

**EFFECTS OF DISEASE INDUCED INFLAMMATION ON REPRODUCTIVE
NEUROENDOCRINOLOGY IN DOMESTIC RUMINANTS**

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Allison Nicole Renwick

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ABSTRACT

Successful reproduction is dependent on the pulsatile release of gonadotropin releasing hormone (GnRH), the final common output from the central nervous system that stimulates luteinizing hormone (LH) secretion to control reproduction. While GnRH, and subsequently LH, is released in an episodic pattern, the “GnRH pulse generator” is believed to be a population of neurons in the arcuate nucleus of the hypothalamus that express kisspeptin, named KNDy neurons. Reproductive success is dependent on various internal and external cues that ultimately regulate GnRH/LH secretion; however, the underlying central mechanisms of inflammation induced suppression of reproduction have yet to be fully elucidated. Chapter I is a review of relevant scientific literature that pertains to the relationship between inflammation and GnRH/LH secretion. Therein, the focus is on the hypothalamic-pituitary-gonadal axis, KNDy neurons, immune response and regulation of GnRH/LH secretion through inflammation. Chapter II focuses on an experiment to elucidate the effects of acute endotoxin-induced inflammation on kisspeptin neurons in adult cows. Chapter III focuses on an experiment to determine the role of cyclooxygenase 1 and 2 in the acute endotoxin induced suppression of reproduction in adult ewes. Chapter IV focuses on an experiment to evaluate the effects of chronic endotoxin induced inflammation on kisspeptin neurons in castrated, male sheep. Each chapter provides a succinct introduction of the current understanding on endotoxin induced suppression of LH secretion and the lack of knowledge surrounding this central regulation in domestic ruminants. Methodologies are provided for our collection and analysis of LH secretion, stress and immune system markers, and immunohistochemistry for reproductively relevant

proteins. Chapters II through IV are concluded with discussions of the findings with those of other researchers and considerations of follow-up experiments to strengthen the current studies. In Chapter V, the implications of this research are discussed and a current working model is provided for inflammation induced regulation of GnRH/LH secretion. In addition, it is postulated that a greater understanding of these underlying mechanisms will 1) aid to increase productivity and profitability in domestic livestock and 2) identify targets useful for therapeutic strategies to prevent or lower the risk of infertility in humans.

TABLE OF CONTENTS

CHAPTER 1: LITERATURE REVIEW	1
Introduction.....	1
Livestock Implications.....	2
Human Implications.....	3
Overview of Hypothalamic-Pituitary-Gonadal Axis Physiology	5
History of GnRH and LH.....	6
Importance of GnRH/LH secretion.....	7
Estrous Cycle	8
Kisspeptin-Neurokinin B-Dynorphin Neurons in Reproduction	9
Kisspeptin	10
Neurokinin B.....	13
Dynorphin	14
KNDy Neurons	15
Immune Response	15
Immune System	15
Prostaglandins	18
Endotoxin Inflammation Impairs Reproduction	20
LPS and Steroid Hormones.....	20
LPS and GnRH/LH Secretion.....	22
LPS and Kisspeptin.....	23
Introduction to Research	24
Cattle as a Model	24
Sheep as a Model	24
Intact vs. Gonadectomized and Male vs. Female	25
Chronic Inflammation.....	26
Non-Steroidal Anti-Inflammatory Drugs.....	28
Experiment 1 – Evaluating an Acute Dose of Endotoxin in Adult Cows.....	29
Experiment 2 – Evaluating a General COX Inhibitor in an Acute Endotoxin Model	30
Experiment 3 – Defining a Chronic Endotoxin Model for Livestock	30
References.....	32
CHAPTER TWO: ACUTE ENDOTOXINEMIA INHIBITS LUTEINIZING HORMONE AND DECREASES HYPOTHALAMIC KISSPEPTIN IN ADULT COWS.	42
Introduction.....	43
Materials and Methods.....	45
Estrous Synchronization	46
Experiment 1	47
Experiment 2.....	47
LPS Preparation	48
Blood Sample Processing	48
Tissue Collection	49

Luteinizing Hormone Analysis	49
Progesterone Analysis.....	50
Cytokine Analysis.....	50
Immunohistochemistry	51
Image Analysis.....	51
Statistical Analysis.....	52
Results.....	52
Experiment 1 Vaginal Temperature.....	52
Experiment 1 Progesterone Concentration	53
Experiment 1 Cytokine Concentration.....	53
Experiment 1 Luteinizing Hormone Analysis	54
Experiment 2 Vaginal Temperature.....	55
Experiment 2 Cytokine Concentration.....	55
Experiment 2 Luteinizing Hormone Analysis	56
Experiment 2 Kisspeptin.....	56
Discussion	56
Acknowledgements.....	59
References.....	61
Appendix.....	65
CHAPTER 3: FLUNIXIN ATTENUATES INFLAMMATION INDUCED SUPPRESSION OF LUTEINIZING HORMONE IN ADULT EWES.	72
Introduction.....	73
Materials and Methods.....	76
Animals.....	76
Experimental Design.....	77
Estrous Synchronization	77
Treatments.....	78
LPS Preparation	78
Blood Sample Preparation	78
Tissue Collection	79
Temperature Analysis	80
Luteinizing Hormone Analysis	80
Progesterone Analysis.....	80
Immunohistochemistry	81
Image Analysis.....	82
Statistical Analysis.....	83
Results.....	83
Temperature	83
Progesterone Concentration.....	84
Luteinizing Hormone Analysis	84
LHbeta Immunopositive Cells	86
ARC Kisspeptin, cFos, and Dual Labeled Immunopositive Cells.....	87
POA Kisspeptin Immunopositive Cells.....	88
Discussion	88

Acknowledgements	95
References	96
Appendix	101
CHAPTER 4: CHRONIC INFLAMMATION DECREASES ARCUATE KISSPEPTIN EXPRESSION IN CASTRATED MALE SHEEP.	110
Introduction	111
Materials and Methods	114
Animals	114
Experimental Design	115
LPS Preparation	116
Blood Sample Processing	117
Tissue Collection	117
Luteinizing Hormone Analysis	118
Steroid Hormone Analysis	118
Immunohistochemistry	119
Image Analysis	120
Statistical Analysis	121
Results	121
Temperature	121
Steroid Hormone Concentrations	122
Luteinizing Hormone Analysis	124
Kisspeptin/cFos	126
LHbeta	126
Discussion	127
Acknowledgements	135
References	136
Appendix	141
CHAPTER 5: FUTURE DIRECTIONS AND CONCLUSIONS	151
Future Directions	151
NKB	152
Dynorphin	152
Dopamine	153
Peripheral Metabolic Hormones	154
Insulin	155
Glucagon-like peptide 1	156
Leptin	156
Appetite Neuropeptides	158
POMC and α -Melanocyte Stimulating Hormone	158
Neuropeptide Y/Agouti-related peptide	160
Conclusions	161
References	162
Appendix	169
List of References	170
Vita	193

LIST OF TABLES

Table 1. Hourly Rectal Temperature. CON (saline pretreatment and treatment). CONF (2.2 mg flunixin/kg BW pretreatment and saline treatment). ACT (saline pretreatment and 400 ng LPS/kg BW treatment). ACTF (2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW treatment). TRT indicates treatment. HR indicates hour relative treatment. Significance $p < 0.05$ with different letters.	101
Table 2. Rectal Temperatures. Rectal temperatures measured with a digital thermometer and analyzed for average on Days 1, 4, and 7; the temperature 3h after treatment on these days and 5h or the peak on day 1. Control (CON, IV saline daily; n=4), single acute dose (SAD, 400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7; n=4), daily acute dose (DAD, 400 ng of LPS/kg BW IV daily; n=4), daily increasing dose (DID, 400 ng of LPS/kg BW IV on Day 1 with 20% increase each day; n=4), and chronic subcutaneous dose (CSD, 20 µg of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump; n=4) and an IV group of the combined IV treatments on Day 1 as they received the same dose of LPS. Significant p-values are bolded ($p < 0.05$) and difference is indicated by different letters within each row. § as peak indicates the highest temperature amongst the measured timepoints for the day. * indicates definition of a fever per Merck Veterinary Manual. 1 p-value indicates the comparison between CON, SAD, DAD, DID, and CSD. 2 p-value indicates the comparison between CON, IV, and CSD.	141

LIST OF FIGURES

Figure 2.1. Experimental Designs.....	65
Figure 2.2. Experiment 1, Plasma Cytokine Concentration. (A) Plasma concentration of IL-6 and (B) concentration of IL-10. Control (CON1; n=3; solid black line) and endotoxin administered (400ENDO; n=3; dashed black line) cows in Experiment 1. Significance determined at $p<0.05$. * indicates the 400ENDO group is different than the CON1.	66
Figure 2.3. Experiment 1, Plasma Progesterone Concentration. (A) Mean plasma progesterone concentration and (B) the number the cows with detectable plasma concentrations of progesterone in three samples – a pre-treatment, 2 hours after treatment, and 4 hours after treatment. Control (CON1; n=3; solid black) and endotoxin administered (400ENDO; n=3; dashed black) cows in Experiment 1. Significance determined at $p<0.05$. * indicates the 400ENDO group is different than the CON1.	66
Figure 2.4. Experiment 1, Plasma Cytokine Concentration. (A) Plasma concentration of IL-6 and (B) concentration of IL-10. Control (CON1; n=3; solid black line) and endotoxin administered (400ENDO; n=3; dashed black line) cows in Experiment 1. Significance determined at $p<0.05$. * indicates the 400ENDO group is different than the CON1.	67
Figure 2.5. Experiment 1, Representative Plasma LH Pulse Profiles from Individuals. (A) Control (CON1; solid black line) and (B) endotoxin administered (400ENDO; dashed black line) cows in Experiment 1. Pulses are identified with filled markers.	67
Figure 2.6. Experiment 1, Plasma Luteinizing Hormone Concentration, Pulse Frequency, and Amplitude. (A) Average hourly plasma LH concentration, (B) average plasma LH concentrations in the 5 hours after treatment and (C) LH pulses per hour in the 5 hours after treatment (D) LH pulse amplitude for control (CON1; n=3; solid black) and endotoxin administered (400ENDO; n=3; dashed black line) cows in Experiment 1. Significance determined at $p<0.05$ indicated by *.	68
Figure 2.7. Experiment 2, Vaginal Temperature. Average hourly ambient temperature indicated by gray line. Average hourly vaginal temperature for control (CON2; n=4; solid black line) and endotoxin administered (600ENDO; n=4 due to recorder issue; dashed black line) cows in Experiment 2. Significance determined at $p<0.05$. * indicates the 600ENDO group is different than the CON2.	69
Figure 2.8. Experiment 2, Plasma Cytokine Concentrations. (A) Concentration of IL-6 and (B) concentration of IL-10. Control (CON; n=4; solid black line) and endotoxin administered (ENDO; n=5; dashed black line) cows in Experiment 2. Significance determined at $p<0.05$. * indicates the ENDO group is different than the CON. + indicates tendency ($p<0.10$) for difference.	70
Figure 2.9. Experiment 2, Plasma LH Concentrations. (A) Average plasma LH concentration each hour relative to treatment and (B) average plasma LH concentrations pre and post treatment for control (CON2; n=4; solid black) and endotoxin administered (600ENDO; n=5; dashed black line) cows in Experiment 2. Significance determined at $p<0.05$ indicated by *.	70

- Figure 2.10. Experiment 2, Kisspeptin. Representative images of ARC kisspeptin staining in CON2 (A) and 600ENDO (B) with mean average number of kisspeptin immunopositive neurons in ARC (C). Representative images of POA kisspeptin staining in CON2 (D) and 600ENDO (E) with mean average number of kisspeptin immunopositive neurons in POA (F). Significance determined at $p < 0.05$ indicated by * 71
- Figure 3.1. Vaginal Temperature. Hourly mean vaginal temperature relative to LPS/saline treatment. CON (saline pretreatment and treatment) is the black solid line. CONF (2.2 mg flunixin/kg BW pretreatment and saline treatment) is dotted black line. ACT (saline pretreatment and 400 ng LPS/kg BW treatment) is solid gray line. ACTF (2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW treatment) is gray dashed line. Black arrow is the LPS/saline treatment administration. White arrow is the flunixin/saline pretreatment administration. Statistical difference ($p < 0.05$) indicated by * for ACT vaginal temperature compared to CON vaginal temperature and + for ACTF vaginal temperature compared to CON vaginal temperature. CON vaginal temperature was not different from CONF at any timepoint. 102
- Figure 3.3. Mean plasma progesterone concentration. Mean plasma progesterone concentration relative to LPS/saline treatment. CON (saline pretreatment and treatment) is the black solid line. CONF (2.2 mg flunixin/kg BW pretreatment and saline treatment) is dotted black line. ACT (saline pretreatment and 400 ng LPS/kg BW treatment) is solid gray line. ACTF (2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW treatment) is gray dashed line. There was no statistical effect, but there were animals in ACT and ACTF that presented detectable progesterone. 103
- Figure 3.4. Representative LH pulse profiles. (A) Control (saline pretreatment and treatment). (B) Control+flunixin (CONF; 2.2 mg flunixin/kg BW pretreatment and saline treatment). (C) Acute LPS (ACT; saline pretreatment and 400 ng LPS/kg BW treatment). (D) Acute LPS + flunixin (ACTF; 2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW). Black arrow is the LPS/saline treatment administration. White arrow is the flunixin/saline pretreatment administration. * indicates the identified pulse. 104
- Figure 3.5. Hourly mean plasma LH concentration. (a) Mean LH plasma concentration described by treatment groups such that Control (saline pretreatment and treatment) indicated by black bar, Control+flunixin (CONF; 2.2 mg flunixin/kg BW pretreatment and saline treatment) indicated by gray bar, Acute LPS (ACT; saline pretreatment and 400 ng LPS/kg BW treatment) indicated by white bar and Acute LPS + flunixin (ACTF; 2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW) indicated by black striped bar. (B) Mean LH plasma concentration described on whether the animal received LPS or saline for their treatment with those that received saline in black bar and those that received LPS in white bar. Significance ($p < 0.05$) indicated by different letters. 105
- Figure 3.6. LH Pulse Frequency. (A) Number of pulses per hour over the 5 hour time period after LPS/saline treatment. (B) Number of pulses per hour from 0 to 2.5 hours after LPS/saline treatment. (C) Number of pulses per hour from 2.5 to 5 hours after

- LPS/saline treatment. Control (saline pretreatment and treatment) indicated by black bar, Control+flunixin (CONF; 2.2 mg flunixin/kg BW pretreatment and saline treatment) indicated by gray bar, Acute LPS (ACT; saline pretreatment and 400 ng LPS/kg BW treatment) indicated by white bar and Acute LPS + flunixin (ACTF; 2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW) indicated by black striped bar. * indicates significance $p < 0.05$ compared to CON. 106
- Figure 3.7. LH β Immunopositive Cells. Representative images of individuals from (A) saline control, (B) Control+flunixin (CONF; 2.2 mg flunixin/kg BW pretreatment and saline treatment), (C) Acute LPS (ACT; saline pretreatment and 400 ng LPS/kg BW treatment) and (D) Acute LPS + flunixin (ACTF; 2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW). (E) Average number of LH β immunopositive cells. There was no statistical difference. 107
- Figure 3.8. Kisspeptin, cFos and Dual Labeled Immunopositive Cells in ARC. Representative images of individuals from (A) saline control, (B) Control+flunixin (CONF; 2.2 mg flunixin/kg BW pretreatment and saline treatment), (C) Acute LPS (ACT; saline pretreatment and 400 ng LPS/kg BW treatment) and (D) Acute LPS + flunixin (ACTF; 2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW). (E) Average number of kisspeptin immunopositive cells. (F) the proportion of dual labeled kisspeptin cells. * indicates significance. SAL is the combined ACT and CON while FLU is the combined ACTF and CONF. 108
- Figure 3.9. Kisspeptin Immunopositive Cells in POA. Representative images of individuals from (A) saline control, (B) Control+flunixin (CONF; 2.2 mg flunixin/kg BW pretreatment and saline treatment), (C) Acute LPS (ACT; saline pretreatment and 400 ng LPS/kg BW treatment) and (D) Acute LPS + flunixin (ACTF; 2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW). (E) Average number of kisspeptin immunopositive cells. There was no significant difference. 109
- Figure 4.1. Experimental Design. Twenty sheep were blocked by body weight and randomly assigned to one of five treatment groups – control (CON, saline daily; n=4), single acute dose (SAD, 400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7; n=4), daily acute dose (DAD, 400 ng of LPS/kg BW IV daily; n=4), daily increasing dose (DID, 400 ng of LPS/kg BW IV on Day 1 with 20% increase each day; n=4), and chronic subcutaneous dose (CSD, 20 μ g of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump; n=4). Serial blood samples were collected every 12 minutes for 6 hours (1 hour before treatment and 5 hours after treatment) on Days 1 and 7 and tissue collection of brain and pituitary occurred on Day 7. 142
- Figure 4.2. Rectal Temperatures. Temperatures recorded on Days 1, 4, and 7. Rectal temperatures measured with a digital thermometer at -1, 1, 3, 5 and 8 hours relative to treatment administration on Days 1 and 4 and -1, 1, and 3 hours relative to treatment administration on day 7. Control (CON, IV saline daily; n=4) depicted as a black solid line, single acute dose (SAD, 400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7; n=4) as a dark gray line, daily acute dose (DAD, 400 ng of LPS/kg BW IV daily; n=4) as a light gray line, daily increasing dose (DID, 400 ng of LPS/kg BW IV on Day 1 with 20% increase each day; n=4) as black dash lines, and

chronic subcutaneous dose (CSD, 20 µg of LPS/kg BW daily delivered subcutaneously via Alzet smotic pump; n=4) as dotted line. Black arrow indicates treatment administration each day. 143

Figure 4.3. Steroid Hormone Analysis from samples -1, 2, and 4 hours relative to treatment. Plasma cortisol concentration on Day 1(A) and Day 7 (B). Plasma progesterone concentrations on Day 1 (C) and Day 7 (D). The proportion of cortisol concentration to progesterone concentration Day 1 (E) and Day 7 (F). Black solid horizontal line indicates the limit of detection for assay. Control (CON, saline daily; n=4) depicted as a black bar, single acute dose (SAD, 400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7; n=4) as a dark gray bar, daily acute dose (DAD, 400 ng of LPS/kg BW IV daily; n=4) as a light gray bar, daily increasing dose (DID, 400 ng of LPS/kg BW IV on Day 1 with 20% increase each day; n=4) as black striped bar, and chronic subcutaneous dose (CSD, 20 µg of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump; n=4) as white bar. Significant differences indicated by different letters at $p < 0.05$. Tendency ($0.05 < p < 0.1$) indicated with *. 144

Figure 4.4. Representative LH pulse profiles from individual animals on Day 1 (1) and Day 7 (2). Blood samples collected and analyzed for LH concentration every 12 minutes for 6 hours (1 hour before treatment; 5 hours after treatment) on Day 1 and Day 7 of the experimental week. Representative animals shown for control (CON, saline daily) (A), single acute dose (SAD, 400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7) (B), daily acute dose (DAD, 400 ng of LPS/kg BW IV daily) (C), daily increasing dose (DID, 400 ng of LPS/kg BW IV on Day 1 with 20% increase each day) (D), and chronic subcutaneous dose (CSD, 20 µg of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump) (E). Black arrow indicates treatment administration (at Time 0). Black filled circles with * indicate a pulse defined by the following criteria: (1) the peak must exceed the sensitivity of the assay, (2) a peak must occur within two data points of the previous nadir, and (3) the peak must exceed a 95% CI of the previous and following nadirs. 145

Figure 4.5. Plasma Luteinizing Hormone Mean Concentration. LH concentration in one hour pretreatment (black), 0-2.5h after treatment (gray), and 2.5-5h after treatment (white) for Day 1 (A) and Day 7 (C). (B) the square root of LH concentration for Day 1 in order to normalize the data before statistical analysis was completed. Control is CON (saline daily; n=4). Single acute dose is SAD (400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7; n=4). Daily acute dose is DAD (400 ng of LPS/kg BW IV daily; n=4). Daily increasing dose is DID (400 ng of LPS/kg BW IV on Day 1 with 20% increase each day; n=4). Chronic subcutaneous dose is CSD (20 µg of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump; n=4). IV (A and B) is the combination of SAD, DAD, and DID as they all received 400 ng LPS/kg BW on Day 1. *indicates significance within the treatment group from the pre-treatment value. 147

Figure 4.6. Plasma Luteinizing Hormone Pulse Frequency. Number of pulses per hour derived from the number of pulses over five hours (after treatment administration). Day 1 (A) and Day 7 (C) shown as control (CON, saline daily; n=4) depicted as a

black bar, single acute dose (SAD, 400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7; n=4) as a dark gray bar, daily acute dose (DAD, 400 ng of LPS/kg BW IV daily; n=4) as a light gray bar, daily increasing dose (DID, 400 ng of LPS/kg BW IV on Day 1 with 20% increase each day; n=4) as black striped bar, and chronic subcutaneous dose (CSD, 20 µg of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump; n=4) as white bar. Intravenous treatments of 400 ng of LPS/kg BW (SAD, DAD, DID) on Day 1 were combined (B) shown as the control (CON, saline daily; n=4) depicted as a black solid bar, intravenous administration (IV; 400 ng of LPS/kg BW; n=12) as dark gray bar, and chronic subcutaneous dose (CSD, 20 µg of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump; n=4) as white bar. Significant difference shown with different letters within each subfigure at a p-value <0.05..... 148

Figure 4.7. Representative 10X images of ARC kisspeptin immunopositive cells on Day 7. A) Control (CON, saline daily; n=4). B) Single acute dose (SAD, 400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7; n=4). C) Daily acute dose (DAD, 400 ng of LPS/kg BW IV daily; n=4). D) Daily increasing dose (DID, 400 ng of LPS/kg BW IV on Day 1 with 20% increase each day; n=4). E) Chronic subcutaneous dose (CSD, 20 µg of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump; n=4). Black arrows indicate immunopositive cells. IIIV indicates third ventricle. F) Number of kisspeptin immunopositive cells in CON (black), SAD (dark gray), DAD (light gray), DID (black striped), and SSD (white). Significant difference shown with different letters at a p-value <0.05. 149

Figure 4.8. Representative 10X images of LHβ immunopositive cells from the anterior pituitary on Day 7. A) Control (CON, saline daily; n=4). B) Single acute dose (SAD, 400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7; n=4). C) Daily acute dose (DAD, 400 ng of LPS/kg BW IV daily; n=4). D) Daily increasing dose (DID, 400 ng of LPS/kg BW IV on Day 1 with 20% increase each day; n=4). E) Chronic subcutaneous dose (CSD, 20 µg of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump; n=4). Black arrows indicate examples of immunopositive cells. 150

Figure 5.1. Working Model. LPS induced inflammation produces an increase in the steroid hormones as well as peripheral appetite hormones that alter the production of various neuropeptides including those involved in appetite regulation, but ultimately suppresses the expression of kisspeptin and potentially neurokinin B (NKB) in order to decrease GnRH secretion..... 169

CHAPTER 1: LITERATURE REVIEW

Introduction

Reproduction is a basic physiological process that is a coordinated effort of multiple organ systems within the body acting together to ultimately produce viable offspring and ensure perpetuation of a species. A key component of the reproductive system includes the hypothalamus-pituitary-gonadal (HPG) axis due to its role in the pulsatile release of gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH). Humans and animals commonly experience a variety of stressors throughout their lifespan, including inflammation associated with infections and non-infectious diseases, that impair an organism's ability to reproduce.

Lipopolysaccharide (LPS; endotoxin), specifically the lipid A component [1], is a common cause for the immune response in many infections and noninfectious diseases. It is a component of the outer cell wall of Gram-negative bacteria, like *Escherichia coli* (*E. coli*) that binds to the toll-like receptor 4 (TLR-4) on the membrane of immune cells facilitated by LPS binding protein (LBP) creating a macromolecular complex called a myddosome complex [1, 2]. The myddosome complex stimulates the activation of NF- κ B and activation protein 1 (AP-1), which in turn causes a hyperexpression of multiple inflammatory genes [2]. This intracellular signaling causes the production and secretion of pro-inflammatory cytokines (specifically interleukin (IL)-1 β , IL-6, and tumor necrosis factor alpha [TNF- α]) [1]. Regardless of the inciting cause, inflammation impairs reproduction and alterations to the HPG axis, specifically within the hypothalamus.

Livestock Implications

The productivity and profitability of domestic livestock is related to their ability to produce viable offspring every year and for multiple years. Once domestic livestock are unable to become pregnant, the animal may be considered to be no longer reproductively competent and sold or slaughtered for meat prematurely. Unfortunately, disease is a stressor that an animal may face more than once in their lifetime and will negatively affect their ability to reproduce and, in turn, remain on a farm. Interestingly, the accepted conception rate for mature dairy cows is around 30%, but dairy heifers typically have a conception rate around 60% [3-6]. This contrast in conception rate may be because many dairy cattle experience inflammatory diseases during their post-partum period. Inflammatory diseases cause a slower return to estrous, lower first artificial insemination (AI) conception rate, and a higher rate of pregnancy loss compared to their healthy counterparts [7].

Endometritis, inflammation of the innermost layer of the uterus, is commonly [7, 8] caused by bacterial infections in the uterus, especially following parturition. Multiple types of Gram-negative bacteria will cause endometritis, but the most common is *E. coli*. It may irrevocably impair fertility [9] in various ways including a reduction in the first service conception rate [10], overall pregnancy risk, prolonged calving interval, and increase of involuntary culling [11]. In addition, metritis (inflammation of the entire uterus) is a common ailment, affecting up to 20% of lactating dairy cows [12], associated with Gram-negative bacteria that also influences fertility in early lactation [8]. Specifically, cows with metritis are 63% less likely to return to estrus within 50 days postpartum, at least 60% less likely to be pregnant following first AI postpartum, and 239% more likely to lose

their pregnancy than if they did not experience metritis [7]. In general, dairy cattle have lower conception rates and submission rates for breeding and increased calving to conception intervals following the resolution of uterine infection [13]. Additionally, there is significant economic loss each year due to metritis through the overall decrease in milk production and reproduction of the herd [14] as costs associated directly and indirectly range from \$156.00 to \$948.00 per cow [14]. Moreover, mastitis is another common [7] inflammatory disease that affects reproduction and is defined by the inflammation of mammary tissue. Like endometritis and metritis, mastitis can be caused by an array of Gram-negative bacteria, including *E. coli*. In cattle and sheep with mastitis, there is delayed timing of ovulation through suppression of LH pulses and a slowed or blocked LH surge [15-17]. Delayed ovulation reduces the chance of successful fertilization, thus creating infertility in animals.

Human Implications

Infertility and subfertility in humans are common occurrences with about one fifth of the global population being affected [18, 19]. While there are a number of causes for infertility and subfertility in human patients, improper secretion of GnRH and/or LH is common, potentially resulting from comorbidities known to induce systemic inflammation. As the global population continues to grow and civilizations become modern, there will likely be an increase in inflammatory infectious and non-infectious diseases that may lead to an infertility epidemic. Wildlife encroachment and climate change, including increased global temperature and deforestation, will facilitate ideal environments for bacterial growth and antimicrobial resistance [20]; more opportunities for viral mutations and animal-to-human diseases all of which will lead to higher incidence infections associated with chronic

systemic inflammation. Furthermore, modernization may facilitate more sedentary lifestyles and consumption of additional highly processed and calorically dense foods, resulting in a greater incidence of metabolic diseases (e.g., type II diabetes) and subsequently more systemic inflammation.

Humans may experience infections such as endometritis, metritis and mastitis and consequently infertility similarly to domestic livestock. For example, pelvic inflammatory disease (PID) is oftentimes caused by infections from sexually transmitted, Gram-negative bacteria, including *E. coli* and in approximately 11-16% of PID cases leads to subfertility and infertility due to inflammation and related damage [21-24]. Furthermore, there are also a variety of non-infectious conditions that serve as the source for infertility. For example, obesity causes chronic activation of the immune system [25] and has been linked to impaired reproduction in humans through anovulation, infertility, miscarriage, and overall poor pregnancy outcomes [26]. It is believed that LPS is responsible for the initiation of inflammation in obesity [25] and the mechanism for impairment of reproduction in obese humans involves reduction in GnRH and LH secretion [27].

Polycystic ovarian syndrome (PCOS) is another common chronic inflammatory condition occurring in 4-20% of women at reproductive age worldwide [28] resulting in infertility and subfertility [29]. In granulosa cell culture, LPS administration causes an increase in TLR-2 expression along with pro-inflammatory cytokines, TNF- α , IL-1 β , IL-6 and IL-8, and the overexpression of TLR-2 promoted an overall inflammatory response within the cell culture [30]. Furthermore, in rat model of PCOS, serum concentration of LPS is elevated and there is an elevated TLR-4 expression in the ovary [31]. The level of

phospho-p65, the dominant transcription factor downstream of LPS/TLR-4 signaling, was increased in the ovarian tissues of PCOS rats [31]. Additionally, women with PCOS experience higher mean LPS and LBP along with a positive correlation between the endotoxemia markers with testosterone level, ovarian volume, number of antral follicles, but a negative relationship with the number of menses per year [32]. Taken together, infertility and subfertility associated with PCOS, and a variety of other chronic inflammatory ailments, is linked to LPS from Gram negative bacteria.

Overview of Hypothalamic-Pituitary-Gonadal Axis Physiology

The HPG axis is comprised of the hypothalamus, anterior and posterior pituitary glands, and gonads (testes in male; ovaries in females). Neurons in the pre-optic area (POA; rats, sheep, primates) and/or mediobasal hypothalamus (MBH; sheep, primates) of the hypothalamus produce and secrete GnRH from terminal projections to the external zone of the median eminence and thus directly into a network of fenestrated capillaries. The hypophyseal portal system carries GnRH to the gonadotropes within the anterior pituitary (adenohypophysis) to stimulate the production and secretion of LH and follicle stimulating hormone (FSH). Luteinizing hormone and FSH enter into peripheral circulation to stimulate the gonads to promote gametogenesis (sperm in testes and ovum in ovary) and steroidogenesis (estrogen, progesterone, testosterone). These steroid hormones provide negative and positive feedback loops to inhibit and stimulate the release of GnRH and, subsequently, LH and FSH.

History of GnRH and LH

The pituitary gland secretes hormones that stimulate or inhibit other endocrine glands and there is a defined relationship between the actions of the pituitary gland and the central nervous system (CNS) through the brain stem and hypothalamus. As early as 1930, the hypophyseal portal system was identified to connect the adenohypophysis to the hypothalamus, thus allowing for further research into the relationships between the two. In 1977, the Nobel Prize for Physiology was awarded to Roger Guillemin, Andrew V. Schally, and Rosalyn Yalow for their work in the discovery of peptide hormone production in the brain (Guillemin and Schally) as well as the radioimmunoassay (RIA) development for peptide hormones (Rosalyn Yalow). The years leading up to this accomplishment, Yalow developed the RIA to measure concentrations through research on peptide hormones like insulin, adrenocorticotrophic hormone (ACTH), and growth hormone. In the late 1950s, Guillemin and Schally extracted three hypothalamic compounds from sheep and pigs that stimulated the release of ACTH, thyroid stimulating hormone (TSH), and LH. In turn, the compounds were named releasing factors. However, the specific sequences and identities of the compounds took an additional twenty years to accomplish with the compounds being identified as corticotropin releasing hormone (CRH), thyrotropin releasing hormone (TRH), and luteinizing releasing hormone (LHRH, later defined as GnRH) respectively. Both of these research accomplishments accelerated the neuroendocrinology field, especially in reproduction.

Gonadotropin releasing hormone is difficult to measure as it is only detectable in the hypophyseal portal system where it is released in very small (picogram/min) amounts

and eventually diluted in peripheral blood. However, nearly a decade after the initial discovery of GnRH, it was determined that GnRH and LH are released in a pulsatile fashion and each pulse of GnRH will produce a pulse of LH [33]. In this seminal work, sheep underwent a neurosurgery to insert two cannulas into the hypophyseal portal system and, following recovery, both blood from the hypophyseal portal system and jugular vein were collected every 15 minutes for several hours from unanesthetized sheep to determine GnRH and LH concentrations, respectively [33]. Therefore, LH pulses are often times utilized as an indirect measure for GnRH pulses (GnRH/LH secretion). Individual pulses are defined by three criteria: 1) the peak exceeds the sensitivity of the assay, 2) a peak occurs within two points of the previous nadir and 3) the peak exceeds a 95% confidence interval of the previous and subsequent nadir [34].

Importance of GnRH/LH secretion

As the final common reproductive output of the CNS, GnRH plays a critical role in the timing of puberty as a marked rise in pulsatile GnRH/LH secretion marks the beginning of neuroendocrine puberty onset in mammals [35]. While, the central mechanism behind this increase varies between species, the theory for puberty in domestic livestock (pigs, cattle, sheep) as well as rodents is the gonadostat hypothesis which states that puberty is the result of a decreased sensitivity to sex steroid negative feedback to allow an increase in GnRH/LH secretion [36-39]. Unlike other animals, primates appear to have a different central mechanism for puberty onset since the suppression of GnRH/LH secretion is not steroid dependent for most of the prepubertal period [40]. The current working model for puberty in primates is that there is a “central brake” independent of sex steroids that inhibits GnRH/LH secretion for the majority of the prepubertal period [40]. However, there is a

brief period around the time of puberty onset (peripubertal period) in which primates exhibit central sensitivity to gonadal sex steroids, but the mechanisms responsible for the transition remain largely unknown [34].

Estrous Cycle

The HPG axis allows for reproductive cycles in females of species through its coordination of multiple organs utilizing the endocrine system. Specifically, female mammals have an estrous cycle and there are variations in cycle length, season, and number of follicular waves depending on the species. Despite these variations, in general estrous cycles can be described as two phases – luteal (diestrus and metestrus) and follicular (proestrus and estrus) – with differences in hormonal profiles that regulate the animal's ability to reproduce. Immediately following ovulation, a corpus luteum (CL) is formed and the small luteal cells (SLC; derived from granulosa cells) and large luteal cells (LLC; derived from theca cells) begin to produce progesterone causing concentration to be high and thus, negative feedback to suppress pulsatile GnRH/LH secretion by decreasing the concentration and pulse frequency. During this time, the animal is in the luteal phase of the estrous cycle.

