Effects of feed additives on uterine morphology and selected reproductive attributes

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The influence of chromium supplementation on aspects of reproduction and lactation in early postpartum dairy cattle

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ABSTRACT

Dairy cattle characteristically exhibit decreased milk yield and reproductive performance, as well as increased uterine infection rates during periods of high stress. Chromium supplementation has demonstrated efficacy as a feed additive capable of reducing the detrimental effects of stress. As such, its application in dairy production may help to alleviate economic losses associated with seasonal heat stress and the stress experienced during the early postpartum period. Therefore, the objective of the work described in this thesis was to evaluate the potential benefits of short-duration, high-dose chromium (Cr) supplementation in early postpartum dairy cows during the summer months. Multiparous, early postpartum cows (20.95 ± 0.21 DIM, 658.29 ± 13.61 kg) were assigned to one of two treatment groups: 1) normal TMR (Con; n=10) and 2) normal TMR + Cr propionate supplementation (CrPro; 12 mg/h/d Cr; n=12). Body weight (BW), milk yield, and feed intake were measured each day of the experiment. Ambient temperature and humidity were monitored, and the temperature-humidity index (THI) was calculated for the duration of the study as an indicator of the severity of the heat stress experienced by the cows. Transrectal ultrasonography was performed every three days to assess ovarian follicular and luteal dynamics. Respiration rates (RR), rectal temperatures (RT), and blood glucose were recorded concurrently with ovarian ultrasonography. Plasma was collected and used for analysis of progesterone concentrations. Every six days in conjunction with ultrasonography, endometrial cytology samples were collected via cytobrush from each cow to determine the incidences of subclinical endometritis (SCE), as determined by polymorphonuclear leukocyte (PMNL) %. There were no treatment-based differences in RR, RT, blood glucose, feed intake, milk yield, or BW. However, the supplementation strategy did improve reproductive parameters. Within the Con group, there was an increase in PMNL % between samples five and six. Furthermore, at cytology sample six, the Con group had a greater percentage of PMNL than the CrPro group (P=0.01). Chromium consumption did not affect the counts or sizes of most follicles, with the exception being the 6-9 mm category where the CrPro group had a greater average diameter and tended to have more follicles in this category. While CL numbers or size did not differ between treatments, the ratio of progesterone (P_4) to corpus luteum (CL) volume was greater in the CrPro group compared to the Con group (P=0.03). The results from this study indicate that the proposed supplementation strategy does not influence DMI or milk yield in cows experiencing stress. Nonetheless, short-duration, high-dose Cr supplementation strategy could benefit reproductive performance and thereby limit economic losses experienced by dairy producers during periods of stress.

The influence of chromium supplementation on aspects of reproduction and lactation in early postpartum dairy cattle

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GENERAL AUDIENCE ABSTRACT

Dairy producers continue to experience monetary losses due to the decrease in production performance by dairy cows resulting from physiological changes in response to stress. Elevated ambient temperature and humidity conditions can lead to heat stress, which has been found to decrease both milk and reproductive performance on dairy operations. The period after calving, known as the postpartum period, can also lead to metabolic changes in a cow due to the stress of giving birth and beginning to produce milk. Chromium is an essential trace mineral that can be supplemented with cattle feed to improve the impacts of such stressful periods on production parameters. This study evaluated the effect of a short-duration, high-dose chromium supplementation strategy on reproduction and lactation of early postpartum dairy cattle during summer months. Twenty-two cows were assigned to two treatment groups: control (standard feed only) and chromium supplement (standard feed plus chromium propionate supplementation). Cows receiving the chromium supplement did not experience any changes in respiration rate, rectal temperature, blood glucose levels, feed intake, milk yield, or body weight. Reproductive analyses determined that cows receiving the chromium supplementation had a lower number of immune cells present in the uterus by the end of the experiment, which may be indicative of a healthier uterine environment. Chromium supplemented cows also had a greater level of progesterone

concentration to corpus luteum volume compared to cows in the control group. These results indicate that the short-duration, high-dose chromium supplementation strategy could benefit reproductive performance and limit losses experienced by dairy cows under stress conditions.

DEDICATION

I would like to dedicate this thesis to my wonderful family for always encouraging me, sharing my excitement over accomplishments and daily victories, and endless love and support throughout this journey. And to the wonderful Holstein ladies, especially Thelma, who made this the experience of a lifetime.

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LIST OF ABBREVIATIONS

AI: Artificial Insemination

BW: Body Weight

CL: Corpus Luteum

Con: Control

Cr: Chromium

CrPro: Chromium Propionate

DIM: Days in Milk

DMI: Dry Matter Intake

E_2: Estradiol

FSH: Follicle Stimulating Hormone

GnRH: Gonadotropin Releasing Hormone

HPO: Hypothalamic-Pituitary-Ovarian

LH: Luteinizing Hormone

NEB: Negative Energy Balance

NEFA: Non-Esterified Fatty Acid

P_4: Progesterone

PGE_2: Prostaglandin E_2

$PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$

PMNL: Polymorphonuclear Leukocyte

RR: Respiration rate

RT: Rectal Temperature

SCE: Subclinical Endometritis

$T_{\text{dew point}}$: Dew Point Temperature

$T_{\text{dry bulb}}$: Dry Bulb Temperature

THI: Temperature-Humidity Index

CHAPTER 1: Review of Literature

Introduction

Across the dairy industry, millions of dollars are lost each year from a combination of reproductive and lactational losses due to various forms of stress. Heat stress and the challenges associated with the postpartum period are two stress conditions that can impact production performance on dairy operations. With seasonal temperatures increasing as a result of global warming, and the ever-present challenges associated with the postpartum period (as it encompasses the change from late pregnancy to early lactation), it is imperative to understand the mechanisms behind stress-induced physiological responses in order to ameliorate their detrimental effects. Thus, there continues to be a necessity for research aimed at elucidating effective management solutions that help to sustain dairy production during both seasonal heat stress and the postpartum period.

A myriad of dairy production parameters are affected by heat stress conditions. Changes in follicular development, endocrine regulation, and the uterine environment can all contribute to reduced reproductive performance, while milk production declines beyond the amount that can be explained by reduced feed intake. These physiological heat stress responses can become particularly exacerbated during the early postpartum period in dairy cattle. The animal's transition from pregnancy to milk production results in increased energy requirements that often exceed the cow's capacity for energy intake. This can cause and/or be concurrent with opportunities for metabolic and infectious diseases (such as ketosis, hypocalcemia, displaced abomasum, mastitis, endometritis, etc.). Successful transition into lactation is fraught with obstacles, even in optimum ambient temperatures. When this transition occurs in combination with the external stress of heat conditions, long-lasting effects on production affect the efficiency and profitability of dairy production.

Knowledge of both heat stress and the postpartum period, and their impacts on reproductive and lactational performance, is continuously developing. In order to make meaningful contributions to this field of research, it is important to understand previously completed investigations. This literature review will begin with general descriptions of the estrous cycle and reproductive system. Subsequent sections will describe heat stress and its impacts on various dairy production parameters, including specific information on the postpartum uterine immune response, and its potential improvement with feed supplementation (specifically, chromium). Together, this literature review will provide the base knowledge needed to fully understand the implications of the subsequent research.

The Estrous Cycle

Overview

The estrous cycle is a repetitive cycle of events involving multiple hormones and their actions on a variety of reproductive structures and organs. Consisting of two phases, divided into 4 stages, each part of the estrous cycle plays a determining role in the success of pregnancy or resumption of a new cycle. Cows have an estrous cycle length averaging 21 days, with individual lengths dependent upon the animal. The follicular phase is composed of both the proestrus and estrus stages, while the luteal phase consists of both the metestrus and diestrus stages. Proestrus is the short period of time between days 18 and 20, where the corpus luteum (CL) has begun to regress, a large follicle is present, and the reproductive tract is preparing for pregnancy. Estrus quickly follows this stage and is classified as the period from the beginning of behavioral estrus until ovulation occurs. This stage is typically four days in length and considered the transition from one cycle to the next. Metestrus follows the estrus stage and is the period during which cells from the previously ruptured follicle transform into a CL. Metestrus is the second longest phase in the estrous cycle, averaging around six days in length. The last stage is diestrus, with a typical duration of seven days, encompassing days 11 through 17 of the estrous cycle. During this period, the CL is fully functioning, producing progesterone (P_4) to support a potential pregnancy. However, if pregnancy is not established and/or maintained, the cow will regress the CL and begin a new estrous cycle (Marshall & Ewart, 1903; Ireland et al., 1980).

The Hypothalamic-Pituitary-Ovarian Axis

The estrous cycle is regulated through the interactions between the hypothalamus, pituitary gland, and ovaries, also known as the hypothalamic-pituitary-ovarian (HPO) axis. Hormones produced by structures within the HPO axis can control the production of other hormones through positive or negative feedback and/or elicit a direct response from a reproductive structure.

The hypothalamus is a portion of the brain within which the neurons responsible for production of gonadotropin releasing hormone (GnRH) can be found (Nett et al., 1987). Gonadotropin releasing hormone is the hormone responsible for stimulating the production of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland. For this to occur, GnRH is released in a pulsatile manner from the hypothalamus to then travel into and through the median eminence (Nett et al., 1987; Clarke & Cummins, 1982; Mori et al., 1991). Within the median eminence lies the hypothalamo-hypophyseal portal system that transports GnRH to the gonadotroph cells, the cells responsible for gonadotropin production (Walters & Schallenberger, 1984; Clarke & Cummins, 1982; Schally et al., 1971; Ohkura et al., 2009).

Within the hypothalamus lies two portions capable of controlling GnRH secretion: the tonic center and the surge center. During the luteal phase, when P_4 concentrations are greater, P_4 alters pulsatile secretion of GnRH, causing pulses to be less frequent with greater amplitude. During this time, estradiol (E_2) can also act as a negative feedback signal to decrease GnRH concentrations in an effort to suppress downstream FSH production (Cox & Britt, 1982; Francis Pau et al., 1986). However, at the proper stage of the cycle, when luteolysis occurs and P_4 concentrations decline, E_2 has a short period of positive feedback on GnRH production. This positive feedback mechanism occurs within the surge center of the hypothalamus, where E_2 can act on GnRH neurons to increase the frequency of GnRH secretion, allowing it to build into a GnRH surge. This surge of GnRH is then able to travel to the pituitary and cause the surge in LH (Hauger et al., 1977; Moenter et al., 1992a).

The pituitary gland is composed of two portions: the adenohypophysis and neurohypophysis. The adenohypophysis, also known as the anterior pituitary, is the main portion involved in estrous cycle regulation through the HPO axis. The anterior pituitary, as previously mentioned, is responsible for synthesis of the gonadotropins LH and FSH, which act on the ovaries. Gonadotropin releasing hormone and E_2 receptors located within the anterior pituitary regulate LH and FSH secretion depending on the stage of the estrous cycle (Nett et al., 1987; Cox & Britt, 1982).

The main function of LH is to cause ovulation through the LH surge that occurs during estrus. Due to the pulsatile manner of GnRH secretion, and synchronicity between GnRH and LH, LH is also produced in pulses. As GnRH pulses increase and extend over time, LH will also increase and prolong production leading to the LH surge and ovulation of the oocyte from the ovulatory follicle. Alternatively, a decrease in GnRH and subsequent LH can lead to an anovulatory state (Clarke & Cummins, 1982; Mori et al., 1991; Moenter et al., 1992b; Savio et al., 1988; Rahe et al., 1980; Ciechanowska et al., 2008). The LH produced by the pituitary can be measured in peripheral circulation and continue to be detected throughout the body for up to four hours post LH surge (Hilliard et al., 1964; Clarke & Cummins, 1982). Once the LH surge has occurred, it is postulated that there is a decrease in the adenohypophysis's response to GnRH so that LH and FSH pulse amplitude and frequency can decrease (Walters & Schallenberger, 1984). Post LH surge, the number of GnRH receptors decrease to help bring LH back to a basal concentration (Nett et al., 1987).

Luteinizing hormone (as well as FSH) is also important for follicular growth and development. Reduced concentrations of LH lead to the turnover of the dominant follicle prior to the LH surge, while increases in FSH initiate development of a new follicular wave (Savio et al., 1993a; Ginther et al., 2001; McNatty et al., 1981; Savio et al., 1988; Adams et al., 1992). The importance of both gonadotropins for follicle development is demonstrated in the two-cell two-gonadotropin model for E_2 production. Luteinizing hormone binds to its receptors on the theca cells of the dominant follicle to convert pregnenolone to androstenedione through the delta5 pathway (Fortune & Armstrong, 1977; Henderson et al., 1984; Fay & Douglas, 1987). This androstenedione travels to the granulosa cells of the dominant follicle where aromatase can convert it to E_2 upon FSH receptor binding on the granulosa cells (pre-selection) (Fortune & Armstrong, 1977; Dorrington et al., 1975). Estradiol production at the proper times in the proper amounts is critical for maintenance of estrous cyclicity. Its modes of action are too numerous to describe in depth in this literature review, but it is perhaps best known for inducing estrus behavior, signaling the hypothalamus for ovulation, and affecting the histoarchitecture and function of reproductive tissues.

Follicular Development

During the estrous cycle, follicular development occurs in waves, with a new wave initiated following the atresia or ovulation of the previous dominant follicle (Merz et al., 1981; Dufour et al., 1972; Lucy et al., 1992). A follicular wave consists of recruitment of a cohort of follicles from a follicular pool, selection of a follicle for dominance and final growth of a dominant follicle that will either undergo atresia or go on to ovulate (Sirois & Fortune, 1988; Sunderland et al., 1994; Austin et al., 2001). The onset of a follicular wave is dependent upon a transient rise in FSH to promote follicular growth, the first of which occurs quickly after the LH surge from the previous ovulation (Adams et al., 1992; Badinga et al., 1992; Sunderland et al., 1994; Crowe et al., 1997; Fricke et al., 1997; Evans et al., 2002). It is the pool of smaller developing follicles that have high numbers of FSH receptors, allowing them to grow in response to FSH, which binds throughout recruitment (Fay & Douglas, 1987). During the next phase of follicular growth (selection), a dominant follicle deviates from the second largest follicle as LH concentrations increase (Bergfelt et al., 2001). As the dominant follicle continues to grow, it becomes less sensitive to FSH concentrations, and relies more on LH concentrations to increase E_2 and inhibin production. These products of the putative dominant follicle decrease FSH availability, thereby inhibiting the growth of the smaller follicles that still depend on FSH (Adams et al., 1992; Ginther et al., 2001; Sunderland et al., 1994; Donadeu & Ginther, 2001; Baird et al., 1981). Ultimately, the dominant follicle outcompetes all other follicles of the cohort due to an increase in LH receptors on the granulosa cells, enabling the dominant follicle to use LH to promote its own growth and development (Sunderland et al., 1994). Upon atresia or ovulation of the dominant follicle, inhibin concentrations decrease, resulting in an increase of FSH which can stimulate growth of the next cohort of follicles (Wrathall et al., 1990; Rivier et al., 1991; Martin et al., 1988; Kaneko et al., 1997).

