THE IMPACT OF CASTRATION ON THE IMMEDIATE INFLAMMATORY RESPONSE TO EARLY-WEANING IN MALE PIGS

By

Andrew Roney

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Comparative Medicine and Integrative Biology—Master of Science

2022

ABSTRACT

THE IMPACT OF CASTRATION ON THE IMMEDIATE INFLAMMATORY RESPONSE TO EARLY-WEANING IN MALE PIGS

By

Andrew Roney

In this study, differences between castrated and intact male piglets in the immediate immune response to early-weaning stress were determined. Testosterone and 17β-estradiol were both higher in intact males than in castrates, while cortisol was not different between gonadal groups. CBC results showed a tendency for castrates to have a greater increase in neutrophil:lymphocyte ratio over 24 hours, indicating greater inflammation. Thymuses tended to be longer in intact males than in castrates. Basal testosterone levels in intact males were strongly associated with increased total mast cell numbers in the small intestinal tissues. Basal testosterone was associated with increased 0-hour neutrophil numbers in intact males and 24-hour 17β-estradiol was associated with decreased 24-hour neutrophil numbers in the villus crypts of the small intestine tissues. This study has revealed gonadal effects on the immediate immune response to early-weaning stress, but the hormonal relationships revealed need to be confirmed by hormone blockade and/or replacement studies

ACKNOWLEDGEMENTS

The author would like to thank Dr. Adam Moeser, Dr. Nathalie Trottier, Dr. Cynthia Jordan, Dr. Narayanan Parameswaran, and Dr. Dale Rozeboom for serving on his guidance committee.The author also wishes to thank all the members of the Gastrointestinal Stress Biology Laboratory for all their help during his graduate career.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Losses due to disease morbidity and mortality continue to be a major cause of economic losses and reduced pig welfare for the swine industry. An increased understanding of factors associated with disease risk would allow for improved management practices that could reduce disease and mortality, thus increasing production. A well-established, but relatively unexplored risk factor for mortality in pigs is biological sex. Compared with female and intact male pigs castrated males experience higher rates of mortality (Jacela et al. 2011) and morbidity (Morales et al. 2002). However, despite the potential economic significance associated with higher mortality in male castrates, the underlying biological mechanisms remain poorly understood. This represents a critical gap in knowledge as it will be difficult to address the issue of higher mortality until its causes are known. The objective here is to provide a review of the current literature on sex differences in disease in mortality in pigs with a specific focus on sex differences in the immune system that could contribute to differences in mortality with a focus on immune function.

SEX DIFFERENCES IN PIG MORTALITY

Several research studies have reported sex-biases in mortality in pigs across different age groups. In a herd with a history of porcine circovirus, barrows were reported to exhibit a higher mortality rate compared with gilts (7.7% vs 4.5%) during the finishing period (Jacela et al. 2011). In another study examining mortality from hemorrhagic bowel syndrome, the authors reported that barrows had a significantly greater mortality rate than gilts during the summer (7.97% vs 6.21%, p=0.0009) winter (Straw et al. 2001). Mortality rate during the and spring

were numerically higher for barrows than gilts (4.87% vs 4.36% and 4.00% vs. 2.99%, respectively), but differences were not statistically significant(p=0.29 and p=0.21, respectively) (Straw et al. 2001). A study by (Nevrkla et al. 2017) showed that that castrated male piglets exhibited a higher mortality rate than female piglets before 14 days of age and across 2 genotypes (9.56% vs 6.82% for Genotype I and 4.49% vs 3.01% for Genotype II). Barrows were observed to have a higher mortality rate than gilts (46 barrows vs 18 gilts) (Baxter et al. 2009), but no such difference was observed between boars and gilts (9 boars vs 4 gilts) (Baxter et al. 2008). In two studies using unweaned piglets less than 28 days of age and raised under similar conditions, (Morales et al. 2017) directly compared pre-weaning mortality between castrated and intact male piglets and found that castrated males exhibited higher mortality than intact males which was observed in light-weight and medium-weight piglets (12.2% vs 6.3%, p=0.017 and 5.5% vs 2.7%, p=0.041, respectively), but not in heavy-weight piglets (1.5% vs 2.4%, p=0.327). Another study found that nursery phase barrows were twice as likely to die as gilts (Larriestra et al. 2006). (Baxter et al. 2012) found that castrated male piglets are more likely than females to die from disease preweaning and, using stillbirths to measure prenatal mortality, found a sex bias in mortality only in the postnatal period, indicating that postnatal factors are responsible for this disparity between the sexes. The effects of sex on various measures of performance are summarized in Table 1.

Table 1. Summary of sex differences in indicators of survival and growth.

Study	Jacela et al., 2011	Straw et al., 2001	Nevrkla et al., 2017
Pig Age	Vaccination during nursery phase, data collection during finishing phase	25-28 weeks in Farm 1. Finishing pigs in Farm 2.	Pigs weaned at 28 +/- 3 days
Mortality Rate: Female	Exp 1: 4.3% Exp 2: 6.5%	Farm 1: 6.21% in the summer	Genotype I: 6.82% by day 14 and 9.22% by weaning Genotype II: 3.01% and by day 14 and 3.76% by weaning
Mortality Rate: Castrated Male	Exp 1: 7.4% Exp 2: 12.0%	Farm 1: 7.97% in the summer Farm 2: 1.7 times more barrows than gilts died from all causes other than intestinal volvulus	Genotype I: 9.56% by day 14 and 10.92% by weaning Genotype II: 4.49% by day 14 and 7.37% by weaning
Mortality Rate: Intact Male	N/A	N/A	N/A
Growth Performance	Growth:Feed ratio of 0.383 and 3.87 in control barrows and 0.398 and 0.403 in control gilts Average Daily Gain of 944 g in vaccinated barrows and 894 g in vaccinated barrows, but 881 g and 895 g in control and vaccinated gilts, respectively (Experiment #2) Carcass weight of 92 kg and 94.3 kg for control and vaccinated barrows respectively, but 89.8 kg and 90.5 for control and vaccinated gilts respectively (Experiment #2)	Not measured	Not measured
Genetics	Breed not stated	Pigs of PIC genetics used on both farms	Danish Duroc Sire "Genotype I" sows hybrids of two synthetic line "Genotype II" sows were two breed sows
Weaning Age	21 days of age	18 days for Farm #1	28+/-3 days

Table 1. (cont'd)

Study	Baxter et al. 2009	Baxter et al., 2008	Larriestra et al., 2006	Baxter et al. 2012
Pig Age	Piglets weaned at 28 days	Piglets weaned at 28 days	17 days to 10 weeks	Pigs weaned no younger than 26 days
Mortality Rate: Female	18 died postnatally and pre-weaning (Causes of death include crushing (42), starvation (10), and "other"(21). Deaths were not broken down by sex X cause, only by sex or cause)	4 died postnatally and pre-weaning	95% survival rate	33
Mortality Rate: Castrated Male	46 died postnatally and pre-weaning	N/A	93% survival rate	57
Mortality Rate: Intact Male	N/A	9 died postnatally and pre-weaning	N/A	N/A
Growth Performance	Not measured	Not measured	Gilts more likely to be lightweight at 10 weeks of age	Not measured
Genetics	Large White X Landrace X Duroc sows and Landrace sires	Landrace X Large White sows	Not stated	Landrace X Large White X Duroc sows
Weaning Age	28 days	28 days	17 days or younger for some animals and older than 17 days for the others	26 days

SEX-BASED DIFFERENCES IN PREDICTORS OF PRE-WEANING SURVIVAL IN PIGLETS

One study found that light-weight pigs were more likely to die compared with heavy weight pigs and that gilts were both more likely to be smaller and yet more likely to survive than barrows, but the authors did not find a statistically significant interaction between the two factors (Larriestra et al. 2006). The idea that males are heavier at birth, heavier pigs are more

likely to survive, and yet male piglets are more likely to die is backed up by others (Baxter et al. 2012). While this may indicate that differences in body weight may not be contributing to sex differences in piglet mortality, further studies that directly compare males and females of similar weights would be required to test this. Another indicator of likelihood to survive to weaning that is interesting is rectal temperature, as surviving pigs have higher rectal temperatures at one hour after birth and females both are more likely to survive, and surviving females tended ($p=0.082$) to have higher rectal temperatures at birth, 1 hour after birth, and 24 hours after birth than surviving males (Baxter et al. 2012). Lower rectal temperature at 24 hours after birth has been associated with piglet mortality in other studies as well (Galiot et al. 2018, Panzardi et al. 2013).

MECHANISMS OF SEX-BASED DIFFERENCES IN IMMUNITY

As indicated above, sex differences in mortality exist in swine, but there are very few studies in swine investigating the mechanisms underlying these sex differences. Here we will review basic biology of sex differences and immunity based from human and rodent research model systems.

SEX CHROMOSOMES AND IMMUNE FUNCTION

Sex-based differences in immunity are driven by two major factors: the sex chromosomes and sex hormones. Sex chromosomes, especially the X chromosome, play a vital role in the sexually dimorphic immune response. Out of all chromosomes, the X chromosome contains the most genes related to immunity (Bianchi et al. 2012). In females, random inactivation of X chromosomes results in mosaicism, where different cells express alleles from

different X chromosomes (Spolarics et al. 2017). Mosaicism can be a double-edged sword in the context of immunity. The combined effects of mosaicism and X chromosome reactivation render females more likely to develop autoimmune disease (Forsdyke 2009, Long et al. 2016, Wang et al. 2016), but also dampen the effects of the disease as not every cell will express the "disease" allele (Spolarics et al. 2017). The lack of mosaicism in males creates an all-or-nothing scenario where they are less likely to develop an autoimmune disorder (Spolarics et al. 2017), but having more severe disease conditions if they do develop an autoimmune disorder as every cell expresses the harmful allele (Margery-Muir et al. 2017, Yacoub Wasef 2004). Mosaicism also has consequences in the context of compromised immunity; for example, females that are mosaic for X chromosomes either containing a wild type IKKY gene or lacking it are immune compromised the lack of it is lethal in males (Smahi et al. 2000, Nelson 2006). Another case where mosaicism is beneficial is when it diminishes the harmful effects of a gene without diminishing its positive effects, as was the case for mice who were mosaic for wild-type X chromosomes and X chromosomes with the CYBB gene knocked-out and were able to retain effective bactericidal activity while sustaining reduced oxidative-stress mediated organ damage (Chandra et al. 2011).

Individuals either possessing extra sex chromosomes or missing one have different immune profiles than XX or XY individuals. For example, males possessing an extra X chromosome, as in Klinefelter Syndrome (Klein and Flanagan 2016), and females whose second X chromosome is either missing entirely or was subjected to extensive deletions, as in Turner Syndrome, are at increased risk of developing certain autoimmune disorders (Bianchi et al. 2012). While the Turner Syndrome example strongly indicates a more direct effect of the X

chromosome, the Klinefelter Syndrome example may be an example of a more indirect effect as these individuals tend to have reduced levels of androgens (Seminog et al. 2015), whose role in immunity will be discussed shortly.

The *sry* gene is located on the Y chromosome and it is responsible for the differentiation of the fetus into a male (Libert, Dejager and Pinheiro 2010). To study chromosome-specific contributions, a genetic-editing model has been established where the sry gene is placed on an autosome in one parent and is absent in the other producing offspring who can be XX females, XY males, XX individuals with testes, or XY individuals with ovaries (Arnold and Burgoyne 2004). In EAE and lupus mouse models, castrated XX *sry* males had more severe disease condition and greater mortality from lupus than castrated XY males (Smith-Bouvier et al. 2008). Higher mortality from lupus was also seen in intact XX females than in intact XY—females, while no significant mortality was seen in intact XX*sry* or XY males (Smith-Bouvier et al. 2008). In mice subjected to a myelin basic protein immunization, ovariectomized XY—females had a stronger immune response, as measured in inflammatory cytokine levels, than ovariectomized XX females did, with a similar result being seen in XY and XX*sry* males castrated 8 weeks before the immunization (Palaszynski et al. 2005). Taken together, these findings indicate that, when gonadal and hormonal differences are accounted for, having XX chromosomes may contribute to stronger Th2 immunity while having XY chromosomes may contribute to stronger Th1 immunity.

SEX HORMONES AND IMMUNE FUNCTION

Androgens tend to have immunosuppressive effects. Testosterone mediates its action by binding to androgen receptors. Testosterone can be converted to estrogen via aromatization and act on estrogen receptors (Bianchi 2019). Cytokine-induced inflammation is greater in men who have low testosterone levels and there is a negative relationship between testosterone levels and inflammatory markers (Bianchi 2019). Testosterone downregulates the production of pro-inflammatory cytokines and upregulates the production of anti-inflammatory cytokines (Mohamad et al. 2019). Castration in mice has been found to increase susceptibility to and increase the severity of experimental autoimmune encephalomyelitis (Palaszynski et al. 2004). Androgens also downregulate T cell proliferation and B cell development (Traish et al. 2018, Altuwaijri et al. 2009). The effect on T cells is likely accomplished via androgen receptors in the thymic stroma (Lai et al. 2012) and castration can increase B cell numbers (Lai et al. 2012). Androgens play an important role in neutrophil production as PCOS women have heightened neutrophil counts and androgen receptor knock-out mice display a 90% reduction in neutrophils (Ibáñez et al. 2005, Chuang et al. 2009). Castration in adults induces thymic enlargement, leading to increased naïve T cell production (Heng et al. 2005, Roden et al. 2004, Sutherland et al. 2005). A study conducted with both general and myeloid-specific androgen receptor knock-out (ARKO) mice indicated that ARKO mice healed wounds faster and that androgen receptors inhibit wound healing by upregulating the recruitment of monocytes to the wound area and increasing TNF-α expression in macrophages (Lai et al. 2009). Absolute and relative spleen weight and splenocyte proliferation were increased by castration in rats and NO

production was increased by testosterone deficiency by castration *in vivo* and culturing of splenocytes in the absence of testosterone *in vitro* (Chen et al. 2016).

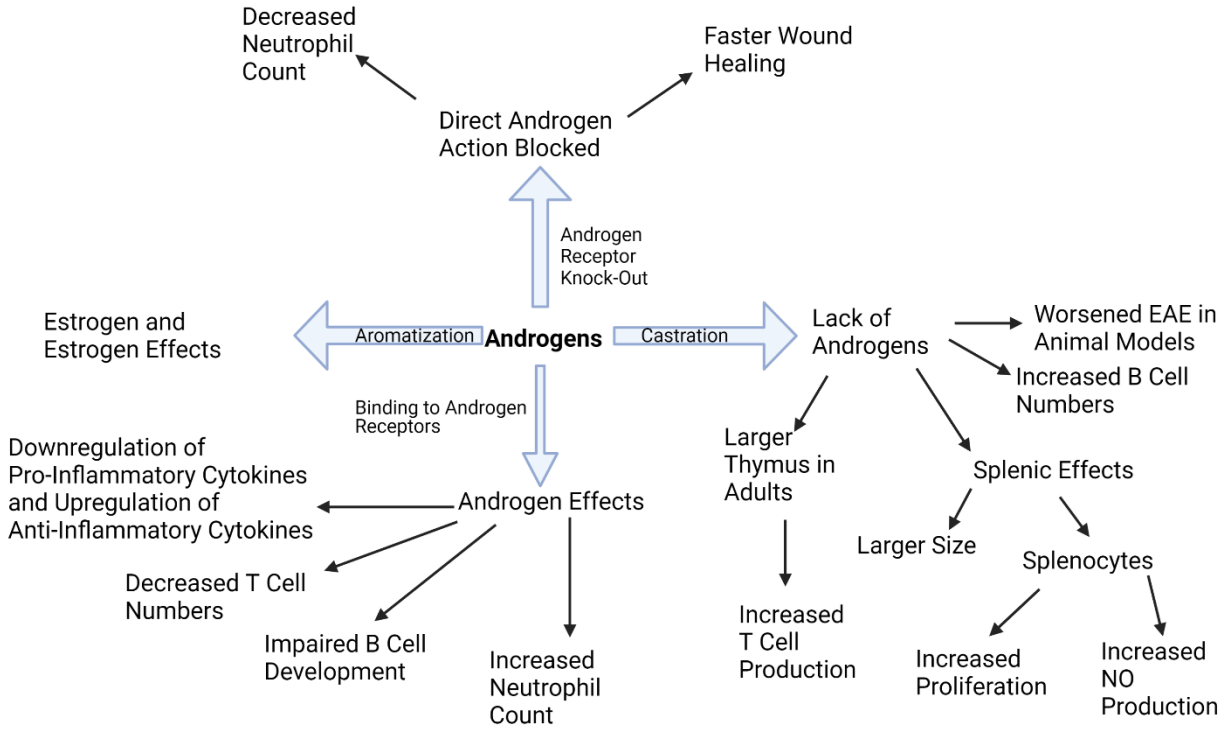

Figure 1. A summary of effects of androgens, androgen receptor knockout, and castration on the immune system. This figure was created using BioRender.com

Estrogens are immunomodulating substances like androgens are but, unlike androgens, their effects are not mostly suppressive. Estrogen treatment *in vitro* increased autoantibody production by murine B cells and oral contraceptive use has been linked to worsened symptoms in systemic lupus erythematosus (SLE) patients or even the onset of SLE symptoms in in previously healthy women(Ansar Ahmed, Penhale and Talal 1985, Ansar Ahmed et al. 1989). Conversely, high levels of estrogens, such as those seen in pregnant humans and animals, have been associated with reduced symptoms of autoimmune diseases such as multiple sclerosis or

rheumatoid arthritis and reduced rates of skin graft rejection in both human patients and animal models (Grossman 1984, Voskuhl 2011, Voskuhl et al. 2016, Calabrese 2001). Estrogen treatment increases autoantibody production by B cells in mice (Verthelyi and Ahmed 1994). Estrogen treatment was also found to accelerate disease progression and increase mortality in a rodent model of lupus (Roubinian et al. 1978). Estrogens also inhibit apoptosis in auto-reactive B cells (Grimaldi et al. 2002) and promote the development of autoimmune disorders associated with a Th2 cytokine profile (González et al. 2010, Wilder 1996). In mice experimentally infected with encephalomyocarditis virus, castrated males exhibited increased survival rates compared with intact males, but this effect was reversed by estradiol treatment (Friedman, Grota and Glasgow 1972). However, there is currently evidence to suggest that estradiol may play a protective role in COVID-19 infections and estradiol administration is being studied as a potential treatment (Mauvais-Jarvis, Klein and Levin 2020).

Estrogens act via estrogen receptors α and β, with α being implicated in inflammation and autoimmune diseases than β. For example, in inflammatory bowel diseases such as ulcerative colitis or Crohn's Disease, estrogen receptor α expression is generally increased while estrogen receptor β expression is either unchanged or decreased (Jacenik et al. 2019). In a mouse model of allergic dermatitis, an agonist for estrogen receptor α, but not β, exacerbated the inflammatory response (Watanabe et al. 2018). Estrogen receptor α, but not β, agonism suppressed the Th1 response (Watanabe et al. 2018). Estrogen receptor α is important for T cell activity as it is required for Th1 and Th17 cell accumulation in lymphopenic environments (Mohammad et al. 2018). T cells lacking estrogen receptor α also have impaired activation in response to TCR-stimulation, decreased activation-induced apoptosis, and proliferation

(Mohammad et al. 2018). In a mouse model for lupus, knock-out of estrogen receptor α ameliorated the disease and improved survival (Scott et al. 2017). However, this protection is lost if the mouse is ovariectomized and estradiol treatment worsens the disease state in the ovariectomized knock-out mice, but not the ovariectomized wild-type mice (Scott et al. 2017). Deficiency of estrogen receptor α impairs T cell stimulation by dendritic cells and its knockout in mice impaired cytokine production and the response to CD40L (Douin-Echinard et al. 2008). Estrogen acts via estrogen receptor α to upregulate dendritic cell activation by TLR-ligands (Siracusa et al. 2008). Estrogen receptor B is also implicated in some autoimmune disorders, as its expression increases relative to estrogen receptor A in lupus patients (Rider et al. 2006) and is found in the synovial tissue of rheumatoid arthritis patients (Ishizuka et al. 2004).

Altogether, these factors act to provide most females a more robust immune system than most males.

PERINATAL ANDROGEN SURGE

The hormonal factors involved in sexual dimorphism include the organizational pre- and postnatal androgen surges as well as the pubertal surge and gonadal hormone activation in adults. The perinatal androgen surge is a phenomenon that occurs in males, but not females, before and shortly after birth. The perinatal androgen surge can be divided into the prenatal surge and the postnatal surge. The postnatal surge starts soon after birth and its length can vary by species.

The prenatal androgen surge in swine drops off at day 60 of gestation and begins around or before day 40 of gestation (Colenbrander, de Jong and Wensing 1978). The postnatal

androgen surge begins quickly in swine, with testosterone beginning to rise at 1 week after birth, peaks at around 2-3 weeks, and although declines to birth levels by about 8 weeks of age (Colenbrander et al. 1978, Schwarzenberger et al. 1993). However, other androgens, such as dehydroepiandrosterone sulphate and 19-nortestosterone, remain elevated until 2 months of age (Schwarzenberger et al. 1993). Figure 2 shows the postnatal pattern of serum testosterone levels in swine. 19-nortestosterone is an androgen that is only found in intact males during early life and peaks at 3 weeks of age in pigs (Choi et al. 2007). It is interesting to note that 19-nortestosterone levels are higher in piglets than in post-pubertal boars (Choi et al. 2007), which is in contrast to testosterone, whose maximum postnatal level is lower than its post-pubertal maximum level (Ford 1983b). As 19-nortestosterone is an intermediary in the aromatization of testosterone to estradiol (Bricout and Wright 2004), this could be a result of increased aromatase activity during this period. The targets of the androgenic activity of 19-nortestosterone may be different from that of testosterone as 19-nortestosterone is more potent in tissues where 5-A reductase activity is low and less potent where the reductase activity is high, while the opposite is true of testosterone (Bergink et al. 1985). Androgen receptor mRNA is elevated in the testes at 2 weeks and is elevated in the muscle and adipose tissue at 3 weeks (Choi et al. 2007).

