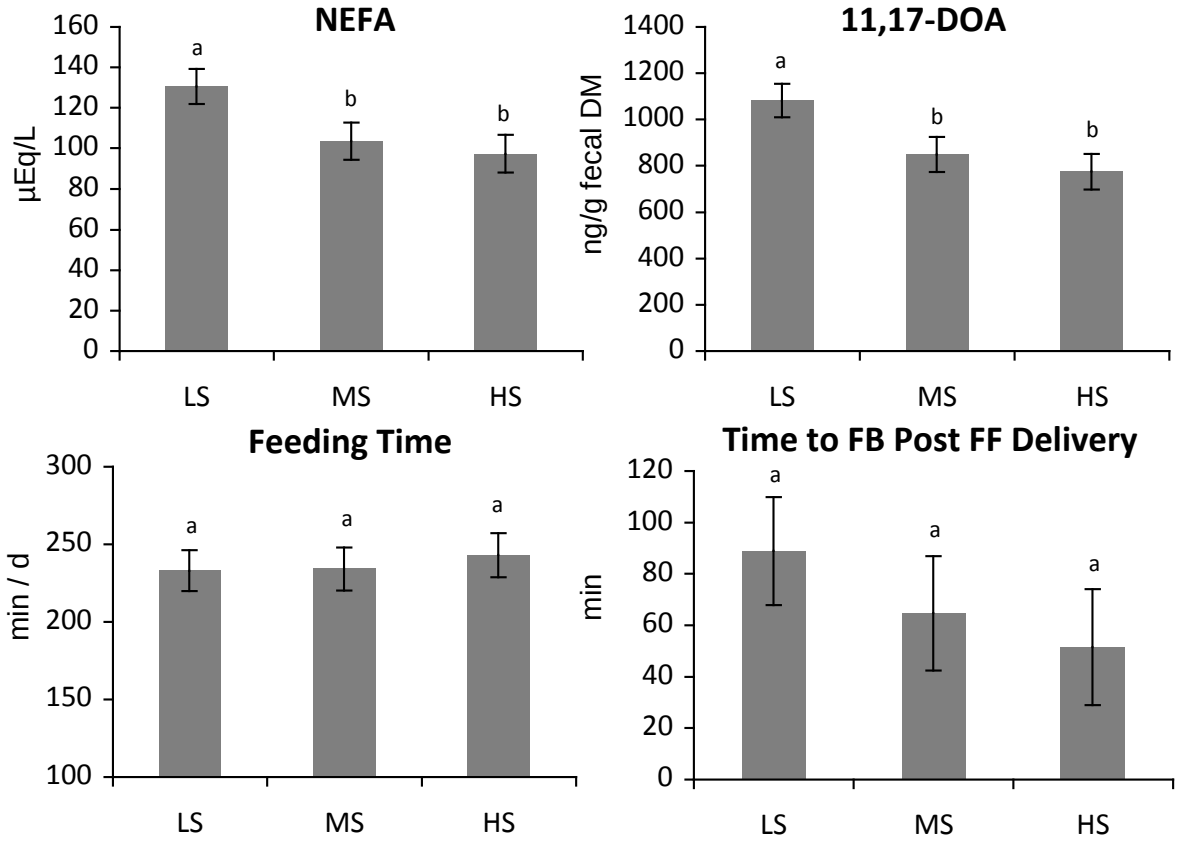
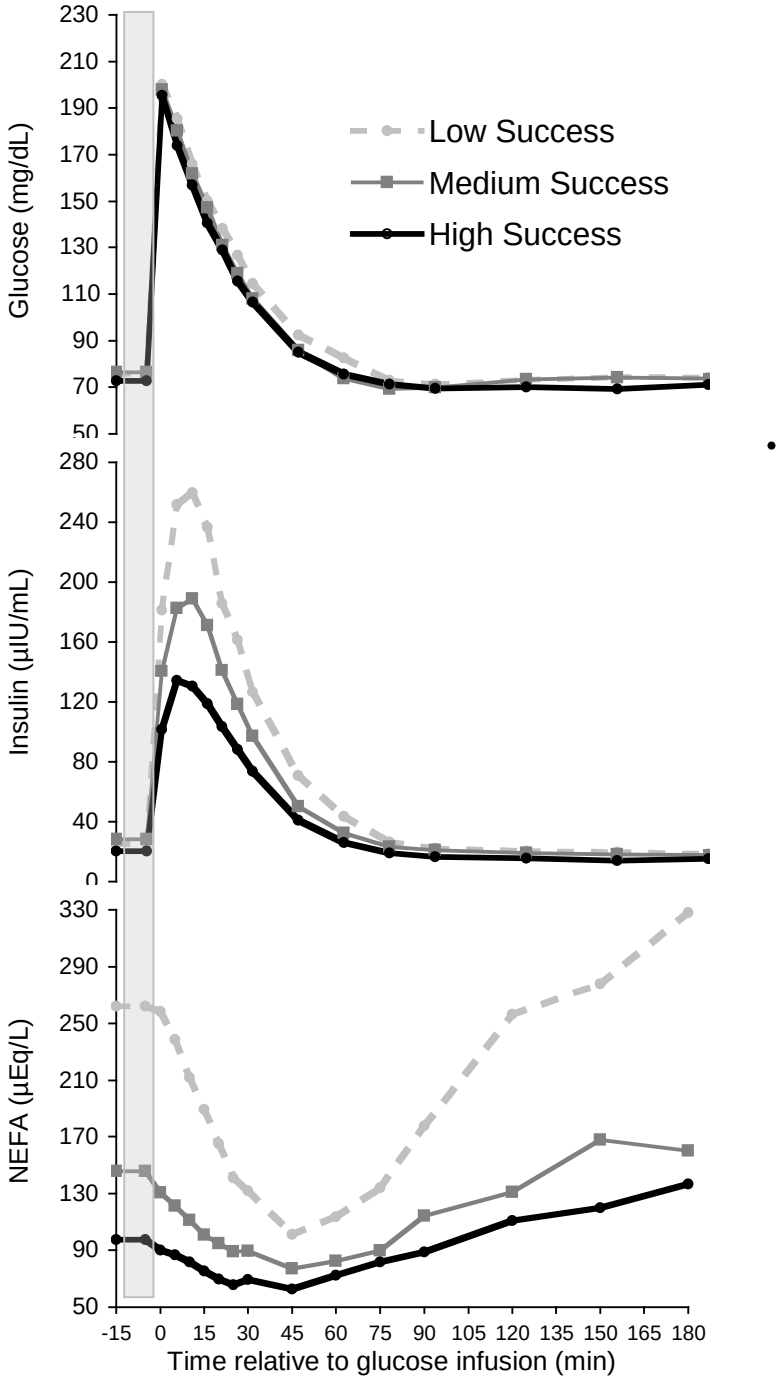


