



12-2015

## Effects of low-dose naltrexone on feed intake, growth, endocrine and immune parameters in the recently-weaned pig

Ashley Christine Carter

*University of Tennessee - Knoxville, [acarte30@vols.utk.edu](mailto:acarte30@vols.utk.edu)*

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To the Graduate Council:

I am submitting herewith a thesis written by Ashley Christine Carter entitled "Effects of low-dose naltrexone on feed intake, growth, endocrine and immune parameters in the recently-weaned pig." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Cheryl Kojima, Major Professor

We have read this thesis and recommend its acceptance:

Henry Kattesh, Brian Whitlock

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

**Effects of low-dose naltrexone  
on feed intake, growth, endocrine and immune  
parameters in the recently-weaned pig**

A Thesis Presented for the  
Master of Science  
Degree  
The University of Tennessee, Knoxville

Ashley Christine Carter  
December 2015

## **DEDICATION**

For my parents, John and Alma Carter

Your beliefs in my abilities have helped me to make my dreams into my reality.

## ACKNOWLEDGMENTS

I would like to thank my major professor and mentor, Dr. Cheryl Kojima for her guidance throughout many degrees at the University of Tennessee. Without her motherly advice and patience (and a few well written recommendation letters) I would not have been able to accomplish all that I have in these few years. I will not only consider her a mentor, but also a great friend always.

A very special thanks goes to Mrs. Denice Milligan. She has been a rock in many storms of personal and professional hardships. I will never forget her words of wisdom, “It’s how you handle plan B.” and “If nobody died, it will all be ok.” Thank you, Denice. So much.

Thank you to my committee members, Drs. Henry Kattesh and Brian Whitlock, for their support of my pursuit of two degrees simultaneously. Thank you to all who worked at JARTU for their help during my project. I would also like to thank Leczek Wojakiewicz and Barbara Gillespie for their technical instruction and support in the laboratory. Thank you to Rekek Negga and Sierra Lockwood for their instruction and friendship during this journey. Without you two, I’d have been way lost, many times.

Thank you to the Department of Animal Science faculty, staff, and graduate students. Additionally, thank you to the College of Agricultural Sciences and Natural Resources, Office of the Dean. It was a wonderful experience to work with a team with such close friendships.

Finally, I’d like to thank my family and closest friends for their love and support. So many long, sleepless nights went into this degree, and they weren’t without their grouchy mornings. For their understanding of my hardships and their encouragements, I can never repay them.

Thank you all.

## ABSTRACT

Weaning is a stressful event for pigs and causes decreased feed intake, poor growth, and increased susceptibility to disease. Previous studies have shown that syndyphalin-33, a synthetic opioid, was effective in increasing feed intake, abrogating the changes in appetite regulating genes during weaning, and abrogating the effects of a salmonella challenge on immune cells in newly-weaned pigs. However, there are several concerns associated with the administration of an opioid in commercial swine operations. Low-dose naltrexone (an opioid antagonist) has been used to alleviate symptoms from fibromyalgia and Crohn's disease in humans. As inflammation is a common factor in both auto-immune diseases in humans and weaning stress in pigs, a logical next-step would be to examine the effects of low-dose naltrexone on pigs at weaning. In this study, low-dose naltrexone administration was evaluated for its effects on feed intake, growth, endocrine, and immune parameters in newly weaned pigs. Four treatments of 0 mg/day (d), 1 mg/d, 5 mg/d, 10 mg/d naltrexone were administered orally daily beginning 2 wk prior to weaning to 48 commercial crossbred pigs. Each treatment group included 12 pigs. Body weights and blood samples were collected d 0 and at 1, 4, and 7 post-weaning. All animals treated with naltrexone had increased total gain as compared to the control animals ( $P < 0.05$ ). A decrease in feed intake was seen in animals treated with 5 mg/d naltrexone as compared to the control animals ( $P < 0.05$ ). Plasma cortisol concentrations were similar to previously published concentrations and increased 1 d post-weaning in the control animals ( $P < 0.01$ ). Animals treated with 10 mg/d naltrexone had higher plasma concentrations of cortisol relative to all other treatments ( $P < 0.01$ ). On 1 d post-weaning, animals treated with 1 mg/d and 5 mg/d naltrexone had lower plasma cortisol concentration than the controls ( $P < 0.01$ ), and by d 4 post-weaning, all animals treated with naltrexone had lower cortisol concentrations relative to the control

animals ( $P < 0.01$ ). As a non-opioid, oral low-dose naltrexone may be a promising therapy to decrease the growth lag associated with weaning.

## TABLE OF CONTENTS

<b>CHAPTER 1: Literature Review</b> .....	<b>1</b>
<b>Introduction: The Issue With Weaning</b> .....	<b>2</b>
<b>Behavior and Growth</b> .....	<b>2</b>
<b>Cortisol: Physiological Stress Indicator</b> .....	<b>3</b>
<b>Corticosteroid-Binding Globulin</b> .....	<b>3</b>
<b>Free Cortisol Index</b> .....	<b>4</b>
<b>Effect of Weaning on Appetite</b> .....	<b>4</b>
<b>Effect of Weaning on Immunological Functions</b> .....	<b>5</b>
<b>Previous Efforts to Relieve the Post-Wean Lag</b> .....	<b>7</b>
<b>Opioids</b> .....	<b>8</b>
<b>Effect of Opioids on Immunological Functions</b> .....	<b>9</b>
<b>Effect of Opioids on Appetite</b> .....	<b>10</b>
<b>Antagonist Induced Up-Regulation</b> .....	<b>11</b>
<b>Low-Dose Naltrexone</b> .....	<b>12</b>
<b>Conclusion</b> .....	<b>13</b>
<b>CHAPTER 2: Manuscript</b> .....	<b>14</b>
<b>Introduction</b> .....	<b>15</b>
<b>Materials and Methods</b> .....	<b>16</b>
<b>Statistical Analysis</b> .....	<b>18</b>
<b>Results</b> .....	<b>19</b>
<b>Discussion</b> .....	<b>22</b>
<b>Conclusion</b> .....	<b>27</b>
<b>LIST OF REFERENCES</b> .....	<b>29</b>
<b>APPENDIX</b> .....	<b>38</b>
<b>VITA</b> .....	<b>43</b>



## LIST OF FIGURES

- Figure 1.** Cumulative feed intake (A), body weights (B) and cumulative gain (C) over the first 7 days post-weaning. Raw means and standard errors are shown. Means with identical letters do not differ. For (B), letters indicate sameness or difference of means relating to day post-weaning; an asterisk indicates a time where there occurred a difference between the mean for control (0 mg/d naltrexone) pigs and pigs from at least one other treatment group. ....**39**
- Figure 2.** Plasma concentrations of cortisol (A) and corticosteroid-binding globulin (B). Calculated free cortisol index (C). Raw means and standard errors are shown. Means with identical letters do not differ; an asterisk indicates a time where there occurred a difference between the mean for control (0 mg/d naltrexone) pigs and pigs from at least one other treatment group. ....**40**
- Figure 3.** Whole blood white blood cell (WBC) concentration (A) and percentage of WBC that were identified as monocytes (B). Raw means and standard errors are shown. Means with identical letters do not differ; an asterisk indicates a time where there occurred a difference between the mean for control (0 mg/d naltrexone) pigs and pigs from at least one other treatment group .....**41**
- Figure 4.** Percentage of WBC that were identified as neutrophils (A) and lymphocytes (B), and the calculated neutrophil:lymphocyte ratio (C). Raw means and standard errors are shown. Means with identical letters do not differ; an asterisk indicates a time where there occurred a difference between the mean for control (0 mg/d naltrexone) pigs and pigs from at least one other treatment group. ....**42**

**CHAPTER 1**  
**Literature Review**

## **Introduction: The Issue with Weaning**

The weaning of swine occurs between twenty one and twenty eight days of age (Ko et al., 2015). At weaning, pigs are exposed to new stressors such as social hierarchy, environments, handlers, pathogens, nutritional factors, and feeding systems. These stressors not only decrease feed intake and cause poor growth, but also increase disease susceptibility (Matteri et al, 2000; Kojima et al., 2007, 2008; Jenkins et al., 2009). The post-wean growth lag causes problems for the swine industry because it reduces post-weaning average daily gain, reduces feed efficacy, and increases the time needed for animals to reach the desired market weight.

This literature review will cover background information regarding the physiological effects of weaning on the pig, current techniques used to reduce the post-weaning growth lag, and the use of low-dose naltrexone as a potential aid in negating the undesirable effects of weaning-related stress.