In the follicular phase of the estrous cycle, circulating levels of progesterone decrease following the stimulation of prostaglandin $F2\alpha$ (PGF 2α) secretion from the uterus coupled with episodic pulses of oxytocin secretion from the CL, which causes luteolysis. As plasma concentrations of progesterone decreases, pulsatile GnRH/LH secretion increases as does FSH secretion, thus allowing for ovarian follicular recruitment and growth as well as the production of estradiol and inhibin A from the ovary. There are a series of follicular waves in which pulsatile GnRH/LH and FSH secretions promote the

growth and development of the follicle to eventually provide one dominant follicle. Inhibin A will inhibit the secretion of FSH from the pituitary, thus allowing LH to be primarily important for ovulation of the dominant ovarian follicle. Estradiol is produced as the theca cells and granulosa cells of the ovarian follicle work together in a two-cell two-gonadotropin theory. The theca cells have the receptor for LH (LHR), but not the receptor for FSH (FSHR), as well as the enzymes required to convert cholesterol to androgen except for aromatase, the enzyme to convert androgen to estradiol. On the other hand, granulosa cells do not have LHR (until it is a dominant follicle), but have FSHR and only the enzymes that are needed to convert androgen into estradiol. Therefore, the two cell types are required for estradiol production. Estradiol provides positive feedback in the POA to increase the secretion of GnRH/LH and the granulosa cells of the dominant ovarian follicle acquire LHR, thus allowing for even more estradiol to be produced by the follicle. The increase in estradiol and activation of neurons in the POA/AVPV causes an LH surge that is required for ovulation, marking the end of the follicular phase.

Lastly, some species experience seasonal anestrus in which they do not experience estrus nor ovulate during specific times of the year primarily driven by photoperiod, such as sheep during late spring through early fall. In turn, the estrus cycle is halted during seasonal anestrus in part because of estrogen negative feedback on GnRH/LH secretion [41].

Kisspeptin-Neurokinin B-Dynorphin Neurons in Reproduction

The pulsatile release of GnRH/LH is highly regulated by various internal and external cues including sex steroid negative feedback from the gonads on the

hypothalamus. A simple explanation for the pulsatile secretion of GnRH/LH is that gonadal sex steroids directly regulate GnRH neurons. However, GnRH neurons are devoid of reproductively relevant sex steroid receptors (estrogen receptor- α (ER- α) [42] progesterone receptor (PR) [43], and androgen receptor (AR) [44]. Thus, there is implication of an upstream mechanism by which sex steroids contribute to the regulation of GnRH/LH secretion.

A population of neurons in the arcuate nucleus (ARC) of the hypothalamus has been identified as a key component in the regulation of pulsatile GnRH/LH secretion. These neurons are named KNDy neurons for the reproductively relevant neuropeptides they produce - kisspeptin, neurokinin B, and dynorphin. The neuropeptide co-expression in these neurons was first described in female sheep with approximately 80% of these neurons expressing kisspeptin along with neurokinin B (NKB) and approximately 94% of kisspeptin expressing neurons expressed dynorphin [45]. However, about 73% of NKB expressing neurons present in the ARC also contained kisspeptin and approximately 95% of dynorphin expressing cells contained kisspeptin [45]. Taken together, there is almost complete colocalization between kisspeptin and dynorphin and the majority also express NKB, even though there are also NKB expressing neurons that do not express kisspeptin [45]. In regards to steroid feedback, approximately 90-95% of KNDy neurons express ER- α [46] and PR [47] and KNDy neurons also contain AR mRNA [48, 49]. Therefore, these neurons likely play a direct role in the sex steroid feedback loops within the hypothalamus.

Kisspeptin

Kisspeptin is a small neuropeptide encoded by the *Kiss1* gene, formerly known as human metastasis suppressor gene produced by neurons in the hypothalamus [50]. This

neuropeptide was discovered in Hershey, PA thus kisspeptin was named after the Hershey's Kisses chocolate candy [50] Originally, kisspeptin was described to suppress melanoma and breast cancer metastasis [51, 52]. However, a loss of function mutation in the gene for kisspeptin receptor, Kiss1R (formerly known as the orphan G-Protein Linked Receptor 54 or GPR54 [52, 53]), defined a role in reproduction as it causes hypogonadotropic hypogonadism in humans [54]. Furthermore, both male and female mice with this gene mutation also experience a lack of puberty onset and hypogonadism [55]. Within two decades of its discovery, kisspeptin has been shown to stimulate LH secretion in all species [56] with its effects directly on GnRH neurons rather on the pituitary gonadotropes [57]. First, the administration of the smallest functional component of kisspeptin, kisspeptin-10, increases plasma concentrations of LH [48, 58], however, prior administration of cetrorelix, GnRH receptor antagonist [59], prevents the kisspeptin-10 induced increase in plasma LH concentration [57]. In contrast, administration of a Kiss1R antagonist, peptide 234, will reduce the concentration of LH in plasma of many species [60]. Kisspeptin in the ARC is involved in the determination of breeding state as the expression is decreased during non-breeding season, however, an infusion of kisspeptin will cause ovulation [41]. Additionally, GnRH neurons express Kiss1R [61, 62], specifically, in rats 77% of GnRH neurons express mRNA for Kiss1R [63]. Finally, it has been shown through high frequency photo-stimulation that the Kiss1 neurons within the ARC of mice release a combination of neuropeptides to excite the Kiss1 neurons and in turn, in a coordinated action, stimulate GnRH neurons [64]. Taken together, pulsatile stimulation of GnRH secretion is primarily through the secretion of kisspeptin from the

KNDy neurons. This population of kisspeptin is likely the gatekeeper for puberty onset as intact female lambs have a greater number of ARC neurons expressing kisspeptin (KNDy neurons) following puberty onset while ovariectomized female lambs did not have the age dependent increase [65] further supporting the idea of sex steroid negative feedback inhibits ARC kisspeptin neurons (KNDy neurons) prior to puberty but not once puberty occurred.

A second population of neurons that express and secrete kisspeptin, but not NKB nor dynorphin, is located in the POA (sheep [46, 61, 66], guinea pigs, monkeys [67], and humans [67]) or the anteroventral periventricular nucleus (AVPV)/rostral periventricular area of the third ventricle (RP3V) in rodents [68, 69]. Kisspeptin expression in the POA in females is 10-20-fold greater than in males, thus identifying this population as a sexually dimorphic region. Furthermore, about 50% of the kisspeptin cells in the POA also express estrogen receptor α [46]. There is a relationship in female animals that this rostral population of kisspeptin neurons is associated with the estradiol positive feedback mechanism that causes the LH surge as kisspeptin antagonist administration will block the LH surge and estrogen administration increases the expression of Kiss1 gene in the POA [70, 71]. Therefore, the more rostral population (AVPV or POA) of kisspeptin producing neurons is likely responsible for the GnRH/LH surge required for ovulation of the ovum while the ARC population is for tonic GnRH/LH pulse secretion. However, there is evidence that in sheep, the ARC kisspeptin neurons and the POA kisspeptin neurons coordinate to facilitate the LH surge [66, 72, 73].

Neurokinin B

Neurokinin B is a tachykinin peptide encoded by Tac2 gene in rodents and Tac3 in humans that binds to Tac3 receptor (aka neurokinin 3 receptor or NK3R) [74]. A mutation in the Tac3 gene will result in lack of puberty onset in humans [75] and to a much lesser degree of reproductive abnormalities in mice [76]. Neurokinin B is colocalized in ARC neurons expressing kisspeptin (KNDy neurons) in several species including mice [77], rats [49, 76], primates [78, 79], and sheep [45, 80].

Similar to kisspeptin, NKB stimulates LH secretion in most species dependent on estrogen presence (sex steroid milieu) [81, 82]. Central administration of senktide, an NK3R agonist that mimics NKB by binding to NK3R, increases the number of LH pulses in sheep [80]. Conversely, central administration of SB222200, an NK3R antagonist that block the effects of NKB by binding to NK3R, decreases the number of LH pulses [80]. Interestingly, there is significantly more mRNA for NKB in ewes during late follicular phase as opposed to the luteal phase [83]. Specifically, central infusion of an NK3R antagonist, MRK-08, did not change the plasma LH surge amplitude nor LH surge onset in ewes, but the time from surge onset to peak plasma concentration of LH was longer [83]. Taken together, it is likely that NKB is not required for estrogen positive feedback that is needed for ovulation, but controls pulsatile secretion of GnRH/LH. Furthermore, GnRH neurons do not express NK3R, but NK3R are expressed in ARC KNDy neurons [84], therefore, NKB acts in an autocrine fashion to stimulate the ARC KNDy neuron kisspeptin secretion.

Dynorphin

Dynorphin is an endogenous opioid peptide produced by neurons within various parts of the nervous system, including the hypothalamus [85]. It is encoded by the PDYN gene in humans, a product of the proopiomelanocortin gene, and it binds with a higher affinity to kappa-opioid receptors (κ -OR) [86]. Contrary to kisspeptin and NKB, dynorphin has been shown to inhibit GnRH/LH secretion specifically through progesterone negative feedback [85, 87, 88]. In ovary intact, luteal phase ewes (high endogenous progesterone concentrations) as well as ovariectomized ewes with progesterone implants, LH secretion is suppressed, however, in ovariectomized ewes without progesterone implants, LH secretion is restored. Upon further investigation, ovariectomized ewes have lower amounts of dynorphin and progesterone in their cerebrospinal fluid than ovariectomized ewes with progesterone implants [85]. Furthermore, the administration of κ -OR antagonist, PF-4455242, in estrogen-treated ovariectomized goats causes an increased frequency of plasma LH pulses and increased frequency of episodic bursts in multiple unit activity in the ARC [89]. Additionally, both GnRH and KNDy neurons express κ -OR [90], however, it is likely that dynorphin acts on the KNDy neurons to terminate GnRH pulse secretion as NKB injections increased the internalization of κ -OR within KNDy neurons during both pulse onset and termination while it only caused κ -OR internalization in GnRH neurons within the MBH only during pulse termination [91]. Taken together, dynorphin is acting to inhibit the secretion of LH and promote LH pulse termination through an autocrine function to inhibit the KNDy neurons and subsequently the secretion of GnRH/LH.

KNDy Neurons

Investigation into the central mechanisms to regulate GnRH/LH pulsatile secretion provides growing evidence for the key role of KNDy neurons. First, the majority of GnRH neurons receive input from KNDy neurons (45% of the soma in the POA and 60% in MBH [92]). Interestingly, approximately 90-95% of KNDy neurons express ER- α , AR and PR, thus providing a mechanism through which sex steroid negative feedback occurs to suppress GnRH/LH secretion [44, 46-48]. Additionally, GnRH neurons express Kiss1R [61] while KNDy express NK3R and κ -OR [84, 90]. Therefore, kisspeptin is the final stimulatory output of the KNDy neurons onto the GnRH neurons while NKB stimulates and dynorphin inhibits KNDy neuron production of kisspeptin [93]. Additionally, these neurons, along with kisspeptin neurons in the POA, serve to induce the LH surge in sheep [66, 72]. Thus, KNDy neurons in the ARC play a more critical role in LH secretion as they control both the LH surge and the pulsatile GnRH/LH secretion.

Immune Response

Disease is defined as abnormal condition that negatively affects the function of an organism and is an umbrella term for multiple ailments that ultimately act as stressors through their disruption to homeostasis that animals encounter throughout their lifetime. There are multiple causes of disease such as exposure to pathogens (bacteria, virus, etc.) and improper function of an organ or organ system. Regardless of the cause, there is likely a response provided by the immune system.

Immune System

The immune system is a system of multiple tissues that recognize, respond to and eliminate potentially harmful pathogens, foreign materials, and abnormal cells. There are

two branches of the immune system that become activated - an innate branch (described below) and an adaptive branch (second line of defense; T cells, B cells; delayed response; antigen specific; creates memory of the antigens for better protection against future infections) -to produce pro-inflammatory and anti-inflammatory responses upon immune system activation [94]. Some of the alterations include fever, inflammation, decreased appetite, and impaired reproduction [95]. While all of these responses are important, inflammation plays a crucial role in the removal of offending factors and restoration of tissue structure and function.

The innate branch of the immune system is the first line of defense for the activated immune system [94]. Compared to the adaptive branch, there is an immediate response, it is not antigen specific, and there is no “memory” needed for response [94]. It consists of both external and internal factors that provide protection. The external factors include mechanical (e.g., skin) and chemical (e.g., hydrochloric acid of stomach) while the internal includes white blood cells (leukocyte; eosinophils, basophils, neutrophils, monocytes/macrophages, natural killer (NK) cells), cytokines (extracellular proteins produced by immune cells that affect the behavior of other cells; e.g., interferons, interleukins (IL 1-37) and TNF- α), and their associated receptors (e.g., toll-like receptor (TLR)) [94].

Toll-like receptors are a class of pattern recognition receptors that recognize pathogen associated molecular patterns (PAMPs; highly conserved common antigens on bacteria and viruses) and danger associated molecular patterns (DAMPs; necrosis, apoptosis, tissue damage) [94]. They are expressed on surfaces and within cells, especially

sentinel cells. Specifically, they are on glial cells during neurodevelopment as well as in the adult CNS [96, 97]. Upon binding, an inflammasome is formed and caspase-dependent production of IL-1 and IL-18 occur to stimulate leukocyte infiltration [97, 98].

Anti-inflammatory (e.g., IL-10) and pro-inflammatory (e.g., IL-1 β , IL-6, TNF- α) cytokines are produced by cells of the immune system in order to modulate the responses of other cells [94, 99]. Specifically, IL-1 promotes inflammation (including collagen synthesis), affect leukocytes, kills cells, impacts brain (fever, drowsiness, loss of appetite), affects cell growth and blood flow, and metabolism [99, 100]. Interleukin-6 interacts with the adaptive immune system through stimulation of pro-inflammatory T cells, suppression of anti-inflammatory T cells, enhancement of T cell cytotoxicity, fever, and B cell activation. Tumor necrosis factor α promotes inflammation (activates adhesion molecules and pro-coagulants, induces IL-1 and -6 productions, etc.), enhances bone resorption and collagen synthesis, causes septic shock and sickness behavior and altered lipid metabolism, and activates numerous cell types [100].

However, there are differences in the inflammatory responses between the CNS and other tissues as the components of adaptive immune system has limited access to brain parenchyma due to the blood brain barrier (BBB) and there are local special innate immune response cell types within the CNS [97, 101] including myeloid cells, astrocytes and microglia [97, 102]. Specifically, there are cytokine receptors that are expressed on sensory neurons of the peripheral nervous system (PNS) which then communicates to the CNS [103, 104]. Additionally, the peripheral nerves can recognize and activate due to bacterial products to modulate pain or inflammation [104, 105]. Cytokines may influence the brain

directly as they are small enough to pass through the BBB at the median eminence [104, 106] as are bacterial toxins [104, 107, 108] during peripheral infection. Cytokines may act on the microglia either directly or indirectly through secondary messengers like prostaglandins [104, 109, 110].

Prostaglandins

Prostaglandins (PGs) are lipid autocoids that are crucial in their role in the inflammatory response. First, in their biosynthesis, arachidonic acid is released from the plasma membrane due to phospholipase activity. Next, cyclooxygenase (COX) converts arachidonic acid to PGG₂ then peroxidase activity creates PGH₂. The final conversions are caused by specific isomerases that convert PGH₂ to produce various prostanoids such as thromboxane (TxA), PGD₂, PGE₂, PGI₂, and PGF₂ α . Overall, prostaglandins are important for the induction of an inflammatory response (redness, heat, swelling, pain) including fever and constitutive tissue protection.

Cyclooxygenase is an enzyme that exists in two isoforms, COX-1 and COX-2, and serves an important role in inflammation as these isoforms are inhibited by different drugs, like non-steroidal anti-inflammatory steroids (NSAIDs) in order to decrease the inflammatory response [111]. Cyclooxygenase-1 is the constitutive form as it is expressed in most cells and is a dominant source of homeostatic prostanoids [112, 113]. Additionally, it couples preferentially with thromboxane synthase, PGF synthase, and cytosolic PGE synthase [112]. Interestingly, COX-2 is the inducible form as it is induced by inflammatory stimuli, hormones, and growth factors thus acting as the main source of prostanoid formation for inflammation [112, 113]. Both COX-1 and COX-2 are constitutively expressed in the brain [114, 115]. Also, COX-2 is preferential for PGI synthase and

microsomal PGE synthase, which are induced by cytokines and tumor promoters [112]. Despite these generalizations, both COX-1 and COX-2 contribute to the autoregulatory and inflammation response prostanoids [112].

Prostaglandin E2 is the most abundant PG and it is important for the regulation of immune response, blood pressure, gastrointestinal integrity and reproduction during physiologic conditions [112]. It is generally produced through both COX-1 and COX-2 in synovium, chondrocytes, bone nociceptors, CNS, platelets, vascular smooth muscle cells, and kidney. However, during inflammation, it leads to redness, swelling and pain associated with inflammation through its role in arterial dilation and increased microvascular permeability and effect on peripheral sensory neurons and CNS [112]. It can regulate many cell types, such as macrophages, dendritic cells, and T and B lymphocytes. It has both pro- and anti-inflammatory effects. Specifically in neuroinflammation, PGE2 can cause lesions on neurons and enhanced pain transmission, but it also has anti-inflammatory and neuroprotective effects through microglial EP2 and EP4 receptors [112].

Prostaglandin D2 is produced in the CNS, and primarily mast cells, but also leukocytes and like the others, it has homeostatic and inflammatory responses through COX-2 specifically. In the brain, it regulates sleep and pain perception while in peripheral tissues, it mediates an inflammatory and immune response such as apoptosis.

Prostaglandin I2 regulates cardiovascular homeostasis and is mostly in endothelial cells, vascular smooth muscle cells, endothelial progenitor cells, platelets, kidneys, nociceptors, and CNS. It is regulated by COX-2 and is a vasodilator and an inhibitor of

platelet aggregation, leukocyte adhesion and vascular smooth muscle cell proliferation and mitosis.

Endotoxin Inflammation Impairs Reproduction

In addition to proinflammatory cytokine release, LPS causes an increase in plasma cortisol and prostaglandin F₂ α (PGF₂ α) concentrations, decreased appetite, and induction of fever when acutely administered [116-118]. Further, LPS causes hyperlipidemia [119, 120], hyperinsulinemia (from insulin resistance, at the liver specifically [121]), and hypoglycemia (failure to produce glucose from insulin resistance at the liver and deposition of glucose from hyperinsulinemia, [122]). However, the central administration of a CD14/TLR-4 antagonist, attenuates these responses associated with endotoxin exposure [123]. There are disruptions at all levels of the HPG axis, but especially at the level of the pituitary and the hypothalamus [124]. Thus, LPS will negatively impact reproduction throughout the HPG axis and likely via redundant mechanisms.

LPS and Steroid Hormones

One of the well-established regulators of GnRH/LH secretion is suppression through steroid hormones including progesterone and cortisol. Sex steroid hormone receptors exist on ARC KNDy neurons [44-47, 125] and allow for sex steroid (e.g. progesterone) negative feedback to inhibit the pulsatile secretion of GnRH, and subsequently LH [36]. Furthermore, these neurons contain glucocorticoid receptors [44, 126-128] thus allowing for cortisol to suppress GnRH secretion [129]. Plasma cortisol concentration increases during LPS induced inflammation through activation of the hypothalamic-pituitary-adrenal (HPA) axis [130] with cytokine stimulation of neurons to

produce corticosteroid releasing hormone (CRH) with subsequent production and secretion of adrenocorticotrophic hormone (ACTH) from the pituitary and finally stimulation of cortisol from the adrenal glands [130]. There are likely ACTH-independent direct actions of LPS on the adrenal gland. First, there are TLR-4 present in adrenal cells [131, 132], thus allowing LPS to bind and to induce cortisol secretion from the adrenal zona fasciculata [133]. In triamcinolone acetonide (a synthetic corticosteroid)-treated female cattle, LPS administration increases cortisol, but not ACTH, thus indicating that the increase in cortisol was due from a source other than through activation of the HPA axis [133]. The mechanism of action is through the COX-2 activation as specific COX-2 inhibitor, but not a specific COX-1 inhibitor, will attenuate the LPS effects on cortisol [131-133]. The increased plasma cortisol concentration in response to LPS induced inflammation ultimately suppresses the HPG axis, thus inhibiting the production and release of reproductive hormones as it suppresses GnRH secretion [43, 125, 129]. Furthermore, the increase in cortisol allows for the potential for increase in progesterone due to an overwhelming ACTH stimulation of the steroidogenesis enzymes within the adrenal glands [134, 135]. In turn, there is potential for an increased progesterone negative feedback onto the kisspeptin system to suppress reproduction [43, 85, 88, 125, 136]. Moreover, LPS induces the production of PGF2 α which also alters the HPG axis. Normal reproductive function of PGF2 α is to lyse the CL thus ceasing ovarian progesterone production [35], however, PGF2 α also serves a mediator of inflammation [137]. Thus, high levels of plasma PGF2 α in response to LPS exposure indirectly interfere with the negative feedback of

progesterone on GnRH/LH secretion as well as the priming of the uterus for pregnancy (normal reproductive roles of progesterone [35]).

LPS and GnRH/LH Secretion

At the level of the pituitary, systemic administration endotoxin will suppress LH production and secretion [138], especially through a reduction in amplitude of the LH pulse [124]. Additionally, acute systemic administration of LPS will prevent high LH pulse frequency by decreasing its pituitary responsiveness to GnRH through a cytokine cascade and decrease in GnRH-receptor expression [124, 139-141]. Interestingly, the decreased responsiveness may occur only during times of a moderate inflammation as a single lower dose of endotoxin (40 ng/kg) solely suppressed LH secretion while a greater dose (400 ng/kg) decreased both GnRH and LH secretion [124]. Contrary to in vivo data, there is evidence that indicates that LH secretion increases due to endotoxin exposure from pituitary cells in vitro [138]. Thus, the impairment caused by endotoxin may be a more direct result of suppression at the level of the hypothalamus.

In the hypothalamus, LPS suppresses GnRH, and subsequently LH, secretion [117, 124]. The disturbance in the GnRH pulse secretion is caused by blunting, eliminating and disrupting the rhythm of the GnRH release [117, 124]. Upon intravenous infusion of LPS, GnRH pulses are disturbed and subsequently, there is a delayed or a complete cessation of estrus in ewes and a decrease in estradiol production, which ultimately impairs ovulation, which is dependent on estradiol rise that is stimulated by high GnRH pulse frequency [117, 124, 142]. Specifically, it has been shown that intravenous administration of LPS will

decrease the expression of GnRH in the POA of rats [143] thus providing a mechanism for anovulation.

LPS and Kisspeptin

As there are alterations of GnRH secretion, it is also important to elucidate if the GnRH pulse generator is also affected or if it is solely the GnRH neurons. There is limited research on the effects of endotoxin on the KNDy neurons. Some research indicates that endotoxin decreases hypothalamic kisspeptin mRNA expression [143-145]. Additionally, acute endotoxin-induced inflammation will cause a temporary decrease in circulating LH and testosterone and about a 50% decrease in the number of immunopositive kisspeptin neurons in the ARC of male rats following LPS administration [146]. The mechanism behind the suppression of LH is likely due to the impact on neurons upstream of the GnRH neurons as administration of kisspeptin will restore the LH secretion [146]. Additionally, it has been shown that an acute intravenous dose of endotoxin prevents the surge of plasma LH required for ovulation at the hypothalamus by preventing kisspeptin and dynorphin positive cell activation [147]. Interestingly, upon intravenous administration of a low dose of LPS (100 ng/kg BW) to ewes in the follicular phase of the estrous cycle, there is a decrease in the proportion of activated kisspeptin neurons in the ARC and medial POA 12 hours after administration of LPS [148]. However, there is limited data in regards to protein expression for kisspeptin in other livestock species, like cattle, and male livestock.

Introduction to Research

Endotoxin induced inflammation will undoubtedly suppress the reproductive neuroendocrine axis (Figure 1). However, there are mechanisms by which this inhibition occurs to further elucidate as well as definition of a true physiological model.

Cattle as a Model

Cattle are a useful model for neuroendocrinology research, especially in regards to activated immune system suppression of reproduction as they experience infectious and non-infectious diseases. They are a major component of U.S. agriculture as they are an important source of meat and milk. Cattle are advantageous over rodents by the amount of tissue and blood that can be collected from them to analyze. Additionally, due to their size, large amounts of blood can be collected in a relatively short amount of time which is ideal for hormone pulse profile evaluation. Therefore, understanding what happens specifically to them when they are exposed to endotoxin may help to elucidate preventative and therapeutic methods in which impaired reproduction may occur.

Sheep as a Model

Sheep are an invaluable and well-defined model for reproductive neuroendocrinology research. First, as mentioned previously, they are the only species in which it is possible to measure both GnRH and LH simultaneously in an un-anaesthetized animal [33]. Additionally, the anatomy of their brains has been well defined over the years and it is similar to that of a human [149].

Similar to cattle, sheep are advantageous over rodents due to their size. Compared to rodents, relatively large amounts of blood can be collected over relatively long periods

of time which allows for LH and GnRH pulse profiles complete with mean concentration, pulse frequency and pulse amplitude. Additionally, there is more tissue for analysis as sheep are larger than rodents. The larger quantity of blood and tissue allow for evaluation of multiple hormones, cytokines, proteins, and neuropeptides within the same animal and sample.

Sheep are also excellent models for cattle as they have physiologic similarities as they are ruminants, have similar placentation, and comparable estrous cycle. However, they are smaller and easier to handle than cattle, but still are physiologically similar; therefore, making them potentially a stronger model to use for neuroendocrine research as often times there is a lot of handling and manipulation (e.g., frequent blood sampling).

Intact vs. Gonadectomized and Male vs. Female

Gonadal intact animals are the most reproductively physiologic, however, gonadectomized animals provide an excellent, simple model to evaluate the impact of LPS on reproductive neuroendocrinology, as the use of a sex steroid free (gonadectomized) model allows for the assessment of differences that arise as the result of organizational actions of sex steroids independent changes that could arise [150]. Gonadectomy generally occurs earlier in life and can be accomplished without “invasive” surgery in males compared to females thus allowing castrated male to be a useful, functional model. Furthermore, males are often utilized in place of female animals during times when there is limited data as the neuroendocrine control of reproduction in males is somewhat less complicated. Males are generally easier to acquire as they are usually less expensive than females and there are more available.

Chronic Inflammation

The majority of research related to the effects of inflammation on reproduction has been completed using an acute LPS intravenous dosing model. However, an acute model, while helpful in providing insight to what is initially occurring with acute inflammatory disease, may not be as physiology relevant as a chronic model of inflammation. Regarding a chronic model of inflammation, there are several possibilities of a physiologically relevant model including intravenous (IV) injections, intramuscular (IM) injections, and subcutaneous (SQ)/intraperitoneal (IP) diffusion as the main modes of administration and repeated acute dose or an increasing dose for the appropriate dosage of LPS.

Firstly, a repeated acute dose of endotoxin is one of the common methods to simulate a chronic model for inflammation. This method has been used in post pubertal, follicular phase gilts. However, a daily repeated low dose of LPS did not impact the steroidogenic signaling as the mRNA for STAR, ESR1, LDLR, YP19A1, CYP17A1 and 3 β -HSD nor the follicular fluid estrogen in the LPS treated group (0.1 μ g/kg BW IV daily for five days) was not different than the control group (treated with saline) [151]. There was an increase in TLR protein and plasma glucose concentration, however, there was not an induction of fever thus indicating that the dose was not sufficient in causing chronic systemic inflammation [151]. In anestrus ewes, an acute dose of 400 ng/kg BW IV daily for six days has been shown to suppress LH concentration and limit the overnight recovery after 4 days [152]. Interestingly, FSH was elevated starting the second day of treatment and was the highest on the fifth day [152]. Additionally, cortisol increased with each LPS injection [152]. Finally, in peripubertal female rats, repeated LPS (50 μ g/kg BW) every other day increased GnRH and glutamate content while it decreased circulating

concentrations of LH, estradiol and delayed vaginal opening, indicating the failure in release of GnRH [153].

Another potential strategy of LPS administration to induce chronic inflammation is a daily increase in the dose concentration. In pigs, increasing the LPS dose by 12% starting at 60 µg/kg BW every other day induced a fever and pro-inflammatory cytokine concentration as well as decreased lipid deposition and average daily gain [154]. However, reproductive parameters were not evaluated in that study. In cattle, a constant daily rising infusion of LPS, starting at 0.017 µg/kg BW/hour and increasing by 20%, 30%, 40%, and so on, for seven days caused a mild fever for Days 1-3 and decreased milk yield on Day 1 [155]. However, there was no difference in follicular growth, dominant follicle size, progesterone serum concentration, progesterone follicular concentration nor estradiol follicular concentration [155]. It is possible that the cattle became refractory to the LPS and so, according to the authors, a higher starting dose as well as a different method of administration may have been needed in order to fully impair the HPG axis [155].

Lastly, there is SQ or IP administration of LPS. In ovariectomized female rats, a moderate (500 µg/kg BW) dose of LPS administered IP induces an immune response with fever and decreased feed intake [144]. Additionally, there is a decrease in the serum LH concentration, however, there was no change in the Kiss1, Kiss1R, nor GnRH mRNA expression [144]. Therefore, the dose of LPS administered may not have been sufficient to induce a change in the hypothalamus. In ovariectomized and gonadal intact female rats, a greater dose of LPS (5 mg/kg) administered IP induced an immune response characterized by fever and decreased feed intake [144]. Interestingly, there was also a decreased amount

of GnRH and Kiss1 mRNA, however the mRNA for Kiss1R did not change [144]. Subcutaneous administration of LPS in mice (600 µg/kg/day) increased blood leukocyte concentration, weight of the spleen (major immune response organ), proinflammatory cytokines in the liver and subcutaneous fat, and increased circulating concentrations of insulin, however, reproductive parameters were not evaluated [156].

Overall, more research is needed to elucidate the most physiologically relevant model of chronic inflammation that will both induce an immune response and suppress the reproductive neuroendocrine axis in livestock species. Additionally, the majority of the research currently does not include data on the kisspeptin and KNDy neurons nor is there data in males.

Non-Steroidal Anti-Inflammatory Drugs

As the mechanisms by which endotoxin suppresses the reproductive axis emerge, it is important to determine specific treatments that could be used to prevent or mitigate the effects caused by LPS. A potential solution for inflammation-induced suppression of reproductive neuroendocrinology is through use of non-steroidal anti-inflammatory drugs (NSAIDs). This class of drugs can be found over the counter or can be prescribed by medical professionals commonly in order to control fever, inflammation and pain. There are many types of NSAIDs, but ones of particular interest for endotoxin-induced inflammation include those that inhibit COX-1 and COX-2 as they bind to and inactivate COX site on one of the COX dimer monomers and ultimately reduce PG production [109, 112, 157, 158]. Specifically, administration of flunixin meglumine, a general COX inhibitor, suppresses the increase in TNF- α , IFN γ , haptoglobin (anti-inflammatory acute

phase protein important to attenuate IL-6 [159]) and serum amyloid A (acute phase protein important for LPS clearance [160]) concentrations associated with the response to LPS [161]. Therefore, NSAIDs could provide an economical and easily accessible treatment for inflammation-induced HPG axis suppression.

Inhibition of COX-1 and COX-2 has been shown to prevent the suppression of GnRH/LH from IL-1 β and TNF- α in rats [162, 163]. Furthermore, flurbiprofen (general COX inhibitor) not only impairs the fever response [157, 158] the increase in cortisol and progesterone concentrations, [157], but also prevent the LPS-induced suppression of GnRH and LH secretion through concentration and pulse frequency [157]. However, flurbiprofen does not prevent the increase in TNF- α [157]. Unfortunately, there is no information on whether NSAIDs could prevent the suppression of GnRH/LH through protection of the kisspeptin in the POA and KNDy neurons. It is possible for NSAIDs to eliminate potential suppression of KNDy neurons, and subsequently, GnRH/LH secretion. Alternatively, there may be a partial protection of KNDy neurons as there is a functional limit to the number of KNDy neurons required for proper GnRH/LH secretion [164].

Experiment 1 – Evaluating an Acute Dose of Endotoxin in Adult Cows

Cattle are vital to the human food chain and often experience endotoxin-induced inflammation. In these experiments, a total of fifteen cows with estrous cycles synchronized so they would be in the proestrus phase, were utilized in order to evaluate the immune response (through changes in vaginal temperature and IL-6 and IL-10 concentrations) and reproductive neuroendocrine changes caused by an acute IV dose of endotoxin. This data will serve as preliminary data for future studies in which endotoxin will be delivered for a longer period of time to be physiologically relevant. We predict that

endotoxin will suppress the overall stimulatory output from the KNDy neurons, thus limiting the secretion of GnRH/LH.

Experiment 2 – Evaluating a General COX Inhibitor in an Acute Endotoxin Model

Understanding the mechanism by which endotoxin causes a suppression of the reproductive neuroendocrine axis is essential in order to develop treatments and preventions. Non-steroidal anti-inflammatory drugs are accessible over the counter or prescribed by a medical professional. In order to evaluate the role COX has in the suppression, a general COX inhibitor (flunixin meglumine) was administered thirty minutes prior to an acute IV dose of LPS while ewes were in the proestrus phase of their estrous cycle. Following treatment administration, immune response (vaginal temperature) and reproductive parameters (kisspeptin, LH) were assessed in these sheep. We hypothesized that flunixin would mitigate the immune response to systemic LPS and prevent suppression of the reproductive parameters induced by an acute model of inflammation.