As mentioned above, testosterone can be converted to estrogen via the actions of aromatase. Testicular aromatase and estrogen receptor expression are greatly elevated in boars at 2 weeks of age (Choi et al. 2007). Inhibition of aromatase at this time delays testicular maturation in boars (At-Taras et al. 2006), which could indicate a role for estrogens in testicular development. Estrone and estrone sulfate also increase during the first 2 weeks of life and

decline by 2 months of age (Ford 1983b). Estradiol is elevated at 12 days of age, but declines by 21 days, and its postnatal maximum is lower than its post-pubertal maximum (Ford 1983b). There is likely role of neonatal estrogens in the development of sexual behavior as male pigs castrated at birth or two months and then post-pubertally treated with estrogen display female-typical sexual behavior, but this does not occur if castration occurs at 4 months of age or later (Ford 1982, Ford 1983a).

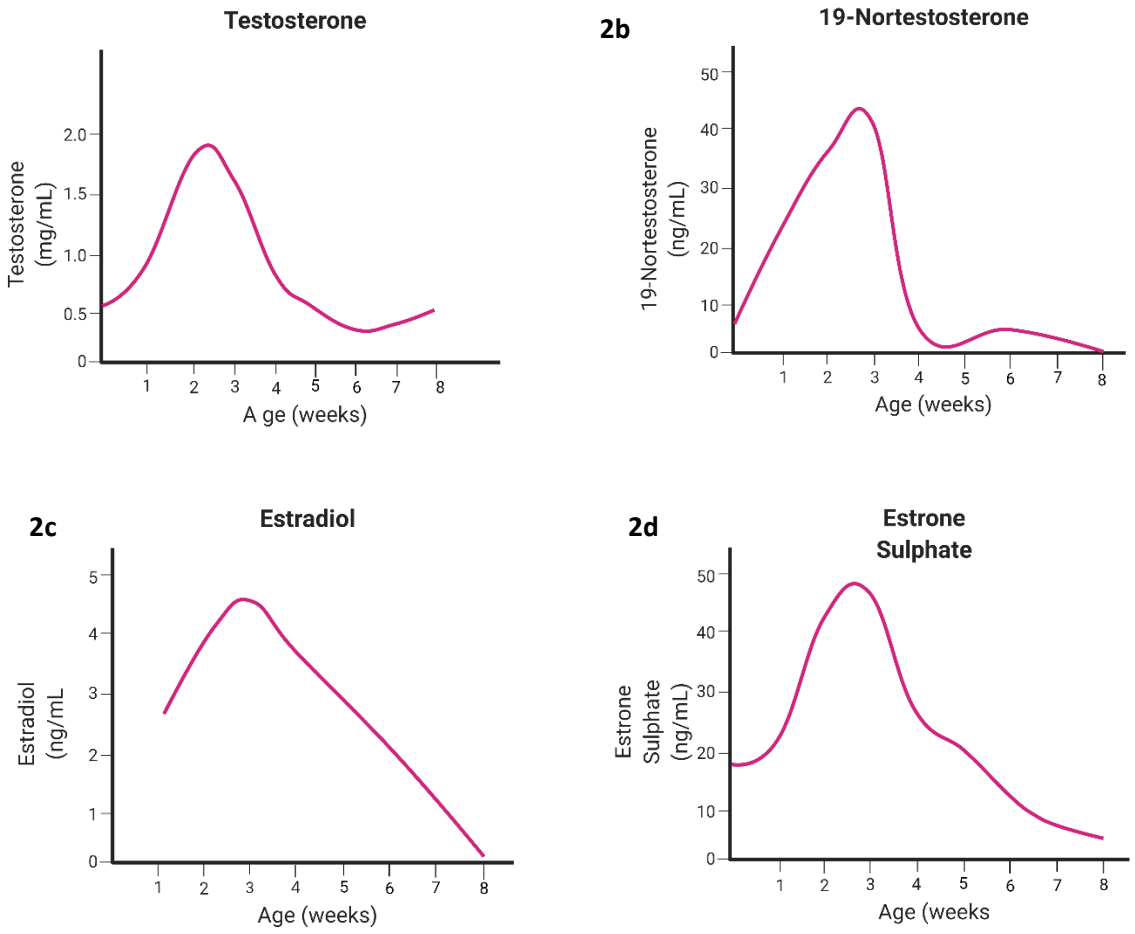

Figure 2. Androgen and estrogen levels in young male pigs during the postnatal androgen surge. (a) Testosterone levels in male pigs during the first eight weeks after birth. This graph was constructed based on data from Colenbrander et al., 1978, Ford, 1983, and Schwarzenberger et al., 1993. (b) 19-nortestosterone levels in male pigs during the first eight weeks after birth. This graph was constructed based on data from Schwarzenberger et al., 1993, and Choi et al., 2007. (c) Estradiol levels in male pigs during the first eight weeks after birth. This graph was constructed based on data from Ford, 1983, and Mariscal et al., 1996. (d) Estrone sulphate levels in male pigs during the first 8 weeks after birth. This graph was constructed based on data from Ford, 1983, Schwarzenberger et al., 1993, and Lanthier et al. 2006. These graphs were made using BioRender.com

SPECIES DIFFERENCES IN THE POSTNATAL ANDROGEN SURGE

The postnatal surge in humans lasts longer than in swine and may have clinical significance. Testosterone begins to rise by 8 days of age (Winter et al. 1976), peaks at 2-3 months of age (Winter et al. 1976), and declines within the first year of life (Winter et al. 1976). The majority of Sudden Infant Death Syndrome, or SIDS, are 2-4 months old (Gordon et al. 1999, Peterson, van Belle and Chinn 1979), which coincides with the peak of the androgen surge in humans, and victims of both sexes who were 1-5 months old had elevated testosterone (Emery et al. 2005). LH and FSH in boys peak at 3 months of age and decline by 9 months of age (Andersson et al. 1998). In boys, FSH levels post-surge are still higher than they were at birth (Winter et al. 1976) and do not decline to prepubertal levels until 4 months of age (Winter et al. 1975). Post-surge FSH and LH levels are higher than birth levels in male rats as well (Dussault et al. 1977). Rats and mice begin their postnatal androgen surges almost immediately after birth and it ends at 4 hours post-birth in rats and 6 hours post-birth in mice (Corbier, Edwards and Roffi 1992).

IMPACT OF CASTRATION ON IMMUNE FUNCTION IN PIGS

Insight into why male castrated pigs exhibit a higher mortality and morbidity compared with gilts may be provided by studies investigating the immunological effects of castration. (Merlot, Thomas and Prunier 2013) found that castrated pigs had heightened catecholamine receptor expression in the liver at 160 days of age, and higher haptoglobin and IgG at 152 days of age, but lower WBC counts at 119 and 152 days of age, lower thymus weight at 160 days of age and increased pneumonia incidence than their intact counterparts. In pigs, castration at 5-6

days of age, which is just before or at the start of the porcine neonatal androgen surge, has been associated with increased thymic lipid content and thymocyte proliferation in response to ConA , as well as decreased thymic weight and YD TCR+ thymocyte percentage, but had no effect on CD1+, CD4+CD8+, CD4+CD8-, or CD4-CD8+ cell frequencies, or basal thymocyte proliferation (Leclercq et al. 2014b) However, another study found no difference in TNF-α, IL-β, CRP, serum amyloid A, or haptoglobin between intact pigs and pigs that were castrated at 5 days of age (Moya et al. 2008). Surgical castration at 5-6 days of age was associated with increased CD3+CD4+CD8+ cells at 4 months, but not 5 months of age (Leclercq, Prunier and Merlot 2014a). This was also associated with decreased lymphocytes and leukocytes at 5 months of age, lower TCRYD+ T lymphocyte percentage at 4 and 5 months, lower basal T cell proliferation at 5 months, lower pneumonia scores, and reduction in T cell and B and NK cell hematopoiesis (Leclercq et al. 2014a). These effects may be dependent on the timing of castration as surgical castration shortly after the end of the neonatal androgen surge in rats or late in life in mice and androgen blockade in adult humans resulted in increased thymic size and thymocyte proliferation (Radojevic et al. 2007, Sutherland et al. 2005) Immunocastration at 3 and 4 months of age was associated with higher CD3+CD4+CD8+ cells than intact males at 5 months of age as well as heavier livers than intact males or males surgically castrated before 26 days of age (Leclercq et al. 2014a). However, immunocastration was associated with decreased lymphocyte and leukocyte numbers compared to intact pigs and a TCRYD+ T cell percentage that were intermediate between intact and surgically castrated males, as well as lower basal T cell proliferation at 5 months of age and a trend for relatively lighter spleens (Leclercq et al. 2014a). Granulocytes were unaffected by surgical castration and CD3+, CD3+CD4+, and

CD3+CD8+ cell percentages, T cell proliferation in response to ConA, and anti-influenza IgG and haptoglobin were unaffected by either surgical castration or immunocastration (Leclercq et al. 2014a). Aluwé, Tuyttens and Millet 2015 found no difference in mortality, from 20 kg to slaughter, between pigs surgically castrated at 4 days and pigs immunocastrated at 15 weeks.

SEX-BASED IMMUNOLOGICAL DIFFERENCES IN SWINE

There are sex-based immunological differences between intact males, castrated males, and females. Females have higher basal serum TNF-α and IL-1β concentrations than castrates (Williams et al. 2009). Although females and castrates have similar temporal patterns of IL-1β, TNF-α, and IL-6 after exposure to 25 μg LPS per kg body weight, females have a higher peak for TNF-α and IL-6 while castrates have a higher peak for IL-1β (Williams et al. 2009). Castrates have higher serum amyloid A and females have higher haptoglobin 24 hours after LPS exposure (Williams et al. 2009). Following *Salmonella* infection in newly weaned pigs, females had a higher intraperitoneal temperature and lymphocyte counts, while castrates had higher total white blood cell and neutrophil counts (Burdick Sanchez et al. 2017). *Salmonella* concentration in the cecum, mesenteric and sub-iliac lymph nodes, liver, spleen, gallbladder, and kidney post-challenge were not affected by sex (Burdick Sanchez et al. 2017). High psychosocial stress induced by mixing at 10 weeks of age and again at 27 weeks and measured by lesion scores, increases mRNA expression for CCRL2,CD1D, IL13RA1, TNFRSF1A, and TNFRSF1B as well as for genes involved in TREM 1, IL-6, TLR, and IL-8 signaling and decreases mRNA expression for CD19, CR2, CXCR7, IL27RA, IL6R, IL7R, and TRAF5 in females, but does not impact the expression of these genes in castrates (Oster et al. 2014). Females exhibited higher IL-2 mRNA

expression and IL-2/IL-4 mRNA expression ratio than intact males as well as higher IL-2/IL-4 ratio in PHA-stimulated PBMC supernatant, but intact males have higher IL-4 concentrations in PHA-stimulated PBMC supernatant (de Groot et al. 2005) In response to resuscitation following ventricular fibrillation, intact males 3-4 months of age had greater increase in TNF-α than females (Niemann et al. 2009). Females subjected to early-weaning stress (weaned at 15 days of age) exhibited more frequent diarrhea than their castrate counterparts when they were 16-20 weeks of age (Pohl et al. 2017). Early-weaned castrates had a higher number of tryptase-positive mast cells than late-weaned castrates at 7 and 20 weeks of age, but no such difference existed between early-weaned and late-weaned females (Pohl et al. 2017). Mast cells from 20-week-old females that were earlier subjected to early-weaning stress released more tryptase upon activation by c48/80 than mast cells from 20-week-old castrates subjected to the same early-weaning stress or from late-weaned control females (Pohl et al. 2017).

CONCLUSION

Overall, the current literature demonstrates a significant impact of sex on morbidity and mortality, and immune measures in swine. Specifically, male castrated pigs exhibit significantly higher mortality compared with females. However, the mechanisms remain poorly understood.

While research on sex differences in immune function has largely focused on adult gonadal hormones in adult animals, it is important to note that the majority of studies in pigs reporting sex differences in mortality and immune responses described above were conducted in neonatal and pre-pubertal piglets. This suggests that sex differences are also a result of factors arising in early in life and not dependent on adult gonadal androgens. The early life factors that

could contribute to this response are genetic or chromosomal influences as discussed above, or the perinatal androgen surge in in males, in which androgens induce sexual dimorphism in the central nervous system and reproductive systems, thus organizing tissues for later life responses to pubertal sex hormones. However, the impact of this surge on the immune system development has yet to be extensively explored and may underly sex differences observed in prepubertal pigs. The previously mentioned differences in the effects of castration on immune parameters based on timing of castration relative to the neonatal androgen surge suggests a potential role of early androgens in immune development in swine, but no studies have directly addressed this to this author's knowledge. Overall, given the significant impact of sex and gonadal status on immune development and disease risk, research into the mechanisms for increased disease risk could reveal potential targets to increase disease resistance and improve pig welfare.

CHAPTER 2

INTRODUCTION

Disease mortality and morbidity represent a significant problem for the swine industry. While morbidity and mortality result from a number stressful challenges in production, a host factor that has been shown to significantly impact disease risk is biological sex. Specifically, castrated male pigs are more susceptible to disease (for example, the higher prevalence of cystercosis in castrated males compared to intact males seen by Morales et al. 2002) and have a higher mortality rate than female pigs or intact male pigs (Larriestra et al. 2006). This sex/gonadal bias for castrates to be more susceptible to morbidity and mortality becomes alarming when one considers that castrated males comprise a significant portion of the commercial swine population. Despite the potential significant economic and welfare implications of sex differences in disease risk in pigs, the biological mechanisms driving the disproportionate mortality rates remain poorly understood.

While many factors could contribute to sex differences in disease risk, sex differences in the immune system has been the most widely studied in humans and rodents. Only a few studies have compared immune system between the sexes in pigs. Comparisons have been made between intact and castrated pig, but these studies have been restricted to comparing blood immune markers under basal, non-stressed conditions, which may not reveal sex differences that may arise in the face of an activated immune response or challenge. Further, the focus on peripheral blood measures may also limit the sensitivity of detecting sex differences, as compared with investigating tissue based immunological investigations.

In the human and rodent literature, mechanisms of sex differences in immunity have focused almost exclusively on the role of adult gonadal sex hormones such as testosterone, estrogen, and progesterone in adult animals. While it is clear that adult sex hormones play a critical role in driving sexually dimorphic immune responses, this does not explain why sex biases in immune-related diseases and immune responses are evident in prepubertal animals and between castrated males and females. In fact, most of the studies reporting sex differences in pigs were conducted with prepubertal animals (Larriestra et al. 2006), suggesting that early life mechanisms are likely playing a key role. One potential sex-related event occurring in early life is the perinatal androgen surge which occurs in males as both a prenatal and postnatal elevation of gonadal androgens. The perinatal androgen surge is critical for the sexually dimorphic development or masculinization of the brain and reproductive system (McCarthy 2008). The postnatal androgen surge in male pigs begins with 24-48h after birth and elevated androgen levels persist for 1-2 months postnatally. While the postnatal androgen surge is playing a key organizational role in reproductive and neurological development, relatively less is known regarding its impact on immune development. Male piglets are routinely castrated at 7-10 days of age which is during the peak phase of the postnatal androgen surge in pigs (Colenbrander, de Jong and Wensing 1978, Schwarzenberger et al. 1993). How castration and ablation of the perinatal androgen surge impacts immune development remains to be fully elucidated. However, in studies in rodents, perinatal androgens have shown to be important for mast cell development (Mackey et al. 2020) supporting the potential impact of perinatal androgens levels on immune development.

Better understanding of the effects of castration on the response to early-weaning could result in significant improvements in swine management, leading to improved herd health and productivity. Therefore, the objective of this study is to characterize the differences between castrated and intact male pigs in their peripheral and intestinal immune and endocrine responses to early weaning stress. Given the inhibitory effects of testosterone on the immune system, and that immunostimulatory effects of castration in male rodents (Pace, Sautebin and Werz 2017, Gubbels Bupp and Jorgensen 2018, Grossman 1984), we hypothesize that castration in male pigs exacerbates the inflammatory response to early weaning, compared with intact males.

MATERIALS AND METHODS

ANIMALS

The University Institutional Animal Care and Use Committee (IACUC) approved all procedures used in this study. A total of 24 Yorkshire-Hampshire cross pigs from multiple litters were used in this study. Sows were parity 2-4. Pigs were weaned between 14-16 days with an average wean age of 15 days. The castrated males were castrated at an average age of 8 days. After weaning, pigs were housed in 1.7 m^2 crates holding up to 6-8 pigs for 24 hours and had ad libitum access to water via a water nipple. To recapitulate industry conditions where weaned piglets often do not consume feed or have access to feed for 24 h during transport and nursery placement, weaned piglets were fasted for 24h prior until collection of tissues. Before euthanasia, body weights of the pigs were measured.

Additional samples from other cohorts were used. These samples came from pigs that were of the same breed, were weaned at the same age, were housed under the same conditions, were castrated (where applicable) at the same age, and whose blood and tissues were collected and processed the same as the pigs in the original cohort. Blood for the 0-hour, 30-minute, and 24-hour time points was collected from three castrated males and three intact males whose tissues were collected at 24 hours after weaning. Blood for the 0-hour and 30-minute time points was collected from an additional three castrated males and three intact males from whom only blood was collected. Additional blood samples were taken from three castrated males and three intact males at 0 hours.

TISSUE SAMPLE COLLECTIONS

12 pigs (six intact males and six castrated males) were euthanized immediately after weaning (this group is henceforward referred to as the 0-hour group) and 12 pigs (six intact males and six castrated males) were euthanized 24 hours after weaning. Before euthanasia, pigs were sedated with a mixture of Telazol, ketamine, and xylazine at 0.03 mL/kg body weight. Once the pigs were sedated, the pigs were euthanized via an intracardiac injection of pentobarbital sodium (Euthasol) at 2.2 mL/kg body weight. Organ tissue samples were collected immediately after euthanasia. Intestinal samples were washed with phosphate buffered saline to remove any digesta. Ileum mucosal scrapings were performed with glass slides. The scrapings and other tissue samples were flash frozen in liquid nitrogen and subsequently stored at –80 degrees Celsius. One castrated male from the 0-hour group was discovered to be a cryptorchid during collection and their data was thus excluded from the analyses.

BLOOD COLLECTION AND CBC

Blood was collected via jugular venipuncture using 20 G vacutainer needles immediately prior to weaning (0 hours), 30 minutes after weaning, and at 24 hours post weaning and prior to euthanasia. Blood was collected into heparinized tubes (Becton, Dickinson and Company, Ref # 366643) and immediately put on ice. 1 tube of heparinized blood was submitted for CBC analysis (see information below). The remaining blood was centrifuged at 2700 rpm at 8 degrees Celsius for 30 minutes to separate out the plasma. The plasma was transferred to 1.7 mL tubules and then stored at –80 degrees Celsius. CBC analysis was performed by the Michigan State Veterinary Diagnostic Laboratory. Blood from the males from the additional cohorts were included in the CBC analysis.

ORGAN MEASUREMENTS

Body weight and organ measurements were taken at 0 hours and at 24 hours. Immediately after euthanasia, the whole spleen and thymus were removed and their weights and lengths were measured and recorded. Relative weights and lengths were calculated by dividing by body weight.

PLASMA HORMONES

Levels of cortisol, testosterone and 17B-estradiol were measured in the plasma.

Cortisol was measured using an Enzo Life Sciences Cortisol ELISA Kit (Catalog #: ADI-900-071) following the manufacturer's instructions using sample diluted 50X with the assay buffer. Plasma from males from the additional 24-hour cohort was included.

Testosterone was measured using an Enzo Life Sciences Testosterone ELISA Kit (Catalog #: ADI-900-065) following the manufacturer's instructions using sample diluted 50X with the assay buffer. Plasma from males from the additional 24-hour cohort was included.

17B-estradiol was measured using an Enzo Life Sciences 17B-Estradiol High Sensitivity ELISA Kit (Catalog #: ADI-901-174) using a 1:8 assay buffer:sample dilution and following the manufacturer's instructions except the assay buffer was not diluted with deionized water. Plasma from both additional male cohorts was included.

C-REACTIVE PROTEIN

Plasma C-reactive protein was measured using a Pig C-Reactive Protein (CRP) ELISA form Life Diagnostics, Inc. (Catalog Number: CRP-9) according to manufacturer's instructions.

INTESTINAL AND MESENTERIC LYMPH NODE CYTOKINES

Levels of IL-1B and TNF-A were measured in the ileum mucosa and mesenteric lymph nodes.

Protein samples were prepared and analyzed before the cytokine levels were measured. 0.5 cm sections were cut off from frozen tissue sample. Cell lysis buffer was prepared by mixing 98 parts of MPER lysis buffer with one part 100X protease inhibitor (Roche Diagnostics GmbH, Ref # 11697498001) and one part 100X phosphatase inhibitor (Thermo Scientific, Ref # 78426). The 0.5 cm pieces of tissue were added with 500 uL of lysis buffer to microfuge tubes containing silicon beads and then homogenized at 6300 rpm for 15 seconds three times, being placed on iced for one to two minutes between homogenizations. An additional 500 uL of lysis

buffer was added to the tubes, which were then placed on ice and gently shaken for 10 minutes. The resulting fluid was then transferred to new microfuge tubes sans beads and centrifuged at 13000 rpm for 15 minutes at four degrees Celsius. The supernatant was transferred to a new tube and stored at –80 degrees Celsius. The protein concentration of the samples was measured using a Thermo Scientific Pierce™ BCA Protein Assay Kit (Catalog #: 23225 or 23227) following the manufacturer's instructions.

TNF-A was measured using a R&D Systems Quantikine ELISA Porcine TNF-A Immunoassay Kit (Catalog #: PTA00) following the manufacturer's instructions and using enough of each sample to load 50 uL of protein into each well. Ileum mucosa and mesenteric lymph node samples from the additional intact female and 24-hour male cohorts were included.