## **Behavior and Growth**

At weaning, pigs are removed from their dams and mixed together in group pens, usually in a separate building from other, older animals. Pigs have a social hierarchy that includes a dominant animal and subordinate animals. Fighting erupts quickly after being placed together, forcing each pig to battle for dominance. Establishment of the new hierarchy is usually complete within 24 hours of mixing (Symoens and Van Den Brande, 1969). During fights, they are not ingesting calories for growth. In fact, they are using energy to fight and heal rather than to grow (Colson et al., 2012). The growth lag continues until the new hierarchy has been established.

## **Cortisol: Physiological Stress Indicator**

Release of cortisol is reviewed in detail by Matousek et al. (2010). A stress stimulus initiates an action potential, transported by sensory nerves to the hypothalamic-pituitary-adrenal (HPA) axis. Activation of the hypothalamus initiates the secretion of corticotrophin-releasing hormone (CRH) from neurosecretory cells into the primary capillary plexus of the hypophyseal portal system. Corticotrophin-releasing hormone exits the portal system at the secondary capillary plexus and stimulates corticotrophs in the anterior pituitary to produce adrenocorticotrophic hormone (ACTH). The corticotrophs release ACTH into the secondary capillary plexus to circulate through the body to the adrenal glands. Activation of cells in the zona fasciculata of the adrenal cortex results in the release of cortisol into systemic circulation. Cortisol is a glucocorticoid-steroid hormone that is involved in many pathways, including immunological functions and appetite regulation (Webster et al., 2002; Liu et al., 2007). Because of its quick release during a stress-stimulus, cortisol measurements are commonly used as an indicator of stress. Weaning results in a transient but marked increase in cortisol concentrations that usually returns to pre-wean levels by four days post weaning (Le Dividich and Seve, 2000; Kojima et al., 2008; Cooper et al., 2009). Cortisol's specific action in the appetite pathway and immunological functions will be discussed in subsequent sections.

## **Corticosteroid-Binding Globulin**

Corticosteroid-binding globulin (CBG) is a transport carrier glycoprotein found in circulation after its production and release from the liver. The majority of cortisol found in blood is bound to CBG (Adcock et al., 2006). Only free cortisol has been shown to be biologically active. When bound to CBG, cortisol is unable to cause physiological changes. (Schroeder and

Henning, 1989; Rosner, 1990; Adcock et al., 2006). When blood cortisol concentrations increase due to stressful situations, CBG concentrations decrease, allowing cortisol to be bio-available (Kojima et al., 2008; Cooper et al., 2009; Henley and Lightman, 2011).

### **Free Cortisol Index**

The free cortisol index (FCI) is an indicator of unbound cortisol in circulation. The FCI is determined for each sample by dividing the concentration of cortisol by the concentration of CBG (Adcock et al., 2007). Previously, FCI has been used as an estimate of free cortisol in humans (le Roux et al., 2002, 2003), and the use of FCI in swine was validated by Adcock and colleagues in 2006. During weaning in pigs, the FCI increases as a result of an increase in serum concentrations of cortisol combined with a decrease in plasma concentrations in CBG (Heo et al., 2003; Kojima et al., 2008; Cooper et al., 2009). It should be noted that the FCI, as a ratio, is not a sensitive indicator of free cortisol; therefore small changes in cortisol concentrations will minimally affect the resulting FCI.

### **Effects of Weaning on Appetite**

Appetite is controlled by orexogenic and satiety centers in the hypothalamus (Wynne et al., 2005). The following is a summary of appetite control from reviews by Wynne et al. (2005) and Stanley et al. (2005). Leptin and insulin function as appetite suppressants through the same pathway. Leptin is produced and released by adipocytes, while insulin is produced by pancreatic beta-cells and released from the liver. Both hormones stimulate pro-opiomelanocortin (POMC) to increase release of alpha-melanocyte stimulating hormone (MSH). Alpha-MSH subsequently stimulates CRH release from the hypothalamus to suppress appetite. In healthy pigs, appetite

stimulators compete with appetite inhibitors to bind the Melanocortin-4 receptor (MC4R) (Wynne et al., 2005; Stanley et al., 2005). Melanocortin-4 receptor is found in the central nervous system and is part of the melanocortin pathway; a major controller of appetite. Agouti-related protein (AgRP) competes with alpha-MSH to bind with MC4R. However, in stressed animals with increased plasma cortisol concentrations, the production of AgRP is decreased and alpha-MSH is allowed to bind to MC4R at a higher rate, therefore, decreasing appetite stimulation (Wynne et al., 2005; Stanley et al., 2005). The post-weaning growth lag in pigs is well recognized and clearly associated with reduced feed intake (Bark et al., 1986; McCracken et al., 1995; Cooper et al., 2009). The increased binding of MC4R by alpha-MSH was seen in experiments involving rats performed by Kas and colleagues (2005) and Liu and others (2007).

### **Effects of Weaning on Immunological Functions**

The immune system is important for removal of foreign cells/material and initiation of healing (Donaldson, 2007). The immune system is composed many types of leukocytes: basophils, neutrophils, eosinophils, monocytes, and lymphocytes. Basophils are mediators of inflammation that produce histamine and bradykinin, while neutrophils are small phagocytic short-lived cells. Eosinophils attack multi-cellular parasites in addition to detoxifying histamines from basophils. Monocytes circulate in the blood and are long-lived phagocytic cells (Donaldson, 2007). Lymphocytes can be roughly classified as one of two different types of cells: B- cells or T-cells. T-cells have three specific actions: recognition of the pathogen, initial release of cytokines, and destruction of virus infected cells. B-cells produce and release antibodies. Antibodies bind to foreign cells/material, marking the pathogen for phagocytosis (Donaldson,

2007; Moore, 2007). The response to infection by release of antibodies is referred to as the humoral response of the immune system.

Leukocytes contain receptors for pathogens in their cell membranes, making them capable of initiating the immune response. When the immune system is activated, many different immune cells can release histamine and chemotaxins, which cause blood vessels in the affected area to dilate and become more porous, allowing increased blood flow and migration of leukocytes into the area (Donaldson, 2007). Chemotaxins promote the release of leukocytes from storage in bone marrow for additional support against the pathogens (Moore, 2007). In addition to the humoral response, a cellular response is also initiated when foreign cells/material enters the body. The cellular response includes the production and release of cytokines. Cytokines can be either pro- or anti-inflammatory in nature and are regulated by a positive feed-back system to further increase the number of circulating cytokines and cytokine receptors. Pro-inflammatory cytokines such as interleukin-1 and interleukin-6 increase the number of migrating leukocytes to the site of infection (Donaldson, 2007). This causes a local increase in temperature and swelling. Interleukin- 1 also binds to the thermoregulatory center of the hypothalamus initiating a febrile response (Eskilsson et al., 2014). Other cytokines, such as interleukin-10 and interleukin-4 are anti-inflammatory. They are released by T cells to stimulate and promote proliferation of B cells and antibody production (Donaldson, 2007; Lilic, 2009). Phagocytosis, swelling, fever, and leukocyte proliferation, commonly referred to as inflammation, will continue until the pathogen is eliminated.

When experiencing stress, such as weaning, tumor necrosis factor alpha (TNF $\alpha$ ), released from macrophages and monocytes, stimulates CRH secretion from the hypothalamus, thus initiating the release of cortisol from the adrenal cortex (Bernardini et al., 1990). The following

section will discuss how cortisol affects the immune system. The glucocorticoid receptor (GR) is found within the cytoplasm of immune cells. Cortisol must diffuse into the cell and bind its receptor in order to evoke a response (Adcock, 2000). Cortisol modulates cytokine production, decreases adhesion, trafficking, maturation, and migration of immune cells, and decrease inflammatory mediators such as prostaglandins. (Webster et al., 2002; Dolan-O'Keefe and Nick, 1999). Generally, cortisol promotes anti-inflammatory responses and suppress pro-inflammatory responses. This is performed by control of cytokine transcription and disruption of signaling mechanisms (Bianchi et al., 2000). Cortisol performs a crucial task of controlling inflammation; therefore preventing self injury during the immune response. However, over stimulation of the HPA axis can result in suppression of the immune system, consequently increasing susceptibility to disease (Craddock, 1978). This is seen in times of intense environmental or physiological stress, such as weaning (McLamb et al., 2013; Bomba et al., 2014). Intense stress jeopardizes immune function and the effects of stress have been observed in human and animal studies. For example, an increase in swine intestinal disease is associated with weaning (McLamb et al., 2013; Bomba et al., 2014) and in humans, chronic stressors such as school exams or work stress increase the risk of acquiring viral infections and prolong wound healing (Rozlog, 1999) and marital distress causes long term immune cell dysfunction (Jaremka et al., 2013).