Experiment 3 – Defining a Chronic Endotoxin Model for Livestock

To date, the majority of the research involving endotoxin-induced inflammation has used an acute IV LPS model. While there is valuable information to be gathered from an acute inflammation model, it may not be the most physiologically relevant. Therefore, it is imperative to evaluate the impacts of endotoxin through a chronic inflammation model. In this experiment, we evaluated parameters of neuroendocrine reproductive physiology (LH profiles including mean concentration, pulse frequency and pulse amplitude; kisspeptin protein expression) as well as other physiologic responses to LPS (rectal temperature; cortisol and progesterone concentrations) in wethers treated with endotoxin.

We utilized five treatment groups (control; a single acute dose of LPS IV on Day 1; daily administration of an acute dose of LPS; daily administration of an increasing dose of LPS; and chronic subcutaneous dose of LPS over seven days) in order to determine the most physiologically relevant to suppress the neuroendocrine reproductive axis. The data from this study will provide information on the route and dosage of LPS administration will provide a physiologically relevant response to inhibit the reproductive neuroendocrine axis. We hypothesized that the daily administration of an increasing dose of LPS group will provide the best chronic endotoxin model to suppress the HPG axis.

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**CHAPTER TWO: ACUTE ENDOTOXINEMIA INHIBITS LUTEINIZING
HORMONE AND DECREASES HYPOTHALAMIC KISSPEPTIN IN ADULT
COWS.**

Abstract

Lipopolysaccharide (LPS) from Gram-negative bacteria induces an immune response and impairs reproduction through suppression of gonadotropin releasing hormone (GnRH), subsequently luteinizing hormone (LH) secretion. Acute LPS administration suppresses kisspeptin in rodents and sheep, however, little is known in regards to other livestock species. Thus, we sought to examine a central mechanism whereby LPS suppresses LH secretion in adult cows. In Experiment 1, 6 cows were randomly assigned to two groups – control (CON1; 2 mL of saline intravenously [IV]; n=3) and acute endotoxin (400ENDO; 400 ng of LPS/kg BW IV; n=3). In Experiment 2, 9 cows were randomly assigned to two groups - control (CON2; 2 mL of saline IV; n=4) and acute endotoxin (600ENDO; 600 ng of LPS/kg BW IV; n=5). Blood was analyzed for LH, interleukin (IL) 6 and IL 10 concentrations. Hypothalamic tissue from cows in Experiment 2 was analyzed for kisspeptin. Concentrations of IL-6 and IL-10 were higher in cows that received LPS cows following treatment. Cows in 400ENDO had fewer LH pulses per hour (0.5 ± 0.1 pulses per hour) compared to CON1 (1.1 ± 0.1 pulses per hour). In Experiment 2, 600ENDO cows had a decreased plasma LH concentration compared to CON2. There were fewer kisspeptin immunopositive cells in the preoptic area (POA; 36.3 ± 8.3 cells) and arcuate nucleus (ARC; 62.3 ± 6.2 cells) of 600ENDO cow hypothalami compared to CON2 (POA, 73.4 ± 9.3 cells; ARC, 87.3 ± 8.0 cells). Taken together, we suggest that LPS will suppress reproduction through the kisspeptin system in cattle.

Introduction

The ability of an animal to produce viable offspring is dependent in part on the pulsatile release of gonadotropin releasing hormone (GnRH) from the hypothalamus to stimulate the episodic secretion of luteinizing hormone (LH) important for gametogenesis and steroidogenesis within the gonads. In domestic livestock, inflammatory diseases such as metritis, mastitis, and subacute rumen acidosis are commonly associated with infertility [1-6]. Specifically, lipopolysaccharide (LPS; endotoxin), a component of the cell wall of Gram-negative bacteria such as *Escherichia coli* (*E. coli*), is associated with chronic inflammation during various infections and non-infectious diseases. The physiologic responses to LPS include activation of the immune system including increase in body temperature (a fever) and an increased production of pro-inflammatory cytokines [7, 8] (e.g. tumor necrosis factor- α [TNF- α], interleukin- [IL] 1 β , IL-6) and a stress response with an increase in plasma cortisol [9] and progesterone [10, 11] concentrations.

In addition to these physiologic alterations, reproduction is impaired by endotoxin induced-inflammation. Luteinizing hormone production and secretion is suppressed following LPS administration through decreased responsiveness of the gonadotropes to GnRH [12], decreased LH pulse frequency and decreased LH pulse amplitude [12-16]. Within the hypothalamus, LPS administration suppresses GnRH secretion by blunting, eliminating, and disrupting the rhythm of GnRH release [16, 17].

Kisspeptin is a key neuropeptide in the stimulation GnRH, and subsequently LH, secretion [18]. In female mammals, there are two distinct populations of kisspeptin neurons – one in the preoptic area (POA) or anteroventral periventricular nucleus (AVPV; rodents)

and another in the arcuate nucleus (ARC) with both populations also expressing estrogen receptor α [19-21]. In female mammals, the population within the POA/AVPV is considered the GnRH surge center as these neurons, in the presence of elevated circulating estradiol, express increased levels of *Kiss1*. Meanwhile, the ARC is considered to be the tonic GnRH pulse center in both male and female mammals [18, 22] with elevated circulating estradiol decreasing expression of *Kiss1*. Importantly, inflammation is believed to act, at least in part, on the kisspeptin neurons to impair reproduction. Kisspeptin expression in the ARC is suppressed in intact male [23] and female [24] rats as well as ovariectomized female rats [25] following LPS administration. In intact ewes, a relatively low acute dose of endotoxin (100 ng LPS/kg BW) inhibits the LH surge and suppresses kisspeptin expression in the POA and ARC [26, 27]. However, there is limited data in regards to other domestic, non-seasonal breeding, livestock species, such as cattle.

The focus of this study was to evaluate the changes to reproductive neuroendocrinology as determined by kisspeptin expression in the ARC and POA and LH concentrations in plasma following systemic, endotoxin-induced inflammation with increased vaginal temperature and cytokine production (IL-6 as proinflammatory and IL10 as anti-inflammatory). We hypothesize that endotoxin-induced inflammation will suppress the kisspeptin expression in the hypothalamus along with LH secretion observed through decreased LH pulse frequency.

Materials and Methods

Fifteen adult Angus cows (multiparous, 2.5 to 4 years old) were utilized in two experiments. Cows in Experiment 1 were group housed outdoors at the East Tennessee

Research and Educational Center at University of Tennessee (ETREC) (UT Blount location) for the duration of the experiment. In Experiment 2, cows were group housed outdoors at ETREC UT Holston location until approximately 2 weeks before the experiment when they were group housed at the University of Tennessee Veterinary Research and Education Center (VREC). At both locations, all animals had open access to water, grass pasture and hay (primarily fescue). Starting about two weeks prior to the studies, all animals were acclimated to handling, serial blood collection, and underwent estrous synchronization. All procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee (IACUC #:2854-0721 and 2958-0223).

Estrous Synchronization

To ensure proestrus phase, all animals underwent estrous synchronization. Animals were administered an intramuscular (IM) injection of prostaglandin F_{2α} (PGF_{2α}; Lutalyse® Injection, dinoprost tromethamine injection; 5 mg/mL; 6 mL or 30 mg IM; Zoetis Inc., Kalamazoo, MI 49007) and estrus detection patch (Kamar® Heatmount® Detectors; Kamar Products, Inc., Zionsville, IN 46077) was applied to the tailhead of each cow in the morning about 11 days prior to experiment. Animals were observed twice a day (morning and afternoon for 30 minutes each) for 72 hours. Following the final observation period, those animals that did not show signs of estrus nor had their heat patch broken were administered GnRH (Cystorelin®, Gonadorelin Diacetate Tetrahydrate; 100 µg; 50 µg/mL; Boehringer Ingelheim Animal Health USA Inc., Duluth, GA 30096) IM and all animals received a controlled intravaginal drug release (CIDR) with progesterone (Eazi-breed CIDR Cattle Insert; 1.38 g; Zoetis Inc., Kalamazoo, MI 49007) which remained in place for 7 days until 24 hours prior to the experiment. Cows received additional doses of

Lutalyse® (30 mg) IM at 60 and 48 hours before the start of the experiment. Twenty-four hours before the experiment, progesterone CIDRs were removed and an intravaginal temperature monitoring probe (embedded into a blank CIDR courtesy of Drs. Jeff Dailey, Nicole Sanchez and Jeff Carroll at USDA-ARS in Lubbock, Texas [28]) were inserted in each cow. Vaginal Temperature was recorded every 5 minutes and reported as an hourly average. An additional temperature sensor (HOBO) was utilized to measure ambient temperature.

Experiment 1

The experimental design for this study is depicted in Figure 2.1. In August 2021, cows (~2.5 years old; 560.5 ± 19.2 kg BW) were randomly assigned to two groups: control (CON1; n=3) and endotoxin (acute endotoxin; 400ENDO; n=3). The CON1 group received 2 mL pyrogen-free saline (50 mL Single-dose 0.9% Sodium Chloride Injection, USP; Hospira Inc., Lake Forest, Illinois 60045 USA) intravenously (IV) and 400ENDO received 400 ng of LPS/kg of BW in 2 mL of saline (same as was used in CON1) IV [13, 29]. On the experiment day, the cows underwent serial blood collection with a sample every 12 minutes for six hours (1-hour pre-LPS/saline treatment and 5 hours post-LPS/saline treatment). Peripheral blood samples were collected via a jugular catheter (16-gauge 13 cm Mila IV catheter, extended use).

Experiment 2

The experimental design for this study is depicted in Figure 2.1. In May 2023, cows (~4 years old; 598.6 ± 17.2 kg BW) were randomly assigned to two groups: control (saline; CON2; n=4) and endotoxin (acute endotoxin; 600ENDO; n=5). Cows in CON2 received 2 mL pyrogen-free saline (50 mL Single-dose 0.9% Sodium Chloride Injection, USP;

Hospira Inc., Lake Forest, Illinois 60045 USA) IV while those in 600ENDO received 600 ng of LPS/kg of BW in 2 mL of saline IV [29]. Unlike what was done in Experiment 1, blood samples were collected from cows by jugular venipuncture hourly for 5 hours (2 samples before treatment administration and 3 samples after treatment). Vaginal temperature monitoring occurred in 8 of the 9 animals (four animals for each treatment) due to recorder issue. Following the final blood sample collection, animals were humanely euthanized for collection of hypothalamic tissue

LPS Preparation

Stock solution of LPS (5 mg/mL) was generated using 100 mg of LPS (Sigma Aldrich, L4005) diluted with 20 mL of sterile water (100 mL Single-dose Sterile Water For Inj., USP; Hospira Inc., Lake Forest, Illinois 60045 USA) in a fume hood and aliquoted into sterile microcentrifuge tubes (0.5 mL vials) and stored at -20°C. All IV treatments of LPS were prepared from one stock solution (5 mg/mL) and diluted to a total volume of 2 mL in saline (50 mL Single-dose 0.9% Sodium Chloride Injection, USP; Hospira Inc., Lake Forest, Illinois 60045 USA) approximately 1 hour prior to administration.

Blood Sample Processing

Blood samples (3-6 mL/each) were collected into glass tubes (12x75 mm, Fisherbrand, Cat #: 14-961-26) each containing 50 µl of 10,000 USP/mL heparin (Heparin Sodium Injection, USP; 1 mL vial; Sagent Pharmaceuticals, Schaumburg, Illinois 60195 USA) for all LH concentration analysis and Experiment 1 cytokine concentration analysis. Blood samples for cytokine concentration analysis were collected into EDTA coated tubes (BD Vacutainer; REF #367861) for Experiment 2. Samples for hormone analysis were

stored less than 48 hours at 4°C until they were centrifuged at 3 relative centrifugal force (rcf) for 20 minutes at 4°C and plasma was aliquoted in duplicate into titer tubes (BioRad Laboratories, Catalog #s: 2239390 & 2239392) and frozen at -20°C until they were assayed for LH and progesterone concentrations. Samples for cytokine analysis were immediately centrifuged at 4 rcf for 10 minutes at 4°C and plasma was aliquoted in duplicate into 1.5 mL microcentrifuge tubes (Fisherscientific, cat #05-408-129) and placed on dry ice until stored at -80°C until processing.

Tissue Collection

Cows in Experiment 2 received two IV injections of 5 ml 10,000 USP heparin 10 min apart within 30 minutes of euthanasia. All animals were euthanized with an IV overdose of Beuthanasia-D (1 mL per 4.54 kg BW; Merck Animal Health, Rahway, NJ, USA). Heads were removed and immediately perfused via the carotid arteries with approximately 6 L of 4% paraformaldehyde (PFA) in 0.1M PBS (pH 7.4) containing 0.1% sodium nitrite. Brains were removed following perfusion and stored in 4% PFA for 24 hours at 4°C then transferred into 20% sucrose solution and stored at 4°C until saturated with the solution. Frozen coronal sections of the hypothalamus were cut at 50 µm in a five-parallel series at -35°C and frozen sagittal sections of the pituitary were cut at 30 µm at -23°C with a cryostat (Microm HM550P, Germany, Model #956424), both stored in cryopreservative solution (1% polyvinylpyrrolidone, 30% ethylene glycol and 30% sucrose in 0.1 M PB) at -20°C until used for immunohistochemistry.

Luteinizing Hormone Analysis

Plasma samples were assessed for LH using a radioimmunoassay (RIA). Luteinizing hormone concentrations were measured in duplicate with an RIA using 50–

100 μ L of plasma and reagents purchased from the National Hormone and Peptide Program (Torrance, CA) as previously described [30]. Analysis of LH data included mean LH concentration, LH pulse frequency, and LH pulse amplitude. Individual LH pulses were identified using previously described criteria [31]. Briefly, there were three main criteria: (1) the peak must exceed the sensitivity of the assay, (2) a peak must occur within two data points of the previous nadir, and (3) the peak must exceed a 95% CI of the previous and following nadirs. Luteinizing hormone RIA sensitivity was 0.2 ng/ml with intra-assay coefficients of variation being 10.97% for Experiment 1 and 9.27% for Experiment 2.

Progesterone Analysis

Plasma samples from Experiment 1 were provided to the Edwards lab in the Department of Animal Science at the University of Tennessee and underwent radioimmunoassay (RIA) in order to obtain progesterone concentrations for three samples—before treatment administration (pre-treatment), 2 hours after treatment and 4 hours after treatment. Progesterone concentrations were measured in duplicate with an RIA kit (Progesterone Double Antibody RIA Kit, MP, SKU: 0717010-CF) with sensitivity of 0.2 ng/mL. Intraassay variation of 5.95% and interassay variation of 2.07%.

Cytokine Analysis

Plasma samples were analyzed in duplicate using a MILLIPLEX Bovine Cytokine/Chemokine Magnetic Bead Panel 1 96 Well Plate Assay (BCYT1-33K, Millipore) for interleukin-6 (long lasting pro-inflammatory cytokine) and interleukin-10 (anti-inflammatory cytokine) per the immunoassay instructions provided by the manufacturer. Plates were analyzed by Luminex device with a sensitivity of 5.2 ng/ml and intraassay variation of 24.13% for Experiment 1 and 34.70% for Experiment 2.

Immunohistochemistry

Four middle ARC and POA hemi-sections from the hypothalamus of cows in Experiment 2 were stained for kisspeptin. Immunohistochemical procedures were performed as previously described [32]. Briefly, tissue was washed in 0.1M phosphate buffered saline (PBS) and incubated in a PBS solution containing 0.4% Triton-X and 4% normal goat serum before an overnight incubation in a solution containing primary antibody (rabbit anti-kisspeptin polyclonal, 1:50K, gift from Belltramo, #566). Following the overnight incubation, tissue was incubated in a secondary antibody (biotinylated goat-anti-rabbit IgG, 1:500, Vector Laboratories, BA-1000), Vectastain ABC-elite, and DAB. Following a series of washes in 0.1 M PB, sections were mounted on microscope slides, air dried overnight, dehydrated using a series of increasing alcohol baths, and coverslipped.

Image Analysis

Images were captured using Leica DMi1 microscope (Leica Microsystems, Switzerland; Leica Application Suite version 4.12.0) with consistent camera settings across all hemi-sections. Kisspeptin labeled cells were quantified by two independent individuals blinded to treatment groups. Immunopositive cells for kisspeptin were identified by brown cytoplasmic staining, and those with defined borders were included in the analysis [32, 33]. GIMP 2.10.34 (GNU Image Manipulation Program; GIMP Development Team; created by Spencer Kimball and Peter Mattis) was utilized to upload images and mark individual cells on an image with a superimposed image layer. Then, Image J (NIH) was used to quantify the number of marked cells within the region of interest.

Statistical Analysis

All statistics were performed in SAS v9.4 (Cary, NC) using GLIMMIX function for two-way ANOVA. Progesterone concentrations (experiment 1) were also analyzed with Kruskal-Wallis test. Significance is determined with p-value < 0.05 and tendency defined as p-value < 0.10. Complete random design with sampling and repeated measures (time relative to treatment) were utilized for vaginal temperature (reported hourly), progesterone concentration (Experiment 1), cytokine concentrations, and LH pulse concentration analysis. Cytokine concentrations in Experiment 1 were normalized by square root for IL-6 and log for IL-10. Meanwhile, complete random design with sampling were utilized for LH pulse frequency and LH pulse amplitude in Experiment 1 and the number of immunopositive cells in Experiment 2.

Results

Experiment 1 Vaginal Temperature

There was an effect of treatment (p=0.01), time (p<0.0001) and an interaction of treatment and time (p<0.0001) on vaginal temperature. The CON1 group had no difference in vaginal temperature at any time point (Figure 2.2). For the 400ENDO group, temperature increased over time with the greatest temperature four hours post treatment (40.03 ± 0.35 °C) (Figure 2.2). There was no difference in vaginal temperature between the CON1 (38.27 ± 0.06 °C) and 400ENDO (38.54 ± 0.30 °C) at the start of the experiment (Figure 2.2). Beginning one hour after treatment administration and continuing until the end of the study, 6 hours later, vaginal temperature was increased in 400ENDO compared to CON1 (Figure 2.2).

Experiment 1 Progesterone Concentration

The Kruskal-Wallis Test provided a significant ($p=0.03$) Chi-Square value of 4.76 and the Wilcoxon Two Sample Test provided a significant ($p=0.02$) Z score of -2.12 between treatments. Following an ANOVA, there were tendencies for an effect of time ($p=0.0674$) and interaction of treatment and time ($p=0.0674$). There was no detectable presence of progesterone (<0.2 ng/ml) in the plasma in the 400ENDO nor CON1 cows in blood samples taken before treatment (Figure 2.3). Two hours after treatment, all three 400ENDO cows had detectable progesterone in the plasma none of the CON1 cows had detectable progesterone (Figure 2.3B). Furthermore, there is a significant increase in progesterone concentration in 400ENDO 2 hours after administration (0.86 ± 0.46 ng/ml) (Figure 2.3A). Meanwhile, there was one cow that received endotoxin that had detectable progesterone (0.34 ng/ml) 4 hours after treatment administration (Figure 2.3B).

Experiment 1 Cytokine Concentration

There was an effect of treatment ($p=0.0028$), time ($p<0.0001$) and an interaction of treatment and time ($p<0.001$) on plasma concentration of IL-6 such that as time following LPS administration elapsed, the concentration of IL-6 increased in 400ENDO compared to that in CON1 (Figure 2.4A). Before treatment administration, the concentration of IL-6 was not different between CON1 (34.01 ± 28.81 ng/ml) and 400ENDO (281.78 ± 240.85 ng/ml) (Figure 2.4A). Concentration of IL-6 was higher in 400ENDO than CON1 from 2 hours after treatment administration through 5 hours after treatment administration (end of the experiment) (Figure 2.4A).

There was an effect of treatment ($p=0.0038$) and an interaction of treatment and time ($p=0.0008$) on plasma concentration of IL-10 (Figure 2.4B), such that LPS

administration increased the concentration of IL-10 over time. Before treatment administration, the concentration of IL-10 was not different between CON1 (49.95 ± 7.97 ng/ml) and 400ENDO (315.68 ± 106.21 ng/ml) (Figure 2.4B). The concentration of IL-10 was greater in 400ENDO compared to CON1 from 1 hour after treatment through 5 hours after treatment (end of the study) (Figure 2.4B).

Experiment 1 Luteinizing Hormone Analysis

Representative LH pulse profiles of individual cows in CON1 (Figure 2.5A) and 400ENDO (Figure 2.5B) are depicted in Figure 5. In 400ENDO cows, there appears to be a suppression within the first 3 hours after endotoxin administration (Figure 2.5B). There was an effect of treatment ($p=0.0328$) on mean plasma concentration of LH, but no effect of time ($p=0.12$) nor an interaction of treatment and time ($p=0.15$) such that LPS elevated the mean plasma LH concentration (Figure 2.6A,B). The 400ENDO (5.12 ± 0.28 ng/mL) animals had a higher concentration of LH than CON1 (4.14 ± 0.28 ng/mL) (Figure 2.6B).

There was a treatment effect ($p=0.0142$) on the number of LH pulses per hour such that LPS administration decreased the number of LH pulses. The 400ENDO animals had fewer LH pulses per hour (0.5 ± 0.1 pulses per hour) following treatment compared to CON1 (1.1 ± 0.1 pulses per hour) (Figure 2.6C).

There was no treatment effect ($p=0.56$) on LH pulse amplitude. The overall LH pulse amplitude in the 5 hours after treatment was not different between 400ENDO (2.48 ± 0.66 ng/ml) and CON1 (2.13 ± 0.71 ng/ml) (data not shown). Within the first 3 hours after treatment, 400ENDO (2.11 ± 0.52 ng/ml) LH pulse amplitude and CON1 (2.52 ± 0.45 ng/ml) (Figure 2.6D) were not different nor from 3 to 6 hours after treatment (400ENDO, 2.66 ± 0.58 ng/ml; CON1, 1.80 ± 0.29 ng/ml) (Figure 2.6D).

Experiment 2 Vaginal Temperature

There was an effect of treatment ($p=0.0167$), time ($p<0.0001$) and an interaction of treatment and time ($p<0.0001$) such that 600ENDO cows had an increase in vaginal temperature over time (Figure 2.7). Before treatment, vaginal temperature in CON2 (38.2 ± 0.2 °C) and 600ENDO (38.39 ± 0.2 °C) were not different (Figure 2.7). From 1 hour after treatment administration through the end of the experiment (4 hours after treatment administration), vaginal temperature in 600ENDO was increased compared to CON2 (Figure 2.7).

Experiment 2 Cytokine Concentration

There was an effect of treatment ($p=0.0017$), time ($p<0.0001$) and an interaction of treatment and time ($p<0.001$) on the plasma concentration of IL-6 such that as time following LPS administration elapsed, the concentration of IL-6 increased in 600ENDO compared to that in CON2 (Figure 2.8A). Before treatment administration, the concentration of IL-6 was not different between CON2 (30.58 ± 20.29 ng/ml) and 600ENDO (167.35 ± 119.29 ng/ml) (Figure 2.8A). The concentration of IL-6 was increased in 600ENDO compared to CON2 beginning 2 hours after treatment administration and remained higher 3 hours after treatment.

There was an effect of time ($p=0.025$) and tendencies for treatment ($p=0.052$) and interaction of treatment and time ($p=0.097$) on the plasma concentration of IL-10 (Figure 2.8B). Before treatment administration, the concentration of IL-10 tended ($p=0.097$) to be increased in 600ENDO (3147.71 ± 1153.63 ng/ml) compared to CON2 (1360.50 ± 519.66 ng/ml) (Figure 2.8B). The concentration of IL-10 tended to be higher in 600ENDO (4449.99 ± 1354.18 ng/ml) compared to CON2 (1271.09 ± 688.02 ng/ml) 1 hour after

treatment, but not in the remainder of the experiment (3 hours after treatment) (Figure 2.8B).

Experiment 2 Luteinizing Hormone Analysis

Average hourly plasma LH concentration are depicted in Figure 2.9A. There was an effect of treatment ($p=0.016$), a tendency for an effect of time ($p=0.068$) and no interaction of treatment and time ($p=0.855$) such that those that received LPS had a decreased plasma LH concentration. Overall, the 600ENDO (2.07 ± 0.25 ng/mL) animals had a decreased concentration of LH compared to CON2 (2.54 ± 0.10 ng/mL) (Figure 2.9B). There was no treatment effect on the pretreatment concentration ($p=0.4514$) such that CON2 (2.72 ± 0.18 ng/mL) mean LH concentration was not different than 600ENDO (2.53 ± 0.16 ng/mL). There was a treatment effect on the post-treatment concentration ($p=0.0028$) such that 600ENDO (1.92 ± 0.07 ng/mL) was decreased compared to CON2 (2.41 ± 0.08 ng/mL).

Experiment 2 Kisspeptin

There was a treatment effect on the number of kisspeptin immunopositive cells in the POA ($p=0.0210$) and the ARC ($p=0.04589$). Within the POA, cows in 600ENDO (36.3 ± 8.3 cells) had fewer kisspeptin immunopositive cells compared to CON2 (73.4 ± 9.3 cells) (Figure 2.10). Also, within the ARC, those in 600ENDO (62.3 ± 6.2 cells) had fewer kisspeptin immunopositive cells compared to CON2 (87.3 ± 8.0 cells) (Figure 2.10).

Discussion

Taken together, the results from these experiments implicate that endotoxin-induced systemic inflammation associated with various infectious and non-infectious diseases impairs reproduction in cattle similarly to other species (sheep, mice, rats) by

suppressing LH secretion through decreased LH pulse frequency [13] likely through kisspeptin system [23, 25-27].

Firstly, endotoxin exposure in Experiment 1 (400 ng LPS/ kg BW) and Experiment 2 (600 ng LPS/ kg BW) induced activation of the immune system as observed by an increase in vaginal temperature with a peak or plateau around 4 hours after treatment [34-36]. Furthermore, there was elevation in proinflammatory cytokine, IL-6, plasma concentration in both experiments beginning 2 hours after endotoxin administration as expected [37-40] as it is a longer lasting proinflammatory cytokine. In the immune response, anti-inflammatory cytokines are also produced, which can be observed with the increased concentration of IL-10 in Experiment 1 and a tendency for an increased concentration in Experiment 2 1 hour following LPS administration as expected [41, 42]. However, in Experiment 2, 600ENDO cows had a tendency for an increased concentration in IL-10 prior to administration as well. The concentrations were different between the two experiments. This difference may have been due to the difference in the anticoagulant utilized and the time of year the experiment occurred. Furthermore, the cows in Experiment 1 were calmer than those in Experiment 2 which may have had higher concentrations of cortisol, thus reducing inflammation or suppressing the immune system. Importantly, the proinflammatory cytokine, IL-6, was similar in concentration and elevation in the two experiments.

Furthermore, LPS administration will increase plasma cortisol concentration [43, 44]. Cortisol is a potential mechanism of suppression in reproduction as ARC kisspeptin neurons contain cortisol receptors and cortisol impairs GnRH and LH secretion [45-48].

While cortisol was not measured in these experiments, plasma progesterone concentration could serve as an indirect measurement for cortisol as an overwhelming in the stimulation of adrenal cortex enzymes will induce an increase in cortisol and steroid precursor hormones, like progesterone [10, 11, 49-51]. Additionally, progesterone provides negative feedback on the ARC kisspeptin neurons to suppress GnRH and LH secretion [52-54]. While progesterone was initially measured in Experiment 1 in order to evaluate the stage of estrous cycle for the cows, it also indicated that 400ENDO cows experienced endotoxemia. At the beginning of the experiment, all cows enrolled in Experiment 1 were in proestrus as no animals had detectable plasma progesterone concentration. All animals remained pre-ovulatory at the end of the experiment as there were no corpus lutea present on either ovary (no ovulation) in all animals and CON1 animals had no detectable plasma progesterone. Two hours after LPS administration, all 400ENDO cows experienced increased progesterone concentration.

In regards to reproduction, there is altered LH secretion in response to endotoxin exposure [12-16]. In Experiment 1, the LH pulse frequency in 400ENDO was suppressed throughout the 5-hour blood collection. There was no significant difference in the hourly mean LH concentration. Mean LH concentration was numerically lower in the first two hours following LPS administration and numerically higher in 400ENDO compared to CON1 3 through 5 hours following LPS administration. As the amplitude in the 3-5 hours after LPS administration was numerically increased in 400ENDO compared to CON1, it was likely that LH secretion was still suppressed with changes in concentration linked to pulse amplitude while pulse frequency remains impaired. Increase in animal numbers will

clarify this potential change, especially as vaginal temperature and plasma cytokine concentrations were elevated in these time frames. Furthermore, in Experiment 2, there was a suppression in LH concentration in the 3 hours after LPS administration, likely within each hour. As LH secretion was described by mean LH concentration, LH pulse frequency, and LH pulse amplitude, a limitation to our analysis was the lack of LH pulse profile data availability, thus there may be alterations in LH secretion that were not observed.

There were fewer kisspeptin immunopositive neurons both in the ARC and POA in the cows which aligns with previous research in rodents [23-25, 55, 56] and sheep [26, 27]. As kisspeptin is a key regulator for GnRH/LH secretion, it is likely the decrease observed in LH concentration is due to this upstream suppression in kisspeptin. While our data was limited to Experiment 2, it was likely that those in Experiment 1 would have similar results as cytokines and temperature were elevated and LH secretion was altered.

Overall, various infectious and non-infectious diseases could greatly plague the cattle and dairy industries through the physiologic alterations caused by endotoxemia along with the immune system activation and subsequent systemic inflammation. Specifically, the suppression central reproduction could lead to infertility due to alterations in kisspeptin. More research is required in order to further investigate mechanisms for potential treatments and preventions.

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Appendix

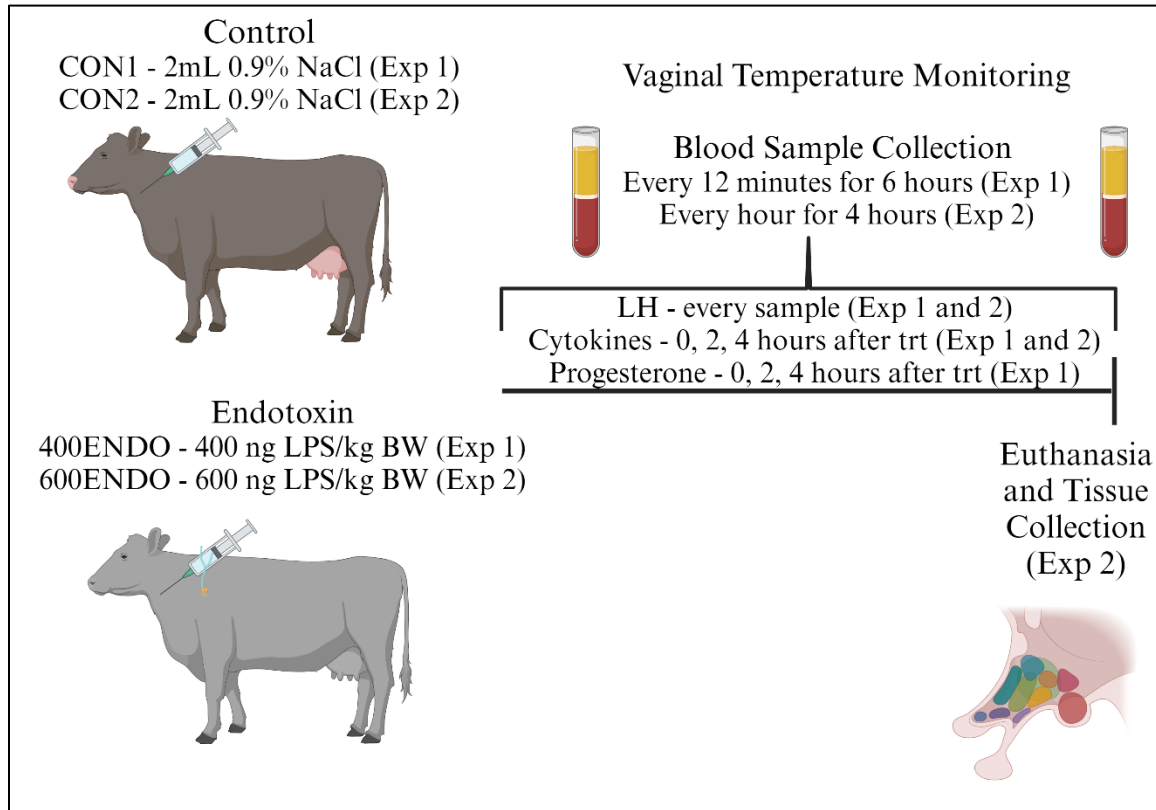


Figure 0.1. Experimental Designs.

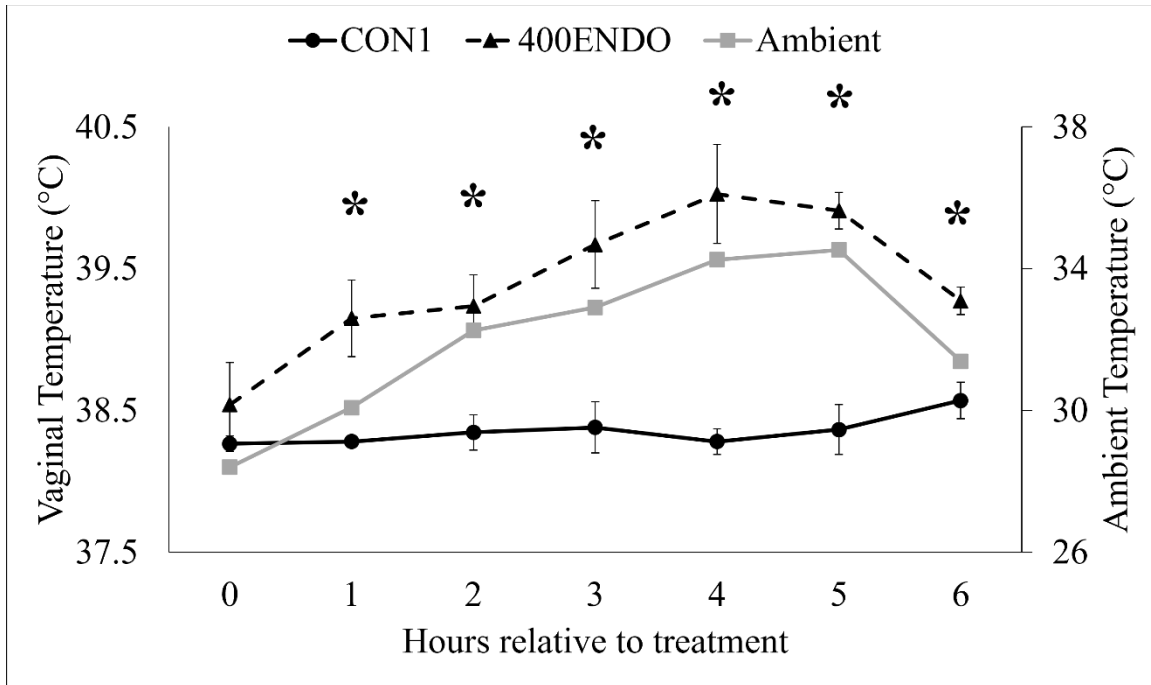


Figure 0.2. Experiment 1, Vaginal Temperature. Control (CON1; n=3; solid black line) and endotoxin administered (400ENDO; n=3; dashed black line) cows in Experiment 1. Significance determined at $p < 0.05$. * indicates the 400ENDO group is different than the CON1 within an hour. Average ambient temperature in gray.