IL-1B was measured using a R&D Systems Quantikine ELISA Porcine IL-1B/IL-1F2 Immunoassay Kit (Catalog #: PLB00B) following the manufacturer's instructions and using enough of each sample to load 50 uL of protein into each well. Ileum mucosa and mesenteric lymph node samples from the additional intact female and 24-hour male cohorts were included.

HISTOLOGICAL ANALYSIS

Immediately after euthanasia, full-thickness ileum and jejunum samples were placed in histology cassettes and fixed in Carnoy's 2000 (StatLab). After 24 hours tissue cassettes were transferred 70% EtOH for long-term storage. Ileum and Jejunum samples from the additional 24-hour male cohort were included.

These samples were delivered to the Michigan State University Investigative HistoPathology Laboratory for Toluidine Blue staining to identify mast cells and H&E staining for morphological analysis and neutrophil counting.

Mast cells were were quantified in the jejunal villi, crypts, and submucosa, the ileal mucosa villi, crypts, and submucosa, and the ileal Peyer's Patches villi, crypts, and submucosa. Total mast cells from five different fields of view per region were counted, summed, and divided between round and elongated and between activated and inactivated.

Morphological measurements taken include villus length, villus crypt depth, submucosa layer width, and muscle layer thickness. Up to 10 and no less than six images were taken of each jejunum, ileum mucosa region, and ileum Peyer's patches region.

Neutrophils of the villi and crypts were counted in the same villi and crypts used for morphological measurements. Neutrophil counts were averaged for each individual.

STATISTICAL ANALYSIS

For plasma hormones, fold change and differences between time points and groups determined using only samples from animals that had data for all three time points. The same criterion was used for samples used for mixed measures analysis. Comparisons between groups at 0 hours made using all individuals, including the animals whose tissues were collected at 0 hours. However, these data points not excluded when performing correlations between hormones and non-hormonal measurements, although outliers were still excluded. 0-hour hormone levels used to correlate with organ measurements and TNF-A and IL-1B and the correlations separated by time point.

GraphPad Prism Version 9 was used to perform all calculations. All graphs are presented as Mean +/-- SD. Outliers were determined via ROUT method using Q=5% and were removed before further analysis. Mixed effects analyses or 2-way ANOVA were used to determine if main effects of weaning and/or castration exist. Mixed effects was used for data obtained from blood samples, where data from the same individual was available for multiple timepoints, while 2-way ANOVA was used for data from all other tissue samples where each individual provided data for only one time point. Normality was assessed via Shapiro-Wilk test, with a further test being made for lognormality when the data was normally distributed. For data that was normally distributed or could be log transformed, Welch's t-test was used to compare between different gonadal/sex groups and paired t-tests were used to compare different time points within the same gonadal/sex group. For data that was not normal and could not be log transformed, Mann-Whitney test was used to compare unpaired data and the Wilcoxon test was used for paired data. All graphs are presented using the untransformed data, even when analyses were made using transformed data. Correlations were examined via simple linear regression. P values less than or equal to 0.05 were considered statistically significant, while P values greater than 0.05 but less than or equal to 0.1 were considered a trend.

RESULTS

BODY WEIGHT

There were no significant differences in bodyweight between timepoints or gonadal groups (Fig. 3).

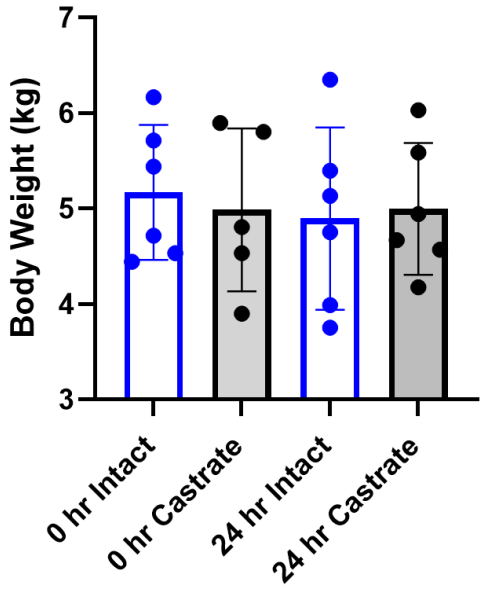

Figure 3. Body Weights. Body weights of piglets at time of collection (n=6 for 0-hour MI, n=5 for 0-hour MC, n=6 for 24-hour MI, n=6 for 24-hour MC. Graphs presented as means +/-- standard deviation with paired t-test for intragroup comparisons or Welch's t-test for inter-group comparisons. #0.1>p>0.05, *p<0.05, **p< 0.01.

COMPLETE BLOOD COUNT

BLOOD NEUTROPHILS

Mixed effects analysis indicated a significant weaning effect for neutrophil number. Mixed effects analysis indicated a trend (p=0.0752) for a weaning effect on neutrophil percent.

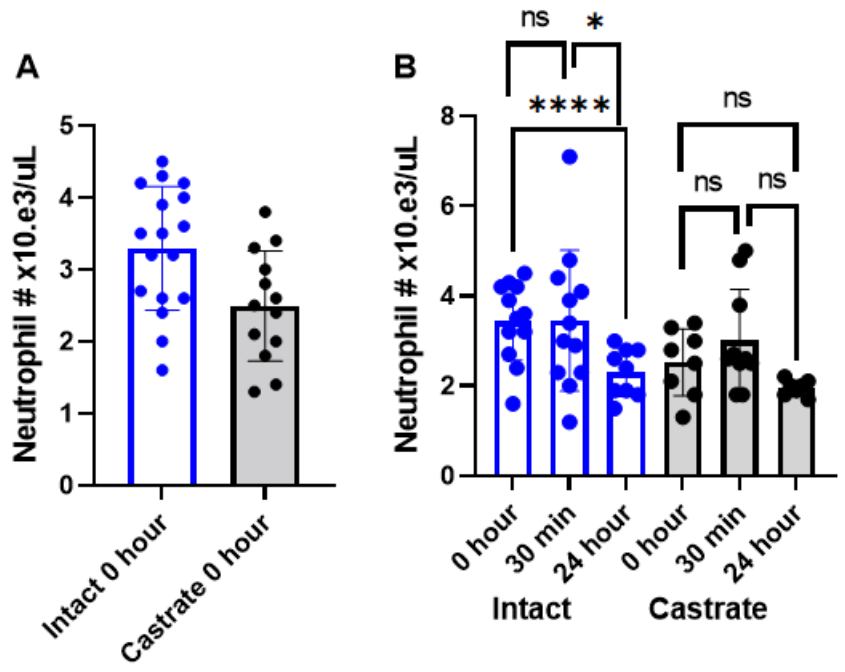

Figure 4. Blood neutrophil numbers at baseline and after weaning. (A) Basal neutrophil numbers (n=17 for 0-hour MI, n=13 for 0-hour MC) (B) Neutrophil numbers in response to weaning (n=12 for 0-hour MI, n=12 for 30-minute MI n=9 for 24-hour MI, n=8 for 0-hour MC, n=10 for 30-minute MC, n=7 for 24-hour MC). Graph A used samples from all animals for whom 0-hour data was available, but 2 castrates were excluded as outliers. Graph B used only individuals whose blood was collected at multiple time points, but 1 castrate at 0 hours and 2 castrates at 24 hours were excluded as outliers. Graphs presented as means +/-- standard deviation mixed effects with paired t-test for intragroup comparisons or Welch's t-test for intergroup comparisons. *p<0.05, **p< 0.01, ***p<0.001, ****p<0.0001. MI, Male intact pigs. MC, male castrated pigs.

Neutrophil numbers decreased significantly in intact males at 24 hours post-weaning; however, this response was not observed in in castrated males (Fig. 4B). Intact males had significantly higher neutrophil numbers compared with castrated males at 0 hours (Fig. 4A).

BLOOD LYMPHOCYTES

Mixed effects analysis indicated a significant weaning effect for lymphocyte number.

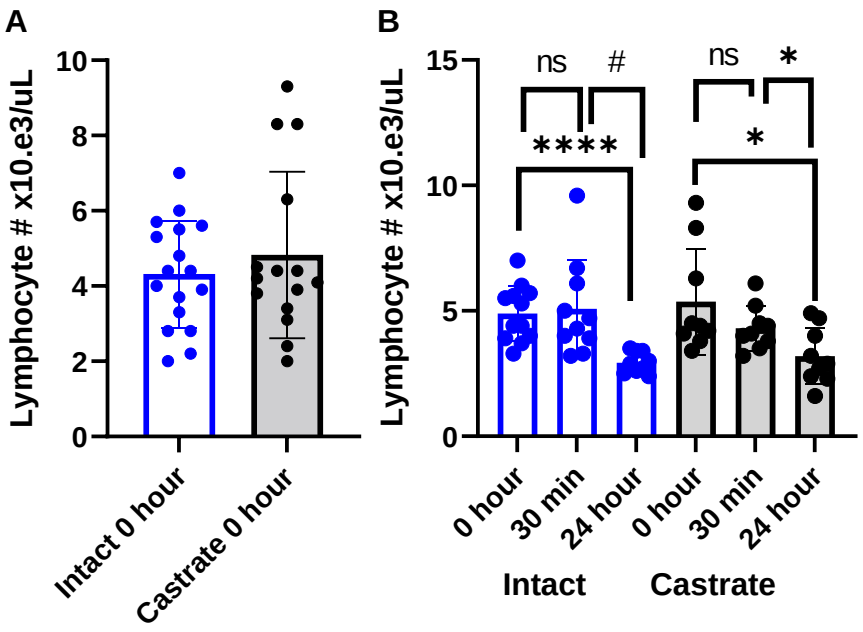

Figure 5. Blood lymphocyte numbers at baseline and in response to weaning. (A) Basal lymphocyte numbers (n=17 for 0-hour MI, n=15 for 0-hourMC). (B) Lymphocyte numbers in response to weaning (n=12 for 0-hour MI, n=10 for 30-minute MI, n=9 for 24-hour MI, n=9 for 0-hour MC, n=9 for 30-minute MC, n=9 for 24-hour MC). Graph A used samples from all animals for whom 0-hour data was available. Graph B used only individuals whose blood was collected at multiple time points, with 1 castrate and 2 intact males at 30 minutes excluded as outliers. Graphs presented as means +/-- standard deviation mixed effects with paired t-test for intragroup comparisons or Welch's t-test for intergroup comparisons. *p<0.05, **p< 0.01, ***p<0.001, ****p<0.0001. MI, Male intact pigs. MC, male castrated pigs.

A significant decline in blood lymphocyte numbers was observed at 24 h post weaning in both intact and castrated males (Fig. 5B). A trend (p=0.0633) for reduced lymphocyte numbers at 24h vs. 30 min was observed in intact males and this reduction reached significance in castrated males. There were no differences in blood lymphocyte levels between intact and castrated male pigs at 0h (Fig. 5A).

Neutrophil:lymphocyte ratios were also evaluated as a marker of immune activation, with a higher ratio indicative of increased immune activation. Data for castrated males at 24 hours was determined to be not normally distributed and so the data was log transformed for comparisons between timepoints Mixed effects analysis indicated a significant weaning effect on neutrophil:lymphocyte ratio. The ratio did not significantly change between in response to weaning in intact males, however, a significantly (p=0.0396) elevated N:L ratio was observed in castrated males indicating a more activated immune response to weaning in castrates (Fig. 6B). The ratio was higher in intact males than in castrated males at 0 hours (Fig. 6A), likely due to the higher number of neutrophils in male intact pigs.

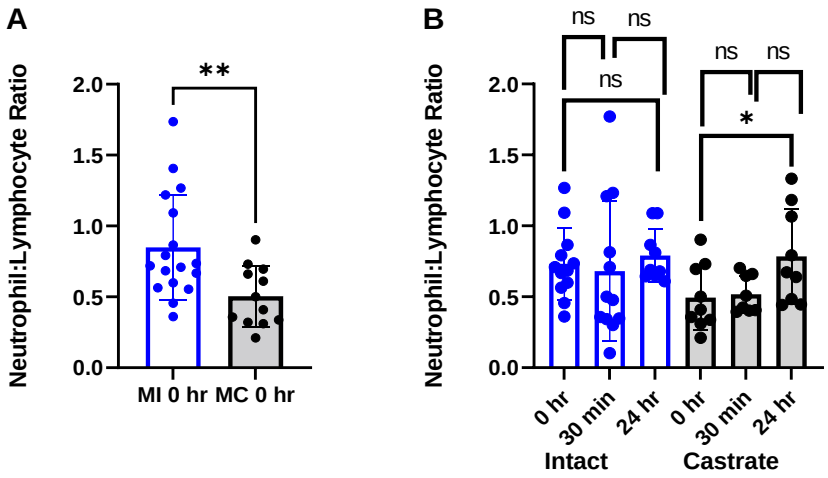

Figure 6. Blood neutrophil:lymphocyte ratio at baseline and in response to weaning. (A) Basal neutrophil:lymphocyte ratio (n=17 for 0-hour MI, n=12 for 0-hour MC). (B) Log of the neutrophil:Lymphocyte ratio in response to weaning (n=12 for 0-hour MI, n=12 for 30-minute MI, n=9 for 24-hour MI, n=9 for 0-hour MC, n=8 for 30-minute MC, n=9 for 24-hour MC). Graph A used samples from all animals for whom 0-hour data was available, with 3 castrates excluded as outliers. Graph B used only individuals whose blood was collected at multiple time points, with 1 castrate at 0 hours excluded as an outlier. Graph B shows the untransformed data. Graphs presented as means +/-- standard deviation mixed effects with paired t-test for intragroup comparisons or Welch's t-test for intergroup comparisons. *p<0.05, **p< 0.01, ***p<0.001, ****p<0.0001. MI, Male intact pigs. MC, male castrated pigs.

OTHER CBC MEASUREMENTS

Total protein, fibrinogen, platelet count, WBC, monocyte numbers, and eosinophil numbers in the blood were also measured. Total protein increased significantly at 24 hours compared to 0 hours in intact males, but not castrated males. There were no significant changes in fibrinogen post-weaning in either group, nor were there differences between groups at 0 hours. Intact males experienced a significant decrease in platelet numbers at 24 hours compared to 0 hours while the castrated males did not, but there were no differences in platelet numbers at 0 hours. Both groups saw a significant decrease in WBC at 24 hours compared to 0 hours and 30 minutes, but there were no differences between groups at 0 hours. There were no changes in monocyte numbers post-weaning , nor were there any differences at 0 hours. Castrated males experienced a significant decrease from 30 minutes to 24 hours while intact males saw only a trend ($p=0.0625$) for a decrease between these time points, but there were no significant differences in eosinophil numbers at 0 hours.

C-REACTIVE PROTEIN (CRP)

Mixed-effects analysis indicated a significant effect of weaning for plasma CRP levels, but it did not indicate a significant castration or weaning by castration effect. Both intact and castrated males tended ($p=0.0851$ and $p=0.0860$, respectively) to have higher CRP at 24 hours than 0 hours (Fig. 7B). Comparing 30 min vs 24 h timepoints, castrated males tended ($p=0.0948$) to have greater CRP responses while intact males did not. A trend ($p=0.0986$) for higher CRP in intact males compared with castrated males was observed at 30 minutes post-weaning.

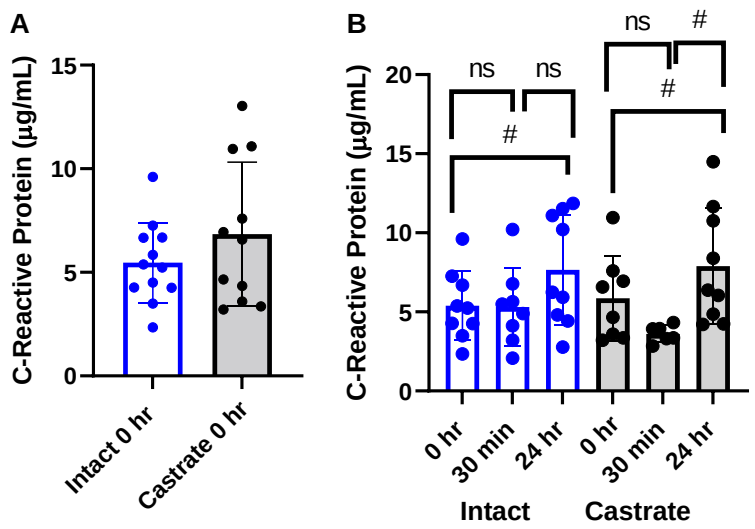

Figure 7. Plasma C-reactive protein at baseline and in response to weaning. (A) Basal plasma C-reactive protein (n=12 for 0-hour MI, n=11 for 0-hour MC). (B) Plasma C-reactive protein response to weaning (n=9 for 0-hour MI, n=9 for 24-hour MI, n=8 for 0-hour MC, n=9 for 24-hour MC). Graph A used samples from all animals for whom 0-hour data was available, with 1 castrate excluded as an outlier. Graph B used only individuals whose blood was collected at multiple time points, with 1 castrate at 0 hours, 1 intact male at 0 hours, and 2 castrates at 30 minutes excluded as outliers. Some individuals were not able to be measured as their plasma samples had been used up by the time C-reactive protein was analyzed. Graphs presented as means +/-- standard deviation mixed effects with paired t-test for intragroup comparisons or Welch's t-test for intergroup comparisons. *p<0.05, **p< 0.01, ***p<0.001, ****p<0.0001. MI, Male intact pigs. MC, male castrated pigs.

ORGAN MEASUREMENTS

THYMUS

2-way ANOVA indicated a trend (p=0.0987) for a weaning effect and a significant castration effect for absolute thymus length. A trend (p=0.0881) for a castration effect for relative thymus length was indicated. There were no significant differences between gonadal groups or time points in absolute or relative thymic weight (Fig. 8A). Nor were there any differences between gonadal groups or time points in relative thymic length. However, there

were trends (p=0.0695 and p=0.0721) for intact males to have longer thymuses than castrates at 0 hours and 24 hours, respectively (Fig. 8B). No differences were seen in relative percentage change for either thymiic weight or length (Fig. 8C-D).

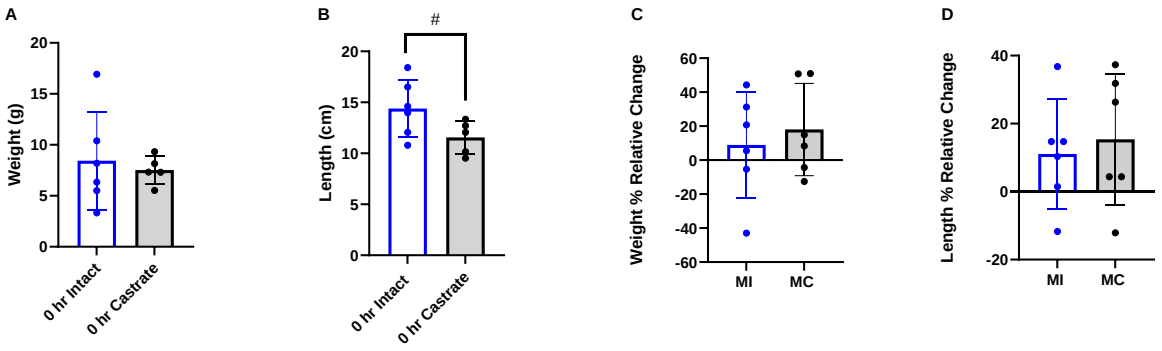

Figure 8. Absolute thymic weight and length at baseline and after weaning. (A) Basal thymic weight (n=6 for 0-hour MI, n=5 for 0-hour MC). (B) Basal thymic length (n=6 for 0-hour MI, n=5 for 0-hour MC). (C) Thymic weight after weaning (n=6 for 0-hour MI, n=6 for 0-hour MC). (D) Thymic length after weaning (n=6 for 0-hour MI, n=6 for 0-hour MC). All graphs used only pigs from the original cohort, excluding the cryptorchid. All graphs presented as means +/-- standard deviation with Welch's t-test. *p<0.05, ***p<0.001, ****p<0.0001. MI, Male intact pigs. MC, male castrated pigs.

SPLEEN

2-way ANOVA indicated a trend (p=0.0524) for weaning effect for absolute spleen length. A trend (p=0.0993) for a weaning effect for relative spleen weight was also indicated. A significant weaning effect for relative spleen length was indicated. There was a trend (p=0.0864) for the spleens of castrated males to be heavier at 24 hours than at 0 hours and splenic length was significantly greater at 24 hours than at 0 hours in castrated males. No differences between time points were seen for either splenic length or weight in intact males. No differences between gonadal groups or time points were observed for relative splenic weight, but relative splenic length increased significantly for castrated males from 0 hours to 24

hours and tended (p=0.0855) to do the same for intact males. Absolute splenic weight was not different between intact and castrated males at either 0 hours or 24 hours (Fig. 9A). No differences were seen between gonadal groups in relative splenic length. No differences were seen in relative percentage change for either splenic length or weight (Fig. 9C-D).