Figure 5.3. The glucose, insulin and NEFA responses curves during a GTT of cows grouped into 3 categories based on their competition index (CI) score: High Success group (CI ≥ 0.6), Medium Success group (0.4 ≥ CI < 0.06), and Low Success group (CI < 0.4). Shaded portion of figure highlights basal analyte concentrations at t = 15 and 5 min prior to glucose infusion.



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CHAPTER SIX

OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

The period around calving is very challenging for dairy cows because they must adapt to numerous environmental and physiological changes in a short period of time. Most diseases affecting cattle occur during the weeks following calving likely because many cows cannot successfully adjust to these changes. This research explored the relationships between physiology, behavior, and management of cows during the period around calving to further our understanding of factors that increase risk for compromised dairy cattle health and productivity.

Measures of Stress and Inflammation as Indicators of Health and Performance

The first major objective of this research was to determine if physiological analytes associated with stress (cortisol) and inflammation (haptoglobin; **Hp**), measured during the period around calving, were associated with health status, milk yield or reproductive performance after calving. Because plasma concentrations of nonesterified fatty acids (**NEFA**) have been shown by several researchers to be useful indicators of risk for disease, reduced milk yield and compromised reproductive performance, the next major objective of this research was to determine whether analytes associated with stress and inflammation could provide additional information (relative to NEFA) about level of risk.

This research supported the conclusions of others that negative energy balance, as measured by increased concentrations of NEFA, is an important risk factor for disease after

calving. Increased prepartum concentrations of fecal cortisol metabolites (**11,17-DOA**) increased the odds of cows developing health disorders after calving, but in multiparous cows only and only when sampled during week -2 relative to calving. On the other hand, higher plasma cortisol concentrations during week -2 were associated with lower odds of disease in primiparous cows. Prepartum Hp was not associated with postpartum disease status after accounting for calving difficulty as a covariate in the analysis. Increased NEFA concentrations were associated with disease regardless of when NEFA was sampled before calving; further, the strength of these associations between NEFA and health status were greater than the strength of the associations observed for plasma cortisol or 11,17-DOA during wk -2 (lower type I error risk). Based on these results, herd-level testing programs (i.e., cross-sectional sampling with an appropriate sample size) focused on identifying disease risk in transition dairy cattle, should consider NEFA as their primary analyte of interest.

Increased NEFA concentrations around calving were also found to be an important risk factor for reduced milk yield and longer days to conception, which supports the findings of others. Perhaps the most novel finding of the current research was in the discovery that both increased Hp and 11,17-DOA concentrations around calving are strongly associated with reduced milk yield and compromised reproductive performance, particularly when sampled during the postpartum period. In fact, measures of stress and inflammation during the postpartum period appeared to be better indicators of subsequent milk yield and reproductive performance than NEFA. For example, cattle with a Hp concentration > 1.1 g/L between day 3 to 10 postpartum, had 947 kg lower projected 305-d milk yield and heifers with Hp > 1.3 g/L during this period, had a 41% lower rate of conception. On the other hand, increased NEFA during the postpartum period was associated with higher milk yield in heifers, only tended to be associated with lower milk yield in multiparous cows, and was associated with a 39% lower rate of

conception for heifers. These results suggest that herd-based analyte testing programs aimed at identifying opportunities to improve milk yield or reproductive performance, may benefit by adding Hp or 11,7-DOA to the analysis.