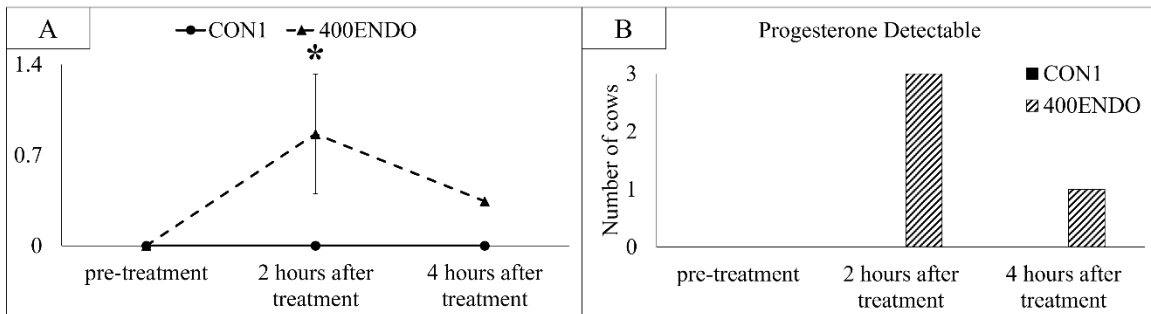


Figure 0.3. Experiment 1, Plasma Progesterone Concentration. (A) Mean plasma progesterone concentration and (B) the number the cows with detectable plasma concentrations of progesterone in three samples – a pre-treatment, 2 hours after treatment, and 4 hours after treatment. Control (CON1; n=3; solid black) and endotoxin administered (400ENDO; n=3; dashed black) cows in Experiment 1. Significance determined at $p < 0.05$. * indicates the 400ENDO group is different than the CON1.

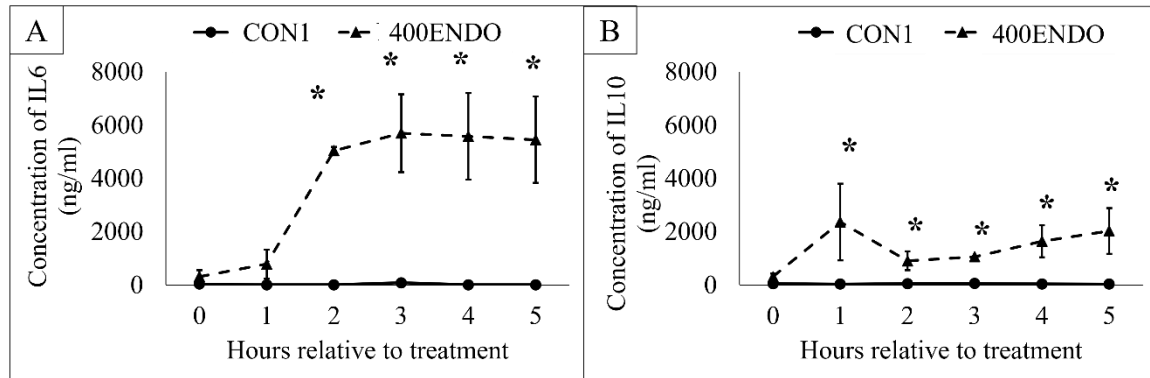


Figure 0.4. Experiment 1, Plasma Cytokine Concentration. (A) Plasma concentration of IL-6 and (B) concentration of IL-10. Control (CON1; n=3; solid black line) and endotoxin administered (400ENDO; n=3; dashed black line) cows in Experiment 1. Significance determined at $p < 0.05$. * indicates the 400ENDO group is different than the CON1.

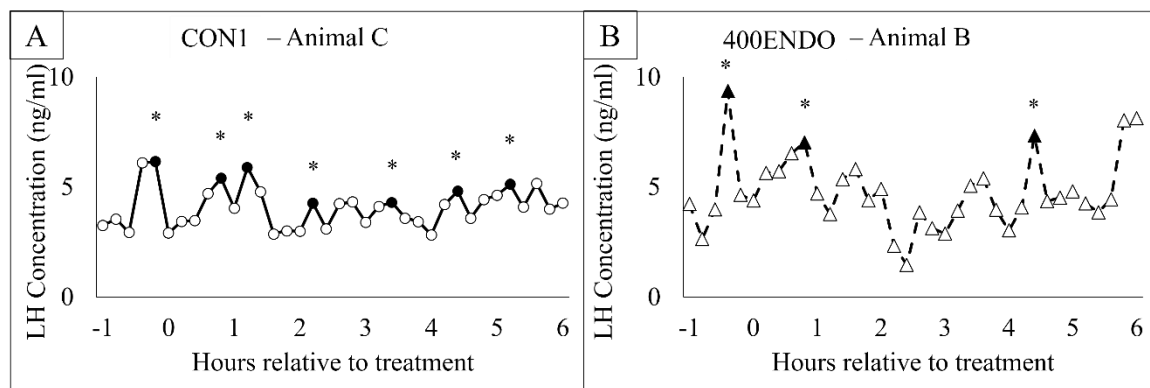


Figure 0.5. Experiment 1, Representative Plasma LH Pulse Profiles from Individuals. (A) Control (CON1; solid black line) and (B) endotoxin administered (400ENDO; dashed black line) cows in Experiment 1. Pulses are identified with filled markers and asterisks above.

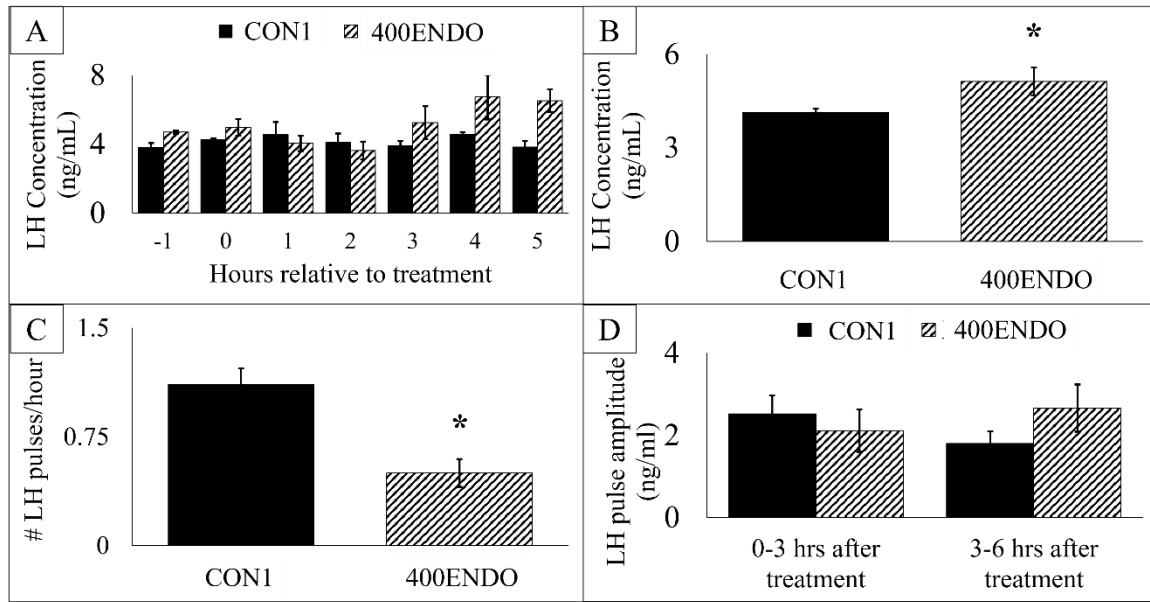


Figure 0.6. Experiment 1, Plasma Luteinizing Hormone Concentration, Pulse Frequency, and Amplitude. (A) Average hourly plasma LH concentration, (B) average plasma LH concentrations in the 5 hours after treatment and (C) LH pulses per hour in the 5 hours after treatment (D) LH pulse amplitude for control (CON1; n=3; solid black) and endotoxin administered (400ENDO; n=3; dashed black line) cows in Experiment 1. Significance determined at $p < 0.05$ indicated by *.

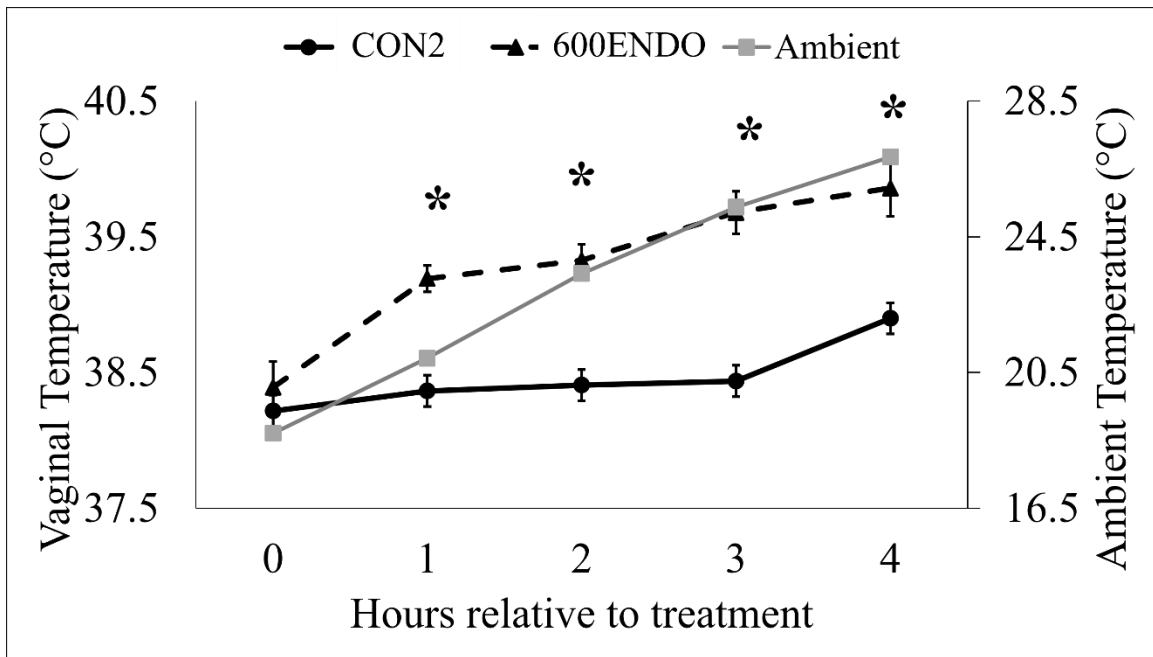


Figure 0.7. Experiment 2, Vaginal Temperature. Average hourly ambient temperature indicated by gray line. Average hourly vaginal temperature for control (CON2; n=4; solid black line) and endotoxin administered (600ENDO; n=4 due to recorder issue; dashed black line) cows in Experiment 2. Significance determined at $p < 0.05$. * indicates the 600ENDO group is different than the CON2 within an hour.

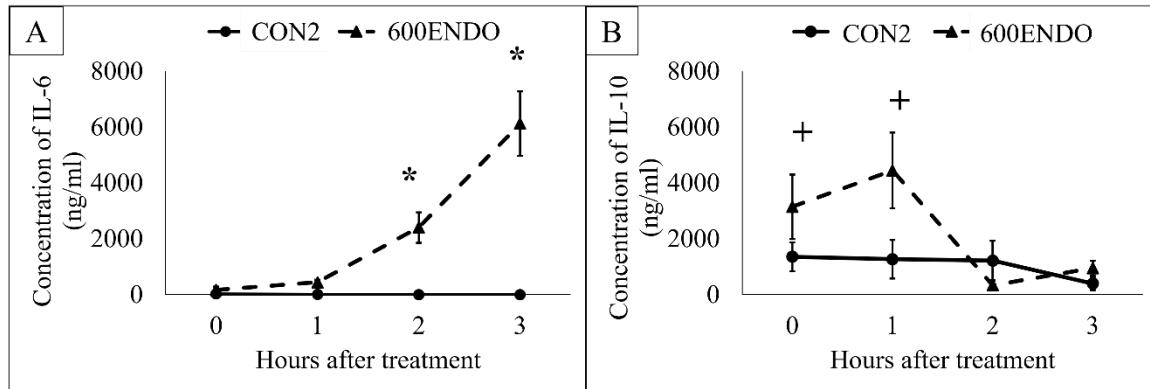


Figure 0.8. Experiment 2, Plasma Cytokine Concentrations. (A) Concentration of IL-6 and (B) concentration of IL-10. Control (CON; n=4; solid black line) and endotoxin administered (ENDO; n=5; dashed black line) cows in Experiment 2. Significance determined at $p < 0.05$. * indicates the ENDO group is different than the CON. + indicates tendency ($p < 0.10$) for difference.

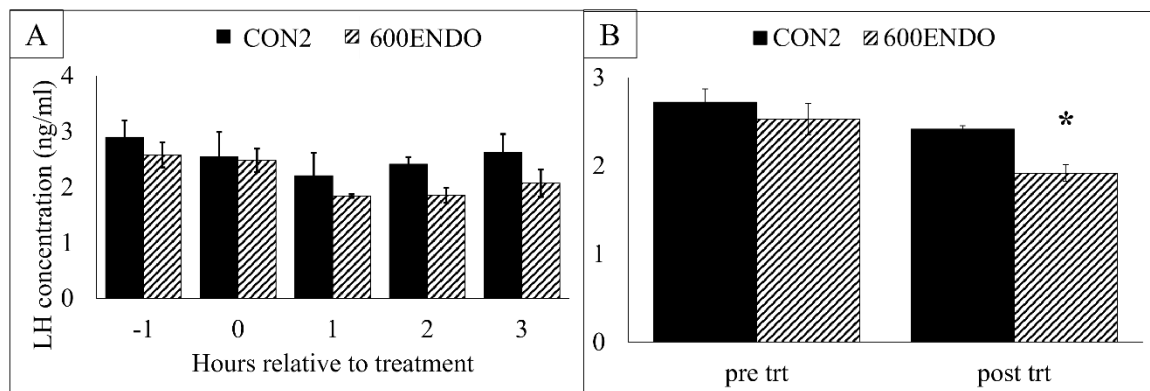


Figure 0.9. Experiment 2, Plasma LH Concentrations. (A) Average plasma LH concentration each hour relative to treatment and (B) average plasma LH concentrations pre and post treatment for control (CON2; n=4; solid black) and endotoxin administered (600ENDO; n=5; dashed black line) cows in Experiment 2. Significance determined at $p < 0.05$ indicated by *.

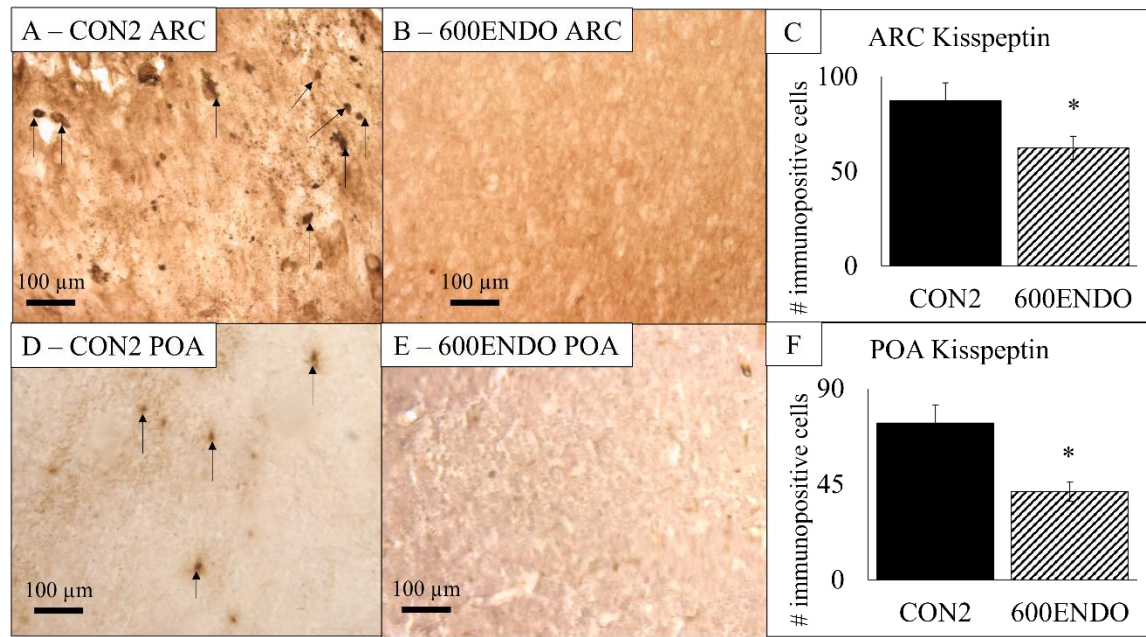


Figure 0.10. Experiment 2, Kisspeptin. Representative images of ARC kisspeptin staining in CON2 (A) and 600ENDO (B) with mean average number of kisspeptin immunopositive neurons in ARC (C). Representative images of POA kisspeptin staining in CON2 (D) and 600ENDO (E) with mean average number of kisspeptin immunopositive neurons in POA (F). Significance determined at $p < 0.05$ indicated by *. Black arrows in A and D indicate immunopositive cells.

**CHAPTER 3: FLUNIXIN ATTENUATES INFLAMMATION INDUCED
SUPPRESSION OF LUTEINIZING HORMONE IN ADULT EWES.**

Abstract

Reproductive capability is dependent on the pulsatile secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus and subsequent release of luteinizing hormone (LH) from the anterior pituitary. However, these hormones are tightly regulated and oftentimes impaired by stressors including activation of the immune system in response to lipopolysaccharide (LPS) during infections and non-infectious diseases. Kisspeptin is a key stimulator of GnRH/LH secretion. Acute LPS administration suppresses kisspeptin in rodents and sheep, however, little is known in regards to the central mechanism(s) by which inflammation suppresses reproduction. It is important to determine specific mechanism(s) that could prevent or mitigate the effects of inflammation on reproduction. Non-steroidal anti-inflammatory drugs (NSAIDs) are a drug class available to control fever, inflammation, and pain that may provide an economical and easily accessible treatment for inflammation induced suppression of reproduction. Thus, we sought to evaluate the influence of cyclooxygenase (COX) 1 and 2, in LPS induced inflammation on reproduction through administration of flunixin meglumine (FLU), an NSAID. Fifteen ewes were randomly assigned to treatment groups – saline control, FLU control, saline acute LPS, and FLU acute LPS. Blood was analyzed for LH concentration and hypothalamic tissue was analyzed for kisspeptin. Flunixin pretreatment attenuated the LPS induced suppression of LH pulse frequency within 3 hours of treatment administration. Animals that received FLU had a decreased proportion of dual kisspeptin and cFos stained cells. Taken together, we suggest that COX 1 and 2 may play a key role

in the LPS induced suppression of central reproduction, however, further investigation is needed

Introduction

Reproduction involves a coordinated system of organs to control the ability to produce viable offspring. Successful reproduction depends on the pulsatile release of gonadotropin releasing hormone (GnRH) from the hypothalamus and, subsequently, the episodic secretion of luteinizing hormone (LH) from the anterior pituitary. Reproduction, specifically through GnRH and LH secretion alterations, is tightly regulated by various internal and external cues such as inflammation due to immune system activation [1, 2]. In humans, chronic inflammation is a mediating factor between subfertility and non-infectious diseases (e.g. polycystic ovarian syndrome (PCOS) [3]) and infections (e.g. pelvic inflammatory disease (PID) [4]). This association between subfertility and chronic inflammation is mirrored in domestic livestock experiencing non-infectious disease such as ovarian follicular cysts [5] and infections like metritis [6]. Lipopolysaccharide (LPS; endotoxin; component of Gram-negative bacteria cell membrane) is involved in the pathophysiology for the aforementioned non-infectious diseases and infections amongst various other conditions [7-11] serves as a valuable approach to examine mechanisms whereby inflammation acts to suppress central reproduction. For example, ovarian cyclicity and estrous/menstrual cycle are disrupted by LPS in rats [12], ewes [1], cows [13], and non-human primates [14]. A single peripheral dose of LPS is sufficient to inhibit GnRH, and subsequently LH, secretion in multiple species (rats [15-19], sheep [2, 20, 21], geese [22], goats [23], cattle [24], non-human primates [14, 25]).

Kisspeptin is the key stimulator of GnRH/LH secretion in numerous species [26-33] with two distinct populations in the hypothalamus in female animals. One population exists in the preoptic area (POA; rostral periventricular area of the third ventricle in rodents) and serves as the LH surge center [32] while the other population in the arcuate nucleus (ARC) is the “GnRH pulse generator” [32, 33]. Importantly, inflammation is believed to impact the brain to impair reproduction via kisspeptin expressing neurons. For example, a single peripheral dose of LPS decreases hypothalamic kisspeptin mRNA and protein expression in rodents [15-17, 34-36] and sheep [37, 38].

Non-steroidal anti-inflammatory drugs (NSAIDs) are economical and easily accessible treatments to control fever, inflammation and pain in both animals and humans by inhibiting prostaglandin (PG) synthesis through inhibition of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). Use of an NSAID to block COX-1 and COX-2 activity is a potential mechanism to alleviate immune mediated suppression of reproduction. Administration of a NSAID will prevent the LPS-induced fever [39] and elevation in plasma proinflammatory cytokines (e.g. interleukin [IL] 6 and tumor necrosis factor α [TNF α][40]), plasma PG [41], and plasma cortisol and progesterone concentrations [39, 42]. Specifically, administration of flunixin meglumine (FLU; NSAID; general COX inhibitor) suppresses the increase in TNF α , haptoglobin (anti-inflammatory acute phase protein important to attenuate IL-6 [43]) and serum amyloid A (acute phase protein important for LPS clearance [44]) concentrations associated with the response to LPS [40]. In terms of reproduction, administration of flurbiprofen (NSAID; general COX inhibitor) prevents the LPS induced inhibition of pulsatile GnRH and LH secretion in ewes [42]. In

ovariectomized female rats, indomethacin (NSAID; general COX inhibitor) administration completely blocks the suppressive effects of LPS on *Kiss1* content and LH secretion [16]. However, there is limited information regarding the effects on the kisspeptin neurons in domestic livestock.

In the experiment detailed in this paper, we aim to elucidate the role of COX-1 and COX-2 in the suppression of KNDy neurons during acute endotoxin inflammation through administration of a general COX inhibitor, flunixin meglumine (FLU) in ewes. We predict that the pre-administration of FLU will be sufficient in attenuating the immune response through lessened degree of fever. In turn, we hypothesize that FLU pre-treatment will protect the reproductive neuroendocrine axis with minimal alterations to LH secretion and kisspeptin immunopositive neurons.

Materials and Methods

Animals

Fifteen intact Suffolk ewes approximately 1-2 years old and weighing approximately the same ($p = 0.3488$) were utilized in analysis from this experiment. Ewes were blocked by weight and randomly assigned to one of four treatment groups – saline control (CON; $n=3$; 65.9 ± 3.5 kg), FLU control (CONF; $n=3$; 68.3 ± 4.2 kg), saline acute endotoxin (ACT; $n=5$; 72.2 ± 4.7 kg) and FLU endotoxin (ACTF; $n=4$; 62.3 ± 2.5 kg).

Ewes were individually housed (approximately 3 m²) indoors with exposure to ambient light and temperature during breeding season (November 2021). All animals had open access to water and received a diet of grass hay (approximately 2 kg per day) which was supplemented with a commercially available pelleted all-purpose sheep feed [Tennessee Farmers Cooperative, La Vergne, TN; Tag Identification 93303; fed at

approximately 2% of bodyweight per day per animal (as fed not based on dry matter) to meet 100% of daily maintenance requirement [45]. Approximately two weeks prior to the experiment, animals were acclimated to daily handling and blood sample collections. Approximately one week prior to the experiment, animals underwent estrous synchronization (described below). All procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee (#2859-0921) for use of animals in research.

Experimental Design

Following estrous synchronization, all animals underwent serial blood collection along with their respective treatments. Blood was collected every twelve minutes for eight hours – three hours prior to and five hours after LPS/saline treatment – via jugular venipuncture and hourly vaginal temperature monitoring [46, 47]. Following the final blood sample, animals were humanely euthanized and brain tissue was collected.

Estrous Synchronization

All ewes underwent an estrous synchronization protocol in order to be in proestrus at the time treatment administration, blood collection and euthanasia [37, 38, 48, 49]. Eight days before the experiment day, all ewes were administered two luteolytic doses (1 mL; 5 mg; intramuscular) of prostaglandin F₂ α (5 mg/mL each dose; Lutalyse; Zoetis) three hours apart. Following the second dose, two progesterone containing CIDRs (Eazi-Breed Small Ruminant CIDR; 0.3 g per CIDR; Premier1) were placed intravaginally. One day before the experiment, ewes received two doses (as described above) of PGF₂ α three hours apart and progesterone CIDRs were removed and vaginal temperature probes (embedded into a blank CIDR courtesy of Drs. Nicole Sanchez, Jeff Dailey, and Jeff Carroll at the USDA-

ARS in Lubbock, TX) were inserted and left in place throughout the remainder of the study [46, 47]. Twenty-four hours later, the experimental treatments were administered.

Treatments

All sheep received an intravenous (IV) injection of 2 ml pyrogen-free saline (50 mL Single-dose 0.9% Sodium Chloride Injection, USP; Hospira Inc.) or FLU (2.2 mg/kg BW, 50 mg/mL; Prevail®; VETone® MWI) approximately 30 minutes before a second treatment of either saline (50 mL Single-dose 0.9% Sodium Chloride Injection, USP; Hospira Inc) or LPS (O55:B5; Sigma Aldrich, L4005; 400ng/kg of BW) administered IV. Those in CON received saline at both treatment points while CONF received flunixin 30 minutes before saline (Figure 1). Those in ACT received saline 30 minutes before LPS and ACTF received FLU 30 minutes before LPS (Figure 1).

LPS Preparation

Stock solution of LPS (5 mg/mL) was generated using 100 mg of LPS (Sigma Aldrich, L4005) diluted with 20 mL of sterile water (100 mL Single-dose Sterile Water For Inj., USP; Hospira Inc., Lake Forest, Illinois 60045 USA) in a fume hood and aliquoted into sterile microcentrifuge tubes (0.5 mL vials) and stored at -20°C. All IV treatments of LPS were prepared from one stock solution (5 mg/mL) and diluted to a total volume of 2 mL in saline (50 mL Single-dose 0.9% Sodium Chloride Injection, USP; Hospira Inc., Lake Forest, Illinois 60045 USA) approximately 1 hour prior to administration.

Blood Sample Preparation

Blood samples (3 ml/each) were collected by venipuncture with a syringe and needle (21-gauge needle, 2.54 cm long) and subsequently placed into glass tubes (12x75 mm, Fisherbrand, Cat #: 14-961-26) each containing 50 µl of 10,000 USP/ml heparin

(Heparin Sodium Injection, USP; 1 mL vial; Sagent Pharmaceuticals) and stored less than 48 hours at 4°C until they were processed for plasma collection. Whole blood was centrifuged at 3 relative centrifugal force for 20 minutes at 4°C and plasma was aliquoted in duplicate into titer tubes (BioRad Laboratories, Catalog #s: 2239390 & 2239392) and frozen at -20°C until they were assayed for LH, cortisol and progesterone concentrations.

Tissue Collection

Following blood collection, ewes received two IV injections of heparin (1 mL, 10,000 USP) 10 min apart within 30 minutes of euthanasia. All animals were euthanized with an IV overdose of pentobarbital (Beuthanasia-®D; 390 mg pentobarbital sodium / mL; dose of 1 mL / 4.54 kg BW; Schering -Plough Animal Health Corp). The order of which animals were euthanized was alternating based upon their assigned treatment groups allowing for roughly an equal time after treatment administration for tissue collection between treatments. Heads were removed by sharp dissection and immediately perfused via the carotid arteries with approximately 6 L of 4% paraformaldehyde (PFA) in 0.1M PBS (pH 7.4) containing 0.1% sodium nitrite using a gravity-flow system (i.e., container of PFA was approximately 1 meter above the head). Brains were removed following perfusion and stored in 4% PFA for 24 hours at 4°C then transferred into 20% sucrose solution and stored at 4°C until saturated with the solution. Frozen coronal sections of the hypothalamus were cut at 30-50 µm in a five-parallel series at -35°C and frozen sagittal sections of the pituitary were cut at 30 µm at -23°C with a cryostat (Microm HM550P, Model #956424), both stored in cryopreservative solution (1% polyvinylpyrrolidone, 30% ethylene glycol and 30% sucrose in 0.1 M PB) at -20°C until used for immunohistochemistry.

Temperature Analysis

Vaginal temperature was recorded every 5 minutes utilizing vaginal temperature loggers [46, 47]. The data was summarized as an average for each hour prior to statistical analysis. Temperature data was analyzed through 4 hours as the numbers of animals in each treatment group did not maintain power due to animal euthanasia.

Luteinizing Hormone Analysis

Plasma samples were assessed for LH using a radioimmunoassay (RIA). LH concentrations were measured in duplicate with an RIA using 50–100 μ L of plasma and reagents purchased from the National Hormone and Peptide Program (Torrance, CA) as previously described [50]. Analysis of LH data included mean LH concentration, LH pulse frequency, and LH pulse amplitude. Hourly mean concentrations determined by average of the concentrations each hour. Individual LH pulses were identified using previously described criteria [51]. Briefly, there were three main criteria: (1) the peak must exceed the sensitivity of the assay, (2) a peak must occur within two data points of the previous nadir, and (3) the peak must exceed a 95% CI of the previous and following nadirs. Luteinizing hormone RIA sensitivity was 0.2 ng/mL with intraassay variation of 11.55% and interassay variation of 9.79%.

Progesterone Analysis

Plasma samples from were provided to the Edwards lab in the Department of Animal Science at the University of Tennessee and underwent radioimmunoassay (RIA) in order to obtain progesterone concentrations for three samples – before treatment administration (pre-treatment), 2 hours after treatment and 4 hours after treatment. Progesterone concentrations were measured in duplicate with an RIA kit (Progesteorne

Double Antibody RIA Kit, MP, SKU: 0717010-CF). Intraassay variation of 5.95% and interassay variation of 2.07%.

Immunohistochemistry

Dual Immunohistochemistry for kisspeptin and cFos: Four full hypothalamic sections containing middle ARC and POA were selected based on a sheep hypothalamic atlas and split midline to produce hemi-sections. Free-floating hypothalamic sections were processed for the protein detection of kisspeptin and cFos. Briefly, following a series of washes in 0.1M phosphate buffered saline (PBS), hemi-sections were blocked in 10% H₂O₂ (diluted in 0.1M PBS) to minimize endogenous peroxidase activity and incubated in a PBS solution containing 0.4% Triton-X (Sigma Aldrich, CAS: 9002-93-1; Code:327371000) and 20% normal goat serum (NGS; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, Cat #: 005-000-121). Following this block incubation, tissue was incubated overnight in a solution containing a primary antibody for kisspeptin (anti-rabbit polyclonal primary antibody, 1:50K, M. Belltrimo, #566). After this overnight incubation, the hemi-sections underwent incubation periods in a secondary antibody (biotinylated goat-anti-rabbit IgG, 1:500, Vector Laboratories, BA-1000), Vectastain ABC-elite (1:500; Vector Laboratories, Burlingame, CA), a 3,3'-diaminobenzidine tetrahydrochloride (DAB; Fisher Scientific, cat# AC328005000), and another block incubation (0.4% Triton-X, 4% NGS) before an overnight incubation in cFos primary antibody (anti-guinea pig polyclonal; 1:10K, Synaptic Systems, 226 004). Next, the tissue was incubated in a secondary antibody (biotinylated goat-anti-guinea pig, 1:500, Vector Laboratories, BA-7000), Vectastain ABC-elite (1:500), and DAB-Nickel (2% solution). Following a series of washes in 0.1 M phosphate buffer (PB), sections were mounted on

microscope slides, air dried overnight, dehydrated using a series of increasing alcohol baths, and coverslipped.

Single Immunohistochemistry for LHbeta: Four mid-sagittal sections of the pituitary were stained for LHbeta. Immunohistochemical procedures were performed as previously described [48]. Briefly, tissue was washed in 0.1M phosphate buffered saline (PBS) and incubated in a PBS solution containing 0.4% Triton-X and 4% NGS before an overnight incubation in a solution containing primary antibody (rabbit anti LHB polyclonal, 1:20K, Golden West BioSolutions, TLIA1042.03). Following the overnight incubation, tissue was incubated in a secondary antibody (biotinylated goat-anti-rabbit IgG, 1:500, Vector Laboratories, BA-1000), Vectastain ABC-elite, and DAB. Following a series of washes in 0.1 M PB, sections were mounted on microscope slides, air dried overnight, dehydrated using a series of increasing alcohol baths, and coverslipped.

