EFFECT OF INTRALITTER SIZE ON 60 AND 95 DAY FETAL MYOGENESIS AND DEVELOPMENT IN THE PIG

by

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Abstract

Piglet birth weight (BtW) is inextricably associated with preweaning survival. The non-linear antagonistic relationship between BtW and mortality risk is more severe in pigs <1.11 kg BtW than in pigs with BtW >1.11 kg. Thus, our research categorized fetal pigs as small (SM), median (MD), or large (LG) size depending on relative crown-rump length at d-60 gestation or BW within litter at d-95 gestation to evaluate differences in fetal myogenesis and development. At both d-60 and d-95, brain weights did not differ but brain weight:liver weight ratio was larger ($P<0.05$) for SM compared MD and LG. Cross-sectional area of the *Longissimus* muscle increased with increasing fetal size so that LG and MD had larger ($P<0.05$) whole muscle cross-sectional areas than SM although number of primary and relative secondary muscle fibers and their respective cross-sectional areas did not differ. Day-60 mRNA abundance of both *IGF-1* and *IGF-2* in SM was greater ($P<0.05$) than in MD and LG. Fetal size had a marginally significant effect ($P=0.103$) on gene expression of *IGF-2 receptor* with expression least in LG. Small fetuses had greater ($P<0.05$) d-60 *MyoD* gene expression and d-95 serum IGF-1 levels than MD and LG. Two experiments were conducted to determine the effects of feeding low or high doses of chlortetracycline (CTC) and antibiotic alternatives (pharmacologic Zn, Cu, and essential oil), alone or in combination, on nursery pig growth performance. Pharmacologic Cu (125 ppm from $CuSO_4$), Zn (2,000-3,000 ppm from ZnO), or increasing CTC level (0-441 ppm) improved growth performance additively while *Origanum* essential oil (0.005%) elicited no benefits and decreased G:F. Interactive effects of supplemental Cu (125 ppm), Zn (150 ppm), and ractopamine HCl (10 ppm for 28 d) on finishing pig growth performance, carcass characteristics, and antimicrobial susceptibility of enteric bacteria was evaluated. Ractopamine increased ($P<0.001$) ADG, G:F, HCW, percentage carcass yield, loin depth, percent fat-free lean, and decreased ($P=0.014$) backfat. Copper and/or Zn did not improve ADG, ADFI, or carcass traits. Fecal *E. coli* and *Enterococcus* bacterial resistance to most antibiotics decreased ($P<0.05$) over time or was stable for those that had a low baseline percentage of resistance.

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"...but those who hope in the Lord will renew their strength. They will soar on wings like eagles; they will run and not grow weary, they will walk and not be faint." Isaiah 40:31

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Chapter 1 - Birth Weight Threshold for Determining At-Risk Piglets.

Birth Weight is Associated with Piglet Mortality

The association of piglet birth weight with mortality risk early in life is strongly supported by research. The relationship between litter sizes > 12 live born piglets, light birth weights, and poor piglet preweaning survival has been known for at least 25 years (Tyler et al., 1990). At that time, Gardner et al. (1989) also reported that the odds ratio for piglet survival to 7 d old was 349 for piglets weighing > 2 kg at birth compared to piglets weighing < 0.601 kg at birth. Similarly, pigs weighing > 2 kg at birth were 20.1 times more likely to survive compared to piglets weighing < 0.801 kg.

More recently, Milligan et al. (2002a) observed 52 sows during 8 consecutive parities and reported larger litters were associated with poorer preweaning survivability, lighter birth weights, and negatively skewed birth weight distributions. Panzardi et al. (2013) concluded that piglets weighing < 1.257 kg at birth had greater likelihoods for mortality in the first 3 and 7 d of life compared to piglets weighing > 1.257 kg at birth. Similarly, Tanghe et al. (2014) reported piglets with birth weights of 1.27 kg had poorer survival to weaning compared to piglets weighing 1.55 kg at birth. Several other studies have reported decreasing preweaning piglet mortality as individual piglet birth weight increased and these observations are summarized in Table 1.1.

Both environmental and genetic components contribute to birth weight's effects on mortality. Based on individual records of 133,004 Danish Landrace and 89,928 Danish Yorkshire piglets, the genetic correlations between birth weight and survival to d 5 were moderately high for both maternal (r = 0.395 and 0.604 for respective breeds) and direct (r = 0.532 and 0.554) correlations (Su et al., 2008). Dufrasne et al. (2013) observed birth weight had

a high negative genetic correlation (-0.52) with preweaning mortality rate based on 24,376 crossbred pig records from a single farm. Regardless of housing system and breeding value for survival rate to 5 d post-partum, Pedersen et al. (2011) reported that lighter birth weight piglets had a greater probability of mortality via crushing or starvation than their heavier birth weight counterparts.