While some studies have determined that the majority of cows experience two follicular waves (up to 90%), others have found that three follicular waves are more common (up to 70%; Bleach et al., 2004; Knopf et al., 1989; Savio et al., 1988; Sirois & Fortune, 1988; Ginther et al., 1989). When two follicular waves occur, the cohort of follicles emerge at day zero and day ten of the estrous cycle. On the other hand, in three follicular waves the first dominant follicle emerges around day 2 while the second dominant follicle emerges around day 9. The third dominant follicle emerges around day 16 with a greater growth rate compared to those of the first and second dominant follicles, with the second dominant follicle having the slowest growth rate due to high P_4 production during the luteal phase (Knopf et al., 1989; Taylor & Rajamahendran, 2011; Savio et al., 1988; Sirois & Fortune, 1988). By day 18 of the cycle, the largest follicle present is most likely the one that will become the ovulatory follicle (Dufour et al., 1972). Once the CL goes through luteolysis, E_2 production from the dominant follicle can positively impact LH leading to ovulation (Savio et al., 1988; Savio et al., 1993b; Savio et al., 1993a; Adams et al., 1992; Badinga et al., 1992; Merz et al., 1981).

Post LH surge and prior to ovulation, the granulosa cells of the dominant follicle will switch from aromatizing production of E_2 to the production of P_4. This shift is associated with the beginning of follicular cell luteinization for subsequent formation of the CL post ovulation (Dieleman & Blankenstein, 1985; Henderson & Moon, 1979). The granulosa cells and theca cells of the dominant follicle will become large luteal cells and small luteal cells, respectively. As the CL continues to grow and develop, the small luteal cells can develop into large luteal cells, which are the main source of P_4 production (Alila & Hansel, 1984). Progesterone is absolutely necessary for the maintenance of pregnancy, and an untimely decrease in P_4 production can lead to pregnancy loss (Grygar et al., 1997; Heap et al., 1976; Mann & Lamming, 1995). As the P_4 concentration increases with luteal development, it will suppress LH production and reduce E_2 production from any dominant follicle present, thereby aiding in dominant follicle turnover (Henderson & Moon, 1979; Badinga et al., 1992; Savio et al., 1993a; Ginther et al., 2001; Kesner et al., 1982). A CL reaches maximum size at about day 8 of the estrous cycle and achieves maximum P_4 production around day 13 of the cycle before P_4 concentrations rapidly decrease around day 15 (Mann, 2009; Taylor & Rajamahendran, 2011; Bjersing et al., 1972).

The CL continues to produce P_4 until it is regressed by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterus. While not technically included in the HPO axis, the uterus plays a vital role in estrous cyclicity through regulation of luteal lifespan. Uterine epithelium produces and secretes $PGF_{2\alpha}$ in response to an increase in oxytocin receptors (Lewis & Warren, 1977; Baird et al., 1981; Robinson et al., 1999; Flint & Sheldrick, 1983; Hansel & Seifart, 1967). Estradiol from the developing dominant follicle participates in this process as it upregulates endometrial oxytocin receptors, thereby further increasing $PGF_{2\alpha}$ production (Beard & Lamming, 1994; Beard et al., 1994; Robinson et al., 1999; Flint & Sheldrick, 1983). As a luteolytic hormone, $PGF_{2\alpha}$ acts on its receptors on large luteal cells to induce CL regression. As a result, P_4 production is drastically reduced, thereby allowing the cow to enter estrus and the new dominant follicle to ovulate (Robinson et al., 1999), thus providing a new opportunity for the animal to become pregnant.

Endometritis in Cattle

Overview

The uterus, comprised of a small body and two large horns, is a major part of the reproductive tract in the cow and is the location where the fetus grows and develops. It is made up of three layers: perimetrium, myometrium, and endometrium. The perimetrium is made up of connective tissue surrounded by mesothelium, while the myometrium consists of both circular and longitudinal smooth muscle. The endometrium is comprised of a basal layer and functional layer containing columnar epithelial cells and uterine glands responsible for secreting uterine histotrophs, which are essential for preimplantation development of the embryo (Thasmi et al., 2018). It is this layer of the uterus that is highly susceptible to histological and pathological changes throughout the estrous cycle during high stress periods for the cow. While metritis is an infection encompassing all of the walls of the uterus, these changes in the endometrial layer, can lead to endometritis, which is defined as inflammation of the endometrium (Sheldon et al., 2006). Endometritis is an extremely common infection diagnosed in postpartum cattle. Bartlett and colleagues (1986) reported an average of $106.00 lost for each cow diagnosed, due to decreases in reproductive and lactational performance, cost of treatment, and increased risk of culling.

Endometritis can be separated into three categories based on its severity and time of incidence: puerperal metritis, subclinical endometritis, and clinical endometritis. Puerperal metritis is an acute form of the uterine bacterial infection that typically occurs within the first three weeks postpartum and is characterized by red-brown uterine discharge, an enlarged uterus, and elevated body temperature. Clinical endometritis, also diagnosed after three weeks postpartum, is the easiest to observe, as it is characterized by purulent (more than 50% pus) or mucopurulent (a 50/50 split of pus and mucus) discharge, or through the presence of uterine fluid upon ultrasonography (Sheldon et al., 2006; Gobikrushanth et al., 2016). Subclinical endometritis (SCE) is when the uterine infection causes inflammation but no other clinical signs, such as uterine discharge, after three weeks postpartum. Therefore, diagnoses are most commonly made through endometrial cytology to determine the amount of polymorphonuclear leukocytes, PMNL (Kasimanickam et al., 2004; Sheldon et al., 2006).

When analyzing endometrial cytology, the main cell type being counted is neutrophils, also known as polymorphonuclear leukocytes (PMNL). This form of white blood cell is considered the uterus's first line of defense, with the infection increasing overall white blood cell presence as well (Hammon et al., 2006; Kim et al., 2005). The prevalence of neutrophils (measured as a percentage of cells) determines whether a cow is diagnosed with SCE, with the number of neutrophils needed for a positive diagnosis changing over lactation. At 40-60 days postpartum, over 5% PMNLs in the cell count indicates infection, while it is 10% between 34-47 days postpartum, and 18% between 21-33 days postpartum (Gilbert et al., 2005; Sheldon et al., 2006). If endometritis continues past 60 days postpartum, it is then considered persistent endometritis, with around 25% of those originally infected continuing on to become persistent (Gautam et al., 2010).

Following parturition, there is a loss in compartmentalization between the uterus and vagina that allows for the microbial colonies to mix. This postponed distinction between the vaginal microbiome and uterine microbiome has been associated with the occurrence of postpartum endometritis. With similar microbiomes between the uterus and vagina in the first week postpartum, there can be increased infections (Miranda-CasoLuengo et al., 2019). Studies have examined the specific pathogenesis of endometritis in its different forms, and it is now believed that SCE is not simply a milder form of clinical endometritis, but rather caused by its own set of bacteria, more specifically an increase in Lactobacillus and Acinetobacter (Galvão et al., 2010; Wang et al., 2018; Dubuc et al., 2010). The most common bacteria found in endometriotic cattle are Arcanobacterium pyogenes, Escherichia coli, Fusobacterium necrophorum, and Prevotella melaninogenicus, where A. pyogenes and E. coli are the most prevalent in clinical endometritis (Sheldon & Dobson, 2004; Brick et al., 2012). However, others believe that the difference between SCE and clinical endometritis is more so related to the number of bacteria present rather than the type of bacteria (Sheldon et al., 2002). No matter the differences, each form of endometritis can negatively impact a cow's overall productivity.

Prevalence Factors

Endometritis can be found in any cattle herd, although the rate of infection can vary widely, typically ranging from 18% - 52% (Cheong et al., 2011; Stevenson, 1987; Bartlett et al., 1986; Markusfeld, 1984; Hammon et al., 2001; Carneiro et al., 2014; Ahmadi et al., 2019; Heuwieser et al., 2000). As previously mentioned, this infection is most common during the early postpartum period, and multiple factors can play a role in increasing or decreasing the incidence rate (Huzzey et al., 2007; Bartlett et al., 1986).

Cheong and others (2011) found that while primiparous and multiparous cows have similar rates of infection, that combinations of parity, milk production, and other diseases can increase rates of infection. Most (Cheong et al., 2011; Duffield et al., 2009; Dohoo & Martin, 1984; Correa et al., 1993), but not all (Cheong et al., 2011; Bruun et al., 2002), agree that high-producing cows, especially those that develop ketosis and those that experience other reproductive issues, are at a greater risk for developing endometritis. Lower body condition scores pre-calving and negative energy balance (NEB) also increase the likelihood of developing endometritis (Duffield et al., 2009; Carneiro et al., 2014). During NEB, concentrations of non-esterified fatty acids (NEFAs) and β-hydroxybutyric acids increase, which suppresses the immune system, therefore, increasing the rate of SCE or clinical endometritis (Hammon et al., 2006; Galvão et al., 2010; Wathes et al., 2009; Huzzey et al., 2007). In addition to these individual, production-based risk factors for endometritis, management and environmental conditions can also affect its incidence. For example, Cheong and colleagues (2011) found an association with type of bedding, where use of free stalls and straw bedding decrease the likelihood of SCE.

As previously mentioned, other reproductive issues that occur around the time of parturition can lead to the onset of endometritis within subsequent weeks. Stillbirth, gestation length, dystocia and assisted calving, retained placenta, hypocalcemia, displaced abomasum, and even birth of a large sized calf, typically male, are most of these key prevalence factors (Correa et al., 1993; Huzzey et al., 2007; Markusfeld, 1984; Kasimanickam et al., 2004; Whiteford & Sheldon, 2005; Bruun et al., 2002; Hossein-Zadeh & Ardalan, 2011; Salasel et al., 2010). Retained placenta, specifically, has been found by Huzzey and colleagues (2007) to drastically impact the severity of endometritis, with 58% of severe incidences and 7% of mild incidences having had a retained placenta. Summer calving, parturition issues, and severity of endometritis early on are all factors that can lead to persistent endometritis; however, Gautam and others (2010) noted that there is around a 10% chance of a new incidence of infection later postpartum as well.

Diagnosing the Disease

Over the years, various methods have been used to diagnosis endometritis and its various forms in cattle. Depending on the study and degree of severity, endometrial cytology, ultrasonography, vaginoscopy, or palpation can be used as diagnostic tools. Endometrial cytology can be accomplished using a cytobrush or lavage technique, which both collect a representative population of cells for assessment (Kasimanickam et al., 2004). Ultrasonography can be used to determine the amount of fluid in the uterus, endometrial thickness, and cervical thickness, all potential indicators of the disease. While these have been used as diagnosing factors, the position of the probe and location being observed can influence the ultrasonography results, deeming it less accurate than other potential techniques (Barlund et al., 2008). Vaginoscopy is particularly useful for clinical endometritis diagnoses where there is significant purulent or mucopurulent discharge present (LeBlanc et al., 2002a). However, when cattle are experiencing SCE, vaginoscopy has been found to lack accuracy in its detection (Kasimanickam et al., 2004).

Endometrial cytology can be collected at multiple visits with much more accuracy compared to vaginoscopy. The cytobrush technique is more accurate than lavage cytology, most likely as a result of a decrease in cell integrity during the lavage process (Barlund et al., 2008). Considering the large impacts on reproductive performance (discussed below), the ability of endometrial cytology to diagnose the lesser symptomatic condition (SCE) is extremely important for early detection and treatment (Gilbert et al., 2005). Barlund and others (2008) determined that the use of cytobrush cytology had the highest repeatability as a reference diagnostic test in early postpartum cows with endometritis. Palpation is also a common method of identification of endometritis in production settings. However, in early lactation cattle, LeBlanc and others (2002a) found that vaginoscopy was able to identify 44% of the infected cattle alone compared to the lower sensitivity of detection by palpation. The use of a combination of diagnostic tools though, is the most beneficial way to truly identify any form of endometritis (Kasimanickam et al., 2004).

Reproductive Impacts

Endometritis can have significant impacts on reproductive performance in cattle that can become very costly for producers. The combination of decreased milk production and reproductive performance from endometritis can lead to increased culling rates by as much as 70% (Huzzey et al., 2007; Bartlett et al., 1986; Galvão et al., 2010; Gilbert et al., 2005; LeBlanc et al., 2002b).

Many studies have specifically examined the detrimental effects of endometritis on pregnancy rates and most agree that endometritis reduces fertility. Median days open increased by an average of 25 days in multiparous cows, and first service pregnancy was lower for cows with

SCE (Cheong et al., 2011; Gilbert et al., 2005; Dubuc et al., 2010; Barlund et al., 2008). Subclinical endometritis was also found to increase the number of inseminations required per pregnancy, with pregnancy rates per insemination reduced up to 60% (Gilbert et al., 2005; Rutigliano et al., 2008; Kasimanickam et al., 2004). Similar increases in time to pregnancy for cows infected with clinical endometritis could be attributed to the associated increase in cervical diameter and purulent discharge (LeBlanc et al., 2002b; Brick et al., 2012). First service conception rates decreased depending on the severity of endometritis present, and cows were 5.3 times less likely to become pregnant, and 2.16 times more likely to lose a pregnancy after the first service (Hammon et al., 2001; Machado et al., 2015). However, other studies have indicated no impact from SCE on reproductive performance including on first service conception rate (Plöntzke et al., 2010; Carneiro et al., 2014).

Previous studies have also specifically looked at the effects of endometritis on ovarian dynamics and oocyte competency. Some evidence indicates that endometritis can negatively impact dominant follicle growth and estradiol production, as well as corpus luteum (CL) size, progesterone (P_4) production, and delay cyclicity (Sheldon et al., 2002; Williams et al., 2007; López-Helguera et al., 2012). However, contradictory studies have shown no influence of endometritis on ovarian dynamics including changes in dominant follicle growth, follicle wave emergence, follicle stimulating hormone concentration, ovulation, or the proportion of ovular cows (Gobikrushanth et al., 2016; Sheldon et al., 2002). Previous studies evaluating reproductive effects after induction of endometritis have also established changes in oocyte competency. The infected uterine environment is associated with decreased embryo development following fertilization in vitro, as well as altered oocyte and granulosa cells transcriptomes, all responses capable of being detected months after the infection has subsided (Dickson et al., 2020; Piersanti et al., 2019;

Horlock et al., 2020). Due to the vast number of effects endometritis can have on reproductive performance, research on its impacts continues to be conducted.

Possible Solutions

With the often-times high incidence rate of endometritis across cattle farms, solutions continue to be developed. Some recent experiments have investigated the use of intramuscular injections of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) as a treatment for endometritis. While not directly affecting the incidence of SCE, $PGF_{2\alpha}$ did increase pregnancy rates and was more beneficial when given after four weeks postpartum (LeBlanc et al., 2002b; Galvão et al., 2009). In a herd with a 34% incidence of endometritis, $PGF_{2\alpha}$ treatment increased estrus detection and decreased days open and the time interval to the first breeding service (Heuwieser et al., 2000). These results are more likely due to the function of $PGF_{2\alpha}$ as an immunomodulator, rather than the actions of P_4 as an immune inhibitor (P_4 is reduced by $PGF_{2\alpha}$).

Prostaglandin $F_{2\alpha}$ has been found to stimulate the influx of neutrophils, while P_4 has been found to inhibit immune responses, thereby leading to increased incidences of infection (Lewis, 2003; Hansen et al., 1986). When P_4 levels exceed basal concentrations, immunosuppressive substances are secreted into the uterus (Hansen et al., 1986). Since $PGF_{2\alpha}$ is luteolytic in cattle, the associated reduction in CL production of P_4 has been identified as one mechanism by which prostaglandin stimulates the immune system. It has also been postulated that increased uterine contractility caused by $PGF_{2\alpha}$ aids in directly expelling infection from the uterus (Del Vecchio et al., 1994).