### **Previous Efforts to Relieve the Post-Wean Lag**

Because of its associated costs, many researchers have investigated methods of alleviating the post-weaning growth lag in pigs. A decrease in serum zinc concentrations at the time of weaning was observed. Because zinc plays a role in growth, experiments were conducted that supplemented zinc by injection prior to weaning (Schell and Kornegay, 1994), but no



difference in weight gain was observed after treatment. Addition of glutamine to feed was successfully used to prevent jejunal atrophy in newly weaned pigs (Wu et al., 1996); however, increased growth was not observed until two weeks after weaning. Addition of spray-dried plasma to nursery diets has been used to increase feed intake after weaning (Touchette et al., 2002; Kojima et al., 2007). Recently, the use of spray-dried plasma was questioned due to possible contamination and subsequent spread of Porcine Epidemic Diarrhea Virus (PED; Polo et al., 2014). Recent research has examined the use of syndyphalin-33 (SD-33; a synthetic opioid) to alleviate the negative effects of weaning (Jenkins et al., 2009; Kojima et al., 2009; Cooper et al., 2011). Syndyphalin-33 did alleviate the post-wean growth lag, but would be difficult to use in production because of its classification as a synthetic enkephalin. However, because of its success, more research is being conducted to examine alternate methods of increasing endogenous opioid tone (see Chapter 2). This literature review will now discuss opioids, their receptors, and their effects on immune modulation and feed intake.

## **Opioids**

Opioids are peptides that can be found within animals and some plants or synthesized in a laboratory for medical use. Endogenous opioids are produced within the body and include endorphins, enkephalins, and dynorphins. They bind to three different types of opioid receptors found on cell membranes throughout the body: mu ( $\mu$ , MOR), kappa ( $\kappa$ , KOR), and delta ( $\delta$ , DOR) (Koneru et al., 2009; Ninkovic and Roy, 2013). When bound to their receptors, opioids can produce analgesic and euphoric effects in addition to affecting the immune system and appetite regulation (Koneru et al., 2009; Ninkovic and Roy, 2013). When bound, MORs are responsible for analgesia, euphoria, reduced GI motility, respiratory depression,

immunosuppression, and physical dependence (Koneru et al., 2009; Al-Hashimi et al., 2013). Activated KORs cause sedation, affect appetite, and inhibit the release of anti-diuretic hormone (ADH, Koneru et al., 2009; Yeomans and Gray, 2002). Bound DORs also produce analgesia, euphoria, and physical dependence. Endorphins bind with high affinity to mu and delta receptors. Enkephalins preferentially bind to delta receptors and dynorphins bind to kappa receptors (Koneru et al., 2009). The following sections will cover how opioids modulate immunological functions and appetite regulation.

### **Effects of Opioids on Immunological Functions**

The effect of opioids on immune function is a topic of high controversy, and has been reviewed by Al-Hashimi and others (2013). Increased incidence of disease has been observed in opioid abusers and medical patients receiving chronic opioid medications (Hussey and Katz, 1950; Risdahl et al., 1998). The effects of opioids on the adaptive immune system are numerous and include a decrease in T-cell viability and T-helper cell function (Bryant et al., 1988a, 1988b, 1991; Roy et al., 2001), decrease in natural killer cell function (Gaveriauz-Ruff et al., 1998), decrease in macrophage activity (Bussiere et al., 1993), and an increase in the anti-inflammatory cytokines TGF-B1 and IL-10 (Pacifici et al., 2000). The innate immune system is also affected. Decreases in leukocyte migration (Casellas et al., 1991), chemotaxis (Simpkins et al., 1984; Perez-Castrillon et al., 1992; Grimm et al., 1998), and phagocytosis (Casellas et al., 1991; Szebo et al., 1993) have been observed with administration of opioids. According to Sacerdote (2006), drug and host factors, in addition to the duration of exposure, all influence how various opioids affect immune function. It is believed that the MOR is primarily responsible for the immunosuppressive effects of opioids (Dietis et al., 2011; Ninkovic and Roy, 2013). This theory

is supported by research using MOR knockout mice, where immune modulation is not observed (Gaveriaux-Ruff et al., 1998; Roy et al., 1998; Nelson et al., 2000).

The locations of immunomodulation are controversial and may include the central neuro-endocrine, central neuro-paracrine, and peripheral mechanisms (Al-Hashimi et al., 2013). The theory of central control is supported by the observation that opioids that are unable to cross the blood-brain barrier show less immune modulation than those that do (Shavit et al., 1986; Hernandez et al., 1993). The theory of peripheral control is supported by research using MOR knockout mice, which reports that no immune modulation occurs without the receptor present on immune cells (Gaveriaux-Ruff et al., 1998; Roy et al., 1998; Nelson et al., 2000). The HPA axis also plays a role in immune modulation. Endogenous and exogenous opioids have been shown to modulate the HPA axis ensuing in varied results that are dependent on an acute or chronic administration of the opioid (Vuong et al., 2010). Acute administration (results measured within 24-hours of a single dose) results in an increase in ACTH and glucocorticoid levels in animals. Chronic administration (results measured after many days of administration) results in a decreased response of glucocorticoids to activation of the HPA axis (Vuong et al., 2010).

### **Effects of Opioids on Appetite**

Opioids are involved in the homeostatic and hedonic (pleasure) mechanisms of feed intake (Nogueiras et al., 2012). The MOR and KOR receptors are most active in feed intake regulation (Yeomans and Gray, 2002). When activated, opioid receptors located on pre- and postsynaptic POMC neurons inhibit firing, therefore increasing feed intake (Pennock and Hentges, 2011). Previous research shows that administration of naloxone, an opioid receptor antagonist, can block the appetite stimulating affects of agouti-related protein and neuropeptide-

Y (Rudski, et al., 1996; Hagan et al., 2001). Opioid administration can also affect the rewarding aspect of feeding. [D-Ala<sup>2</sup>, N-MePhe<sup>4</sup>, Gly-ol]-enkephalin (DAMGO), a MOR agonist, was injected into the nucleus brain of rats, resulting in increased intake of saccharin and increased feeding in satiated rodents, respectively (Zhang and Kelley, 2002; MacDonald et al., 2003). Obese et al. (2006) reported that syndyphalin-33, a synthetic opioid, increased feed intake in sheep and demonstrated that the effects of syndyphalin-33 were temporarily canceled by administration of naloxone at 6 and 8 hours post-treatment. Cooper et al. (2011) observed an increase of MOR expression within the hypothalamus of control animals at the time of weaning, but not in pigs treated with 0.5µmole/kg syndaphalin-33. Also, treatment with syndyphalin-33 abrogated the increase of MC4R expression in control animals caused by weaning. Finally, Cooper et al. (2011) observed an increase in AGRP expression 4 days post-weaning in animals treated with syndyphalin-33. All of these changes in appetite regulating genes seen after syndyphalin-33 treatment support the theory that opioids are important regulators of appetite.

### **Antagonist Induced Endogenous Opioid and Receptor Up-regulation**

Weaning affects appetite regulation and immunological function. Specifically, cortisol released during times of stress decreases expression of appetite stimulating genes and decreases immune cell adhesion, trafficking, maturation, and migration. Because of their ability to increase appetite and decrease inflammation, opioids have been considered as a means to alleviate the post-weaning growth lag in pigs. However, opioid administration in commercial swine production is not feasible due to strict regulations and human abuse potential. Zagon and McLaughlin (1989) proposed a theory saying that a small, transient administration of an opioid antagonist, such as naltrexone, will compel the body to compensate by up-regulating endogenous

opioids and their receptors. This hypothesis has been supported by others (Brown and Panksepp, 2009; Younger et al., 2014). The increase of opioid concentrations and receptors would supersensitize opioid function within the body (Zukin et al., 1982; Brown and Panksepp, 2009); potentially resulting in effects similar to exogenous opioid administration. These effects may include increasing anti-inflammatory cytokines such as TFG-B1 and IL-10 and decreasing leukocyte migration and chemotaxis. Recent research has observed that low-doses of naltrexone can relieve the symptoms of human auto-immune diseases. Since inflammation is a common factor between auto-immune diseases in humans and weaning stress in pigs, a logical next step would be to examine the effects of low-dose naltrexone on pigs at weaning.

