OXYTOCIN AND THE STRESS RESPONSE IN BEEF CATTLE: OPPORTUNITIES AND LIMITATIONS

Dissertation

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ABSTRACT

Changes in the physiological, psychological, and behavioral manifestations of stress have been observed in association with increases in circulating oxytocin. Providing oxytocin intra-nasally has been shown to attenuate stressor-induced hypothalamo-pituitary-adrenal (HPA) axis activation in humans and non-human primates, however anxiolytic effects may be context and species specific.

The first study, presented in Chapter Two, aimed to investigate the effect of intra-nasal oxytocin supplementation on stressor-induced activation of the HPA axis in beef cattle. I hypothesized that oxytocin would attenuate activation of the HPA axis, ultimately decreasing plasma cortisol and adrenocorticotropic hormone concentrations. This study demonstrated that oxytocin treatment did not affect measured indicators of the HPA axis activation and confirmed that restraint and isolation stress increases cortisol in *Bos taurus* heifers compared with heifers subjected only to isolation. In addition, an endogenous release of oxytocin was detected in response to restraint stress. The extreme nature of restraint and isolation stress may have overshadowed any effect of intra-nasal oxytocin.

The second study, presented in Chapter Three, aimed to investigate the effect of intra-nasal oxytocin on cattle subjected to a more intermediate stressor – transportation. I hypothesized that *Bos indicus* cattle treated intra-nasally with oxytocin would have a less extreme increases in cortisol concentrations and changes in immune parameters when subjected to handling and 6 h road transportation compared with cattle treated intra-nasally

with saline. This study confirmed that short-duration road transportation induces signs of an acute inflammatory response, however no effect on the HPA axis was detected. In addition, intra-nasally administered oxytocin altered leukogram numbers such that specific leukocytes returned to baseline more quickly in calves given oxytocin. The low dose and mild nature of the stressor may explain lack of substantial effects HPA axis activation.

The third study, presented in Chapter Four, aimed to evaluate the pharmacokinetics of three different doses of oxytocin administered intra-nasally. Using a continuous sampling timeline was crucial in assessing an accurate dose-response curve for intra-nasal oxytocin. Results from this study coincide with previous studies investigating different intra-nasal doses in that pharmacokinetic parameters remained the same across doses. An intermediate half-life of oxytocin was established for oxytocin administered intra-nasally in *Bos taurus* steers. Furthermore, this study added to the limited existing knowledge regarding appropriate doses and sampling timelines necessary to obtain meaningful results in studies utilizing intra-nasal oxytocin.

The final study, presented in Chapter Five, aimed to investigate oxytocin and cortisol in lactating and non-lactating cows. Previous authors have reported greater oxytocin concentrations paired with attenuated increases in cortisol concentrations in lactating sheep; non-lactating sheep were shown to have greater cortisol concentrations in response to restraint stress. I hypothesized that intra-nasally administered oxytocin would attenuate activation of the HPA axis in non-lactating beef cattle comparable to attenuation observed in lactating cows. Results indicated that lactating and non-lactating cows had similar plasma concentrations of oxytocin. However, an attenuated increase in cortisol concentration was observed in lactating cows compared with non-lactating cows.

Hormones other than oxytocin, such as prolactin, may be involved in facilitating the stress hypo-responsive state observed in lactating mammals.

Overall, the present work does not support intra-nasal oxytocin as an attenuator of the HPA axis in beef cattle subjected to restraint and isolation or transportation stress. The rapid disappearance of oxytocin from plasma following intra-nasal administration, confirmed by experiments presented in Chapters Four and Five, indicates that any action of oxytocin is most likely not at the adrenal level; however, more investigations would be needed to confirm this notion.

This dissertation is dedicated to:

Grandpa, the coolest guy I know.

I cannot thank you enough for always encouraging me to ask questions.

You inspire me every day to never stop learning.

Mom and Grandma

Thank you for showing me, every day, what unconditional love really is.

~

“If I have seen further than others, it is by standing upon the shoulders of giants.”

-Sir Isaac Newton

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LIST OF ABBREVIATIONS

Acute phase protein.....APP

Bovine respiratory disease.....BRD

Cerebral spinal fluid.....CSF

Corticotropin releasing hormone.....CRH

Dark cutting caracass.....DCC

Day.....d

Hour……h

Hypothalamo-Pituitary-Adrenal……HPA

Intra-nasal…….....IN

Non-transported……NT

Oxytocin……OXT

Restraint and Isolation Stress……...……RIS

Saline……...S

Transported…….....T

Vasopressin.....VP

CHAPTER ONE: REVIEW OF LITERATAURE

THE STRESS RESPONSE

Background

The term ‘stress’ is often considered ambiguous and a universal definition of stress has proved to be difficult to attain. An ‘everyday definition’ states that stress is a “physical, mental or emotional strain or tension” (Fraser, 2008). Stress is often brought on by stressors, which can be defined as adverse forces that pose a challenge to homeostasis; stressors can be either extrinsic or intrinsic (Tsigos and Chrousos, 2002). A more scientific definition describes stress as “the biological response elicited when an individual perceives a threat to its homeostasis,” (Moberg and Mench, 2000). Homeostasis refers to maintenance of a stable environment, in this case physiologically maintaining stable conditions within the body. Another, more recent definition describes stress as “a complex physiological state that embodies a range of integrative and behavioral processes when there is a real or perceived threat to homeostasis,” (Tilbrook and Ralph, 2017). A stress response, or the “…coordinated physiological responses within the body…” activated, “…in an attempt to reestablish homeostasis,” (Carroll and Forsberg, 2007), then ensues following the body’s recognition that a stressful event has

occurred. An activated stress response can have direct and/or indirect effects on other systems, such as but not limited to metabolic, immune, and neuroendocrine pathways (Moberg and Mench, 2000). This coordinated reaction of the body to maintain homeostasis makes the study of stress equally as complex as the stress response itself, if not more so - the body knows what to do, while researchers are trying to understand, predict, and in some cases manipulate the overall system. Categorizing types of stress is helpful when investigating stress and the stress response.

An activated stress response is not inherently negative as colloquial connotation would have us believe. Positive stimuli, such as mating, can show a similar physiological consequences as negative stimuli, such as restraint (Fraser, 2008). The terms 'eustress' and 'distress' are often used to distinguish between 'good' and 'bad' stress, respectively (Selye, 1975; Moberg and Mench, 2000). Differentiating a non-threatening stress can be done by taking into account the biological cost of the stress (Moberg and Mench, 2000). Distress will be the main focus of this dissertation.

Distress can be further subdivided into two distinct categories: acute and chronic. Acute stress is considered a short, intense stress experience, while chronic stress indicates a more prolonged circumstance that may threaten or exceed coping resources (Miller et al., 2007). Chronic stress increases the chance of adverse medical outcome in humans (Miller et al., 2007) and increases the activation of the stress response in response to novel, acute stress in rodents (Bhatnagar and Dallman, 1998). Stressors that fall within these two groups can be further subdivided by type of stressor: physical or psychological. Physical stressors greatly impact the immune response, while psychological stressors are greater activators of the pituitary-adrenocortical system (Moberg and Mench, 2000).

Making clear distinctions between different types of stressors is necessary due to ample evidence demonstrating that stress type and duration impacts the biological response to stress.

There are two primary pathways activated in response to stress: the hypothalamo-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary (SAM) axis. For brevity, the HPA stress axis will be the primary focus of this dissertation.

The hypothalamo-pituitary-adrenal (HPA) axis

The HPA axis involves a complex cascade of carefully regulated biological pathways and is essential for maintaining homeostasis and proper body function. Many different stressors can signal the hypothalamus to secrete corticotropin-releasing hormone (CRH) and/or vasopressin (VP); both CRH and VP are capable of stimulating the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland (Carroll and Forsberg, 2007; Miller et al., 2007; Hall, 2016). Under non-stressful conditions, CRH and VP are secreted in a circadian rhythmic manner, subsequently increasing ACTH and cortisol concentrations in the early morning for both humans (Horrocks et al., 1990) and cattle (Thun et al., 1981). Stress, as well as other factors such as light and feeding schedule, can disrupt the natural rhythm of CRH and VP secretion, often leading to amplified concentrations of ACTH and cortisol (Tsigos and Chrousos, 1994). In addition, neurohormones CRH and VP have been shown to be species-specific; CRH is a more potent stimulator of ACTH secretion in bovine, while VP is more potent within the HPA axis response in ovine species (Carroll and Forsberg, 2007).

Corticotropin-releasing hormone is consistently regarded as the primary player in mounting a stress response. Administering CRH directly to non-human primates and rodents has been shown to elicit an inflammatory response, a common indicator of stress (Shibasaki et al., 1993), while CRH antagonists suppress these responses (Ward et al., 2000; Bakshi et al., 2002; Tsigos and Chrousos, 2002). Smith et al. (1998) utilized knockout mice lacking CRH type 1 receptors to investigate the importance of the role of CRH in mounting a stress response. Mice without CRH type-1 receptors were found to be unable to effectively mount stress responses and, even more interestingly, all homozygous mutant mice died within 48 hours following birth from neonatal respiratory distress (Smith et al., 1998). The authors suggest that ACTH, resulting from a functional HPA axis, is vital to early development of the adrenal gland in neonates (Smith et al., 1998). Even though neonatal adrenal development is not of specific concern when attempting to better understand and characterize stress in cattle, this study demonstrates the importance of CRH within the stress response, as well as the essentiality of the HPA axis to proper body function.

Similarly, ACTH - the single chain hormone consisting of 39 amino acids (Carroll and Forsberg, 2007; Hall, 2016) - is released in a pulsatile manner and has a half-life of roughly 10-15 minutes (Yalow et al., 1964). Increases in plasma concentrations of ACTH signal the zona fasciculata of the adrenal cortex to produce and release glucocorticoids (Carroll and Forsberg, 2007; Miller et al., 2007), therefore ACTH and cortisol are often discussed in unison. Previous research in humans has shown a direct correlation between ACTH and cortisol concentrations, as well as a pulsatility over a twenty-four hour period (Horrocks et al., 1990). In 1994, Verkerk et al. found that cows, in response to an ACTH

challenge, reached peak plasma cortisol concentration more quickly (30 minutes) compared with humans (20 minutes), indicating greater sensitivity of cattle and sheep to increases in ACTH (Verkerk et al., 1994) compared with humans. The pulsatility of ACTH and cortisol, as well as species differences, should be considered when selecting and evaluating sampling timelines in different species.

Increases in plasma concentrations of ACTH signal the adrenal cortex to produce and release glucocorticoids (Carroll and Forsberg, 2007). Cortisol is the primary glucocorticoid produced in the adrenal cortex in most mammals, while rodents primarily produce corticosterone (Carroll and Forsberg, 2007). Plasma cortisol concentrations are predominately measured as an indicator of activation for the HPA axis in beef cattle under transportation stress (Parker et al., 2007; Hulbert and Moisá, 2016), weaning stress (Enríquez et al., 2011), and various husbandry procedures (Mellor et al., 2002). Baseline plasma cortisol concentrations range from 0.4 to 9.7 ng/mL in cattle (Thun et al., 1981) and 10 to 25.0 ng /mL in sheep (Apple et al., 1993a). Cortisol has a longer half-life of 60 minutes, compared with ACTH, and is considered one of the "final effectors of the HPA axis and participate in the control of whole-body homeostasis" (Tsigos and Chrousos, 2002), most notably by stimulating gluconeogenesis (Carroll and Forsberg, 2007).

Glucocorticoids stimulate the conversion of fat and protein to intermediate metabolites which are subsequently converted to glucose (Carroll and Forsberg, 2007; Cohen et al., 2012), therefore blood glucose concentrations increase in response to increases in glucocorticoid concentrations. Baseline glucose concentrations in ruminants are notably lower than in other species, typically ranging from 45 to 80 mg/dL (Frandson et al., 2009). In order to return the body to a homeostatic blood glucose concentration, a

high blood glucose concentration inhibits glucagon secretion and increases the release of insulin (Hall, 2016).

It is also important to note that various stressors can affect the natural rhythm of the HPA axis, the consequences of which need further investigation. One meta-analysis, which included 107 human studies, found that chronically stressed individuals had significantly lower concentrations of cortisol in the morning, greater concentrations of cortisol in the afternoon/evening, and an overall flatter diurnal pulsatility (Miller et al., 2007). The time of sampling should be carefully considered when conducting experiments and interpreting results in stress studies.

Measuring glucocorticoids

Glucocorticoids, such as cortisol, are the most commonly measured indicator of HPA axis activation in mammals. A prolonged increase in cortisol concentration is associated with immunosuppression and excessive protein catabolism (Carroll and Forsberg, 2007), which can contribute to decreased animal efficiency. Cortisol can be measured in the blood, saliva, urine, feces, or milk; each of these methods offers a different understanding of the HPA axis (Miller et al., 2007). Diurnal variation can play a role in cortisol concentrations, making time of day and frequency of sampling key factors when relying on cortisol as an indicator of adrenal activity. Sampling the blood offers the most immediate assessment of changes in cortisol concentrations, but often involves some amount of handling of the animal which may have an effect on cortisol concentrations (Moberg and Mench, 2000). Other methods, such as collection of saliva, offer a less invasive alternative for measurement of cortisol.

Compared with blood, saliva samples take slightly longer to collect (~0.5 minutes compared with 3 minutes, respectively) and plasma cortisol concentrations are consistently greater than salivary cortisol concentrations in cattle (Negrão et al., 2004). In a review by Hellhammer et al. (2009), some dissociations between salivary cortisol and HPA axis function are discussed. It is noted that the relationship between plasma and salivary cortisol may not always be linear depending on saturation of cortisol binding globulin; saturation is influenced by circulating gonadal steroids (Hellhammer et al., 2009). Several factors, such as type of stressor, sex of animal, and time of day, can influence salivary cortisol and should be taken into consideration when designing studies. Overall, salivary cortisol concentrations have been shown to correlate with plasma cortisol concentrations in several species (Parrott et al., 1989; Vincent and Michell, 1992; Negrão et al., 2004) and is generally accepted as a less sensitive but still useful indicator of adrenal activity.

Two alternative, non-invasive techniques for monitoring the HPA axis activation are measuring cortisol concentrations in urine or in feces. Typically, a 24-hour urinary collection is used to determine free cortisol concentrations and roughly 1% of cortisol in the blood is excreted in the urine (Hellhammer et al., 2009). In Marmoset monkeys, free cortisol concentrations increased in the urine following exposure to restraint and isolation stress (Smith and French, 1997). In addition, cortisol/creatinine in urine have been used to assess the hormonal stress response in dogs given insulin; greater urinary cortisol concentrations were found in dogs following insulin treatment (Beerda et al., 1996). Fecal cortisol has been assessed and correlated to urinary cortisol in captive chimpanzees exposed to anesthesia (Whitten et al., 1998). In dairy cattle, fecal cortisol concentrations

were elevated 12-24 hours following an ACTH challenge that resulted in peak cortisol concentration in the plasma (Morrow et al., 2002). Morrow et al. (2002) establish a direct but delayed correlation between adrenal activity and fecal cortisol excretion. However, as with any measurement of the HPA axis, there is considerable inter-individual variation in measurement of cortisol in the urine or feces. Overall, urine and fecal collections offer a general understanding of cortisol concentrations when compared with the more immediate sampling methods such as looking at changes in blood and saliva cortisol concentrations.

Measuring milk cortisol is a less common measurement of stress, as it is specific to lactating females. Milking is a part of every-day life for dairy animals, therefore measuring cortisol in the milk is non-invasive and may be useful for measuring short-term stress. Verkerk et al. (1998) used an ACTH challenge to induce peak cortisol concentrations in the plasma at different times prior to milking (1, 3, and 4 hours before afternoon milking). Equilibrium of cortisol between plasma and alveolar milk was re-established within one hour of peak plasma cortisol concentrations (Verkerk et al., 1998). Neither composite milk nor foremilk indicated any differences in cortisol concentrations in animals challenged with ACTH three or four hours prior to milking (Verkerk et al., 1998). Authors suggest milk cortisol concentrations may only be useful at indicating exposure to stress shortly before milking (Verkerk et al., 1998).

Summary

Stress induces a coordinated reaction of the body to maintain homeostasis. The integration of this response in all other body systems makes the study of stress equally as

complex as the stress response itself, if not more so. One system in particular, the HPA axis, carefully regulates biological pathways and aides in maintaining homeostasis and proper body function. Researchers can measure certain parameters, such as glucocorticoids, to assess HPA axis activation. Various stressors can activate the HPA axis in unique ways, therefore the type of stressor selected should be carefully considered when investigating stress.

EXPERIMENTAL STRESS MODELS

Restraint and isolation

Use of concurrent restraint and isolation has been intermittently explored as a stress model in ruminants for nearly 50 years. The 'restraint and isolation stress' (RIS) model offers more control than other stress models (e.g. transportation, weaning), increasing overall consistency and reliability of the experimentally induced stress response. Inherent features of ruminant species make RIS particularly stressful to ruminants; specifically, cattle and sheep are highly gregarious animals that become distressed when isolated from their herd or flock mates (Boissy and Le Neindre, 1997; Napolitano et al., 2008). Restraining isolated cattle further activates a fear or anxiety response (Apple et al., 1995; Boissy and Le Neindre, 1997), while still allowing control over stress conditions and ease of sampling. Several studies have explored the effects of RIS on indicators of HPA axis activation, carcass quality, and immune function, with most reporting results supportive of RIS as an effective stress model in ruminants.

Plasma cortisol concentrations are the most commonly reported measurement in stress research and even the earliest RIS studies measured plasma cortisol concentrations

(Moberg et al., 1980; Minton and Blecha, 1990; Coppinger et al., 1991). Coppinger et al. (1991) report consistently greater plasma cortisol concentrations in ewe lambs exposed to 6 h bouts of RIS on three consecutive days compared with ewe lambs that were not restrained. A decrease in overall cortisol responsiveness on d 3 of the RIS treatment, compared with d 1, was detected suggesting some habituation to stressor (Coppinger et al., 1991). However, cortisol concentrations were still greater in restrained and isolated lambs on d 3 compared with control lambs that also exhibited a similar decrease in responsiveness (Coppinger et al., 1991). The following year, Minton et al. (1992) conducted a similar study in wether lambs exposed to 6 h RIS for three consecutive days and report similar increases in plasma cortisol concentrations. Overall, early literature supports 6 h RIS as a reliable stress model in ruminants to yield increases in cortisol, with some habituation over time.

Apple et al. (1993a) examined the effects of RIS on pituitary-adrenal secretions in ewe and wether lambs, finding greater plasma cortisol, lactate, and ACTH concentrations in lambs subjected to 6 h RIS; several similar RIS studies support these findings (Apple et al., 1995; Minton et al., 1995; Apple et al., 2005). Apple et al. (1993a) also measured serum glucose concentrations, which were unaffected by stress treatment. In contrast, Chen et al. (2016) report a strong correlation between serum concentrations of glucose and cortisol. With the use of chute behavioral scores and exit velocities, authors suggest that serum glucose may be better than serum cortisol as a predictor of behavioral responses to acute stressors (Chen et al., 2016). Inconsistencies within the literature may be attributable to several factors such as length of RIS or the number of RIS bouts. In addition, time since last feeding, body condition, and existing glycogen stores directly

impact serum glucose concentrations, and are therefore potential confounding factors for interpretation of glucose data (Chen et al., 2016). Still, cautious interpretation of previous literature indicates more consistently that blood metabolites indicative of HPA axis activation increase in response to RIS, providing support for the use of RIS as a stress model in ruminants.

Although there is robust literary support for the current understanding that cortisol plays an important role in maintaining homeostasis under stress conditions, cortisol alone (in the absence of either a physiological or a psychological stressor) does not yield the same effects observed during stressful experiences. One study infused cortisol to one group, imposed 6 h RIS to another group, and compared these two groups to a non-stressed control group (Minton et al., 1995). Using assessment of lymphocyte proliferation, known to be altered by stress conditions (Coppinger et al., 1991; Minton et al., 1992), Minton et al. (1995) conclude that cortisol alone cannot be responsible for all effects of stress on immune function. Authors suggest that a mediated interaction between cortisol and epinephrine may be responsible for the effects of stress on immune function in restrained and isolated animals (Minton et al., 1995). Minton et al. (1995) report findings that support the concept that activation of the HPA axis attained using RIS cannot be fully simulated by cortisol infusion alone.

The RIS model offers a consistent stress model for the study of the 'dark-cutting condition' (DCC), also known as DFD for dark, firm, and dry carcass characteristics. Extreme glycogen depletion in the muscle is often associated with extreme stress pre-harvest and directly leads to DCC (Apple et al., 2005). In 2000, an estimated 2.3% of beef cattle in the United States were categorized as cutting dark (Miller, 2007). Several

studies spanning over a decade extensively investigated DCC and the use of RIS as a model for consistently producing DCC in sheep (Apple et al., 1993b; Apple et al., 1995; Apple et al., 2005). Exposing lambs to 6 h RIS decreased muscle glycogen stores compared to a non-stressed control group in some studies (Apple et al., 1993b; Apple et al., 1995), while a more recent study found no differences in muscle glycogen content, but still report an increase in the number of carcasses presenting with DCC (Apple et al., 2005).

There are conflicting results concerning meat tenderness. Apple et al. (1993b) report no differences in Warner-Bratzler shear force values for lambs exposed to 6 h RIS compared with non-stressed control lambs, while more recent studies found that RIS decreased the Warner-Bratzler shear force values (Apple et al., 1995; Apple et al., 2005). Authors suggest that the observed increase in muscle tenderness with RIS could be caused by the higher ultimate pH activating calpain proteases (Apple et al., 1995). Even with this increased tenderness, Commission Internationale de L'Eclairage L*a*b* values were lower in RIS lambs compared to control lambs indicating that the muscle was darker, less red, and less yellow, respectively (Apple et al., 1995). Also, lambs exposed to RIS yielded carcasses with lower USDA quality grade compared with non-stressed control lambs (Apple et al., 1993b). Using RIS, Apple et al. (1995; 2005) were able to consistently produce DCC in sheep. This condensed discussion of the effects of RIS on carcass quality provide evidence of the stress-inducing qualities of RIS that present as decreased meat quality, further supporting the use of RIS as a practical and reliable stress model in ruminants.