Uterine infections have also been associated with increases in lipopolysaccharides which increases the production of prostaglandin E_2 (PGE_2) as opposed to $PGF_{2\alpha}$. Prostaglandin E_2 acts luteotropically to prolong CL lifespan, thereby maintaining P_4 production (Herath et al., 2006;

Mateus et al., 2003). Therefore, these increased levels of PGE_2 could be counteracting the luteolytic function of $PGF_{2\alpha}$ to maintain P_4 concentrations at levels capable of having immunosuppressive abilities. For these reasons, $PGF_{2\alpha}$ injections are commonly used as a treatment option for endometritis (Galvão et al., 2009; Kasimanickam et al., 2005; Knutti et al., 2000; Heuwieser et al., 2000). While this treatment is not always effective (Galvão et al., 2009; Dubuc et al., 2010; LeBlanc et al., 2002b), it is a popular choice because it is a relatively inexpensive treatment that can be easily administered.

Antibiotics can also be administered as a treatment for endometritis. Antibiotics such as cephapirin and oxytetracycline are typically administered through intrauterine infusion, but their use is controversial. While the antibiotics have been found to improve reproductive performance in some instances (LeBlanc et al., 2002b; Ahmadi et al., 2019), others indicate no differences in cure rates (Makki et al., 2017). If ineffective, antibiotic administration should be avoided as a safeguard against the development of antibiotic-resistant strains of bacteria. The presence of these antibiotics in milk products is also of concern for producers and contributes to increased economic loss as this milk cannot be sold (Ahmadi et al., 2019; Dinsmore et al., 1996).

The disadvantages associated with antibiotic treatment of endometritis have prompted the development of non-antibiotic solutions such as dextrose or liquid paraffin. Unfortunately, the efficacy of these treatments is variable or non-existent. While one study determined that dextrose could be a beneficial alternative to antibiotic treatments and increase first service conception rate (Ahmadi et al., 2019), another found that dextrose decreased clinical endometritis incidence but had no impact on first service conception rate (Machado et al., 2015). Makki and colleagues (2017) evaluated the use of $PGF_{2\alpha}$, cephapirin, dextrose or liquid paraffin and determined that there was no cytological or clinical difference in cure rate. In search of another solution, Yasui and others

(2014) found that chromium-propionate supplementation could be one useful tool in reducing cytological endometritis in dairy cattle. The feed additive seemed to cause an early penetration of PMNL into the uterus after parturition that led to a decrease in cytological endometritis later in lactation. These results are promising and may be indicative of an effective preventative strategy that is inexpensive and easily administered.

Heat Stress

Overview

Heat stress is an escalating issue on dairy cattle operations around the world. Every year, the United States experiences an annual loss of around $897 million due to the heat stress impacts on dairy production parameters (St-Pierre et al., 2003). With the increase in global ambient temperatures over the years, heat stress is and will continue to be a problem for producers. Heat stress has been defined as the "sum of external forces on an animal that leads to a physiological response", including an increase in body temperature (Becker et al., 2020; Dikmen & Hansen, 2009; Bernabucci et al., 2014). Not only can heat stress increase rectal temperature (RT), but it can also increase respiration rate (RR), decrease feed intake, reduce productivity (lactation and reproduction), exacerbate negative energy balance (NEB), and decrease immune function (Min et al., 2015; Wilson et al., 1998b; Fabris et al., 2019; Roman-Ponce et al., 1978; Al-Katanani et al., 2002; Rhoads et al., 2009; Berman et al., 1985; Min et al., 2016). Ultimately, these effects alone, or in combination, increase culling rates (Brown et al., 2016).

Many have reported that multiparous, high-yielding, and early postpartum cows are more susceptible to the detrimental effects of heat stress (Müschner-Siemens et al., 2020). Heat stress conditions can be evaluated through the temperature-humidity index (THI) or by simply using ambient temperatures. The THI is advantageous because it takes into account the relative humidity.

The THI is calculated using one of several formulas, such as: THI = $T_{\text{dry bulb}} + ((0.36 * T_{\text{dew point}}) + 41.2)$ (Brown et al., 2016). In general, THI values at 68 or above are considered heat stress conditions for lactating dairy cattle (Zimbelman et al., 2009). Ambient temperatures between 25-29°C are considered the upper critical temperature for lactating dairy cows, while it is around 33°C for heifers; with both thresholds capable of increasing RR to as much as 80 breaths per minute (Berman et al., 1985; Garcia et al., 2015; Wilson et al., 1998a). The fact that a greater ambient temperature is required to cause the same responses in heifers as lactating cows is likely due to a lower metabolic rate and the associated internal heat production (Wilson et al., 1998a). While heifers are less susceptible than cows, they are not immune to the effects of heat stress, and effective management strategies are still needed for every animal in the industry (Davidson et al., 2021).

Feed Intake and Energy Balance

Along with decreasing milk production in cows, heat stress can also impact feed intake and metabolism. During the periparturient period when cows are transitioning into high milk production, the incidence of NEB increases as their energy requirements typically exceed the amount they are able to consume. When heat stress is added on top of this stressful period, maintenance requirements increase as much as 25% (due to efforts to dissipate heat), thereby exacerbating NEB (Wheelock et al., 2010; Min et al., 2015; Wilson et al., 1998b; NRC, 1989). The concomitant decrease in dry matter intake (DMI) can be as much as 28%, which further contributes to NEB (Baumgard et al., 2011; O'Brien et al., 2010; Wilson et al., 1998a). The change in feed intake can lead to a decrease in body weight and body condition score of the cows, which in turn, can negatively impact production performance (Cavestany et al., 1985; Seyed Almoosavi et al., 2021; Baumgard et al., 2011).

Many investigations have focused on heat-stress-induced changes in DMI and the resulting impacts on insulin and glucose metabolism. Studies using heat stress chambers found that glucose concentrations decrease in response to heat stress, most likely due to the reduction in DMI associated with heat stress (Rhoads et al., 2009; Shwartz et al., 2009). Similar studies also noted that heat stress typically increases insulin concentrations, which is contrary to the expected response since DMI is reduced by heat stress (Wheelock et al., 2010). In a related fashion, heat-stressed dairy cows do not mobilize non-esterified fatty acids (NEFAs) as would be expected during periods of reduced DMI (Cowley et al., 2015; Seyed Almoosavi et al., 2021). The absence of a NEFA response to heat stress is particularly surprising since it is known to increase circulating concentrations of catabolic hormones such as norepinephrine, epinephrine, and cortisol (Wheelock et al., 2010). Baumgard and others (2011) postulated that these unique changes in metabolic hormones and metabolites were indicative of a metabolic shift to prioritize the use of glucose by the peripheral tissues at the expense of milk synthesis. The mechanisms responsible and the implications of this unique metabolic status continue to be investigated.

Milk Production

Impacts of heat stress on milk production are easily observed on-farm and directly affect the profitability of dairy operations. The amount of milk lost to seasonal heat stress varies widely from farm to farm and is dependent upon a multitude of factors, including the microclimate within the pens, the type and number of cooling devices, the ration formulation, and the susceptibility of individual animals. Irrespective of these variables, however, only a portion (35% in Rhoads et al., 2009) of the reduction in milk production is due to the decrease in DMI associated with heat stress (Rhoads et al., 2009; Min et al., 2015; Wilson et al., 1998b; Bernabucci et al., 2014). Therefore, further research to elucidate other factors causing the remaining (65%) reduction in milk yield is needed.

For milk production, heat stress can have both immediate and long-lasting effects as it's also known to affect subsequent lactations. Multiple studies have reported that late gestation heat stress decreases early- and mid- lactation performance in the following lactation (Moore et al., 1992; Fabris et al., 2019; Tao et al., 2011). This long-lasting reduction in milk yield could be the result of inhibited mammary gland development during the dry period, specifically related to a decrease in epithelial cell proliferation (Tao et al., 2011).

Reproductive Performance

Just as heat stress can drastically impact milk production in dairy cattle, it can also alter reproductive performance. Within the dominant follicle, theca and granulosa cell function is affected by heat stress, resulting in altered steroidogenic capacity (Wolfenson et al., 1997; Roth et al., 2001; Li et al., 2016). Studies have demonstrated that heat stress can decrease estradiol (E_2) production from the dominant follicle, decreasing its size and increasing the number of subordinate follicles that can continue to grow (Wilson et al., 1998b; Wolfenson et al., 1997; Bridges et al., 2005; Roth et al., 2001; Schüller et al., 2017). Reduced E_2 production by the dominant follicle could be due to decreased blood flow to the reproductive tract as well as effects on luteinizing hormone (LH) secretion and receptor function, as LH from the anterior pituitary is needed to stimulate E_2 production (Roth et al., 2001; Critser et al., 1983; Schüller et al., 2017). Regardless of the mechanism by which heat stress affects E_2 production, the consequences are problematic. Amongst other concerns, expression of estrus behavior is reduced by heat stress, which can be a detriment for producers that use visual signs of estrus as their only form of heat detection (Polsky et al., 2017; H. M. Silva et al., 1992; Schüller et al., 2017).

In addition to its impacts on ovarian follicles, heat stress alters the corpus luteum (CL); although, there is a lack of agreement regarding the specific heat-stress-induced effects. Heat stress has been found to decrease progesterone (P_4) production during chronic or prolonged heat stress (Alhussien et al., 2018; Wolfenson et al., 2002; Schüller et al., 2017), and also shorten the length of the luteal phase (Wolfenson et al., 1988). In other studies, acute heat stress either did not impact P_4 production, or actually increased P_4 production (de Castro e Paula et al., 2008; Wolfenson et al., 2002). Due to the dual functionality of P_4 as both an immunosuppressor and maintainer of pregnancy, these conflicting results indicate that heat stress could be either beneficial or harmful, depending on the reproductive and immune status of the cow. Some previous research has also shown that heat stress can delay luteolysis; therefore, increasing the number of follicular waves and the length of the luteal phase (Wilson et al., 1998b; Wilson et al., 1998a). In contrast, an in vitro study using bovine endometrial cells found that heat stress increased prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) concentrations which could result in reduced P_4 concentrations and earlier luteolysis (Putney et al., 1988). Although often contradictory, these findings indicate that heat stress alters CL function and P_4 concentrations which could ultimately disrupt cyclicity, reduce pregnancy rates and/or increase uterine infection rates.

For dairy cattle, the 3-5 days prior to and succeeding breeding are critical for good conception rates and pregnancy outcomes. Heat stress experienced around the time of breeding can decrease conception rates as much as 23% depending on the length and severity of stress experienced by the cow (Cavestany et al., 1985; Schüller et al., 2017; Polsky et al., 2017; Badinga et al., 1985; Morton et al., 2007; Ealy et al., 1994; Gwazdauskas et al., 1975; Ryan et al., 1993; Guinn et al., 2019). The reduction in conception rate can be even greater in high-producing cows due to the energy demands of milk production (Djelailia et al., 2020). Temperatures between 30-

35°C on the day of breeding or day after breeding can cause pregnancy rates to approach 0% in both cows and heifers (Cavestany et al., 1985; Badinga et al., 1985).

The observed effects of elevated ambient temperatures are mainly due to an increase in early embryonic loss (Cavestany et al., 1985; El-Tarabany & El-Tarabany, 2015; Djelailia et al., 2020; Biggers et al., 1987; Silva et al., 1992). During the first 30 days of pregnancy, early embryonic loss is already high, and heat stress can increase it further. This could be due to elevated temperature in the reproductive tract, as the embryo is sensitive to the maternal body temperature during early development (Roman-Ponce et al., 1978; Ealy et al., 1993; Edwards & Hansen, 1996). During heat stress, the competency of the embryo can decline by as much as 50%, decreasing viability and blastocyst rate past day 7 of development by around 85% (Putney et al., 1989; Al-Katanani et al., 2002; Sartori et al., 2002; Ryan et al., 1993; Silva et al., 2013). Amongst other effects, it is postulated that heat stress impacts nuclear maturation by decreasing genomic activation and increasing morphological imperfections, to increase apoptosis of the cells (Putney et al., 1989; Edwards & Hansen, 1996; Krininger III et al., 2002; Pinedo & De Vries, 2017). An increase in apoptotic cells in response to heat stress conditions has also been associated with an increase in nuclear fragmentation and caspase activity, both representing DNA damage (Ferreira et al., 2011; Paula-Lopes & Hansen, 2002; Roth & Hansen, 2004), ultimately causing the demise of the embryo.

There are a multitude of effects of heat stress on reproductive parameters; far more than can be described in this literature review. Ultimately, however, those effects alone, or in combination, can be devastating for dairy farm productivity. For these reasons, management strategies aimed at improving reproductive performance during periods of heat stress have the potential to increase both profitability and sustainability.

Chromium Supplementation in Cattle

Overview

As the dairy industry continues to endure major monetary losses due to poor reproductive performance, new strategies are being investigated to improve overall fertility. One such strategy is the use of chromium (Cr) supplementation. Chromium is a crucial trace mineral important for proper carbohydrate, lipid, and protein metabolism (Mertz, 1993; Pechova & Pavlata, 2007). Chromium has been found to bind to an oligopeptide known as low molecular weight chromium binding substance. This complex is able to help facilitate the entrance of Cr into tissues and play a role in the activation of the insulin receptor tyrosine protein kinase activity to potentiate insulin actions (Davis & Vincent, 1997). In recent years, it has been studied as both a metabolic and reproductive influencer.

Chromium supplementation can be given in a variety of forms, and currently, Kemin Industries is the only United States Food and Drug Administration (USFDA) approved producer of a chromium-propionate (CrPro) supplement for livestock (Baggerman et al., 2020). In an initial study, CrPro was fed to pigs and its effects were compared to those of both a control and chromium picolinate supplement. Investigators found that Cr, specifically in the form of CrPro, increased glucose clearance in pigs during an insulin challenge. This study was one of the first to directly compare the efficacy of different forms of Cr. The results demonstrated the ability of Cr supplementation to alter glucose metabolism, and furthermore, indicated that the specific form of the supplement was key to its effectiveness (Matthews et al., 2001).

Actions as an Insulin Co-Factor

As an insulin-cofactor, Cr has a large role in regulating insulin and glucose concentrations. The scientific community's understanding of its actions continues to develop. When glucose concentrations increase within the bloodstream, there is a rapid release of insulin. This increase in blood insulin concentrations causes Cr to be mobilized from the blood to insulin-dependent cells. There it acts to enhance insulin signaling, in order to reduce blood glucose concentrations and bring them back to basal levels. This fundamental action of Cr has been demonstrated in numerous species, including ruminants. In growing heifers, Cr supplementation increased the utilization of glucose in a dose-dependent manner (Sumner et al., 2007).

Chromium also increases the number of insulin receptors on the cell surface, as well as receptor sensitivity (Pechova & Pavlata, 2007; Spears et al., 2012), thereby resulting in an overall increase in insulin sensitivity. Insulin sensitivity goes hand in hand with insulin resistance; when one is high, the other is low, and vice versa. In general, insulin resistance reduces the productivity of farm animals, which further emphasizes the importance of Cr as a regulator of insulin-mediated glucose uptake. Previous research evaluating insulin secretion after a glucose challenge in growing beef heifers determined that Cr supplementation increased insulin sensitivity. The study found that the area under the glucose curve was reduced in the heifers fed Cr, indicating glucose uptake was enhanced (Spears et al., 2012). This conclusion was further supported in two separate studies, where Leiva and colleagues (2015 and 2017) used non-lactating dairy cows to show that Cr supplementation prevented the expected increase in insulin resistance caused by excessive energy intake, and enhanced insulin sensitivity, respectively. Together, this research demonstrates the efficacy of Cr as a regulator of insulin function in cattle.