Birth weight is one of many maternal and neonatal physiological factors associated with piglet survival rate. The relationships between survival-to-weaning and birth weight, individual placental weight, and placental efficiency (ratio of pig weight to placental weight) were evaluated for 1,036 piglets from 118 litters. Birth weight proved to be the best predictor of survival-to-weaning (van Rens et al., 2005). Other factors associated with survival to weaning at 28 d of age have been identified as vigor regardless of birth weight, and latency to first suckling (Baxter et al., 2008).

Multiple Factors Affect Piglet Birth Weight and Mortality

Piglet birth weight is a multifactorial trait influenced by physiological and environmental factors that directly or interactively affect the growth and development of fetuses. Factors such as maternal parity and birth litter size affect birth weight interactively (Bergstrom, 2011). Considered on a common litter size basis, individual piglet birth weight has been shown to be affected by both the genotype of the maternal uterine environment and piglet genotype (Miles et al., 2012).

In the same way, preweaning mortality is the compounded result of physiological and environmental factors. For instance, Casellas et al. (2005) reported preweaning mortality varied by month, was greater for offspring of primiparous dams, and, according to Bergstrom (2011), is affected by the parity of the sow that piglets suckled. Because birth weight accounts for several

variables, the measure is a strong indicator of neonatal mortality risk. However, there are some instances where individual birth weight may not be the sole reliable predictor of preweaning mortality such as when there are large differences in genetic background. For example, offspring with a greater percentage of Meishan breeding have better survivability than Large White offspring when considered at the same body weight and litter size (Lee and Haley, 1995).

Birth Weight is Associated with Poor Lifetime Performance

Not only does birth weight have a large impact on piglet survival to weaning, but it also predicts subsequent growth performance and muscle fiber characteristics. Peterson (2008) reported that lighter birth weight pigs had decreased growth rates from birth to market compared to heavier birth weight pigs. Likewise, Douglas et al. (2013) used various statistical analyses (logistic regression, continuous linear plateau model, categorical analysis) to examine 2 databases (40,000 and 90,000 pigs) and observed that poor birth to slaughter growth rates were associated with low birth weight. Moreover, increasing birth weight was linearly associated with increased lifetime growth rate for piglets with birth weights up to 1.8 to 1.9 kg. However, increased birth weight was not associated with faster growth for pigs weighing > 1.8 to 1.9 kg. These data indicate that there may be a threshold above which further increases in birth weight do not predict increased growth rate.

Reduced growth rates mean that lighter birth weight pigs require more time to reach the target market weight. The probability of a pig attaining a full market value weight of at least 98 kg BW by 180 days of age becomes greater with increasing birth weight (Bergstrom, 2011). Among piglets originating from litters ≥ 12 born alive, lighter birth weight pigs had decreased BW at weaning, at 5 and 7 weeks post-weaning, and at the first marketing pull; thereby having increased days to market as compared to heavier littermates (Beaulieu et al., 2010). Specifically,

Quiniou et al. (2002) observed poorer growth rate of lighter birth weight pigs with pigs weighing 1 kg at birth requiring almost 14 more d to reach 105 kg BW than required by pigs weighing 2 kg. Observing a smaller number of animals, Gondret et al. (2005) similarly reported that pigs weighing between 0.8 and 1.1 kg at birth reached 102 kg BW, 12 d later than pigs with birth weights between 1.75 and 2.05 kg. The slower growth rates of lighter birth weight pigs is consistent with the observation that the genetic correlation between birth weight and HCW on a common age basis was observed to be 0.55 based on 13,029 crossbred pig records originating from a single farm (Dufrasne et al., 2013).

Additionally, Gondret et al. (2005) observed that, compared to heavier birth weight pigs, lower birth weight pigs exhibited 24% lower plasma IGF-1 levels, 14 to 20% greater cross-sectional area of muscle fibers, and tended to have a corresponding 13 to 20% reduction in total muscle fiber number. However, they did not observe an effect of birth weight on HCW, percent carcass yields, final backfat depths, or tissue lipid content. In agreement, Beaulieu et al. (2010) observed that birth weight had minimal effects on carcass quality.

In addition to exhibiting decreased growth performance and muscle growth potential, some data suggests birth weight is also associated with decreased reproductive performance and poorer growth potential of future offspring. Corson et al. (2009) monitored sow offspring over 2 generations by identifying the lightest and heaviest BW first generation females and subsequently observing their offspring (second generation). The lighter BW mothers had decreased litter sizes along with decreased average piglet weights and large litter weight variations compared to the heavier BW sows.