### **Low Dose Naltrexone**

Naltrexone is a synthetic opioid-receptor antagonist. It binds with high affinity to MOR and DOR and with a lower affinity to KOR within the body (Weerts et al., 2008). It is orally effective and has a 4 hour half-life, reaching peak levels one hour after administration (Kleber, 1985; Lobmaier et al., 2010). Its major metabolite, 6-beta-naetrexol, has a half-life of 13 hours, allowing the antagonistic effects of naltrexone to last for days (Kleber, 1985). When administered for treatment of narcotic addiction and alcoholism, normal dosages range from 50-100 mg daily (Weinrieb and O'Brien, 1997; Lobmaier et al., 2010; Younger et al., 2014). An average low-dose naltrexone treatment in human medicine is 4.5 mg daily, though the dose can vary within a few milligrams (Younger et al., 2014). Low-dose naltrexone has been used to reduce symptoms of fibromyalgia (Younger and Mackey, 2009; Younger et al., 2013), Crohn's disease (Smith et al., 2007), and multiple sclerosis (Cree et al., 2010). In the pilot study by Younger and Mackey (2009), human participants were given 4.5 mg naltrexone/ day for a period

of two weeks resulting in decreased levels of pain and stress. Furthermore, the effects of naltrexone were still being observed two weeks after cessation of treatment (Younger and Mackey, 2009). The symptoms of Crohn's disease were reduced in patients receiving 4.5 mg naltrexone/ day, and 67% of the patients went into complete remission (Smith et al., 2007). Naltrexone given at 4.5mg/d improved mental health and pain in multiple sclerosis patients (Cree et al., 2010). Low-dose naltrexone administered at 0.1, 1.0, and 3.0 mg was found to decrease feed intake and to increase lying time in rats (Wright and Rodgers, 2013, 2014a, 2014b). To date, the Food and Drug Administration (FDA) has not done any testing to determine the withdrawal period of naltrexone in food animals.

## **Conclusion**

Weaning causes disruptions in immunity and appetite. The post-weaning growth lag has been a subject of research over the past few years, and low-dose naltrexone shows promise in alleviating the negative effects of weaning. It has been shown to reduce inflammation and stress in human studies and alter feed intake and behavior in rodent studies. The following chapter explores a research project with newly weaned pigs and low-dose naltrexone.

## **CHAPTER 2**

### **Manuscript**

## **Effects of low-dose naltrexone on feed intake, growth, endocrine and immune parameters in the recently-weaned pig**

### **Introduction**

Weaning stress causes decreased feed intake, poor growth, and increased susceptibility to disease in pigs (Matteri et al, 2000; Kojima et al., 2007, 2008; Jenkins et al., 2009). These responses to stress can manifest as a growth lag, but may also manifest as increased herd morbidity or mortality, particularly in conditions of poor herd health and management. Previous researchers have published findings that syndyphalin-33, a synthetic enkephalin, can negate the negative effects of weaning by increasing feed intake, plasma growth hormone levels, and circulating leukocyte numbers (Kojima et al., 2009). However, syndyphalin-33 is an opioid and would therefore be difficult to approve for production use.

Naltrexone is a long acting (4 hour half-life), orally effective opioid antagonist that binds with high affinity to  $\mu$  receptors throughout the body. It also binds  $\kappa$  and  $\delta$  receptors but with lower affinity (Shader, 2003). Often, naltrexone is used for treatment of alcohol and narcotic addiction (Davidson et al., 1999); additionally, in low doses, naltrexone has been used to reduce symptoms of fibromyalgia (Younger and Mackey, 2009; Younger et al., 2013) and Crohn's disease (Smith et al., 2007). In the pilot study by Younger and Mackey (2009), human participants were given 4.5 mg naltrexone/d for a period of two weeks resulting in decreased levels of pain and stress. Furthermore, Younger and Mackey (2009) published findings that the effects of naltrexone were still being observed two weeks after cessation of treatment. The most prevalent hypothesis is that a temporary blockade of opioid receptors on immune cells and neurons by naltrexone prompts the body to compensate by up-regulating endogenous opioids and



their receptors, resulting in an overall increase in opioid tone (Zagon and McLaughlin, 1989, 1995; Tempel et al., 1985; Brown and Panksepp, 2009).

The hypothesis is that administering low doses of naltrexone orally to pigs two weeks prior to weaning would result in altered feed intake and weight gain due to the up-regulation of endogenous opioids and their receptors. This research examined the effects of low-dose naltrexone on growth, feed intake, and circulating measures of cortisol, corticosteroid-binding globulin, and immune cell populations in the recently weaned pig.

## **Materials and Methods**

Forty-eight pigs (3.7 +/- 0.1 kg) were randomly assigned to four treatment groups of 12 pigs per group at approximately 10 days of age. The four treatment groups included a control (0 mg/d; N0) and three naltrexone treatments: 1 mg/d (N1), 5 mg/d (N5), and 10 mg/d (N10). Naltrexone was compounded with dextrose into size 4 capsules by the University of Tennessee College of Veterinary Medicine pharmacy and was administered orally once daily at the same time each morning for 14 days prior to weaning. Control animals received dextrose capsules to prevent differences in results due to handling. The pigs were manually restrained, dosed using a pill gun, and immediately returned to their dam and litter. At weaning, (d 0, approximately 28 days of age) the pigs were weighed and blood samples collected via jugular venipuncture. Pigs were weaned into twelve 16 m<sup>2</sup> pens by treatment (4 pens per treatment group, 3 pigs per pen). On d 1, 4, and 7 after weaning, the pigs were weighed and blood samples were collected. Feed and water were provided ad libitum. Cumulative post-weaning feed intake per pen was calculated at the end of the study (d0- d7).

Blood samples (approximately 4 mL) were collected via anterior venipuncture and allotted as follows: 1 mL into a tube spray-coated with 5.4 mg K<sub>2</sub>-EDTA (BD Vacutainer, Franklin Lakes, NJ), and 3 mL into a tube spray-coated with 86 IU heparin (BD Vacutainer) for plasma collection. Blood collected into the EDTA- containing tubes was used to create white blood cell (WBC) differential slides and was also used in a hematology analyzer for measurement of white blood cell count (WBC). The heparinized blood samples were centrifuged at 801 g for 15 minutes and the recovered plasma was used for measurement of total cortisol concentrations and corticosteroid-binding globulin (CBG) concentrations (see below). The free cortisol index was calculated for each sample.

During blood collection, animals were temporarily placed in a V-trough and restrained by hand. Collection of the sample was achieved within 1 minute of initial restraint to reduce restraint-related increases in cortisol concentration within the sample.

Slides for WBC differentials were made from whole blood smears (approximately one cell layer thick), fixed in methanol, and stained with a Wright-Giemsa I and II Solution (Fisher Scientific, Houston, TX) according to the manufacturer's directions. One hundred leukocytes were counted using light microscopy at 600x total magnification. The following leukocytes were counted: monocytes, lymphocytes, immature neutrophils (bands), neutrophils, eosinophils, and basophils. This paper will discuss the percentage of leukocytes identified as monocytes, neutrophils, and lymphocytes. The neutrophil/lymphocytes ratio (NLR) was calculated for each sample.

A hematology analyzer (Vet ABC by Scil, Gurnee, IL) was used to quantify WBC. This machine was operated according to the manufacturer's instructions using blood from EDTA-containing tubes.

Plasma total cortisol concentration was determined using the Coat-a-Count radioimmunoassay procedure (Diagnostic Products, Los Angeles, CA) as described previously (Scroggs et al, 2002). Intra- and interassay CV were 10.0% and 9.2% respectively for low and 3.0% and 5.6% respectively for high cortisol quality control standards. The sensitivity of this assay ranged from 9.66 to 551.8 nmol/L. Plasma CBG concentration (mg/L) was measured by direct enzyme-linked immuno-absorbent assay (ELISA) as described previously (Roberts et al., 2003). Intra- and interassay CV were 5.86% and 15.31%, respectively for CBG quality control standards. The sensitivity of this assay ranged from 0.05 mg/L to 400 mg/L. The free cortisol index (FCI, nmol/mg), was calculated using the ratio of concentrations of plasma total cortisol to CBG (Adcock et al., 2007).

### **Statistical Analysis**

All statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC). A mixed model ANOVA with repeated measures was used to analyze body weight, cortisol, CBG, FCI, and all other blood values with pig as the experimental unit. All variables using repeated measures had time, treatment, and interaction as fixed effects. Pre-treatment weight was used as a covariate for body weight, and FCI required a log transformation to maintain homogeneity of variance. Cumulative feed intake was analyzed using a one-way ANOVA with pen as the experimental unit and treatment as the main effect. Total weight gain over 7 days post-weaning was analyzed using a one-way ANOVA with pig as the experimental unit and treatment as the main effect. Least squares means were compared using Fisher's protected LSD for all analyses, and a significance level of  $P < 0.05$  was used. Trends were reported where  $0.10 > P > 0.05$ .

Graphs depict raw means with standard errors. Results were reported as mean  $\pm$  standard deviation.

## **Results**

### Feed Intake:

Cumulative feed intake from d0- d7 differed between treatments ( $P= 0.0472$ ), but was not different between N0 and N1 (Figure 1A). A decrease in feed intake was found in N5 ( $15.35 \pm 0.54$  kg) as compared to N0 ( $17.61 \pm 0.54$  kg;  $P= 0.0121$ ) and N1 ( $17.27 \pm 0.54$  kg;  $P= 0.0278$ ). Cumulative feed intake of N10 did not differ from other treatment groups.