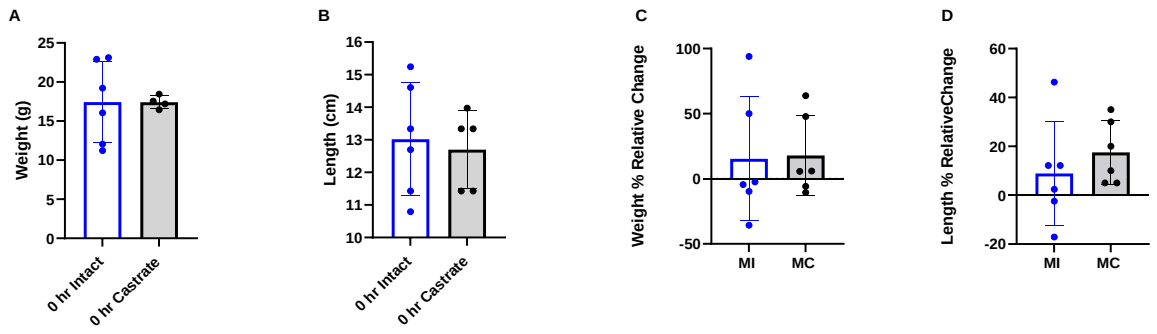

Figure 9. Absolute splenic weight and length at baseline and after weaning. (A) Basal splenic weight (n=6 for 0-hour MI, n=4 for 0-hour MC). (B) Basal splenic length (n=6 for 0-hour MI, n=5 for 0-hour MC). (C) Relative change in splenic weight (n=6 for MI, n=6 MC). (D) Relative change in splenic length (n=6 for MI, n=6 MC). All graphs used only pigs from the original cohort, excluding the cryptorchid and 1 castrate at 0 hours that was removed as an outlier. All graphs presented as means +/-- standard deviation with Welch's t-test. *p<0.05, ***p<0.001, ****p<0.0001. MI, Male intact pigs. MC, male castrated pigs.

INTESTINAL MORPHOLOGY

ILEUM MUCOSA

2-way ANOVA indicated a trend (p=0.0926) for a weaning by castration effect on villus length and a trend (p=0.0539) for castration effect on villus crypt depth. No significant effects of castration were indicated for submucosa width or muscle layer width. No significant differences were found between castration and intact males or weaning for villus length, villus crypt depth, submucosa width, or muscle layer width. However, a significant difference was

found in villus length relative change, with intact males showing 17% decrease in villi length post-weaning and castrated males showing a 9% increase in villi length.

ILEUM PEYER'S PATCHES

2-way ANOVA indicated a trend ($p=0.0954$) for a weaning effect on submucosa width. Villus crypt depths were significantly reduced at 24h post weaning compared with 0h in intact males. There was a significant difference in villus length relative change with intact males exhibiting a reduction in villus length while castrates exhibited an increase. A similar significant difference was seen in crypt depth relative change.

JEJUNUM

2-way ANOVA indicated a significant weaning effect on villus length. No other significant effects were indicated. Intact males had significantly shorter villi lengths at 24 hours than at 0 hours. Castrated males also had significantly shorter villi lengths at 24 hours than at 0 hours. There were no significant differences in relative change between groups.

INTESTINAL MUCOSA NEUTROPHIL NUMBERS

ILEUM MUCOSA

2-way ANOVA indicated a significant weaning effect, but no other significant effects were indicated. Both gonadal groups had higher neutrophil numbers at 24 hours than at 0 hours in both the villi and the crypts. There were no differences in neutrophil number between gonadal groups (Fig. 10A, D). There were no differences between groups in 0-hour to 24-hour relative percentage change (Fig. 11A, D).

ILEUM PEYER'S PATCHES

2-way ANOVA indicated a significant weaning effect, but no other significant effects were indicated. Both gonadal groups had higher neutrophil numbers at 24 hours than at 0 hours in the crypts, while only intact males saw significantly higher neutrophil numbers in the villi at 24 hours compared to 0 hours. There were no differences in neutrophil number between gonadal groups (Fig. 10B, E). There were no differences between groups in 0-hour to 24-hour relative percentage change (Fig. 11B, E).

JEJUNUM

2-way ANOVA indicated a significant weaning effect for neutrophil numbers in both the villi and crypts, but no other significant effects were indicated. Both gonadal groups had higher neutrophil numbers at 24 hours than at 0 hours in the crypts, while only intact males saw significantly higher neutrophil numbers in the villi at 24 hours compared to 0 hours. There were no differences in neutrophil number between gonadal groups (Fig. 10C, F).

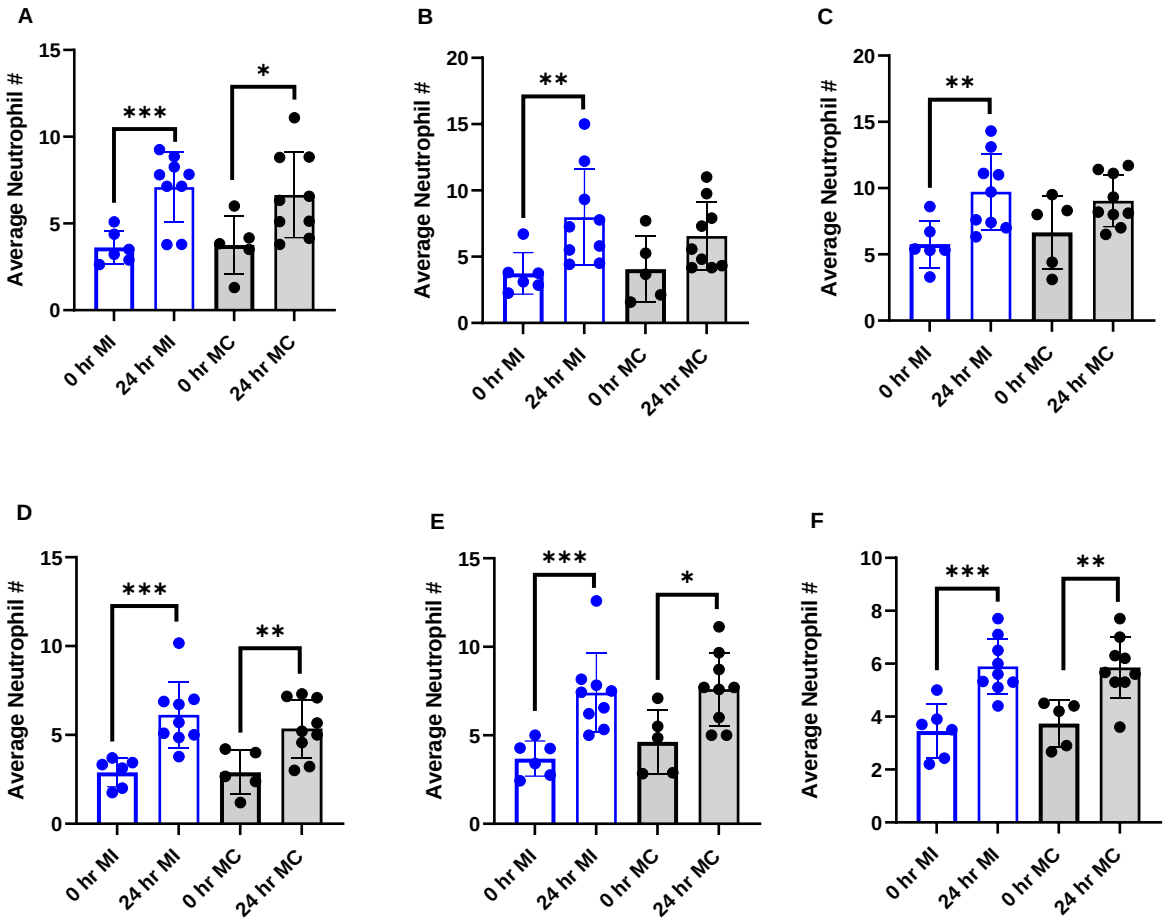

Figure 10. Neutrophil numbers of the small intestine. (A) Ileum mucosa villus tips (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). (B) Ileum Peyer's patches villus tips (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). (C) Jejunum villus tips (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). (D) Ileum mucosa villus crypts (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). (E) Ileum Peyer's patches villus crypts (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). (F) Jejunum villus crypts (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). All values are averages of multiple images. All graphs presented as means +/-- standard deviation with Welch's t-test. ns p>0.1, *p<0.05, **p< 0.01,***p<0.001, ****p<0.0001. IM, ileum mucosa, MI, male intact pigs. MC, male castrated pigs.

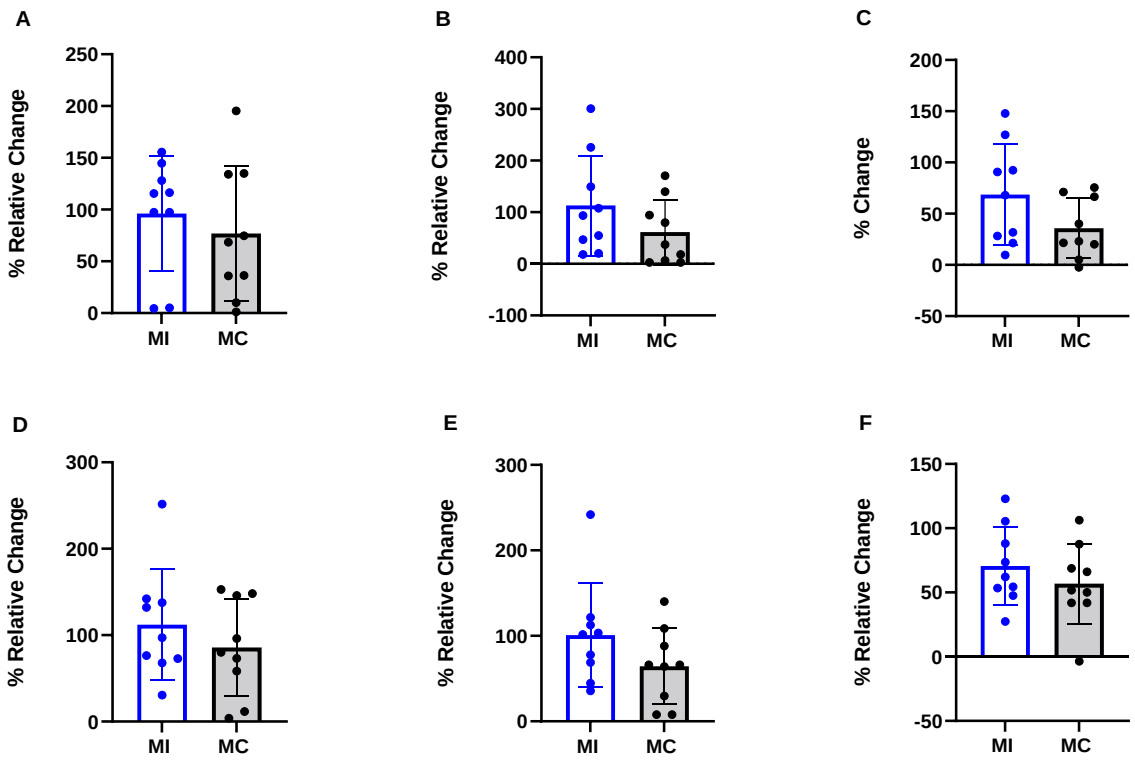

Figure 11. Change in intestinal neutrophil numbers 24 hours after weaning. (A) Ileum mucosa villus tips (n=9 for 0-hour MI, n=9 for 0-hour MC). (B) Ileum Peyer's patches villus tips (n=9 for 0-hour MI, n=9 for 0-hour MC). (C) Jejunum villus tips (n=9 for 0-hour MI, n=9 for 0-hour MC). (D) Ileum mucosa villus crypts (n=9 for 0-hour MI, n=9 for 0-hour MC). (E) Ileum Peyer's patches villus crypts (n=9 for 0-hour MI, n=9 for 0-hour MC). (F) Jejunum villus crypts (n=9 for 0-hour MI, n=9 for 0-hour MC).All graphs presented as means +/-- standard deviation with Welch's t-test. ns p>0.1, *p<0.05, **p< 0.01,***p<0.001, ****p<0.0001. IPP, ileum Peyer's patches, MI, male intact pigs. MC, male castrated pigs.

INTESTINAL MAST CELLS

ILEUM MUCOSA SUBMUCOSA

Both intact and castrated males did not have normal distributions for total mast cell numbers at 0 hours, so the data was log transformed. 2-way ANOVA indicated a trend (p=0.0609) for a castration effect on total mast cell numbers, but no significant effects were indicated for activation. There was a trend (p=0.0839) for castrated males to have a higher

activated mast cell percentage than intact males at 24 hours. No other significant differences were found.

ILEUM MUCOSA VILLUS TIPS

2-way ANOVA did not indicate any significant effects of weaning or castration. Castrated males had significantly higher total mast cell numbers at 24 hours than at 0 hours. Intact males had a significantly higher activated mast cell percentage at 24 hours than at 0 hours. There were no other significant differences.

ILEUM MUCOSA VILLUS CRYPTS

2-way ANOVA indicated a significant weaning effect on total mast cell numbers. Intact males tended ($p=0.0658$) to have a higher total mast cell number at 24 hours than at 0 hours. Castrated males had significantly higher total mast cell numbers at 24 hours than at 0 hours. There was a trend ($p=0.0764$) for intact males to have a higher activated mast cell percentage at 24 hours than at 0 hours.

ILEUM PEYER'S PATCHES SUBMUCOSA

2-way ANOVA indicated a significant weaning effect and a trend ($p=0.0606$) for a castration effect on total mast cell numbers. There was a significant weaning effect on activated mast cell percentage. Castrated males had significantly higher total mast cell numbers at 24 hours than at 0 hours. Castrated males had borderline significantly ($p=0.0552$) lower total mast cell numbers than intact males at 0 hours (Fig. 12H).

ILEUM PEYER'S PATCHES VILLUS TIPS

2-way ANOVA indicated a trend (p=0.0833) for a weaning effect on total mast cell numbers. Significant weaning, castration, and weaning by castration effects were found for activated mast cell percentage. Castrated males tended (p=0.0619) to have higher total mast cell numbers at 24 hours than at 0 hours. There were no other significant differences found

ILEUM PEYER'S PATCHES VILLUS CRYPTS

2-way ANOVA did not indicate any significant effects on total mast cell number or activation. Castrated males had significantly higher total mast cell numbers at 24 hours than at 0 hours. There were no other significant differences

JEJUNUM SUBMUCOSA

Intact males did not have a normal distribution for total mast cell numbers at 24 hours, so the data was log transformed. 2-way ANOVA indicated a significant castration effect on total mast cell number and a trend (p=0.0887) for a weaning by castration effect on activated mast cell percentage. Castrated males tended (p=0.0924) to have higher total mast cell counts at 24 hours than at 0 hours. Castrated males tended (p=0.0795) to have a higher activated mast cell percentage at 24 hours than at 0 hours. Castrated males had significantly lower total mast cell numbers than intact males at 0 hours (Fig. 12I). Castrated males had a borderline significantly (p=0.0546) greater increase in total mast cells in response to weaning than intact males (Fig. 13I).

JEJUNUM VILLUS TIPS

2-way ANOVA indicated significant weaning and castration effects on total mast cell numbers. There were significant weaning, castration, and weaning by castration effects on activated mast cell percentage. Intact males had borderline significantly (p=0.0598) higher total mast cell numbers at 24 hours than at 0 hours. Castrated males had significantly higher total mast cell numbers at 24 hours than at 0 hours. Castrated males tended (p=0.0995) to have lower total mast cell numbers than intact males at 0 hours (Fig. 12C). Castrated males had borderline significantly (p=0.0557) lower total mast cell numbers than intact males at 24 hours. There were no significant differences in activated mast cell percentages.

JEJUNUM VILLUS CRYPTS

2-way ANOVA indicated a significant weaning effect on total mast cell numbers. There was a borderline significant (p=0.0518) castration effect on activated mast cell percentage. Castrated males had significantly higher total mast cell numbers at 24 hours than at 0 hours. Castrated males had a borderline significantly (p=0.0557) lower activated mast cell percentage than intact males at 0 hours. Castrated males had a significantly greater increase in total mast cells in response to weaning than intact males (Fig. 13F).

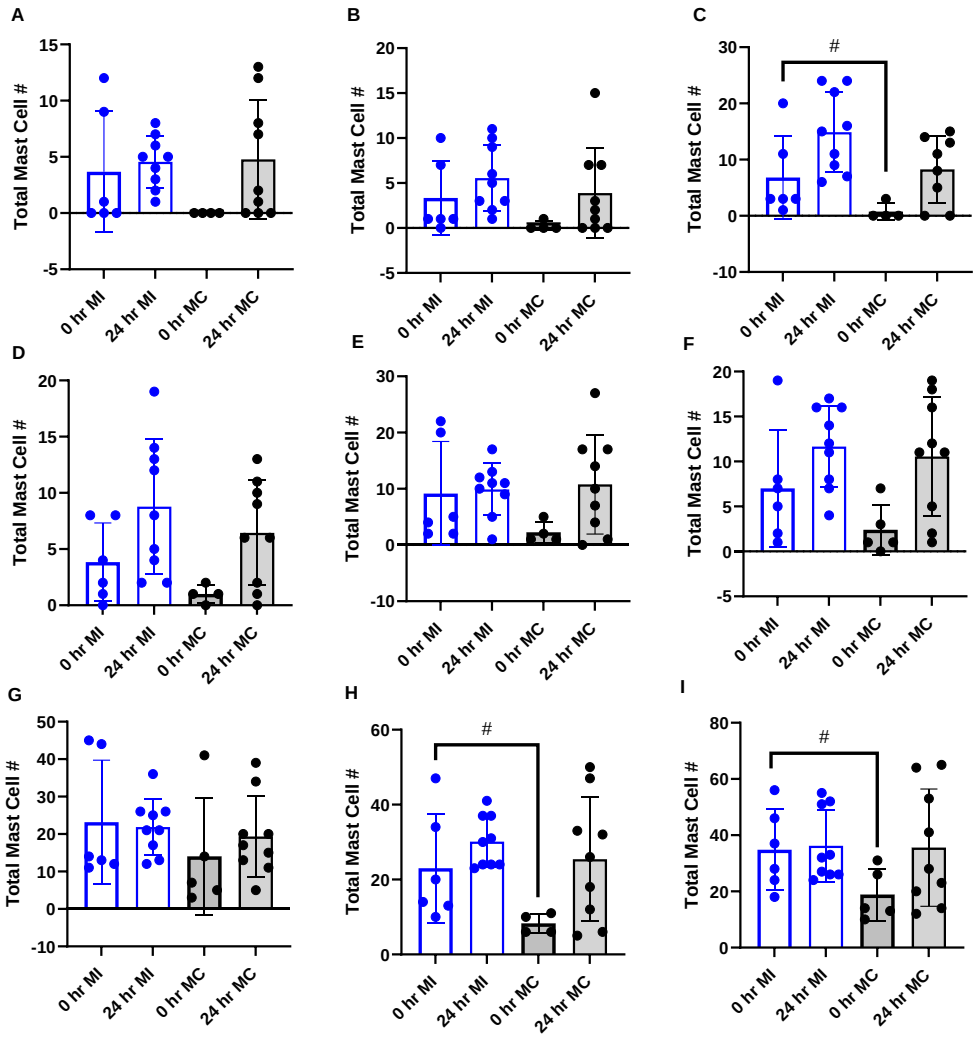

Figure 12. Total mast cell numbers in the small intestine. (A) Ileum mucosa villus tips (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). (B) Ileum Peyer's patches villus tips (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). (C) Jejunum villus tips (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). (D) Ileum mucosa villus crypts (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). (E) Ileum Peyer's patches villus crypts (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). (F) Jejunum villus crypts (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). (G) Ileum mucosa submucosa (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). (H) Ileum Peyer's patches submucosa (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). (I) Jejunum submucosa (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). All data are the averages of five images per animal. All graphs presented as means +/-- standard deviation with Welch's t-test. *p<0.05,**p< 0.01,***p<0.001, ****p<0.0001. MI, male intact pigs. MC, male castrated pigs.

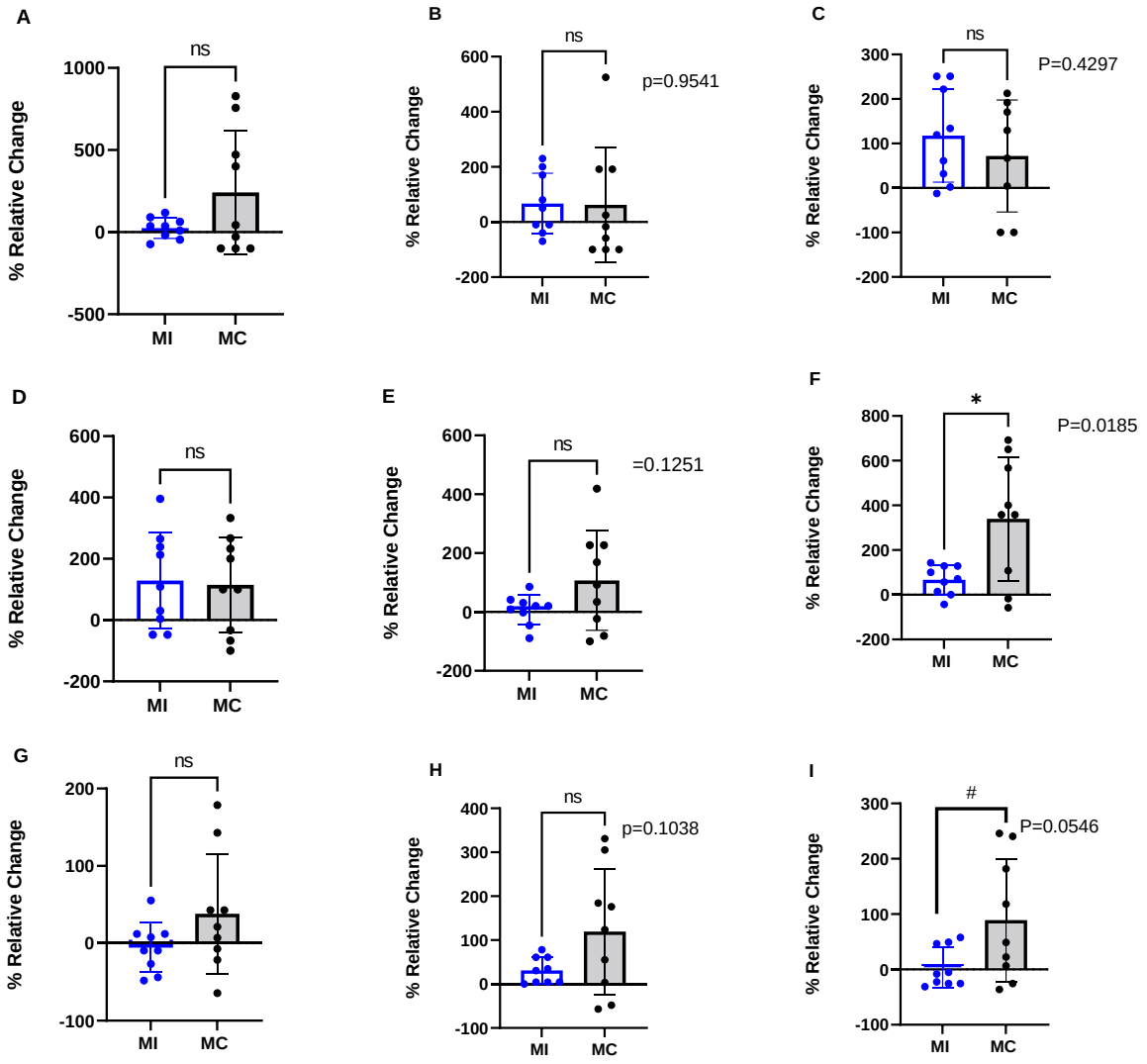

Figure 13. Weaning-induced change in total mast cell numbers in the small intestine. (A) Ileum mucosa villus tips (n=9 for MI, n=9 for MC). (B) Ileum Peyer's patches villus tips (n=9 for MI, n=9 for MC). (C) Jejunum villus tips (n=9 for MI, n=8 for MC). (D) Ileum mucosa villus crypts (n=9 for MI, n=9 for MC). (E) Ileum Peyer's patches villus crypts (n=9 for MI, n=9 for MC). (F) Jejunum villus crypts (n=9 for MI, n=9 for MC). (G) Ileum mucosa submucosa (n=9 for MI, n=9 for MC). (H) Ileum Peyer's patches submucosa (n=9 for MI, n=9 for MC). (I) Jejunum submucosa (n=9 for MI, n=9 for MC). One castrated male was excluded from the results for jejunum villus tips as an outlier. All graphs presented as means +/-- standard deviation with Welch's t-test. *p<0.05, **p< 0.01,***p<0.001, ****p<0.0001. MI, male intact pigs. MC, male castrated pigs.