In addition, distress can have a negative impact on the immune system's ability to mount an immune response to prevent infection and disease (Moberg and Mench, 2000; Tsigos and Chrousos, 2002). Is the restraint and isolation stress model appropriate for evaluating the effect of stress on immunity? For brevity, I will not go into the details of the many complexities of different immune responses. Rather, I will focus my efforts on evaluating past RIS research that measured different immune parameters (e.g. lymphocyte proliferation, T-cell activation, and cell-mediated adaptive response) to answer the question of appropriateness of the stress model.

A reduction in the lymphocyte proliferative response to mitogens phytohemagglutinin and Con A was detected in lambs exposed to 6 h RIS for three consecutive days when compared with non-stressed lambs (Coppinger et al., 1991; Minton et al., 1992; Minton et al., 1995). Lambs infused with cortisol (at concentrations consistent with RIS) had lymphocyte proliferative responses to mitogens most similar to the control group, rather than the RIS group (Minton et al., 1995); this indicates that cortisol alone did not cause the observed reduction in lymphocyte proliferative function observed in lambs exposed to RIS. Minton et al. (1995) offer several possible explanations as to the mechanism of stress-induced inhibition of lymphocyte proliferative function, including a mediated interaction between cortisol and epinephrine. The authors also acknowledge that measuring lymphocyte proliferative response to mitogens only provides insight into a small aspect of immune function (Minton et al., 1995). Cortisol may be exerting a greater effect on other features of the immune system.

Production of interleukin-2 (IL-2), responsible for T-cell activation and cell-mediated adaptive immunity (Hall, 2016), can also be reflective of immunocompetence.

Coppinger et al. (1991) report decreased IL-2 production in lambs exposed to 6 h RIS when compared to non-stressed lambs. In contrast, follow-up studies found no differences in IL-2 production (Minton et al., 1992; Minton et al., 1995). Minton et al. (1995) highlight the consensus that reduction of lymphocytes, specifically IL-2, does not consistently occur with use of the RIS model. Currently there is not enough evidence to support the use of RIS as a model for investigating the effects of distress on immunocompetence. Instead, transportation of young animals is often used as a stress model to evaluate immune compromised animals at high risk for disease contraction (i.e. simulating a scenario known to induce shipping fever).

With the limited amount of literature available on the use of RIS as a stress model in ruminants it is difficult to assess the validity and potential applications of RIS in research. Though RIS may not be an ideal model for the study of the effects of stress on immunocompetence, as suggested by inconsistent results within the limited research, its usefulness in other areas of stress research is promising. The ability of RIS to consistently produce lower quality carcasses and increase the prevalence of DCC supports the effectiveness of RIS to induce mobilization of body stores. In addition, cortisol and ACTH are consistently increased with the use of RIS. Overall, current literature supports RIS as an effective stress model in ruminants, however further research in this area is needed.

Transportation

Transportation of cattle has progressed significantly over the past 300 years, from sea transport to rail and now road. Even with notable advancements, the stress of

transport remains both an animal welfare and an economic loss issue (Swanson and Morrow-Tesch, 2001). Factors contributing to distress and associated with transport include, but are not limited to, pre-transport handling, loading and unloading, regrouping, loading density, feed and water deprivation, and novelty (Swanson and Morrow-Tesch, 2001; Schwartzkopf-Genswein et al., 2012). Calves are more susceptible to complications during transport due to their lack of exposure to different environments and naïve immune systems (Swanson and Morrow-Tesch, 2001). Greater morbidity and mortality rates in newly received calves are frequently attributed to Bovine Respiratory Disease (BRD), commonly known as 'shipping fever' (Phillips, 2002). Infectious agents typically associated with BRD (e.g. bacterial species, parainfluenza-3, bovine viral diarrhea virus, and bovine respiratory syncytial virus) are often not capable of causing BRD without the presence of other contributing factors, most notably psychological and physical stressors associated with pre- and post-weaning management (Duff and Galyean, 2007). Roughly 14% of calves are affected by BRD, with 70% of feedlot calf deaths being attributable to BRD (Fike and Spire, 2006). Shipping fever costs the beef industry alone upwards of $750 million annually (Griffin et al., 2010), or an estimated $49.55-$151.18 USD/head (Smith, 2009). Investigating strategies to decrease stress on the animal during transportation has the potential to reduce economic losses in various ways (e.g. decreased mortality, decreased morbidity, decreased cost of disease treatment, increased feed intake and average daily gain in feedlot phase). Transportation can be used as a model to alter immunocompetence and stress in young cattle.

Immunological parameters have been measured to assess the effects of transportation on the immune system's capacity to protect against disease, specifically

respiratory infections when discussing calves. Long-term (14 h) and short-term (30 minutes) transport were both investigated in heifers by Dixit et al. (2001) who report greater lymphocyte ACTH secretion following 14 h transport, but no differences with short-duration transport. Another study found no differences in total neutrophil or total lymphocyte numbers following a 72 h transportation period, however a decrease in mitogen-stimulated lymphocyte proliferation was detected (Stanger et al., 2005). A decrease in the activity or functionality of immune cells, rather than a decrease in total number of immune cells, is one possible explanation for compromised immune systems which leads to BRD. Though previous research found little change in commonly measured immune parameters with transportation, the development of new techniques to measure immune function (e.g. techniques to easily measure lymphocyte proliferation) may help to reveal more information to better assess the usefulness of transportation as an intermediate stress model.

In addition, physiological indicators of HPA axis activation are commonly measured to evaluate stress associated with transportation, however duration and type of transport, along with the sampling timeline, need to be considered when interpreting results of studies utilizing transportation as a stress model. Previous literature indicates that cortisol concentrations increase with transportation, across many different conditions, durations, ruminant species, and experimental practices (Kannan et al., 2000; Buckham Sporer et al., 2008; Tadich et al., 2009; Kang et al., 2017). Duration of transport and timing of sampling play a key role in results interpretation, as Parker et al. (2009) found that cortisol concentrations return back to baseline by 2.5 hours into transport. Some increases in cortisol reported in other studies following longer duration

transport (i.e. 6 – 8 hours) may be more attributable to handling rather than transport stress (Grandin, 1997).

Increased circulating cortisol concentrations have been linked to diuresis (Parker et al., 2003a), further dehydrating feed and water deprived animals. In a study by Marques et al. (2012) feed and water deprivation were shown to have a similar effect on average daily gain and gain to feed ratio as seen with transported cattle, when compared with control cattle that were neither transported nor deprived of feed and water. In addition, acid-base balance is negatively altered (i.e. mild metabolic acidosis) similarly for feed and water deprivation and transportation (Parker et al., 2003b), indicating a significant role of physiological changes from feed and water deprivation in overall transport stress. As a stress model, transportation consistently produces an HPA axis response, however partitioning the effect of transportation from the effect of feed and water deprivation remains a challenge.

In general, transportation stress remains a difficult stress model to use due to the combination of stressors and the inability to isolate one stressor at a time (i.e. all road-based transportation studies inadvertently have a loading and unloading component). Limitations in transport research are also present due to difficulty of maintaining continual access to animals for sampling.

Weaning

In most animal production systems, weaning involves the separation of dam and offspring, as well as simultaneous transition from milk as the young animal's primary source of nutrients to consumption of solid feed (Weary et al., 2008). In a natural setting,

the gradual reduction of milk production by the dam throughout lactation facilitates weaning (Pond et al., 2005; Weary et al., 2008), and occurs between 7 and 14 months of age in cattle (Enríquez et al., 2010) and between 4 and 7 months of age in sheep (Arnold et al., 1979). In contrast, conventional production systems often abruptly wean between 5 and 8 months of age, resulting in a combination of physiological and nutritional changes (Haley et al., 2005; Pond et al., 2005); these changes are often accompanied by both environmental (Weary et al., 2008) and social (Veissier and Le Neindre, 1989) changes associated with moving newly weaned animals to a new location. Due to the high number of stressors imposed concurrently, weaning is often considered the most stressful time in a production animal's life (Stookey and Watts, 2007). An alternative weaning strategy called two-stage weaning aims to separate the nutritional transition period from the dam separation transition period with the use of a nose-clip or fence-line contact (Price et al., 2003; Haley et al., 2005; Enríquez et al., 2010). Behavioral observations, specific stress-related blood metabolites, and immune function are the most commonly measured parameters used to evaluate weaning stress.

One of the most common behavioral observations reported in weaning animals is number of vocalizations, based on the ideology that animal calls are reflective of biological status (Watts and Stookey, 2000). Several studies indicate increases in high frequency vocalizations in young ruminants being associated with abrupt weaning (Haley et al., 2005; Schichowski et al., 2008; Enríquez et al., 2011). Recent studies have also found that abrupt weaning increases pacing, in comparison with two-stage weaning methods in calves (Haley et al., 2005; Enríquez et al., 2010). Pacing has also been reported in lambs during weaning or temporary separation (Napolitano et al., 2008).

Abrupt weaning also decreases time spent lying (Price et al., 2003) and eating (Ungerfeld et al., 2016). Agitation scores, given on a one to three scale (one being no agitation and three being extremely agitated) based on the combined assessment of all previously discussed behavioral parameters, increased in lambs when abruptly weaned (Schichowski et al., 2008). Increases in behavioral manifestations of stress in young ruminants during weaning support the understanding that weaning induces stress, however utilizing weaning as a stress model in ruminants would require the disentanglement of stressors, the desire to study non-specific stress, or specific interest in overall weaning stress.

Plasma cortisol concentrations increase in response to weaning in some studies (Lay et al., 1998) and not in others (Hickey et al., 2003; O'Loughlin et al., 2014). Similar inconsistencies are reported for norepinephrine. Hickey et al. (2003) report increases in plasma norepinephrine in association with weaning, whereas no differences were reported in a study by Lefcourt and Elsasser (1995). Variations in sampling timelines and specific handling or husbandry practices are two factors that may explain some of the inconsistencies in results of previous studies. Lack of consistent practices in published weaning studies makes it difficult to draw conclusions on the effectiveness of weaning as a stress model in ruminants. Immune function and/or disease prevalence may serve as better indicators of stress in weaning research; these parameters are less subject to minute-by-minute or hour-by-hour changes, which are difficult to detect without continuous blood sampling.

Weaning, especially when combined with other stressors, can compromise immunocompetence of production animals and ultimately increase susceptibility to infection and disease (Lynch et al., 2010). Traditionally regarded as an indicator of

inflammatory response (Gabay et al., 1999), acute phase proteins (APP) are commonly measured in weaning research as an indicator of immunocompetence. However, the reliability of APP in the assessment of weaning stress has recently been called into question (O'Loughlin et al., 2014). Arthington et al. (2005) report that abrupt weaning, regardless of age, increases APP concentrations in Brahman steer calves, while other studies found no effect of weaning on APP concentrations (Hickey et al., 2003; Carroll et al., 2009; Lynch et al., 2010). A recent study by O'Loughlin et al. (2014) also found no effect of weaning on APP concentrations, and ultimately suggest that APP may not be a reliable biomarker of weaning stress in cattle.

Neutrophils, leukocytes, and lymphocytes may be more useful indicators of immune function within a weaning stress model in ruminants, as they have been shown to yield more consistent results. Lynch et al. (2010) evaluated numerous immune response parameters in abruptly weaned beef calves compared with non-weaned control calves, finding both an increase in total leukocytes and a decrease in the number of lymphocytes two days post-weaning. In addition, the concentration of neutrophils increased in weaned calves two days post-weaning, yet the percentage of neutrophils performing phagocytosis decreased compared to baseline in weaned calves only (Lynch et al., 2010). Hickey et al. (2003) also report increases in neutrophil concentration and decreases in lymphocyte concentration, reporting an overall increase in the neutrophil:lymphocyte during weaning. Authors conclude that abrupt weaning impairs immune function, however it should be noted that the majority of cells associated with immune function returned to baseline concentrations seven days post-weaning (Lynch et al., 2010). Although abruptly weaned calves appear to experience only short-lived decreases in immunocompetence, infection

can still occur within this seven-day period. Introduction to a new environment and/or new herd mates during this critical period following weaning can increase the prevalence of disease and infection.

Regardless of the parameter measured to assess the effects of weaning, utilizing weaning as a stress model in ruminants would require the disentanglement of stressors, the desire to study non-specific stress, or specific interest in overall weaning stress.

Summary

In general, utilizing restraint and isolation as a model for stress offers the most consistent activation of the HPA axis and optimal experimental control. In addition, with the use of jugular catherization, continuous sampling is most practical within an RIS model. However, RIS may be more extreme than the typical stressful situations encountered throughout production that researchers want to investigate. Utilizing a stressor more common to production, such as transportation, may offer better evaluation of strategies to decrease stress. However, using transportation stress as a stress model comes with its own set of obstacles. First, it is typically impractical to have continuous sampling during transportation. Secondly, specifically isolating transport stress from the stress that is imposed during loading and unloading (i.e. handling stress) remains a challenge. Another stress that is experienced by all production animals is weaning. Weaning is extremely stressful and involves a multitude of concurrent stressors experienced over a short period of time. However, a desire to specifically study weaning stress is needed to properly utilize weaning as a stress model.

Overall, each stress model comes with advantages and disadvantages and a somewhat unique physiological reaction. The nature of the research questions being investigated should be considered when choosing which stress model to use.

OXYTOCIN

Background

Primarily known for its involvement in labor and parturition, this peptide hormone was given the name 'oxytocin' or "quick birth" (Gimpl and Fahrenholz, 2001; Hall, 2016). Oxytocin has a longer half-life in cerebral spinal fluid (CSF; 19-28 minutes), compared with a half-life of 3-8 minutes in blood (Gimpl and Fahrenholz, 2001; Leng and Ludwig, 2016). Oxytocin is stored and secreted from the posterior pituitary gland (Onaka, 2004; Leng and Ludwig, 2016). Stimulation of the paraventricular nuclei, located in the hypothalamus, triggers the production and release of oxytocin into peripheral circulation (Hall, 2016). Classically, oxytocin is released in response to suckling and causes contraction of myoepithelial cells in the mammary tissue resulting in milk excretion (Hall, 2016). Stimulation can also occur in response to various stressors such as conditioned fear, unconditioned fear, novelty, and other noxious stimuli (Onaka, 2004). The magnitude of central and/or peripheral oxytocin release is highly dependent upon type of stressor and species (Onaka, 2004).

Peripherally circulating oxytocin concentrations range from 1-10 pg/mL in humans and rodents (Leng and Ludwig, 2016). In cattle, 1-5 pg/mL is considered a normal circulating range and a circulating concentration of 10 pg/mL induces maximum milk ejection (Bruckmaier, 2013). However, the action of oxytocin goes beyond

parturition and lactation, as oxytocin receptors can be found in virtually all tissues within the body and expression is highly dependent on steroid concentrations (e.g. estrogen and testosterone) (Gimpl and Fahrenholz, 2001). Because of oxytocin's widespread dispersal and involvement in numerous body systems, the full action of oxytocin has yet to be elucidated.

Even still, oxytocin has been shown to be involved in the reduction of both anxiolytic behaviors (Windle et al., 1997) and indicators of HPA axis activation (Neumann et al., 2000; Amico et al., 2004) in rodents. Therefore, some have chosen to investigate the stress response when oxytocin is provided exogenously. Both invasive (e.g. intra-cerebral, subcutaneous, and intra-venous) and non-invasive (e.g. intra-nasal) approaches have been used to provide exogenous oxytocin in previous research. The focus of this dissertation will be intra-nasal oxytocin; however, evidence from studies using more invasive techniques of administration will be included for discussion of the general functionality of exogenous oxytocin due to the small number of studies available that use intra-nasal oxytocin.

Intra-nasal administration

The blood-brain barrier is crucial for the maintenance of CNS homeostasis and restricting entry of neurotoxins. Oxytocin cannot readily cross the blood-brain barrier (Lee et al., 2009; Lochhead and Throne, 2012). The nasal cavity represents a large absorptive surface and is highly vascular, providing a route for some compounds to bypass the blood-brain barrier (Lochhead and Throne, 2012). Intra-nasal administration is non-invasive and has been shown to initiate effects within the brain rapidly (Carson et al.,

2016). The olfactory region, making up roughly 10% of the nasal epithelium, allows direct access to the brain via olfactory nerves (Lochhead and Throne, 2012). The amount of a compound that reaches the brain tissue following intra-nasal administration still remains to be elucidated.

There is some debate regarding the volume of injected compound that actually reaches the brain and how to measure this in experimental investigations. Commonly, the CSF is used as an indicator of what is present in the brain tissue. Specifically, compound concentrations measured within the CSF are thought to be proportional to compound concentrations found in brain tissue following systemic administration (Carson et al., 2016). A review by Leng and Ludwig (2016) states that only 0.002% and 0.005% of administered oxytocin actually reaches the CSF following subcutaneous and intra-nasal administration, respectively. Striepens et al. (2013) report elevated concentrations of oxytocin in the CSF of humans 75 minutes following intra-nasal administration. However, it has been shown that intra-nasal delivery of large molecules can result in widespread dispersal within the brain, while CSF and blood concentrations remain relatively unchanged (Carson et al., 2016). In addition, plasma concentrations cannot be relied upon to predict concentrations of neuropeptides in CSF (Kagerbauer et al., 2013; Striepens et al., 2013). These inconsistencies make the study of intra-nasal delivery particularly difficult, as removal of the brain or immediate brain scans would be necessary to assess exactly if, where, and how a compound is affecting the brain tissue.

A study by Gossen et al. (2012) provided 26 IU of oxytocin intra-nasally to adult men and measured changes in plasma concentrations of oxytocin. This study revealed that providing oxytocin elevated oxytocin concentrations in the plasma for up to 30

minutes (Gossen et al., 2012). Similarly, piglets administered 24 IU of oxytocin had CSF concentrations of oxytocin that were increased for 30 minutes; oxytocin concentrations peaked 10 minutes following intra-nasal administration (Rault, 2016). Dal Monte et al. (2014) report increases in both plasma and CSF 40 minutes following intra-nasal administration of oxytocin in male rhesus monkeys. Furthermore, Neumann et al. (2013) report increased oxytocin concentrations in both the brain and plasma of rats provided oxytocin intra-nasally. Peak oxytocin concentrations in the amygdala, hippocampus, and plasma were reached between 30 and 60 minutes following intra-nasal administration (Neumann et al., 2013). A positive correlation between the absolute peak of oxytocin concentration in brain regions (i.e. the hippocampus and the amygdala) and plasma was established (Neumann et al., 2013). Therefore, measuring changes in plasma oxytocin concentrations may act as a sufficient indicator of changes in CSF and brain oxytocin concentrations.

Before moving forward, it is worth quickly noting that different species have differently shaped airways, potentially effecting the absorption rate of various compounds. For example, humans and primates have L-shaped cavities, while rats have a more linear airway that would be similar to cattle (Lochhead and Throne, 2012). These passage way differences are accompanied by difference in mucosa and therefore absorption rates; mucociliary clearance is slower in humans compared to rodents (Quintana et al., 2015). Species differences should be considered when evaluating research in intra-nasally administered compounds. Overall, intra-nasal delivery offers an exciting new area of research as it offers direct and rapid access to the brain.

Exogenous oxytocin and stress

In general, administration of exogenous oxytocin has an attenuating effect on the HPA axis in mice (Windle et al., 1997; Petersson et al., 2005) and humans (Legros et al., 1984; Heinrichs et al., 2003). Furthermore, greater oxytocin concentrations in plasma have been linked to beneficial changes in the behavior and physiology of mammals confronted with various stressors (Ditzen et al., 2009; Ralph and Tilbrook, 2016). The pattern of exogenous oxytocin treatment to attenuate the HPA axis (Legros et al., 1984; Windle et al., 1997; Heinrichs et al., 2003; Petersson et al., 2005) supports further investigation of oxytocin administration in mammals.

An early study by Legros et al. (1984) report an inverse relationship between oxytocin and cortisol following an intravenous infusion of oxytocin in humans. Individuals in this study were not purposefully stressed, but were intravenously catheterized (Legros et al., 1984) which may inadvertently cause stress. Cortisol concentrations decreased over time, as oxytocin concentrations increased over time from infusion (Legros et al., 1984). It is possible that reductions in cortisol concentrations over time were due to participants becoming used to the catheter and not specifically the result of greater concentrations of oxytocin. However, findings in other species support oxytocin as an anxiolytic agent and show lesser cortisol concentrations with oxytocin infusions.

Windle et al. (1997) utilized ovariectomized female rats that were centrally treated with different oxytocin infusion rates (1, 10, or 100 ng/h) or isotonic saline and subsequently put through stress tests. The magnitude of increase in corticosterone concentrations following exposure to noise stress was significantly blunted by oxytocin

infusion at 10 and 100 ng/h (Windle et al., 1997); these anxiolytic effects of oxytocin were only seen in stressed rats. Decreased corticosterone concentrations have also been observed in more recent studies that injected oxytocin subcutaneously (Petersson et al., 2005), indicating that central infusion may not be necessary to attain anxiolytic effects of exogenous oxytocin.

Furthermore, Windle et al. (1997) also made behavioral observations and noted two important findings. First, there was no reduction in total activity (i.e. total time spent in activities such as locomotion, burrowing, and grooming) with oxytocin administration, confirming that a sedative effect of oxytocin is unlikely (Windle et al., 1997). More recent research in ovariectomized rats subcutaneously injected with oxytocin support this finding (Petersson et al., 2005). Secondly, the prevalence of anxiety-based behaviors was reduced with oxytocin infusion (Windle et al., 1997), further supporting oxytocin as an anxiolytic neuropeptide.

Similar stress attenuative effects have been shown with the use of intra-nasal oxytocin. Ditzen et al. (2009) report reduced salivary cortisol concentrations in men and women given 40 IU of oxytocin intra-nasally 45 minutes prior to discussing a conflict issue. During discussion of the conflict issue, positive behavior relative to negative behavior was increased in couples receiving intra-nasal oxytocin indicating increased positive communication (Ditzen et al., 2009). Participants of a similar study were given 24 IU of oxytocin intra-nasally 50 minutes prior to being exposed to the Trier Social Stress Test (Heinrichs et al., 2003). This study revealed that social support and oxytocin interact to produce an attenuated cortisol response to stress in humans (Heinrichs et al., 2003). In addition, Cardoso et al. (2013) investigated the effect of providing either 24 or

48 IU of oxytocin intra-nasally on salivary cortisol concentrations measured during physical stress (i.e. running on a treadmill). Results indicated that 24 IU of intra-nasal oxytocin decreased the salivary cortisol response to physical stress (Cardoso et al., 2013). In humans, the activation of the HPA axis via physical or psychological stressors appears to be attenuated with the administration of intra-nasal oxytocin.