### Body Weight:

Body weight differed by time ( $P < 0.0001$ ) and a trend toward a time by treatment interaction was observed ( $P = 0.0581$ ). Body weights did not differ between d 0 ( $8.04 \pm 0.15$  kg) and d 1 ( $7.99 \pm 0.15$  kg), but all four treatment groups gained weight from d 1 to d 4 ( $8.33 \pm 0.15$  kg) and from d 4 to d 7 ( $8.99 \pm 0.15$  kg; Figure 1B). Body weight did not differ across treatment groups on d 0 or d 1. Body weight on d 4 differed between N5 and N10 where animals in N5 ( $8.81 \pm 0.29$  kg) weighed more than the animals in N10 ( $7.85 \pm 0.29$  kg;  $P = 0.0239$ ). On d 7, body weight differed between N0 and N5 such that animals in N5 ( $9.40 \pm 0.29$  kg) weighed more compared with animals in N0 ( $8.36 \pm 0.29$  kg;  $P=0.0148$ ).

### Total Gain:

Total gain over 7 days post-weaning was greater in all naltrexone treatments ( $1.3 \pm 0.21$ ;  $1.08 \pm 0.21$ ; and  $1.04 \pm 0.21$  kg for N1, 5, and 10, respectively) as compared to the control group ( $0.33 \pm 0.21$  kg;  $P= 0.0154$ ; Figure 1C). There was no difference observed in total gain between N1, N5, or N10.

### Blood Characteristics:

Plasma cortisol concentrations (PCC) are depicted in Figure 2A. An effect of time was observed ( $P < 0.0001$ ) such that PCC increased from d 0 ( $89.40 \pm 6.62$  nmol/L) to d 1 ( $145.57 \pm 6.80$  nmol/L), and then decreased to d 0 concentrations on d 4 ( $89.34 \pm 6.62$  nmol/L). Concentrations remained unchanged from d 4 to d 7 ( $79.94 \pm 6.55$  nmol/L). A time by treatment interaction was noted ( $P < 0.0001$ ). On d 0, PCC did not differ between treatment groups. On d 1, N10 ( $203.65 \pm 13.11$  nmol/L) had greater PCC than all other treatments. N0 had greater PCC on d 1 than N1 and N5 ( $153.81 \pm 13.11$ ;  $114.97 \pm 13.11$ ; and  $109.87 \pm 14.95$  nmol/L for N0, 1, and 5 respectively). By d 4, PCC in N1, N5, and N10 ( $77.47 \pm 13.11$ ;  $84.72 \pm 13.11$ ;  $75.80 \pm 13.64$  nmol/L for N1, 5, and 10 respectively) decreased and were less than concentrations observed in N0 ( $119.36 \pm 13.11$  nmol/L). No differences in PCC were observed among treatments on d 7.

Plasma CBG concentrations are depicted in Figure 2B. An effect of time was observed ( $P < 0.0001$ ) such that CBG decreased from d 0 ( $1.64 \pm 0.09$  mg/L) to d 1 ( $0.92 \pm 0.09$  mg/L). Plasma concentrations of CBG did not differ between d 1 ( $0.92 \pm 0.09$  mg/L) and d 4 ( $0.77 \pm 0.09$  mg/L) or between d 4 ( $0.77 \pm 0.09$  mg/L) and d 7 ( $0.88 \pm 0.09$  mg/L).

The FCI is depicted in Figure 2C. An effect of time was observed ( $P < 0.0001$ ) such that the FCI increased from d 0 ( $58.71 \pm 6.10$  nmol/mg) to d 1 ( $168.96 \pm 20.40$  nmol/mg). The FCI decreased from d 1 to d 4 ( $127.33 \pm 15.09$  nmol/mg) and decreased from d 4 to d 7 ( $101.73 \pm 11.99$  nmol/mg). A time by treatment interaction was also observed ( $P = 0.0099$ ). There was a decrease in FCI from d 1 to d 4 in N1 and N10 ( $P = 0.0352$ ;  $P = 0.0002$  for N1 and 10 respectively).

Total WBC concentrations are depicted in Figure 3A. There was an effect of time ( $P < 0.0001$ ) such that WBC concentrations were not different between d 0 and d 1; however, d 7 ( $10.26 \pm 0.39 \text{ } 10^3/\text{mm}^3$ ) concentrations were greater than d 0 ( $8.03 \pm 0.4 \text{ } 10^3/\text{mm}^3$ ) and d 1 ( $8.24 \pm 0.38 \text{ } 10^3/\text{mm}^3$ ) in all treatments.

The percentage of leukocytes that were identified as monocytes (%mono) are depicted in Figure 3B. An effect of time was observed ( $P < 0.0001$ ) such that from d 0 ( $9.93 \pm 0.86 \%$ ) to d 1 ( $14.15 \pm 0.84 \%$ ), %mono increased, but was unchanged from d 1 to d 4 ( $13.98 \pm 0.84 \%$ ). An increase in %mono occurred from d 4 to d 7 ( $17.92 \pm 0.84 \%$ ). An interaction between time and treatment was also observed ( $P = 0.0073$ ). On d 0 and d 1, %mono did not differ between treatment groups. On d 4, N0 and N1 differed in that N1 ( $10.75 \pm 1.67 \%$ ) animals had lower %mono than those in N0 ( $16.67 \pm 1.67 \%$ ;  $P = 0.0133$ ). On d 7, animals in N10 differed from animals in N0 in that %mono was less in N10 ( $14.61 \pm 1.74 \%$ ) animals as compared to N0 ( $21.0 \pm 1.67 \%$ ;  $P = 0.0089$ ).

The percentage of leukocytes identified as neutrophils (%neut) differed by time ( $P < 0.0001$ ; Figure 4A). An increase from d 0 ( $24.16 \pm 1.61 \%$ ) to d 1 ( $45.38 \pm 1.57 \%$ ) was observed in %neut. The percentage of neutrophils decreased from d 1 to d 4 ( $39.38 \pm 1.57 \%$ ), and continued to decrease from d 4 to d 7 ( $35.48 \pm 1.57 \%$ ). An interaction between time and treatment was also observed ( $P = 0.0287$ ). No differences between treatment groups were observed on d 0 or d 1. On d 4, %neut was less in N0 ( $34.83 \pm 3.14 \%$ ) as compared to N1 ( $46.17 \pm 3.14 \%$ ;  $P = 0.0118$ ). N5 and N10 were not different than N0 or N1 on d 4 ( $38.08 \pm 3.14 \%$  and  $38.42 \pm 3.14 \%$  for N5 and 10 respectively). On d 7, no differences between treatment groups were observed.

The percentage of leukocytes that were identified as lymphocytes (%lymp) differed by time ( $P < 0.0001$ ; Figure 4B). A decrease in %lymp occurred on d 1 ( $39.50 \pm 1.64$  %) relative to d 0 ( $63.80 \pm 1.67$  %). An increase in %lymp was observed on d 4 ( $45.06 \pm 1.64$  %) relative to d 1 but did not differ between d 4 to d 7 ( $45.0 \pm 1.64$  %).

The neutrophil to lymphocyte ratio (NLR) did not differ by treatment but an effect of time was observed ( $P < 0.0001$ ; Figure 4C) such that ratios increased from d 0 ( $0.39 \pm 0.09$ ) to d 1 ( $1.34 \pm 0.09$ ) and decreased from d 1 to d 4 ( $1.01 \pm 0.09$ ). The NLR was unchanged from d 4 to d 7 ( $0.86 \pm 0.09$ ). An interaction between time and treatment was also noted ( $P = 0.0255$ ). On d 0, NLR did not differ between treatment groups. On d 1, NLR was less in N1 ( $1.06 \pm 0.17$ ) relative to N0 ( $1.73 \pm 0.17$ ;  $P = 0.0056$ ). On d 4 and d 7, there were no differences in NLR observed between treatments.

## **Discussion**

Recently researchers have observed the potential of low-dose naltrexone to alter pain, stress, and immune function (Smith et al., 2007; Younger and Mackey, 2009; Younger et al., 2013). A theory has been put forth stating that low-dose naltrexone up-regulates endogenous opioids and opioid receptors (Zagon and McLaughlin, 1989). In the present study, a low-dose therapy of naltrexone prior to weaning was hypothesized to improve post-wean performance in pigs. The effects of low-dose naltrexone on growth, feed intake, and circulating measures of cortisol, corticosteroid-binding globulin, and immune cell populations were examined in the recently weaned pig. The various dosages were selected based on previous low-dose naltrexone research performed by Younger and Mackey (2009) in which naltrexone (4.5 mg) was administered orally to human participants with fibromyalgia. The authors concluded that the

participants had less pain, fatigue, and stress. Ramanathan and others concluded that it takes approximately two weeks to alter the endorphin system; therefore, we elected to administer the naltrexone treatment two weeks prior to weaning.