Image Analysis

Images were captured using Leica DMi1 microscope (Leica Microsystems, Switzerland; Leica Application Suite version 4.12.0) with consistent camera settings across all hemi-sections. Kisspeptin, LHbeta, cFos, and dual labeled cells were quantified by two independent individuals blinded to treatment groups. Immunopositive cells for kisspeptin and LH β were identified by brown cytoplasmic staining, and those with defined borders were included in the analysis [48, 52] while cFos immunopositive cells were identified with black nuclear staining. GIMP 2.10.34 (GNU Image Manipulation Program; GIMP Development Team; created by Spencer Kimball and Peter Mattis) was utilized to upload images and mark individual cells on an image with a superimposed image layer. Then,

Image J (NIH) was used to quantify the number of marked cells within the region of interest.

Statistical Analysis

All statistical analysis was executed in SAS 9.4 (Cary, NC) utilizing GLIMMIX function to perform two-way ANOVA. Vaginal temperature, plasma progesterone concentration, and plasma LH concentration were analyzed as a complete random design with sampling (multiple individuals), factorial (two treatments per individual) and repeated measures (value measured multiple times). Luteinizing hormone pulse frequency and LH pulse amplitude were analyzed as a complete random design with sampling (multiple individuals in each group) and factorial (two treatment groups per individual). The number of immunopositive cells from IHC were analyzed as a complete random design with sampling (multiple individuals in each group) and factorial (pretreatment and treatment). Significant difference was determined if $p\text{-value} < 0.05$ and tendency for difference was determined if $0.05 < p\text{-value} < 0.1$.

Results

Temperature

There was no effect of FLU ($p=0.8365$) nor an interaction of LPS and FLU (0.4879) on vaginal temperature. There was an effect of LPS ($p=0.0033$), time ($p<0.0001$), an interaction of LPS and time ($p<0.0001$), an interaction of FLU and time ($p=0.0225$) and an interaction of LPS, FLU, and time (0.0070). There was no difference in vaginal temperature prior to LPS/saline treatment (Figure 3.1; Table 1). Vaginal temperature was significantly increased at 2 hours after LPS/saline treatment in ACT ($39.91 \pm 0.22^{\circ}\text{C}$) compared to other ewes that was maintained through the duration of the experiment (Figure 3.1, Table 1).

Vaginal temperature for ACTF ($40.53 \pm 0.25^{\circ}\text{C}$) increased 3 hours after LPS/saline administration compared to CON ($38.93 \pm 0.28^{\circ}\text{C}$) and CONF ($39.07 \pm 0.28^{\circ}\text{C}$) (Figure 3.1, Table 1).

Progesterone Concentration

The Kruskal Wallis Test Chi Square value (3.6504) was not significant ($p=0.561$) such that LPS did not have an effect on progesterone concentration. The Kruskal Wallis Test Chi Square value (1.5645) was not significant ($p=0.2110$) such that FLU did not have an effect on progesterone concentration. The Kruskal Wallis Test Chi Square value (5.8590) was not significant ($p=0.1187$) when the pretreatment and treatment were analyzed as one treatment on plasma progesterone concentration. However, of note, there were individual animals in the ACT and ACTF that had detectable amounts of progesterone after the administration of LPS while there was undetectable progesterone concentration or no presence of progesterone in animals from CON and CONF (0 ng/ml as reported value) (Figure 3.2). The mean progesterone concentration for ACT was 0.05 ± 0.05 ng/mL (in 2 of the 5 sheep) and ACTF was 0.25 ng/mL (in 1 of 4 sheep) 2 hours after endotoxin exposure (Figure 3.2). Four hours after treatment the mean progesterone concentrations for ACT was 0.16 ± 0.13 ng/mL (in 2 of the 5 sheep; 1 sheep detectable at 2h) meanwhile ACTF did not have detectable progesterone concentration (Figure 3.2).

Luteinizing Hormone Analysis

Representative LH pulse profiles are depicted in Figure 3.3. Plasma concentrations of LH were no different prior to administration of treatments ($p=0.1969$). There was no difference between the mean plasma LH concentration for CON (5.6 ± 0.8 ng/ml), CONF (4.0 ± 0.8 ng/ml), ACT (5.2 ± 0.6 ng/ml) nor ACTF (5.7 ± 0.7 ng/ml) during pre-treatment

period. There was an effect of LPS ($p=0.0235$), an interaction of LPS and FLU ($p=0.0311$), an effect of time ($p=0.0021$), and an interaction of LPS and time ($p=0.0155$) on plasma concentrations of LH. There was no effect of FLU ($p=0.6389$), no interaction of FLU and time ($p=0.7273$), and no interaction of LPS, FLU, and time on plasma concentration of LH ($p=0.4569$). Hourly mean LH concentration is depicted in Figure 3.4A. Plasma concentrations of LH was elevated in those that received endotoxin (LPS; ACT and ACTF) at 3 hours (7.9 ± 0.5 ng/ml) and 4 hours (7.9 ± 0.5 ng/ml) after LPS administration compared to those that received saline (SAL; CON and CONF) at 3 hours (4.9 ± 0.7 ng/ml) and 4 hours (5.3 ± 0.7 ng/ml) as well as the pretreatment concentration (5.4 ± 0.5 ng/ml) (Figure 3.4B).

In the pre LPS treatment period, there was no effect of FLU ($p=0.948$). There was no difference in the pulse frequency between CON (1.3 ± 0.1 pulses per hour), CONF (1.2 ± 0.1 pulses per hour), ACT (1.1 ± 0.7 pulses per hour) nor ACTF (1.2 ± 0.1 pulses per hour) prior to LPS/saline administration (data not shown). There was an effect of FLU ($p=0.0019$) and an interaction of LPS and FLU ($p=0.0132$) on plasma LH pulses per hour in the 5 hours following LPS/saline treatment. ACT had fewer pulses per hour (0.7 ± 0.1 pulses per hour) compared to CON (0.9 ± 0.1 pulse per hour), CONF (0.9 ± 0.1 pulse per hour) and ACTF (1.1 ± 0.1 pulse per hour) (Figure 3.5A). From hour 0 to 2.5 following LPS administration, there was an effect of FLU ($p=0.0037$), but not an effect of LPS ($p=0.6612$), or an interaction of LPS and FLU on plasma LH pulses per hour ($p=0.2975$). During the first 2.5 hours following LPS/saline treatment, those that received saline as a pretreatment (ACT and CON) had fewer LH pulses per hour (0.7 ± 0.1 pulse per hour)

compared to those that received FLU (ACTF; 1.3 ± 0.1 pulse per hour) (Figure 3.5B). Every animal in CON had 0.8 pulses per hour and in CONF had 1.2 pulses per hour within the first 2.5 hours after treatment while those in ACT had an average pulse per hour of 0.56 ± 0.16 pulses per hour and those in ACTF had an average of 1.3 ± 0.19 pulses per hour (Figure 3.5B). However, from 2.5 to 5 hours after LPS, there were no effects of LPS ($p=0.7052$), FLU ($p=0.5308$) or interaction of LPS and FLU ($p=0.1823$). During those last 2.5 hours, there was no difference in the number of plasma LH pulses per hour between CON (0.9 ± 0.1 pulse per hour), CONF (0.7 ± 0.1 pulse per hour), ACT (0.8 ± 0.1 pulse per hour) nor ACTF (0.9 ± 0.1 pulse per hour) (Figure 3.5C).

There were no treatment effects ($p=0.7050$) on the plasma LH pulse amplitude during the pretreatment period such that there was not difference in LH pulse amplitude between CON (1.8 ± 0.5 ng/ml), CONF (2.0 ± 0.5 ng/ml), ACT (2.7 ± 0.4 ng/ml), nor ACTF (2.8 ± 0.4 ng/ml) (data not shown). There was no effect of FLU ($p=0.5121$), LPS ($p=0.1389$), nor an interaction of LPS and FLU ($p=0.5829$) on plasma LH pulse amplitude in the 5-hour blood collection after LPS treatment administration (data not shown). There was no difference between the LH pulse amplitude for CON (3.0 ± 1.0 ng/ml), CONF (1.9 ± 1.0 ng/ml), ACT (3.9 ± 0.7 ng/ml) nor ACTF (3.8 ± 0.8 ng/ml) (data not shown).

LHbeta Immunopositive Cells

Representative images of LHbeta immunopositive cells from individuals are depicted in Figure 3.6. There was no effect of FLU ($p=0.2411$) or an interaction of LPS and FLU ($p=0.4392$) on the number of LHbeta immunopositive cells. However, there was a tendency for an effect of LPS ($p=0.0868$) on the number of LHbeta immunopositive cells in the pituitary of sheep. The number of immunopositive cells was not different between

CON (56.2 ± 5.8 cells), CONF (45.3 ± 5.8 cells), ACT (62.0 ± 4.5 cells) or ACTF (59.6 ± 5.0 cells) (Figure 3.6). Those that received LPS tended to have increased LHbeta expression.

ARC Kisspeptin, cFos, and Dual Labeled Immunopositive Cells

Representative images of kisspeptin and cFos immunopositive cells from individuals are depicted in Figure 3.7. Within the ARC, there was no effect of LPS ($p=0.9659$), FLU ($p=0.2130$), nor an interaction of LPS and FLU ($p=0.0802$) on the number of kisspeptin immunopositive cells. There was no difference in the number of ARC kisspeptin immunopositive cells between CON (23.3 ± 6.6 cells), CONF (42.7 ± 6.6 cells), ACT (34.5 ± 5.1 cells) and ACTF (30.9 ± 5.7 cells) (Figure 3.7E). There was no effect of LPS ($p=0.9123$), FLU ($p=0.9129$) nor an interaction of LPS and FLU ($p=0.0513$) on the number of cFos immunopositive cells in the ARC. There was no difference in the number of cFos immunopositive cells in CON (37.5 ± 14.4 cells), CONF (64.8 ± 14.4 cells), ACT (67.7 ± 11.1 cells), and ACTF (37.5 ± 12.5 cells) (data not shown). There was no effect of LPS ($p=0.5147$) or FLU ($p=0.2624$) on the number of dual kisspeptin-cFos cells, but there was an interaction of LPS and FLU ($p=0.0440$). There were fewer dual stained cells in ACTF (2.9 ± 0.9 cells) compared to ACT (6.3 ± 0.8 cells), however, there was no difference in ACTF from CON (3.4 ± 1.1 cells) and CONF (4.5 ± 1.1 cells) (data not shown). There was no effect of LPS ($p=0.5268$) or an interaction of LPS and FLU ($p=0.4065$) on the proportion of dual stained cells, but there was an effect of FLU ($p=0.0248$). Animals that received FLU had a decreased proportion of dual stained cells (10.5 ± 1.5) compared to those that received saline (16.0 ± 1.5) (Figure 3.7F).

POA Kisspeptin Immunopositive Cells

Representative images of kisspeptin cells in the POA from individuals are depicted in Figure 3.8. There is no effect of LPS ($p=0.3866$), FLU ($p=0.9744$), nor an interaction of LPS and FLU ($p=0.4326$). There is no difference in the number of kisspeptin immunopositive cells between CON (11.2 ± 2.4 cells), CONF (13.0 ± 2.4 cells), ACT (15.3 ± 2.4 cells) and ACTF (13.3 ± 2.1 cells) (Figure 3.8E).

Discussion

Peripheral inflammation associated with various diseases and infections is a key initial induction factor for central inflammation through production of cytokines that cross the blood brain barrier [53, 54] to directly bind to afferent nerves and endothelial cells or to activate local microglia and astrocytes in the brain to synthesize additional cytokines to cause febrile, hormonal, and behavioral effects associated with infectious disease, such as impaired reproduction [55]. It is established that endotoxin induced inflammation causes a suppression in reproduction through impaired GnRH/LH production and secretion [20, 36, 56, 57] and acute endotoxin administration suppresses kisspeptin mRNA and protein expression in rodents [16, 34, 37, 38], however, the responsible central mechanism (s) remain to be elucidated. As shown herein, the activation of the immune system (increased vaginal temperature) and suppression in reproduction (initial decreased LH a general COX inhibitor. However, there was “recovery” of reproduction by the time tissue was collected (about 5 hours after LPS administration) as there was no difference in kisspeptin expression nor LH pulse frequency.

Overall, the ewes that received LPS, ACT and ACTF, both experienced an immune and stress response following their exposures to endotoxin while CON and CONF did not

as observed through temperature and progesterone. As expected, the ewes in ACT and ACTF had an increase in vaginal temperature [58, 59] compared to the CON and CONF ewes. However, pre-treatment with FLU delayed, reduced overall the increase in vaginal temperature [60] caused by LPS in ACTF ewes as seen with the increase occurring at 2 hours after endotoxin administration compared to 1 hour in ACT ewes, a lower average temperature, and an earlier time to peak in vaginal temperature.

Initially, we measured progesterone concentrations to ensure that the ewes were in proestrus during the experiment. As expected, all ewes did not have detectable progesterone prior to treatment and, anecdotally, there were no corpus luteum present on any of the ovaries at the time of euthanasia. The CON and CONF ewes did not have detectable progesterone at 2 hours and 4 hours after LPS/saline treatment thus indicating no progesterone present throughout the duration of the experiment. While there was no statistical increase in progesterone, there were individual animals that received LPS with detectable levels of progesterone following endotoxin exposure. An increase in animal numbers could provide statistical significance. Additionally, the progesterone concentration in ACTF after 4 hours of endotoxin exposure was numerically lower than that of the ACT group, thus indicating a potential attenuation due to the FLU pretreatment. While the changes in progesterone concentrations were not significant, it is important to note that there was a physiological change in some ACT and ACTF ewes that would likely be significant with more animals. Endotoxin produces an increase in plasma cortisol [60]. During a stress response, cholesterol is converted into various intermediate steroid hormones, such as progesterone, by specific enzymes prior to final output of cortisol [61-

64]. Therefore, when there is overwhelming activation the steroidogenesis pathway for cortisol, there can be an increase in the production of the intermediate hormones as well as cortisol [61-64].

Endotoxin impairs reproduction throughout the HPG axis [2]. There was a suppression in LH secretion over the course of 5 hours after LPS administration, specifically within the first 2.5 hours following LPS administration as ACT ewes had fewer LH pulses per hour compared to CON that aligns with previous literature [21, 57]. From 2.5 to 5 hours after LPS treatment, there was no difference in LH pulse frequency in ACT. In contrast to previous literature [2], there was no reduction in LH pulse amplitude after LPS treatment in ACT ewes. Furthermore, there is an increase in LH concentration in the later hours following LPS administration in animals in ACT compared to those in CON and CONF. Treating anterior pituitary explants from ewes with LPS inhibits the elevation of LH β (gonadotropin subunit specific for LH) gene expression induced by GnRH administration [56]. However, there was no difference in the number of LH β immunopositive gonadotropes amongst the groups at the time of tissue collection in the study reported here (5 hours after LPS treatment). Taken together, there is likely a recovery of LH secretion in ACT ewes in this present study.

As expected, the ACTF ewes had normal LH pulse frequency throughout the duration of the experiment likely due to the COX system as inhibition of COX-1 and COX-2 will prevent inflammation-induced suppression of GnRH and LH secretions in rats [18, 19]. There was also no effect on LH pulse amplitude nor mean LH concentration when evaluated as its own group. However, when combined with ACT, LPS treatment induced

an increase in LH concentration in this present study. This effect was likely influenced by the ACT group as there was a clear suppression in LH pulse frequency initially followed by a recovery. Additional animals in each treatment may allow for a more noticeable between groups.

Furthermore, this suppression in reproduction is “echoed” in the hypothalamus as the rhythm of GnRH release is blunted, eliminated or disrupted by LPS [2, 20] and there is decreased mRNA expression of GnRH [36]. Interestingly, LH secretion can be restored by the administration of kisspeptin, therefore, GnRH neurons retain their ability to respond appropriately and the impairment is likely through the kisspeptin system [34]. A limit of this experiment is that GnRH was not measured in plasma nor hypothalamic tissue.

Endotoxin decreases hypothalamic mRNA for kisspeptin and kisspeptin receptor [15-17, 35, 36] as well as about a 50% decrease in the number of kisspeptin immunopositive neurons in the ARC in rats [34]. In ewes, a relatively low acute dose of endotoxin prevents kisspeptin secretion and dynorphin cell activation as described by a decreased proportion of activated kisspeptin neurons in the ARC [37, 38]. In contrast, there was no difference in kisspeptin expression nor activated kisspeptin neurons in the ARC in ACT ewes in this experiment. In the current study, ACTF and CONF combined had a smaller proportion of activated (dual stained) kisspeptin neurons compared to the combined group of FLU and ACT in ARC. Again, as there was a recovery in LH secretion and no difference in the number of kisspeptin immunopositive cells in ACT, it is likely that the ACT influenced the group of those that received saline as a pretreatment. Additionally, ACT had greater number of dual stained neurons compared to ACTF and CON thus

indicating an increase in the amount of kisspeptin being produced as dual stained are kisspeptin neurons that are actively producing kisspeptin. The number of activated neurons was not greater than CONF, thus the effect seen in ACT may be fading as the LH secretion is recovered. In the POA, a relatively low dose of LPS prevents the LH surge in part through the suppression of kisspeptin secretion in sheep [37, 38]. In contrast to this previous research, we did not have a difference in the number of kisspeptin immunopositive neurons within the POA. A limitation to the POA data is that we do not provide cFos, dual labeled, nor proportion of cells activated.

Ultimately, the alterations to the hypothalamus and the pituitary induced by endotoxin-induced inflammation decrease estradiol production and impair ovulation within the ovary as defined by a delay or complete cessation of estrus in ewes [2, 20, 37, 38, 65]. While we did not evaluate ovulation, the recovery in LH secretion and the lack of difference in ARC and POA kisspeptin expression amongst the groups indicates that all ewes could have normal estrous cycles and appropriately ovulate.

Taken together, more research is required in order to better elucidate the role of the COX system in inflammation-induced suppression of central reproduction. Utilization of a higher dose of LPS, a shorter time between administration and tissue collection following acute endotoxin administration, or tissue collection during the surge rather than proestrus could accomplish this task. First, it is possible that the amount of endotoxin was not sufficient enough to produce a prolonged effect on the hypothalamus due to insufficient suppression of kisspeptin below the threshold required for proper LH secretion [66]. It is possible that the pituitary was mainly affected by the dose and mode by which endotoxin

was administered herein [1, 2]. Alternatively, a chronic model of inflammation may be more physiologically relevant for evaluating the inflammation induced suppression of reproduction as seen in infections and non-infectious diseases. For example, in a daily LPS administration, suppression of LH concentration was sustained 5 days after the first LPS administration [67]. We have shown that chronic LPS administration with a daily increasing dose of LPS will continue to suppress kisspeptin and LH pulse frequency after 7 days, thus eliciting a response as well as overwhelming or avoiding immunotolerance [68].

Effects of endotoxin on LH suppression, pro-inflammatory cytokines [69], and cortisol production [70], occur, and potentially peak, within the first 2 hours of LPS administration as observed in this study and others. Therefore, collecting tissue closer to 2-3 hours after LPS administration may provide clearer insight. Alternatively, evaluating the effects through the LH surge may be more beneficial as the recovered LH pulse frequency and similar ARC kisspeptin expression in ACT could be due to the time samples were collected as there are differences between proestrus and estrus [37, 38]. It is possible that the LH surge in ACT may have been eliminated, blunted, or delayed due to an insufficient increase in kisspeptin immunopositive cells and activity in the POA [32, 38, 48, 49, 68, 69]. Altering the stage of the estrous cycle would influence the amount of estrogen present. In mice, hypothalamic macrophage infiltration (immune cell that enters tissue and phagocytizes) occurs during diet induced obesity (inflammatory condition) in males, but not females, thus indicating a potential for estrogen to provide protection against

an immune response [70, 71]. As proestrus and estrus are marked by elevated levels of estrogen, potential sex steroid protection may have occurred in the ACT animals.

Another limitation of this study is that it is unknown whether these animals had previous exposure to LPS. The ewes also may have experienced immunotolerance with inhibited cellular respiration and altered secretomes of macrophages due to immunity developed from previous exposures to endotoxin [72, 73]. While we did remove animals from the study that may have had previous exposure, all of these ewes were from the same source so it is possible additional ewes did have immunity against LPS due to previous exposures which would alter their response to our exogenous LPS administration.

Acute LPS administration suppresses kisspeptin in rodents and sheep, however, little is known in regards to the central mechanism(s) by which inflammation suppresses reproduction. It is important to determine specific mechanism(s) that could prevent or mitigate the effects of inflammation on reproduction. Non-steroidal anti-inflammatory drugs are available over the counter or by prescription to control fever, inflammation, and pain that may provide an economical and easily accessible treatment for inflammation induced suppression of reproduction. Thus, further investigation into the influence of cyclooxygenase (COX) 1 and 2 and other NSAID pathways may provide potential preventions and treatments for LPS induced inflammation suppression of reproduction in order to increase the productivity and profitability of domestic livestock as well as human subfertility and infertility.

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Appendix

Table 1. Hourly Rectal Temperature. CON (saline pretreatment and treatment). CONF (2.2 mg flunixin/kg BW pretreatment and saline treatment). ACT (saline pretreatment and 400 ng LPS/kg BW treatment). ACTF (2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW treatment). TRT indicates treatment. HR indicates hour relative treatment. Significance $p < 0.05$ with different letters.

TRT	HR -1	HR 0	HR1	HR2	HR3	HR4
CON	38.88 $\pm 0.27^{\circ}\text{C}^{\text{a}}$	38.88 $\pm 0.28^{\circ}\text{C}^{\text{a}}$	38.90 $\pm 0.28^{\circ}\text{C}^{\text{a}}$	38.88 $\pm 0.28^{\circ}\text{C}^{\text{a}}$	38.93 $\pm 0.28^{\circ}\text{C}^{\text{a}}$	38.92 $\pm 0.28^{\circ}\text{C}^{\text{a}}$
CONF	38.94 $\pm 0.27^{\circ}\text{C}^{\text{a}}$	39.04 $\pm 0.28^{\circ}\text{C}^{\text{a}}$	38.96 $\pm 0.28^{\circ}\text{C}^{\text{a}}$	38.98 $\pm 0.28^{\circ}\text{C}^{\text{a}}$	38.99 $\pm 0.28^{\circ}\text{C}^{\text{a}}$	39.07 $\pm 0.28^{\circ}\text{C}^{\text{a}}$
ACT	38.78 $\pm 0.21^{\circ}\text{C}^{\text{a}}$	39.09 $\pm 0.22^{\circ}\text{C}^{\text{a}}$	39.65 $\pm 0.22^{\circ}\text{C}^{\text{ab}}$	39.91 $\pm 0.22^{\circ}\text{C}^{\text{b}}$	40.36 $\pm 0.22^{\circ}\text{C}^{\text{c}}$	40.61 $\pm 0.22^{\circ}\text{C}^{\text{c}}$
ACTF	38.82 $\pm 0.23^{\circ}\text{C}^{\text{a}}$	38.90 $\pm$ $0.25^{\circ}\text{C}^{\text{a}}$	39.12 $\pm 0.25^{\circ}\text{C}^{\text{a}}$	39.69 $\pm 0.25^{\circ}\text{C}^{\text{ab}}$	40.53 $\pm 0.25^{\circ}\text{C}^{\text{c}}$	40.30 $\pm 0.25^{\circ}\text{C}^{\text{c}}$

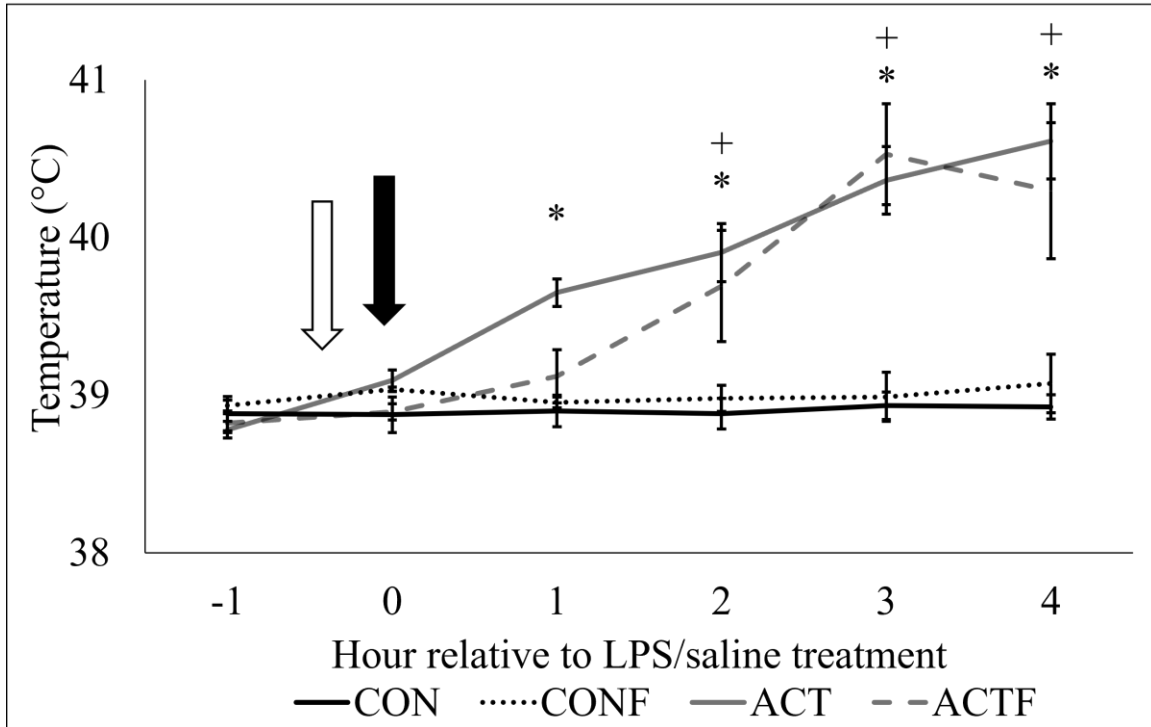


Figure 0.1. Vaginal Temperature. Hourly mean vaginal temperature relative to LPS/saline treatment. CON (saline pretreatment and treatment) is the black solid line. CONF (2.2 mg flunixin/kg BW pretreatment and saline treatment) is dotted black line. ACT (saline pretreatment and 400 ng LPS/kg BW treatment) is solid gray line. ACTF (2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW treatment) is gray dashed line. Black arrow is the LPS/saline treatment administration. White arrow is the flunixin/saline pretreatment administration. Statistical difference ($p < 0.05$) indicated by * for ACT vaginal temperature compared to CON vaginal temperature and + for ACTF vaginal temperature compared to CON vaginal temperature. CON vaginal temperature was not different from CONF at any timepoint.

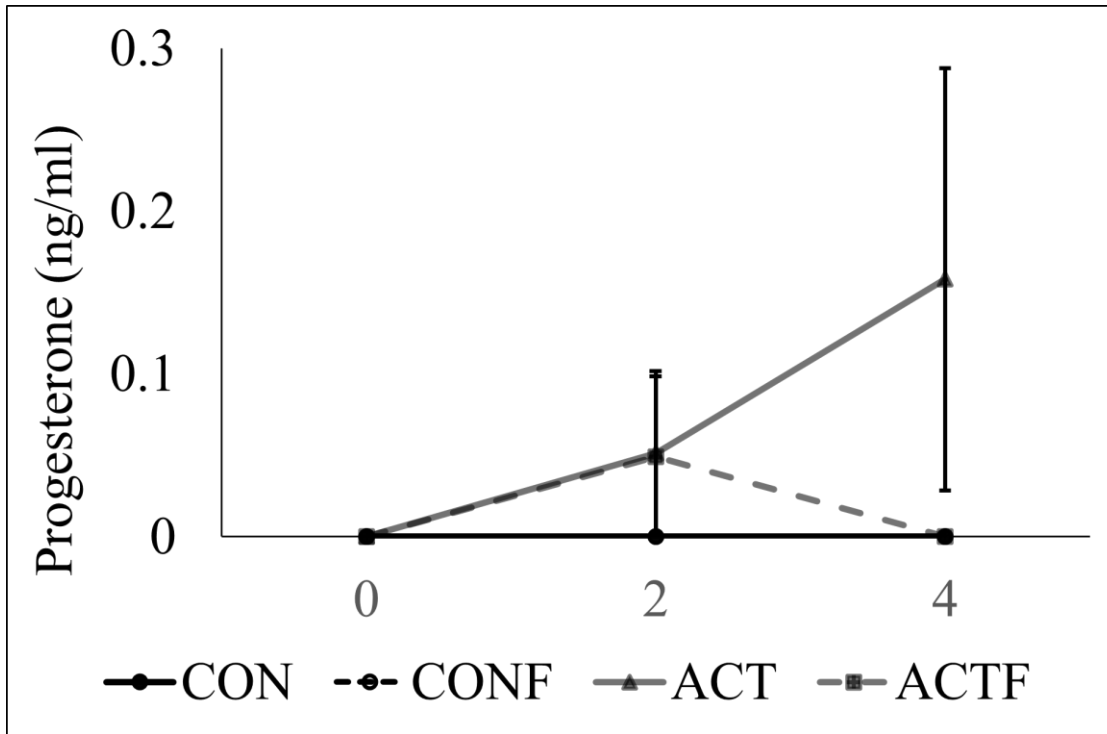


Figure 0.2. Mean plasma progesterone concentration. Mean plasma progesterone concentration relative to LPS/saline treatment. CON (saline pretreatment and treatment) is the black solid line. CONF (2.2 mg flunixin/kg BW pretreatment and saline treatment) is dotted black line. ACT (saline pretreatment and 400 ng LPS/kg BW treatment) is solid gray line. ACTF (2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW treatment) is gray dashed line. There was no statistical effect, but there were animals in ACT and ACTF that presented detectable progesterone.

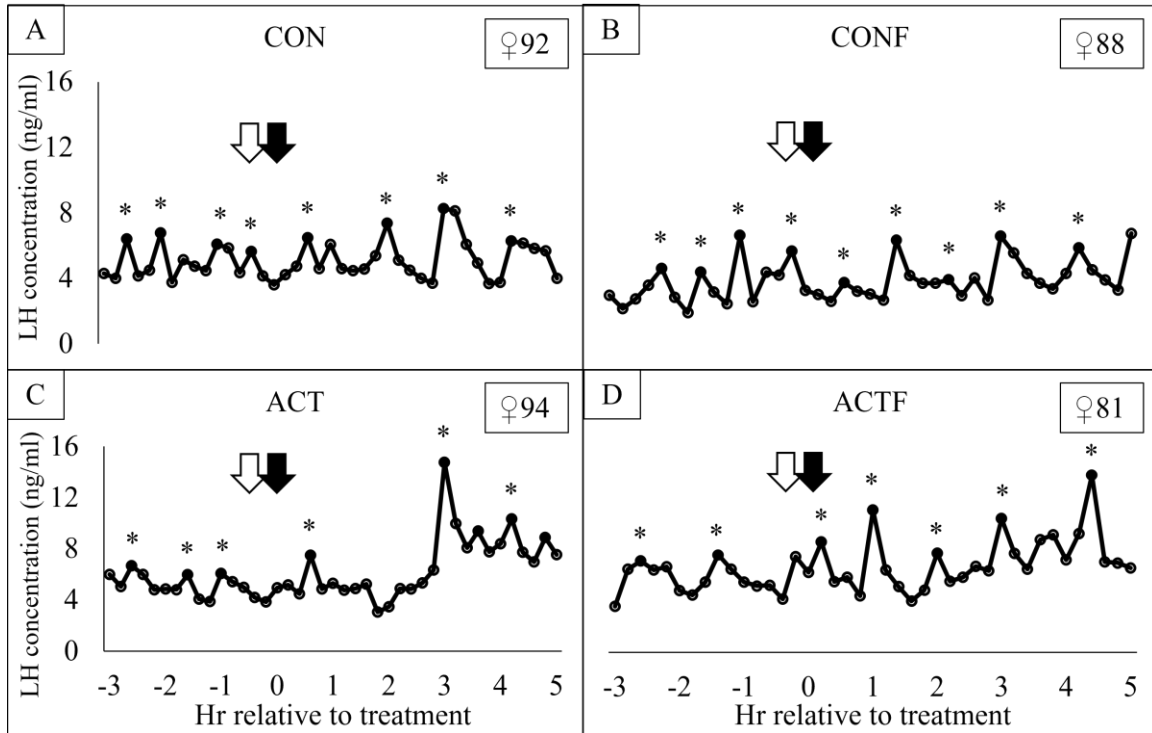


Figure 0.3. Representative LH pulse profiles. (A) Control (saline pretreatment and treatment). (B) Control+flunixin (CONF; 2.2 mg flunixin/kg BW pretreatment and saline treatment). (C) Acute LPS (ACT; saline pretreatment and 400 ng LPS/kg BW treatment). (D) Acute LPS + flunixin (ACTF; 2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW). Black arrow is the LPS/saline treatment administration. White arrow is the flunixin/saline pretreatment administration. * indicates the identified pulse.

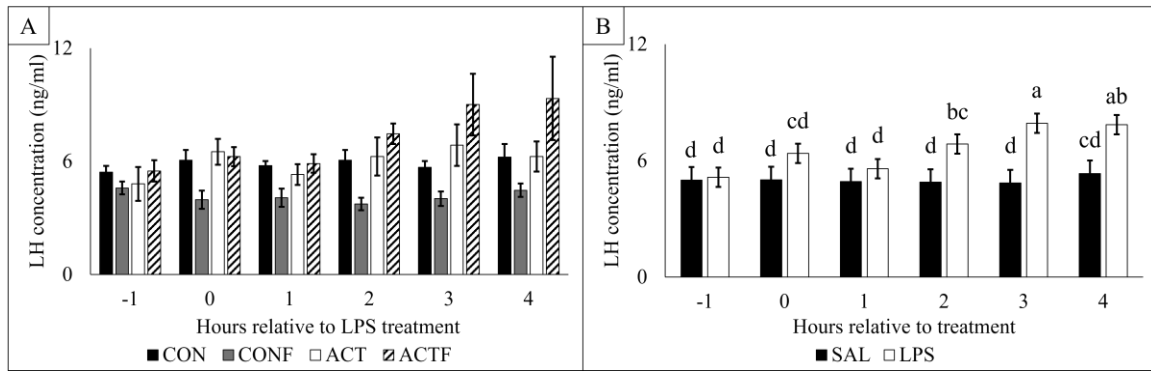


Figure 0.4. Hourly mean plasma LH concentration. (a) Mean LH plasma concentration described by treatment groups such that Control (saline pretreatment and treatment) indicated by black bar, Control+flunixin (CONF; 2.2 mg flunixin/kg BW pretreatment and saline treatment) indicated by gray bar, Acute LPS (ACT; saline pretreatment and 400 ng LPS/kg BW treatment) indicated by white bar and Acute LPS + flunixin (ACTF; 2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW) indicated by black striped bar. (B) Mean LH plasma concentration described on whether the animal received LPS or saline for their treatment with those that received saline in black bar and those that received LPS in white bar. Significance ($p < 0.05$) indicated by different letters.