In non-human mammals, intra-nasal oxytocin has a paradoxical effect on the HPA axis and there are relatively few studies exploring this relationship. Piglets given multiple doses of intra-nasal oxytocin (24 IU per administration) early in life demonstrate greater cortisol concentrations in response to a dexamethasone challenge (Rault et al., 2013). In comparison, adult monkeys administered oxytocin intra-nasally had lesser ACTH concentrations following the imposition of mild restraint stress (Parker et al., 2005). Differences between species and stressor type across studies make drawing conclusions about the efficacy of exogenous oxytocin to attenuate stress particularly difficult.

Summary

Oxytocin is an important neuropeptide released in mammals during parturition and lactation. Recently, oxytocin has been implicated in HPA axis hypo-responsiveness. Providing exogenous oxytocin has an anxiolytic effect in rodents. Intra-nasal delivery of neuropeptides offers a non-invasive route of administration that increases concentrations of the applied neuropeptide in the brain, the CSF, and the plasma. The effects of providing oxytocin intra-nasally on the HPA axis are paradoxical, however only a few studies have explored this relationship to date. More investigations are needed to elucidate the mechanisms involved in stress hypo-responsive conditions.

LACTATION AND STRESS

Background

Lactation is considered a hypo-responsive stress condition (Slattery and Neumann, 2008; Hillerer et al., 2014), making it a potentially useful model for studying attenuation of the stress response. Specifically, in terms of the HPA axis, expression of CRH in the paraventricular nucleus and pituitary sensitivity to CRH are both reduced during lactation (Slattery and Neumann, 2008); the mechanism behind this is unknown but some suggest it is a result of glucocorticoid negative feedback (Slattery and Neumann, 2008). However, anxiolytic effects of oxytocin observed in behavioral studies have sparked questions of oxytocin's potential involvement within the HPA axis cascade.

Oxytocin and prolactin

Oxytocin is known for its involvement in parturition and milk ejection, or milk letdown. A suckling event, or stimulation of the teat by the offspring, signals the release of oxytocin via impulses that are transmitted to oxytocinergic neurons in the hypothalamus (Gimpl and Fahrenholz, 2001). Oxytocin knock-out mice fail to nurse their offspring due to the essentiality of oxytocin in triggering milk excretion (Gimpl and Fahrenholz, 2001) and continuous elevation of oxytocin concentrations (~10 pg/mL) is necessary for complete milk removal in dairy cows (Bruckmaier et al., 1994). In addition to oxytocin's role in milk excretion, some studies have begun considering a possible role of oxytocin in the hypo-responsive stress condition commonly observed during lactation.

Ralph and Tilbrook (2016) report greater plasma oxytocin concentrations (~1.9 times greater) paired with less extreme increases in plasma cortisol concentrations in

response to restraint stress in lactating ewes compared with non-lactating ewes from the same herd. In a similar study, lactating ewes exposed to isolation and restraint stress displayed a blunted HPA axis response when compared with non-lactating ewes (Tilbrook et al., 2006). Authors conclude that the extent of stress attenuation is dependent on suckling, however number, time of suckling, and duration of suckling bouts are not reported or discussed. Presence of the lamb, not lactation alone, may have caused the increase in oxytocin concentrations (Ralph and Tilbrook, 2016); previous literature has elucidated that olfactory and visual stimuli by an offspring is enough to alter hormone concentrations. Evaluating the effects of lactation in other species where offspring are typically not present (e.g. rodents) may be more useful for initial investigation into oxytocin's role in the stress hypo-responsive condition observed during lactation.

In rodents, an oxytocin antagonist was used to investigate the role of reproductive status on the effectiveness of oxytocin as a mediator of the stress response (Neumann et al., 1999). Neumann et al. (1999) report that virgin rats infused with an oxytocin antagonist had greater ACTH and corticosterone concentrations when exposed to an elevated plus maze (i.e. psychological stressor) compared with virgin rats that received a control vehicle. In addition, there were no differences in ACTH or corticosterone in pregnant or lactating females that received either the control vehicle or the oxytocin antagonist (Neumann et al., 1999). These findings suggest that the dampened responsiveness of the HPA axis observed in peripartum and lactating mammals is not solely a result of oxytocin (Neumann et al., 1999). The concomitant presence of high concentrations of prolactin may be necessary to facilitate the stress attenuation observed during lactation.

Prolactin is essential for the initiation and maintenance of lactation. Like oxytocin, greater concentrations of prolactin observed during lactation have been shown to accompany a stress hypo-responsive state (Torner et al., 2002; Slattery and Neumann, 2008). Torner et al. (2002) report impaired maternal behavior and increased anxiety-related behaviors in lactating rats given a prolactin anti-sense treatment compared with a control treatment. It is suggested that a reduction in stress perception is necessary to reduce fearfulness in the mother to allow proper protection of the offspring (Torner and Neumann, 2002). In addition, greater increases in ACTH concentrations were observed following exposure to an elevated plus maze (i.e. psychological stressor) in rats given the prolactin anti-sense treatment compared with rats that received the control treatment (Torner et al., 2002). This indicates some involvement of prolactin in the attenuation of the HPA axis response to stress during lactation. Prolactin has also been shown to have a positive effect on oxytocin secretion at the level of the hypothalamus (Torner and Neumann, 2002); also, oxytocin acts as a releasing factor for prolactin at the anterior pituitary (Leng and Ludwig, 2016). These two hormones may work in concert to facilitate the stress hypo-responsive state observed during lactation (Cook, 1997; Slattery and Neumann, 2008).

Summary

Lactation is considered a hypo-responsive stress condition. Oxytocin is an important neuropeptide present during lactation and may be involved in the hypo-responsive state of the HPA axis observed during lactation. However, paradoxical accounts of oxytocin in non-lactating mammals eludes to the involvement of other

lactational neuropeptides. Prolactin has been implicated in stress hypo-responsiveness in non-lactating rodents due to greater concentrations of prolactin observed during lactation. Oxytocin and prolactin likely work together during lactation to promote a euthymic state in mothering mammals.

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CHAPTER TWO: INTRA-NASAL OXYTOCIN TREATMENT DOES NOT ATTENUATE THE HYPOTHALAMO-PITUITARY-ADRENAL AXIS IN BEEF HEIFERS SUBJECTED TO ISOLATION STRESS OR RESTRAINT AND ISOLATION STRESS

"Intra-nasal oxytocin treatment does not attenuate the hypothalamo-pituitary-adrenal axis in beef heifers subjected to isolation stress or restraint and isolation stress" has been submitted to *Domestic Animal Endocrinology* by authors B.K. Wagner, A.E. Relling, J.D. Kieffer, and A.J. Parker.

ABSTRACT

Changes in the physiological, psychological, and behavioral manifestations of stress have been observed in association with increases in circulating oxytocin. Providing oxytocin intra-nasally has been shown to attenuate stressor-induced hypothalamo-pituitary-adrenal (HPA) axis activation in humans and rodents, however anxiolytic effects may be context and species specific. The present study aimed to investigate the effect of intra-nasal oxytocin supplementation on stressor-induced activation of the HPA axis in beef cattle. I hypothesized that oxytocin would attenuate activation of the HPA axis, ultimately decreasing plasma cortisol and adrenocorticotropic hormone (ACTH). Twenty-eight *Bos taurus* heifers were blocked by bodyweight and randomly allocated to one of four treatment groups, in a 2 × 2 factorial arrangement: 1) Saline, isolated, standing, and

unrestrained (S-isolation stress (IS), 0.015mL/kg BW 0.9% isotonic saline, n =7); 2) Saline, isolated, and restrained (S-restraint and isolation stress (RIS); 0.015 ml/kg BW 0.9% isotonic saline; n = 7); 3) OXT, IS (OXT-IS, 0.3 IU/kg BW oxytocin; n = 7); and 4) OXT and RIS (OXT-RIS, 0.3 IU/kg BW oxytocin; n = 7). Oxytocin and saline were administered intra-nasally. Intra-Intra-nasal treatments were given followed by a waiting time of 30 minutes when each of the stress treatments were applied for two hours. Blood samples were collected via jugular catheters directly following stressor application and every 10 minutes thereafter, for two hours. Cortisol concentrations increased over time in animals exposed to RIS ($P < 0.01$) and decreased over time in animals exposed to IS ($P < 0.01$). Concentrations of ACTH decreased over time for the IS treated heifers but remained elevated for the RIS treated heifers ($P < 0.01$). Under the conditions of the present study, OXT treatment did not affect measured indicators of HPA axis activation. A treatment × time interaction ($P < 0.01$) was detected for oxytocin, such that OXT heifers exhibited greater initial oxytocin concentrations followed by a decline; saline treated heifers had consistently stable oxytocin concentrations. The RIS treated heifers increased their glucose ($P < 0.01$) and lactate ($P < 0.01$) concentrations throughout the application of the stressors compared with the IS treated heifers. Overall, restraint stress increased cortisol and oxytocin in *Bos taurus* heifers compared with heifers subjected only to isolation. Finding a more intermediate stress model may better allow for detection of the effects of oxytocin on ACTH and cortisol concentrations.

INTRODUCTION

Oxytocin is established as a hormone associated with lactation, maternal and sexual behavior, sleep cycles, and social perception in mammals (Gimpl and Fahrenholz, 2001; Lancel et al., 2003; Calcagnoli et al., 2015). Oxytocin also appears to have a role in the regulation of the hypothalamo-pituitary-adrenal (HPA) axis in a number of species (Gibbs, 1986; Neumann et al., 2000; Parker et al., 2005). Generally, endogenous oxytocin secretion is enhanced with neurogenic or behavioral stressors in laboratory rodents such as swimming or social defeat however, oxytocin is also released with physical stimuli such as ether inhalation and electric shock in rats (Gibbs, 1986). In lactating sheep, greater concentrations of oxytocin correlate with an attenuated response in cortisol concentrations when confronted with a predator (Ralph and Tilbrook, 2016). In addition, oxytocin antagonists, centrally infused, yield increased activation of the HPA axis in mice (Neumann et al., 2000). Although central oxytocin administration has an inhibitory effect on the HPA axis in animals, peripheral administration of oxytocin also attenuates the HPA axis in mice (Uvnas-Moberg et al., 1993; Petersson et al., 2005) and humans (Heinrichs et al., 2003). Conversely oxytocin and cortisol may be interdependent at the onset of a novel stressor whereby oxytocin may potentiate the HPA axis response but overtime has been shown to have an attenuating effect on the HPA axis (Brown et al., 2016). The inhibitory or excitatory effects of blood oxytocin concentrations on the HPA axis are dependent upon the species studied, the stressor used and the environmental conditions of the study

(Neumann et al., 2000; Brown et al., 2016). Beef cattle face neurogenic, behavioral and physical stressors throughout their life that vary in intensity however, there is no information available on how oxytocin may interact with the HPA axis in beef cattle exposed to different stressors. The welfare of beef cattle may be improved by manipulating the concentration of oxytocin in blood when a stressor is imposed upon them. The administration of intra-nasal (IN) oxytocin may be a novel strategy to attenuate the animal's HPA axis response to various stressors and minimize common stress-related effects on growth rate and immunity. Therefore, our hypothesis was that intra-nasally administered oxytocin would attenuate activation of the HPA axis in beef cattle, decreasing plasma adrenocorticotropic hormone (ACTH) and cortisol when subjected to isolation stress and restraint and isolation stress.

METHODS

Animal management

Twenty-eight *Bos taurus* heifers of angus genetic type (278 ± 31.47 kg mean ± SEM bodyweight, < 2 years of age) were used in the present study. The heifers were randomly selected from the Ohio Agricultural Research and Development Center's beef herd at the Eastern Agricultural Research Station in Caldwell, Ohio. On d -15, the heifers were acclimated to the working environment and human contact twice per day, for a total of 5 days. The acclimation protocol involved walking the heifers through the animal handling facilities twice a day. The animals were made to stand in a race and chute and were gently touched and spoken to by the herdsman as they walked beside the heifers. The heifers were caught in the head gate of the chute and a halter was applied each morning and removed in the afternoon. The heifers were returned to their pens at the completion of

the acclimation procedure each day. On d -7, a controlled intravaginal drug releasing device (CIDR, Eazi-Breed™ Zoetis, Parsippany, NJ; active ingredient is progesterone, at 1.38g per insert) was inserted into each heifer to avoid the confounding effects of estrus. During the acclimation period the heifers had ad libitum access to fescue hay and water when in their pens. Twenty-four hours prior to catheterization, all animals had their feed and water withdrawn. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (#2017A00000012).

Experimental design

Animals were ranked and blocked by bodyweight and randomly allocated to one of four treatment groups, in a 2 × 2 factorial arrangement: 1) Saline, isolated, standing, and unrestrained (S-IS, 0.015mL/kg BW 0.9% isotonic saline, n =7); 2) Saline, restrained and isolated, (S-RIS; 0.015 ml/kg BW 0.9% isotonic saline; n = 7); 3) Oxytocin, isolated, standing, and unrestrained (OXT-IS, 0.3 IU/kg BW oxytocin; n = 7); and 4) Oxytocin and restrained and isolated (OXT-RIS, 0.3 IU/kg BW oxytocin; n = 7). Four heifers were tested at a time (i.e. one from each treatment group), either in the morning at 0800 hours or afternoon at 1200 hours, over a five-day period. Dose rate of oxytocin (0.3 IU/kg bodyweight) was based on previously reported doses used in humans, assuming an average bodyweight of 70 kg for humans (Gossen et al., 2012; Striepens et al., 2013; Spengler et al., 2017),

Intravenous catheterization and pre-treatment

Two hours and thirty minutes prior to intra-nasal treatment, heifers were moved into a chute and haltered. Their heads were then restrained to the side of the head chute to

allow open access to the jugular vein. A 5 cm × 5 cm area was clipped over the jugular vein and a baseline blood sample was collected. To begin catheter placement, a local anesthetic (3 mL, 2% Lidocaine Hydrochloride injectable, Vedco Inc., St. Joseph, Missouri, USA) was injected subcutaneously at the site of catheter placement. Betadine® surgical scrub (Purdue Pharma L.P., Stamford, CT, USA) was used to clean the area, followed by the removal of surgical scrub by alcohol (70% ethanol). A small stab incision was then made with a sterile #10 blade scalpel and a 14-gauge × 5.25" indwelling intravenous catheter (Becton, Dickinson, and Co., Franklin Lakes, NJ, USA) was placed into the jugular vein. Catheters were secured to the skin with sutures and extension sets were secured to the top of the neck using superglue (Scotch™ Brand, Maplewood, MN, USA) and Elastikon® (Thermo Fisher Scientific, Waltham, MA, USA). After catheterization was complete (–2 h), animals were moved to a chute (IS) or a section of the race-way (RIS) and loosely halter tied to the side of the area. The heifers were able to move their head from side to side until the start of the study.

Treatment administration and stressor application

At –0.5 h, the heifers head was restrained and each heifer was administered their assigned intra-nasal treatments (S, isotonic saline or OXT, Oxytocin, 20 IU/mL Vetone®, Bimeda-MTC Animal Health Inc, Ontario, Canada) with a mucosal atomization device (Nasal™ Teleflex® Inc., Morrisville, NC) by the attending veterinarian. Doses ranged from 3 to 5 mL, dependent upon bodyweight. Half of each dose was administered into each nostril. The veterinarian was the only person present on farm that was aware of treatment assignments to avoid any unintended carer bias. All other attending herdsman and investigators were blinded to the treatments. Black shade cloth was secured to four

sides of the standing areas for animals allocated to the IS treatment to avoid visual contact between animals. Twenty-five minutes following administration of assigned treatments, herdsmen prepared the animals allocated to the RIS treatment by loosely applying a soft rope in accordance with Reuff's method of casting (Miller and Robertson, 1959). The waiting time until the start of the stressor treatments was based on previous data that demonstrated a return to basal cortisol concentrations within a two-hour period (Parker et al., 2009). At h 0, Reuff's method was used to lay the RIS allocated heifers onto prepared bedding consisting of 40 cm of sawdust. Rubber mats (1m^2) were then placed under the animal's head and neck. The heifer's legs were first bandaged with vet wrap around the left and right metacarpus and metatarsus. Both front legs were then secured together by wrapping Elastikon® over the bandaged sites. The hind limbs were secured with Elastikon® in the same way. The animal's four legs were then secured using vinyl cow hobbles such that the front and back legs on each side were secured together. The heifers then lay on the bedding for two hours.

Sampling

The heifers were paired for IS and RIS to enable the same timing of sampling. Serial blood sampling of both animals began immediately after the RIS animal was safely restrained. Twenty millilitres of blood was collected via the jugular vein catheter every 10 minutes for 2 h. Specifically, to attain fresh blood, a small sample (4 mL) was pulled through the extension line and discarded prior to taking the full blood sample; following collection, 2 mL of heparinized saline was used to clear the line and maintain patency. Collected blood was distributed into three Vacutainer™ tubes (10 mL lithium heparin, 5 mL EDTA, 5 mL fluoride oxalate), inverted a minimum of eight times, and immediately

placed on ice (-4°C, until centrifugation). After sampling was complete, catheters and CIDRs were removed and animals were released back into the herd after visual assessment by the attending veterinarian.

Sample analyses

All blood tubes were centrifuged at 3,000 × g for 15 minutes, after which plasma was removed with disposable pipette and stored at -20°C until required. Recovered plasma from lithium heparin tubes was used, along with a commercially available RIA kit (MP Biomedicals, LLC., Solon, OH, USA), to measure cortisol concentrations per the guidelines provided. The intra-assay and inter-assay variations were 9.2 and 15.7%, respectively. The minimum and maximum concentrations of detection were 10.0 and 1000.0 ng/mL, respectively.

Plasma ACTH concentration were measured using a commercially available double-antibody RIA kit (MPBiomedicals, LLC., Solon, OH, USA) and plasma recovered from EDTA tubes. The intra-assay variation is 5.7%.

Oxytocin concentrations were also determined using a commercially available double-antibody RIA kit (Oxytocin (Human, Rate, Mouse, Bovine) RIA Kit, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) using plasma recovered from EDTA tubes. The intra-assay variation ranged from 3.1 to 9.6% and the inter-assay variation was 7.5%. The minimum concentration of detection was 2.5 pg/mL.

Commercially available colorimetric kits were used to determine glucose (Sigma-Aldrich, St. Louis, MO, USA) and lactate (Lactate Assay Kit II, Sigma-Aldrich, St. Louis, MO) from plasma recovered from fluoride oxalate tubes.

Data analysis

Data were analyzed using MIXED procedure in SAS 9.4 Software (SAS Institute, 1999). Block and heifer were treated as random effects; treatment (S or OXT), stress (IS or RIS), time, and their interaction were treated as fixed effects. Day (1, 2, 3, 4, or 5) and time of day (morning or afternoon) were non-significant and subsequently removed from the final model. Repeated measures were assessed using the first-order autoregressive covariance structure. Selection of this covariance structure was based on lowest Bayesian information criterion between first-order autoregressive, compound symmetry, and heterogenous compound symmetry structures. Significance was determined at $P \leq 0.05$ and trends are reported at $0.05 < P \leq 0.10$. A log transformation was used on oxytocin data due to non-normality; results are presented as back transformed values.

RESULTS

A stress by time interaction ($P < 0.01$) was detected that indicated an increase in plasma cortisol concentrations over the course of the 2 h sampling period in RIS heifers; in contrast, plasma cortisol concentrations in IS heifers declined over the same time period ($P < 0.01$; Figure 1). Intra-nasal treatment of oxytocin had no effect on plasma cortisol concentrations in heifers ($P = 0.54$) and no interactions with intra-nasal treatment ($P > 0.10$) were detected.

A stress by time interaction ($P < 0.01$) was detected for ACTH, such that plasma ACTH concentrations decreased over time in IS animals and showed no distinct pattern over time in RIS animals (Figure 2). No effect of intra-nasal oxytocin treatment, or its interaction with stressor or time, was detected for plasma ACTH concentrations ($P = 0.56$).

Average plasma oxytocin concentrations were greater in RIS heifers (6.46 pg/mL) compared with IS heifers (3.43 pg/mL; $P < 0.01$), irrespective of intra-nasal treatment. A treatment by time interaction ($P < 0.01$) was detected for oxytocin, such that OXT heifers exhibited a greater oxytocin concentration initially followed by a decline over time; in contrast, S heifers had consistently stable oxytocin concentrations throughout the sampling period (Figure 3).

Glucose did not show the same stress by time interaction ($P = 0.36$) as observed in cortisol, there was an effect of stress such that RIS heifers showed an overall increase in plasma glucose concentration compared with IS animals (7.18 ± 0.28 and 5.11 ± 0.28 mM, respectively; $P < 0.01$; Figure 4). No effect of intra-nasal treatment, or its interaction with stressor or time, was detected for glucose ($P = 0.89$). Similarly, there was an effect of stress such that RIS heifers showed an overall increase in plasma lactate concentration compared with IS animals (0.89 ± 0.08 and 0.41 ± 0.28 mM, respectively; $P < 0.01$; Figure 5), while no stress by time interaction ($P = 0.06$) was detected. No effect of intra-nasal treatment, or its interaction with stressor or time, was detected for lactate ($P = 0.61$).

DISCUSSION

Intra-nasal oxytocin treatment had no effect on the ACTH or cortisol concentrations in *Bos taurus* heifers subjected to either restraint and isolation stress or isolation stress in our study. This was unexpected because oxytocin treatment has been reported to attenuate plasma glucocorticoid concentrations after exposure to various stressors in rodents (Neumann et al., 2000; Amico et al., 2004) and humans (Heinrichs et al., 2003; Ditzen et al., 2009). Therefore, I reject the hypothesis stated in the introduction. The imposition of successive stressors in this study may have influenced the effects of oxytocin treatment on

the heifers ACTH and cortisol concentrations. The HPA axis is known to become sensitized to stressors as dehydration ensues primarily due to elevated concentrations of arginine vasopressin (Matthews and Parrott, 1991; Parker et al., 2004). Twenty-four hours of water deprivation is not a prototypical stressor that will activate the HPA axis in the dromedary (Finberg et al., 1978), sheep (Parker et al., 2003) or cattle (Parker et al., 2004). Furthermore, the study was conducted inside a building during mild environmental conditions where the risk of rapid dehydration was minimal to the heifers. The catheterization process and the two-hour waiting period after catheterization is unlikely to affect the activity of the HPA axis in this study because of the animal's experience to previous handling and due to the care taken to avoid pain during the catheterization process.