#### Feed Intake (FI):

Inhibition of piglet growth rate caused by weaning is well recognized and clearly associated with reduced FI (Bark et al., 1986; McCracken et al., 1995; Cooper et al., 2009). In this experiment, cumulative FI decreased in animals treated with 5 mg/d naltrexone during the two weeks prior to weaning as compared to the control. Opioid antagonist promote POMC expression and blocks AGRP binding to MC4R. Both of these effects inhibit appetite. Although we hypothesized that low-dose naltrexone would increase feed intake by promoting endogenous opioid concentrations, it seems that administration of even low-doses of an antagonist can still inhibit appetite. This study did not observe a difference in FI in animals treated with 1 mg/d or 10 mg/d naltrexone as compared to control animals. In studies examining the effects of low-dose naltrexone on FI in rats (Wright and Rodgers, 2013, 2014a, 2014b), researchers administered 0.1 mg/kg, 1.0 mg/kg, or 3.0 mg/kg of naltrexone by a single IP injection. Behavior and FI were examined for one hour starting fifteen minutes after drug administration. A decrease in FI was observed at all dosages. In a study by Avena and others (2014), rats were given an IP injection of 0.1 mg/kg naltrexone. FI was measured hourly after injections at 1, 2, 3, 4 and 12 hr. FI decreased at 12 hr post injection of 0.1mg/kg naltrexone, and increased at 1 hr post injection of 0.1mg/kg naltrexone. It should be noted that these studies examined the effects of naltrexone immediately after 1 dose, whereas, this study administered naltrexone daily through two weeks prior to weaning and examined results over 7 days post-weaning. The mechanism by which



naltrexone affects feed intake is complicated. It has been shown that endogenous opioids are an important part of appetite regulation and that administration of an opioid antagonist, such as naltrexone, decreases appetite (Nogueiras et al., 2012).

#### Body Weight (BW):

After an initial lag in growth from d 0 to d 1 caused by weaning, all treatment groups recovered so that weights were greater on d 4 and still increasing on d 7. This pattern of growth after weaning has been observed by many researchers (Bark et al., 1986; McCracken et al., 1995; Cooper et al., 2009; Cooper et al., 2011). Four days after weaning, animals treated with 5 mg/d naltrexone pre-weaning weighed more than those treated with 10 mg/d naltrexone before weaning, and 7 days post-weaning animals treated with 5 mg/d naltrexone before weaning weighed more than the control animals. To date, no previous research has been published evaluating the long term effect of low-dose naltrexone on weight gain in any species.

Interestingly, animals treated with 5 mg/d naltrexone also had decreased FI. An increase in feed efficiency is possible; the animals could be partitioning energy toward growth rather than behaviors such as fighting. Wright and Rodgers (2013) observed an increase in resting behavior in rats fifteen minutes after being treated with 3.0 mg/kg naltrexone as compared to the control group. It would be beneficial to observe post-weaning piglet behavior in future studies to establish if low-dose naltrexone decreases energy-wasting fighting behavior. Animals treated with 10 mg/d naltrexone experienced effects similar to animals treated with high doses of naltrexone. Typical daily dosages of naltrexone used for opioid addiction range from 50 mg/d to 100 mg/d (Younger et al., 2014) and have been shown to decrease appetite and weight gain (Billes et al., 2014). The dosage of 10mg/d used in this study is lower than average naltrexone dosages, but appears to be too high of a dose to elicit the desired effects in weaned pigs.

### Total Gain:

All the animals in this study receiving naltrexone treatments gained more weight over seven days post-weaning than animals in the control group. As mentioned above, no previous research has examined the long term effects of low-dose naltrexone on weight gain. The animals administered 10 mg/d naltrexone did not differ in BW from the control at any point in the study; however they ended the study weighing slightly more on average than the control animals. This difference caused a significant increase in gain in the animals treated with 10mg/d naltrexone. Also, those animals experienced an extended growth lag until d 4, suggesting that 10mg/d is not ideal for the purposes of increasing post-wean performance in pigs.

### Plasma Cortisol:

Plasma cortisol concentrations (PCC) increased 24 hr after weaning as expected and previously reported by many researchers (Le Dividich and Seve, 2000; Kojima et al., 2008; Cooper et al., 2009). The effects of weaning on PCC were abrogated by treatment with 1mg/d naltrexone and 5mg/d naltrexone 1 d after weaning. Furthermore, PCC in animals treated with 10 mg/d naltrexone were higher than PCC in control animals 1d after weaning. This result is consistent with the concept that 10 mg/d naltrexone is too high a dose, resulting in increased cortisol levels similar to research using high doses (50-100mg daily) of naltrexone (Kosten et al., 1986; Ray et al., 2009). Previous research has shown that opioid drugs have the ability to affect adrenal gland function, therefore decreasing PCC (Pirnik et al, 2001; Daniell, 2006). However, no reports have been published describing the effects of low-dose naltrexone on PCC.

### Corticosteroid-Binding Globulin (CBG):

Plasma corticosteroid-binding globulin concentrations decreased 1 d after weaning in all treatment groups. This decrease is consistent with other reported observations of CBG

concentrations at weaning (Kojima et al., 2008; Cooper et al., 2009). Concentrations remained lower than pre-weaning levels seven days post-weaning. Nock and colleagues (1997) found that administration of the opioid morphine could up-regulate CBG levels in rats. The morphine treatment was administered by a subcutaneous pellet up to 14 days. In the present study, administration of low-dose naltrexone did not elicit similar effects; no previous research has examined the effects of low-dose naltrexone on CBG concentrations.

#### Free Cortisol Index (FCI):

The free cortisol index increased 1 d post-weaning in all treatment groups. After this initial increase, the FCI steadily decreased from d 1 to d 7 of this study. This increase of FCI at weaning has been observed previously (Kojima et al., 2008; Cooper et al., 2009). Only animals receiving 1 mg/d naltrexone returned to pre-weaning levels of FCI by d 7. It is important to mention that the FCI is a ratio of plasma cortisol concentrations and corticosteroid-binding globulin concentrations. Because of this, the FCI is not very sensitive to changes in PCC. To date, no research has been published showing the effects of opioids or low-dose naltrexone on the FCI.

#### White Blood Cells:

Weaning stress causes increases in total WBC count, percentage of neutrophils and monocytes, and an increase in the neutrophil to lymphocyte ratio (Kojima et al., 2008, 2009; Jenkins et al., 2009; Cooper et al., 2009; Bomba et al., 2014). In the present study, no differences were seen across treatment groups over 7 d post-weaning in any counted WBC population. Total WBC count did not change from d 0 to d 1, but increased from d 1 to d 7. The percentage of neutrophils and monocytes increased from d 0 to d 1, similar to results published by Cooper and others (2009) and commonly seen at the time of weaning. The percentage of lymphocytes

initially decreased on d 1, but increased from d 1 to d 4, a trend also seen by Cooper and others (2009).

Opioids, such as morphine, are considered immunosuppressive, and have also been shown to decrease inflammation by decreasing leukocyte migration and chemotaxis, leukocyte proliferation, and inflammatory cytokine release including IL-1 and IL-6 (Ninkovic and Roy, 2013). Low-dose naltrexone has been used in research for treatment of auto-immune diseases such as fibromyalgia and Crohn's disease. It has been theorized to increase B-endorphin, mu opioid receptor ( $\mu$ , MOR), kappa opioid receptor ( $\kappa$ , KOR), and delta opioid receptor ( $\delta$ , DOR) by temporarily blocking opioid receptors, resulting in down regulation of TNF $\alpha$ , IL-1 and IL-6 (Ninkovic and Roy, 2013). This causes a shift from T-helper 1 cells (TH1) to T-helper 2 cells (TH2). Since T-helper 2 cells produce anti-inflammatory cytokines such as IL-4 and IL-10, low-dose naltrexone has been termed anti-inflammatory (Brown and Panksepp, 2009; Younger et al., 2014).

#### Neutrophil to Lymphocyte Ratio (NLR):

Stress can cause a systemic inflammatory response (Sutherland et al., 2006). During inflammation, numbers of circulating neutrophils increase causing the NLR to increase. The NLR has been considered an indicator of stress in many species including cattle, rhesus macaques, and swine (Sutherland et al., 2013; Lee et al., 2013; Khafipour et al., 2014; O'Driscoll et al., 2015). In the present study, no differences between treatment groups were present, and the NLR increased from d 0 to d 1 in all groups. This increase in the NLR was also observed by Cooper et al (2009). In the present study, the NLR on d 1 was less in animals given 1 mg/d naltrexone than in the control animals. A decrease in the NLR was observed in all treatment groups from d 1 to d 4, and the ratio remained the same through d 7. Cooper et al. (2009) also

observed this decrease in the NLR 7 days post weaning. The effects of low-dose naltrexone and opioids on the NLR are largely unknown, but appear to be driven through increases in % neutrophils.