TNF-α

ILEUM MUCOSA

A 2-way ANOVA indicated a significant weaning by castration effect on ileum mucosa TNF-α. Ileal TNF-α levels in castrated males, but not in intact males, were significantly higher at 24 hours than at 0 hours. No statistically significant differences were observed between intact and castrated males for TNF-α levels in Ileal mucosa at 0 hours (Fig. 14A). The percentage relative change for ileal mucosa TNF-α was significantly higher in castrated males than in intact males (Fig. 14B).

MESENTERIC LYMPH NODE

A 2-way ANOVA did not indicate any significant weaning, castration, or weaning by castration effect on mesenteric lymph node TNF-α. There were no differences in mesenteric lymph node TNF-α between time points within gonadal groups. Nor were there any differences in mesenteric lymph node TNF-α between gonadal groups (Fig. 15A). There was a significant difference in relative change between intact males and castrated males, with castrated males having an increase and intact males having a decrease (Fig. 15B).

IL-1β

ILEUM MUCOSA

2-way ANOVA indicated a trend (p=0.0782) for a weaning effect. There was a trend (p=0.0943) for higher IL-1β at 24 hours than at 0 hours in intact males. There was no difference between intact males and castrated males in relative change (Fig. 14D).

MESENTERIC LYMPH NODE

2-way ANOVA did not indicate a significant, weaning, castration, or weaning by castration effect on mesenteric lymph node IL-1β. There were no differences in mesenteric lymph node IL-1β between time points, between gonadal groups, or in fold change (Fig. 15C-D).

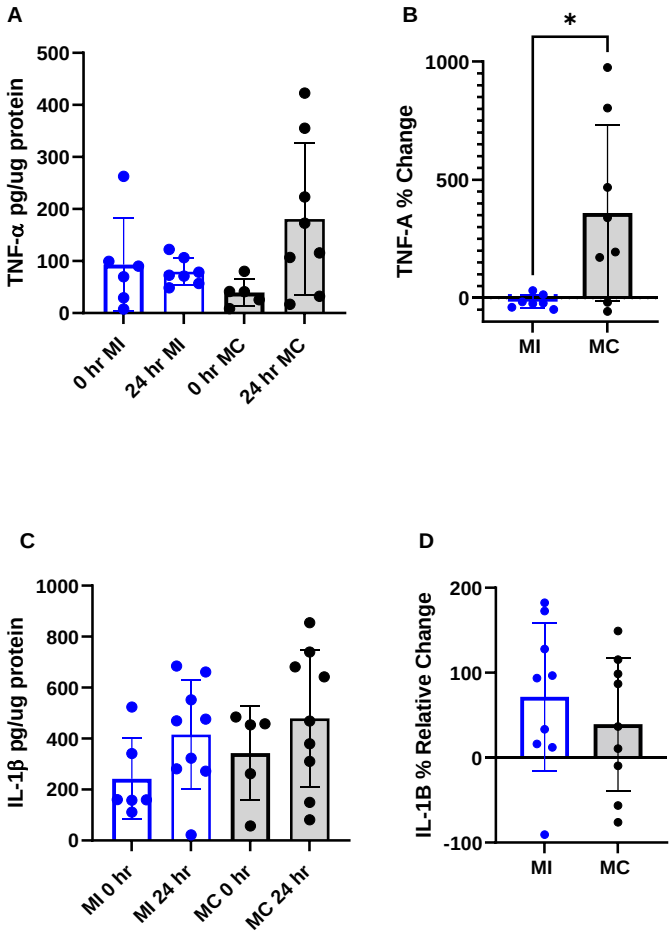

Figure 14. Ileum mucosa TNF-α and IL-1β. (A) Basal TNF-α levels (n=6 for 0-hour MI, n=7 for 24-hour MI, n=5 for 0-hour MC, n=8 for 24-hour MC). (B) TNF-α response to weaning (n=7 for 0-hour MI, n=8 for 0-hour MC.). (C) Basal IL-1β levels (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). (D) IL-1β response to weaning (n=9 for 0-hour MI, n=9 for 0-hour MC). For graph B, two intact males were excluded as outliers and data could not be gathered for one castrated male. All graphs presented as means +/-- standard deviation Welch's t-test. *p<0.05, **p< 0.01, ***p<0.001, ****p<0.0001. MI, Male intact pigs. MC, male castrated pigs.

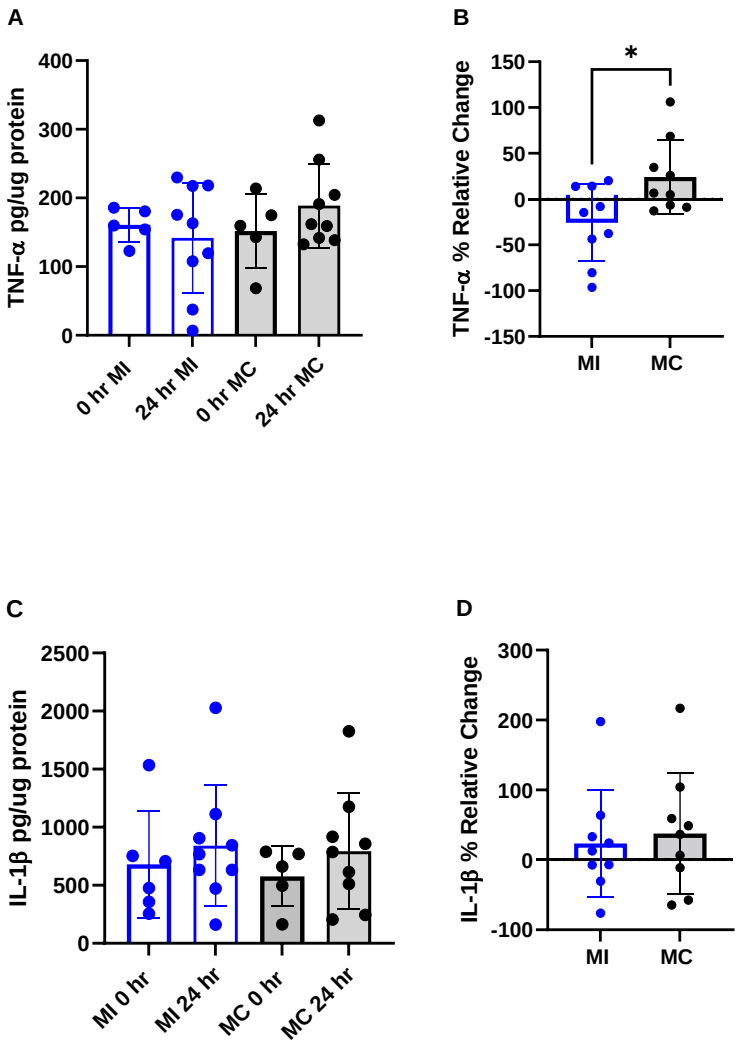

Figure 15. Mesenteric lymph node TNF-α and IL-1β. (A) Basal TNF-α levels (n=5 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). (B) TNF-α response to weaning (n=9 for 0-hour MI, n=9 for 0-hour MC). (C) Basal IL-1β levels (n=6 for 0-hour MI, n=9 for 24-hour MI, n=5 for 0-hour MC, n=9 for 24-hour MC). (D) IL-1β response to weaning (n=9 for 0-hour MI, n=9 for 0-hour MC). All graphs presented as means +/-- standard deviation Welch's t-test. *p<0.05, **p< 0.01, ***p<0.001, ****p<0.0001. MI, Male intact pigs. MC, male castrated pigs.

PLASMA CORTISOL

As cortisol is a hormone released in response to stress and it can downregulate, and be downregulated by (Rubinow et al. 2005), testosterone via the hypothalamic-pituitary-gonadal

axis, its plasma concentrations were measured in order to determine if castration affected its levels. Mixed effects analysis indicated a significant weaning effect, but no castration or weaning by castration effects on cortisol. Cortisol was significantly elevated at 30 minutes in both groups and decreased by 24 hours, but cortisol remained elevated above 0-hour levels in intact males while it returned to 0-hour levels in castrated (Fig. 16B). Cortisol levels were not different between the two groups at any timepoint (Fig. 16B). No differences were seen between the two groups in relative change between time points. However, when cortisol relative change was correlated with testosterone relative change, a significant negative correlation was found in intact males alone or when both gonadal groups were pooled together, but not in castrated males alone.

The levels of cortisol and changes in cortisol were not significantly different between the two groups and so cortisol is not thought to significantly contribute to any differences in immune measurements between the two groups at the time points examined.

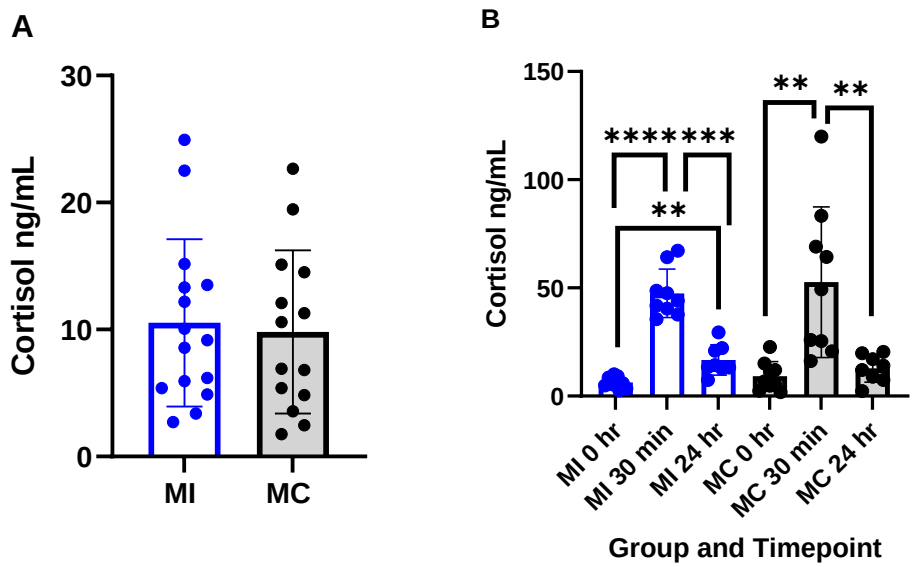

Figure 16. Plasma cortisol at baseline and over time. (A) Basal plasma cortisol (n=15 for MI, n=14 for MC) (B) Cortisol levels over time (n=9 for MI 0-hour, n=9 for MI 30-minute, n=8 for MI 24-hour, n=9 for MC 0-hour, n=9 for MC 30-minute, n=8 for MC 24-hour). Graph A used data from all animals sampled at 0 hours while graph B used only those animals sampled at all time points. One castrate and one intact male from the 24-hour groups were excluded as outliers. Graphs presented as means +/-- standard deviation with paired t-test for intragroup comparisons or Welch's t-test for inter-group comparisons. *p<0.05, **p< 0.01, ***p<0.001, ****p<0.0001. MI, Male intact pigs. MC, male castrated pigs.

PLASMA TESTOSTERONE AND ESTRADIOL

Testosterone is a hormone primarily produced by the testes in males and drives masculinization. Plasma testosterone was measured to confirm ablation of androgens in castrated males. Mixed effects analysis indicated significant weaning, castration, and weaning by castration effects on testosterone. While testosterone significantly decreased from 0 hours to 30 minutes in both groups, testosterone in intact males rose significantly from 30 minutes to 24 hours to be borderline significantly (P=0.0541) higher at 24 hours than at 0 hours (Fig. 17B). Such a rise did not occur in the castrated males (Fig 17B). Testosterone was higher in intact

males than in castrated males at all time points observed (Fig. 17A-B). No differences were seen between the two groups in fold change between time points.

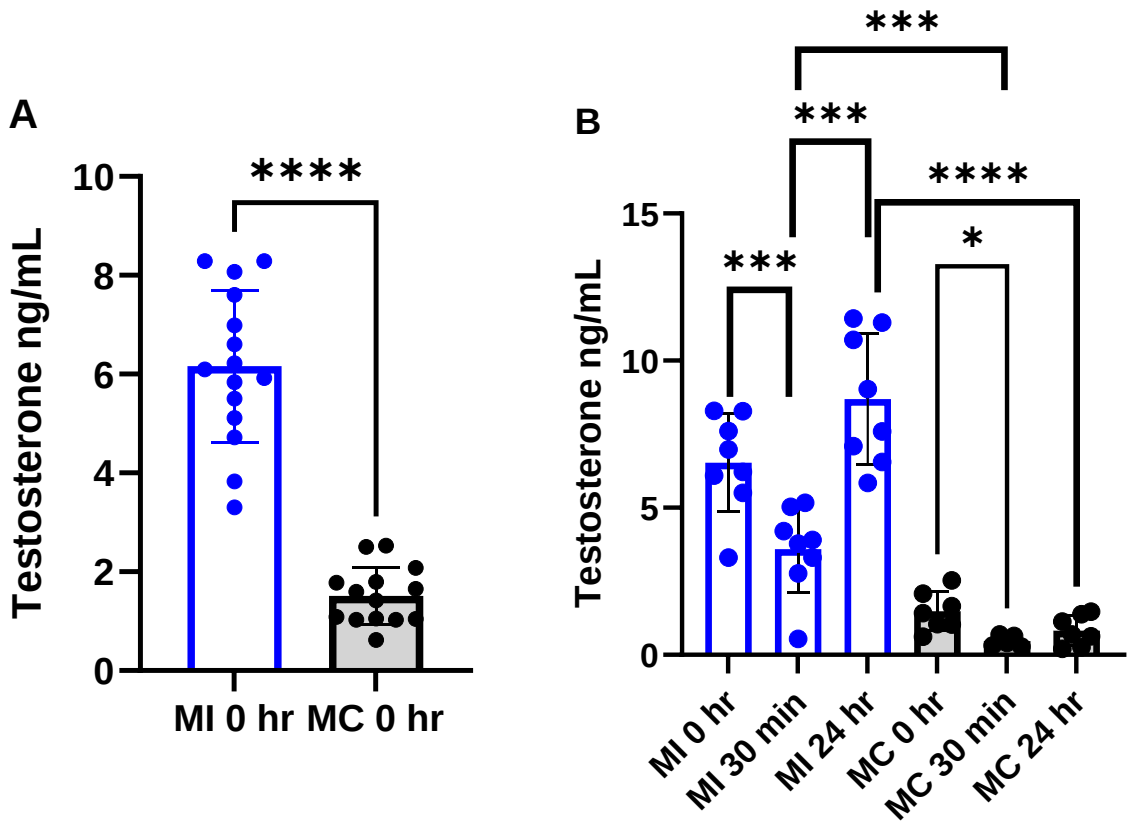

Figure 17. Plasma testosterone at baseline and over time. (A) Basal testosterone (n=15 for MI, n=14 for MC (B) Testosterone levels over time (n= 8 for MI 0-hour, n=8 for MI 30-minute, n=8 for MI 24-hour, n=7 for MC 0-hour, n=5 for MC 30-minute, n=7 for MC 24-hour). Graph A used data from all animals sampled at 0 hours while graph B used only those animals sampled at all time points and from whom data was successfully obtained. Two 30-minute castrates were excluded as outliers. Graphs presented as means +/-- standard deviation with paired t-test for intragroup comparisons or Welch's t-test for inter-group comparisons. *p<0.05, ***p<0.001, ****p<0.0001. MI, Male intact pigs. MC, male castrated pigs.

17β–estradiol can be produced via aromatization of testosterone and it can play a role in masculinization in some species (McCarthy 2008). Therefore, the plasma concentration of

estradiol was measured to confirm ablation of neonatal estradiol in castrated males. Mixed effect analysis indicated significant castration and weaning by castration interaction effects, but no significant weaning effect on 17β-estradiol. The results for castrated males at 0 hours were not normally distributed and thus the data was log transformed. In intact males, there were no significant differences in estradiol concentrations between time points (Fig. 18B). However, castrates saw a significant rise at 24 hours compared to 0 hours (Fig. 18B). 17β-estradiol was significantly higher in intact males than in castrated males at 0 hours and 24 hours, but not at 30 minutes (Fig. 18A-B). There was a slight trend (p=0.0951) for a difference between the groups in 0 hours to 24 hours fold change, with intact males having a decrease and the castrated males having an increase. No other differences in fold change between time points was seen between the two groups.

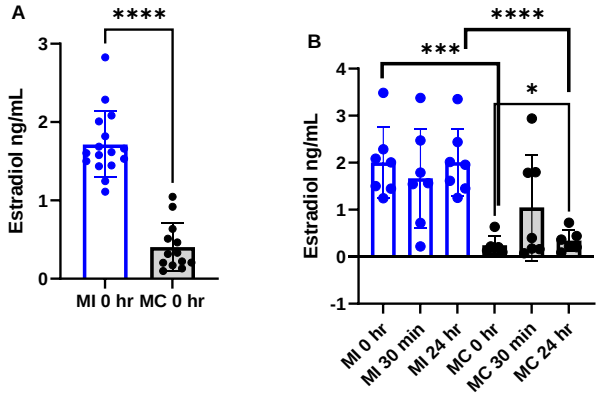

Figure 18. Plasma 17β-estradiol at baseline and over time. (A) Basal 17β-estradiol levels (n=16 for MI 0-hour, n=13 for MC 0-hour). (B) 17β-estradiol levels over time (n=7 for MI 0-hour, n=7 for MI 30-minute, n=7 for MI 24-hour, n=6 for MC 0-hour, n=7 for MC 30-minute, n=6 for MC 24 -hour). Graphs presented as means +/-- standard deviation with paired t-test for intragroup comparisons or Welch's t-test for inter-group comparisons. Graph A used all animals sampled at 0 hours and 2 intact males and 3 castrates were excluded as outliers. Graph B used only animals that were sampled and from whom data was successfully collected. One castrate from both the 0-hour and 24-hour timepoints was excluded as an outlier. *p<0.05, ***p<0.001, ****p<0.0001. MI, Male intact pigs. MC, male castrated pigs.

SEX HORMONE AND IMMUNE MARKER CORRELATIONS

SYSTEMIC MARKERS

Neutrophil numbers and percentages were correlated with plasma testosterone and 17β-estradiol concentrations at the same time points to determine if there were possible relationships between hormone levels and neutrophils.

When broken down by time point and gonadal group, a significant negative correlation was found between plasma testosterone and neutrophil number and percentage at 0 hours in intact males (Fig. 19A). No correlations were found between testosterone and neutrophil measurements in castrated males.

When broken down by time point and gonadal group, a significant positive correlation was found between plasma testosterone and lymphocyte percentage at 0 hours in intact males, but no correlations were found between plasma testosterone and lymphocyte number in intact males. No correlations between plasma testosterone and lymphocyte measurements were found in castrated males,

When broken down by time point and gonadal group, plasma testosterone had a significant negative correlation with the neutrophil:lymphocyte ratio at 0 hours in intact males (Fig. 19B). No correlations were found between plasma testosterone and the neutrophil:lymphocyte ratio in castrated males.

When broken down by gonadal status and time point, there were no significant correlations between plasma testosterone and plasma C-reactive protein.