Intra-nasal oxytocin dose rate

A similar dose rate (0.3 IU/kg bodyweight) to that reported in other studies (Gossen et al., 2012; Striepens et al., 2013; Spengler et al., 2017) was used in the present study. However, it is unknown if a greater or lesser concentration of oxytocin would have an effect on the HPA axis of cattle exposed to either the RIS or IS treatments in the present study. Furthermore, the time delay of thirty minutes from intra-nasal treatment with oxytocin to the start of the stress treatment and subsequent blood sampling may have affected the lack of response on measured indicators of HPA axis activation due to the rate of clearance from blood. Studies in rats and mice however, have demonstrated a peak oxytocin concentration in the extra cellular fluid of the brain at 30 minutes after intra-nasal treatment (Neumann et al., 2013). Although oxytocin has a short half-life of 3-8 minutes in the blood of mammals (Gimpl and Fahrenholz, 2001; Leng and Ludwig, 2016), intra-nasal oxytocin treatment has been demonstrated to increase concentrations of oxytocin in

the CSF for up to 30 minutes in swine (Rault et al., 2013) and 60 minutes in rhesus macaques (Lee et al., 2018). In addition, Rault et al. (2016) report oxytocin concentrations in cerebral spinal fluid to be above basal concentrations for 60 minutes. Oxytocin and other neuropeptides (e.g. vasopressin) administered intra-nasally have demonstrated differing rates of disappearance in the CSF and blood with the CSF exhibiting a slower rate of disappearance (Born et al., 2002; Striepens et al., 2013). Furthermore, binding of oxytocin to receptors in brain tissue are increased and CRF receptors decreased at 50 and 30 minutes after intra-nasal oxytocin treatment in rats and mice, respectively (Neumann et al., 2013). In the present study, heifers treated with oxytocin prior to RIS application had greater plasma oxytocin concentrations (8 pg/mL) before the start of stress treatments compared with saline treated heifers (5 pg/mL), indicating that oxytocin concentrations were still elevated in the blood at the start of stressor application.

The type of stress in our study had an effect on plasma oxytocin concentrations. Heifers allocated to the RIS treatment had greater plasma oxytocin concentrations compared with the heifers allocated to the IS treatment. Moreover, greater oxytocin concentrations were found in the heifers treated with intra-nasal saline and subjected to RIS compared with heifers that were given intra-nasal oxytocin and isolation stress at 30 minutes after treatment. The SIS treated heifers had no change in their endogenous oxytocin concentrations throughout the study. Although restraint stress has been shown to increase endogenous oxytocin concentrations in the blood of rats (Neumann et al., 2013), endogenous oxytocin concentrations did not change from pre-stressor concentrations in neither lactating nor non-lactating sheep exposed to standing restraint or barking dogs (Ralph and Tilbrook, 2016). Our data and the reports on rats and sheep may suggest that

the endogenous release of oxytocin occurs when an animal is fearful of a stressor. Conversely, intra-nasal oxytocin treatment appears to attenuate the HPA axis at less fearful interactions than the RIS and IS models used in this study (Amico et al., 2004; Parker et al., 2005).

RIS v IS model responses

Heifers exposed to RIS had greater concentrations of plasma ACTH and cortisol throughout the study when compared with heifers in the IS group. The RIS treatment induced peak cortisol concentrations in heifers (108 ng/mL) that were far greater than peak cortisol concentrations reported by others using RIS in calves (50 ng/mL; Apple et al., 2005). In heifers exposed to the IS treatment, ACTH and cortisol concentrations responded with an initial increase but decreased to basal concentrations over the two-hour sampling period, suggesting adaptation to isolation stress. Physical and visual isolation from conspecifics in cattle is believed to induce an activation of the HPA axis (Boissy and Le Neindre, 1997). The response of ACTH and cortisol concentrations from the RIS heifers compared with the IS heifers is interesting in that the magnitude of activation remained at a maximum throughout stressor application, suggesting that this model of stress was unrelenting for the heifers.

Data of the present study on ACTH and cortisol agrees with the data published in sheep and cattle using a RIS model (Minton et al., 1992; Apple et al., 1995). In addition, Apple et al. (2005) report restrained and isolated steers had plasma glucose and lactate concentrations greater than unstressed control steers; this is in agreement with results of the present study. Increased lactate is often an indicator of muscle fatigue, as lactic acid is necessary for the resynthesis of ATP following muscle contraction (Frandson et al., 2009).

Increased circulating glucose can indicate mobilization of body stores and is known to occur when cortisol concentrations increase, as cortisol stimulates the conversion fat and protein to glucose intermediates (Carroll and Forsberg, 2007; Cohen et al., 2012).

CONCLUSIONS

Restraint and isolation stress caused greater concentrations of ACTH and cortisol in heifers compared with isolation stress alone supporting previous literature. Supplementing oxytocin to heifers at a dose rate of 0.3 IU/kg bodyweight did not attenuate the increases in ACTH and cortisol concentrations under conditions of the present study. The stress model used and delayed start of sampling relative to intra-nasal administration in the present study are potential explanations for unexpected results. However, oxytocin concentrations were greater in restrained and isolated heifers compared with isolated heifers, indicating an endogenous release of oxytocin and likely some involvement of oxytocin in the HPA axis. Utilizing intra-nasal oxytocin to attenuate the stress response has yielded promising results in humans and rodents, however more research is needed in cattle due to the species dependent nature of the stress response. In addition, finding a more intermediate stress model, such as transportation, may better allow for detection of the effects of oxytocin on hormones of interest.

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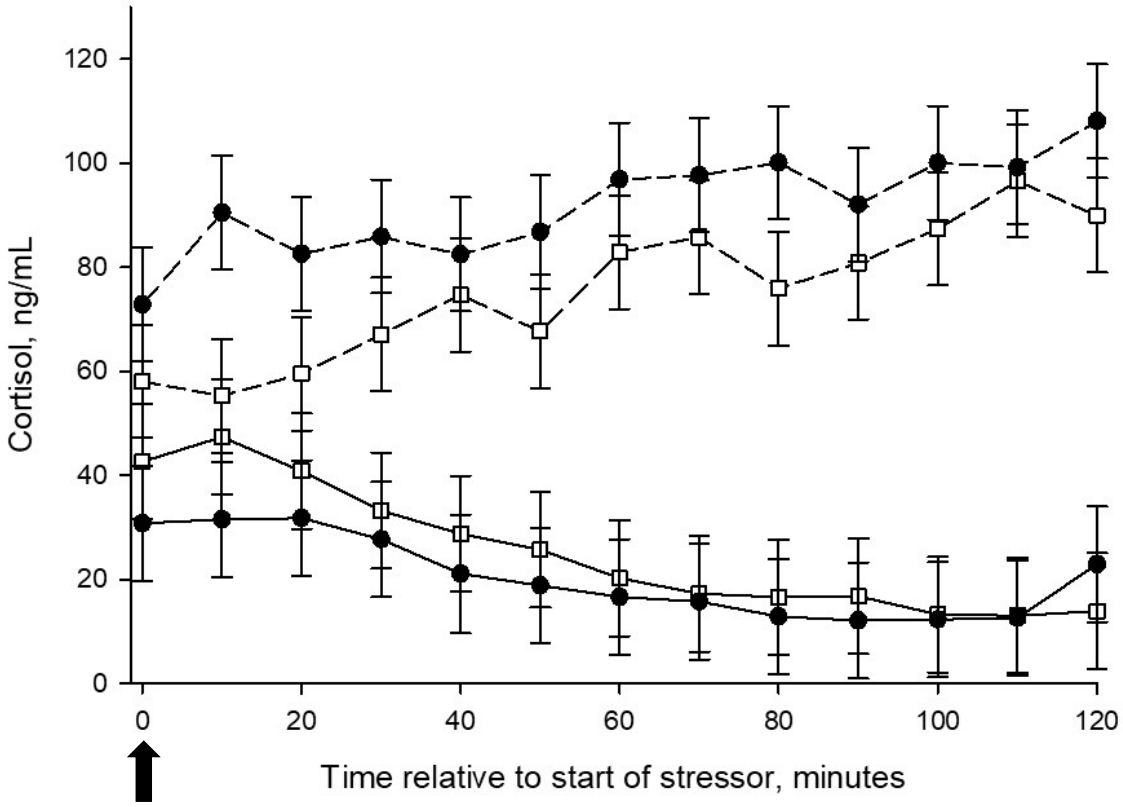

Figure 1. Mean (± SEM) plasma cortisol concentrations in *Bos taurus* heifers subjected to restraint and isolation stress (RIS, – – –; n = 14) or isolation stress (IS, ── ; n = 14), and given either isotonic saline (□; n = 14) or oxytocin (0.30 IU/kg bodyweight, • ; n = 14) intra-nasally. The black arrow indicates the start of the stress treatments and intra-nasal treatments were imposed 30 minutes prior to the start of the stress.

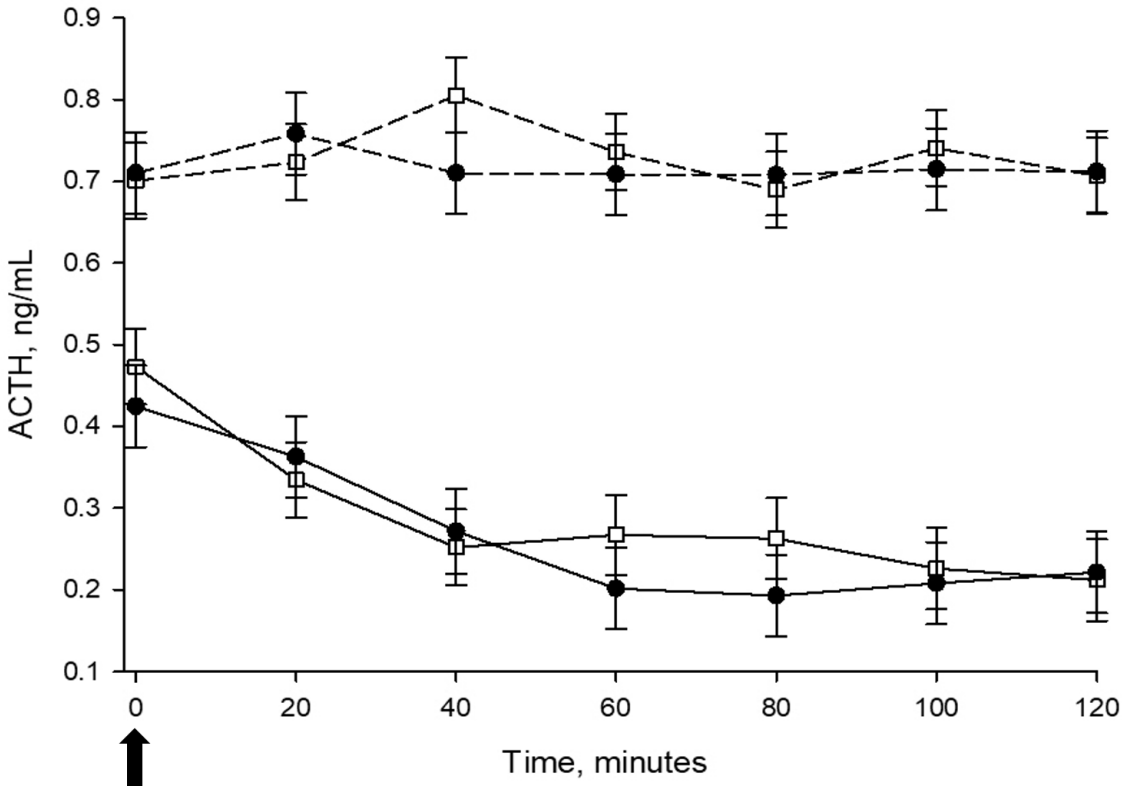

Figure 2. Mean (± SEM) plasma Adrenocorticotropic hormone (ACTH) concentrations in *Bos taurus* heifers subjected to restraint and isolation stress (RIS, – – –; n = 14) or isolation stress (IS, ── ; n = 14), and given either saline (S, □ ; n = 14) or oxytocin (OXT, • 0.30 IU/kg bodyweight; n = 14) intra-nasally at time -30 min. The black arrow indicates the start of the stress treatments and intra-nasal treatments were imposed 30 minutes prior to the start of the stress.

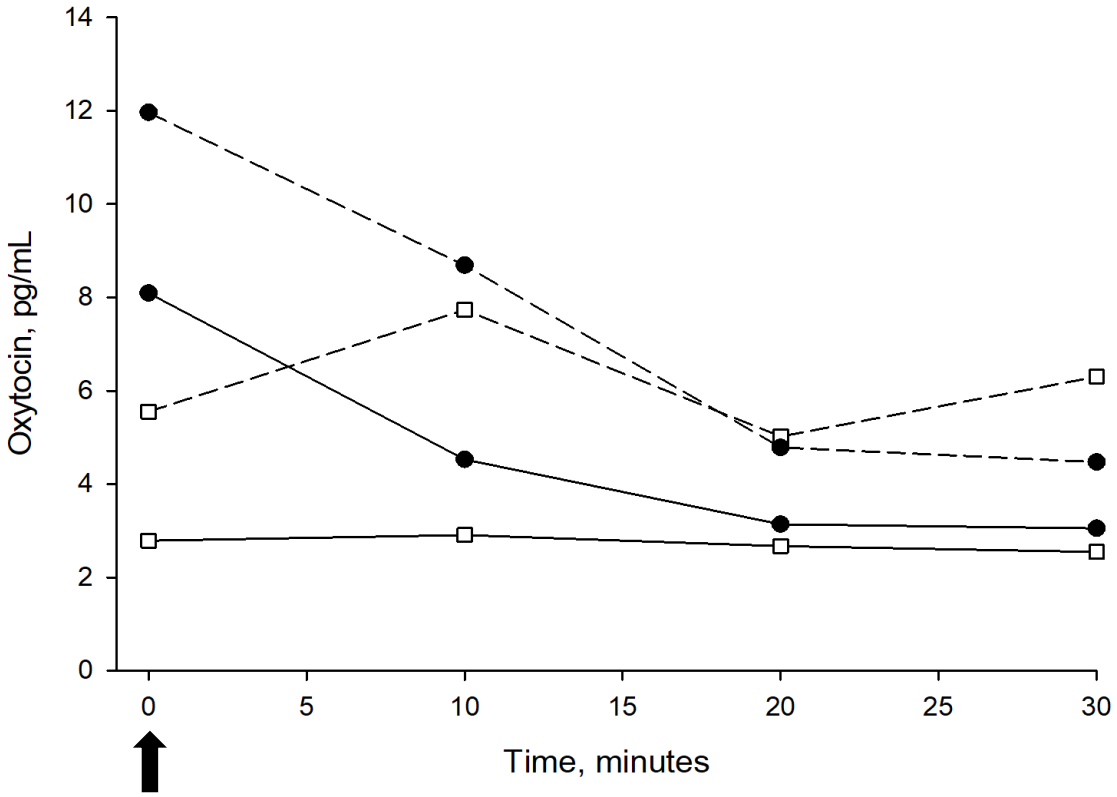

Figure 3. Back transformed mean plasma oxytocin concentrations in *Bos taurus* heifers subjected to restraint and isolation stress (RIS, – – –; n = 14) or isolation stress (IS, ——— ; n = 14), and given either saline (S, □ ; n = 14) or oxytocin (OXT, • ; n = 14) intra-nasally at time -30 min. Oxytocin was administered intra-nasally at a rate of 0.30 IU/kg bodyweight. The black arrow indicates the start of the stress treatments and intra-nasal treatments were imposed 30 minutes prior to the start of the stress.

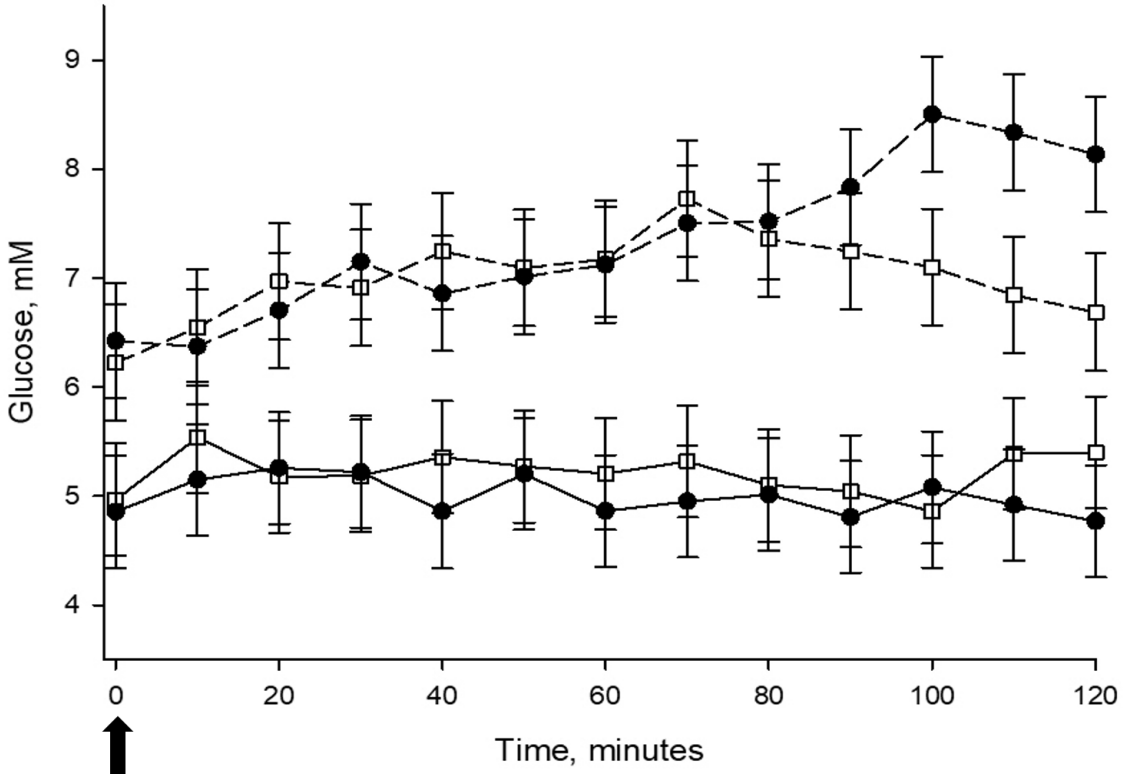

Figure 4. Mean (± SEM) plasma glucose concentrations in *Bos taurus* heifers subjected to restraint and isolation stress (RIS, – – –; n = 14) or isolation stress ¬¬(IS, ── ; n = 14), and given either saline (S, □ ; n = 14) or oxytocin (OXT, • ; n = 14) intra-nasally at time -30 min. Oxytocin was administered intra-nasally at a rate of 0.30 IU/kg. The black arrow indicates the start of the stress treatments and intra-nasal treatments were imposed 30 minutes prior to the start of the stress.

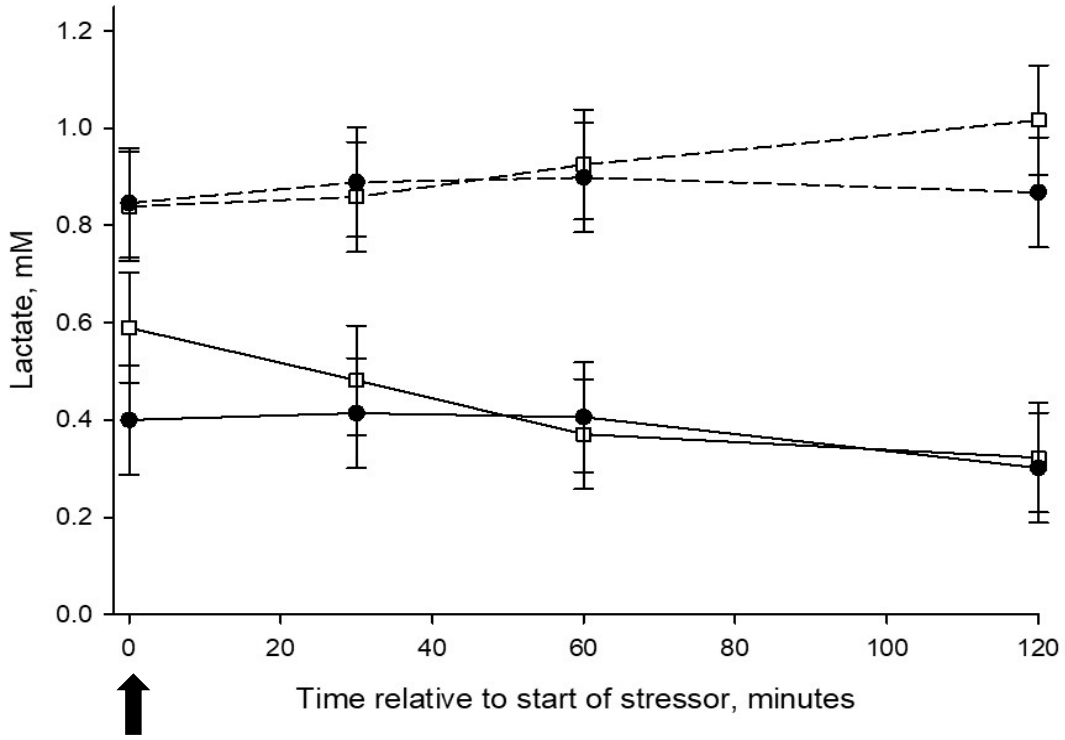

Figure 5. Mean (± SEM) plasma lactate concentrations in *Bos taurus* heifers subjected to restraint and isolation stress (RIS, – – –; n = 14) or isolation stress (IS, ── ; n = 14), and given either saline (S, □ ; n = 14) or oxytocin (OXT, • ; n = 14) intra-nasally at time -30 min. Oxytocin was administered intra-nasally at a rate of 0.30 IU/kg. The black arrow indicates the start of the stress treatments and intra-nasal treatments were imposed 30 minutes prior to the start of the stress.