Cr Supplementation during Stress Periods

As previously discussed, the onset of stressful periods can alter physiological responses, exacerbate negative energy balance (NEB), and reduce the magnitude of an immune response. The postpartum period and heat stress conditions are particularly concerning for dairy cattle, and cows exposed to either (or both) stressor may benefit from Cr supplementation.

The periparturient period, encompasses the time surrounding parturition through early lactational and postpartum stages. Events associated with this time frame can affect the incidence of infection. Early postpartum risk factors associated with infection include (but are not limited to) increased hepatic lipidosis, decreased serum insulin, and the onset of NEB (Guidry et al., 1976; Kehrli et al., 1989). Previous research has demonstrated that Cr supplementation can ease some of the maladies associated with this risky phase of production. For example, when fed during early lactation, cows regained body condition score more quickly and were then more fertile following artificial insemination (Ferguson, 2016).

Negative energy balance during early lactation is associated with high serum concentrations of non-esterified fatty acids (NEFAs) (Ospina et al., 2010). Chromium supplementation can improve the energetic status of early lactation dairy cows by decreasing NEFA concentrations and blood serum cortisol, while also increasing insulin concentrations, to improve dry matter intake (DMI) (Westwood et al., 2002; Hayirli et al., 2001; Bryan et al., 2004). Many studies have found that the addition of Cr to diets for postpartum cows can also increase milk yields during periods of NEB (Yang et al., 1996; Hayirli et al., 2001; Smith et al., 2005). Unfortunately, the milk yield response to Cr supplementation is inconsistent, sometimes resulting in an increase and sometimes associated with no change (Bryan et al., 2004). Overall, however, results are indicative of a positive metabolic effect of Cr supplementation during stressful periods.

Any observed improvements in physiological status following Cr consumption may be the result of satisfying a deficiency, particularly during early lactation. Research has demonstrated that an increase in milk production in combination with husbandry/management stress can lead to a Cr deficiency in cattle (Burton et al., 1996). High stress periods increase glucose metabolism, therefore increasing Cr mobilization and leading to Cr deficiency (Borel et al., 1984; Mertz, 1993; Hayirli et al., 2001). Stress also increases blood cortisol levels which are antagonistic for insulin action so that glucose can be spared for organs, like the brain, with greater demands. Cortisol, a glucocorticoid, also negatively affects the immune response by decreasing neutrophil function. Chromium consumption improves these aspects of the stress response by reducing blood cortisol levels, while acting as an immunomodulator in high producing cows (Chang & Mowat, 1992; Moonsie-Shageer & Mowat, 1993; Burton et al., 1996; Soltan, 2010).

Impacts on Reproductive Performance

As the dairy industry continues to strive to improve reproductive performance, many experiments have been aimed at developing management strategies to lessen the negative impacts of common stressors (such as heat stress and the transition into lactation). Specifically, the use of Cr supplementation has received little, but growing, attention (Pechova & Pavlata, 2007).

Chromium supplementation is advantageous for reproductive performance for many reasons, not the least of which is its ability to improve energetic status. Energy balance plays a crucial role in reproductive performance. Butler and others (2006) have demonstrated that it can predetermine the function of the hypothalamic-pituitary-ovarian axis, as well as the fate of the first postpartum follicular wave. Periods of excessive negative energy balance associated with reductions in DMI and greater concentrations of NEFAs have been associated with a decrease in reproductive performance, including lowered estrus detection and conception rates (Westwood et al., 2002). The use of Cr supplementation can improve DMI and reduce NEFA concentrations observed during poor energy balance to thereby increase pregnancy rates (Hayirli et al., 2001;

Bryan et al., 2004). However, additional research is needed to elucidate the exact mechanisms by which Cr directly and indirectly improves reproductive performance during periods of NEB.

In conjunction with improved energetic status, Cr is also known to promote insulin sensitivity. Theoretically, this improvement in insulin sensitivity should result in increased fertility in early lactation dairy cattle as insulin resistance has been found to decrease oocyte competence and embryonic development (Sinclair, 2010; Baruselli et al., 2016; Oliveira et al., 2016; Laskowski et al., 2016). While Cr supplementation does not always increase reproductive performance (Leiva et al., 2015; Baruselli et al., 2016), improvements in the percent pregnant in the first 28 days of breeding as well as a reduction in the proportion of non-cyclic cows have been previously demonstrated. Interestingly, at days 49 and 60 post-breeding, Cr no longer had an effect on the percent pregnant, further indicating it is most beneficial during the periparturient, early lactation period (Soltan, 2010).

In addition to the previously mentioned problems associated with early lactation, cows commonly exhibit a reduced capacity for immune response that can ultimately influence reproductive performance (Kehrli et al., 1989; Kehrli & Goff, 1989). In 1993, Burton and others found that periparturient and early lactation dairy cattle fed a Cr supplement could alter their specific immune response during that stressful period. The group evaluated multiple in vivo and in vitro indicators of immune response following an immune challenge, finding that Cr was able to enhance both cell-mediated and humoral immunities during the periparturient period. Therefore, this group concluded that Cr supplementation may help dairy cows overcome stress-associated immunosuppression (Burton et al., 1993).

The cow’s ability to respond to an infection can affect subsequent fertility in a number of ways, one of which involves endometritis. Subclinical or clinical endometritis, forms of uterine disease that causes inflammation, are common infections afflicting cattle during the postpartum period. The characteristics and diagnostic procedures for endometritis were previously described. A study examining the effects of Cr supplementation on endometritis found that Cr increased neutrophil influx to the uterus in the early postpartum period (7 d post-calving). If only one sample had been collected, this influx of neutrophils could have been interpreted as a detriment to fertility. By analyzing multiple samples, however, Yasui and others (2014) noted that this early influx of neutrophils decreased the incidence of endometritis later in lactation, when cows were approaching the end of the voluntary waiting period (40-60 d post-calving). Taken together, these results indicate CrPro has positive effects on uterine health during the postpartum and early lactational period (Cheong et al., 2011; Yasui et al., 2014).

While clearly beneficial for aspects of the transition period, Cr supplementation also improves the physiological response to heat stress. The ability of Cr to increase insulin-stimulated glucose uptake parallels the metabolic shift during heat stress that prioritizes the use of glucose by the peripheral tissues. And while the mechanisms are not yet understood, the ability of Cr to reduce body temperature could improve reproduction alone. Heat stress increases body temperatures, which can result in decreased estrus detection, embryo quality, and conception rates (Rhoads et al., 2009; Polsky et al., 2017; Ealy et al., 1994; Cavestany et al., 1985; Edwards & Hansen, 1996). Therefore, by simply returning body temperature to values typical of thermoneutral conditions, Cr supplementation could improve overall reproductive performance.

As the dairy industry continues to navigate the challenges associated with reproductive performance, there will be a constant demand for development of novel, inexpensive and effective management strategies for the periparturient period. Supplementation with Cr is an easy approach that meets all of these important criteria. Its multi-faceted benefits, including effects on insulin resistance, responses to high stress periods, and the immune response, can act independently or synergistically to improve reproductive performance in dairy cattle.

Summary

As temperatures continue to rise and heat stress abatement becomes harder to achieve, dairy cattle will continue to experience reproductive and lactational losses. The effects on subsequent estrous cyclicity, uterine immune response, and pregnancy rates will perpetuate the current high culling rates brought on by stress conditions. It is imperative for researchers to continue elucidating the mechanisms and subsequent physiological responses involved in stress responses in order to mitigate the detrimental effects on animal productivity. The research described in the chapters hereafter will add to the current base of knowledge surrounding the effects of heat stress in dairy cattle. The subsequent work focuses on chromium supplementation as a potential management solution to combat the negative effects of stress induced by elevated ambient temperatures during the early postpartum period.

CHAPTER 2: The influence of chromium supplementation on aspects of reproduction and lactation of early postpartum dairy cattle

Introduction

During periods of stress, such as those of heat stress and the early postpartum period, individual physiological responses can reduce productivity, thereby decreasing economic profit for dairy producers. The severity of the responses to stress can be diminished with chromium (Cr) supplementation. Chromium is an essential trace mineral (Mertz, 1993) that has received increasing attention in recent years for its ability to enhance insulin-mediated glucose uptake in dairy cattle, which, in practice, often results in an increase in milk production. The improvement in energetic status following Cr consumption can also positively affect reproductive performance, immune function, and responses to stressors.

The physiological responses to elevated ambient temperatures are a major concern for the dairy industry, as they are associated with approximately 1.5-billion-dollars in economic losses each year for dairy operations in the United States. These losses result from the combined effects of heat stress on milk yield, reproductive performance, disease incidence and culling rates (St-Pierre et al., 2003). Previous studies have shown that Cr supplementation moderates the response to heat stress in several species (Kumar et al., 2015; Sahin et al., 2002; Liang et al., 2022; Jamal et al., 2021; Hung et al., 2021). For example, pigs fed a Cr supplement under heat stress conditions tended to have greater average daily gain and final body weight (BW), as well as increased blood neutrophils percent compared to heat-stressed contemporaries not consuming additional Cr (Liu et al., 2017; Mayorga et al., 2019). Heat-stressed dairy cows likewise benefit from Cr supplementation, as they exhibit improved dry matter intake (DMI) and milk yield, as well as reduced cortisol, respiration rate (RR), and rectal temperature (RT) (Moonsie-Shageer & Mowat, 1993; Soltan, 2010). These beneficial effects of Cr consumption during periods of heat stress are particularly important for dairy production as climate change increases exposure to extreme ambient temperatures (Hempel et al., 2019; Key et al., 2014).

Beyond the stress imposed by elevated ambient temperatures, the postpartum period is also a time during which dairy cows are susceptible to a variety of disorders. The transition in homeorhetic priority from maintaining pregnancy to producing substantial quantities of milk is associated with a state of negative energy balance (NEB) during which cows are unable to consume enough feed to satisfy their energy requirements (Mann et al., 2015; McNamara et al., 2003; Grummer et al., 2004; Marquardt et al., 1977). Negative energy balance during the postpartum period is associated with a plenitude of maladies, including displaced abomasum, ketosis, fatty liver, and hypocalcemia (Cameron et al., 1998; McArt et al., 2012; Bertics et al., 1992; Bobe et al., 2004; Caixeta et al., 2015). The stress experienced during the postpartum period also increases the incidence of endometritis, which can significantly decrease reproductive performance (Bartlett et al., 1986; Kasimanickam et al., 2004). Subclinical endometritis (SCE) is a specific form of endometritis present during the first two months postpartum and is not associated with clinical signs of infection (Sheldon et al., 2006). This condition can be improved by Cr supplementation of dairy cattle, although, to the best of our knowledge, it has not been tested in conjunction with seasonal heat stress (Yasui et al., 2014).

The ability of Cr to improve postpartum energetic status and the physiological responses to stressors could prove economically beneficial for dairy operations. Previous research studies have typically relied on lengthy supplementation periods during which Cr treatment was initiated prior to calving (Yasui et al., 2014; Burton et al., 1996; Soltan, 2010; Yang et al., 1996; Bryan et al., 2004; Hayirli et al., 2001). On farm, pre-calving supplementation can sometimes be troublesome, depending on how dry cows are housed and managed. Therefore, the objective of this study was to monitor milk yield and aspects of reproductive performance during short-term, postpartum Cr supplementation over the summer months. We hypothesized that short-duration, high-dose Cr propionate (CrPro) supplementation strategy would improve the selected production parameters in early postpartum, dairy cows during the summer months.

Materials and Methods

Animals and Treatments

All animal procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC). Twenty-two multiparous (2.86±0.34 lactations), lactating Holstein cows (658.29 ± 13.61 kg BW, 20.95 ± 0.21 DIM) with clinically normal periparturient periods were selected from the Virginia Tech Dairy Science Complex on a rolling basis. Cows were housed in one of two 12-stall pens within a freestall barn for the duration of the experiment, spanning the months of June through September. Milk production was recorded twice daily (0000 h and 1200 h), and BW were collected after the second milking each day. Cows were individually fed once daily (1100 h -1400 h) using a Calan gate system (American Calan, Inc., Northwood, NH). Cows were trained to use the Calan gates for 4-days prior to the treatment period.

Throughout the experiment, all cows were fed the same base total mixed ration (TMR) formulated to meet or exceed the nutrient requirements of early lactation (Table 1). Cows were fed for ad libitum intake and feed refusals were collected and weighed daily. On d 0, each cow was randomly assigned to either the control (Con; n=10) or Cr propionate (CrPro; n=12) treatment group, based upon parity and average milk production over the three previous days (Figure 1). Cows assigned to the CrPro group, received 30g of CrPro daily for 24 d (12 mg Cr/h/d; KemTRACE chromium propionate; Kemin Industries Inc., Des Moines, IA). The supplement was added to each cow's ration individually as a topdressing, and then hand-mixed into the top one-third of the TMR. The 30-g dose was chosen based upon the manufacturer's recommendations, which took into account estimated BW and DMI.

All cows were subjected to the OvSynch estrus synchronization protocol upon enrollment, prior to treatment initiation (Figure 1). Briefly, at -10d, each cow received an initial dose of 2mL of gonadotropin releasing hormone (GnRH) (Cystorelin; 50mcg/mL; Merial, Duluth, GA, USA) via intramuscular (i.m.) injection in the neck. Seven days later (-3d), each cow received a 5mL i.m. injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Lutalyse; 5mg/mL; Zoetis Inc. Kalamazoo, MI). Approximately 56 h later (-1d), each cow received a second 2mL injection of GnRH.

Data Collection and Ultrasonography

Beginning at -6d relative to treatment initiation, data and sample collection commenced, and continued until the end of the experiment (24d) when the cows returned to the herd (Figure 1). In the morning of every third day, transrectal ultrasonography was conducted (IBEX PRO portable ultrasound with a L7HDi linear transducer; E.I. Medical Imaging, Loveland, CO) in order to obtain ovary maps containing measurements of all luteal structures and all follicular structures >5 mm in diameter. Visible follicles ≤5mm in diameter were counted, but not measured. Follicles were categorized by size according to Lucy et al., 1991. The number of follicles within each category and their average diameter were used for statistical analyses. Likewise, the number and average diameter of all luteal structures were evaluated. The volume of the CL was calculated from the diameter using the formula: $(4/3)\ \Pi\ (d/2)^3$, where d = the diameter of the CL. Fluid-filled cavities within the CL were noted, but not measured, and were therefore not included in the calculation of CL volume.

Rectal temperature, RR, and a blood sample (coccygeal venipuncture into sodium heparin vacutainers Becton, Dickinson and Company, Franklin Lakes, NJ)) were also collected from each cow on the same days, prior to transrectal ultrasound. Blood glucose concentrations were immediately determined using a hand-held glucometer (Contour Next EZ; Ascensia Diabetes Care US, Inc, Parsippany, NJ). The remainder of each blood sample was put on ice until centrifugation at 3000 x *g* for 15 minutes. After centrifugation, plasma was collected, frozen and stored at -20°C until subsequent analyses.