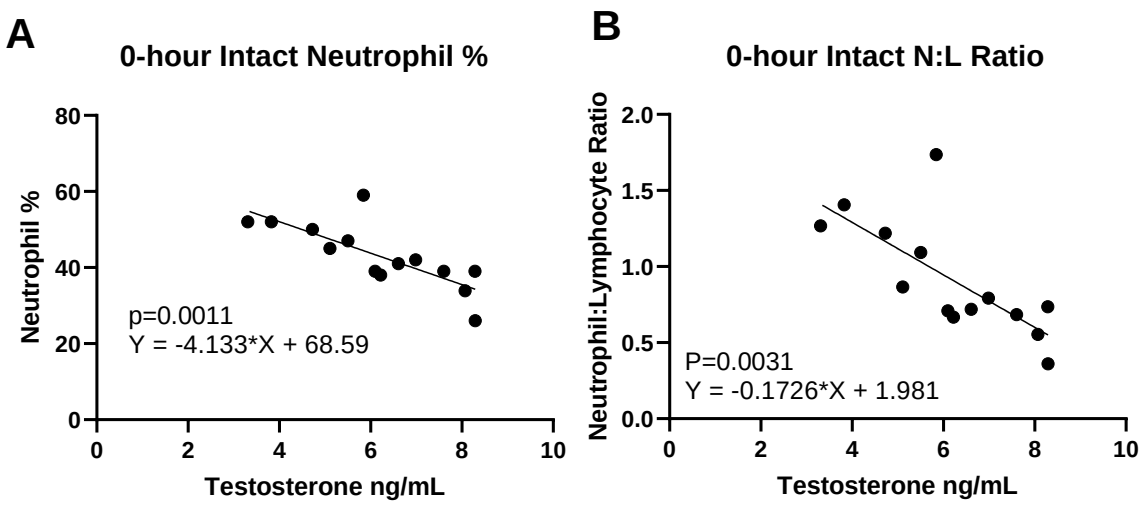

Figure 19. Basal testosterone and basal blood neutrophil percentage and neutrophil: lymphocyte ratio in intact males. (A) Basal plasma testosterone and blood neutrophil percentage in intact males (n=17). (B) Basal plasma testosterone and blood neutrophil:lymphocyte ratio in intact males (n=17). Graphs presented as simple linear regression.

No significant correlation was found between 17β-estradiol and neutrophil measurements in intact males. However, a significant correlation was found between 17β-estradiol and neutrophil percentage at 0 hours in castrated males and a trend (p=0.0877) for a positive correlation with neutrophil percentage at 24 hours in castrated males.

At 30 minutes in intact males, a significant positive correlation was found between plasma 17β-estradiol and lymphocyte number and there was a trend (p=0.0899) for a positive correlation between plasma 17β-estradiol and lymphocyte percentage. No correlations were found between plasma 17β-estradiol and lymphocyte number in castrated males. In castrated males there were trends (p=0.0670, p=0.0645, and p=0.0740) for a negative correlation between plasma 17β-estradiol and lymphocyte percentage at 0 hours, 30 minutes, and 24 hours, respectively.

No correlations were found between plasma 17β-estradiol and the neutrophil:lymphocyte ratio in intact males.

A trend (p=0.0615) existed for a positive correlation between 24-hour C-reactive protein and 24 hour 17β-estradiol in intact males. A significant correlation existed between 24-hour C-reactive protein and 24 hour 17β-estradiol in castrated males. No other significant correlations were found between plasma C-reactive protein and 17β-estradiol.

LYMPHOID ORGANS

Correlations between organ measurements and plasma hormones were calculated using 0-hour hormone levels to determine relationships between basal hormone levels and organ weights and lengths.

There was a borderline significant (p=0.0538) negative correlation between 0-hour plasma testosterone and 0-hour thymus weight in castrated males. However, intact males had significant positive correlations between 0-hour plasma testosterone and 0-hour absolute and relative thymic weight and absolute thymic length (Fig. 20 A-B). No other correlations were found between 0-hour plasma testosterone and thymic measurements.

0-hour plasma testosterone was positively correlated with 0-hour absolute spleen weight and length in intact males (Fig. 20C-D). 0-hour plasma testosterone was also negatively correlated with 24-hour relative spleen length in intact males. No other significant correlations were found between 0-hour plasma testosterone and splenic measurements.

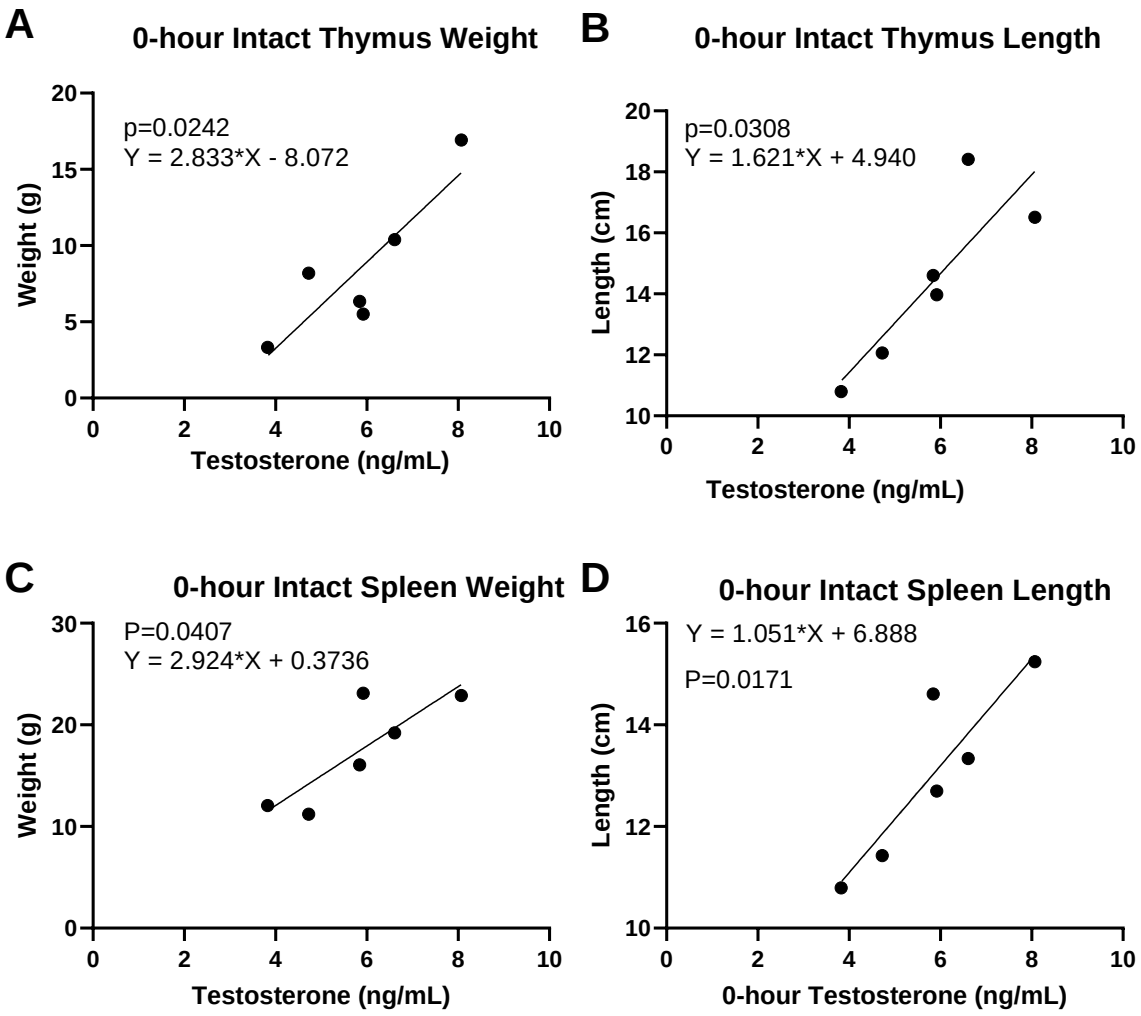

Figure 20. Basal testosterone and lymphoid organ measurements in intact males. (A) Basal testosterone and thymus weight (n=6). (B) Basal testosterone and thymus length (n=6). (C) Basal testosterone and spleen weight (n=6). (D) Basal testosterone and spleen length (n=6). All graphs presented as simple linear regression.

No correlations were found between 0-hour plasma estradiol and thymic measurements.

There was a trend (p=0.0657) for a negative correlation between 0-hour plasma 17β-estradiol and 0-hour relative spleen length in intact males. No other significant correlations were found between 0-hour plasma 17-estradiol and splenic measurements.

INTESTINAL MORPHOLOGY

Correlations were made between 0-hour hormone levels and morphological measurements to determine if any relationships existed between basal hormones and morphology.

A borderline significant (p=0.0581) positive correlation existed between 0-hour plasma testosterone and 24-hour villus length in the ileum mucosa of intact males. No other significant correlations were found between 0-hour plasma testosterone and morphological measurements of the ileum mucosa.

A significant positive correlation was seen between 0-hour plasma testosterone and 0-hour crypt depth in the ileum Peyer's patches of intact males. 0-hour plasma testosterone tended (p=0.0753) to be positively correlated with 0-hour submucosa width in the ileum Peyer's patches of intact males. There was a trend (p=0.0632) for 0-hour plasma testosterone to be negatively associated with 0-hour villus length in the ileum Peyer's patches of castrated males. 0-hour plasma testosterone had a significant positive correlation with 24-hour villus length in the ileum Peyer's patches of intact males.

There was a significant positive correlation between 0-hour plasma testosterone and 0-hour villus length in the jejunum of intact males. A significant negative correlation between 0-

hour plasma testosterone and 0-hour submucosa width was found in the jejunum of intact males.

A trend (p=0.0923) for a negative correlation existed between 0-hour plasma estradiol and 0-hour submucosa width in the ileum mucosa of intact males. Castrated males tended (p=0.0898) to have a positive correlation between 0-hour plasma estradiol and 0-hour villus length in the ileum mucosa.

No significant correlations with estradiol were found in either the ileum Peyer's patches or jejunum.

INTESTINAL NEUTROPHILS

Correlations were made using 0-hour a 24-hour hormone levels to determine what relationships existed between intestinal neutrophil numbers and basal and contemporary hormones.

Basal testosterone levels tended (p=0.0900) to be and were significantly positively correlated with 0-hour neutrophil numbers in the villi and crypts, respectively, in the ileum mucosa of intact males (Fig. 21A-B). The crypts at the same timepoint in castrated males saw a trend (p=0.072) for a negative correlation with basal testosterone. No significant correlations between 24-hour testosterone and 24-hour neutrophil numbers in the ileum mucosa were seen.

No significant correlations between basal testosterone and 0-hour or 24-hour neutrophil numbers in the ileum Peyer's patches were seen. No significant correlations

between 24-hour testosterone and 24-hour neutrophil numbers in the ileum Peyer's patches were seen.

There was a significant positive correlation between basal testosterone and 0-hour neutrophils in the villi of the jejunum intact males. There were no significant correlations between basal testosterone and 24-hour neutrophils in the jejunum. A significant positive correlation between 24-hour testosterone and 24-hour neutrophils was seen in the villi of the jejunum of castrated males.

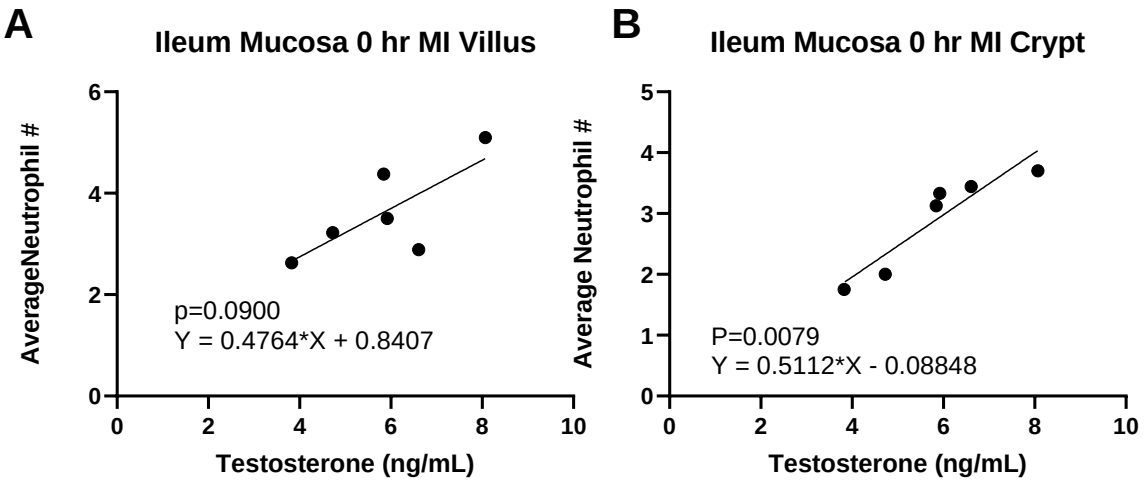

Figure 21. **Basal testosterone and ileal mucosa neutrophil numbers in intact males.** (A) Basal testosterone and neutrophils in the villi (n=6). (B) Basal testosterone and neutrophil in the villus crypts (n=6). All graphs presented as simple linear regression.

For intact males, the relative percentage change in testosterone and intestinal neutrophils were correlated to determine if any relationships existed between the weaning response in testosterone and the response in intestinal neutrophil numbers. Significant ad borderline significant (p=0.0536) negative correlations were found between the change in

testosterone and the change in neutrophil numbers in the villi of the ileum mucosa and ileum Peyer's patches, respectively (Fig. 22A-B). No significant correlations were found for the crypts or the jejunum.

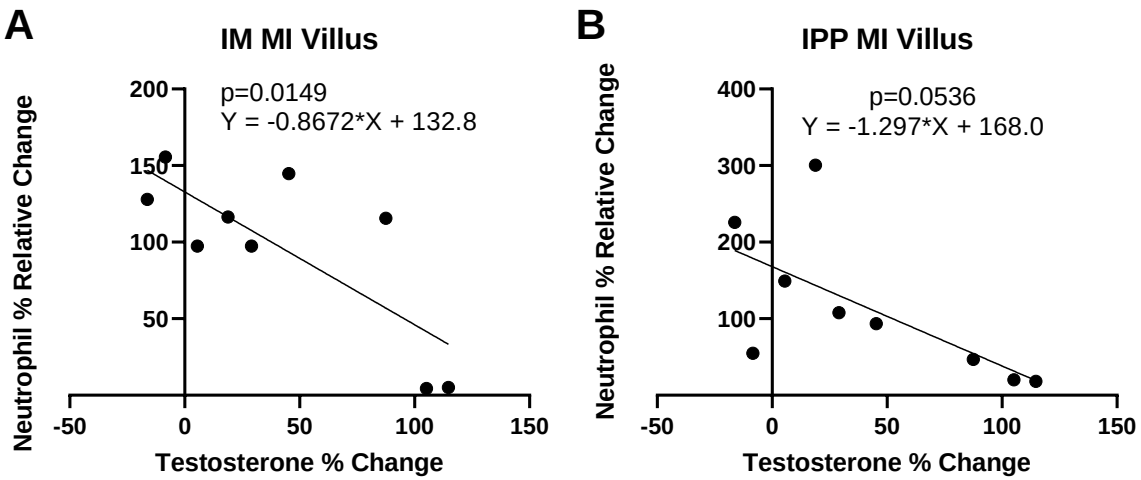

Figure 22. **Response to weaning in testosterone and neutrophils of the ileal villi.** (A) Change in testosterone and neutrophils of the ileal mucosal villi (n=9). (B) Change in testosterone and neutrophils of the villi of the ileum Peyer's patches (n=9). All graphs presented as simple linear regression.

No significant correlations between basal estradiol and 0-hour or 24-hour neutrophil numbers in the ileum mucosa were seen. Intact males saw a significant negative correlation between 24-hour estradiol and 24-hour neutrophils in the crypts, but no correlations were seen in castrated males.

There were no significant correlations between basal estradiol and 0-hour neutrophil numbers in the ileum Peyer's patches. Intact males at 24 hours tended (p=0.0822) to have a negative correlation between basal estradiol and neutrophils in the villi of the ileum Peyer's patches. Intact males saw a significant and a trend (p=0.0885) for a negative correlation

between 24-hour estradiol and 24-hour neutrophils in the villi and crypts, respectively, of the ileum Peyer's patches. No correlations were seen in castrated males.

There were no significant correlations between basal estradiol and 0-hour neutrophils in the jejunum. However, at 24 hours, basal estradiol was significantly and borderline significantly ($p=0.0559$) negatively correlated with neutrophils in the villi and crypts, respectively, in the jejunum of intact males. Similar results were seen in castrated males with a trend ($p=0.0890$) for and a borderline significant ($p=0.0523$) negative correlation between basal estradiol and neutrophils of the villi and crypts, respectively, of the jejunum. Intact males had a significant negative correlation between 24-hour estradiol and 24-hour neutrophils in the crypts of the jejunum, but no significant correlations were seen in castrated males.

INTESTINAL MAST CELLS

Correlations with hormones were made using the 0-hour values of the hormones to determine any relations between mast cell measurements and basal hormones.

Testosterone tended ($p=0.0638$) to be positively associated with 0-hour total mast cell numbers in the ileum mucosa submucosa of intact males (Fig. 23A). There were no other significant associations for the ileum mucosa submucosa.

Testosterone had a significant positive correlation with 0-hour total mast cell numbers in the ileum mucosa villus tips of intact males (Fig.23B). Testosterone had a significant negative association with 24-hour activated mast cell percentage in the ileum mucosa villus tips castrated males.

Testosterone had a significant positive correlation with 0-hour total mast cell numbers in the ileum mucosa villus crypts of intact males (Fig. 23C).

Testosterone had a significant positive correlation with 0-hour total mast cell numbers in the ileum Peyer's patches submucosa of intact males (Fig. 23D).

Testosterone had a significant positive correlation with 0-hour total mast cell numbers in the ileum Peyer's patches villus tips of intact males (Fig. 23E). Testosterone tended ($p=0.0727$) to be positively correlated with 0-hour total mast cell numbers in the ileum Peyer's patches villus tips of castrated males.

Testosterone tended ($p=0.0722$) to be positively associated with 0-hour total mast cell numbers in the ileum Peyer's patches villus crypts of intact males (Fig.23F).

Testosterone had a borderline significant ($p=0.0587$) positive association with 0-hour total mast cell numbers in the jejunum submucosa of intact males (Fig.23G). There were no other significant associations in the jejunum submucosa.

Testosterone had a significant positive correlation with 0-hour total mast cell numbers in the jejunum villus tips of intact males (Fig. 23H). Testosterone had a significant positive correlation with 24-hour activated mast cell percentage in the jejunum villus tips of intact males.

Testosterone tended ($p=0.0637$) to be positively associated with 0-hour total mast cell numbers in the jejunum villus crypts of intact males (Fig. 23I).

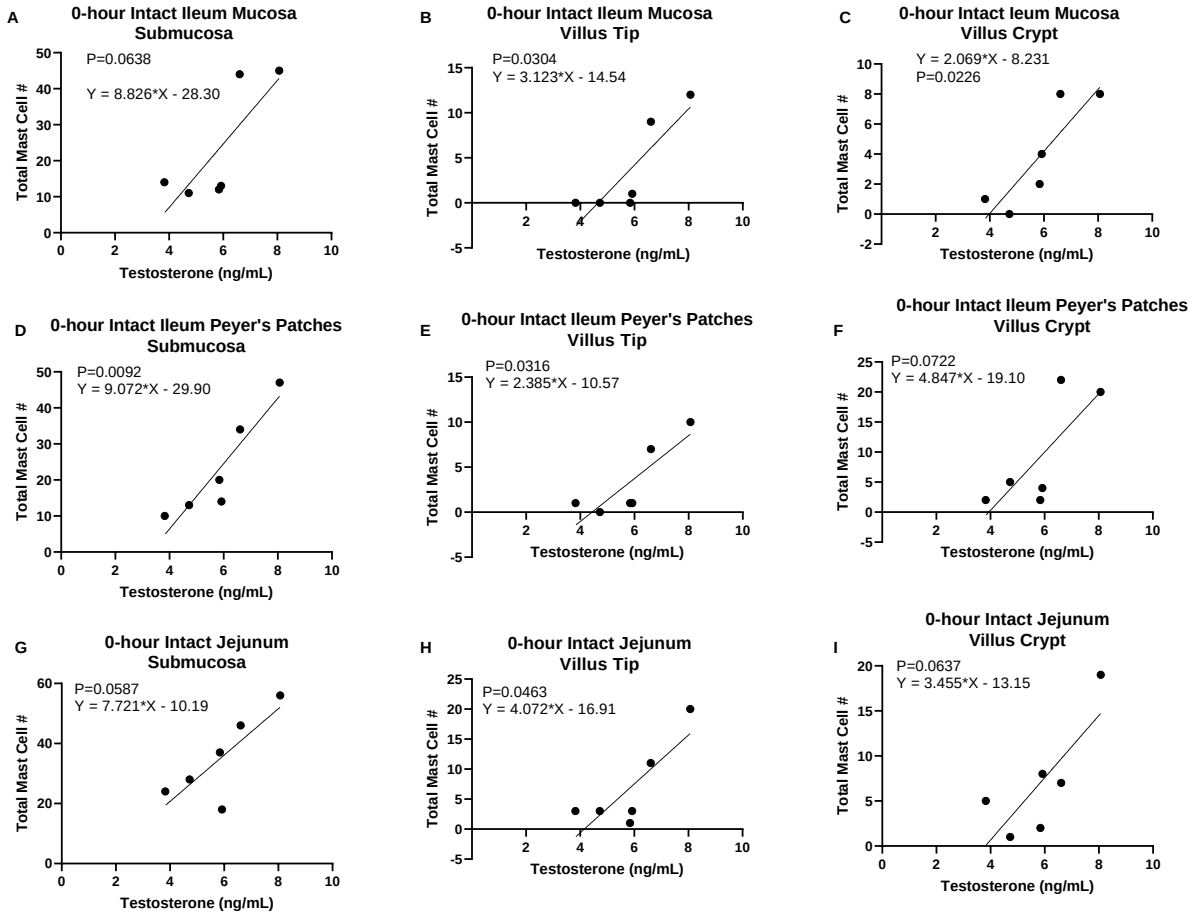

Figure 23. **Basal testosterone and intestinal mast cells in intact males.** (A) Ileum mucosa submucosa (n=6). (B) Ileum mucosa villus tips (n=6). (C) Ileum mucosa submucosa (n=6). (D) Ileum Peyer's patches submucosa (n=6). (E) Ileum Peyer's patches villus tips (n=6). (F) Ileum Peyer's patches villus crypts (n=6). (G) Jejunum submucosa (n=6). (H) Jejunum villus tips (n=6). (I) Jejunum villus crypts (n=6). All graphs presented as simple linear regression.