CHAPTER THREE: OXYTOCIN ALTERS LEUKOGRAM COMPOSITION IN *BOS INDICUS* CATTLE EXPOSED TO SHORT-DURATION TRANSPORT STRESS

ABSTRACT

Oxytocin attenuates the hypothalamo-pituitary-adrenal (HPA) axis in mammals. Recently, intra-nasal oxytocin supplementation has been investigated in humans and rodent models, revealing anxiolytic effects. Transportation activates the HPA axis, ultimately increasing glucocorticoids and altering immune function in cattle. Thirty, recently weaned *Bos indicus* steers were blocked for bodyweight and randomly allocated to one of three treatment groups: 1) intra-nasally dosed with sterile saline and held in the yard for 6 h (S-NT; n = 10), 2) intra-nasally dosed with sterile saline and transported for 6 h (S-T; n = 10), and 3) intra-nasally dosed with oxytocin (0.3 IU/kg bodyweight) and transported for 6 h (OXT-T; n = 10). Briefly, steers were moved into the chute, weighed, and blood sampled at hours 0 (before transport), hour 6 (after transport), hour 48, and hour 72. Intra-nasal treatments were administered at hour 0 directly following blood collection. A group by time effect ($P < 0.05$) was detected for lymphocytes and basophils, such that oxytocin helped maintain baseline counts. A group by time effect was detected for neutrophils and eosinophils directly following transport ($P < 0.01$). Total leukocyte counts were not different between treatment groups ($P = 0.96$). No differences were distinguished between treatment groups or over time for plasma cortisol concentrations ($P = 0.46$). A group by time interaction ($P < 0.03$) was detected for bodyweight such that transportation, independent of intra-nasal treatment, yielded lesser bodyweights compared with the non-transported group. Oxytocin altered specific

leukocytes in *Bos indicus* cattle exposed to short-duration transport stress indicating a protective effect of oxytocin. Although no effect on the cortisol concentrations was detected, road transport induced some signs of an acute inflammatory response directly following transportation.

INTRODUCTION

Oxytocin attenuates the hypothalamo-pituitary-adrenal (HPA) axis in mammals (Amico et al., 2004; Parker et al., 2005). Oxytocin-deficient mice have greater increases in circulating glucocorticoids when confronted with various stressors compared with wild-type mice possessing a normal oxytocinergic system (Amico et al., 2004). In lactating ruminants, increases in circulating oxytocin concentrations have been correlated with attenuated increases in circulating cortisol concentrations observed under stress conditions (Ralph and Tilbrook, 2016). Recently, intra-nasal oxytocin supplementation has been investigated in humans (Kosfeld et al., 2005; Ditzen et al., 2009) and rodent models (Kent et al., 2016), revealing anxiolytic effects. Providing oxytocin intra-nasally has been shown to attenuate increases in ACTH concentrations observed in non-human primates subjected to restraint stress (Parker et al., 2005). Overall, stress attenuative effects of oxytocin noted in other species (Amico et al., 2004; Parker et al., 2005) suggest that intra-nasal oxytocin supplementation is worth investigating in production animals to aide during unavoidable stress events that have the potential to be detrimental to the animal health and efficiency.

Extreme or prolonged activation of the HPA axis can affect immunocompetence, potentially causing subsequent losses in production as a result of disease and/or decreased animal efficiency (Swanson and Morrow-Tesch, 2001; Stookey and Watts, 2007). Changes in leukogram composition have been reported in conjunction with greater circulating cortisol concentrations in cattle (Murata et al., 1987; Hulbert et al., 2011). Transportation activates the HPA axis (Swanson and Morrow-Tesch, 2001), ultimately increasing glucocorticoids and altering immune function in cattle (Dixit et al., 2001;

Stanger et al., 2005; Hulbert et al., 2011), and was therefore selected as an appropriate stress model for the present study.

Oxytocin, in addition to attenuating increases in glucocorticoids, has an inhibitory effect on the pro-inflammatory response, exerting protective effects during immunologic disturbances (Wang et al., 2015). We hypothesized that *Bos indicus* cattle treated with intra-nasal oxytocin would have a less extreme increases in cortisol concentrations and immune parameters closer to baseline when subjected to handling and 6 h road transportation compared with cattle treated intra-nasally with saline.

METHODS

Animal management and experimental design

Thirty, recently weaned *Bos indicus* steers were randomly selected from the herd at James Cook University's Fletcherview Research Station. Twenty-four hours prior to the start of the transportation, steers were weighed and had their feed and water withdrawn. Animals were blocked by bodyweight and randomly assigned to one of three treatment groups: 1) intra-nasally dosed with sterile saline and held in the yard for 6 h (S-NT; n = 10), 2) intra-nasally dosed with sterile saline and transported for 6 h (S-T; n = 10), and 3) intra-nasally dosed with oxytocin (0.3 IU/kg bodyweight) and transported for 6 h (OXT-T; n = 10).

Treatment administration and sample collection

At h 0, steers were moved into the chute, weighed, and restrained to the side of the head chute to allow open access to the left jugular vein. A total of 22 mL of blood was then collected into four Vacutainer™ tubes (5 mL serum, 10 mL lithium heparin, 5 mL fluoride oxalate, and 2 mL EDTA) via the jugular vein. All blood tubes were

immediately inverted a minimum of eight times; tubes containing lithium heparin, fluoride oxalate, or EDTA were kept cool (-4°C) for a maximum of 12 hours before centrifugation and plasma separation.

Directly following blood collection, previously allocated intra-nasal treatments were administered with a Mucosal Atomization Device (MAD Nasal™, McFarlane Medical Equipment Pty Ltd., Surrey Hills, VIC, AUS). Doses of oxytocin (Syntocinon, 10 IU oxytocin/mL, Ilium, Troy Laboratories Australia Pty. Ltd., New South Wales) and saline ranged from 6 to 8 mL, dependent upon bodyweight. Half of each dose was administered into each nostril. Animals were then loaded onto the truck at a density of 0.88 m2 / animal and transported, or moved to a nearby dry lot, for 6 hours. Six-hour transportation was selected as the transportation time due to the majority of transportation events (86%) for cattle being ≤ 8 hours (Schwartzkopf-Genswein et al., 2012). Immediately following the conclusion of transportation (h 6), and again at 48 and 72 h, blood was collected from all animals using the same procedures as described above. With the exception of the 6 h of transportation or dry lot treatments, all treatment groups were held together in a pen for the duration of the experiment.

Sample analyses

Samples in serum Vacutainer™ tubes were allowed time to clot and then centrifuged at 3,000 × g for 10 minutes. A commercially available solid-phase, competitive chemiluminescent enzyme immunoassay (IMMULITE®/IMMULITE 1000 Cortisol; Siemens Healthcare Pty Ltd, Bayswater, Victoria, AUS) was then used to measure cortisol from sera samples. Fluoride oxalate blood tubes were centrifuged at

3,000 × g for 15 minutes, after which the plasma was removed and stored at -20°C until needed. Plasma was then thawed and measured for glucose (OSR6121) and lactate (OSR6193) with an automated chemistry analyzer (AU480; Beckman Coulter, Inc., Lane Cove, NSW, AUS). In addition, a complete cell blood count was performed on whole blood samples from EDTA Vacutainer™ tubes using a Sysmex XT-2000i Automated Hematology Analyzer (Sysmex Australia Pty Ltd, Macquarie Park, NSW, AUS).

Statistical analysis

Data were analyzed using MIXED procedure in SAS 9.4 Software (SAS Institute, 1999). Animal was treated as a random effect; treatment group and time were treated as fixed effects. Repeated measures were assessed using the first-order autoregressive covariance structure for leukocyte counts, cortisol concentrations, and bodyweight results. The heterogenous compound symmetry covariance structure was utilized for glucose and lactate results. The appropriate covariance structures were selected based on lowest Bayesian information criterion between first-order autoregressive, compound symmetry, and heterogenous compound symmetry structures. Hour 0 values were used in each statistical model as a covariate. Significance was determined at $P \leq 0.05$ and trends are reported at $0.05 < P \leq 0.10$. A log transformation was used on eosinophil data due to non-normality; results are presented as back transformed values.

RESULTS

Mean counts for differentiated leukocytes are reported in Table 1. A treatment group by time effect was detected for lymphocytes and basophils counts ($P < 0.01$; Figure 6). A treatment group by time effect was also detected for neutrophils and eosinophils, such that counts increased and decreased ($P < 0.01$), respectively, directly

following transport (Figure 7). Total leukocyte counts were not different between treatment groups ($P = 0.96$). An effect of time ($P < 0.01$) was found for white blood cells, red blood cells, and hemoglobin (Table 2).

There were no differences between treatment groups or over time for plasma cortisol concentrations (S-NT = 21.5 ng/mL, S-T = 23.9 ng/mL, and OXT-T = 20.8 ng/mL; $P = 0.46$). An effect of time was found for glucose and lactate concentrations ($P < 0.01$). The concentration of glucose was greater at hour 6 (0.72 mM) compared with hours 0 (0.64 mM), 48 (0.63 mM), and 72 (0.65 mM). The concentration of lactate was greater at hour 6 (0.47 mM) and lesser at hour 48 (0.31 mM) compared with hour 0 (0.40 mM).

A treatment group by time interaction ($P < 0.03$) was detected for bodyweight such that transportation, independent of intra-nasal treatment, yielded lesser bodyweights at 6 and 72 h compared with the non-transported group (Figure 8). Overall, treatment with intra-nasal oxytocin did not yield differences between treatment groups.

DISCUSSION

Basophil and lymphocyte counts returned to baseline more quickly in steers that were administered oxytocin intra-nasally prior to short-duration transport compared with steers that received saline intra-nasally, suggesting oxytocin may be having a protective effect during transportation-induced immunologic disturbance. In addition, basophil counts were greater in transported steers that received oxytocin intra-nasally compared with transported steers that received saline intra-nasally directly following transportation. However, basophils are rarely found in circulation and interpretation of changes in

basophil counts can be challenging (Parkinson et al., 2010). Greater basophil counts within normal ranges (Roland et al., 2014) can indicate a system primed for dealing with infection; maintenance of basophil counts, as seen in the present study, is optimal compared with increases that may exceed normal ranges and initiate an allergic reaction (Hall, 2016). In contrast, lymphopenia is commonly associated with increased glucocorticoids and systemic reactions in cattle (Parkinson et al., 2010) and was observed in both transported groups directly following transportation at 6 h. Lymphocyte counts returned to baseline at 48 and 72 hours relative to the start of transport in the treatment group that received oxytocin compared with the two treatment groups that received saline, independent of transportation; again, this rapid return to baseline suggests a protective effect of oxytocin. In general, increased oxytocin release has been shown to have inhibitory effects on the pro-inflammatory response, exerting protective effects during various immunologic disturbances (Wang et al., 2015). To the authors' knowledge, no direct correlation between oxytocin and leukocytes has been investigated previously.

Neutrophilia is commonly reported in transported cattle (Murata et al., 1987; Earley et al., 2006; Lomborg et al., 2008), supporting findings in the present study and indicating an inflammatory response associated with transportation stress. Eosinophil counts were lesser directly following transportation in the present study, supporting previous reports in goats (Nwe et al., 1996) and cattle (Stanger et al., 2005; Earley et al., 2006). In contrast, no differences in eosinophil counts were detected following 4-hour transportation in calves (Murata et al., 1987). Eosinopenia is commonly noted as occurring in response to increases in blood concentrations of glucocorticoids (Parkinson

et al., 2010). Duration of transport and sampling timeline may affect these parameters, explaining variation across studies. Overall, several indicators of an acute inflammatory response were detected in response to 6-hour road transportation in the present study.

Unexpectedly, total leukocyte counts were not different between treatment groups. Lack of a substantial protective effect of intra-nasally provided oxytocin on total leukocyte counts may be attributable to the short half-life of oxytocin in plasma (3-12 minutes; Leng and Ludwig, 2016; Chapter 4) following intra-nasal administration. A more sustained increase, mediated centrally, may be necessary to observe more substantial immunologic effects. In addition, oxytocin has been shown to have substantial effects when animals are confronted with a psychosocial stress (e.g. elevated plus maze in rodents) (Amico et al., 2004). A different stress-model that includes a psychological component may better uncover differences between intra-nasal treatments in cattle.

In terms of the effects of transportation, Stanger et al. (2005) report decreases in total leukocyte counts 72 hours post-transport following 72 hours of transportation. However, no sample was taken directly following transportation, therefore comparisons are limited (Stanger et al., 2005). In contrast, Buckham Sporer et al. (2008) report increases in total leukocyte counts following nine hours of transportation. Still, results from the present study most closely support those reported by Murata et al. (1987); no differences in total leukocyte counts were detected directly following four-hour transport (Murata et al., 1987). Again, differences in immediacy of sampling and duration of transport may explain variation across studies. It is also important to note that when measuring immune parameters, we are only able to get a snapshot without collecting

many samples over several days for proper and complete leukogram interpretation (Parkinson et al., 2010).

Cattle used in the present study were acclimated to handling and less excitable than the average for the genotype. Novelty and sufficient duration of transportation in young calves was expected to increase cortisol concentrations due to activation of the HPA axis. Elevations in plasma cortisol concentrations have been reported in cattle transported for four hours (Murata et al., 1987; Hulbert et al., 2011). However, in the present study, plasma cortisol concentrations were not different between transported and non-transported animals after road transportation for six-hours. Similarly, beef cattle have exhibited cortisol concentrations similar to baseline concentrations after 3 hours of transportation (Arthington et al., 2003; Parker et al., 2009), suggesting a diminishing response of the HPA axis to road transport. It is noteworthy that eosinopenia, observed in transported calves in the present study, is commonly seen in healthy animals in response to increases in glucocorticoid concentrations (Parkinson et al., 2010); this suggests cortisol may have been elevated at some point but not detected due to discontinuous sampling. Additionally, there were no differences in cortisol concentrations between steers given oxytocin or saline prior to transport. More continuous sampling during transportation may be necessary to detect any differences between intra-nasal treatments that may have occurred during peak stress (i.e. first 2 hours of transport).

A significant effect of time was found for glucose and lactate, such that concentrations were greater directly following transportation. Transportation, and not feed and water deprivation, caused detected increases; non-transported calves were also

deprived of feed and water throughout the study and displayed no increase in glucose or lactate. Previous studies report conflicting results. One such study reports reduced glucose concentrations 24 hours following transportation in calves transported for four hours (Hulbert et al., 2011). In contrast, Parker et al. (2007) report no differences in plasma glucose or lactate concentrations following 24- or 48-hour transport. It is possible that glucose and lactate concentrations had a chance to stabilize in animals transported for longer durations, as in previous studies.

Reduced bodyweight observed directly following transport (h 6) was expected as part of typical live-weight loss associated with transportation (Arthington et al., 2003; Earley et al., 2006). As little as three hours of transportation can cause a 7.6% decrease in bodyweight in weaned beef calves (Arthington et al., 2003). Natural variation in bodyweight between animals and across days due to feed and water intake is one thing to consider when assessing differences at h 72.

CONCLUSIONS

In conclusion, oxytocin altered specific leukocytes in *Bos indicus* cattle exposed to short-duration transport stress indicating a protective effect of oxytocin. Although no effect on the HPA axis was detected via changes in cortisol concentration, road transport induced some signs of an acute inflammatory response directly following transportation. The short half-life of oxytocin or mild nature of short-duration transport may explain lack of effect of intra-nasal oxytocin.

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Table 1. Mean counts for differentiated leukocytes from *Bos indicus* calves that have been treated with intra-nasal oxytocin or saline and transported for 6 hours or treated with intra-nasal saline and not transported

	Treatment Groups[1]				P-values		
	S-NT	S-T	OXT-T	SEM	Group	Time	Group x Time
Leukocytes, 10^9/L							
Neutrophils	2.63[b]	3.52[a]	3.12[a]	0.1	< 0.01	< 0.01	< 0.01
Lymphocytes	7.98	7.55	7.94	0.2	NS	NS	< 0.01
Monocytes	0.349	0.364	0.382	0.02	NS	< 0.01	NS
Eosinophils[2]	0.114[a]	0.075[b]	0.079[c]	-	< 0.01	< 0.01	< 0.01
Basophils	0.054	0.053	0.056	0.00	NS	< 0.01	< 0.01

[1]S-NT = saline, non-transported (n=10); S-T = saline, transported 6 h (n=10); OXT-T = oxytocin given intra-nasally at 0.3 IU/kg bodyweight, transported 6 h (n=10)

[2]Back-transformed data presented

[a-c] Means within a row differ between treatment groups ($P < 0.05$); NS = non-significant ($P > 0.05$)

Table 2. Mean complete blood counts from *Bos indicus* calves over time			
	Complete Blood Count		
	WBC, 10^9/L[1]	RBC, 10^{12}/L[2]	HGB, g/L[3]
Time, hours			
0	11.2[b]	13.2[a]	150.6[a]
6	13.3[a]	12.5[b]	142.2[b]
48	11.2[b]	12.4[b]	141.3[b]
72	10.9[b]	12.6[b]	142.5[b]

[1] WBC = white blood cells; pooled SEM = 0.4

[2] RBC = red blood cells; pooled SEM = 0.4

[3] HGB = hemoglobin; pooled SEM = 2.2

[a-b] Means within a column differ ($P < 0.05$)

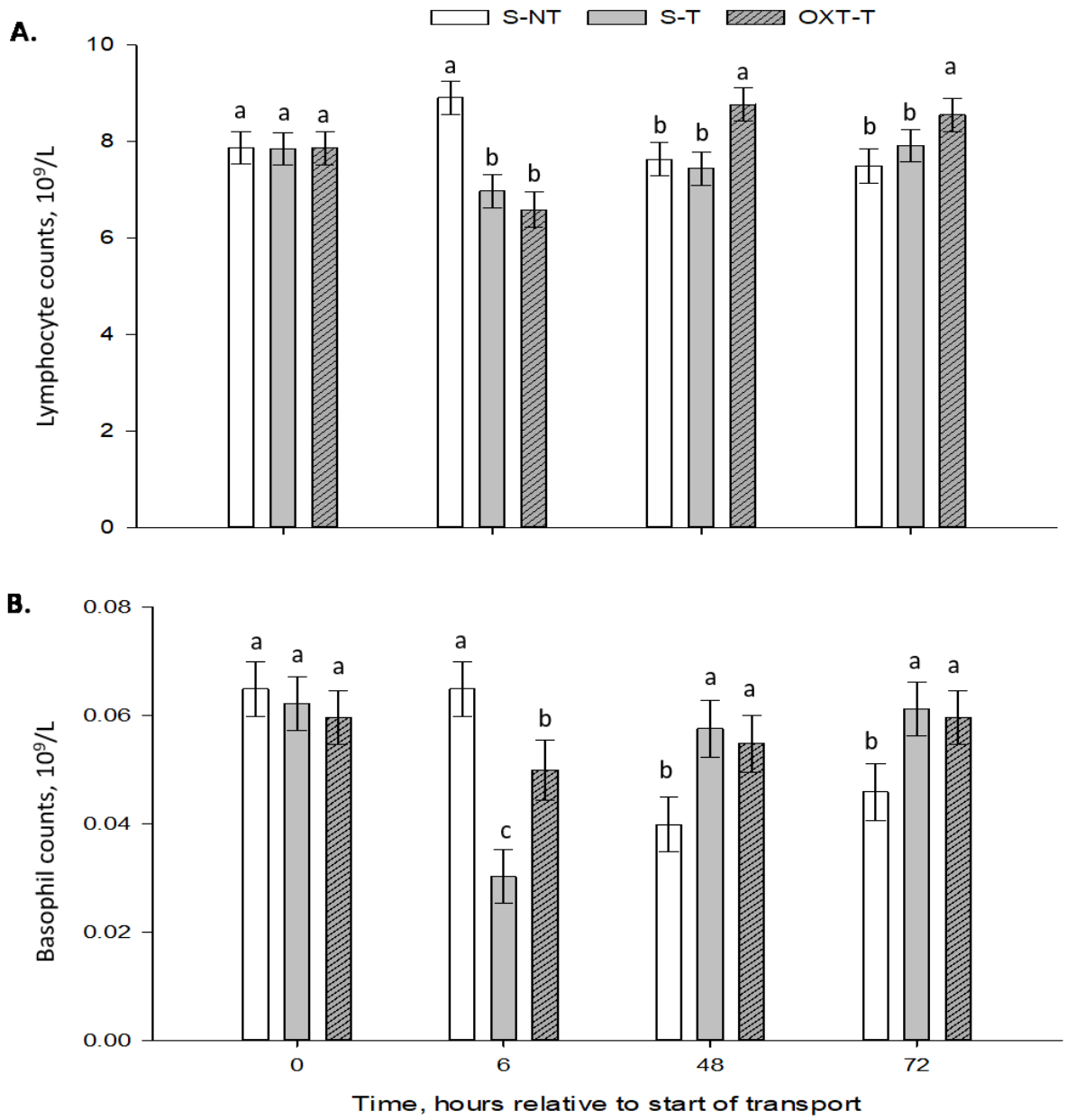

Figure 6. Mean lymphocyte (A) and basophil (B) counts from *Bos indicus* calves given intra-nasal saline and transported (S-T; n = 10), intra-nasal oxytocin and transported (OXT-T; n = 10), or intra-nasal saline and not transported (S-NT; n = 10) at 0 h before transport, after 6 h of transport and at 48 h and 72 h after transport [a-c] Means differ between treatment groups.