Endometrial Cytology Sampling

Every other procedure day (every 6 days), starting at -6d, uterine cytology samples were also collected prior to transrectal ultrasonography as described in Kasimanickam et al., 2004 (Figure 1). A cytology brush (Cytobrush Plus GT; CooperSurgical, Inc, Trumbull, CT) was attached to the plunger of an artificial insemination (AI) rod that had been sterilized in a chlorhexidine solution. The cytology brush was withdrawn into the AI rod and covered in a protective chemise for passage through the vagina. Upon reaching the cervix, the rod was pushed through the chemise, but the brush remained protected within the AI rod for passage through the cervix and into the uterine horn. The AI rod was advanced to a position approximately 8 cm past the uterine bifurcation in the left or right uterine horn, at which time the cytobrush was exposed, rolled against the uterine endometrium, and then reinserted into the AI rod. The AI rod was then moved to the opposite uterine horn, and the process was repeated before removal from the tract. Swabs were immediately rolled onto a glass slide and allowed to air dry. Each slide was sprayed with a fixative (CytoPrep Fixative; Electron Microscopy Sciences, Hatfield, PA) and then stored in a slide holder until staining.

After all slides were collected from all cows, they were stained with a modified Giemsa stain (Differential Quik Stain Kit (Modified Giemsa); Electron Microscopy Sciences, Hatfield, PA) with slight modifications to the manufacturer's recommended procedures (necessary for identification of cells of interest). Briefly, each slide was first dipped for 10 seconds in a fixative solution, followed by 1 minute in an Eosin Y-based solution. The slides were then dipped 2x in the fixative solution, before being dipped 2x in a methylene blue trihydrate-based solution, then rinsed in DI water and allowed to air dry. The slides were then dipped 10x each in 100% ethanol and xylene, before 2 drops of a toluene-based histological mounting medium (Permount Mounting Medium; Electron Microscopy Sciences, Hatfield, PA) was placed on the sample for coverslip mounting. Coverslips were allowed to dry overnight.

Cytological Assessment

An EVOS xl Core Imaging System (Life Technologies Corporation, Carlsbad, CA) was used to capture images from each slide. The number of images collected varied based upon the number of cells present in the field of view, as the goal was to count 200 cells. Cell counts were conducted using ImageJ Software (National Institutes of Health, Bethesda, MD) and representative images are presented in Figure 2. Two hundred cells were randomly counted on each slide by two observers blinded to the treatments. The number of PMNL within the 200 cells counted by the two observers were averaged and the percent PMNL was calculated for use during statistical analyses.

In addition to the PMNL prevalence, red blood cell (RBC) contamination was assessed as described in (Pascottini et al., 2015). Briefly, the quantity of erythrocytes was observed, and a subjective score was assigned as having no RBCs (score of 0), low RBCs (score of 1), moderate RBCs (score of 2), or high RBCs (score of 3). As with the PMNL counts, the scores were averaged between two observers.

Plasma Progesterone Analyses

The progesterone (P_4) concentrations within plasma samples were determined using an IMMULITE 2000 Progesterone solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite 2000 XPi platform; Siemens Medical Solutions Inc, USA)(Podico et al., 2020). All samples were run in a single assay with an intra-assay CV of 1.99%.

Temperature-humidity Index

Climatic data was obtained (NOAA) and included dry bulb temperature ($T_{\text{dry bulb}}$) and dew point temperature ($T_{\text{dew point}}$) observed at 15-minute intervals for the full duration of the study. Values for $T_{\text{dry bulb}}$ and $T_{\text{dew point}}$ were averaged for each hour for the length of the experiment. Temperature-humidity index (THI) values were calculated from these means using the formula: $THI = T_{\text{dry bulb}} + ((0.36 * T_{\text{dew point}}) + 41.2$(Brown et al., 2016). These THI values were then used to determine the mean THI for each hour in a 24h day.

Statistical Analyses

One cow was removed from the experiment for reasons unrelated to the treatment. Data were analyzed using the MIXED procedure of SAS (SAS Institute, Inc, Cary, NC). Independent variables were treatment (Con or CrPro), day of experiment, and their interaction. Cow was included in the model as the repeated variable. When day of experiment or the interaction between treatment and day of experiment were not significant, they were removed from the model. For analysis of PMNL percent, RBC score was included as a covariate. For each analysis, eight covariance structures were tested and the most appropriate was selected based upon Akaike's information criterion, Akaike's information criterion with correction, and Bayesian information criterion values. Pairwise comparisons were conducted using Tukey's procedure. The probabilities of the presence of a CL or SCE on sample collection days was analyzed using the FREQ procedure of SAS. Results are reported as least square means ± standard errors of the means. Statistical significance was declared at $P \leq 0.05$ and tendencies to differ at $0.05 \leq P \leq 0.10$.B

Results and Discussion

Previous research has evaluated the use of different doses of Cr across various lengths of supplementation (Moonsie-Shageer & Mowat, 1993; Soltan, 2010; Kafilzadeh et al., 2012; Yasui et al., 2014). There are also different forms of Cr supplementation, including CrPro, Cr methionine, Cr picolinate, and Cr yeast (Yasui et al., 2014; Soltan, 2010; Kafilzadeh et al., 2012; An-qiang et al., n.d.; Moonsie-Shageer & Mowat, 1993). Currently, KemTRACE Chromium is the only USFDA-reviewed form of CrPro for feed supplementation in cattle (Baggerman et al., 2020), and has been shown to have a greater bioavailability compared to other forms of Cr when fed to growing barrows (Matthews et al., 2001). In this study, cows in the CrPro group received 12 mg/h/d of Cr (30 g of CrPro) for 24 d. Previous studies have reported changes in production parameters at concentrations as low as 6mg/h/d when supplementation was initiated during the dry period (Soltan, 2010). The amount of Cr administered in this study is greater than those in previous experiments in order to evaluate the efficacy of short-duration, high-dose supplementation (Moonsie-Shageer & Mowat, 1993; Soltan, 2010; Kafilzadeh et al., 2012; Yasui et al., 2014).

The current study was conducted in early lactation dairy cows that were presumably exposed to heat stress, as the treatment and sample collection periods spanned the summer and early fall. In order to determine the severity of heat stress to which the cows were exposed, the $T_{\text{dry bulb}}$ and $T_{\text{dew point}}$ were assessed (Figure 1a). These parameters were also used to calculate the mean THI for each hour per day for the duration of the trial. Previous work reported that a THI value of 68 or greater is indicative of heat stress conditions that can reduce productivity in dairy cattle

(Zimbelman et al., 2009). Cows enrolled in this study were experiencing THI levels of 68 or greater for approximately 14h per day (Figure 1b).

Cows were moved into Calan gate pens approximately 14 d prior to being assigned to their treatment group. Pre-treatment feed intake, milk yield and the feed intake to milk yield ratio were all similar between cows that were ultimately assigned to Con and CrPro treatments. Initial BW did tend to differ (642.1 ± 19.4 kg and 688.3 ±17.7 kg BW for Con and CrPro, respectively; P=0.09) and this tendency was maintained throughout the experiment (632.1 ± 19.2 kg and 675.7 ± 17.5 kg BW during treatment for Con and CrPro, respectively; P=0.11). As such, the change in BW was evaluated and did not differ between treatment groups (Table 1). Likewise, feed intake, milk yield and the feed intake to milk yield ratio were unaffected by treatment (Table 1). These results contrast many previous experiments that report an increase in both DMI and milk yield as a result of Cr supplementation (Al-Saiady et al., 2004; An-qiang et al., 2009; Soltan, 2010; Mirzaei et al., 2011). Others, however, have reported similar results, finding no change in DMI and/or milk yield following Cr supplementation (Yasui et al., 2014; Bryan et al., 2004; Yang et al., 1996), indicating that Cr-specific effects on DMI and milk yield are variable.

Due to scheduling constraints, RR and RT were only able to be collected in the mornings (between 0600 and 1100 h) in conjunction with transrectal ultrasound. Even though these measurements were collected in the morning hours following the overnight THI nadir, they were greater than would be expected during thermoneutral conditions and consistent with morning measurements of lactating dairy cattle exposed to controlled heat stress (Table 1; Rhoads et al., 2009). Some previous studies have found that when livestock were subjected to heat stress, Cr supplementation reduced RR and RT, particularly during the hottest hours of the day (Hung et al., 2021; Ribeiro et al., 2020; Liu et al., 2017). Unfortunately, Cr supplementation did not improve

RR or RT in the current experiment (Table 1). The lack of difference in either parameter during Cr supplementation could have been the result of the time of day at which RR and RT measurements were conducted (not during maximum THI) or could be the consequence of a balance of contradicting factors. Other heat-stress studies have also failed to detect an improvement in RR or RT (An-qiang et al., 2009; Mirzaei et al., 2011), and some have even reported an increase in RR and RT in association with Cr consumption (Mirzaei et al., 2011; Mayorga et al., 2019). Interestingly, in both studies where Cr treatment increased RR or RT, feed intake was also greater. Mayorga and co-workers (2019) suggested that a direct relationship between the two caused the elevated RT, as greater feed intake would presumably increase metabolic heat production, thereby increasing body temperature indices. Taken together, the results of these studies indicate that effects of Cr on feed intake, RR, RT, and other production-related parameters are based upon the prevailing balance of factors related to the physiological state of the animal.

Chromium is a potent regulator of insulin-mediated glucose uptake, which typically causes circulating glucose concentrations to be lesser in Cr-supplemented animals (Depew et al., 1998; Stahlhut et al., 2006; Chang et al., 1995; Mowat et al., 1993). This is not always the case, however, particularly for those studies involving heat stress (Mayorga et al., 2019; Soltan, 2010; Mirzaei et al., 2011). Indeed, blood glucose concentrations in the current experiment did not differ between treatment groups (Table 1). Although not directly measured herein, this could be related to the altered glycemic status typically observed in heat-stressed lactating dairy cattle. Early- to mid-lactation dairy cattle exposed to high ambient temperatures typically become hyperinsulinemic and hypoglycemic (Rhoads et al., 2009; Baumgard et al., 2011). Interestingly, unlike underfed animals, heat-stressed dairy cattle generally remain insulin sensitive (Xie et al., 2016). When these conditions co-exist, the abundance of circulating insulin coupled with unchanged insulin sensitivity (and resulting hypoglycemia) indicates that insulin-stimulated glucose uptake is already maximized. Consequently, Cr is unable to further increase glucose uptake under these conditions.

Studies have shown that both heat stress and energetic status can affect circulating P_4 concentrations and the length of the luteal phase (Alhussien et al., 2018; Wolfenson et al., 2002; Schüller et al., 2017; Wolfenson et al., 1988). If disruptions to P_4 production are severe enough, estrous cyclicity and/or the establishment of pregnancy will be detrimentally affected. In the current study, neither the number of CL nor their average diameter were affected by Cr supplementation (Table 2). Unfortunately, blood samples were not collected frequently enough to determine maximum P_4 concentrations (samples were collected every three days), thus preventing an analysis of absolute progesterone values. Since blood samples were collected in conjunction with transrectal ultrasonography, however, the ratio of P_4 concentration to corpus luteum (CL) volume was determined and analyzed. The cows consuming the CrPro produced more P_4 per unit of CL volume than the Con group. In fact, the mean P_4 produced per cm^3 of CrPro CL was nearly double that of the Con CL (Figure 3). Thus, Cr supplementation improved P_4 production per CL volume which could aid in delaying the onset of luteolysis, thereby increasing the likelihood of pregnancy recognition.

Antral follicle count is correlated with lifetime fertility and can be used as an indicator of reproductive fitness (Ireland et al., 2008; Martinez et al., 2016; McNeel & Cushman, 2015). Furthermore, follicle development specifically during the early postpartum period is linked with subsequent fertility in lactating dairy cows (Sood et al., 2022; Furukawa et al., 2020) and can be detrimentally affected by heat stress (Wilson et al., 1998b; Wolfenson et al., 1997; Bridges et al., 2005; Roth et al., 2001; Schüller et al., 2017). Unfortunately, Cr supplementation in the current study affected few follicular measurements (Table 2). There was a tendency for Cr supplementation to increase the number of small follicles (6-9 mm in size), and CrPro treatment increased their average diameter (Table 2). This size category is representative of the recruited cohort of follicles that have not yet been subjected to selection (Hampton et al., 2004). This increase in number and size of the recruited follicles was not sustained, however, as the numbers and diameters of larger follicle categories did not differ between treatments (Table 2).

Heat stress and the early postpartum period are both associated with an increased risk for metritis in dairy cattle. Some studies have found the incidence of endometritis within dairy herds to be over 50% (Cheong et al., 2011; Stevenson, 1987; Bartlett et al., 1986; Markusfeld, 1984; Hammon et al., 2001; Carneiro et al., 2014; Ahmadi et al., 2019; Heuwieser et al., 2000). Endometritis is problematic for a number of reasons, but is perhaps best known for its impact on postpartum fertility, as indicated by reduced first service pregnancy rates, increased services per conception, and greater median days open (Cheong et al., 2011; Gilbert et al., 2005; Dubuc et al., 2010; Barlund et al., 2008; Rutigliano et al., 2008; Kasimanickam et al., 2004). In this study, the incidence of uterine disease was monitored by collecting endometrial swabs every six days from d -6 until d 24 of the experiment, from which the PMNL percentage was determined. The first three samples were collected prior to 34 DIM, which requires an 18% PMNL count for SCE diagnosis. The last three samples were collected at or after 34 DIM, which requires a 10% PMNL count for SCE diagnosis (Gilbert et al., 2005; Sheldon et al., 2006; Gautam et al., 2010). While individual cows did meet or exceed the thresholds for SCE diagnosis, at no point did mean PMNL percent for either treatment group exceed the threshold for SCE diagnosis (Figure 2). The incidence of SCE also did not differ between treatments (data not shown).

Within the Con group, the percentage of PMNL increased between samples 5 and 6 (P=0.02; Figure 2). The increase in PMNL percent at the end of the sampling period was indicative of a resurgence in endometrial inflammation. While we cannot rule out the possibility that this was the result of repeated endometrial swabs, it is important to note that the cows in the CrPro treatment were subjected to the same number (and timing) of procedures and did not exhibit an increase in PMNL percent. Thus, the percentage of endometrial PMNL was greater in the Con group compared to the CrPro group at sample timepoint 6 (P=0.01; Figure 2). The difference in PMNL percent between Con and CrPro-treated cows is supported by previous work where the incidence of SCE at 40-60 d postpartum was reduced by CrPro supplementation (Yasui et al., 2014). Together, these results indicate that Cr supplementation aids in improving the uterine environment in postpartum dairy cows. Any strategy capable of improving the uterine environment also has the potential to increase fertility. Unfortunately, there were not enough cows available for the current study to evaluate pregnancy rates following supplementation.

Conclusions

The short-duration, high-dose CrPro supplementation strategy implemented in this study did not impact many of the physiological parameters measured in this study. This is seen here in the lack of treatment-based changes in blood glucose concentrations, RR, RT, feed intake, and milk yield. Although these indicators of metabolic status were not affected by CrPro supplementation, it did improve reproductive parameters, including P_4 production, PMNL percent, and characteristics of small ovarian follicles. These results indicate that CrPro supplementation could act to improve subsequent pregnancy recognition, lower SCE rates, and ultimately improve fertility. Together, the results indicate that a short-duration, high-dose CrPro supplementation strategy could benefit the reproductive performance of dairy cattle during periods of stress, thereby limiting economic losses experienced by dairy producers.

Table 1. Base TMR concentrate composition for early lactation dairy cows.