## **Conclusion**

This study has demonstrated that pre-weaning treatment with low-dose naltrexone can result in increased weight gain and modulation of the commonly-observed immune and stress responses to weaning. The possibility that a low-dose pre-weaning naltrexone regimen could cause increased post-weaning feed efficiency through modulating behaviors should be examined further. The use of low-dose naltrexone in commercial swine production may represent a means to manifest the positive changes associated with opioid treatments without the negative aspects of narcotic regulation and potential for human abuse.

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## APPENDIX



## Figures

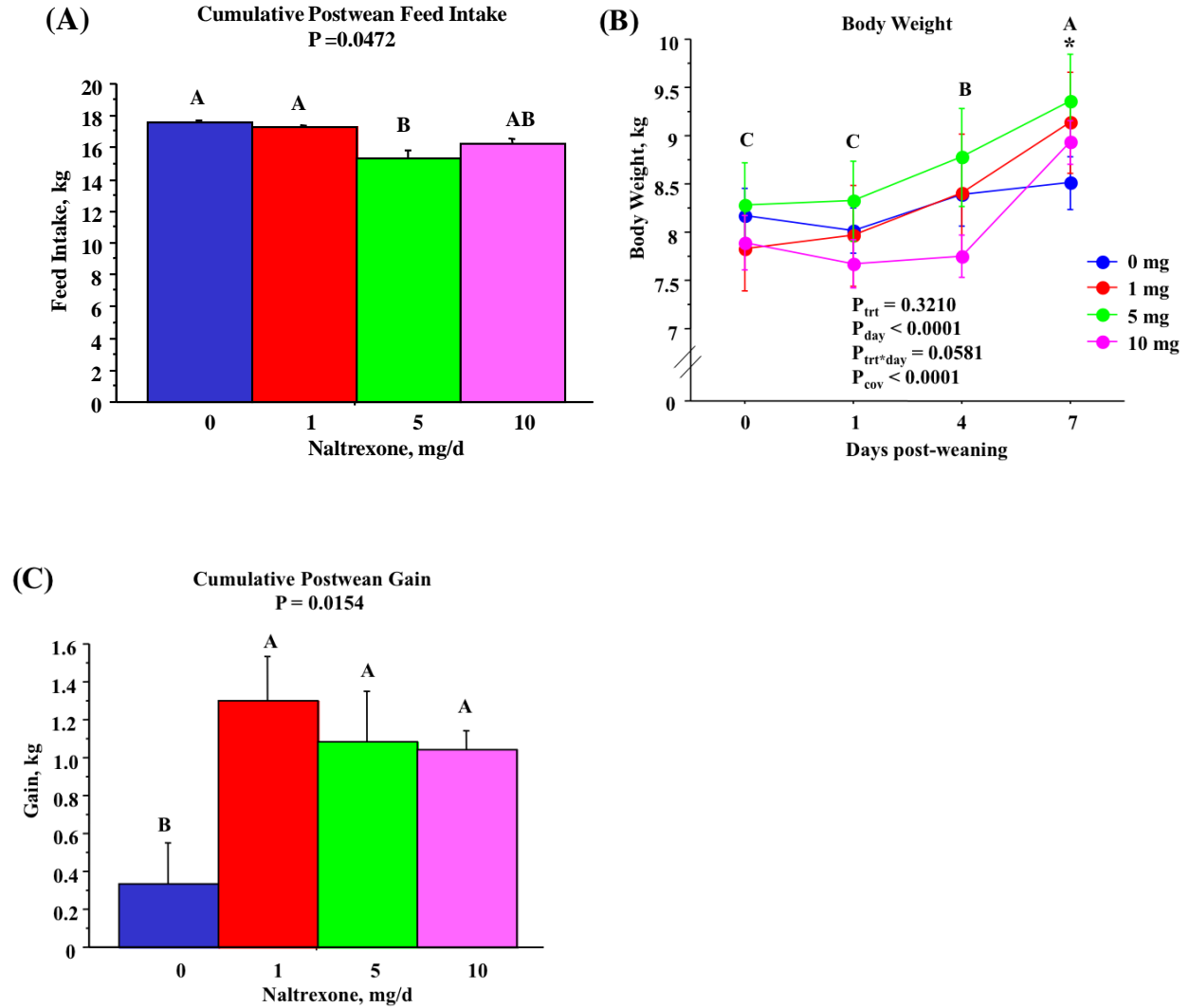


Figure 1. Cumulative feed intake (A), body weights (B) and cumulative gain (C) over the first 7 days post-weaning. Raw means and standard errors are shown. Means with identical letters do not differ. For (B), letters indicate sameness or difference of means relating to day post-weaning; an asterisk indicates a time where there occurred a difference between the mean for control (0 mg/d naltrexone) pigs and pigs from at least one other treatment group.

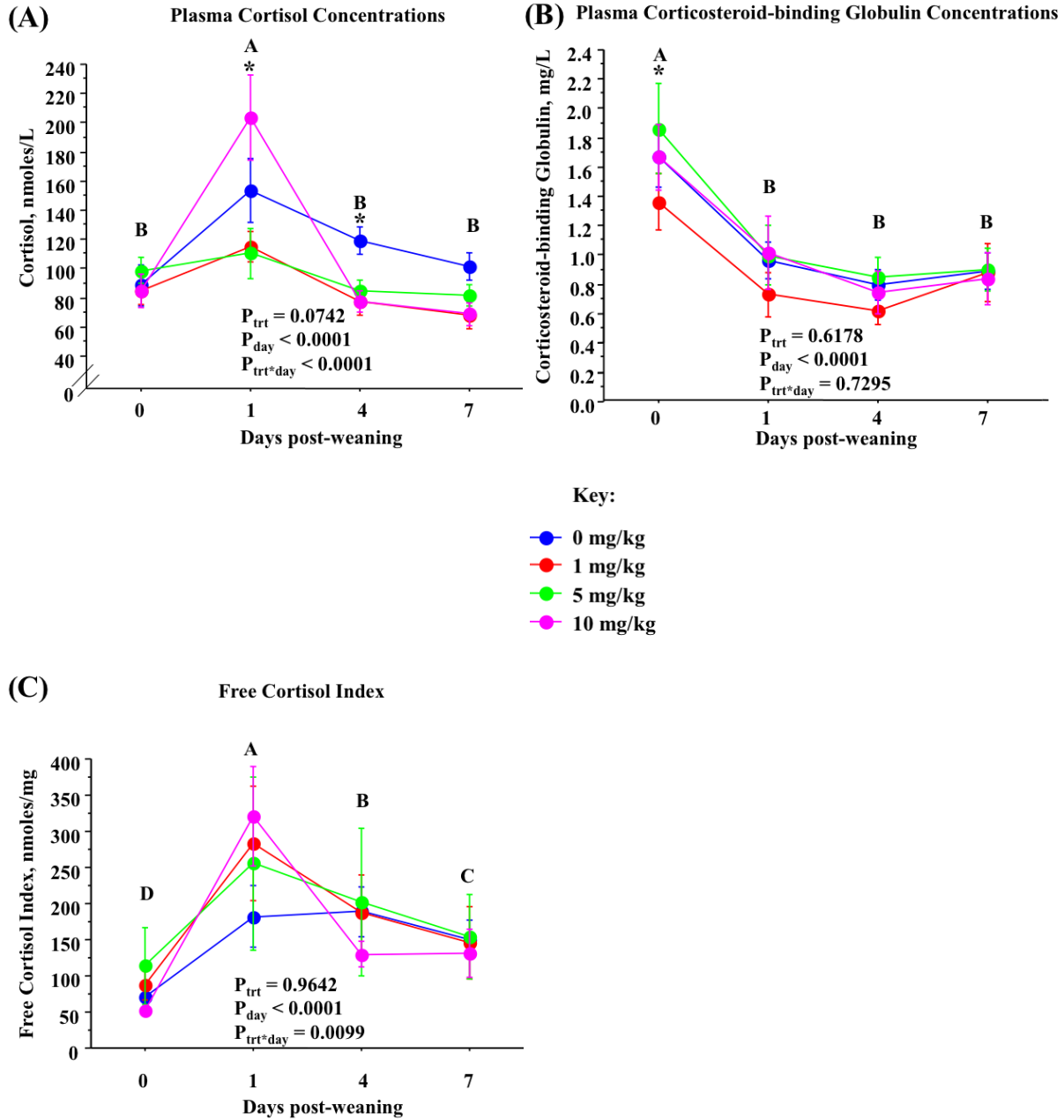


Figure 2. Plasma concentrations of cortisol (A) and corticosteroid-binding globulin (B). Calculated free cortisol index (C). Raw means and standard errors are shown. Means with identical letters do not differ; an asterisk indicates a time where there occurred a difference between the mean for control (0 mg/d naltrexone) pigs and pigs from at least one other treatment group.