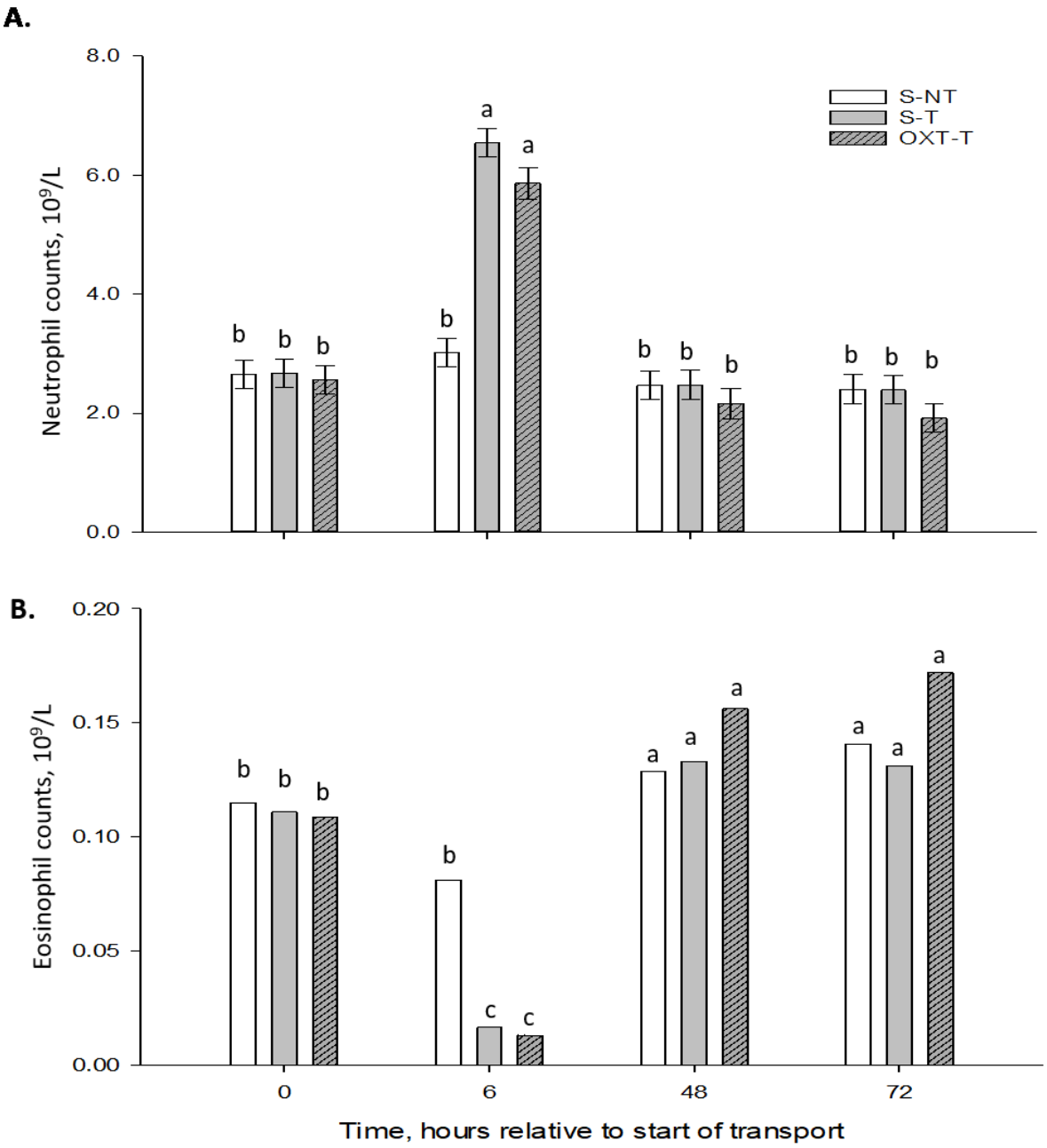

Figure 7. Mean neutrophil (A) and eosinophil (B) counts in *Bos indicus* calves given intra-nasal saline and transported (S-T; n = 10), intra-nasal oxytocin and transported (OXT-T; n = 10), or intra-nasal saline and not transported (S-NT; n = 10) at 0 h before transport, after 6 h of transport and at 48 h and 72 h after transport [a-c] Means differ between treatment groups.

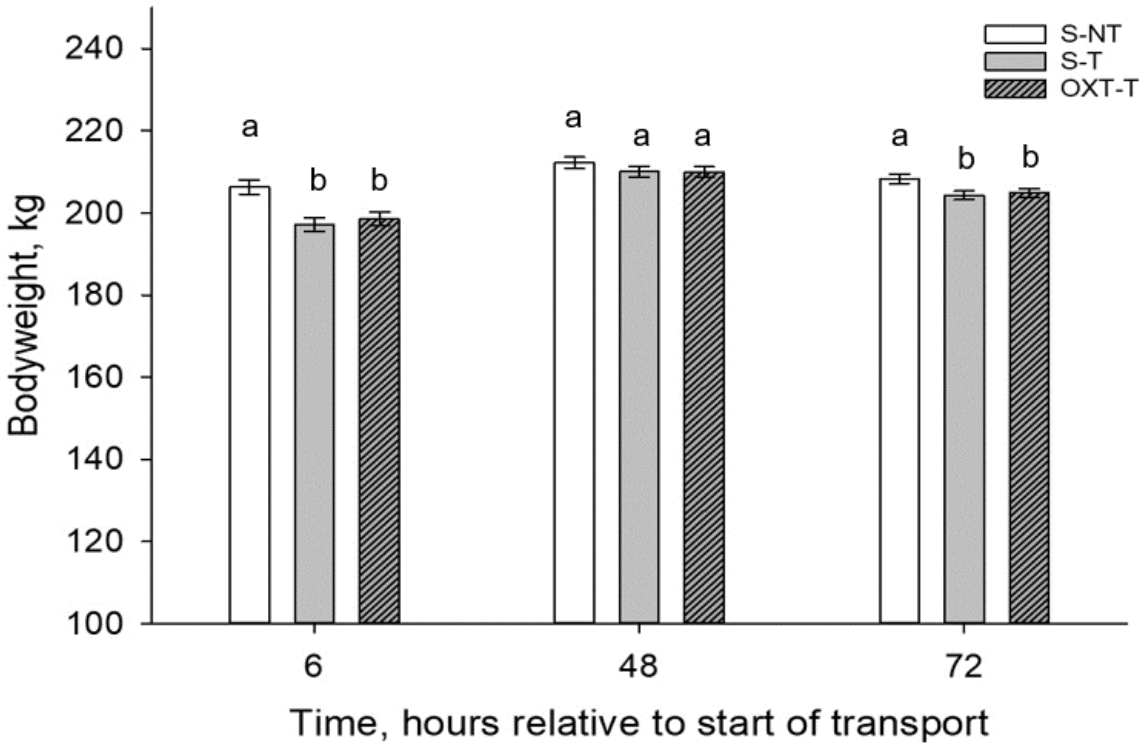

Figure 8. Mean bodyweight for *Bos indicus* calves given intra-nasal saline and transported (S-T; n = 10), intra-nasal oxytocin and transported (OXT-T; n = 10), or intra-nasal saline and not transported (S-NT; n = 10) after 6 h of transport and at 48 h and 72 h after transport [a-c] Means differ between treatment groups.

CHAPTER FOUR: PHARMACOKINETICS OF OXYTOCIN ADMINISTERED INTRA-NASALLY TO BEEF CATTLE

"Short Communication: Pharmacokinetics of oxytocin administered intra-nasally to beef cattle" has been submitted to *Domestic Animal Endocrinology* by authors B.K. Wagner, A.E. Relling, J.D. Kieffer, L.E. Moraes, and A.J. Parker.

ABSTRACT

Providing the hormones oxytocin and vasopressin intra-nasally increased concentrations in plasma and cerebral spinal fluid, respectively, in humans and primates. This is of interest due to the documented anxiolytic effects of oxytocin observed in humans and rodents. To date, a transnasal approach of hormone administration has not been investigated in beef cattle. Defining the pharmacokinetics of intra-nasal oxytocin in cattle is necessary for determining optimum sampling and dosing timelines for future investigations. Five, weaned *Bos taurus* steers were used in a 3 × 3 Latin square design. Treatments included: 1) 0.33 IU oxytocin/kg bodyweight (A, n = 5), 2) 0.66 IU oxytocin/kg BW (B, n = 5), and 3) 1.32 IU oxytocin/kg BW (C, n = 5). Steers were acclimated to handling and restraint procedures for four weeks leading up to the start of the experiment. Blood was collected from jugular catheters at -10, 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 35, 40, 45, 50, 55, and 60 minutes relative to administration of intra-nasal treatment. No treatment by time interaction was detected, however there was an effect of time ($P < 0.001$) and treatment ($P = 0.002$) on oxytocin concentrations. Pharmacokinetic parameters, determined by PKSolver excel add-in, demonstrated an average maximum concentration (C_{MAX}) of 63.3 pg/mL at 3.5 minutes post intra-nasal dose administration.

An average half-life ($T_{1/2}$) of 12.1 minutes post intra-nasal administration was determined. Pharmacokinetic parameters were not different between doses.

INTRODUCTION

Oxytocin is known to be involved in parturition, lactation, maternal, and social behaviors (Gimpl and Fahrenholz, 2001; Calcagnoli et al., 2015). Oxytocin has recently been shown to have anxiolytic properties in several species (Neumann et al., 2000; Parker et al., 2005). In humans and primates, providing the neuropeptides oxytocin and vasopressin intra-nasally increased concentrations in plasma (Gossen et al., 2012) and cerebral spinal fluid (Born et al., 2002; Lee et al., 2018). The dose rate of intra-nasal oxytocin and the time course for the clearance of the hormone is rarely justified in the literature for each of the species where an effect on behavior or anxiety has been reported (Gossen et al., 2012; Calcagnoli et al., 2015; Spengler et al., 2017). Furthermore, dose rates of 24 and 26 IU oxytocin given to an adult human via the intra-nasal route is cited a number of times in the literature as an anxiolytic dose (Gossen et al., 2012; Spengler et al., 2017). This same dose rate however, has been given to 13- and 18-day-old piglets (Rault, 2011) with equivocal effects on the HPA axis. Species differences may prevail in the pharmacokinetics of oxytocin and its effects on the HPA axis. To date, an intra-nasal approach to the administration of neuropeptide hormones has not been investigated in beef cattle. Defining a dose-response curve is necessary for determining optimum sampling and dosing timelines for any future investigation of the effects of intra-nasal oxytocin in cattle. Therefore, the objective of the current study is to evaluate the pharmacokinetics of oxytocin given intra-nasally to cattle.

METHODS

Animal management

Five weaned *Bos taurus* steers of angus genetic type (220 ± 11.9 kg mean ± SEM bodyweight, < 1 year of age) were used in the present study. Steers were selected for temperament based on chute scores from the Ohio Agricultural Research and Development Center's beef herd at the Eastern Agricultural Research Station in Caldwell, Ohio. One month prior to the start of the experiment, steers were acclimated to the working environment and human contact for 2 h per d, for a total of 10 d. Briefly, the acclimation protocol involved working steers calmly through the race and chute while being gently touched by the experimenters. In addition, steers were caught in the head gate and halters were applied and then removed at the end of the acclimation session. Steers were returned to their pen at the completion of the acclimation session each day. During the acclimation period, steers had ad libitum access to fescue hay and water when in their pens. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (#2017A00000130).

Experimental design

Steers were allocated to one of three treatments in a 3 × 3 Latin square design. Steers were randomly allocated to the order in which they received each of the three treatments. A total of three runs were used to allow each steer to receive each of the three treatments. Treatments included: 1) 0.33 IU oxytocin/kg bodyweight (A, n = 5), 2) 0.66 IU oxytocin/kg BW (B, n = 5), and 3) 1.32 IU oxytocin/kg BW (C, n = 5). Different volumes of oxytocin (20 IU/mL, Vetone®, Bimeda-MTC Animal Health Inc., Ontario, Canada), ranging from 3.4 to 16.2 millilitres, were used to obtain the three treatments. The treatment

dose rates were selected from Spengler et al. (2017) and Gossen et al. (2012) who report administering 24 and 26 IU oxytocin /adult male human as an anxiolytic dose, respectively. Assuming the adult male humans were of average size (70 kg), this equates to 0.34 and 0.37 IU/kg bodyweight respectively. The other dose rates of 0.66 and 1.32 IU/kg bodyweight were included to cover the reported dose rates used in humans when calculated to a standard unit based on bodyweight.

Collection procedures

Steers were moved into the chute and catheterized via the jugular vein by the attending veterinarian. Briefly, their heads were restrained to the side of the head chute to allow open access to the jugular vein. A 5 cm × 5 cm area was clipped over the jugular vein and a local anesthetic (3 mL, 2% Lidocaine Hydrochloride injectable, Vedco Inc., St. Joseph, Missouri, USA) was injected subcutaneously at the site of catheter placement. Betadine® surgical scrub (Purdue Pharma L.P., Stamford, CT, USA) was used to clean the area for a total of three passes, followed by the removal of surgical scrub by alcohol (70% ethanol). A 14-gauge × 5.25" indwelling intravenous catheter (Becton, Dickinson, and Co., Franklin Lakes, NJ, USA) was placed into the jugular vein. Catheters were secured to the skin with sutures and extension sets were secured to the top of the neck with Elastikon® (Thermo Fisher Scientific, Waltham, MA, USA). After catheterization was complete (–2 h), animals were rested for two hours.

Following a two-hour rest period in the chute, during which patency of catheter lines were maintained with heparinized saline as needed, intra-nasal treatments were administered with the use of a mucosal atomerization device (Nasal™ Teleflex® Inc., Morrisville, NC) by the attending veterinarian. A 20 mL blood sample was taken 10

minutes prior to (t -10 min) and directly after (t 0 min) treatment administration. A 10 mL sample was taken every two-minutes for the first 30 minutes and then every five minutes for the remainder of the one-hour sampling period. Each sample was distributed into an EDTA tube and immediately put on ice following a minimum of eight inversions.

Sample analyses

Blood tubes were centrifuged within six hours at 3,000 × g for 15 minutes, after which plasma was removed with disposable pipette and stored at -20°C until required. Recovered plasma from EDTA tubes was used to measure oxytocin concentrations via a commercially available double antibody RIA kit (Oxytocin (Human, Rat, Mouse, Bovine) RIA Kit, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA). The intra-assay variation ranged from 3.1 to 9.6%. The minimum and maximum concentrations of detection were 2.5 and 160.0 pg/mL, respectively.

Recovered plasma from lithium heparin tubes was used, along with a slightly modified commercially available RIA kit (MP Biomedicals, LLC., Solon, OH, USA), to measure cortisol concentrations. Following kit instructions for volume of sample (25 µL) yielded results below the normal limit of detection (< 10.0 ng/mL). Therefore, I evaluated different volumes of sample (25, 50, 100, and 150 µL). Using parallel displacement and recovery (101.59 ± 10%) I determined that using 50 µL of sample yielded results between minimum and maximum concentrations of 5.0 and 500.0 ng/mL, respectively. The intra-assay and inter-assay variations were 5.8 and 10.6%, respectively.

Statistical analyses

Data were analyzed using MIXED procedure in SAS 9.4 Software (SAS Institute, 1999). Steer was treated as a random effect, while run (1-3), treatment (A-C), time of sampling, and their interaction were treated as fixed effects. Repeated measures were assessed using the compound symmetry covariance structure. A log transformation was used on oxytocin data due to non-normality; results are presented as back-transformed values. The log transformed, -10 min timepoint was used as a covariate for analysis of log transformed oxytocin data. The -10 timepoint was used as a covariate for analysis of cortisol data. One animal was removed from the study for abnormally fractious behavior. Significance was determined at $P < 0.05$ and trends are reported at $0.05 < P < 0.10$.

Pharmacokinetic parameters were determined using a PKSolver add-in, in Microsoft Excel, per methods described in Zhang et al. (2010). One-compartmental analysis was performed using the "Compartmental Analysis of Plasma Data After Extravascular Input" function within the PKSolver add-in. Parameters included the maximum plasma concentration (C_{MAX}), the time of maximum plasma concentration (T_{MAX}), the area under the curve from time zero to infinity (AUC_{INF}), the clearance by fraction dose (Cl/F), the half-life ($T_{1/2}$), and the mean residence time (MRT). Data were analyzed using MIXED procedure in SAS 9.4 Software. Steer was treated as a random effect, while run (1-3), treatment (A-C), time of sampling, and their interaction were treated as fixed effects.

RESULTS

There was no treatment by time interaction for oxytocin concentrations ($P = 0.15$), however an effect of treatment and time were detected (Figure 9; $P < 0.003$). Treatment C

had a greater average oxytocin concentration (18.9 pg/mL) compared with treatments A (13.2 pg/mL) and B (14.0 pg/mL). Pharmacokinetic parameters (Table 3) demonstrated an average C_{MAX} of 63.3 pg/mL at an average T_{MAX} of 3.5 minutes post intra-nasal administration. An average $T_{1/2}$ of 12.1 minutes post intra-nasal administration was determined. None of the measured pharmacokinetic parameters (i.e. C_{MAX}, T_{MAX}, AUC_{INF}, Cl/F, $T_{1/2}$, and MRT) differed between treatments ($P \geq 0.17$).

A run by treatment interaction was detected for cortisol concentrations, as well as an effect of treatment (Figure 10; $P < 0.01$) and run (Figure 11; $P < 0.01$). Run one had overall greater ($P < 0.01$) cortisol concentrations (13.2 ng/mL) compared with runs two (8.9 ng/mL) and three (8.9 ng/mL). In addition, treatment C had overall greater ($P < 0.01$) cortisol concentrations (12.4 ng/mL) compared with treatments A (8.8 ng/mL) and B (9.8 ng/mL).

DISCUSSION

The present study is the first to identify a C_{MAX} and T_{MAX} for intra-nasal oxytocin supplementation utilizing greater sampling frequency than that which has been published for other mammals (Gossen et al., 2012). Previous studies investigating oxytocin supplementation have yielded variable results in animals (Parker et al., 2005) and humans (Gossen et al., 2012; Striepens et al., 2013), possibly due to unidentified pharmacokinetics and subsequently insufficient sampling timelines.

Oxytocin

It is understood that oxytocin has a short half-life of 3-8 minutes in the blood of mammals (Gimpl and Fahrenholz, 2001; Leng and Ludwig, 2016), however different rates

of disappearance have been reported between blood and cerebral spinal fluid (CSF) when oxytocin is provided intra-nasally (Freeman et al., 2016). Intra-nasal delivery alters CSF and brain tissue concentrations for up to 75 minutes following intra-nasal administration in humans (Striepens et al., 2013; Carson et al., 2016), potentially explaining behavioral effects being observed beyond 8-16 minutes (Kent et al., 2016). Variability in the half-life of oxytocin in plasma has been reported in cattle intravenously infused with oxytocin (0.5-1.0 IU/min), ranging from 3.5 to 23.5 minutes (Gorewit, 1984; Wachs et al., 1984). In the present study, a half-life of 12.1 minutes in plasma suggests an intermediate half-life with intra-nasal administration and therefore may be longer acting than intravenous administration. Peptides, such as oxytocin, administered intra-nasally exhibit slower rates of disappearance in the CSF compared with blood (Born et al., 2002; Striepens et al., 2013), which may explain the intermediate half-life observed in the present study. Thicker mucous or differences between species in mucociliary clearance rates (Quintana et al., 2015) may affect the pharmacokinetics and half-life of intra-nasally supplemented compounds. To date, no studies have reported CSF concentrations of oxytocin in ruminants.

With milking, endogenous oxytocin concentrations can range from 10-100 pg/mL (Bruckmaier, 2013). In the present study, all C_{MAX} concentrations were within this range. However, endogenous releases of oxytocin have only been shown to reach maximum concentrations of 30-40 pg/mL in ruminants (Bruckmaier et al., 1994a; Bruckmaier et al., 1994b). Therefore, I would like to highlight that all doses reached concentrations greater than typical endogenous concentrations, suggesting potential therapeutic qualities of all doses investigated in the present study.

Results of the present study coincide with previous pharmacokinetic studies investigating different intra-nasal doses in that pharmacokinetic parameters remained the same across doses (Moore et al., 1997). In contrast, no differences were detected between doses for C_{MAX} or AUC_{INF} in the present study; this may be due to high variation between individuals and the conservative number of animals utilized.

Cortisol

Unexpectedly, greater doses of intra-nasal oxytocin correlated with greater concentrations of cortisol, contradicting the current understanding that exogenously provided oxytocin attenuates activation of the HPA axis in mammals. Greater cortisol concentrations with oxytocin administration have been reported previously (Rault et al, 2013), however this study was conducted in piglets receiving approximately 2 IU/kg bodyweight. It is possible that significant doses of oxytocin, ≥ 1.32 IU/kg bodyweight, dysregulate the HPA axis and actually potentiate the cortisol response. However, cortisol concentrations in the present study were all within baseline biological ranges and more likely do not constitute biologically significant differences between treatments.

Of perhaps greater significance, an effect of run on cortisol concentrations was detected in the present study. Cortisol concentrations were below the detectable limit in runs two and three, indicating that steers became accustomed to the handling and catheterization procedures during the first run. To the author's knowledge there are no published studies that offer insight into acclimation to catheterization and isolation specifically. However, a study by Cooke et al. (2009) found that cattle acclimated to handling over a four-week period displayed lesser cortisol concentrations compared with cattle that had not been acclimated to handling. Steers in the present study were acclimated

to handling for four weeks prior to the start of the experiment; this may explain the relatively low concentrations of cortisol found in the present study compared with the experiment presented in Chapter 2 that also utilized catheterization and isolation. In addition, repeated exposure to catheterization and isolation decreased cortisol concentrations. It is likely that steers learned from the first run that following these procedures they would be safely returned to their pen with their herd mates.

CONCLUSIONS

Overall, there is a lack of scientific information on the effects of oxytocin, administered intra-nasally, in ruminant species. The present study adds to the limited existing knowledge regarding appropriate doses and sampling timelines necessary to obtain meaningful results. Oxytocin responds in a dose-dependent manner in the bovine when administered intra-nasally, however T_{MAX} and C_{MAX} are the same regardless of dose. An intermediate half-life of 12.1 minutes in plasma has been established for intra-nasal administration of oxytocin in *Bos taurus* steers. In addition, there is evidence to support that animals acclimate to catheterization and handling procedures after a single exposure.

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Table 3. Mean pharmacokinetic parameters for oxytocin in the plasma of *Bos taurus* steers after intra-nasal administration at three doses (A = 0.33 IU/kg bodyweight, B = 0.66 IU/kg bodyweight, and C = 1.32 IU/kg bodyweight).