Ingredients	kg/cow/day[1]	%
Corn grain	5.670	34.90
Soybean meal	1.552	9.55
SoyPlus	3.364	20.71
Dried distillers' grains w/ solubles	3.838	23.63
Soybean hulls	0.768	4.73
Energy Booster 100	0.206	1.27
Bentonite	0.201	1.24
Calcium carbonate / Limestone	0.155	0.95
Salt	0.125	0.77
Sodium bicarbonate	0.251	1.55
Magnesium oxide	0.040	0.25
EMCO Trace Min PMX	0.030	0.18
Sodium selenite (0.06% Se)	0.010	0.06
EMCO Vitamin ADE PMX	0.010	0.06
EMCO Vitamin E (20,000 IU/lb)	0.014	0.09
Clarifly (0.67%)	0.010	0.06
Rumensin 90.7 g/lb	0.002	0.01

[1] kg/cow/day on an as fed basis

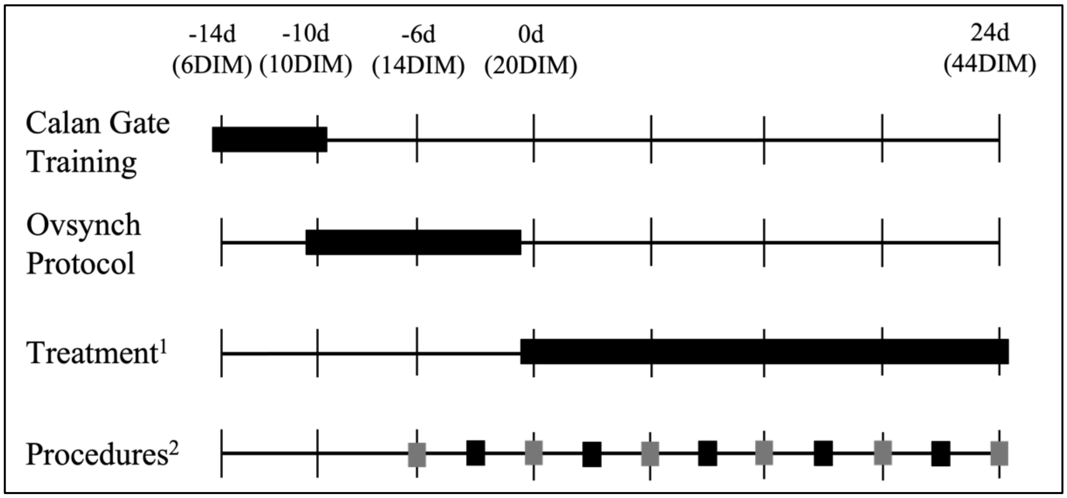

Figure 1. Experimental timeline indicating the duration as d of experiment (d) and (DIM) of each event of interest. Black bars span the length of each indicated method.

[1] Treatments were either 1) control (Con; base TMR only) or 2) chromium propionate (CrPro; base TMR plus 12 mg/h/d of Cr).

[2] Black boxes indicate individual procedure dates that included transrectal ultrasound, collection of blood sample, blood glucose measurement, measurement of respiration rate and measurement of rectal temperature. Grey boxes indicate individual procedure dates that included the same procedures as black boxes plus endometrial cytology sampling.

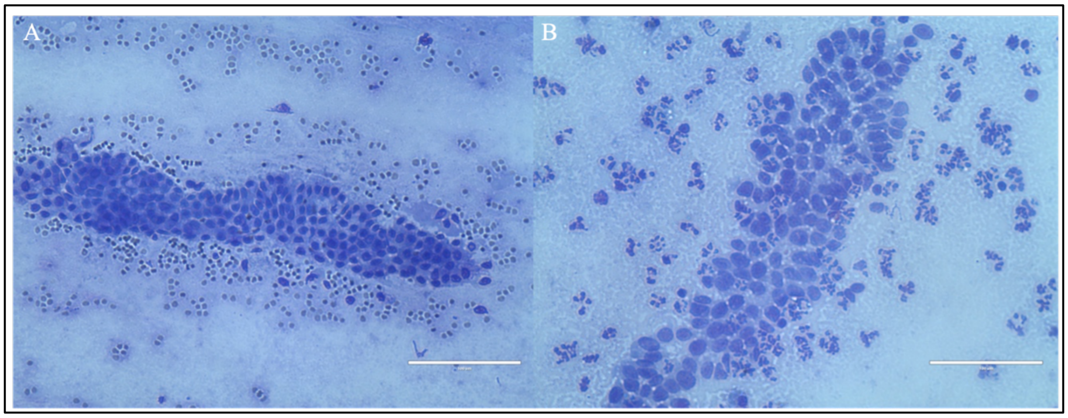

Figure 2. Representative images of endometrial cytology samples from early lactating cows. Panel A represents a sample with little to no polymorphonuclear leukocytes (PMNL) present. Panel B represents a sample with a high number of PMNLs present. Scale bar = 100µm.

Table 2. Ovarian dynamics between treatments groups in early lactating dairy cows.

Parameters	Con[1]	CrPro[2]	P-value
Number of CLs[3]	1.27 ± 0.08	1.38 ± 0.08	P=0.34
Average CL Diameter (mm)	15.37 ± 0.72	15.65 ± 0.67	P=0.78
Number of Follicles <5mm	5.18 ± 0.47	5.56 ± 0.45	P=0.56
Number of Follicles 6-9mm	1.88 ± 0.17	2.26 ± 0.16	P=0.09[a]
Average Diameter of 6-9mm Follicles (mm)	6.95 ± 0.09	7.21 ± 0.08	P=0.04[b]
Number of Follicles 10-15mm	0.87 ± 0.09	0.95 ± 0.08	P=0.52
Average Diameter of 10-15mm Follicles (mm)	11.85 ± 0.17	11.54 ± 0.16	P=0.18
Number of Follicles >15mm	0.35 ± 0.06	0.24 ± 0.06	P=0.17
Average Diameter of >15mm Follicles (mm)	16.46 ± 0.42	16.61 ± 0.43	P=0.79

[1] Con = control treatment group (base TMR only)

[2] CrPro = chromium propionate treatment group (base TMR plus 12 mg/h/d Cr)

[3] CL = corpus luteum

[a] denotes tendency (P<0.10) between treatment groups

[b] denotes significance (P<0.05) between treatment groups

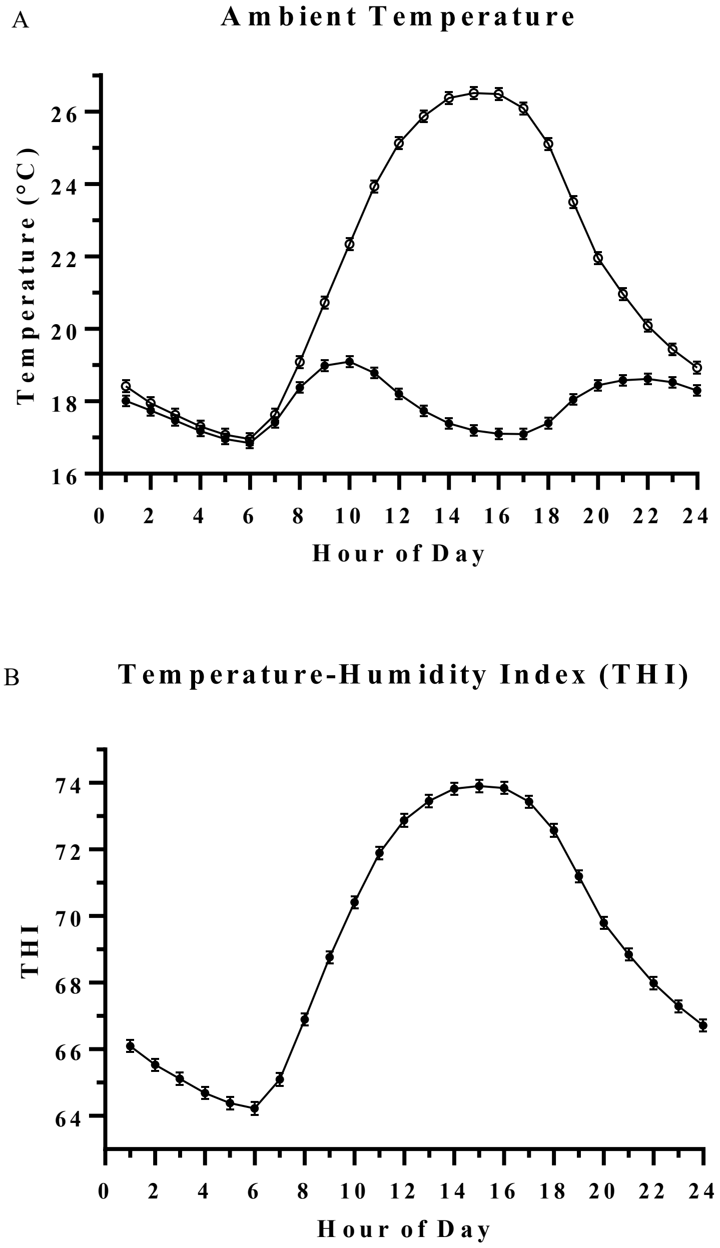

Figure 3. Hourly means for dew point (●) and dry bulb (○) temperatures (C°) collected from a nearby weather station (Panel A). Dew point and dry bulb values were used to calculate temperature-humidity index hourly means (Panel B). $THI = T_{\text{dry bulb}} + ((0.36 * T_{\text{dew point}}) + 41.2)$.

Table 3. Mean physiological parameters of early lactating cows.

Parameters	Con[1]	CrPro[1]	P-value
Feed Intake (kgs)[2]	44.66 ± 1.78	42.33 ± 1.63	P=0.35
Milk Yield (kgs)	57.75 ± 2.28	57.12 ± 2.09	P=0.84
Feed Intake: Milk Yield (kgs)	0.79 ± 0.03	0.75 ± 0.03	P=0.39
Change in BW (kgs)	-1.18 ± 4.56	-0.63 ± 4.56	P=0.93
Respiration Rate (bpm)	66.97 ± 1.40	68.19 ± 1.31	P=0.52
Rectal Temperature (C°)	39.20 ± 0.04	39.16 ± 0.04	P=0.46
Blood Glucose (mg/dL)	58.96 ± 0.52	58.41 ± 0.49	P=0.44

[1] Control (Con; n=10); Chromium supplementation (CrPro; 12 mg/h/d Cr; n=12)

[2] Feed Intake on an as-fed basis for the duration of the study

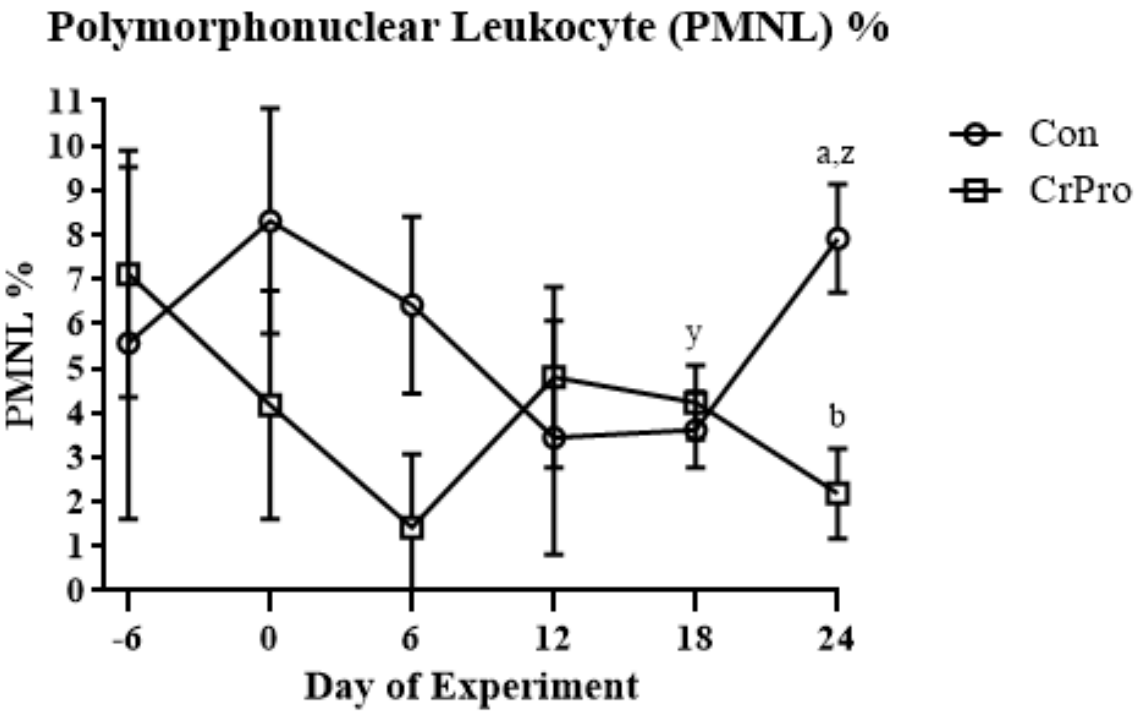

Figure 4. Mean percentage of polymorphonuclear leukocytes (PMNL) of cells counted in the endometrial cytology sample differed (treatment x sample interaction P=0.01). Con = control treatment group of base TMR only. CrPro = chromium propionate treatment group of base TMR plus 12 mg/h/d of Cr.

^{a,b}denotes significance (P=0.01) between treatment groups

^{y,z}denotes significance (P=0.02) within the Con group

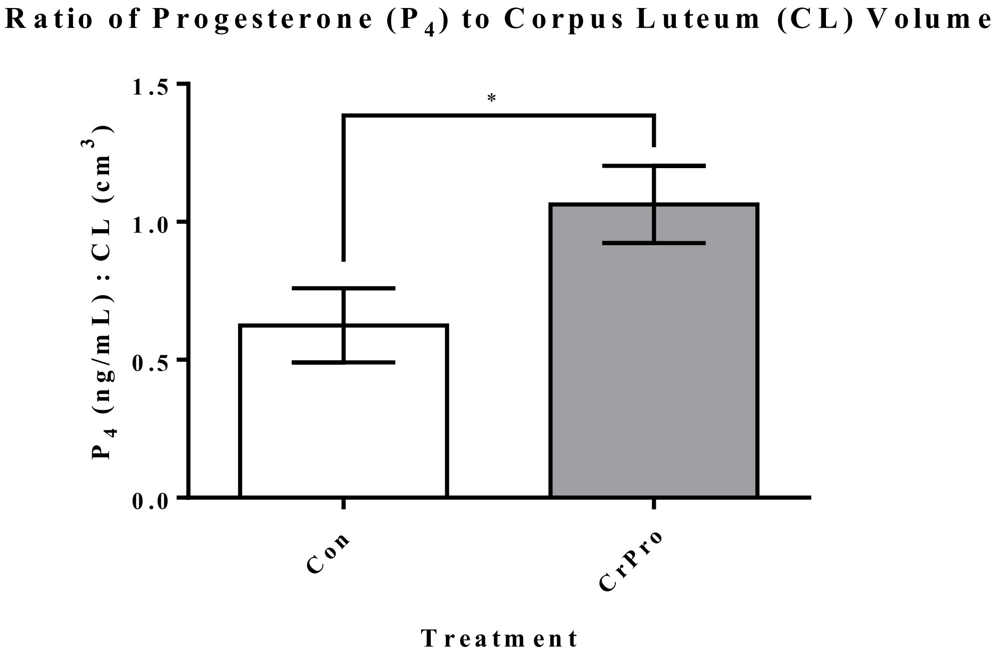

Figure 5. Mean ratio of progesterone (P_4; ng/mL) to corpus luteum (CL) volume (cm^3) between treatment groups. Con = control treatment group of base TMR only. CrPro = chromium propionate treatment group of base TMR plus 12 mg/h/d of Cr. *denotes significance ($P=0.03$) between treatment groups

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APPENDIX 1: Short-term consumption of the mycotoxin zearalenone by pubertal gilts causes persistent changes in the histoarchitecture of reproductive tissues

Introduction

The mycotoxin zearalenone (ZEN) is produced by fungi in the genus *Fusarium* (Gajęcki et al., 2009), and can develop during any stage of livestock feed production or storage (Kabak et al., 2006; Bryden, 2012; Leslie and Logrieco, 2014). While the FDA has not established a limit for ZEN contamination of livestock feed components (Liu and Applegate, 2020), research has suggested that the ingestion of ZEN-contaminated cereal grains is temporarily tolerable up to 0.2 µg/kg of body weight (Gajecki, 2002). At or above this concentration, ZEN can detrimentally affect feed intake, average daily gain and multiple aspects of swine reproductive efficiency.