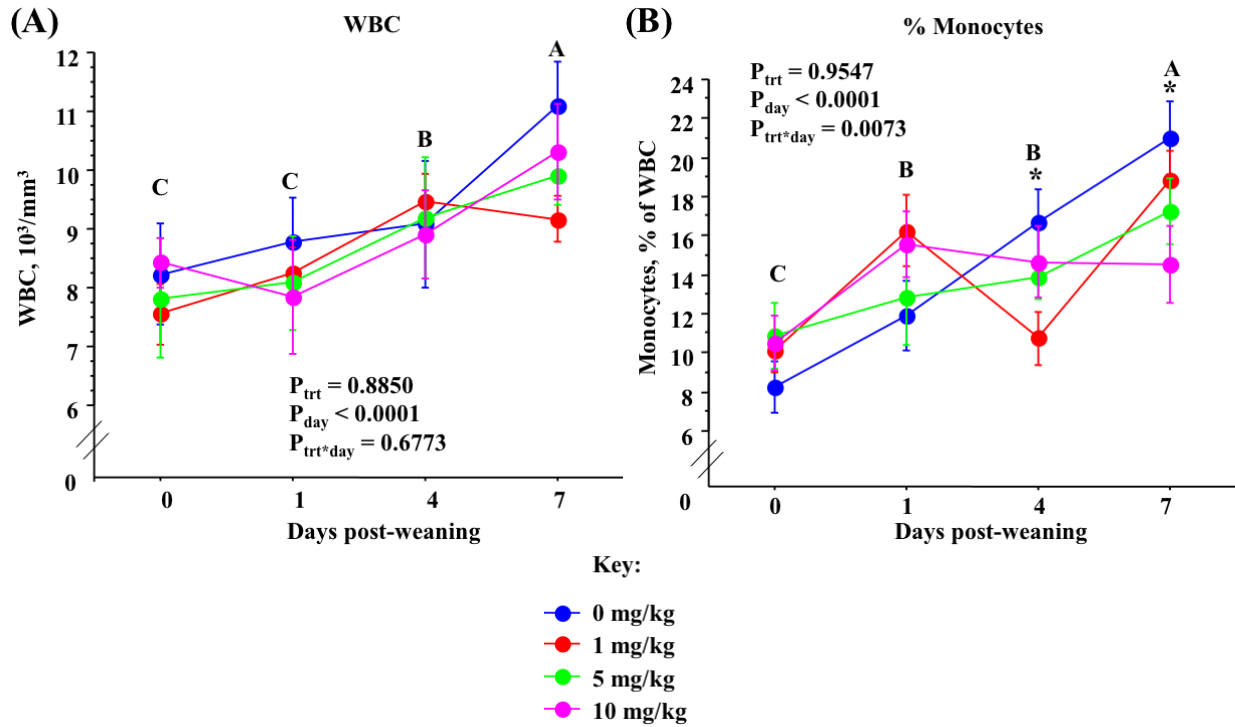


Figure 3. Whole blood white blood cell (WBC) concentration (A) and percentage of WBC that were identified as monocytes (B). Raw means and standard errors are shown. Means with identical letters do not differ; an asterisk indicates a time where there occurred a difference between the mean for control (0 mg/d naltrexone) pigs and pigs from at least one other treatment group.

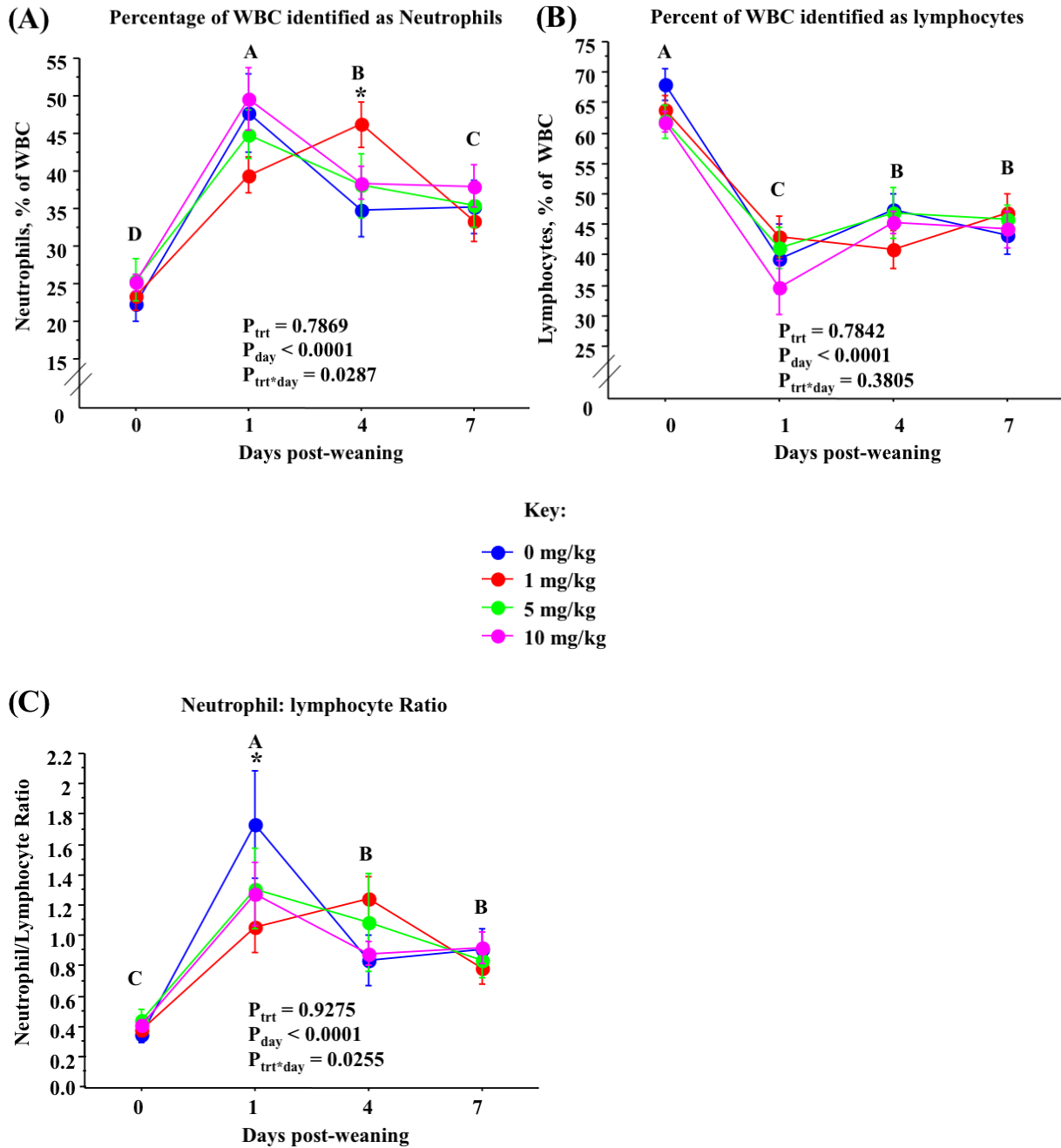


Figure 4. Percentage of WBC that were identified as neutrophils (A) and lymphocytes (B), and the calculated neutrophil:lymphocyte ratio (C). Raw means and standard errors are shown. Means with identical letters do not differ; an asterisk indicates a time where there occurred a difference between the mean for control (0 mg/d naltrexone) pigs and pigs from at least one other treatment group.

## VITA

Ashley Christine Carter was born in Phoenix, Arizona on September 25<sup>th</sup>, 1991 to John and Alma Carter. Her initial years of life found her traveling around the United States before landing in Kingston, Tennessee. Ashley graduated from Roane County High School in 2009. She obtained her Bachelor of Science at the University of Tennessee, Knoxville in Animal Science with a focus on pre-veterinary medicine in the spring of 2013. She obtained two minors during her studies: one in Biology and a second in Psychology. Ashley was a member of Sigma Alpha Professional Sorority until she graduated with Summa Cum Laude. In the fall of 2013, she started her Masters in Animal Science with a focus in physiology. She was accepted to the University of Tennessee, College of Veterinary Medicine in the class of 2018.