		Treatments					
Parameter[1]	Units	A	B	C	Geometric Mean	SEM[2]	P-value
C_{MAX}	pg/mL	48.0	64.6	77.3	63.3	26	0.74
T_{MAX}	min	2.4	4.3	3.6	3.5	2	0.71
AUC_{INF}	min x pg/mL	828	654	1726	1070	370	0.17
Cl/F	mL/min/kg	0.002	0.002	0.002	0.002	0.001	0.92
$T_{1/2}$	min	10.5	15.7	9.9	12.1	5.23	0.61
MRT	min	16.2	25.0	20.0	20.4	7.4	0.69

[1]C_{MAX} = maximum concentration, T_{MAX} = time of C_{MAX}, AUC_{INF} = area under the curve extrapolated to infinity, Cl/F = clearance by fraction dose, $T_{1/2}$ = half-life, and MRT = mean residence time

[2]Standarad error of the mean

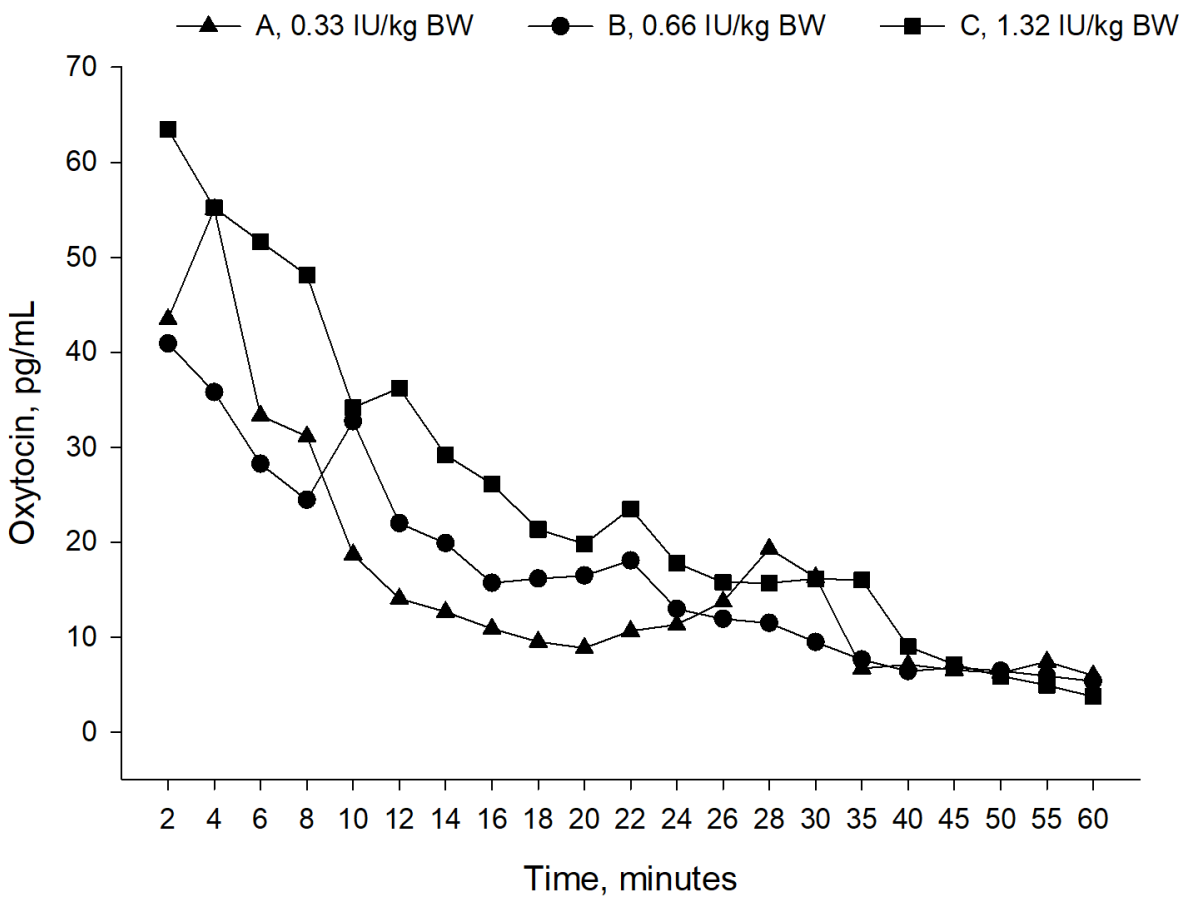

Figure 9. Back transformed mean plasma oxytocin concentrations in *Bos taurus* steers administered oxytocin intra-nasally at three different rates (0.33, 0.66, and 1.32 IU/kg bodyweight. Treatments were administered at time 0 (not shown).

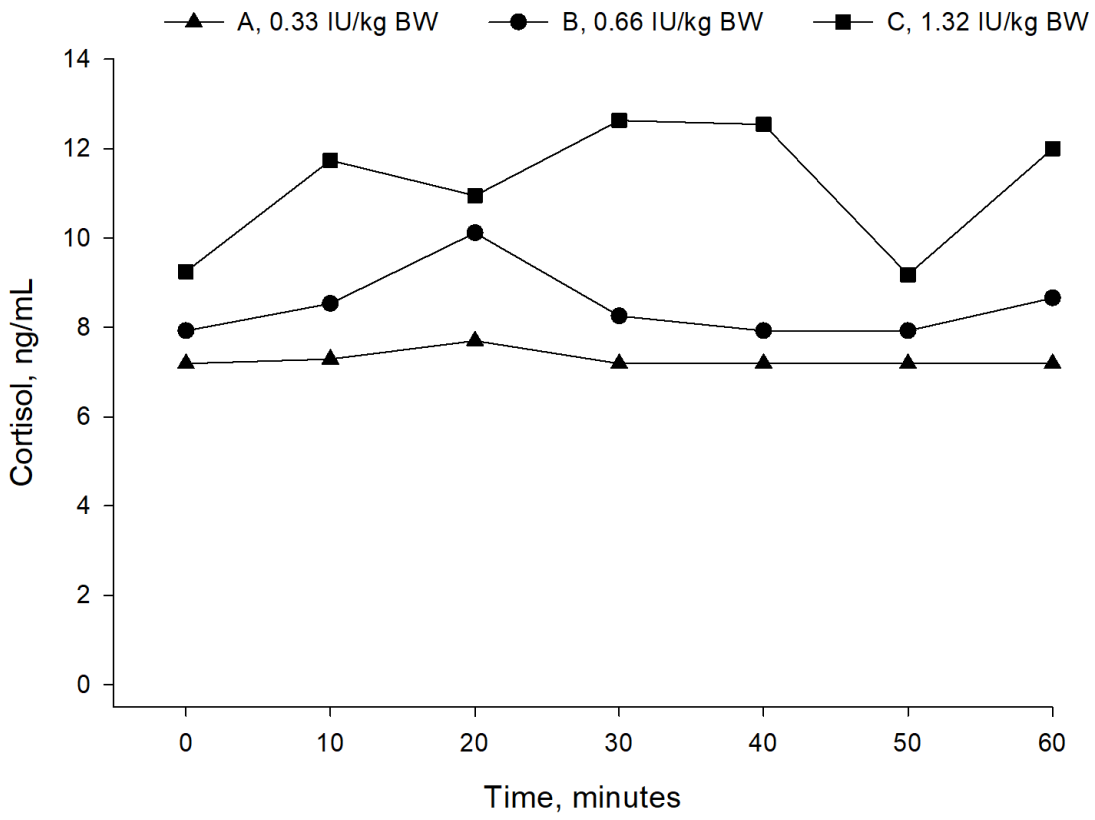

Figure 10. Mean plasma cortisol concentrations in *Bos taurus* steers administered oxytocin intra-nasally at three different rates. Treatments were administered at time 0. The pooled SEM is 1.9. An effect of treatment was detected such that treatment C had overall greater *(P* < 0.01) cortisol concentrations compared with treatments A and B.

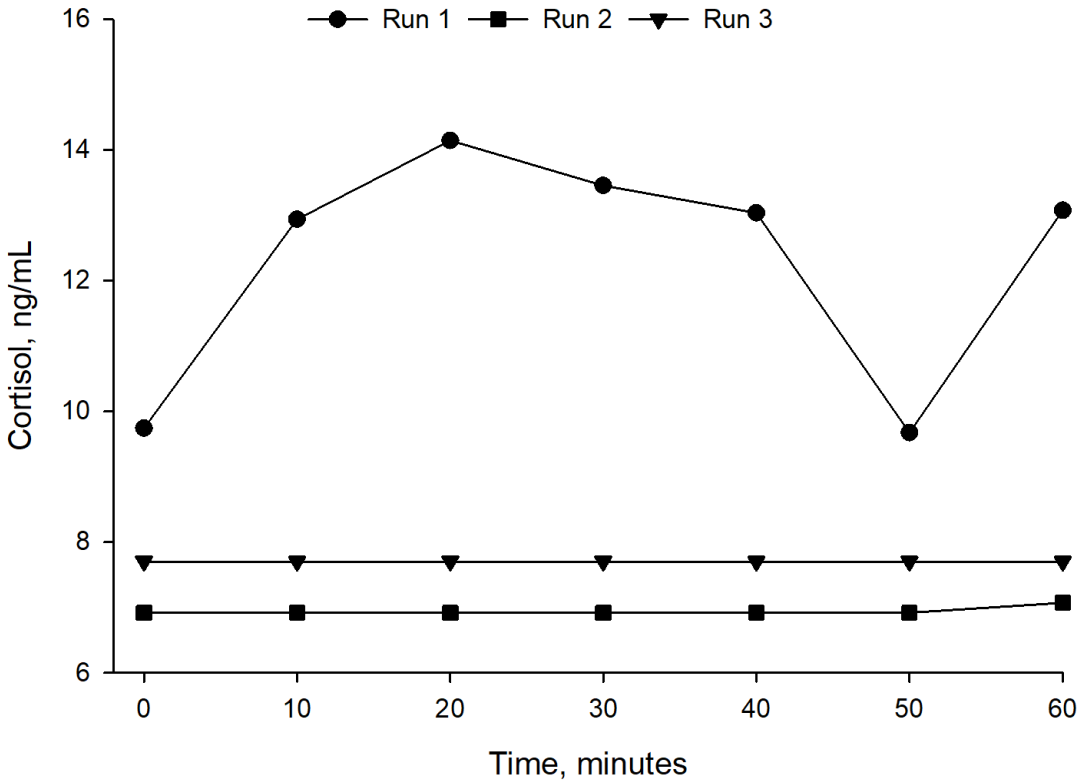

Figure 11. Mean plasma cortisol concentrations in *Bos taurus* steers administered oxytocin intra-nasally over three runs. Treatments were administered at time 0. The pooled SEM is 1.6. An effect of run was detected such that run one had greater *(P* < 0.01) cortisol concentrations compared with runs two and three.

CHAPTER FIVE: LACTATION BUT NOT EXOGENOUS OXYTOCIN ATTENUATES THE HPA AXIS IN BEEF CATTLE SUBJECTED TO RESTRAINT STRESS

ABSTRACT

Oxytocin appears to have a role in the regulation of the hypothalamo-pituitary-adrenal (HPA) axis in a number of species. The attenuated responses of the HPA axis was paired with greater plasma oxytocin concentrations, was reported in lactating ewes compared with non-lactating ewes. Oxytocin may be involved in the stress attenuation commonly observed in lactating females. I hypothesized that intra-nasally administered oxytocin would attenuate restraint-induced activation of the HPA axis in non-lactating beef cattle comparable to attenuation observed in lactating cows. Twenty *Bos taurus* cows were blocked by lactational status and paired by age. By pair, cows were randomly allocated to one of four treatment groups, in a 2 × 2 factorial arrangement: 1) Non-lactating, saline (NL-S; n = 5); 2) Non-lactating, oxytocin (NL-OXT; n = 5); 3) Lactating, saline (L-S; n = 5); and 4) Lactating, oxytocin (L-OXT; n = 5). There was no effect of lactation on plasma oxytocin concentrations ($P = 0.34$). Non-lactating cows had greater ($P < 0.01$) cortisol concentrations compared with lactating cows. There was no effect of intra-nasal treatment or time on plasma cortisol concentrations ($P = 0.99$). Overall, intra-nasal oxytocin did not attenuate the HPA axis in lactating or non-lactating cows. Results of the present study contradict other reported studies that lactating mammals have consistently greater circulating oxytocin concentrations. Other hormones may be involved in the stress attenuation observed during lactation.

INTRODUCTION

In mammals, lactation attenuates the hypothalamo-pituitary-adrenal (HPA) axis (Slattery and Neumann, 2008; Hillerer et al., 2014; Ralph and Tilbrook, 2016). One important neuropeptide present in circulation during lactation is oxytocin (Gimpl and Fahrenholz, 2001). As evidence of the importance of oxytocin during lactation, oxytocin knock-out mice fail to nurse their offspring (Gimpl and Fahrenholz, 2001) and continuous elevation of oxytocin concentrations in plasma is necessary for complete milk removal in dairy cows (Bruckmaier et al., 1994). In addition, greater oxytocin concentrations and lesser cortisol concentrations were found in lactating sheep compared with non-lactating sheep exposed to predator stress (Ralph and Tilbrook, 2016). Oxytocin likely plays a role in the stress attenuative effect of lactation and the euthymic state of nursing mothers. However, paradoxical effects of oxytocin indicate other factors may be involved in the stress hypo-responsive state observed during lactation.

In a number of species, oxytocin attenuates stressor-induced activation of the HPA axis (Windle et al., 1997; Neumann et al., 2000; Ditzen et al., 2009; Ralph and Tilbrook, 2016); however, oxytocin potentiates the HPA axis in other species (Rault et al., 2013; Chapter Two). In addition, central administration of oxytocin has an attenuative effect on the HPA axis (Windle et al., 1997), while peripheral administration of oxytocin has demonstrated equivocal effects (Parker et al., 2005; Petersson et al., 2005; Rault et al., 2013). Providing oxytocin intra-nasally results in widespread dispersal within the brain in rodents and non-human primates (Lochhead and Throne, 2012; Carson et al.,

2016) and has been shown to attenuate increases in adrenocorticotropin hormone in non-human primates (Parker et al., 2005). However, intra-nasal oxytocin was not stress attenuative in non-lactating beef cattle exposed to restraint and isolation stress (Chapter Two). Considering all factors, the consequence of oxytocin is at least partially dependent upon species (Parker et al., 2005; Rault et al., 2013; Chapter Two), stressor (Windle et al., 1997; Ralph and Tilbrook, 2016), physiological state (Neumann et al., 1999), and route of administration (Windle et al., 1997; Petersson et al., 2005). This led us to question the potential use of intra-nasal oxytocin as an attenuator of stress in beef cattle, as has been investigated prior in non-lactating cattle (Chapter Two). Therefore, the hypothesis was that there would be no difference in the response of the HPA axis to restraint between non-lactating beef cows intra-nasally administered oxytocin and lactating beef cows intra-nasally treated with saline.

METHODS

Animal management and experimental design

Twenty *Bos taurus* cows of angus genetic type (2-10 years of age, 510 ± 72 kg mean ± SEM bodyweight) were used for the present study. Lactating cows were 4 to 6 weeks into lactation. Cows were selected from the Ohio Agricultural Research and Development Center's beef herd at the Eastern Agricultural Research Station in Caldwell, Ohio. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (#2017A00000012).

Cows were blocked by lactational status and paired by age. By pair, cows were randomly allocated to one of four treatment groups, in a 2 × 2 factorial arrangement: 1) Non-lactating, saline (NL-S; 0.015 mL/kg of bodyweight 0.9% isotonic saline; n = 5); 2)

Non-lactating, oxytocin (NL-OXT; 0.60 IU/kg of bodyweight oxytocin; n = 5); 3) Lactating, saline (L-S; n = 5); and 4) Lactating, oxytocin (L-OXT; n = 5). Four cows were tested at a time (i.e. one from each treatment group), either in the morning or afternoon, over a three-day period. Calves 1-2 months of age and were kept in front of their dam, allowing visual and nose-to-nose contact for the duration of the study.

Intravenous catheterization and pre-treatment

Two hours prior to treatment, cows were catheterized and then moved to their assigned chute area. Briefly, their heads were restrained to the side of the head chute to allow open access to the jugular vein. A 5 cm × 5 cm area was clipped over the jugular vein and a baseline blood sample was collected. To begin catheter placement, a local anesthetic (3 mL, 2% Lidocaine Hydrochloride injectable, Vedco Inc., St. Joseph, Missouri, USA) was injected subcutaneously at the site of catheter placement. Betadine® surgical scrub (Purdue Pharma L.P., Stamford, CT, USA) was used to clean the area (minimum of three passes), followed by the removal of surgical scrub by alcohol (70% ethanol). A 14-gauge × 5.25" indwelling intravenous catheter (Becton, Dickinson, and Co., Franklin Lakes, NJ, USA) was placed into the jugular vein. Catheters were secured to the skin with sutures and extension sets were secured to the top of the neck using Elastikon® (Thermo Fisher Scientific, Waltham, MA, USA). Cows were then moved to their appropriate chute and allowed to rest for one hour, during which time cows were able to move their head from side to side.

Treatment administration, stressor application, and sampling

Baseline blood samples were taken prior to intra-nasal administration and restraint application at 0, 30, 45, and 60 minutes. Directly following the 60-minute sample, the cow's head was restrained and their assigned intra-nasal treatment (S, isotonic saline or OXT, Oxytocin, 20 IU/mL Vetone®, Bimeda-MTC Animal Health Inc, Ontario, Canada) was administered with a mucosal atomization device (Nasal™ Teleflex® Inc., Morrisville, NC) by the attending veterinarian. The dose rate of oxytocin (0.60 IU/kg of bodyweight) was chosen based on a previous report beef cattle (Chapter 4). Doses ranged from 12 to 19 mL, dependent upon bodyweight. Half of each dose was administered into each nostril. Subsequently, ten millilitres of blood was collected via the jugular vein catheter at 62, 64, 66, 68, 70, 80 90, 100, 110, 120, 130, 140, 150, 160, 170, and 180 minutes. To obtain a blood sample, the extension line was cleared by removing 4 mL of blood and discarded prior to taking the full blood sample; following collection, 2 mL of heparinized saline was used to clear the line and maintain patency. Collected blood was distributed into two Vacutainer™ tubes (lithium heparin and EDTA), inverted a minimum of eight times, and immediately placed on ice (-4°C, until centrifugation). After sampling was complete, catheters were removed and animals were released back into the herd after visual assessment by the attending veterinarian.

Sample analyses

All blood tubes were centrifuged at 3,000 × g for 15 minutes, after which plasma was removed with disposable pipette and stored at -20°C until required. Recovered plasma from EDTA tubes was used to determine oxytocin concentrations using a commercially available double-antibody RIA kit (Oxytocin (Human, Rate, Mouse,

Bovine) RIA Kit, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA). The inter-assay variation was 19.3%. The intra-assay variation ranged from 3.1 to 9.6%.

Recovered plasma from lithium heparin tubes was used, along with a slightly modified commercially available RIA kit (MP Biomedicals, LLC., Solon, OH, USA), to measure cortisol concentrations. Following kit instructions for volume of sample (25 µL) yielded results below the normal limit of detection (< 10.0 ng/mL). Therefore, I evaluated different volumes of sample (25, 50, 100, and 150 µL). Using parallel displacement and recovery (101.59 ± 10%) I determined that using 50 µL of sample yielded results between minimum and maximum concentrations of 5.0 and 500.0 ng/mL, respectively. The intra-assay and inter-assay variations were 2.8 and 11.1%, respectively.

Data analysis

Data were analyzed using MIXED procedure in SAS 9.4 Software (SAS Institute, 1999). Cow was treated as random effects; treatment (S or OXT), lactation (NL or L), time, and their interactions were treated as fixed effects. Repeated measures were assessed using the first-order autoregressive covariance structure. Selection of this covariance structure was based on lowest Bayesian information criterion between first-order autoregressive, compound symmetry, and heterogenous compound symmetry structures. Two animals were removed from analyses for uncharacteristically fractious behavior throughout sampling. Significance was determined at $P < 0.05$ and trends are reported at $0.05 < P \leq 0.10$. A log transformation was used on oxytocin data due to non-normality; results are presented as back transformed values.

RESULTS

There was no effect of lactation on plasma oxytocin concentrations ($P = 0.34$). An effect of time ($P < 0.001$) and a treatment by time interaction ($P < 0.001$) were detected for plasma oxytocin concentrations (Figure 12). Non-lactating cows had greater (Figure 13 and 14; $P = 0.02$) cortisol concentrations (25.3 ng/mL) compared with lactating cows (12.9 ng/mL). There was no effect of intra-nasal treatment ($P = 0.93$) or time ($P = 0.53$) on plasma cortisol concentrations.

DISCUSSION

Lactating vs. Non-lactating - Oxytocin

Results of the present study contradict the reported phenomenon that lactating mammals have consistently greater circulating oxytocin concentrations to assist with lactogenesis and milk letdown (Cook, 1997; Ralph and Tilbrook, 2016). It has been suggested that oxytocin may be responsible for the stress hypo-responsive condition observed during lactation (Ralph and Tilbrook, 2016), however prolactin is a likely co-facilitator (Slattery and Neumann, 2008). In addition, factors such as suckling, species differences, and stressor differences may explain contradictory results in oxytocin concentrations in lactating animals compared with non-lactating animals.

Slattery and Neumann (2008) state that the hypo-responsive state of the HPA axis observed during lactation is not due solely to inhibition by oxytocin. Prolactin is a likely co-facilitator of the hypo-responsive, or decreased degree of responsiveness, stress condition observed in lactating mammals (Slattery and Neumann, 2008). As with oxytocin, prolactin is essential for the maintenance of lactation and greater concentrations of prolactin accompany the stress hypo-responsive state of lactation (Torner and

Neumann, 2002). Prolactin has also been shown to have a positive effect on oxytocin secretion at the level of the hypothalamus (Torner and Neumann, 2002); also, oxytocin acts as a releasing factor for prolactin at the anterior pituitary (Leng and Ludwig, 2016). These two hormones likely work in concert to facilitate the stress hypo-responsive state observed during lactation (Cook, 1997; Slattery and Neumann, 2008). However, suckling, species, and stressor type have all been shown to effect stress attenuation (Tilbrook et al., 2006).

Oxytocin release is known to be pulsatile in nature and often coincides with suckling bouts or tactile stimulation of the teat (Folley and Knaggs, 1966; Lupoli et al., 2001; Uvnas-Moberg et al., 2001). In the present study, calves were present but unable to suckle for the duration of the experiment (~4 hours). Low pulsatile release of oxytocin due to lack of exposure to suckling may explain the relatively low concentrations of oxytocin in the plasma of our lactating cows. However, Ralph and Tilbrook (2016) report consistent oxytocin concentrations in plasma between 40 and 50 pg/mL for 300 minutes in lactating ewes with lambs present but unable to suckle. In the study of Ralph and Tilbrook (2016), non-lactating ewes exhibited oxytocin concentrations between 0 and 5 pg/mL. In comparison to the study of Ralph and Tilbrook (2016), oxytocin concentrations prior to and shortly after intra-nasal administration at 60 minutes were between 2.5 and 5 pg/mL in both lactating and non-lactating cows. The data presented here may support the need for further investigation into the notion that suckling within 30 minutes is needed to induce detectable increases in endogenous plasma oxytocin concentrations (Heinrichs et al., 2002). Results of the present study contradict the current understanding that oxytocin

concentrations are consistently greater in all lactating mammals compared with their non-lactating counterparts (Cook, 1997; Ralph and Tilbrook, 2016).