Estradiol tended (p=0.0999) to be negatively associated with total mast cell numbers at 24 hours in the ileum mucosa submucosa of castrated males. Estradiol tended (p=0.0877) to be negatively associated with 0-hour activated mast cell percentage in the ileum mucosa submucosa of intact males. Estradiol had a significant negative relationship with 24-hour activated mast cell percentage in the ileum mucosa submucosa castrated males.

There were no significant correlations between estradiol and mast cells in the ileum mucosa villus tips.

Estradiol tended (p=0.0772) to be negatively associated with total mast cell numbers at 24 hours in the ileum mucosa villus crypts of castrated males.

Estradiol had a significant negative relationship with total mast cell numbers at 24 hours in the ileum Peyer's patches submucosa of castrated males. Estradiol tended (0.0921) to be negatively associated with 0-hour activated mast cell percentage in the ileum Peyer's patches submucosa of intact males.

Estradiol had a significant positive relationship with 24-hour activated mast cell percentage in the ileum Peyer's patches villus tips of intact males. Estradiol had a significant negative relationship with total mast cell numbers at 0 hours in the ileum Peyer's patches villus crypts of castrated males. Estradiol had a borderline significantly (p=0.0528) negative relationship with total mast cell numbers at 24 hours in the ileum Peyer's patches villus crypts of castrated males. Estradiol had a significant positive relationship with 24-hour activated mast cell percentage in the ileum Peyer's patches villus crypts of intact males.

There were no significant correlations between estradiol and mast cells in the jejunum submucosa.

Estradiol had a significant negative relationship with total mast cell numbers at 24 hours in the jejunum villus tips of castrated males.

Estradiol had a significant negative association with 0-hour activated mast cell percentage in the jejunum villus crypts of intact males. Estradiol tended (p=0.0603) to be negatively associated with 24-hour activated mast cell percentage in the jejunum villus crypts of castrated males.

TNF-α AND IL-1β

Correlations were made between 0-hour plasma hormones and TNF-α levels at both 0- and 24-hours to determine any relations between TNF-α and basal hormones. 24-hour hormone correlations were made to determine any relations between TNF-α and post-weaning hormones.

There was a significant negative correlation between 0-hour plasma testosterone and 0-hour ileal mucosa TNF-α in intact males. There were no significant correlations between 24-hour plasma testosterone and ileal mucosa TNF-α. There were no significant correlations between 0-hour plasma testosterone and ileal mucosa IL-1β.

There was a trend (p=0.0840) for a positive correlation between 0-hour plasma testosterone and 24-hour mesenteric lymph node TNF-α in intact males. There were no significant correlations between 24-hour plasma testosterone and mesenteric lymph node TNF-α. In castrated males, 0-hour plasma testosterone had a negative correlation with mesenteric lymph node IL-1β at 0 hours and a positive correlation at 24 hours. No significant correlations were found between basal plasma testosterone and mesenteric lymph node IL-1β in intact males.

Percentage relative change in testosterone was correlated with percentage relative change in both TNF-α and IL-1β in the ileum mucosa and ileum mucosa in intact males to determine if there were any relationships between the responses to weaning. There were no correlations for the ileum mucosa. However, changes in both TNF-α and IL-1β tended (p=0.0943 and p=0.0983, respectively) to be negatively correlated with changes in testosterone (Fig. 24A-B).

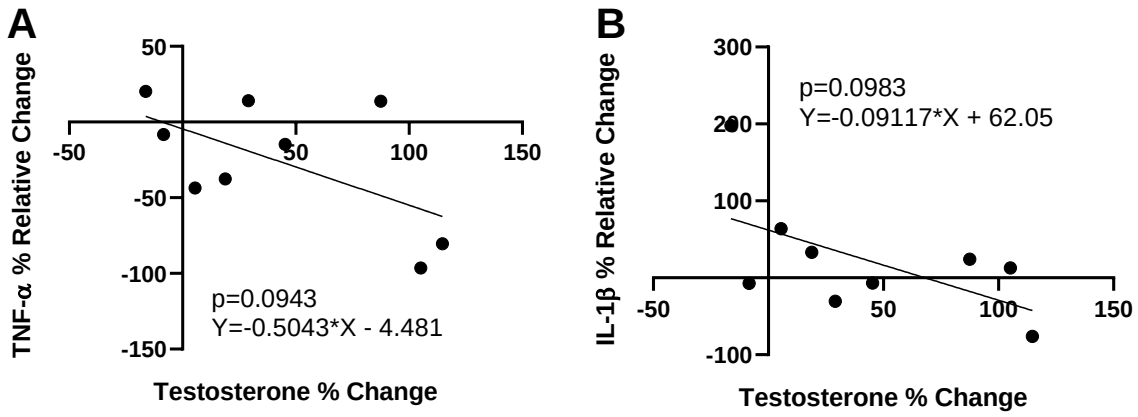

Figure 24. **Relative changes in plasma testosterone and mesenteric lymph node TNF-α and IL-1β in intact males.** (A) Testosterone and TNF-α (n=9). (B) Testosterone and IL-1β (n=9). All graphs presented as simple linear regression.

There were no significant correlations between 0-hour plasma 17β-estradiol or 24-hour 17β-estradiol and ileal mucosa TNF-α. There were no significant correlations between 0-hour plasma 17β-estradiol and ileal mucosa IL-1β.

There were no significant correlations between 0-hour plasma 17β-estradiol or 24-hour plasma 17β-estradiol and mesenteric lymph node TNF-α. No significant correlations were found between 0-hour plasma 17β-estradiol and mesenteric lymph node IL-1β.

DISCUSSION

This study sought to determine if castration is associated with a stronger inflammatory response to early-weaning in male piglets and to determine if sex hormones a potentially played an inhibitory role in the inflammatory response, but a supportive role in immune development. The results of this study indicate that castrated males have a stronger inflammatory response to early-weaning, possibly due to their lack of androgens, while androgens may promote immune development, but not activation, in intact males.

Testosterone, a hormone produced primarily by the testes in males, and 17B-estradiol, a hormone produced by aromatization of testosterone, were both reduced in castrated males compared to intact males. In response to weaning, plasma testosterone levels were significantly elevated 24h post-weaning in intact piglets indicating the weaning activated the hypothalamic gonadal axis. The coinciding of the cortisol peak with the testosterone trough in intact males are likely related to the well-established, opposing relationship between testosterone and cortisol which are both capable of downregulating the other's secretion, as previously stated. Unfortunately, blood samples were only taken at the 0-hour, 30-minute, and 24-hour timepoints, so there remains the distinct possibility that differences that appeared at other time points may have been missed (e.g., cortisol may have declined more quickly in one group).

The CBC data associated castration with stronger inflammatory response to early-weaning. Intact males had reduced neutrophils at 24 hours compared to baseline while the castrated males did not. It is not clear at this point what the cause is, but one possible

explanation is that intact males experience greater migration of neutrophils into the tissue Tfm rodents, which exhibit nonfunctional androgen receptors have significantly reduced neutrophil migration into tissue(Lai et al. 2012). In the present study, neutrophil numbers in intestinal tissues were not significantly different between intact and castrated males; however, in response to weaning, intact males exhibited increased neutrophils numbers at 24 h post-weaning in ileal Peyer's patch mucosa and villi jejunum, while this response was not significant in male castrates Opposite reactions to early weaning were seen in 0-hour to 24-hour lymphocyte percent fold change, with intact males showing an increase in lymphocytes and castrated males showing a decrease. This response contributed to a trend for increased neutrophil:lymphocyte ratio fold change in castrated piglets suggesting that castrated males have a greater inflammatory response to early weaning than intact males. Correlation analyses revealed negative associations between testosterone levels and blood neutrophils and a positive relationship with lymphocytes in intact males. Testosterone is known to have immunosuppressive properties (Bianchi 2019) and thus elevated testosterone in intact males may have contributed to the reduced N:L ratio and overall immune activation. In contrast, 17B-estradiol levels were positively correlated with increased blood neutrophils and negatively correlated with lymphocytes in castrated males, which may have contributed to the increased neutrophil:lymphocyte ratio in castrated males compared to intact males. Work by other researchers has established that estrogens have a bimodal effect on inflammation, with low doses promoting inflammation while high doses suppress inflammation (Ansar Ahmed et al. 1989, Ansar Ahmed et al. 1985, Voskuhl et al. 2016, Grossman 1984, Calabrese 2001, Voskuhl 2011). Therefore, it is expected that estradiol would be associated with a more inflammatory

profile in castrated males, who have low plasma estradiol, and why they would not be associated with an inflammatory profile in intact males, who have higher plasma estradiol.

Castration also had a significant influence on lymphoid organs, spleen, and thymus measurements. At 0 hours (pre-weaning), testosterone was positively associated with 0-hour spleen weight and length, but negatively associated with 24-hr spleen relative length. Intact males tended to have longer thymuses than castrates males at both time points, which is in agreement with the work of Leclercq et al. 2014b, who found that neonatal castration reduces thymic growth in pigs**.** This is further supported by the significant positive correlations between 0-hour plasma testosterone and 0-hour thymus absolute length and absolute and relative weight. The effect of castration on thymic growth seems to be heavily dependent on the timing of castration, as castration during adulthood (Heng et al. 2005, Sutherland et al. 2005) or in prepubertal adolescence (Grossman 1984), prevent or undo the thymic involution caused by androgens in males, while both this study and the aforementioned work by Leclercq et al., 2014 indicated the castration near the start of the neonatal androgen surge results in smaller thymuses. Plasma 17B-estradiol tended to be negatively associated with 0-hour relative spleen length in intact males but had no associations with any thymic measurements. This would indicate that 17B-estradiol is of low importance in the regulation splenic and thymic growth, which is in contradiction to existing evidence indicating that estrogens contribute to thymic involution (Greenstein, Mander and Fitzpatrick 1988, Tanriverdi et al. 2003) Differences in the response to weaning were also observed. Spleen size was increased in response to weaning in castrated males, but not in male castrates, which may suggest another indicator of elevated immune response to weaning in male castrates. Overall, these data suggest that while gonadal

androgens may support thymic and spleen development, castration and loss of androgens may lead to enhanced immune activation in response to weaning stress.

The changes in TNF-α supported the hypothesis that castration led to a stronger inflammatory response. Castrated males displayed a significant increase in ileum mucosa TNF-α, while intact males showed no significant change, and the fold change was significantly different between the groups suggesting an enhanced release of this pro-inflammatory cytokine in castrated pigs. 0-hour plasma testosterone was also negatively associated with ileum mucosa TNF-α in intact males. Although TNF-α levels in the mesenteric lymph node did not differ significantly between groups at either time point, or between timepoints within either group, the same difference in fold change was seen as with the ileum mucosa. These results are in agreement with previous findings on the negative relationship between testosterone and TNFα (Mohamad et al. 2019, Bianchi 2019). Plasma 17B-estradiol did not correlate with any TNF-α measurements in either tissue. This is in contradiction to the work of Watanabe et al., 2018 who found that an estrogen receptor alpha agonist increased TNF-α secretion in lymph nodes (Watanabe et al. 2018).

Castration did not appear to either increase or decrease in IL-1β measurements in the ileum mucosa and mesenteric lymph node. Although there was a trend for IL-1β in the ileum mucosa to increase over 24 hours in intact males, the fold change was not different between the gonadal groups. Castrated males did not differ from each other in IL-1β levels at either timepoint or in fold change in the ileum mucosa. Similar results were seen in the mesenteric lymph node. 0-hour plasma 17B-estradiol did not correlate with any measurements of IL-1B in either tissue. 0-hour plasma testosterone did not correlate with any measurements in the ileum

mucosa, but it did have positive association with 24-hour IL-1β and fold change and a negative association with 0-hour IL-1β in castrates. Overall, these results call into question the idea that gonadal hormones are regulating IL-1β levels in these tissues at this stage of development in the pig. This contradicts the current literature surrounding IL-1β, which indicates that testosterone suppresses IL-1β (Traish et al. 2018, Mohamad et al. 2019)and that estradiol has a bimodal effect with high doses being suppressive and low doses promoting IL-1β (Straub 2007).

Significant differences between gonadal groups did not manifest in C-reactive protein measurements. This is in agreement with the findings of Moya et al. 2008, who found no effect of castration at five days of age on C-reactive protein levels in swine. However, both Moya et al. 2008 and this study conflict with Mohamad et al. 2019, who found a negative relationship between testosterone and C-reactive protein in adult men. This could indicate that testosterone has a regulatory role towards C-reactive protein in adults, but not young prepubertal individuals.

This study produced evidence that indicates that neonatal testosterone may promote mast cell development. Most notably, each tissue section examined found either a significant or a trending positive association between 0-hour testosterone and 0-hour total mast cell numbers in intact males. This, combined with the lack of other consistently appearing correlations between mast cell measurements and testosterone, might indicate that testosterone at this age may promote mast cells proliferation, but not necessarily the immune response. 17β-estradiol was associated mainly with decreased mast cell numbers and activation in castrated males and was inconsistent in its relationships in intact males. This result is

interesting as (Lenz et al. 2018) had found that estradiol treatment increased both mast cell number and mast cell degranulation in the neonatal rat brain.

Intestinal neutrophil numbers generally increased post-weaning in both intact and castrated males, which was expected for animals undergoing an inflammatory response. The relationships between basal testosterone and 0-hour neutrophils in the ileum mucosa may indicate that testosterone may promote immune development in this region of the small intestine and that, because testosterone had opposite correlations in the two groups, its effects may either be dependent on the presence of some other testicular factor or that it may be bimodal in effect with low levels inhibiting development while high doses promote development. The negative correlations between change in testosterone and change in neutrophils of the ileal villi indicate that testosterone may play an immunosuppressive role during an on-going immune response. Estradiol appears to be uninvolved in development of gut neutrophils as no correlations were seen between basal estradiol and 0-hour neutrophils. However, estradiol appears to be potentially involved in regulation of neutrophils during future immune responses in the jejunum as basal estradiol was negatively associated with 24-hour neutrophils in the jejunal villi and crypts of both groups. 24-hour estradiol was also negatively associated with 24-hour neutrophils in the crypts of all three regions, indicating that estradiol could be downregulating neutrophils during an on-going immune response. Interestingly, these 24-hour relationships were only apparent in intact males, and this could possibly be explained by the bimodal actions of estrogens on the immune system (Ansar Ahmed et al. 1989, Ansar Ahmed et al. 1985, Voskuhl et al. 2016, Grossman 1984, Calabrese 2001, Voskuhl 2011).

Overall, the intestinal neutrophil findings suggest that testosterone promotes immune development in a basal state but is immunosuppressive during an inflammatory response.

Intestinal mucosal morphological analyses indicated potential protective and promotional effects of testosterone on intestinal villi. Basal testosterone in intact males was associated with greater villus length in all three intestinal locations examined. Estradiol was also associated with greater villus length at 0 hours in the ileum mucosa of castrated males. Differences between groups in changes in villus length appeared dependent on tissue, as the same type of difference (or similarity) did not appear in more than one group. It is interesting that the villus crypts of the ileum Peyer's patches region were reduced in length after 24 hours in intact males but not in castrated males), as the crypts are responsible for replenishing the epithelium and this decreased depth would indicate a decreased capability to replenish.

In summary, the results of this study strongly indicate that testosterone has an almost yin-yang role in the neonatal immune system: on one hand, it promotes immune development in an unperturbed animal, while on the other it suppresses the immune response when the animal is subjected to a stressor such as early-weaning. Intact males had higher basal immune markers. Testosterone was positively associated with higher basal intestinal and lymphoid organ immune markers. Testosterone was also associated with lower basal blood neutrophils, but higher intestinal neutrophil numbers, suggesting a possible basal immune trafficking role of androgens in the neonatal pig. Meanwhile, intact males had weaker inflammatory responses to early-weaning compared to castrated males and elevated testosterone post-weaning was associated with reduced immune and inflammatory markers.

This is a novel study exploring a poorly understood subject and its findings that gonadal androgens may support development of the peripheral and intestinal immune system but at the same time negatively regulate the activation of the immune system in response to weaning stress are of great significance. For example, this study's findings indicate that castration in piglets may be contributing to overactive inflammatory responses to common production stressors such as weaning. Further, given the impact of stress-associated inflammation on development and long-term health, it is possible that castration-associated enhanced immune responses may contribute to the increased mortality and morbidity observed in castrated piglets. There is also the potential that similar findings may be found in other meat-producing livestock who undergo early weaning and whose neonatal androgen surges are ablated.

While this novel study has helped illuminate a relatively untouched subject, it had its imperfections and there is much information that still needs to be uncovered. Although this study's findings indicate important relationships between testosterone and immune development and activation in pigs, further studies are required to test for a causal role of androgens in regulation of immune development in male pigs and to better understand the mechanisms responsible for the impact of castration on long-term immune development. For example, studies involving hormone replacement and blockade would better test if modulation of neonatal androgens can impact early immune develop and protect against weaning-associated inflammation in pigs. Secondly, although this study represents a first step towards understanding the role of neonatal androgens in immune development, it focused mainly on innate immunity. However, studies are currently underway to characterize B and T cell numbers and populations in the intestinal mucosa and other immune organs. If this study could be

repeated, a higher “n” for the 0-hour groups would be used to better demonstrate differences between groups and correlations with testosterone. Finally, this study examined the effects of castration on short-term immune development and response, but future studies will be needed to assess the long-term effects and further explore this relatively untouched region of scientific knowledge.

BIBLIOGRAPHY

BIBLIOGRAPHY

Altuwaijri, S., K. H. Chuang, K. P. Lai, J. J. Lai, H. Y. Lin, F. M. Young, A. Bottaro, M. Y. Tsai, W. P. Zeng, H. C. Chang, S. Yeh & C. Chang (2009) Susceptibility to autoimmunity and B cell resistance to apoptosis in mice lacking androgen receptor in B cells. *Mol Endocrinol,* 23**,** 444-53.

Aluwé, M., F. A. Tuyttens & S. Millet (2015) Field experience with surgical castration with anaesthesia, analgesia, immunocastration and production of entire male pigs: performance, carcass traits and boar taint prevalence. *Animal,* 9**,** 500-8.

Andersson, A. M., J. Toppari, A. M. Haavisto, J. H. Petersen, T. Simell, O. Simell & N. E. Skakkebaek (1998) Longitudinal reproductive hormone profiles in infants: peak of inhibin B levels in infant boys exceeds levels in adult men. *J Clin Endocrinol Metab,* 83**,** 675-81.

Ansar Ahmed, S., M. J. Dauphinée, A. I. Montoya & N. Talal (1989) Estrogen induces normal murine CD5+ B cells to produce autoantibodies. *J Immunol,* 142**,** 2647-53.

Ansar Ahmed, S., W. J. Penhale & N. Talal (1985) Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. *Am J Pathol,* 121**,** 531-51.

Arnold, A. P. & P. S. Burgoyne (2004) Are XX and XY brain cells intrinsically different? *Trends Endocrinol Metab,* 15**,** 6-11.

At-Taras, E. E., T. Berger, M. J. McCarthy, A. J. Conley, B. J. Nitta-Oda & J. F. Roser (2006) Reducing estrogen synthesis in developing boars increases testis size and total sperm production. *J Androl,* 27**,** 552-9.

Baxter, E., S. Jarvis, L. Sherwood, S. Robson, E. Ormandy, M. Farish, K. Smurthwaite, R. Roehe, A. Lawrence & S. Edwards (2009) Indicators of piglet survival in an outdoor farrowing system. *Livestock Science,* 124**,** 266-276.

Baxter, E. M., S. Jarvis, R. B. D'Eath, D. W. Ross, S. K. Robson, M. Farish, I. M. Nevison, A. B. Lawrence & S. A. Edwards (2008) Investigating the behavioural and physiological indicators of neonatal survival in pigs. *Theriogenology,* 69**,** 773-83.

Baxter, E. M., S. Jarvis, J. Palarea-Albaladejo & S. A. Edwards (2012) The weaker sex? The propensity for male-biased piglet mortality. *PLoS One,* 7**,** e30318.

Bergink, E. W., P. S. Janssen, E. W. Turpijn & J. van der Vies (1985) Comparison of the receptor binding properties of nandrolone and testosterone under in vitro and in vivo conditions. *J Steroid Biochem,* 22**,** 831-6.

Bianchi, I., A. Lleo, M. E. Gershwin & P. Invernizzi (2012) The X chromosome and immune associated genes. *J Autoimmun,* 38**,** J187-92.

Bianchi, V. E. (2019) The Anti-Inflammatory Effects of Testosterone. *J Endocr Soc,* 3**,** 91-107.

Bricout, V. & F. Wright (2004) Update on nandrolone and norsteroids: how endogenous or xenobiotic are these substances? *Eur J Appl Physiol,* 92**,** 1-12.

Burdick Sanchez, N. C., P. R. Broadway, J. A. Carroll, E. V. Gart, L. K. Bryan & S. D. Lawhon (2017) Weaned pigs experimentally infected with Salmonella display sexually dimorphic innate immune responses without affecting pathogen colonization patterns. *Transl Anim Sci,* 1**,** 69-76.

Calabrese, E. J. (2001) Estrogen and related compounds: biphasic dose responses. *Crit Rev Toxicol,* 31**,** 503-15.

Chandra, R., S. Federici, Z. H. Németh, B. Horváth, P. Pacher, G. Haskó, E. A. Deitch & Z. Spolarics (2011) Female X-chromosome mosaicism for NOX2 deficiency presents unique inflammatory phenotype and improves outcome in polymicrobial sepsis. *J Immunol,* 186**,** 6465-73.

Chen, C.-W., C.-Y. Jian, P.-H. Lin, C.-C. Chen, F.-K. Lieu, C. Soong, C.-C. Hsieh, C.-Y. Wan, G. Idova, S. Hu, S.-W. Wang & P. Wang (2016) Role of testosterone in regulating induction of TNF-α in rat spleen via ERK signaling pathway. *Steroids,* 111**,** 148-154.