Plasma cortisol concentrations were difficult to interpret because relationships to milk yield and reproductive performance were inconsistent. For example, increased concentrations of plasma cortisol during the postpartum period (3 to 10 d postpartum) were associated with greater projected 305ME milk yield in both MP and PP cows while postpartum plasma cortisol concentration was not related to reproductive performance. Despite its widespread use in research as a measure of stress, plasma cortisol concentrations can be easily confounded by the stress of handling and sample collection. This research highlights the fact that fecal sampling is a minimally invasive and practical tool that producers can use to both measure stress in their cows and identify which animals are at the greatest risk for lower milk yield and compromised reproductive performance.

These associations were frequently influenced by parity and time of sample collection relative to calving. For herd-based testing programs, this information is important because it suggests that specific predictive thresholds for identifying level of risk cannot always be generalized across parity and that sampling during specific periods relative to calving may be beneficial. A major challenge for prepartum blood testing programs is the inability to know exactly when parturition will occur; the actual date of calving can vary by ± 5 d from the predicted date. Testing programs considering Hp or 11,17-DOA as analytes of interest, should focus on collecting postpartum samples (3 to 10 d postpartum) as this data will likely provide more reliable estimates of risk for reduced milk yield and lower conception rates because knowledge of sampling time relative to calving is assured.

Overstocking – Consequences for Physiological Health

In order to improve transition cow well-being early identification of cattle at high risk for disease or production complications is important; however, understanding how external factors (i.e. management strategies such as overstocking) may contribute to this increased risk is equally important. Therefore, another major objective of this research was to describe the physiological effects of overstocking, including the behavioral and physiological mechanisms by which health may be affected. This research is the first to provide evidence that overstocking is associated with changes in energy metabolism in dairy cattle. Concentrations of plasma NEFA and glucose are increased in cattle during overstocking, despite these animals also having greater dry matter intake. Concentrations of 11,17-DOA also tend to be higher during overstocking. This research also found that overstocking is associated with slightly slower glucose clearance from circulation but a much more produced decrease in pancreatic insulin secretion following a glucose challenge. Changes in glucose uptake may be mediated by alterations in pancreatic secretion of insulin or peripheral tissue responses to insulin. The role of stress hormones (i.e. glucocorticoids) in mediating these changes in energy metabolism are still unclear as overstocking did not influence the amount of cortisol secretion from the adrenal gland following ACTH stimulation.

This research also suggests that younger animals (heifers or first lactation cows) may be at the greatest risk for these metabolic disturbances when they are overstocked with older multiparous cows. When exploring the interactions between behavior and physiology during overstocking, it was found that those animals that were the least successful at competing for access to an overstocked feed bunk had the highest concentrations of 11,17-DOA and NEFA; this may suggest greater stress-loads and more physiological consequences for cows that cannot compete with others in a crowded environment. This low success group of cows consisted almost entirely of heifers. This research provides useful insights that can inform management strategies

aimed at improving cattle well-being during overstocking. For example, finding ways to reduce displacement frequency, such as by using headlock feed barriers or feeding partitions that extend from the feed bunk into the pen, might be a strategy producers can use to improve the overall well-being of low success cows, possibly by making it more difficult for others to displace them.

Directions for Future Research

A reoccurring observation from this research is that primiparous animals (heifers) respond differently than multiparous animals (cows) both behaviorally and physiologically during the transition period. For example, this research demonstrated that when heifers and cows are grouped together in an overstocked environment, the heifers are less successful at competitive interactions at the feed bunk (they are displaced from the feeding area more often than they displace others) and have greater NEFA and fecal cortisol metabolite concentrations in response to overstocking, suggesting increased susceptibility to health or production complications associated with negative energy balance or higher stress-load. An improved understanding of how heifers and cows differ in their behavioral responses to challenging management situations (e.g., overstocking, regrouping, pen moves, etc.) and how this in turn affects biological functioning will be an important area of future research.

The strong associations between increased postpartum Hp concentrations and reduced milk yield or decreased reproductive performance warrants further investigation into the on-farm practicality of using plasma Hp in herd testing programs. While fecal cortisol metabolites were also associated with these performance outcomes, fecal sample processing and laboratory assays to measure 11,17-DOA are time consuming, complicated, and likely to be costly thus limiting the practicality of this measure for wide-spread herd-testing programs. Analyzing Hp concentrations is a relatively simpler process and there are veterinary diagnostic laboratories that

routinely run Hp assays. The next step for this research will be to characterize the inter- and intra-herd variation in Hp concentrations around calving by sampling a much larger population of animals across many farms. This information can then be used to identify herd alarm levels (i.e. proportion of animals above an indicated Hp threshold) that would indicate whether opportunities existed at the herd-level for improving overall transition cow health and productivity.

Final Remarks

Ensuring high standards for animal welfare have been and will continue to be an important component of animal agriculture. The results presented in this dissertation provide a potential basis for the development of practical strategies that can be applied on-farm to improve management and thus overall dairy cow well-being.