A recent survey of swine feeds and feedstuffs in the U.S. found that nearly half of the analyzed samples contained ZEN (Pack et al., 2021). Zearalenone is problematic for a number of reasons but is probably best known as a potent phytoestrogen (Chang et al., 1979; Etienne and Jemmali, 1982; Farnworth and Trenholm, 1983; Gajęcki et al., 2009; Kanora and Maes, 2010). Its structural similarity to estrogen allows it to competitively bind to estrogen receptors, thus disrupting reproductive function (Gajęcki et al., 2009). As a result, consumption of ZEN has been associated with altered estrous cyclicity, reduced embryonic and fetal development, pseudopregnancy and pelvic organ prolapse (Etienne and Jemmali, 1982; Edwards et al., 1987a; Edwards et al., 1987b; Gajęcka et al., 2011). These consequences of ZEN consumption contribute to reproductive failure, which is a significant source of profit loss on swine farms (Arango et al., 2005), thereby impacting the productivity and profitability of swine operations.

Beyond its effects on reproduction, ZEN detrimentally affects a multitude of other organs and systems throughout the body. Zearalenone consumption induces stress and causes inflammation and/or deterioration of the liver and kidneys (Jiang et al., 2010). Hepatic lesions, structural abnormalities and cellular damage are commonly reported (Skiepko et al., 2020; Dolenšek et al., 2021; Papatsiros et al., 2021; Zhang et al., 2021). It is also known to affect hematocrit, white blood cell count, hemoglobin and platelet count (Maaroufi et al., 1996; Jiang et al., 2010). Within the cell, ZEN exposure adversely affects mitochondria, lysosomes, endoplasmic reticula, Golgi bodies, DNA synthesis, protein synthesis and protein degradation (Kouadio et al., 2005; Wang et al., 2022). Furthermore, in long-term studies involving the consumption of high doses of ZEN, rodents developed hepatic and pituitary adenomas and carcinomas (1982). These are just some of the numerous whole-body and tissue-specific effects of ZEN. In addition to the consequences reported in the literature, there are likely a myriad of other effects that have yet to be elucidated.

Many of the known detrimental actions of ZEN exposure are microscopic or molecular in nature, and therefore, not immediately apparent in production settings. For this reason, it is important to learn as much as possible about the effects of ZEN in controlled research studies using concentrations that could be experienced on-farm. In order to broaden the current understanding of the detrimental effects of ZEN, the objective of this study was to monitor whole-body responses to ZEN consumption, with an emphasis on the morphology and histology of gilt reproductive tissues following short-term exposure. Growth, carcass weight, liver weight and reproductive tissues were evaluated after 7 d of ZEN consumption followed by a 14-d recovery, or after a full 21 d of ZEN exposure. We hypothesized that even short-term exposure to moderate concentrations of ZEN would detrimentally affect the liver and female reproductive tract, but that these effects would be transient and resolve in 14 d or less.

Materials and Methods

Animals and Treatments

All animal procedures were approved by the Virginia Tech Institutional Care and Use Committee (IACUC). Thirty cross-bred pubertal gilts (107.25 ± 2.69 kg) were selected for two replicate experiments, during which they were housed individually at the Virginia Tech Swine Center. Animals were randomly assigned to have one of three treatments added to their daily feed ration: 1) solvent only for 21 d (CON; n=10), 2) ZEN for 7 d followed by 14 d of solvent (ZEN-7; n=10), and 3) ZEN for 21 d (ZEN-21; n=10). Commercially available swine grower feed (Big Spring Mill, Elliston, VA) was the base feed to which all treatments were added.

Description of treatment and feed preparation is provided in detail in previous work (Pack et al., 2020a; Pack et al., 2020b). Briefly, the ZEN solution was prepared by dissolving crystalline ZEN (≥98% purity, J&K Scientific, Beijing, China) in acetonitrile (Thermo-Fisher, Waltham, MA, USA) to produce a working solution of 10 mg/mL. Individual doses were prepared by adding the working solution to small quantities of feed (600 □L working solution to 20 g of feed) and placing it under a fume hood overnight to allow the solvent to evaporate. Solvent-only feed was prepared in a similar manner using equivalent amounts of acetonitrile. In total, gilts receiving ZEN treatment were fed 6 mg of ZEN per d.

In order to ensure consumption of the entire treatment (solvent or solvent + ZEN), the daily ration was offered in a stepwise manner. At feeding, gilts were initially offered 227 g of feed containing their assigned treatment (solvent or solvent + ZEN). After the animals had consumed the treated feed, the remainder of their daily ration was offered (2.04 kg). The gilts were observed throughout the feeding process to ensure the entirety of the treatment and daily ration were consumed. Animals were examined daily for symptoms typically associated with ZEN exposure including decreased feed intake, vulva swelling, and vulva reddening.

Tissue Collection

At the end of the 21 d experiment, the gilts were harvested at the Virginia Tech Meat Center. Reproductive tracts and livers were collected. Ovaries were examined to determine whether gilts were luteal or non-luteal at the time of sacrifice. Liver weight, overall tract weight and individual tissue weights, lengths, and widths were measured and recorded immediately after harvest.

Histology and Morphometry

After the uterine and ovarian tissues were weighed and measured, samples from the left and right uterine horns, as well as the left and right oviduct were dissected and fixed for 24 h in 10% formalin. The preserved tissues were then trimmed, transferred to 70% ethanol and shipped to Histo-Scientific Research Laboratories, Inc. (Mount Jackson, VA) for paraffin embedding, sectioning and staining with hematoxylin and eosin (H&E). An EVOS xl Core Imaging System (Life Technologies Corporation, Carlsbad, CA) was used to capture images from multiple fields of view on one slide of each of the four tissues from each pig. Each image represented one field of view. The number of images collected varied based upon the size of the tissue, or the specific structure being evaluated. Tissue-specific elements were measured using ImageJ Software (National Institutes of Health, Bethesda, MD) and representative images are presented in Figure 1. For uterine tissue, epithelial cell height (6 images x 10 measurements/image; 400x), endometrial thickness (3 images x 10 measurements/image; 400x), myometrial thickness (3 images x 10 measurements/image; 400x), and uterine gland density (uterine glands/image, 6 images; 200x) were measured and recorded. Measurements of the oviductal tissue included epithelial cell height (6 images x 10 measurements/image; 400x), submucosal thickness (1 image x 15 measurements/image; 100x for isthmus; 200x for ampulla), and thickness of the muscularis (1 image x 15 measurements/image; 100x for ampulla; 200x for isthmus).

Statistical Analyses

Three gilts were removed from the experiment for reasons unrelated to the treatments. Data were analyzed using the MIXED procedure of SAS (SAS Institute, Inc, Cary, NC). Independent variables were treatment (control, ZEN-7 or ZEN-21), luteal status (luteal or non-luteal) and their interaction. Experimental replicate and harvest group were included in the model as covariates. When replicate, group and/or luteal status were not significant, they were removed from the statistical model. The change in body weight was calculated by subtracting initial body weight from the final body weight. Dressing percent was calculated by dividing carcass weight by final body weight. Organ weights were analyzed in their raw form (absolute weight) as well as calculated as a percent of body weight and percent of carcass weight. Pig was included as the repeated variable. For each analysis, eight covariance structures were tested and the most appropriate was selected based upon Akaike's information criterion, Akaike's information criterion with correction and Bayesian information criterion values. Results are reported as least squares means ± standard errors of the means. Statistical significance was declared at $P \leq 0.05$ and tendencies to differ at $0.05 \leq P \leq 0.10$.

Results and Discussion

In this study, the pigs assigned to ZEN treatment received 6 mg of ZEN per d in 2.27 kg of feed, thus equating to 2.64 ppm (2.64 mg/kg of feed; Pack et al., 2020a). While low in comparison to concentrations administered in some published studies (Farnworth and Trenholm, 1981; Etienne and Jemmali, 1982; Gao et al., 2022), this amount is at the high end of concentrations typically found in ingredients used for livestock feeds (Khatibi et al., 2014; Pack et al., 2021). Major disruptions in reproductive function such as abnormal estrous cycles and pelvic organ prolapse have been observed when gilts consumed feed containing as little as 1 ppm of ZEN (J.J. Zimmerman, 2019), while molecular and histological changes in the reproductive tract have been demonstrated at even lower doses (Song et al., 2021; Wan et al., 2022). In the current study, animals were examined each day throughout the experiment for symptoms associated with ZEN consumption, but no gross morphological changes such as vulva swelling or reddening were observed. Furthermore, no clinical abnormalities of tissues were found at the time of harvest and tissue collection.

Whole Body and Liver Weights

At the beginning of the study, gilts were randomly assigned to treatments based on body weight, which ultimately did not differ between treatment groups (Table 1). Final body weight, change in body weight over the 21 d of the experiment, carcass weight and dressing percentage were also similar between treatment groups (Table 1). These results are consistent with the results of many previous studies. Although sometimes associated with reduced feed intake and average daily gain (Diekman and Green, 1992), consumption of ZEN-contaminated diets often has no impact on growth and body weight (Young and King, 1986; Jiang et al., 2009).

Following consumption, ZEN can be found in liver tissue (Pack et al., 2020b) where it affects liver structure and functional competence (Tiemann et al., 2006). Zearalenone alone, or in combination with other fusariotoxins induces hepatic cell apoptosis and necrosis, increases inflammatory infiltrates, causes dilation of hepatic sinusoids, and alters the amount of interlobular connective tissue (Smith et al., 2017; Skiepko et al., 2020; Dolenšek et al., 2021). In light of these previously demonstrated effects of ZEN consumption on liver structure and function, liver weights were collected at harvest in the current study and analyzed as absolute weight as well as a percentage of body weight and a percentage of carcass weight. No treatment-based differences existed in any of the liver weight parameters (Table 1). The lack of differences was not unexpected based on previous work (Wang et al., 2012; Denli et al., 2015). Furthermore, liver weight is a gross measurement where changes could be masked by the balance of consequences that would increase liver weight (such as inflammation) versus those consequences that would reduce liver weight (such as apoptosis and necrosis).

Gross Measurements of the Reproductive Organs

The combined and individual weights of the reproductive tissues as well as the length of the uterine horns are presented in Table 2. Similar to body and liver weight analyses, no significant differences were observed in the weight or length measurements of the reproductive tissues across treatment groups. While ZEN consumption does not always cause enlargement of the reproductive tract (Etienne and Jemmali, 1982), the results of this study conflict with studies showing increased edema, protein synthesis and cell proliferation in the tubal portions of the reproductive tract (Obremski et al., 2003; Zhou et al., 2018a). Furthermore, previous analyses of the tissues collected from these gilts found that ZEN and/or its metabolite, α-zearalenol, was present in measurable quantities within many of the reproductive tissues (Pack et al., 2020b). In previous studies where differences in reproductive tract or organ weight were reported, the direction of the change was towards heavier tissue weights in those pigs consuming ZEN (Wang et al., 2012; Denli et al., 2015; Song et al., 2021), consistent with the phytoestrogenic properties of ZEN.

Histology of the Reproductive Organs

While there were no gross differences in the reproductive tissues, the histological analyses revealed differences that were not initially apparent. Treatment-based differences were observed in both the oviduct and uterus. In some cases, those differences were independent of luteal status, while for others there was an interaction between the treatments and luteal status.

In the ampulla, the thicknesses of the epithelial and submucosal layers were similar between treatments (P=0.94 and P=0.49, respectively), while the thickness of the muscularis decreased with ZEN exposure. The ampullary muscularis was thickest in CON pigs, intermediate in ZEN-7 pigs and lowest in ZEN-21 pigs (Figure 2). Intermediate thickness in the ZEN-7 treatment group could indicate less severe consequences of the shorter duration of ZEN exposure or partial recovery since tissues were collected 14 d after the cessation of ZEN treatment. Within the isthmus of the oviduct, epithelial cell height was low in CON pigs and greater in ZEN-7 and ZEN-21 pigs (Figure 2). The similarity in epithelial cell height between the ZEN-7 and ZEN-21 treatments suggests that the effects of ZEN consumption on this layer of the isthmus are long lasting, as they persisted for 14 d after the termination of ZEN treatment. The submucosa of the isthmus did not differ between treatments (P=0.52). The muscularis of the isthmus differed by treatment as it was greater in the CON pigs (1094.2 ± 34.4) than ZEN-21 pigs (920.7 ± 32.8; P<0.01), and also differed based on the interaction between treatment and luteal status. In the absence of corpora lutea, the thickness of the isthmic muscularis was similar between all treatments. It was only during the luteal phase that differences emerged. In the luteal phase, pigs that had consumed ZEN (ZEN-7 and ZEN-21) exhibited thinner muscularis layers in the isthmus than CON pigs (Figure 3A). These luteal-phase-specific findings are noteworthy as there are fewer numbers of estrogen receptors in porcine oviductal cells in the presence of progesterone (Chen et al., 2013). Consequently, gilts have comparatively limited ability to respond to phytoestrogens during the luteal phase, yet this was the phase during which differences were detected.

All layers of the uterus were affected by the treatment and/or the interaction between the treatment and luteal status. Uterine gland density, however, was similar across all analyses (P=0.14), which agrees with previously published work (Döll et al., 2004). The uterine endometrium was thicker in those pigs that had consumed ZEN (Figure 2) but was not affected by luteal status or the interaction. The ZEN-induced increase in thickness of the endometrium is consistent with previous reports (Gajęcka et al., 2012; Zhou et al., 2018b; Zhou et al., 2019). There was no main effect of treatment on uterine epithelial cell height (P=0.15). Uterine epithelial cell height following ZEN exposure has been previously measured in pregnant or prepubertal females with some reporting an increase in cell height (Wu et al., 2020) and others reporting no change (Long et al., 1992; Döll et al., 2004) as was demonstrated in the current study by the lack of main effect of treatment. Despite the absence of main effect of treatment, an interaction between treatment and luteal status was evident. For CON and ZEN-7 pigs, the uterine epithelial cell height decreased from non-luteal to luteal status. This difference is consistent with previously reported cycle-based fluctuations in uterine epithelial cell height (Walter and Bavdek, 1997; Kangawa et al., 2017). In contrast, the uterine epithelial cell height in ZEN-21 pigs was nearly identical for non-luteal and luteal stages (Figure 3B). The uterine myometrium differed in an inverse manner. The thickness of the myometrium was similar for non-luteal and luteal pigs in the CON and ZEN-7 treatment groups. Pigs consuming ZEN for 21 d had a thicker myometrium during the luteal phase (Figure 3C). Similar findings have been reported in pre-pubertal gilts exposed to less than half of the ZEN dose used in the current study (Zhou et al., 2019). However, much like the previously described findings in the isthmic muscularis, these changes were unexpected as they occurred in the presence of progesterone, when sensitivity to phytoestrogen exposure would likely be limited by estrogen receptor populations (Koziorowski et al., 1984). In uterine myometrium, there was also a tendency for a main effect of treatment (P=0.06), with myometrial thickness being greater in ZEN-21 pigs than in CON pigs. This increase with ZEN exposure is similar to previous reports (López et al., 1988; Gajęcka et al., 2012; Zhou et al., 2018b; Zhou et al., 2019).