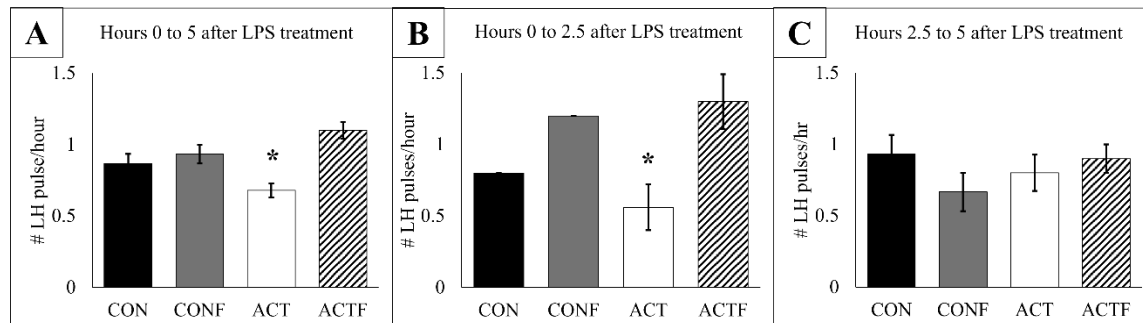


Figure 0.5. LH Pulse Frequency. (A) Number of pulses per hour over the 5 hour time period after LPS/saline treatment. (B) Number of pulses per hour from 0 to 2.5 hours after LPS/saline treatment. (C) Number of pulses per hour from 2.5 to 5 hours after LPS/saline treatment. Control (saline pretreatment and treatment) indicated by black bar, Control+flunixin (CONF; 2.2 mg flunixin/kg BW pretreatment and saline treatment) indicated by gray bar, Acute LPS (ACT; saline pretreatment and 400 ng LPS/kg BW treatment) indicated by white bar and Acute LPS + flunixin (ACTF; 2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW) indicated by black striped bar. * indicates significance $p < 0.05$ compared to CON.

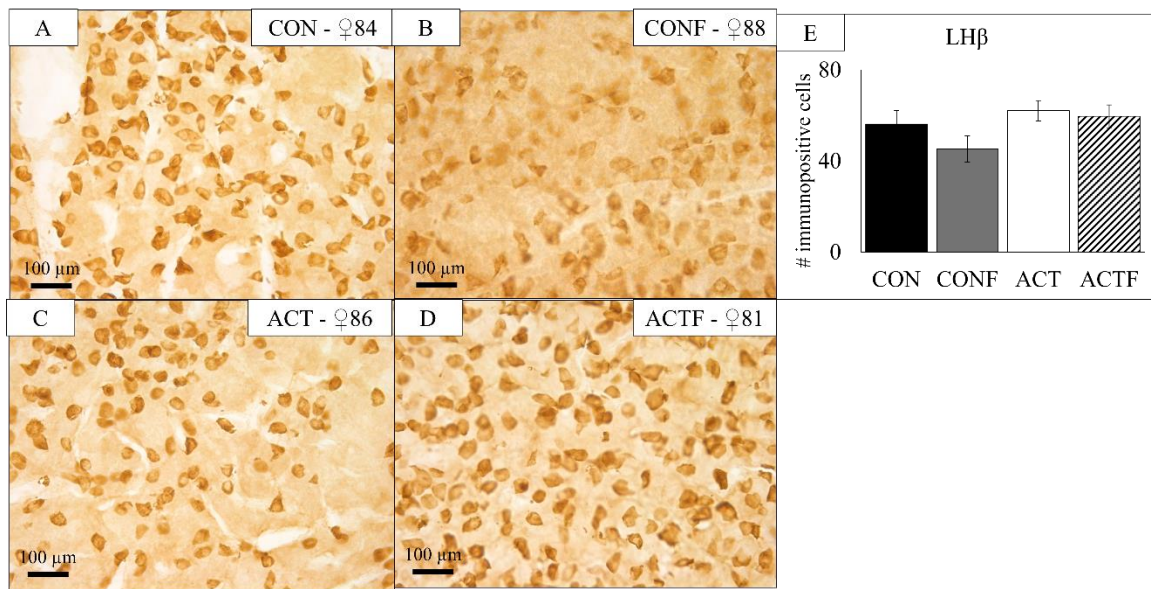


Figure 0.6. 20X LHbeta Immunopositive Cells. Representative images of individuals from (A) saline control, (B) Control+flunixin (CONF; 2.2 mg flunixin/kg BW pretreatment and saline treatment), (C) Acute LPS (ACT; saline pretreatment and 400 ng LPS/kg BW treatment) and (D) Acute LPS + flunixin (ACTF; 2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW). (E) Average number of LHβ immunopositive cells. There was no statistical difference.

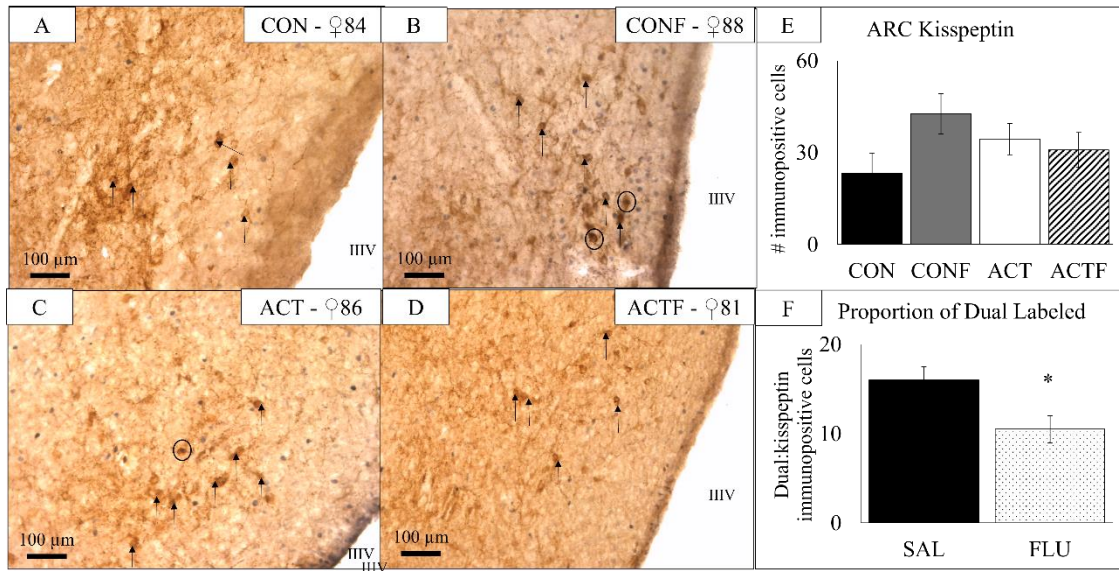


Figure 0.7. Kisspeptin, cFos and Dual Labeled Immunopositive Cells in ARC. Representative images of individuals from (A) saline control, (B) Control+flunixin (CONF; 2.2 mg flunixin/kg BW pretreatment and saline treatment), (C) Acute LPS (ACT; saline pretreatment and 400 ng LPS/kg BW treatment) and (D) Acute LPS + flunixin (ACTF; 2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW). (E) Average number of kisspeptin immunopositive cells. (F) the proportion of dual labeled kisspeptin cells. * Indicates significance. SAL is the combined ACT and CON while FLU is the combined ACTF and CONF. Black arrow indicates kisspeptin positive cell and black circle indicates representative dual labeled.

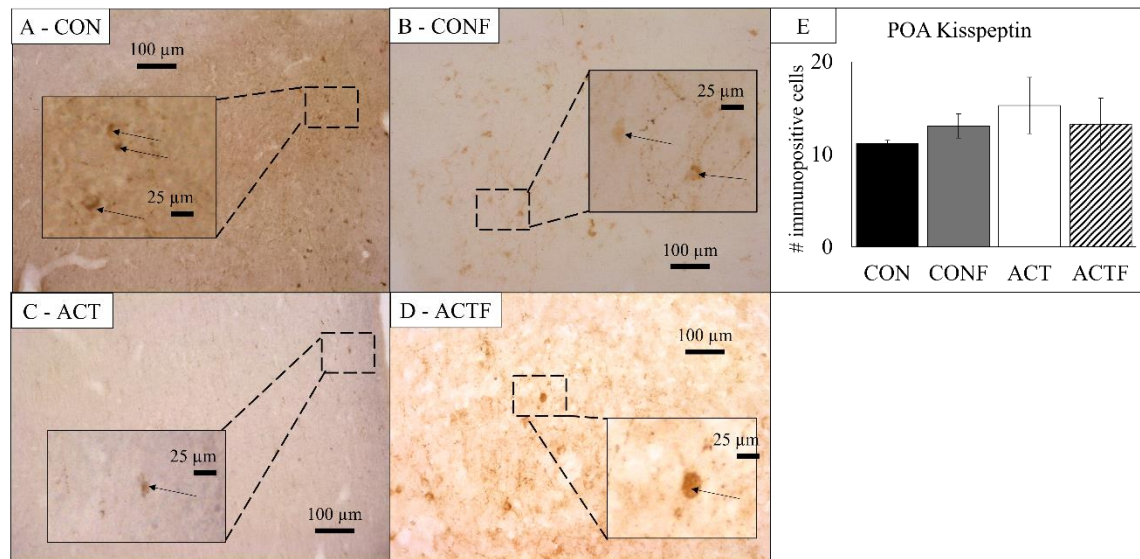


Figure 0.8. Kisspeptin Immunopositive Cells in POA. Representative images of individuals from (A) saline control, (B) Control+flunixin (CONF; 2.2 mg flunixin/kg BW pretreatment and saline treatment), (C) Acute LPS (ACT; saline pretreatment and 400 ng LPS/kg BW treatment) and (D) Acute LPS + flunixin (ACTF; 2.2 mg flunixin/kg BW pretreatment and 400 ng LPS/kg BW). (E) Average number of kisspeptin immunopositive cells. Black arrow indicates cells. There was no significant difference.

**CHAPTER 4: CHRONIC INFLAMMATION DECREASES ARCUATE
KISSPEPTIN EXPRESSION IN CASTRATED MALE SHEEP.**

Abstract

Lipopolysaccharide (LPS) from Gram-negative bacteria induces an immune response and impairs reproduction through suppression of gonadotropin releasing hormone (GnRH), subsequently luteinizing hormone (LH) secretion. While there is evidence that acute inflammation inhibits kisspeptin, little is known about the impact of chronic inflammation on this key reproductive neuropeptide in livestock species. Thus, we sought to examine a central mechanism whereby LPS suppresses LH secretion in sheep. Twenty wethers were randomly assigned to one of five treatment groups: control (CON; n=4), single acute IV LPS dose (SAD; n=4), daily acute IV LPS dose (DAD; n=4), daily increasing IV LPS dose (DID; n=4), and chronic subcutaneous LPS dose (CSD; n=4). On Days 1 and 7, blood samples were collected every 12 minutes for 360 minutes using jugular venipuncture. Following blood collection on Day 7, all animals were euthanized, brain tissue was perfused with 4% paraformaldehyde, and hypothalamic blocks were removed and processed for immunohistochemistry. On Day 1, LH pulse frequency was significantly lower ($p=0.02$) in SAD (0.25 ± 0.1 pulses/hour), DAD (0.25 ± 0.1 pulses/hour), DID (0.35 ± 0.1 pulses/hour), and CSD (0.40 ± 0.1 pulses/hour) compared to CON (0.70 ± 0.1 pulses/hour). On Day 7, only DID animals (0.35 ± 0.1 pulses/hour) had significantly lower ($p=0.049$) LH pulse frequency compared to controls (0.85 ± 0.1 pulse/hour). Furthermore, only DID animals (33.3 ± 10.9 cells/section/animal) had significantly fewer ($p=0.001$) kisspeptin-immunopositive cells compared to controls (82.6 ± 13.6 cells/section/animal). Taken together, we suggest that daily increasing doses of LPS is a powerful inhibitor of

kisspeptin neurons in young male sheep and a physiologically relevant model to examine the impact of chronic inflammation on the reproductive axis in livestock.

Introduction

Reproduction is an important physiological function that can be negatively impacted by the stress of inflammation. Successful reproduction depends on the pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, which, in turn, stimulates episodic secretion of luteinizing hormone (LH) from the anterior pituitary. In humans, chronic inflammation is a mediating factor between polycystic ovarian syndrome (PCOS) and subfertility [1]. In domestic livestock, chronic inflammatory diseases such as metritis, mastitis, and subacute rumen acidosis are commonly associated with infertility [2-7]. The administration of lipopolysaccharide (LPS), component of Gram-negative bacteria cell wall associated with various infections and non-infectious diseases [8], to induce systemic inflammation has been an extremely valuable approach to examine the mechanisms whereby inflammation acts to suppress the reproductive axis. For example, work in non-human primates has shown that repeated administration of LPS for five days is capable of disrupting menstrual cycles [9] and in sheep using a continuous infusion of LPS for 4 hours and for 26 hours disrupted LH secretion in the follicular phase causing delayed or blocked LH surge secretion [10]. Moreover, a single peripheral dose of LPS is sufficient to inhibit GnRH, and subsequently LH, secretion in ovariectomized ewes [11, 12].

While a considerable amount of research in numerous species has sought to identify the “GnRH pulse generator” (reviewed in Nestor et al., 2023 [13]), there exists a broad

body of evidence supporting the idea that kisspeptin-neurokinin B-dynorphin (KNDy) neurons located in the arcuate nucleus (ARC) of the hypothalamus are a key component of the central mechanism of pulsatile GnRH/LH secretion [13]. Given that essentially all GnRH neurons express Kiss1R [14, 15] and that up to 60% of GnRH neurons receive synaptic input from KNDy neurons [16], kisspeptin is believed to be the primary output of KNDy neurons stimulating GnRH cells [13]. Importantly, inflammation is believed to act, at least in part, at the level of the brain to impair reproduction via kisspeptin neurons. For example, a single dose of LPS decreases the hypothalamic kisspeptin mRNA and protein expression in rodents [17-22] and in sheep [23, 24]. However, there is limited evidence in livestock as to the effects of chronic endotoxemia, like seen in diseases and infections, on hypothalamic kisspeptin expression.

Therefore, the purpose of this study was to identify a physiologically relevant model of chronic inflammation that will induce an immune response, a stress response and suppress the reproductive neuroendocrine axis using castrated male sheep model. Common administration routes of intravenous (IV) and subcutaneous (SQ) as well as dosing strategies of a daily repeated acute dose [25, 26] and daily increase in dose [27] were utilized in this study. We hypothesize that a chronic inflammation induced by LPS will provide a physiologically relevant model as described with fewer activated ARC kisspeptin immunopositive neurons, decreased LH pulse frequency and mean concentration, and fewer LH β immunopositive cells as well as a prolonged elevation in rectal temperature (RT), and plasma concentrations of cortisol and progesterone. To test our hypothesis, we will compare five groups receiving LPS and evaluate changes in circulating LH, cortisol

and progesterone after LPS administration and changes in expression of kisspeptin in the ARC and LH β (subunit of LH) in the anterior pituitary. We determined kisspeptin expression along with cFos (immediate early gene commonly used for neuronal activity) to evaluate part of the central control of reproduction. In addition, LH concentration and the expression of LH α , the identifying protein of LH produced in gonadotropes, were examined to determine the effects at the level of the pituitary gland. We evaluated the hypothalamic-pituitary-adrenal axis response caused by LPS exposure through evaluating plasma concentrations of cortisol and RT, which both increase following LPS administration [5, 11, 28]. Furthermore, we determined plasma progesterone concentration as it is possible to have an increase in other steroid hormones due to overwhelming adrenocorticotrophic hormone (ACTH) stimulation of the steroidogenesis enzymes within the adrenal glands [29-33] and progesterone negative feedback may provide another mechanism for LPS induced suppression of reproduction.

Materials and Methods

Animals

Twenty Suffolk wethers (typically banded 2-14 days post-birth for castration) approximately 1 year of age and weighing approximately the same ($p=0.9238$;) were utilized in this experiment. Wethers were blocked by body weight and randomly assigned to one of five groups: control (CON; $n=4$; 62.3 ± 1.6 kg), single acute LPS dose (SAD; $n=4$; 60.4 ± 2.9 kg), daily acute LPS dose (DAD; $n=4$; 59.9 ± 2.8 kg), daily increasing LPS dose (DID; $n=4$; 61.8 ± 1.2 kg), and chronic subcutaneous LPS dose (CSD; $n=4$; 60.7 ± 1.5 kg).

All sheep were group housed indoors with a light cycle of 8 hours of light and 16 hours of dark to simulate breeding season photoperiod at approximately 22 to 24°C. All animals had open access to water and received a diet of grass hay (approximately 45 kg per day) which was supplemented with a commercially available pelleted all-purpose sheep feed [Tennessee Farmers Cooperative, La Vergne, TN; Tag Identification 93303; fed at approximately 2% of bodyweight per day per animal (as fed not based on dry matter) to meet 100% of daily maintenance requirement [34]. Starting four weeks prior to the study, all animals were acclimated to handling which included serial blood collection by jugular venipuncture and RT monitoring. Two weeks prior to the study, animals were individually housed (approximately 3 m².) Once moved to individual housing, animals were fed (twice a day; approximately 12 hours between feedings) the aforementioned pelleted all-purpose sheep feed at approximately 2% of bodyweight per day. All procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee (#2803-0121).

Experimental Design

The experimental design for this study is depicted in Figure 4.1. Animals in the CON group and the CSD group received daily IV injections of 2 mL pyrogen-free saline (50 mL Single-dose 0.9% Sodium Chloride Injection, USP; Hospira Inc., Lake Forest, Illinois 60045 USA). Animals in the SAD group received one IV injection of 400 ng LPS/kg of BW [12] on the first day of the experiment (Day 1) followed by daily IV injections of 2 mL saline (as described above) through the end of the study. Animals in the DAD group received daily IV injections of 400 ng LPS/kg of BW [25] throughout the study. Animals in the DID group received an IV injection of 400 ng LPS/kg of BW on Day 1 followed by a daily IV injection of LPS that increased by 20% from the previous

day administration for 7 days [27]. Animals in the CSD received a subcutaneous osmotic pump (Alzet 2ML1) surgically implanted subcutaneously into the right axillary region on Day 1 which released 20 µg LPS/day [35]. On Days 1 and 7, blood samples were collected every 12 minutes for 6 hours using jugular venipuncture [36], with IV administrations of saline or LPS occurring 1 hour after serial blood collection began. Daily experimental treatments were administered at the same time of day for the other Days of the study (2 to 6). In addition, RT were determined at -1, 1, 3, and 5 hours relative to treatment for Day 1 and -1, 1, and 3 hours relative to treatment on Day 7. Following blood collection on Day 7, all animals were humanely euthanized.

LPS Preparation

Stock solution of LPS (5 mg/mL) was generated using 100 mg of LPS (Sigma Aldrich, L4005) diluted with 20 mL of sterile water (100 mL Single-dose Sterile Water For Inj., USP; Hospira Inc., Lake Forest, Illinois 60045 USA) in a fume hood and aliquoted into sterile microcentrifuge tubes (0.5 mL vials) and stored at -20°C. All IV treatments of LPS were prepared from one stock solution (5 mg/mL) and diluted to a total volume of 2 mL in saline (50 mL Single-dose 0.9% Sodium Chloride Injection, USP; Hospira Inc., Lake Forest, Illinois 60045 USA) approximately 1 hour prior to administration. Osmotic pumps for chronic LPS delivery were loaded with 5 mg/mL LPS stock solution per the instructions 24 hours prior to use and submerged in 0.9% sterile saline overnight at 37°C. Prior to insertion, the osmotic pumps were sterilized according to recommendations by the manufacturer.

Blood Sample Processing

Blood samples (3 mL/each) were collected by venipuncture with a syringe and needle (21-gauge needle, 2.54 cm long) and subsequently placed into glass tubes (12x75 mm, Fisherbrand, Cat #: 14-961-26) each containing 50 µl of 10,000 USP/mL heparin (Heparin Sodium Injection, USP; 1 mL vial; Sagent Pharmaceuticals, Schaumburg, Illinois 60195 USA) and stored less than 48 hours at 4°C until they were processed. Whole blood was centrifuged at 3 relative centrifugal force for 20 minutes at 4°C and plasma was aliquoted in duplicate into titer tubes (BioRad Laboratories, Catalog #s: 2239390 & 2239392) and frozen at -20°C until they were assayed for LH, cortisol, and progesterone concentrations.

Tissue Collection

Following blood collection on Day 7, wethers received two IV injections of heparin (1 mL, 10,000 USP) 10 min apart within 30 minutes of euthanasia. All animals were euthanized with an IV overdose of pentobarbital (Beuthanasia-®D; 390 mg pentobarbital sodium / mL; dose of 1 mL / 4.54 kg BW; Schering -Plough Animal Health Corp., New Jersey 07083 USA). Heads were removed by sharp dissection and immediately perfused via the carotid arteries with approximately 6 L of 4% paraformaldehyde (PFA) in 0.1M PBS (pH 7.4) containing 0.1% sodium nitrite using a gravity-flow system (i.e. container of PFA was approximately 1 meter above the head). Brains were removed following perfusion and stored in 4% PFA for 24 hours at 4°C then transferred into 20% sucrose solution and stored at 4°C until saturated with the solution. Frozen coronal sections of the hypothalamus were cut at 50 µm in a five-parallel series at -35°C and frozen sagittal sections of the pituitary were cut at 30 µm at -23°C with a cryostat (Microm HM550P, Germany, Model

#956424), both stored in cryopreservative solution (1% polyvinylpyrrolidone, 30% ethylene glycol and 30% sucrose in 0.1 M PB) at -20°C until used for immunohistochemistry.

Luteinizing Hormone Analysis

Plasma samples were assessed for LH using a radioimmunoassay (RIA). LH concentrations were measured in duplicate with an RIA using 50–100 µL of plasma and reagents purchased from the National Hormone and Peptide Program (Torrance, CA) as previously described [37]. Analysis of LH data included mean LH concentration, LH pulse frequency, and LH pulse amplitude. Individual LH pulses were identified using previously described criteria [38]. Briefly, there were three main criteria: (1) the peak must exceed the sensitivity of the assay, (2) a peak must occur within two data points of the previous nadir, and (3) the peak must exceed a 95% CI of the previous and following nadirs. Luteinizing hormone RIA sensitivity was 0.2 ng/mL with intra- and inter-assay coefficients of variation being 10.3% and 19.5% respectively.

Steroid Hormone Analysis

Plasma samples were analyzed for cortisol and progesterone using IMMULITE/IMMULITE 2000 Xpi Cortisol chemiluminescent enzyme immunoassay (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) as used in [39]. The IMMULITE 2000 Xpi (Siemens Healthineers) has an analytical sensitivity of 0.2 ug/dL for cortisol [40] and 0.1 ng/mL for progesterone [41]. Plasma samples were placed into the machine where 10 uL and 25 uL of plasma were distributed into separate tubes for analysis of cortisol and progesterone concentrations, respectively. The samples underwent a

chemical reaction that produced a light which is then converted into a concentration. Each sample is read 10 times and produces an average for that sample.

Immunohistochemistry

Dual Immunohistochemistry for kisspeptin and cFos: Four full hypothalamic sections containing middle ARC were selected based on a sheep hypothalamic atlas and split midline to produce hemi-sections. Free-floating hypothalamic sections were processed for the protein detection of kisspeptin and cFos. Briefly, following a series of washes in 0.1M phosphate buffered saline (PBS), hemi-sections were blocked in 10% H₂O₂ (diluted in 0.1M PBS) to minimize endogenous peroxidase activity and incubated in a PBS solution containing 0.4% Triton-X (Sigma Aldrich, CAS: 9002-93-1; Code:327371000) and 20% normal goat serum (NGS; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, Cat #: 005-000-121). Following this block incubation, tissue was incubated overnight in a solution containing a primary antibody for kisspeptin (anti-rabbit polyclonal primary antibody, 1:50K, gift from M. Belltrimo, #566). After this overnight incubation, the hemi-sections underwent incubation periods in a secondary antibody (biotinylated goat-anti-rabbit IgG, 1:500, Vector Laboratories, BA-1000), Vectastain ABC-elite (1:500; Vector Laboratories, Burlingame, CA), a 3,3'-diaminobenzidine tetrahydrochloride (DAB; Fisher Scientific, cat# AC328005000), and another block incubation (0.4% Triton-X, 4% NGS) before an overnight incubation in cFos primary antibody (anti-guinea pig polyclonal; 1:10K, Synaptic Systems, 226 004). Next, the tissue was incubated in a secondary antibody (biotynlated goat-anti-guinea pig, 1:500, Vector Laboratories, BA-7000), Vectastain ABC-elite (1:500), and DAB-Nickel (2%

solution). Following a series of washes in 0.1 M phosphate buffer (PB), sections were mounted on microscope slides, air dried overnight, dehydrated using a series of increasing alcohol baths, and coverslipped.

Single Immunohistochemistry for LHbeta: Four mid-sagittal sections of the pituitary were stained for LHbeta. Immunohistochemical procedures were performed as previously described [42]. Briefly, tissue was washed in 0.1M phosphate buffered saline (PBS) and incubated in a PBS solution containing 0.4% Triton-X and 4% NGS before an overnight incubation in a solution containing primary antibody (rabbit anti LH β polyclonal, 1:20K, Golden West BioSolutions, TLIA1042.03). Following the overnight incubation, tissue was incubated in a secondary antibody (biotinylated goat-anti-rabbit IgG, 1:500, Vector Laboratories, BA-1000), Vectastain ABC-elite, and DAB. Following a series of washes in 0.1 M PB, sections were mounted on microscope slides, air dried overnight, dehydrated using a series of increasing alcohol baths, and coverslipped.

Image Analysis

Images were captured using Leica DMi1 microscope (Leica Microsystems, Switzerland; Leica Application Suite version 4.12.0) with consistent camera settings across all hemi-sections. Kisspeptin, LH β , cFos, and dual labeled cells were quantified by two independent individuals blinded to treatment groups and animals from which sections originated. Immunopositive cells for kisspeptin and LHbeta were identified by brown cytoplasmic staining, and those with defined borders were included in the analysis [42, 43] while cFos immunopositive cells were identified with black nuclear staining. GIMP 2.10.34 (GNU Image Manipulation Program; GIMP Development Team; created by

Spencer Kimball and Peter Mattis) was utilized to upload images and mark individual cells on an image with a superimposed image layer. Then, Image J (NIH) was used to quantify the number of marked cells within the region of interest.

Statistical Analysis

All statistics were performed in SAS v9.4 (Cary, NC) using GLIMMIX function for two-way ANOVA. Significance is determined with $p\text{-value} < 0.05$ and tendency defined as $p\text{-value} < 0.10$. Body weight prior to the start of the experiment was analyzed as a complete random design with sampling. Rectal temperature was analyzed as a complete random design with sampling and repeated measure. Cortisol and progesterone concentrations were analyzed with Kruskal-Wallis test and two-way ANOVA as a complete random design with sampling and repeated measure. Luteinizing hormone pulse frequency and pulse amplitude were analyzed as a complete random design with sampling. Luteinizing hormone concentration was normalized by square root and analyzed with a complete random design with sampling and repeated measures. For IHC results, the number of immunopositive cells were analyzed as a complete random design with sampling.

Results

Temperature

In this study, there was no difference between the RT amongst the groups before treatment administration on Day 1 ($p=0.6896$; Table 1). Rectal temperature peaked 5 hours after all administration of LPS on Day 1, while on Day 4, the RT peaked 1 hours after IV administration of LPS (Figure 2). There was an effect of treatment ($p<0.05$) on the average

RT on Days 1, 4 and 7; the RT 5 hours after treatment administration on Day 1; the RT 3 hours after treatment administration on Day 1; and the RT 3 hours after treatment administration on Day 4 (Table 1). On Day 1, the average RT, the RT at 5 hours on Day 1, and the 3 hours after treatment administration on Day 1 and 4 were increased in all groups that received LPS compared to CON (Table 1). On Day 7, there was a tendency ($p=0.0689$) for an effect of treatment on RT on Day 7 such that the temperature of DAD and DID are increased compared to CON (Table 1).

Steroid Hormone Concentrations

Plasma cortisol concentrations on Day 1 described by treatment was significantly different ($p=0.0046$) and plasma progesterone concentrations on Day 1 described by treatment tended to be different ($p=0.06$). On Day 1, the proportions of plasma concentrations of cortisol to progesterone described by treatment was also significantly different ($p=0.006$). Unlike Day 1, there were no differences on Day 7 for plasma cortisol concentrations ($p=0.9365$), the progesterone concentrations ($p=0.3436$), nor the proportions of cortisol to progesterone described by treatment ($p=0.9224$). Utilizing a two-way ANOVA, there was a significant effect of LPS ($p=0.0001$), time ($p<0.0001$), and LPS treatment over time on Day 1 ($p<0.0001$) on plasma cortisol. For plasma progesterone concentration, there was no effect of treatment ($p=0.2836$), a time effect ($p=0.0092$), and a tendency for effect of treatment by time ($p=0.0940$). On Day 1, there was a treatment effect ($p=0.0004$), a time effect ($p<0.0001$), and a treatment by time effect ($p<0.0001$) on the proportion of cortisol to progesterone.

On Day 1, all animals that received LPS had a significant increase ($p < 0.0001$) in mean plasma cortisol concentration compared to those that did not receive LPS (Figure 3A). There was no difference between any groups in mean plasma cortisol concentration prior to LPS treatment. Increased mean cortisol concentration began 2 hours after LPS administration on Day 1 in SAD ($5.38 \text{ ng/mL} \pm 0.85$), DAD ($6.10 \pm 1.12 \text{ ng/mL}$), and DID ($4.2 \pm 1.11 \text{ ng/mL}$) compared to CON ($0.36 \pm 0.10 \text{ ng/mL}$), while CSD ($0.27 \pm 0.04 \text{ ng/mL}$) was not different than CON (Figure 3A). There was no detectable cortisol on Day 7 prior to treatment administration in any group. Two hours after treatment administration, but not 4 hours after administration, DAD ($1.61 \pm 0.28 \text{ ng/mL}$) and DID ($2.56 \pm 0.79 \text{ ng/mL}$) had detectable cortisol concentration (Figure 3B).

On Day 1, there was a tendency ($p = 0.09$) for LPS to increase progesterone concentration over time. Four hours after treatment, SAD ($0.44 \pm 0.14 \text{ ng/mL}$), DAD ($0.38 \pm 0.10 \text{ ng/mL}$) and DID ($0.38 \pm 0.08 \text{ ng/mL}$) tend to be elevated compared to CON ($0.2 \pm 0 \text{ ng/mL}$; all at or below the level of detection) (Figure 3C). On Day 7, there was no significant difference ($p = 0.44$) in mean progesterone concentration amongst the groups (Figure 3D).

There was an effect of LPS on Day 1 such that the proportion of plasma cortisol to progesterone was higher ($p = 0.0004$). Two hours after treatment, SAD ($23.0 \pm 2.9 \text{ ng/mL}$), DAD ($17.4 \pm 2.7 \text{ ng/mL}$), and DID ($17.3 \pm 3.7 \text{ ng/mL}$) began to have a higher proportion of plasma cortisol to progesterone compared to CON ($1.7 \pm 0.5 \text{ ng/mL}$) and CSD ($1.3 \pm 0.2 \text{ ng/mL}$) (Figure 3E). The proportions 2 hours after treatment administration on Day 7

include CON at 1.2 ± 0.3 ng/mL, SAD at 1.2 ± 0.3 ng/mL, DAD at 8.0 ± 1.4 ng/mL, DID at 12.8 ± 4.0 ng/mL, and CSD at 1.1 ± 0.1 ng/mL (Figure 3F).

Luteinizing Hormone Analysis

Representative LH pulse profiles for each group on Day 1 and Day 7 are depicted in Figure 5. The CON animal (Figure 4A) pulse profile appears as expected with approximately one pulse every hour while LH pulse profiles of those that received IV treatments (SAD, DAD, and DID; Figure 4B-D) appear to have decreased LH pulses and that of an animal from CSD (Figure 4E) appear to have decreased pulse activity later in the collection. For Day 7, the pulse profiles for CON, SAD, DAD and CSD (Figure 4A-C,E) appear as expected while DID has decreased pulse activity (Figure 4D).