However, differences between species may also explain discrepancies between studies in basal oxytocin concentrations (Parker et al., 2005; Chapter Two). Cook (1997) and Ralph and Tilbrook (2016) investigated endogenous oxytocin concentrations in sheep and report greater oxytocin concentrations in lactating ewes compared with non-lactating ewes. This was not observed in the present study; therefore, sheep may have a notably different oxytocinergic system compared with cattle. Moreover, oxytocin administered intra-nasally attenuated stress-induced increases in ACTH concentrations in primates (Parker et al., 2005), whereas the cortisol and ACTH concentrations were not affected by intra-nasal oxytocin in cattle (Chapter Two); these paradoxical effects indicate potential differences between species.

Differences between stressors may be another factor influencing concentrations of oxytocin. Restraint and isolation stress has been shown to potentiate an endogenous release of oxytocin in cattle (Chapter Two). Rodents exposed to a maternal defense test also exhibited an increase in oxytocin concentrations from basal (Neumann, 2002). Furthermore, lactating ewes exposed to restraint stress exhibited greater oxytocin concentrations compared with lactating ewes exposed to predator stress (Ralph and Tilbrook, 2016). Increases in concentrations of oxytocin in response to restraint stress were not observed in the present study, therefore the restraint applied may have been too mild to activate stress attenuating oxytocinergic pathways in lactating or non-lactating cows.

Lactating vs. Non-lactating - Cortisol

Attenuated activation of the HPA axis has been observed in lactating mammals exposed to various stressors (Neumann et al., 1999; Toufexis et al., 1999; Tilbrook et al., 2006; Ralph and Tilbrook, 2016), supporting the concept that lactation is a stress hypo-responsive condition. Results of the present study support previous findings in ruminants (Tilbrook et al., 2006; Ralph and Tilbrook, 2016), as lactating cows had lesser plasma cortisol concentrations compared with non-lactating cows throughout the experiment. In contrast to results of the present study, greater basal concentrations of cortisol have been reported in lactating sheep compared with non-lactating sheep (Cook, 1997). Milk may be acting as a sink for cortisol, as cortisol can readily pass from the blood into the milk in lactating mammals (Bremel and Gangwer, 1978; Termeulen et al., 1981). Verkerk et al. (1998) report rapid changes in cortisol concentrations in the composite milk of dairy cows that coincide with changes in plasma cortisol concentrations. Equilibrium between plasma and alveolar milk can be re-established within one hour of peak cortisol concentrations (Verkerk et al., 1998). A close relationship between average cortisol concentrations in blood and milk has been established (Bremel and Gangwer, 1978). It is possible that milk may be acting as a sink for cortisol in lactating mammals. Consider a beef cow producing 10 liters of milk per day with a blood volume of 48 liters (Parkinson et al., 2010). Assume a plasma cortisol concentration of 40 ng/mL (Tilbrook et al., 2006; Ralph and Tilbrook, 2016; Chapter Two) and a milk cortisol concentration of 10 ng/mL (Bremel and Gangwer, 1978). In this scenario, milk cortisol represents roughly 5% of the total cortisol present in the system. However, in this scenario I am assuming concentrations of milk cortisol in beef cows are similar to milk cortisol concentrations in

dairy cows (Bremel and Gangwer, 1978) because, to the authors' knowledge, there are no reports of milk cortisol concentrations in beef cows. It is possible that the smaller volume of milk produced by beef cows results in greater or lesser concentrations of cortisol in the milk, ultimately changing the proportion of total cortisol present in the milk. To the authors' knowledge, this has not yet been fully explored.

In addition, type of stressor can affect lactational attenuation of the HPA axis (Ralph and Tilbrook, 2016). Cook (1997) used an acute psychological stressor and reported no differences between lactating and non-lactating ewes in attenuation of cortisol. In comparison, Ralph and Tilbrook (2016) used a restraint and isolation stress paradigm similar to the present study and report a greater attenuation of plasma cortisol concentrations in lactating sheep compared with non-lactating sheep. The present study did not find a pattern over time to suggest differences in stress attenuation between lactating and non-lactating cows; however, lactating cows had overall lesser cortisol concentrations compared with non-lactating cows. Hormones other than oxytocin, such as prolactin as discussed previously, may be involved in stress attenuation during lactation. Oxytocin, prolactin, and the combination of oxytocin and prolactin suppressed cortisol in a previous study (Cook, 1997).

Intra-nasal oxytocin

Intra-nasally provided oxytocin has been shown to attenuate the HPA axis activation and non-human primates (Parker et al., 2005), while yielding no detectable differences in cattle (Chapter Two). Increases in plasma oxytocin concentrations resulting from intra-nasal administration did not affect cortisol concentrations in lactating or non-lactating cows, supporting previous finding in heifers (Chapter Two). Contradictory

results across studies may be attributable to differences in sampling frequency or stressor differences.

Differences in sampling frequency may explain inconsistencies across studies in the effectiveness of intra-nasal oxytocin to attenuate the HPA axis. Parker et al. (2005) intra-nasally administered oxytocin and applied restraint for 75 minutes. In this time frame, samples were only collected at 15 and 75 minutes into stress exposure and it was concluded that ACTH concentrations are attenuated in animals that received oxytocin intra-nasally (Parker et al., 2005). However, there may have been an effect of time which would be difficult to investigate with only two sampling timepoints. It is equally likely that concentrations of ACTH were increasing or decreasing prior to the 75-minute sample, of which I cannot know. In contrast, in Chapter Two of this dissertation I collected samples every ten minutes for the duration of the two-hour stress exposure. As with the present study, no effect of intra-nasal oxytocin was detected (Chapter Two). Frequent blood collection is necessary for accurate assessment of the HPA axis.

Furthermore, the stress response, and specifically attenuation during lactation, is stressor specific (Ralph and Tilbrook, 2016). Greater cortisol concentrations have been reported in ewes exposed to stressors in which their offspring may be threatened (Cook, 1997; Ralph and Tilbrook, 2016). In the present study, the stressor imposed was mild restraint and no threat to the calf was posed. A more psychological stressor may have brought forth different results and a potential action of oxytocin. The results of the present study however, are in support of earlier published studies that state oxytocin infusion has no detectable effects on plasma cortisol concentrations (Cook, 1997; Chapter Two).

CONCLUSION

Overall, intra-nasal oxytocin did not attenuate the HPA axis in lactating or non-lactating cows. Additionally, lactating and non-lactating cows had similar circulating oxytocin concentrations, while non-lactating cows had greater circulating cortisol concentrations. Hormones other than oxytocin may be involved in the attenuation of cortisol concentrations observed in lactating ruminants. Furthermore, interpretation of the effects of oxytocin remain dependent upon the occurrence of suckling, species, sampling frequency, and stressor.

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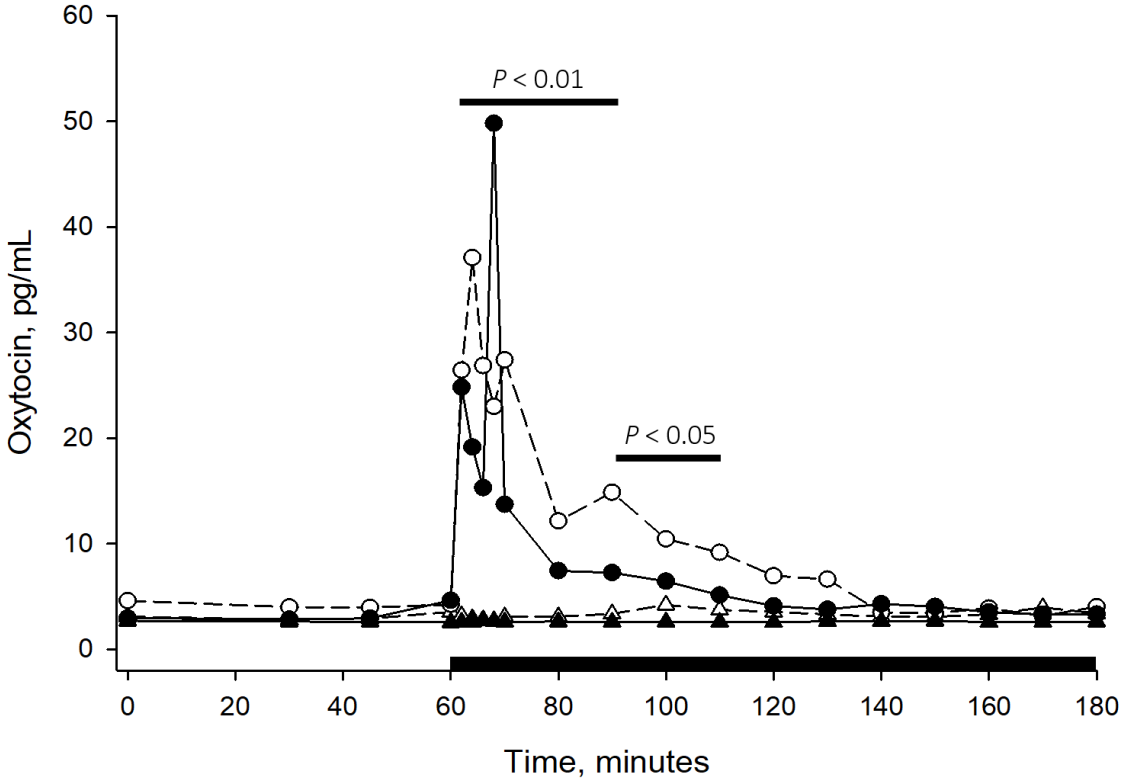

Figure 12. Back transformed mean plasma oxytocin concentrations in *Bos taurus* cows assigned to one of the following treatments: **1)** Non-lactating, saline (NL-S, – – Δ – –; n = 5), **2)** Non-lactating, oxytocin (NL-OXT, – – ○ – –; n = 5); **3)** Lactating, saline (L-S, ——▲——; n = 5); and **4)** Lactating, oxytocin (L-OXT, ——●——; n = 3). Intra-nasal treatments (S or OXT) were administered at time 60 and head restraint was applied for two hours (indicated by the black bar). Oxytocin was administered intra-nasally at a rate of 0.60 IU/kg bodyweight. A treatment by time interaction was detected from 60 to 90 minutes ($P < 0.01$) and from 90 to 110 minutes ($P < 0.05$).

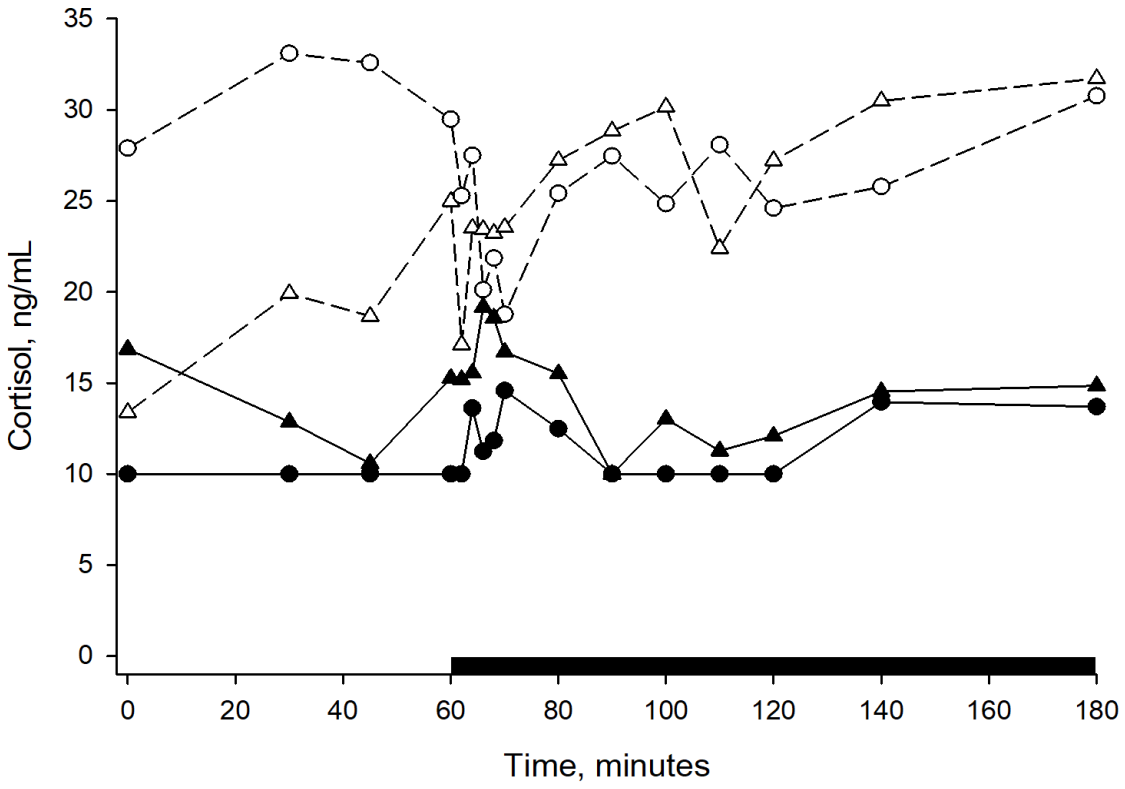

Figure 13. Mean plasma cortisol concentrations in *Bos taurus* cows assigned to one of the following treatments: **1)** Non-lactating, saline (NL-S, – – Δ – –; n = 5), **2)** Non-lactating, oxytocin (NL-OXT, – – ○ – –; n = 5); **3)** Lactating, saline (L-S, ——▲——; n = 5); and **4)** Lactating, oxytocin (L-OXT, ——●——; n = 3). Intra-nasal treatments (S or OXT) were administered at time 60 and head restraint was applied for two hours (indicated by the black bar). Oxytocin was administered intra-nasally at a rate of 0.60 IU/kg bodyweight. The pooled SEM was 5.6. Only an effect of lactation (P = 0.02) was detected (see Figure 14).

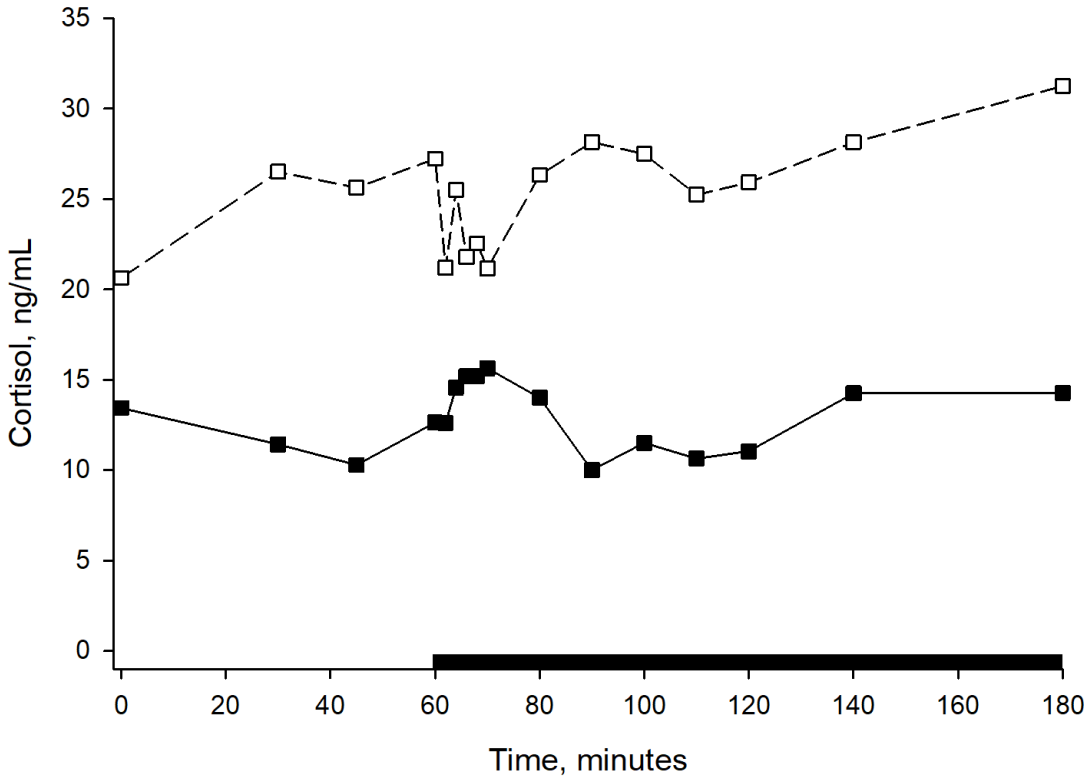

Figure 14. Mean plasma cortisol concentrations in non-lactating (NL, – – □ – –; n = 10) or lactating (L, ——■——; n = 8) *Bos taurus* cows. Time and duration of head restraint is indicated by the black bar. The pooled SEM was 4.0. An effect of lactation ($P = 0.02$) was detected.

CHAPTER SIX: GENERAL DISCUSSION

The present work sought out to investigate the effects of intra-nasal oxytocin as a potential attenuator of stress in beef cattle. Oxytocin appears to have a role in the regulation of the HPA axis in a number of species and peripheral administration of oxytocin attenuates the HPA axis in mice and humans. In addition, oxytocin is a major hormone associated with lactation which is considered to be a stress hypo-responsive state in mammals. Lactating ewes have been shown to have greater concentrations of oxytocin paired with lesser cortisol concentrations when faced with various stressors. Similar to sheep, beef cattle face neurogenic, behavioral and physical stressors throughout their life that vary in intensity and affect overall animal productivity. Prior to the studies presented in this dissertation, there was no information available on how oxytocin may interact with the HPA axis in beef cattle. It was hypothesized that the administration of intra-nasal oxytocin may be a novel strategy to attenuate the HPA axis response in beef cattle exposed to various stressors.

The first study, presented in Chapter Two, confirmed that restraint and isolation stress does activate the HPA axis in *Bos taurus* heifers. Furthermore, supplementing oxytocin to heifers did not affect the HPA axis in restrained and isolated animals or isolated animals. Following previously published protocols, I waited thirty minutes following treatment with intra-nasal oxytocin to begin stress treatments. Delayed stressor application and blood collection may have concealed any effect of oxytocin on the HPA axis. I also used a similar dose rate to that which has been reported in humans. Different species, such as beef cattle, may require a different dose of intra-nasal oxytocin to produce detectable differences in the response of the HPA axis.

In addition, multiple successive stressors (i.e. catheterization, isolation during the waiting period, and intra-nasal administration) could impact results. However, previous experience has shown that waiting two hours after catheterization allows the system adequate time to return to baseline concentrations of cortisol. Taking a sample directly before stressor application would have strengthened this study, likely indicating that cortisol concentrations had returned to baseline. Furthermore, the restraint and isolation stress model used may have been too extreme, obscuring any effect of intra-nasal oxytocin. A more intermediate stress model may have better allowed for detection of the effects of oxytocin on the HPA axis. The next two studies set out to answer these queries.

In the second study, presented in Chapter Three, transportation was used as an intermediate stress model. In addition to attenuating activation of the HPA axis, oxytocin has also been shown to exert protective effects during immunologic disturbances. Transportation both activates the HPA axis and alters immune function in cattle. I aimed to evaluate if providing oxytocin intra-nasally would attenuate the increase in cortisol observed with short-duration transport in *Bos indicus* steers. Cortisol concentrations were not different between transported and non-transported steers, suggesting that the cattle were not especially stressed by the short-duration road transportation. It is possible that significantly greater cortisol concentrations were reached at some point during transportation, but lack of continuous sampling makes this impossible to elucidate from the present study. Furthermore, the stressor may have been too mild in these steers, as they were highly acclimated to handling procedures.

Additionally, leukocytes were evaluated to determine if oxytocin would alter specific immune cells. This study established that oxytocin did have an effect on specific

leukocytes in steers subjected to short-duration transport. Data in basophils and lymphocytes suggest a protective effect provided by intra-nasal oxytocin supplementation. Different results may be seen in *Bos taurus* cattle, as the lipid status and breed differences play a role in the immune response.

Overall, six-hour road transport induced some signs of an acute inflammatory response directly following transportation, however no effect on the HPA axis was detected with changes in cortisol concentrations. The short half-life of oxytocin or mild nature of short-duration transport may explain lack of effects of intra-nasal oxytocin on the HPA axis.

In the third study, presented in Chapter Four, I set out to identify the pharmacokinetic parameters and a specific dose-response curve for intra-nasal oxytocin in cattle. Defining a dose-response curve was necessary for determining optimum sampling and dosing timelines for any future investigation on the effects of intra-nasal oxytocin in beef cattle. This study adds to the limited existing knowledge regarding appropriate doses and sampling timelines necessary to obtain meaningful results. In addition, an intermediate half-life of 12.1 minutes in plasma was established for intra-nasal administration of oxytocin in *Bos taurus* steers. More immediate application of the stressor following intra-nasal oxytocin administration and continuous sampling may have been necessary in the previous studies to see an effect of oxytocin.

In the final study, presented in Chapter Five, the effect of intra-nasal oxytocin was investigated in lactating and non-lactating *Bos taurus* cows. Contrary to our hypothesis, lactating and non-lactating cows had similar plasma concentrations of oxytocin. However, cortisol concentrations were attenuated in lactating cows compared with non-lactating

cows. Intra-nasal oxytocin did not have an effect on the HPA axis. It is possible that hormones other than oxytocin, such as prolactin, may be involved in facilitating the stress hypo-responsive state observed in lactating mammals. In addition, milk may be acting as a sink for cortisol. Furthermore, basal oxytocin concentrations and stress attenuation can be affected by suckling, species, and stressor. To fully explain lactational hypo-responsiveness, the role of prolactin and the effects of offspring presence must be investigated more thoroughly.

Future research involving the use of intra-nasal oxytocin in cattle should avoid only measuring parameters of the HPA axis. Previous reports in other species indicate oxytocin may be having an effect in the brain instead at the adrenal level. In addition, effects of oxytocin, independent of where it is having an effect, may manifest themselves in behavioral changes. Measurement of the potential effects of oxytocin on behavior in cattle is missing from the present work. Furthermore, previous reports suggest that social support and oxytocin may work together to alter physiological indicators of stress. It is possible that an attenuating effect on cortisol concentrations may have been observed if animals were not isolated from herd mates as in Chapters Two, Four, and Five.

In summary, the present work does not support intra-nasal oxytocin as an attenuator of the HPA axis in beef cattle subjected to restraint and isolation or transportation. The quick disappearance of oxytocin from plasma following intra-nasal administration, confirmed by experiments presented in Chapters Four and Five, indicates that any action of oxytocin is most likely not at the adrenal level; however, more investigations would be needed to confirm this notion.

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