To our knowledge, this is the first report of reproductive tract histology during both the non-luteal and luteal phase following exposure to a phytoestrogenic mycotoxin. In general, it appeared that ZEN consumption interrupted expected fluctuations in the respective tissues; appearing to either cause or block changes compared to what was observed in CON pigs. It is notable that where interactions between treatment and luteal status were detected, the differences were always found in tissues from the luteal phase. As previously mentioned, during the luteal phase, estrogen receptor populations are typically low in oviductal (Chen et al., 2013) and uterine cells (Koziorowski et al., 1984), thereby limiting the ability of these reproductive tissues to respond to the phytoestrogenic properties of ZEN. Regardless of tissue sensitivity, ZEN consumption caused changes in the histoarchitecture of the oviduct and uterus in the current study. These effects were likely a consequence of untimely receptor activation. Normally, the reduction in receptor numbers during the luteal phase would coincide with relatively low levels of estrogen production. In the presence of ZEN, however, estrogen receptors are aberrantly activated in the luteal phase, resulting in the histological differences in reproductive tissues observed herein.

Unfortunately, elucidation of the molecular mechanisms responsible for the ZEN-induced changes in the oviduct and uterus was beyond the scope of this study. Irrespective of the ontogenesis, histomorphological differences in reproductive tissues are problematic as variations are associated with subsequent fertility (Małopolska et al., 2021). The thinned muscularis of the isthmus during the luteal phase as well as the overall thinner muscularis of the ampulla is concerning considering the importance of these structures for luteal-phase events such as sperm and oocyte transport leading up to fertilization and subsequent transport of embryos towards the uterus. Likewise, the observed differences in the uterine layers (most of which were specific to the luteal phase) indicate detrimental changes in uterine competency and function that are essential to early embryo development and implantation (Wu et al., 2020). While ZEN-induced changes in the oviductal and uterine histoarchitecture were evident in the present study, additional work is needed to confirm the impact of ZEN on the functional capacity of these tissues.

Conclusions

The amount of ZEN consumed by gilts in the present study did not produce any phenotypic or morphologic changes in their external genitalia, reproductive organs, liver or overall growth. Closer examination of the reproductive tissues revealed changes in the histoarchitecture of portions of the tract in both groups of pigs consuming ZEN. Many of these changes were evident after only 7 d of ZEN exposure and then persisted through the 14-d recovery period, indicating that the effects of ZEN exposure can develop quickly and be long-lasting. Taken together, the results of this experiment are proof that ZEN consumption, even at levels below the threshold for phenotypic and morphologic symptoms, can affect reproductive tissues long-term. Ultimately, these findings underscore the insufficiency of visible symptoms as the sole indicators of harmful levels of ZEN contamination in swine feed.

Figures and Tables

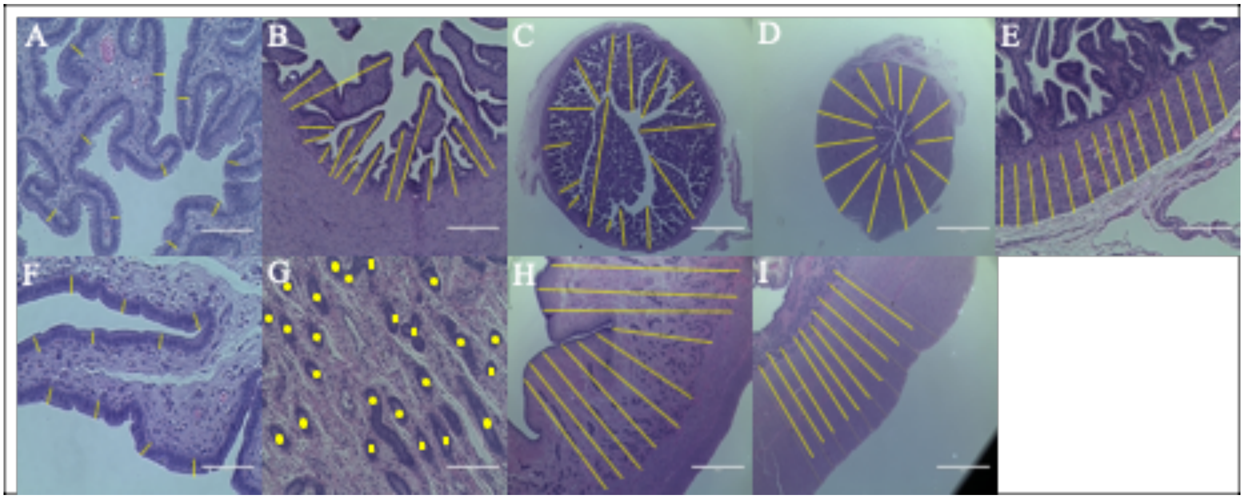

Figure 1. Examples of histological measurements conducted in oviductal and uterine tissues of pubertal gilts. Cross-sections were H&E stained, and measurements were taken with ImageJ software. Oviduct epithelial cell height (A), isthmus submucosal thickness (B), ampulla submucosal thickness (C), isthmus muscularis thickness (D), and ampulla muscularis thickness (E) measurements were recorded. Uterine epithelial cell height (F), gland density number (G), endometrial thickness (H), and myometrial thickness (I) measurements were recorded. Yellow lines or dots indicate points of measurement or count. Scale bar = 1,000 μm (C, D, H, I), 200 μm (B, E, G), or 100 μm (A, F).

Table 1. Measurements of body, carcass and liver characteristics of gilts consuming zearalenone (ZEN).

Component	Control[1]	ZEN-7[1]	ZEN-21[1]	P-value
Initial BW (kg)	104.29±5.33	109.73±5.44	107.77±4.78	P=0.78
Final BW (kg)	115.29±5.70	112.66±5.33	115.56±6.74	P=0.92
Change in BW (kg)	7.98±2.18	5.37±2.07	10.33±2.14	P=0.31
Carcass Wt. (kg)	86.69±3.45	83.53±3.69	85.91±3.27	P=0.81
Dressing %	73.88±0.76	73.86±0.69	72.88±0.87	P=0.63
Liver Wt. (kg)	1.72±0.08	1.54±0.08	1.73±0.07	P=0.20
Liver % of BW	1.62±0.08	1.40±0.07	1.45±0.10	P=0.13
Liver % of Carcass	2.09±0.10	1.86±0.11	2.01±0.09	P=0.31

[1] Control, solvent only for 21 d (CON; n=9); ZEN for 7 d followed by 14 d of solvent (ZEN-7; n=8); ZEN for 21 d (ZEN-21; n=10).

Table 2. Morphological characteristics of reproductive tissues of gilts consuming zearalenone (ZEN).

Component	Control[1]	ZEN-7[1]	ZEN-21[1]	P-value
Total Tract Wt. (g)	471.1±80.5	598.8±85.3	668.0±76.3	P=0.22
Tract % of BW	0.36±0.08	0.51±0.07	0.58±0.09	P=0.16
Tract % of Carcass	0.54±0.08	0.70±0.09	0.76±0.08	P=0.16
Ovary Wt. (g)	13.3±5.3	13.8±5.6	21.0±5.0	P=0.51
Ovary % of BW	0.01±0.01	0.01±0.01	0.02±0.01	P=0.28
Ovary % of Carcass	0.01±0.01	0.02±0.01	0.02±0.01	P=0.41
Total UT[2] Wt. (g)	367.8±69.1	472.5±73.3	466.0±65.5	P=0.50
UT % of BW	0.27±0.06	0.41±0.06	0.43±0.07	P=0.18
UT % of Carcass	0.42±0.07	0.55±0.08	0.52±0.07	P=0.41
Cervix Wt. (g)	57.8±10.1	61.3±10.7	82.0±9.5	P=0.19
Cervix % of BW	0.05±0.01	0.05±0.01	0.08±0.01	P=0.25
Cervix % of Carcass	0.07±0.01	0.07±0.01	0.10±0.01	P=0.17
UT Horn Length (cm)	211.1±33.3	213.4±35.6	191.0±31.5	P=0.87

[1] Control, solvent only for 21 d (CON; n=9); ZEN for 7 d followed by 14 d of solvent (ZEN-7; n=8); ZEN for 21 d (ZEN-21; n=10).

[2] UT=uterine

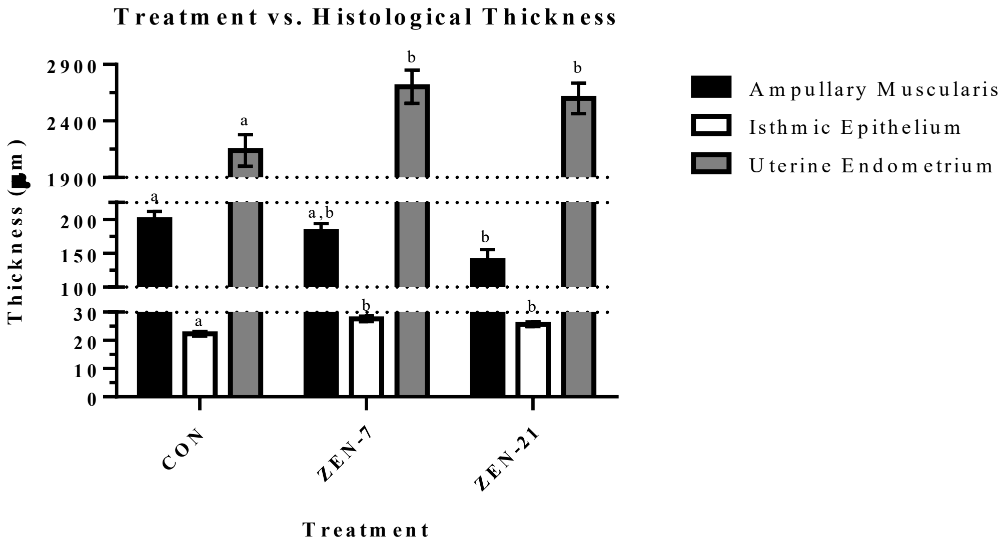

Figure 2. Thickness of the ampullary muscularis, isthmic epithelium and uterine endometrium of gilts assigned to control (CON), ZEN for 7 d followed by 14 d of solvent (ZEN-7) or ZEN for 21 d (ZEN-21). $^{a,b}P<0.05$

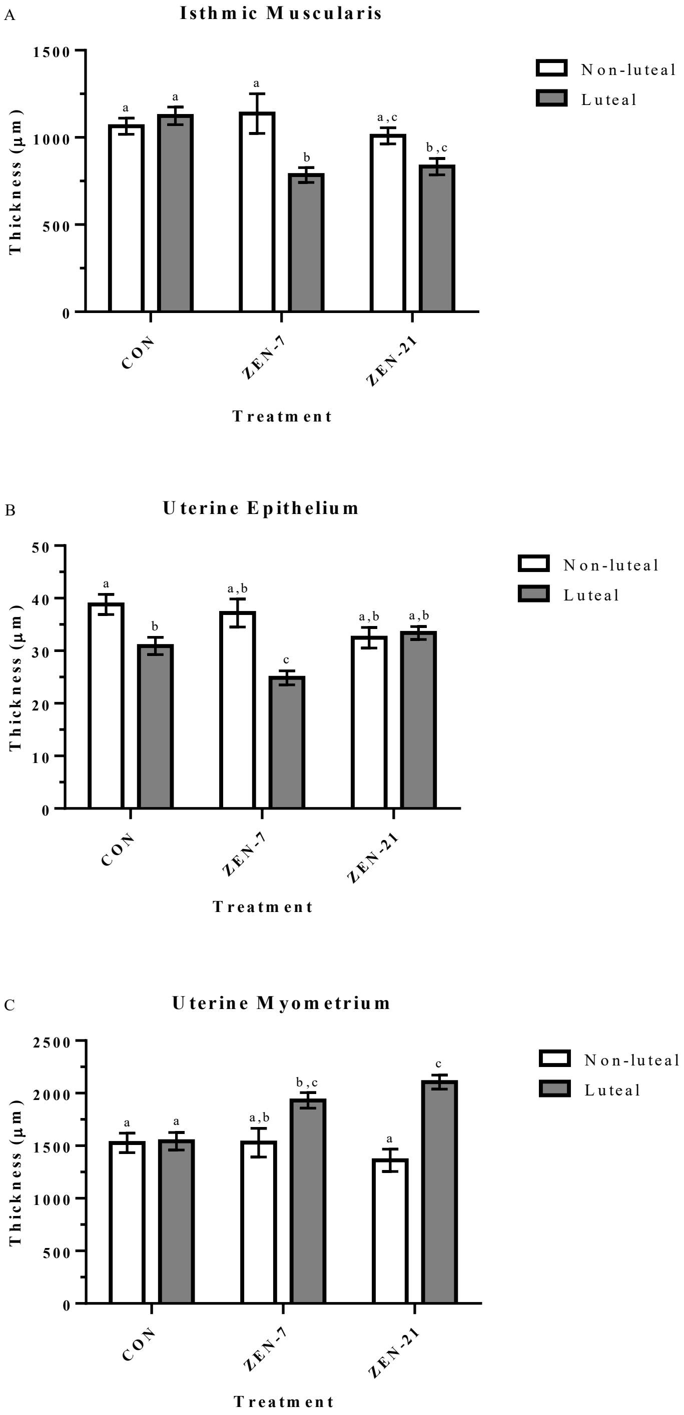

Figure 3. Thickness of the isthmic muscularis (A), uterine epithelium (B) and uterine myometrium (C) during the non-luteal and luteal phase of gilts assigned to control (CON), ZEN for 7 d followed by 14 d of solvent (ZEN-7) or ZEN for 21 d (ZEN-21). $^{a,b,c}P<0.05$

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APPENDIX 2: Overarching Conclusions

Taken together, the results reported in this thesis demonstrate the ability of feed additives (whether they be contaminants or purposely added minerals) to affect the reproductive tracts of livestock, implying that they could directly affect reproductive performance. These results contribute to the base of knowledge already present in published literature concerning the detrimental effects of zearalenone and the potential benefits of chromium. Overall, it is important for scientists, industry professionals, and producers to consider the reproductive impact of management decisions related to feed additives. Tightening profit margins and climate-related production challenges require that livestock operations be as efficient as possible. The feed quality and nutrients offered to livestock is one aspect of production that can be tightly monitored and controlled. As such, any benefit we can glean from minimizing contaminants and enriching specific nutrients should be exploited to improve efficiency.