Choi, N.-J., J. H. Hyun, J. M. Choi, E. J. Lee, K. H. Cho, Y. Kim, J. Chang, I. B. Chung, C. S. Chung & I. Choi (2007) Testicular Expression of Steroidogenic Enzyme Genes Is Related to a Transient Increase in Serum 19-nortestosterone during Neonatal Development in Pigs. *Asian-Aust. J. Anim. Sci.,* 20**,** 1832-1842.

Chuang, K. H., S. Altuwaijri, G. Li, J. J. Lai, C. Y. Chu, K. P. Lai, H. Y. Lin, J. W. Hsu, P. Keng, M. C. Wu & C. Chang (2009) Neutropenia with impaired host defense against microbial infection in mice lacking androgen receptor. *J Exp Med,* 206**,** 1181-99.

Colenbrander, B., F. H. de Jong & C. J. Wensing (1978) Changes in serum testosterone concentrations in the male pig during development. *J Reprod Fertil,* 53**,** 377-80.

Corbier, P., D. A. Edwards & J. Roffi (1992) The neonatal testosterone surge: a comparative study. *Arch Int Physiol Biochim Biophys,* 100**,** 127-31.

de Groot, J., L. Kruijt, J. W. Scholten, W. J. Boersma, W. G. Buist, B. Engel & C. G. van Reenen (2005) Age, gender and litter-related variation in T-lymphocyte cytokine production in young pigs. *Immunology,* 115**,** 495-505.

Douin-Echinard, V., S. Laffont, C. Seillet, L. Delpy, A. Krust, P. Chambon, P. Gourdy, J. F. Arnal & J. C. Guéry (2008) Estrogen receptor alpha, but not beta, is required for optimal dendritic cell differentiation and [corrected] CD40-induced cytokine production. *J Immunol,* 180**,** 3661-9.

Dussault, J. H., P. Walker, J. D. Dubois & F. Labrie (1977) The development of the hypothalamo-pituitary axis in the neonatal rat: sexual maturation in male and female rats as assessed by hypothalamic LHRH and pituitary and serum LH and FSH concentrations. *Can J Physiol Pharmacol,* 55**,** 1091-7.

Emery, M. J., H. F. Krous, J. M. Nadeau-Manning, B. T. Marck & A. M. Matsumoto (2005) Serum testosterone and estradiol in sudden infant death. *J Pediatr,* 147**,** 586-91.

Ford, J. J. (1982) Testicular control of defeminization in male pigs. *Biol Reprod,* 27**,** 425-30.

--- (1983a) Postnatal differentiation of sexual preference in male pigs. *Horm Behav,* 17**,** 152-62.

--- (1983b) Serum estrogen concentrations during postnatal development in male pigs. *Proc Soc Exp Biol Med,* 174**,** 160-4.

Forsdyke, D. R. (2009) X chromosome reactivation perturbs intracellular self/not-self discrimination. *Immunol Cell Biol,* 87**,** 525-8.

Friedman, S. B., L. J. Grota & L. A. Glasgow (1972) Differential susceptibility of male and female mice to encephalomyocarditis virus: effects of castration, adrenalectomy, and the administration of sex hormones. *Infect Immun,* 5**,** 637-44.

Galiot, L., I. Lachance, J. P. Laforest & F. Guay (2018) Modelling piglet growth and mortality on commercial hog farms using variables describing individual animals, litters, sows and management factors. *Anim Reprod Sci,* 188**,** 57-65.

González, D. A., B. B. Díaz, M. e. C. Rodríguez Pérez, A. G. Hernández, B. N. Chico & A. C. de León (2010) Sex hormones and autoimmunity. *Immunol Lett,* 133**,** 6-13.

Gordon, A. E., O. Al Madani, D. M. Weir, A. Busuttil & C. Blackwell (1999) Cortisol levels and control of inflammatory responses to toxic shock syndrome toxin-1 (TSST-1): the prevalence of night-time deaths in sudden infant death syndrome (SIDS). *FEMS Immunol Med Microbiol,* 25**,** 199-206.

Greenstein, B. D., B. J. Mander & F. T. Fitzpatrick (1988) Effects of an aromatase inhibitor on testosterone-induced inhibition of thymus growth in immature female rats. *J Endocrinol,* 119**,** 65-7.

Grimaldi, C. M., J. Cleary, A. S. Dagtas, D. Moussai & B. Diamond (2002) Estrogen alters thresholds for B cell apoptosis and activation. *J Clin Invest,* 109**,** 1625-33.

Grossman, C. J. (1984) Regulation of the immune system by sex steroids. *Endocr Rev,* 5**,** 435-55.

Gubbels Bupp, M. R. & T. N. Jorgensen (2018) Androgen-Induced Immunosuppression. *Front Immunol,* 9**,** 794.

Heng, T. S., G. L. Goldberg, D. H. Gray, J. S. Sutherland, A. P. Chidgey & R. L. Boyd (2005) Effects of castration on thymocyte development in two different models of thymic involution. *J Immunol,* 175**,** 2982-93.

Ibáñez, L., A. M. Jaramillo, A. Ferrer & F. de Zegher (2005) High neutrophil count in girls and women with hyperinsulinaemic hyperandrogenism: normalization with metformin and flutamide overcomes the aggravation by oral contraception. *Hum Reprod,* 20**,** 2457-62.

Ishizuka, M., M. Hatori, T. Suzuki, Y. Miki, A. D. Darnel, C. Tazawa, T. Sawai, M. Uzuki, Y. Tanaka, S. Kokubun & H. Sasano (2004) Sex steroid receptors in rheumatoid arthritis. *Clin Sci (Lond),* 106**,** 293-300.

Jacela, J., S. Dritz, J. DeRouchey, M. D. Tokach, R. D. Goodband & J. L. Nelssen (2011) Field evaluation of the effects of a porcine circovirus type 2 vaccine on finishing pig growth performance, carcass characteristics, and mortality rate in a herd with a history of porcine circovirus-associated disease. *Journal of Swine Health and Production,* 19**,** 10-18.

Jacenik, D., A. I. Cygankiewicz, A. Mokrowiecka, E. Małecka-Panas, J. Fichna & W. M. Krajewska (2019) Sex- and Age-Related Estrogen Signaling Alteration in Inflammatory Bowel Diseases: Modulatory Role of Estrogen Receptors. *Int J Mol Sci,* 20.

Klein, S. L. & K. L. Flanagan (2016) Sex differences in immune responses. *Nat Rev Immunol,* 16**,** 626-38.

Lai, J. J., K. P. Lai, K. H. Chuang, P. Chang, I. C. Yu, W. J. Lin & C. Chang (2009) Monocyte/macrophage androgen receptor suppresses cutaneous wound healing in mice by enhancing local TNF-alpha expression. *J Clin Invest,* 119**,** 3739-51.

Lai, J. J., K. P. Lai, W. Zeng, K. H. Chuang, S. Altuwaijri & C. Chang (2012) Androgen receptor influences on body defense system via modulation of innate and adaptive immune systems: lessons from conditional AR knockout mice. *Am J Pathol,* 181**,** 1504-12.

Lanthier, F., Y. Lou, M. A. Terner & E. J. Squires (2006) Characterizing developmental changes in plasma and tissue skatole concentrations in the prepubescent intact male pig. *J Anim Sci,* 84**,** 1699-708.

Larriestra, A. J., S. Wattanaphansak, E. J. Neumann, J. Bradford, R. B. Morrison & J. Deen (2006) Pig characteristics associated with mortality and light exit weight for the nursery phase. *Can Vet J,* 47**,** 560-6.

Leclercq, C., A. Prunier & E. Merlot (2014a) Effects of neonatal surgical castration and immunocastration in male pigs on blood T lymphocytes and health markers. *Animal,* 8**,** 836-43.

Leclercq, C., A. Prunier, F. Thomas & E. Merlot (2014b) Neonatal surgical castration of male pigs reduces thymic growth but has moderate consequences on thymocytes. *J Anim Sci,* 92**,** 2415-21.

Lenz, K. M., L. A. Pickett, C. L. Wright, K. T. Davis, A. Joshi & M. M. McCarthy (2018) Mast Cells in the Developing Brain Determine Adult Sexual Behavior. *J Neurosci,* 38**,** 8044-8059.

Libert, C., L. Dejager & I. Pinheiro (2010) The X chromosome in immune functions: when a chromosome makes the difference. *Nat Rev Immunol,* 10**,** 594-604.

Long, H., H. Yin, L. Wang, M. E. Gershwin & Q. Lu (2016) The critical role of epigenetics in systemic lupus erythematosus and autoimmunity. *J Autoimmun,* 74**,** 118-138.

Mackey, E., K. M. Thelen, V. Bali, M. Fardisi, M. Trowbridge, C. L. Jordan & A. J. Moeser (2020) Perinatal androgens organize sex differences in mast cells and attenuate anaphylaxis severity into adulthood. *Proc Natl Acad Sci U S A,* 117**,** 23751-23761.

Mariscal, D. V., P. L. Wolfe, E. G. Bergfeld, A. S. Cupp, F. N. Kojima, K. E. Fike, T. Sánchez, M. E. Wehrman, R. K. Johnson, R. J. Kittok, J. J. Ford & J. E. Kinder (1996) Comparison of circulating concentrations of reproductive hormones in boars of lines selected for size of testes or number of ovulations and embryonal survival to concentrations in respective control lines. *J Anim Sci,* 74**,** 1905-14.

Margery-Muir, A. A., C. Bundell, D. Nelson, D. M. Groth & J. D. Wetherall (2017) Gender balance in patients with systemic lupus erythematosus. *Autoimmun Rev,* 16**,** 258-268.

Mauvais-Jarvis, F., S. L. Klein & E. R. Levin (2020) Estradiol, Progesterone, Immunomodulation, and COVID-19 Outcomes. *Endocrinology,* 161.

McCarthy, M. M. (2008) Estradiol and the developing brain. *Physiol Rev,* 88**,** 91-124.

Merlot, E., F. Thomas & A. Prunier (2013) Comparison of immune and health markers in intact and neonatally castrated male pigs. *Vet Rec,* 173**,** 317.

Mohamad, N. V., S. K. Wong, W. N. Wan Hasan, J. J. Jolly, M. F. Nur-Farhana, S. Ima-Nirwana & K. Y. Chin (2019) The relationship between circulating testosterone and inflammatory cytokines in men. *Aging Male,* 22**,** 129-140.

Mohammad, I., I. Starskaia, T. Nagy, J. Guo, E. Yatkin, K. Väänänen, W. T. Watford & Z. Chen (2018) Estrogen receptor α contributes to T cell-mediated autoimmune inflammation by promoting T cell activation and proliferation. *Sci Signal,* 11.

Morales, J., A. Dereu, A. Manso, L. de Frutos, C. Piñeiro, E. G. Manzanilla & N. Wuyts (2017) Surgical castration with pain relief affects the health and productive performance of pigs in the suckling period. *Porcine Health Manag,* 3**,** 18.

Morales, J., T. Velasco, V. Tovar, G. Fragoso, A. Fleury, C. Beltrán, N. Villalobos, A. Aluja, L. F. Rodarte, E. Sciutto & C. Larralde (2002) Castration and pregnancy of rural pigs significantly increase the prevalence of naturally acquired Taenia solium cysticercosis. *Vet Parasitol,* 108**,** 41-8.

Moya, S., L. Boyle, P. Lynch & S. Arkins (2008) Effect of surgical castration on the behavioural and acute phase responses of 5-day-old piglets. *Applied Animal Behaviour Science,* 111**,** 133-145.

Nelson, D. L. (2006) NEMO, NFkappaB signaling and incontinentia pigmenti. *Curr Opin Genet Dev,* 16**,** 282-8.

Nevrkla, P., Z. Hadas, P. Horky & V. Kamanova (2017) Effect of Genotype and Sex of Piglets on Their Losses Before Weaning. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis,* 65**,** 893-897.

Niemann, J. T., J. P. Rosborough, S. Youngquist, A. P. Shah, R. J. Lewis, Q. T. Phan & S. G. Filler (2009) Cardiac function and the proinflammatory cytokine response after recovery from cardiac arrest in swine. *J Interferon Cytokine Res,* 29**,** 749-58.

Oster, M., E. Muráni, S. Ponsuksili, R. B. D'Eath, S. P. Turner, G. Evans, L. Thölking, E. Kurt, R. Klont, A. Foury, P. Mormède & K. Wimmers (2014) Transcriptional responses of PBMC in psychosocially stressed animals indicate an alerting of the immune system in female but not in castrated male pigs. *BMC Genomics,* 15**,** 967.

Pace, S., L. Sautebin & O. Werz (2017) Sex-biased eicosanoid biology: Impact for sex differences in inflammation and consequences for pharmacotherapy. *Biochem Pharmacol,* 145**,** 1-11.

Palaszynski, K. M., K. K. Loo, J. F. Ashouri, H. B. Liu & R. R. Voskuhl (2004) Androgens are protective in experimental autoimmune encephalomyelitis: implications for multiple sclerosis. *J Neuroimmunol,* 146**,** 144-52.

Palaszynski, K. M., D. L. Smith, S. Kamrava, P. S. Burgoyne, A. P. Arnold & R. R. Voskuhl (2005) A yin-yang effect between sex chromosome complement and sex hormones on the immune response. *Endocrinology,* 146**,** 3280-5.

Panzardi, A., M. L. Bernardi, A. P. Mellagi, T. Bierhals, F. P. Bortolozzo & I. Wentz (2013) Newborn piglet traits associated with survival and growth performance until weaning. *Prev Vet Med,* 110**,** 206-13.

Peterson, D. R., G. van Belle & N. M. Chinn (1979) Epidemiologic comparisons of the sudden infant death syndrome with other major components of infant mortality. *Am J Epidemiol,* 110**,** 699-707.

Pohl, C. S., J. E. Medland, E. Mackey, L. L. Edwards, K. D. Bagley, M. P. DeWilde, K. J. Williams & A. J. Moeser (2017) Early weaning stress induces chronic functional diarrhea, intestinal barrier defects, and increased mast cell activity in a porcine model of early life adversity. *Neurogastroenterol Motil,* 29.

Radojevic, K., N. Arsenovic-Ranin, D. Kosec, V. Pesic, I. Pilipovic, M. Perisic, B. Plecas-Solarovic & G. Leposavic (2007) Neonatal castration affects intrathymic kinetics of T-cell differentiation and the spleen T-cell level. *J Endocrinol,* 192**,** 669-82.

Rider, V., X. Li, G. Peterson, J. Dawson, B. F. Kimler & N. I. Abdou (2006) Differential expression of estrogen receptors in women with systemic lupus erythematosus. *J Rheumatol,* 33**,** 1093-101.

Roden, A. C., M. T. Moser, S. D. Tri, M. Mercader, S. M. Kuntz, H. Dong, A. A. Hurwitz, D. J. McKean, E. Celis, B. C. Leibovich, J. P. Allison & E. D. Kwon (2004) Augmentation of T cell levels and responses induced by androgen deprivation. *J Immunol,* 173**,** 6098-108.

Roubinian, J. R., N. Talal, J. S. Greenspan, J. R. Goodman & P. K. Siiteri (1978) Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F1 mice. *J Exp Med,* 147**,** 1568-83.

Rubinow, D. R., C. A. Roca, P. J. Schmidt, M. A. Danaceau, K. Putnam, G. Cizza, G. Chrousos & L. Nieman (2005) Testosterone suppression of CRH-stimulated cortisol in men. *Neuropsychopharmacology,* 30**,** 1906-12.

Schwarzenberger, F., G. S. Toole, H. L. Christie & J. I. Raeside (1993) Plasma levels of several androgens and estrogens from birth to puberty in male domestic pigs. *Acta Endocrinol (Copenh),* 128**,** 173-7.

Scott, J. L., J. R. Wirth, J. Eudaly, P. Ruiz & M. A. Cunningham (2017) Complete knockout of estrogen receptor alpha is not directly protective in murine lupus. *Clin Immunol,* 183**,** 132-141.

Seminog, O. O., A. B. Seminog, D. Yeates & M. J. Goldacre (2015) Associations between Klinefelter's syndrome and autoimmune diseases: English national record linkage studies. *Autoimmunity,* 48**,** 125-8.

Siracusa, M. C., M. G. Overstreet, F. Housseau, A. L. Scott & S. L. Klein (2008) 17beta-estradiol alters the activity of conventional and IFN-producing killer dendritic cells. *J Immunol,* 180**,** 1423-31.

Smahi, A., G. Courtois, P. Vabres, S. Yamaoka, S. Heuertz, A. Munnich, A. Israël, N. S. Heiss, S. M. Klauck, P. Kioschis, S. Wiemann, A. Poustka, T. Esposito, T. Bardaro, F. Gianfrancesco, A. Ciccodicola, M. D'Urso, H. Woffendin, T. Jakins, D. Donnai, H. Stewart, S. J. Kenwrick, S. Aradhya, T. Yamagata, M. Levy, R. A. Lewis & D. L. Nelson (2000) Genomic rearrangement in NEMO impairs NF-kappaB activation and is a cause of incontinentia pigmenti. The International Incontinentia Pigmenti (IP) Consortium. *Nature,* 405**,** 466-72.

Smith-Bouvier, D. L., A. A. Divekar, M. Sasidhar, S. Du, S. K. Tiwari-Woodruff, J. K. King, A. P. Arnold, R. R. Singh & R. R. Voskuhl (2008) A role for sex chromosome complement in the female bias in autoimmune disease. *J Exp Med,* 205**,** 1099-108.

Spolarics, Z., G. Peña, Y. Qin, R. J. Donnelly & D. H. Livingston (2017) Inherent X-Linked Genetic Variability and Cellular Mosaicism Unique to Females Contribute to Sex-Related Differences in the Innate Immune Response. *Front Immunol,* 8**,** 1455.

Straub, R. H. (2007) The complex role of estrogens in inflammation. *Endocr Rev,* 28**,** 521-74.

Straw, B., C. Dewey, J. Kober & S. Henry (2001) Factors Associated with Death Due to Hemorrhagic Bowel Syndrome in Two Large Commercial Swine Farms. *Journal of Swine Health and Production,* 10**,** 75-79.

Sutherland, J. S., G. L. Goldberg, M. V. Hammett, A. P. Uldrich, S. P. Berzins, T. S. Heng, B. R. Blazar, J. L. Millar, M. A. Malin, A. P. Chidgey & R. L. Boyd (2005) Activation of thymic regeneration in mice and humans following androgen blockade. *J Immunol,* 175**,** 2741-53.

Tanriverdi, F., L. F. Silveira, G. S. MacColl & P. M. Bouloux (2003) The hypothalamic-pituitary-gonadal axis: immune function and autoimmunity. *J Endocrinol,* 176**,** 293-304.

Traish, A., J. Bolanos, S. Nair, F. Saad & A. Morgentaler (2018) Do Androgens Modulate the Pathophysiological Pathways of Inflammation? Appraising the Contemporary Evidence. *J Clin Med,* 7.

Verthelyi, D. & S. A. Ahmed (1994) 17 beta-estradiol, but not 5 alpha-dihydrotestosterone, augments antibodies to double-stranded deoxyribonucleic acid in nonautoimmune C57BL/6J mice. *Endocrinology,* 135**,** 2615-22.

Voskuhl, R. (2011) Sex differences in autoimmune diseases. *Biol Sex Differ,* 2**,** 1.

Voskuhl, R. R., H. Wang, T. C. Wu, N. L. Sicotte, K. Nakamura, F. Kurth, N. Itoh, J. Bardens, J. T. Bernard, J. R. Corboy, A. H. Cross, S. Dhib-Jalbut, C. C. Ford, E. M. Frohman, B. Giesser, D. Jacobs, L. H. Kasper, S. Lynch, G. Parry, M. K. Racke, A. T. Reder, J. Rose, D. M. Wingerchuk, A. J. MacKenzie-Graham, D. L. Arnold, C. H. Tseng & R. Elashoff (2016) Estriol combined with glatiramer acetate for women with relapsing-remitting multiple sclerosis: a randomised, placebo-controlled, phase 2 trial. *Lancet Neurol,* 15**,** 35-46.

Wang, J., C. M. Syrett, M. C. Kramer, A. Basu, M. L. Atchison & M. C. Anguera (2016) Unusual maintenance of X chromosome inactivation predisposes female lymphocytes for increased expression from the inactive X. *Proc Natl Acad Sci U S A,* 113**,** E2029-38.

Watanabe, Y., E. Makino, R. Tajiki-Nishino, A. Koyama, H. Tajima, M. Ishimota & T. Fukuyama (2018) Involvement of estrogen receptor α in pro-pruritic and pro-inflammatory responses in a mouse model of allergic dermatitis. *Toxicol Appl Pharmacol,* 355**,** 226-237.

Wilder, R. L. (1996) Hormones and autoimmunity: animal models of arthritis. *Baillieres Clin Rheumatol,* 10**,** 259-71.

Williams, P. N., C. T. Collier, J. A. Carroll, T. H. Welsh & J. C. Laurenz (2009) Temporal pattern and effect of sex on lipopolysaccharide-induced stress hormone and cytokine response in pigs. *Domest Anim Endocrinol,* 37**,** 139-47.

Winter, J. S., C. Faiman, W. C. Hobson, A. V. Prasad & F. I. Reyes (1975) Pituitary-gonadal relations in infancy. I. Patterns of serum gonadotropin concentrations from birth to four years of age in man and chimpanzee. *J Clin Endocrinol Metab,* 40**,** 545-51.

Winter, J. S., I. A. Hughes, F. I. Reyes & C. Faiman (1976) Pituitary-gonadal relations in infancy: 2. Patterns of serum gonadal steroid concentrations in man from birth to two years of age. *J Clin Endocrinol Metab,* 42**,** 679-86.

Yacoub Wasef, S. Z. (2004) Gender differences in systemic lupus erythematosus. *Gend Med,* 1**,** 12-7.