On Day 1, there was no treatment effect ($p=0.88$), but there was an effect of time ($p=0.0005$) and an interaction of treatment and time ($p=0.03$) on mean LH concentration. Mean plasma LH concentration reported in Figure 5A while the normalized data (square root of concentration) are reported in Figure 5B. There was no difference in LH concentration for the CON animals prior to treatment (3.99 ± 0.54 ng/mL), 0-2.5 hours following treatment administration (4.02 ± 0.58 ng/mL) nor 2.5-5h following treatment administration (4.03 ± 0.48 ng/mL; Figure 5B). Combining the IV LPS treatment groups (SAD, DAD, and DID) as they received the exact same dose and route on Day 1, there was a decrease in LH concentration within the 2.5-5 hours following IV LPS administration (3.62 ± 0.23 ng/mL) compared to their pre-treatment LH concentration (4.48 ± 0.27 ng/mL; Figure 5B). The percent change in LH concentration 3 hours after LPS administration for IV treatment was $-24.3 \pm 7.6\%$ and by 5 hours after treatment the percent

change in LH concentration was $-42.6 \pm 5.0\%$ (data not shown). Those in CSD had a decreased LH concentration within the 2.5-5h following LPS administration (3.94 ± 0.24 ng/mL) compared to pretreatment LH concentration (4.51 ± 0.43 ng/mL; Figure 5B). The percent change in plasma LH concentration 3 hours after LPS administration for CSD was $-12.64 \pm 4.64\%$ and $-32.71 \pm 8.05\%$ 5 hours after treatment administration (data not shown). Furthermore, on Day 7, there was no treatment effect ($p=0.3485$) nor time effect ($p=0.8004$), but there was an interaction of treatment and time ($p=0.0081$) on mean plasma LH concentration. Mean plasma LH concentration for Day 7 is reported in Figure 5C at pretreatment, 0-2.5h after treatment and 2.5-5 hours after treatment. There is no difference in the concentration of LH in CON pre-treatment (15.04 ± 4.44 ng/mL), 0-2.5 hours after treatment (16.16 ± 4.40 ng/mL) nor 2.5-5 hours after treatment (17.25 ± 5.08 ng/mL; Figure 5B). The DID had decreased LH mean concentration 0-2.5 hours after treatment administration (19.60 ± 1.74 ng/mL) and 2.5-5 hours after treatment administration (20.97 ± 3.89 ng/mL) compared to the pre-treatment value (25.59 ± 3.44 ng/mL; Figure 5B). The percent change in plasma LH concentration for DID was $-29.5 \pm 5.2\%$ 3 hours after treatment and $-28.2 \pm 14.3\%$ 5 hours after treatment (data not shown).

For plasma LH pulse frequency, LPS had an effect on the number of pulses per hour over the course of the week (treatment by day interaction effect, $p=0.03$). On Day 1, SAD (0.25 ± 0.1 pulses per hour), DAD (0.25 ± 0.1 pulses per hour), DID (0.35 ± 0.1 pulses per hour), and CSD (0.40 ± 0.1 pulses per hour) had fewer LH pulses per hour compared to CON (0.70 ± 0.1 pulses per hour; Figure 6A). On Day 7, DID (0.35 ± 0.1 pulses per hour) had fewer pulses per hour than CON (0.85 ± 0.1 pulses per hour) while

SAD (0.90 ± 0.1 pulses per hour), DAD (0.65 ± 0.1 pulses per hour), and CSD (0.90 ± 0.1 pulses per hour) were not different than CON (Figure 6C). Additionally, DAD was not different than DID on Day 7 (Figure 6C). Additionally, the pulses per hour on Day 7 for SAD, DAD, and CSD were not different from those for CON on Day 1 (Figure 6C). There was no significant effect of LPS on plasma LH pulse amplitude ($p > 0.05$) on Day 1 nor Day 7 (data not shown).

Kisspeptin/cFos

Representative images of dual-labeled ARC hemi-sections from representative animal of each group are depicted in Figure 7. Dark brown staining indicates a cell that expresses kisspeptin while those with black staining indicate cFos expression and those that have both are classified as dual labeled. There was an effect of treatment ($p = 0.001$) on the number of kisspeptin-immunopositive cells, such that DID had fewer cells (33.3 ± 10.9 cells) compared to all other groups (Figure 7). There were no differences amongst the CON (82.6 ± 13.6 cells), SAD (83.8 ± 11.8 cells), DAD (113.7 ± 10.4 cells), and CSD (107.7 ± 13.4 cells) (Figure 7). There was no effect of treatment on the number of cFos immunopositive cells nor an effect on the proportion of kisspeptin cells with cFos (data not shown).

LHbeta

Representative images of single-labeled midsagittal sections of the anterior pituitary from representative animal of each group are depicted in Figure 8. Dark brown staining indicates a cell that expresses LHbeta. There was no effect of treatment ($p = 0.4531$) on the number of LH β immunopositive cells. There were 258.6 ± 21.2 cells for

CON, 210.1 ± 21.7 cells for SAD, 205.1 ± 21.4 cells for DAD, 225.9 ± 18.9 cells for DID, and 228.7 ± 21.1 cells for CSD (Figure 8).

Discussion

Growing evidence indicates that peripheral inflammation associated with various diseases is a key/critical induction factor for central inflammation. During infection, physiological processes, such as reproduction, are impaired by peripherally produced cytokines, crossing the blood brain barrier [44, 45] to directly bind to afferent nerves and endothelial cells activating local microglia and astrocytes in the brain to synthesize additional cytokines resulting in febrile, hormonal, and behavioral effects [46]. Furthermore, alterations to physiology can occur in response to the elevation in cortisol production following LPS response [5, 11, 28]. It is established that LPS induced inflammation causes a suppression in reproduction through impaired GnRH/LH secretion, however, the responsible central mechanism(s) remain largely unknown.

While it has been shown that acute LPS administration suppresses kisspeptin expression and LH secretion [21-24], herein we report that elevated RT and reduced LH secretion following acute activation of the immune system were attenuated within a week of exposure. However, a daily increasing dose of LPS is reliable in maintaining both an immune response (increased RT, tendency for elevated cortisol concentration) as well as suppressed the reproductive axis reproduction (decreased LH pulse frequency and kisspeptin expression) throughout the experiment. Furthermore, on Day 1, all of those that received LPS had an increase in plasma cortisol concentration, with the increase in cortisol observed 2 to 4 hours after IV administration of LPS and 4 hours following CSD

administration of LPS, while CON did not increase cortisol at any time point, which agrees with previous studies as endotoxemia produces an increase in plasma cortisol concentrations [5, 11, 28]. Chronic stress was observed in DAD and DID groups as they had an increase in cortisol concentration throughout the week-long study, while the other groups did not. Cortisol provides a potential mechanism by which reproduction is suppressed as ARC kisspeptin neurons contain cortisol receptors [47-49]. Additionally, there was a slight increase in progesterone concentration for the SAD, DAD, and DID animals. In castrated rodents and sheep, progesterone concentration is negligible or below the limit of assay detection [50, 51], which agrees with the concentrations found in CON. This slight increase could result as the enzymes of the steroidogenesis pathway become overwhelmed by the amount of ACTH released from the anterior pituitary, therefore, precursors of cortisol, such as progesterone, are produced thus providing an indicator of the degree of ACTH release [29-31, 33]. Furthermore, progesterone could influence ARC kisspeptin neurons as they contain progesterone receptors and are implicated in progesterone negative feedback on GnRH secretion [52-55].

Physiologic RT for a sheep ranges from 38.3 to 39.9 °C according to Merck Veterinary Manual [56, 57] with temperatures above these ranges being considered hyperthermia or a fever. The wethers in CON had similar RT in accordance to these standards. Meanwhile, those in CSD and those in SAD developed a fever only on Day 1 and DAD and DID were hyperthermic on Days 1 and 4. Taken together, the wethers in SAD and CSD experienced an acute inflammatory response on Day 1 while those in DAD

and DID experienced a chronic inflammatory response through the majority of the experimental week.

Wethers that received an acute dose of LPS on Day 1 (SAD, DAD, DID) had impaired LH pulse frequency as seen in other experiments [12, 58]. They experienced a decrease in plasma LH pulse frequency as observed with fewer pulses per hour as well as a decrease in mean plasma LH concentration seen with decrease in square root of concentration. Furthermore, the CSD also had a suppression in LH secretion shown as a lower mean plasma LH concentration, specifically during the first 2.5 hours following implant administration. Despite the plasma LH pulse frequency being not different from the CON nor the IV administration, it is numerically between the two groups indicating that the frequency could be slowing down. Collecting blood for a longer period of time in an CSD model would make it possible to evaluate potential difference in plasma LH concentrations.

An acute model is not most physiologically relevant for evaluating the effects of diseases as most diseases produce chronic inflammation [59-61]. Firstly, an immune response was detected in SAD only on Day 1 as described by the presence of a fever as well as an increase in plasma cortisol and progesterone concentrations. Furthermore, models of acute inflammation that collect tissue more immediately after LPS administration observe not only suppression of LH secretion, but also reduce kisspeptin mRNA in the hypothalamus [22-24]. However, in our study, the suppression of LH secretion in SAD was observed on Day 1, but not by Day 7, as the animals in SAD had recovered their LH pulse frequency and concentration. The recovery is reflected in

kisspeptin expression as there was no difference between the number of immunopositive cells between SAD and CON on Day 7 when the tissues were collected. Notably, most previous models investigating the effects of LPS-induced inflammation on reproduction administered LPS in an intravenous (IV) acute dose (400 ng LPS/kg BW [12]). An acute model, while helpful in providing insight to what occurs initially, may not be physiologically applicable to inflammation caused by naturally occurring diseases and/or chronic conditions. For example, the decreased responsiveness of the gonadotropes to GnRH to a single low dose of LPS (40 ng/kg) only suppressed LH in ewes, while a single high dose (400 ng/kg) suppressed both GnRH and LH secretion [62].

Osmotic pumps have been used for SQ and IP administration of LPS. In ovariectomized female rats, a moderate (500 µg/kg BW) dose of LPS administered IP induced an immune response with fever and decreased feed intake [17]. Additionally, there was a decrease in the serum LH concentration, however, there was no change in the Kiss1, Kiss1R, nor GnRH mRNA expression in the hypothalamus [17]. In the present study, the CSD wethers had a blunted immune response (as determined by RT and compared to the other groups receiving LPS), with the LPS being absorbed slower when administered SQ as the RT peak occurred later than in the groups that received LPS IV. Also, CSD wethers did have a reduction of LH secretion on Day 1 as the LH pulse frequency was numerically lower than the CON, even though not significant, but also not significantly different from the IV LPS groups on Day 1. However, by Day 7, there is a recovery of LH secretion and kisspeptin expression as neither LH pulse frequency nor the number of immunopositive neurons in CSD were different from CON. It is possible that SQ administration of LPS

requires a greater sustained dose or a larger starting or loading/priming dose of LPS in order to continuously induce an immune response and suppress reproduction. In ovariectomized and sham female rats, a high dose (5 mg/kg) of LPS administered IP induced an immune response characterized by fever and decreased feed intake [17]. Within the hypothalamus, there was also a decreased amount of GnRH and Kiss1 mRNA, however the mRNA for Kiss1R did not change [17]. Taken together, IV administration of LPS has a more acute effect on the animals while in SQ administration, the effects of LPS are delayed and blunted.

Another potential strategy of LPS-induced chronic inflammation is administration of a repeated acute dose of LPS. In gilts, after five days of treatment, there was no difference in the ovarian mRNA for enzymes important for reproductive hormone steroidogenesis nor the follicular fluid estrogen concentration between the daily LPS treated group (100 ng/kg BW IV daily for five days) and the control group (treated with saline) [26]. While there was an increase in toll like receptor (receptor LPS binds to) protein, there was not an increase in body temperature [26, 63]. Thus, in the gilt, a daily acute dose of LPS was not sufficient in causing chronic inflammation that could suppress reproduction [26]. In anestrous ewes, daily administration of an acute dose of LPS (400 ng LPS/kg) for 6 days caused an increase in circulating cortisol concentration following each dose of LPS and the amount of cortisol stimulation was reduced after Day 3 [25]. Contrary to results reported in this manuscript, it was sufficient enough to produce a decrease in circulating LH concentration compared to pre-treatment every day, 3 hours after LPS administration [25]. Finally, in peripubertal female rats, repeated LPS (50 µg/kg BW),

every other day increased GnRH and glutamate content in the hypothalamus while it decreased plasma LH and estradiol and delayed vaginal opening, thus delayed puberty onset all of which indicate the failure in release of GnRH [64]. In the present study, the immune response is continued almost throughout the week duration as there is the continued increase in cortisol concentration and fever. However, LH pulse frequency is numerically between CON and DID on Day 7 and not significantly different from either group. Additionally, the number of kisspeptin immunopositive cells in DAD is not different from CON. The DAD wethers are likely becoming refractory as the animals develop resistance through LPS immunotolerance with inhibited cellular respiration and altered secretomes of macrophages [65, 66]. These results could be because the hypothalamus, the origin of LH pulse generation, is resistant and the pituitary, specifically the gonadotropes, is the source/cause of LH suppression or, alternatively, LH secretory activity has been suppressed and what is being observed is an intermediary, recovery, period/phase. Taken together, this data suggests that a chronic model of inflammation induced by daily LPS administration suppresses reproduction through effects on the hypothalamus, however, the effect is at risk of development of immunotolerance. Unfortunately, a limitation of this study is that it is unknown whether or not animals had a previous endogenous LPS challenge, such as rumen acidosis, thus allowing the possibility of some animals becoming refractory to our exogenous LPS challenge earlier than others.

Lastly, a daily increasing dose of LPS is a model of chronic inflammation. In pigs, increasing the dose of LPS by 12% every other day (starting dose at 60 µg/kg) induced a fever and an increased pro-inflammatory cytokine concentrations as well as decreased lipid

deposition and average daily gain [67]. Unfortunately, reproductive parameters were not measured, but it can be inferred that reproduction may have been impaired, to some capacity as there was a decreased lipid deposition and average daily gain [67]. In lactating cattle, a continuous infusion of LPS increasing each day (starting at 0.017 µg/kg BW/hour; increase by 20%, 30%, 40% and so) for seven days induced a prolonged immune response with a mild hyperthermia, an increase in serum amyloid A and LPS binding protein concentrations, and an increase in white blood cell counts and decreased milk yield on Day 1 [27]. However, there was no difference in follicular growth, dominant follicle size, progesterone serum concentration, progesterone follicular concentration nor estradiol follicular concentration [27]. It is possible that the cattle became refractory to the LPS and a higher starting dose as well as a different method of administration may have been needed in order to fully impair the HPG axis [27]. A potential reason for not seeing more changes in reproduction, as proposed by Dickson et al., 2019, is that the cattle became tolerant to chronic and exponentially increasing LPS infusion, and in turn, a higher initial dose may be necessary.

In the present study, the DID group likely maintained an immune response as well as suppression in reproductive parameters. While an increase in temperature dissipated by Day 7, there was a tendency for increased cortisol, thus indicating that the immune system is still activated by LPS as cortisol is elevated with endotoxemia [68]. However, RT and steroid hormone concentration may not serve as the best measures to indicate impairment of reproduction as by Day 7 temperature and there was no statistical difference in cortisol nor progesterone concentration amongst the groups. There was a continued suppression of

LH secretion in DID as seen with fewer pulses per hour than CON, SAD and CSD. Additionally, the mean LH concentration remains suppressed on Day 7 in DID compared to CON, SAD, DAD, and CSD. Interestingly, the suppression in LH mean concentration on Day 7 appeared to occur earlier than it did on Day 1 as both the second and third timepoints were lower than the concentration in the first timepoint. Thus, there is likely residual effects of LPS. However, this change was not observed in any other groups. In congruence with the LH data, the number of kisspeptin immunopositive neurons is fewer in DID than those of CON, SAD, DAD, and CSD. Furthermore, there was no difference in the number of LH β thus indicating that the effect on LH secretion is likely through alterations within the hypothalamus. Taken together, out of these described models, a daily increasing dose of LPS is possibly the best model to bring about chronic or subacute inflammation to induce an immune response and suppress reproduction in part that the cause of immunotolerance is overwhelmed in DID.

Overall, LPS will induce an immune response as well as suppress reproduction in domestic livestock, specifically through the kisspeptin system and ultimately decrease LH secretion. Various infections, such as metritis [61, 69, 70], and non-infectious diseases, like PCOS [1, 71, 72], are associated with LPS and chronic inflammation [8]. In turn, one of the major causes of infertility in animal agriculture may be the suppression in central reproduction in diseased animals. Therefore, identifying central mechanisms by which chronic inflammation may lead to future preventions and treatments for infertility to increase the productivity and profitability of domestic livestock.

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Appendix

Table 2. Rectal Temperatures. Rectal temperatures measured with a digital thermometer and analyzed for average on Days 1, 4, and 7; the temperature 3h after treatment on these days and 5h or the peak on day 1. Control (CON, IV saline daily; n=4), single acute dose (SAD, 400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7; n=4), daily acute dose (DAD, 400 ng of LPS/kg BW IV daily; n=4), daily increasing dose (DID, 400 ng of LPS/kg BW IV on Day 1 with 20% increase each day; n=4), and chronic subcutaneous dose (CSD, 20 µg of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump; n=4) and an IV group of the combined IV treatments on Day 1 as they received the same dose of LPS. Significant p-values are bolded (p<0.05) and difference is indicated by different letters within each row. § as peak indicates the highest temperature amongst the measured timepoints for the day. * indicates definition of a fever per Merck Veterinary Manual. 1 p-value indicates the comparison between CON, SAD, DAD, DID, and CSD. 2 p-value indicates the comparison between CON, IV, and CSD.

	CON	SAD	DAD	DID	CSD	pvalue ¹	IV	pvalue ²
Pre-experiment	38.7±0.2 °C	38.8±0.2°C	38.8±0.2°C	39.0±0.2°C	39.0±0.2°C	0.6896	38.9±0.1°C	0.5999
Ave. Day 1	38.9±0.2°C ^c	39.9±0.2°C ^{ab *}	40.1±0.2°C ^{ab *}	40.2±0.2°C ^{a *}	39.5±0.2°C ^b	0.0018	40.1±0.1°C ^{a *}	0.0002
Ave. Day 4	38.6±0.1°C ^c	38.6±0.1°C ^c	39.4±0.1°C ^{ab}	39.6±0.1°C ^a	39.1±0.1°C ^b	<0.0001		
Ave. Day 7	38.4±0.1°C ^c	38.6±0.1°C ^{bc}	39.2±0.1°C ^a	39.3±0.1°C ^a	38.9±0.1°C ^{ab}	0.0019		
5h after trt Day 1 (~peak§)	39.0±0.3°C ^c	41.1±0.3°C ^{ab *}	41.3±0.3°C ^{a *}	41.8±0.3°C ^{a *}	40.5±0.3°C ^{b *}	<0.0001	41.4±0.2°C ^{a *}	<0.0001
3h after trt Day 1	38.9±0.4°C ^c	40.5±0.4°C ^{ab *}	40.5±0.3°C ^{ab *}	40.9±0.3°C ^{a *}	39.6±0.4°C ^{bc *}	0.0051	40.6±0.2°C ^{a *}	0.0006
3h after trt Day 4 (~peak§)	38.7±0.2°C ^b	38.7±0.2°C ^b	39.8±0.2°C ^{a *}	39.9±0.2°C ^{a *}	39.1±0.2°C ^b	<0.0001		
3h after trt Day 7	38.8±0.2°C	38.8±0.2°C	39.6±0.2°C	39.5±0.2°C	39.3±0.2°C	0.0689		

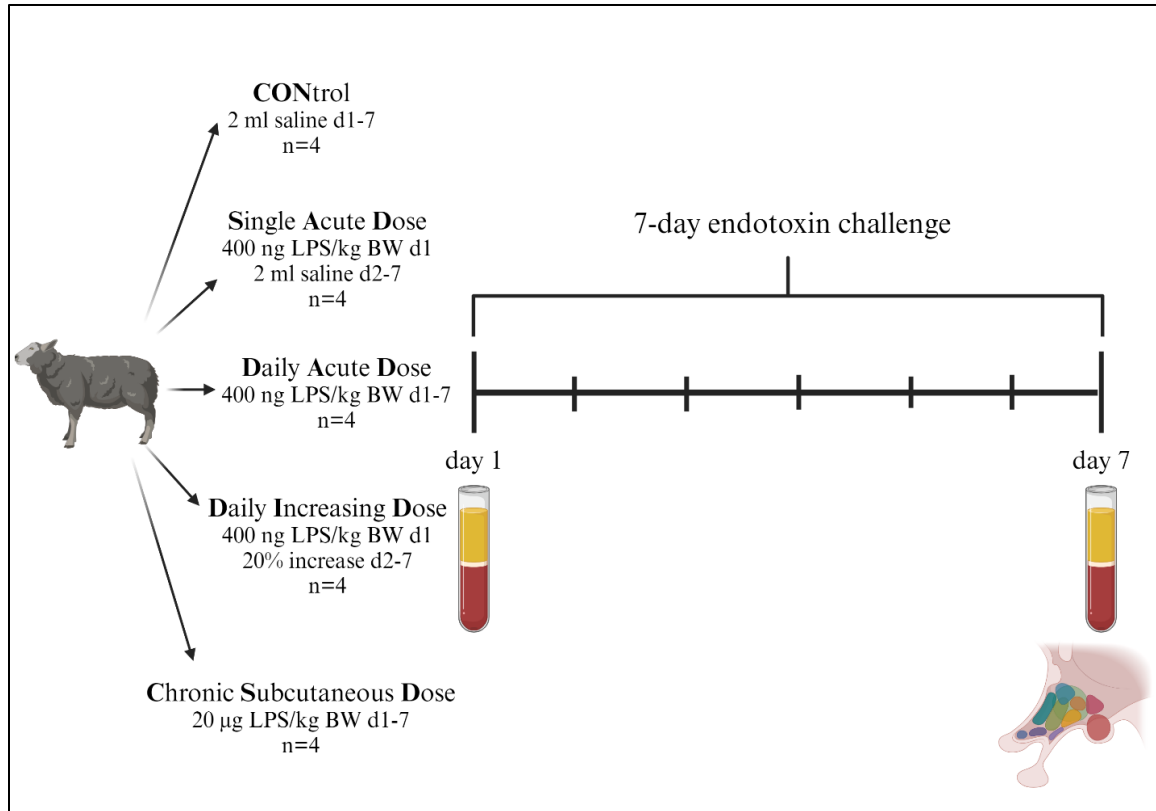


Figure 0.1. Experimental Design. Twenty sheep were blocked by body weight and randomly assigned to one of five treatment groups – control (CON, saline daily; n=4), single acute dose (SAD, 400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7; n=4), daily acute dose (DAD, 400 ng of LPS/kg BW IV daily; n=4), daily increasing dose (DID, 400 ng of LPS/kg BW IV on Day 1 with 20% increase each day; n=4), and chronic subcutaneous dose (CSD, 20 µg of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump; n=4). Serial blood samples were collected every 12 minutes for 6 hours (1 hour before treatment and 5 hours after treatment) on Days 1 and 7 and tissue collection of brain and pituitary occurred on Day 7.

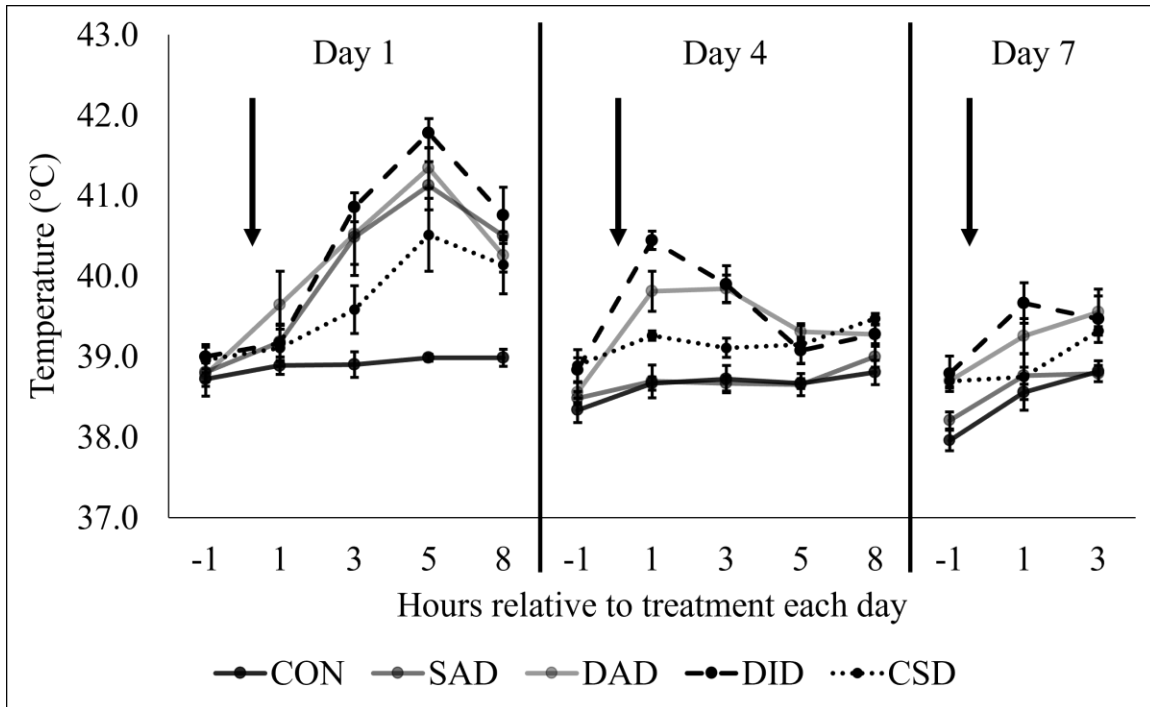


Figure 0.2. Rectal Temperatures. Temperatures recorded on Days 1, 4, and 7. Rectal temperatures measured with a digital thermometer at -1, 1, 3, 5 and 8 hours relative to treatment administration on Days 1 and 4 and -1, 1, and 3 hours relative to treatment administration on day 7. Control (CON, IV saline daily; n=4) depicted as a black solid line, single acute dose (SAD, 400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7; n=4) as a dark gray line, daily acute dose (DAD, 400 ng of LPS/kg BW IV daily; n=4) as a light gray line, daily increasing dose (DID, 400 ng of LPS/kg BW IV on Day 1 with 20% increase each day; n=4) as black dash lines, and chronic subcutaneous dose (CSD, 20 μ g of LPS/kg BW daily delivered subcutaneously via Alzet smotic pump; n=4) as dotted line. Black arrow indicates treatment administration each day.

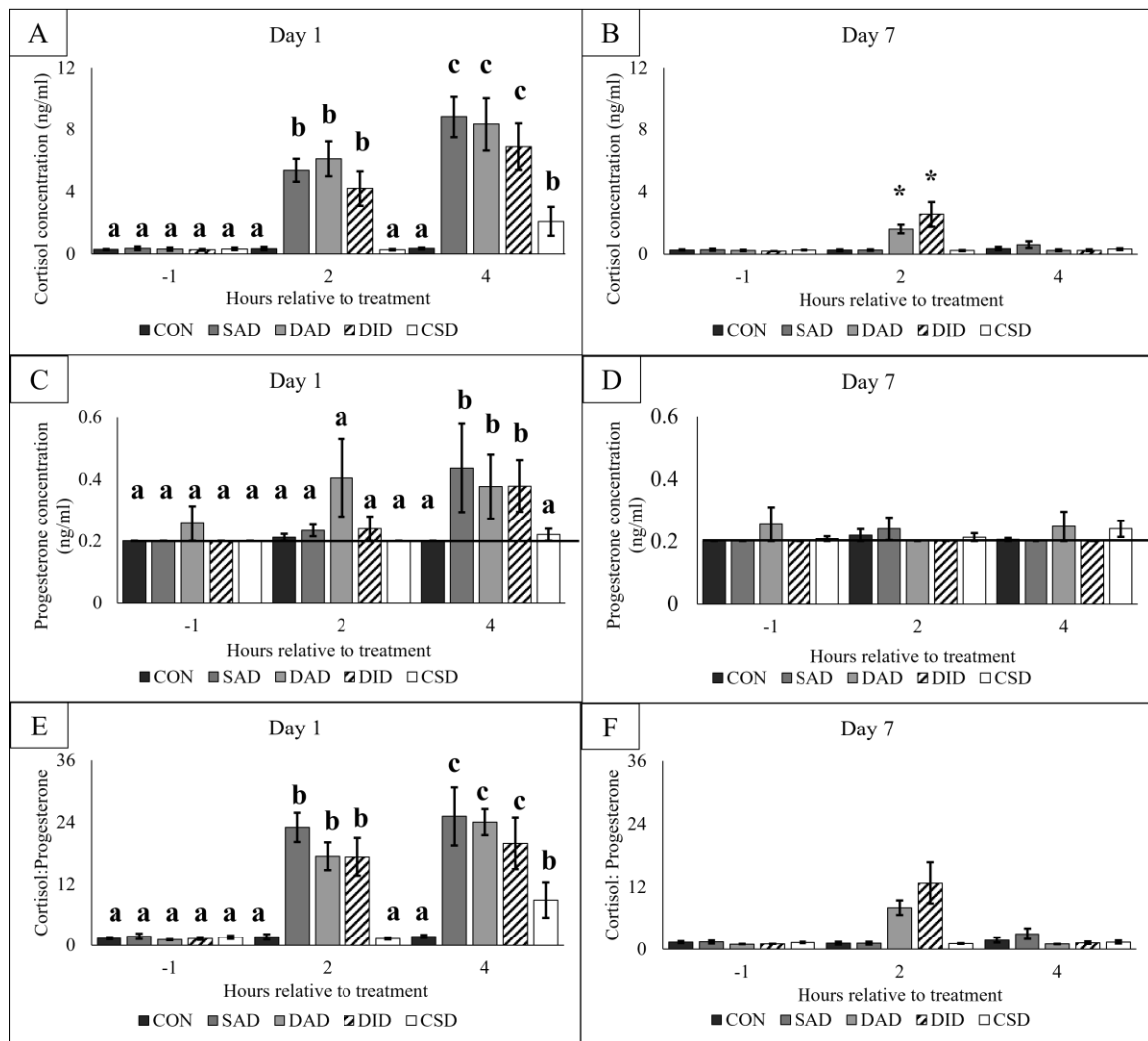
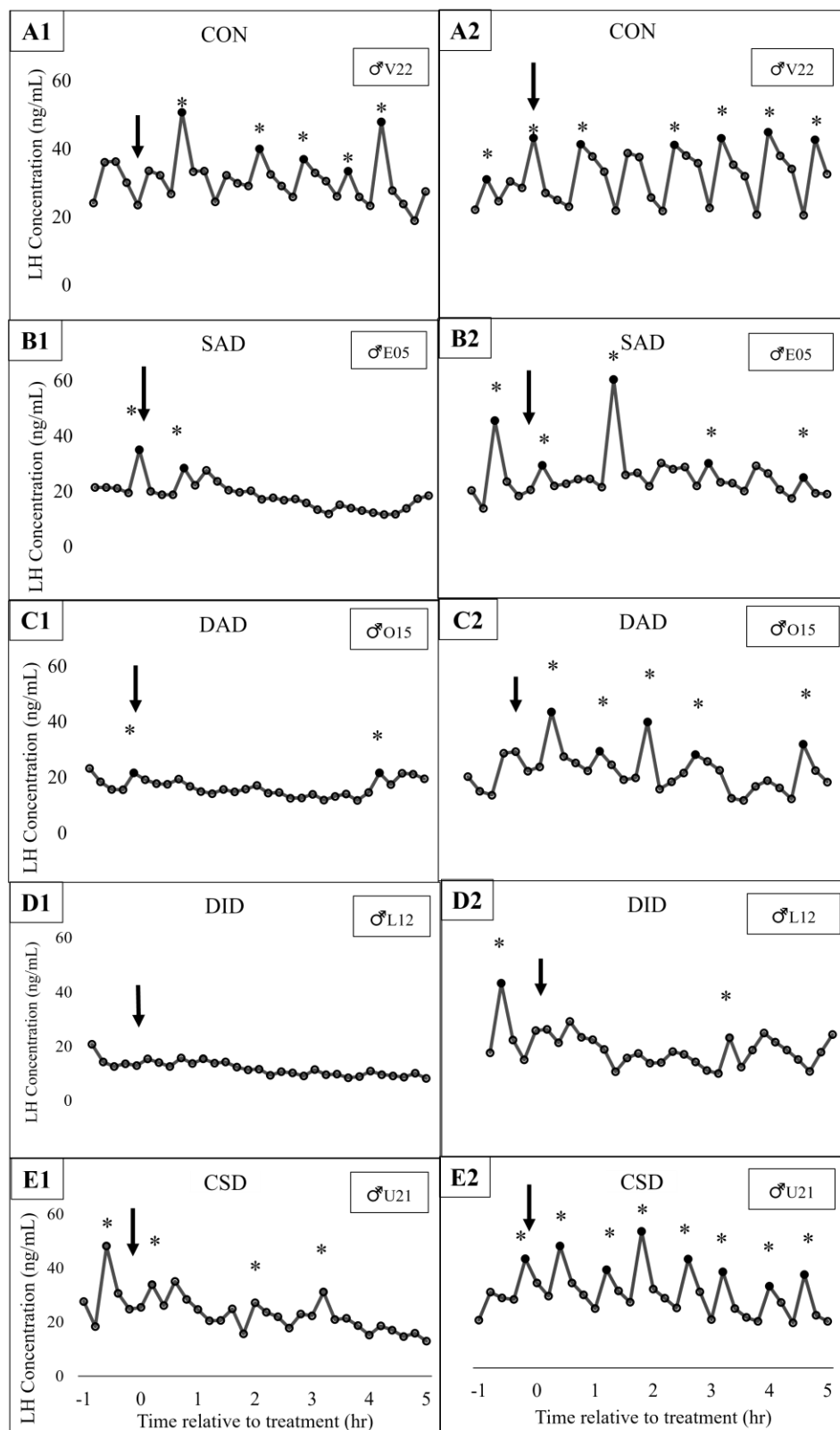


Figure 0.3. Steroid Hormone Analysis from samples -1, 2, and 4 hours relative to treatment. Plasma cortisol concentration on Day 1(A) and Day 7 (B). Plasma progesterone concentrations on Day 1 (C) and Day 7 (D). The proportion of cortisol concentration to progesterone concentration Day 1 (E) and Day 7 (F). Black solid horizontal line indicates the limit of detection for assay. Control (CON, saline daily; n=4) depicted as a black bar, single acute dose (SAD, 400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7; n=4) as a dark gray bar, daily acute dose (DAD, 400 ng of LPS/kg BW IV daily; n=4) as a light gray bar, daily increasing dose (DID, 400 ng of LPS/kg BW IV on Day 1 with 20% increase each day; n=4) as black striped bar, and chronic subcutaneous dose (CSD, 20 μ g of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump; n=4) as white bar. Significant differences indicated by different letters at $p < 0.05$. Tendency ($0.05 < p < 0.1$) indicated with *.

Figure 0.4. Representative LH pulse profiles from individual animals on Day 1 (1) and Day 7 (2). Blood samples collected and analyzed for LH concentration every 12 minutes for 6 hours (1 hour before treatment; 5 hours after treatment) on Day 1 and Day 7 of the experimental week. Representative animals shown for control (CON, saline daily) (A), single acute dose (SAD, 400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7) (B), daily acute dose (DAD, 400 ng of LPS/kg BW IV daily) (C), daily increasing dose (DID, 400 ng of LPS/kg BW IV on Day 1 with 20% increase each day) (D), and chronic subcutaneous dose (CSD, 20 µg of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump) (E). Black arrow indicates treatment administration (at Time 0). Black filled circles with * indicate a pulse defined by the following criteria: (1) the peak must exceed the sensitivity of the assay, (2) a peak must occur within two data points of the previous nadir, and (3) the peak must exceed a 95% CI of the previous and following nadirs.



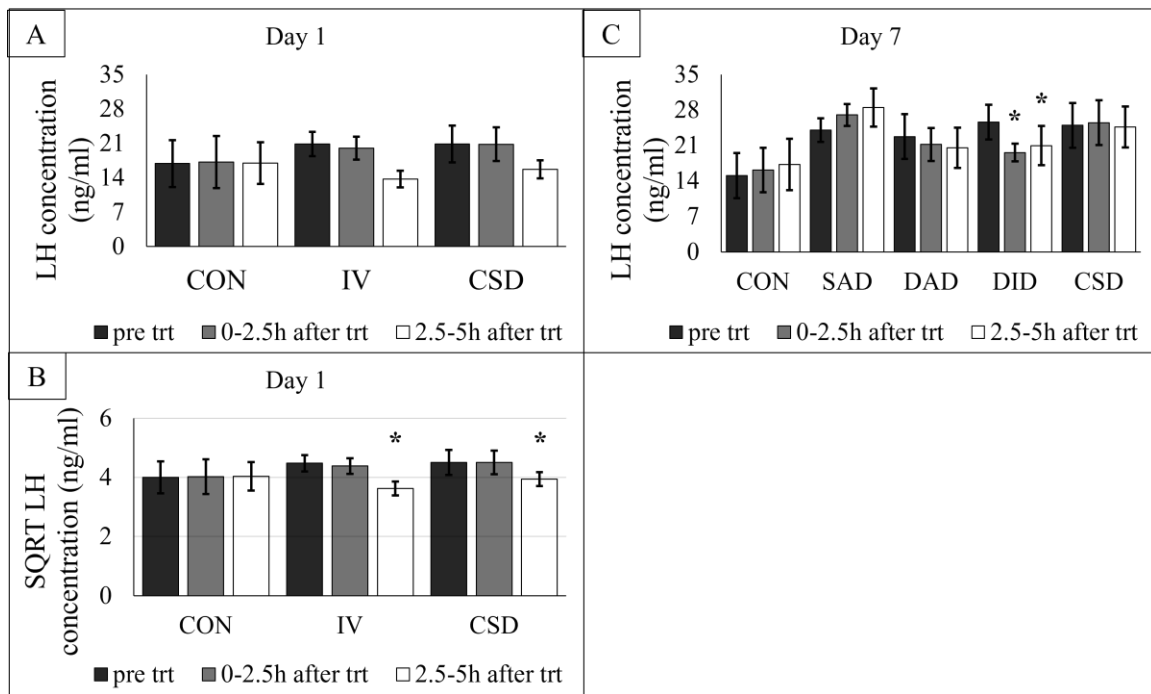


Figure 0.5. Plasma Luteinizing Hormone Mean Concentration. LH concentration in one hour pretreatment (black), 0-2.5h after treatment (gray), and 2.5-5h after treatment (white) for Day 1 (A) and Day 7 (C). (B) the square root of LH concentration for Day 1 in order to normalize the data before statistical analysis was completed. Control is CON (saline daily; n=4). Single acute dose is SAD (400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7; n=4). Daily acute dose is DAD (400 ng of LPS/kg BW IV daily; n=4). Daily increasing dose is DID (400 ng of LPS/kg BW IV on Day 1 with 20% increase each day; n=4). Chronic subcutaneous dose is CSD (20 µg of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump; n=4). IV (A and B) is the combination of SAD, DAD, and DID as they all received 400 ng LPS/kg BW on Day 1. *indicates significance within the treatment group from the pre-treatment value.

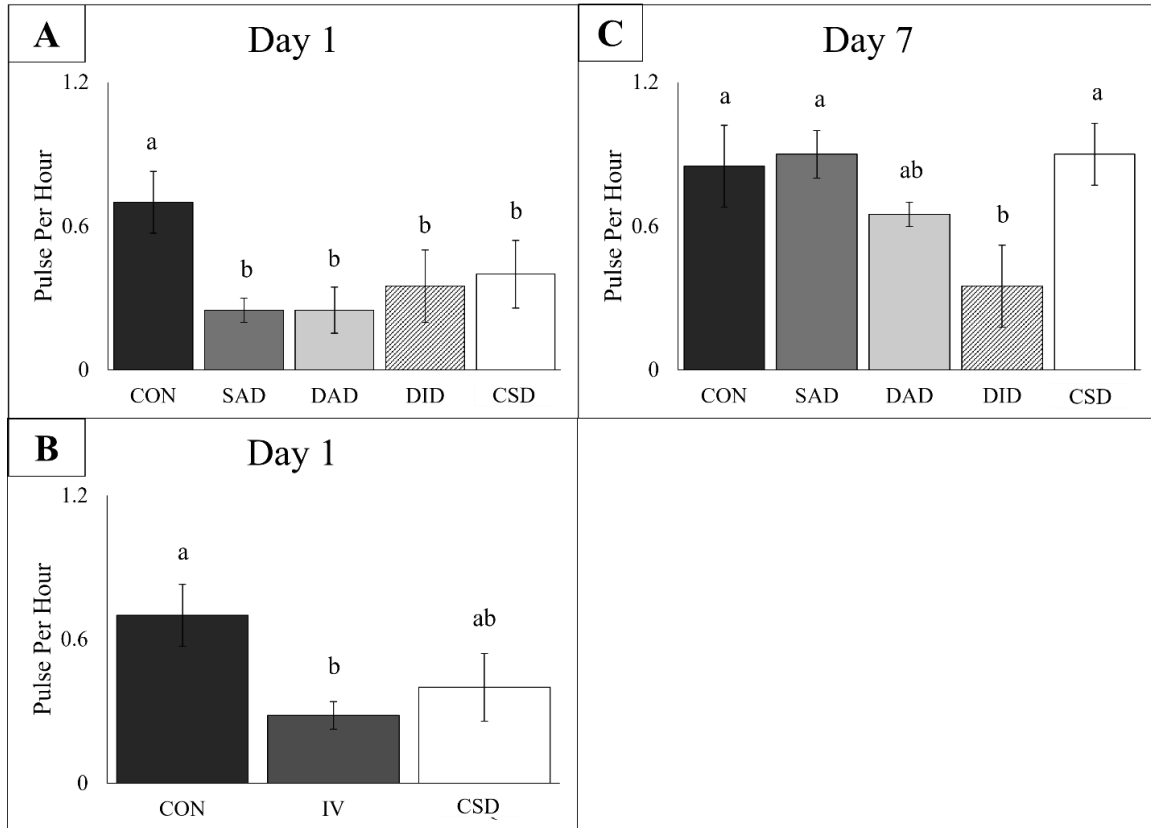


Figure 0.6. Plasma Luteinizing Hormone Pulse Frequency. Number of pulses per hour derived from the number of pulses over five hours (after treatment administration). Day 1 (A) and Day 7 (C) shown as control (CON, saline daily; n=4) depicted as a black bar, single acute dose (SAD, 400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7; n=4) as a dark gray bar, daily acute dose (DAD, 400 ng of LPS/kg BW IV daily; n=4) as a light gray bar, daily increasing dose (DID, 400 ng of LPS/kg BW IV on Day 1 with 20% increase each day; n=4) as black striped bar, and chronic subcutaneous dose (CSD, 20 μ g of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump; n=4) as white bar. Intravenous treatments of 400 ng of LPS/kg BW (SAD, DAD, DID) on Day 1 were combined (B) shown as the control (CON, saline daily; n=4) depicted as a black solid bar, intravenous administration (IV; 400 ng of LPS/kg BW; n=12) as dark gray bar, and chronic subcutaneous dose (CSD, 20 μ g of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump; n=4) as white bar. Significant difference shown with different letters within each subfigure at a p-value <0.05.

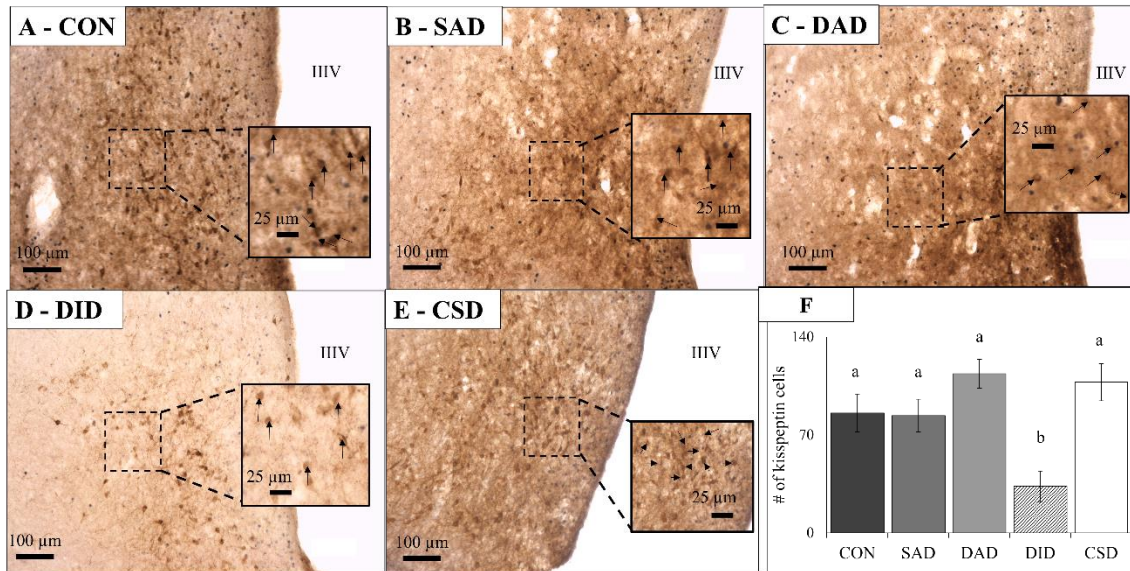


Figure 0.7. Representative 10X images of ARC kisspeptin immunopositive cells on Day 7. A) Control (CON, saline daily; n=4). B) Single acute dose (SAD, 400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7; n=4). C) Daily acute dose (DAD, 400 ng of LPS/kg BW IV daily; n=4). D) Daily increasing dose (DID, 400 ng of LPS/kg BW IV on Day 1 with 20% increase each day; n=4). E) Chronic subcutaneous dose (CSD, 20 μ g of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump; n=4). Black arrows indicate immunopositive cells. IIIIV indicates third ventricle. F) Number of kisspeptin immunopositive cells in CON (black), SAD (dark gray), DAD (light gray), DID (black striped), and SSD (white). Significant difference shown with different letters at a p-value <0.05.

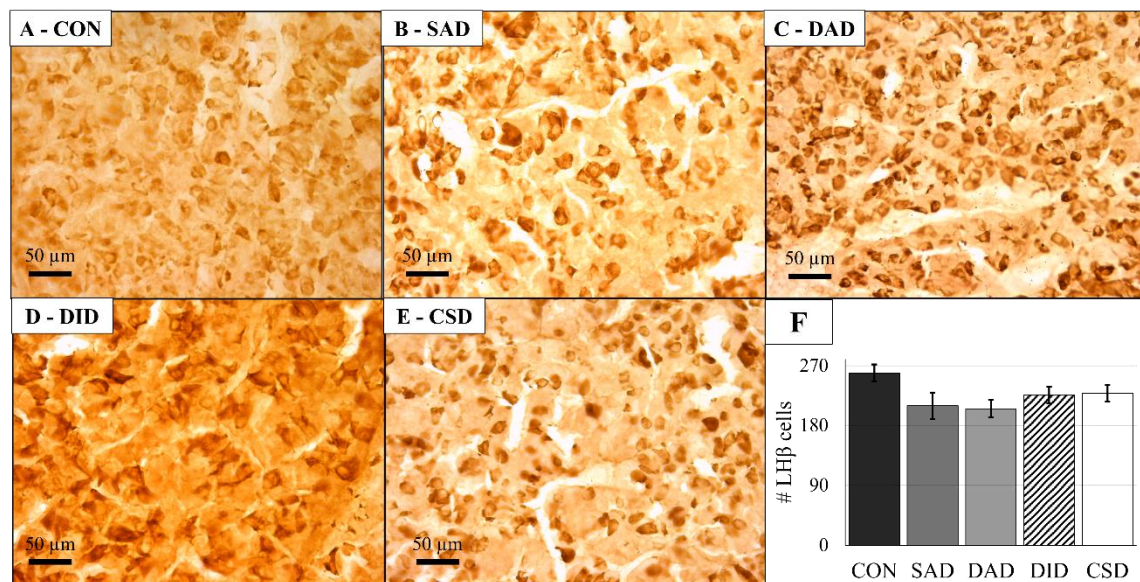


Figure 0.8. Representative 20X images of LHβ immunopositive cells from the anterior pituitary on Day 7. A) Control (CON, saline daily; n=4). B) Single acute dose (SAD, 400 ng of LPS/kg BW IV on Day 1 and saline Days 2-7; n=4). C) Daily acute dose (DAD, 400 ng of LPS/kg BW IV daily; n=4). D) Daily increasing dose (DID, 400 ng of LPS/kg BW IV on Day 1 with 20% increase each day; n=4). E) Chronic subcutaneous dose (CSD, 20 μg of LPS/kg BW daily delivered subcutaneously via Alzet osmotic pump; n=4).

CHAPTER 5: FUTURE DIRECTIONS AND CONCLUSIONS

Future Directions

From literature discussed in Chapter I, it is evident that the mechanisms whereby inflammation impairs reproduction are complex with central regulation of GnRH/LH secretion being an important component. Research described in Chapter II provides a greater breadth in knowledge of the impacts of endotoxin induced inflammation on kisspeptin expression, and subsequently LH secretion, in adult cows. Data depicted in Chapter III suggests that COX-1 and 2 may play a crucial role in mechanisms of inflammation induced suppression of GnRH/LH secretion via the kisspeptin neurons. From work detailed in Chapter IV, we now have a greater understanding that chronic inflammation suppresses GnRH/LH secretion via the kisspeptin system; chronic inflammation varies from acute inflammation in the duration of suppression in reproductive neuroendocrinology; and mode of chronic inflammation will determine the sustained dysregulation.

While these works are novel and add to the overall knowledge of how systemic inflammation alters reproduction through its impact on the hypothalamus, several questions still remain and form the basis for future investigation into the central mechanisms that underlie reduced GnRH/LH secretion. First, there are other regulators of reproduction including nutrition [1-3] and multiple mechanisms may converge at or act through KNDy neurons in order to alter reproduction [4]. Second, additional neuropeptides that may influence the effects of inflammation on reproduction. Therefore, rationale for the exploration of these unanswered questions and predictions of what these avenues of investigation might yield are provided in this chapter.

NKB

Herein, our examination was restricted to one of the three reproductively relevant neuropeptides in KNDy neurons, leaving open the possibility for NKB to play a role during LPS induced inflammation suppression of reproduction. As briefly mentioned in Chapter I, NKB is stimulatory of the GnRH/LH secretion specifically through its autocrine role on KNDy neurons and is responsible for GnRH/LH pulse initiation [5]. Administration of NK3R antagonist blocks the suppressive effects of LPS on LH pulse frequency [6]. As GnRH/LH pulse frequency [7] and kisspeptin expression [8-12] are both suppressed following LPS administration as shown herein, NKB expression in the ARC may also decrease in response to LPS administration due to its role in GnRH/LH pulse initiation and autocrine stimulation of kisspeptin (see Figure 1 for working model).

Dynorphin

Similar to NKB, our examination left dynorphin as a possible key neuropeptide for the suppression of reproduction due to LPS induced inflammation. As briefly discussed in Chapters 2, 3, and 4, LPS administration induces adrenal production of progesterone through overwhelming the HPA axis stimulation [13-16]. Administration of progesterone will increase the cerebrospinal fluid concentration of dynorphin [17]. Dynorphin plays a role in progesterone negative feedback [17-20] in part to suppress the GnRH/LH secretion [5]. Furthermore, dynorphin fibers provide close contacts with the majority (87.5%) of GnRH neurons [21] and GnRH neurons express the receptor for dynorphin [22]. Conversely, maximal co-expression of cFos and dynorphin neurons occurs during early follicular phase, however, LPS decreases the percent of activated dynorphin neurons in ewes [12]. In rodents, pretreatment of κ OR antagonist fails to prevent the suppressive

effects of LPS on LH pulse frequency [6], but it also enhances microglia (macrophage of the brain) mediated proinflammatory responses [23]. Dynorphin activation of κ OR promotes the anti-inflammatory phenotype of microglia by blocking the TLR4/NF κ B pathway in LPS stimulated microglial cells [24]. In epileptic rats, overexpression of the dynorphin precursor protein alleviated neuronal apoptosis, promoted the anti-inflammatory phenotype of microglia and inhibited the TLR4/NF κ B pathway [24]. Therefore, dynorphin expression may increase during times of endotoxin induced inflammation in order to mediate immune response, however, the role in reproduction may occur through the impact on multiple glial and/or neurons rather than KNDy neurons (see Figure 1 for working model).

Dopamine

Dopamine is a neuropeptide produced by dopaminergic neurons in the hypothalamus via tyrosine hydroxylase activity [25] and it is considered inhibitory of reproduction through its role in seasonal anestrus [26] as well as prolactin (milk production) inhibition [27]. During seasonal anestrus, dopamine acts via kisspeptin neurons to regulate GnRH, and subsequently LH, pulse frequency [26, 28]. First, blocking dopamine signaling through administration D2 dopamine receptor antagonist [26] or administration of dopamine derivatives [28] will increase hypothalamic GnRH and kisspeptin content [28] and increase LH pulse frequency [26] in anestrus sheep. Conversely, administration of Kiss1R antagonist will block the increase in LH pulse frequency in response to inhibiting the dopaminergic system [26]. Approximately 31% of dopaminergic neurons in the ARC contain PR with approximately 22% providing

retrograde tracer labeling towards GnRH neurons [29]. Thus, these neurons may transduce progesterone mediated inhibition of GnRH neurons. Dopamine is likely acting through the KNDy neurons as approximately 80% of these neurons contain D2 dopamine receptor during anestrus and 40% of these neurons contain D2 receptors during the breeding season [26].

In rats, administered LPS causes an increase in extracellular dopamine in the brain [30]. In sheep, acute endotoxin does not significantly affect the number of dopamine immunopositive cells nor the percent of dopamine-cFos dual labeled immunopositive cells [12]. However, there is a numerical increase in the percent of activated dopamine immunopositive cells during proestrus and estrus and a numerical suppression in the number of immunopositive dopamine cells through the estrus [12]. As dopamine plays a role in seasonal anestrus and is elevated following LPS administration, it is a possible mechanism for the LPS induced suppression of reproduction (see Figure 1 for working model).

Peripheral Metabolic Hormones

There is a clear necessity for proper nutrition in order to achieve successful reproduction [1, 3, 31-33]. The brain receives information regarding nutritional status through metabolic hormones that can cross the blood-brain barrier and in doing so these hormones are involved in the regulation of other physiological functions, including reproduction. Endotoxin administration increases the systemic circulation of metabolic hormones, insulin (in rodents, not in sheep) [34, 35] and leptin [36-39], that also are involved in the regulation of appetite and reproduction. As metabolic status (undernutrition

and overnutrition) may induce and/or occur alongside chronic inflammation, the relationship between these metabolic hormones and inflammation is important to understand. However, more work is required to fully understand how these hormones work to regulate and interact with the immune response and reproduction.

Insulin

Insulin is a peptide hormone produced by the beta cells in the islets of Langerhans of the pancreas that is important for glucose homeostasis as it promotes the uptake of glucose into cells for storage. It is also thought to be a satiety hormone as circulating levels are high after meals when the body has excess amounts of glucose flowing in the blood stream. While GnRH neurons do not have the insulin receptor, ARC kisspeptin neurons do contain the receptor [40, 41]. Interestingly, in female sheep with an ideal body fatness, insulin administration reduces circulating concentrations of LH. However, in female sheep with lesser body fat, administration of exogenous insulin increases LH secretion [37, 42, 43].

Insulin may serve an anti-inflammatory role during or following exposure to LPS [44]. First, insulin can disrupt the LPS induced COX-2 expression and NF- κ B activation in alveolar macrophages [45, 46] and inhibits the expression of TLR-4 in acute lung injury [47]. Furthermore, insulin administration inhibits the LPS induced activation of microglia and subsequent release of TNF- α [48, 49]. Insulin alleviates the LPS induced cognition impairment, neuronal injuries and death, and disruption to the blood brain barrier [48]. Additionally, the immune response within the brain induced by LPS is inhibited with insulin administration [48]. However, hyperinsulinemia (from insulin resistance, at the

liver specifically [35]), hypoglycemia (failure to produce glucose from liver insulin resistance and deposition of glucose from hyperinsulinemia, [50]), and central insulin resistance [34] are consequences of LPS administration. Ultimately, chronic LPS exposure can lead to insulin resistance and be initiated by chronic low grade LPS induced inflammation associated with metabolic disease [34, 35] and initiate obesity [35, 51-53], which impairs fertility [51, 52, 54].

Glucagon-like peptide 1

Glucagon-like peptide 1 (GLP-1) is a peptide hormone produced and secreted by intestinal enteroendocrine L-cells upon food consumption. This hormone decreases blood glucose level as it promotes insulin secretion by increasing gene transcription, mRNA stability and biosynthesis as well as inhibits apoptosis of β islet cells [55-57]. Within the brain, GLP-1 provides neurogenesis and neuroprotection by reducing necrotic signaling [58] and cell death [59]. Due to these roles, GLP-1 can be utilized as a treatment for obesity and insulin resistance [56, 57, 60] as well as Parkinson's disease [61, 62], Alzheimer's disease [63], stroke [62], traumatic brain injury [58], and multiple sclerosis [64]. In culture, GLP-1 significantly increases the kisspeptin expression [65]. In ovariectomized mice, GLP-1 receptor signaling will activate ARC KNDy neurons, however, it is not sufficient enough to overcome the LH suppression during negative energy balance [66].

Leptin

Leptin is a glycoprotein produced and secreted by adipocytes with the primary role of energy balance as it inhibits hunger and initiates the utilization of fat stores for energy. The receptor for leptin exist throughout the hypothalamus including on neurons in the ARC

in sheep [67] and rodents [68], such as KNDy neurons [69]. In terms of reproduction, leptin administration can stimulate LH secretion [70, 71], prevent the suppression of circulating concentrations of LH in fasted lambs [71, 72], and restore kisspeptin mRNA in undernourished sheep [69, 70]. Conversely, leptin signaling in ARC kisspeptin neurons [73] and deletion of the receptor fails to impact reproduction [40]. Taken together, it is likely that the role of leptin in reproduction is permissive as it is dependent on metabolic status.

Leptin also serves as an adipocytokine that exists at the interface between immune system and metabolism as it facilitates the release of proinflammatory cytokines from adipose tissue [39, 74, 75], but also serves as an anti-inflammatory cytokine [76]. Systemic administration of endotoxin increases leptin expression in both the adipose tissue and serum [36, 38, 39, 77]. Interestingly, leptin will induce the IL-6 production, NF- κ B pathways and IL-1 β production in microglia similarly to the changes typically associated with systemic administration of LPS [39]. Similar to its role in reproduction, leptin has a dual role in immune response, potentially mediated by sex steroids [78, 79] and season [80]. Leptin also may play a role in the protection as *ob/ob* (leptin deficient) mice are highly susceptible to LPS induced lethality, but leptin administration reverses this effect [39, 81, 82]. During endotoxemia in humans, there is a positive correlation between circulating leptin levels and the survival after sepsis [39, 81-83]. However, leptin resistance occurs within the hypothalamus in obesity and in turn leptin is not a sufficient treatment for obesity [84, 85]. There is no alteration to serum leptin in sheep following endotoxin administration [77, 86] despite plasma concentrations of TNF- α being elevated by administration of LPS

[77]. Furthermore, obese mice exhibit decreased hypothalamic leptin receptor, kisspeptin, and GnRH; increased serum leptin and increased circulating TNF- α [87].

Appetite Neuropeptides

Two types of neurons also reside in the ARC that could be of interest in the inflammation induced suppression of GnRH/LH secretion through KNDy neurons as they serve a role in metabolism: POMC and neuropeptide Y (NPY)/agouti related peptide (AgRP). Both of these receive peripheral metabolic hormones that could regulate reproduction.

POMC and α -Melanocyte Stimulating Hormone

Proopiomelanocortin is a precursor polypeptide for melanocyte stimulating hormone α (α -MSH), ACTH, and β -endorphin and is produced by POMC neurons in the ARC. Its products are important for maintaining homeostasis as they maintain skin, stress and immune responses, and sexual function, respectively. These POMC neurons facilitate an important role in appetite and feeding behavior as they cause satiety and inhibit feed intake [88]. Furthermore, KNDy neurons provide glutaminergic input onto POMC neurons [89] and POMC neurons provide input to approximately 32-44% of the KNDy neurons [69, 90]. Thus, POMC and KNDy neurons may have a reciprocal communication in order to regulate GnRH/LH secretion through leptin and satiety. Leptin binds to the long form of its receptor which is present on POMC neurons and stimulates satiety [88] mediated through α -MSH as the cleavage of POMC into α -MSH is in proportion to plasma leptin concentrations and body adiposity (i.e. produced primarily during periods of positive energy balance) [31].

Out of the products of POMC, melanocortins have been shown to stimulate the reproductive axis in various species [91] through GnRH/LH secretion [92]. For example, administration of melanotan II, an α -MSH analog that primarily binds to α -MSH receptor, melanocortin 3 receptor (MC3R), will stimulate LH secretion in adult ewes regardless of their body condition [90, 93]. It is believed α -MSH stimulates GnRH/LH secretion through the KNDy neurons as shown by the role of α -MSH in mediating the permissive effects of leptin during puberty onset [94] and approximately 66% of the KNDy neurons contain MC3R [95].

Endotoxin and α -MSH share an inverse relationship as administration of LPS will reduce the mRNA for POMC and the percent of active α -MSH neurons [96] thus indicating that POMC may be depleted as it is being converted to ACTH rather than α -MSH. The suppression of α -MSH is likely due to the effects IL-1 β [97]. Conversely, α -MSH attenuates the proinflammatory concentration of IL-6 and IL-1 in vitro [98] and is considered anti-inflammatory when administered systemically or locally [99]. Specifically, α -MSH inhibits NF- κ B activation and TLR-4 to promote the anti-inflammatory phenotype of microglia [100, 101]. Further, α -MSH inhibits COX expression within the hypothalamus by binding to MC4R in male rats [102]. In sheep, there is a decrease in activated α -MSH and POMC gene content following LPS administration [96]. Taken together, LPS induced suppression of reproduction may occur due to the inhibition of POMC neurons, decrease in α -MSH, and increased proinflammatory microglia thus suppressing kisspeptin and GnRH neuronal activation.

Neuropeptide Y/Agouti-related peptide

Neuropeptide Y (NPY) and AgRP are neuropeptides co-expressed by NPY/AgRP neurons within the ARC. These neuropeptides increase appetite and feeding behavior while decreasing metabolism, essentially working in opposite “direction” of POMC/ α MSH with causing hunger and stimulation of feeding behavior [88]. A potential mechanism in which NPY/AgRP stimulate feed intake is through inhibition of ARC POMC expression and acting as an antagonist for MC3R and MC4R [88]. These neurons also contain the long form of the leptin receptor; however, leptin will inhibit the actions of these neurons and stimulate POMC neurons. Similar to POMC neurons, NPY/AgRP neurons in the ARC provide input onto KNDy neurons [69] thus allowing both POMC and NPY/AgRP neurons potentially regulate reproduction.

In mice, LPS administration blunts fasting induced activation of NPY/AgRP neurons [103]. In rats, NPY content in the ARC does not change significantly following LPS treatment [104] while POMC content in the ARC increases [104, 105]. Interestingly, NPY administration will restore feed intake that is suppressed with LPS administration [106]. Endotoxin administration oftentimes leads to lack of appetite due to activation of MC4R in the hypothalamus [96]. In sheep, administration of AgRP will prevent the reduction in feed intake secondary to LPS administration [96]. Six hours after LPS administration, there is increased AgRP gene and activated AgRP neurons [96]. Taken together, there may be a link between NPY/AgRP neurons and the suppression of reproduction through inflammation.

Conclusions

Taken together, the suppression of GnRH/LH secretion through endotoxin induced inflammation, as associated with various infectious and non-infectious diseases in domestic livestock and humans, is most likely a combination of suppression in factors, such as nutrition. Additionally, effects of inflammation due to an infection may be compounded by other regulatory factors, including metabolic status. Therefore, there is a need for greater investigation into the interactions and relationship between metabolism and inflammation from immune system activation.

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Appendix

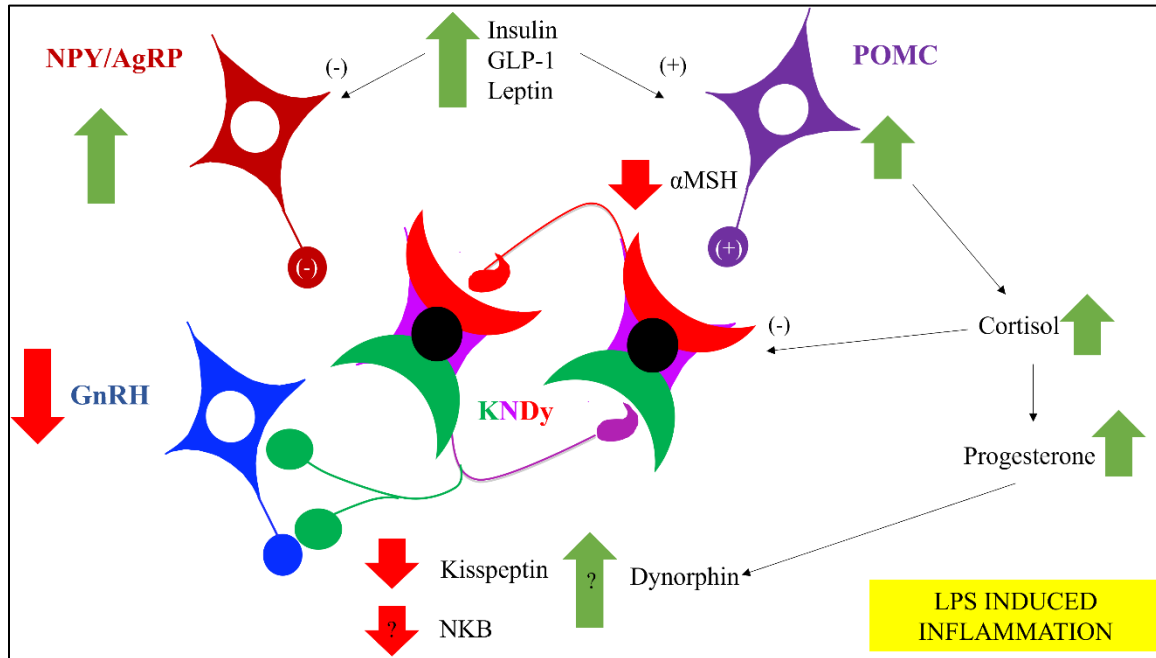


Figure 0.1. Working Model. LPS induced inflammation produces an increase in the steroid hormones as well as peripheral appetite hormones that alter the production of various neuropeptides including those involved in appetite regulation, but ultimately suppresses the expression of kisspeptin and potentially neurokinin B (NKB) in order to decrease GnRH secretion.

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VITA

Allison Nicole Renwick was born in Farmington, Connecticut on January 23, 1996 to Thomas and Teresa Renwick. She grew up and lived in Simsbury, Connecticut with her parents and older sister, Taylor Waddle, with minimal exposure to agriculture contrary to the name of the town she was born. Allison graduated from Simsbury High School in June of 2014 and continued her education that fall at North Carolina State University. While in undergrad, Allison volunteered at the Small Ruminant Educational Unit for about 2.5 years where she not only discovered a love for sheep, but also research. She joined Dr. Casey Nestor's laboratory as an undergraduate researcher in March of 2017 and assisted in research evaluating the effects of negative energy balance via undernutrition on reproductive neuroendocrinology in sheep. Allison graduated in May of 2018 with a bachelor's in Animal Science and a minor in Genetics. In the fall of 2018, Allison began her Master's continuing and focusing her studies in reproductive physiology and research in reproductive neuroendocrinology. While a graduate student, Allison served the Animal Science Department as a research assistant and teaching assistant, both positions facilitating her love for learning and teaching. In April of 2020, Allison successfully defended her thesis, despite delays and complications due to the COVID-19 Pandemic. She moved to Powell, Tennessee in July of 2020 to begin her work towards a Doctorate of Philosophy in Comparative and Experimental Medicine through the University of Tennessee College of Veterinary Medicine in Knoxville under the guidance of Dr. Brian Whitlock. During her time at UTCVM, Allison has successfully obtained small internal grants in order to assist in funding her research projects as well as mentored approximately

35 undergraduate students through her work as a graduate research assistant. She has also participated as a graduate teaching assistant for Anatomy Physiology in the Animal Science Department.