Biological Importance of Absolute or Relative Birth Weights

Based on the association of birth weight with both litter size and preweaning mortality, the attribution of decreased piglet survivability to either large birth litter sizes (and correspondingly low birth weights due to intrauterine growth restriction) or attribution to inherent physiological characteristics of smaller body size (regardless of birth littermate number) is somewhat confounded. The effect of uterine crowding associated with large litter sizes negatively impacts fetal development and the performance of the fetus later in life (Foxcroft et al., 2006). Consequently, a 1 kg birth weight piglet originating from a crowded uterine environment could have poorer subsequent performance than a 1 kg birth weight piglet with fewer littermates and originating from a litter not subjected to uterine crowding.

Hence, studies have sought to clarify whether growth potential is different among piglets with similar absolute birth weights but having either a relatively heavy or light birth weight within the piglet's birth litter. Berard et al. (2008) observed no interactive effects of litter size and birth weight upon growth and carcass traits thus indicating that expected performance inferences made on the absolute birth weight of pigs are valid, even when pigs originate from litters of different sizes.

Nonetheless, evaluating effects of individual weights within litters differing in numerical size is complicated by differences in prenatal mortality (0 to > 50%) and the timing of prenatal losses. Therefore, some litters with small numbers of pigs may have experienced relatively greater crowding than apparent at birth so caution must be employed when examining the effect of birth weight in relation to the size of the farrowed litter on different offspring performance indicators. Pardo et al. (2013) evaluated the performance and characteristics of piglets with common average birth weights but compared those having a heavy or intermediate intralitter

relative birth weight in litters characterized by light average birth weight to those having intermediate or low intralitter relative birth weight in litters characterized by heavy average birth weights. Impaired myogenesis and greater brain-sparing was in piglets originating from light average birth weight litters and categorized as heavy weight when compared to equivalent birth weight pigs originating from heavy average birth weight litters and categorized as light weight.

Physiological Pathways Affecting Light Birth Weight

Recently, Zhang et al. (2014) screened animals with high or low EBV's for individual birth weight. "Differentially selected regions" were identified, some of which contained genes from MDFIC (MyoD family inhibitor domain containing). These genes had functions in protein, metal, ion and ATP binding, viral process, and innate immune response. As reviewed by Herpin et al. (2002), greater skeletal muscle metabolic development and piglet birth weight are associated with improved thermoregulatory ability which corresponds to lower mortality rates for neonatal pigs.

In another attempt to identify physiological differences among piglets with different genetic potential for survival, Leenhouwers et al. (2002) explored the development of fetuses originating from litters of primiparous sows and having up to a 16.4% difference in piglet survival EBVs. Genetic selection for greater piglet survivability to weaning did not appear to be associated with litter average d 111 fetal body weight or within-litter fetal body weight variation, but actually favored piglets of litters with greater litter average cortisol levels and indicators of greater physiological maturity. It is important to note that these observations were based on litter averages and did not consider the relationship of individual fetal weight to survivability.

Other studies have established a negative relationship between piglet plasma cortisol and birth weight. Klemcke et al. (1993) reported a negative relationship between piglet plasma

cortisol levels and individual birth weight. Additionally, the adrenocortical response of smaller pigs when subjected to in vitro ACTH administration was greater than the response exhibited by larger piglets. Kranendonk et al. (2006) administered hydrocortisone-acetate to sows from either gestational d 21 to 50, 51 to 80, or from 81 to 110. The resulting sow salivary cortisol levels were elevated and no difference in total piglet number was observed; however, compared to offspring of sows not receiving hydrocortisone-acetate, the pooled offspring of hydrocortisone-acetate treated sows were characterized by fewer mummies, greater number born alive, smaller birth weights, and slower preweaning ADG. Given this negative relationship between plasma cortisol levels and birth weight as well as the strong positive relationship between birth weight and subsequent neonatal survivability, it therefore seems unlikely that birth weight and survival outcomes are mediated directly through elevated adrenocortical activity.

Leenhouwers et al. (2002) also reported increased litter average placental efficiency, measured as the ratio of birth weight to placental weight, tended to be associated with improved survival expectancies. Furthermore, Rootwelt et al. (2013) conducted an analysis of several piglet characteristics to determine which were associated with survival to weaning at d 35. They observed that in addition to decreased body mass index, increased mortality rate was also associated with decreased average birth weight, placental area, and placental weight.

While greater placental efficiency may reflect improved birth weight and thus be associated with lessened preweaning mortality, the relationship is not likely a causal one. Rootwelt et al. (2013) also observed that birth weight and placental area decreased as litter size increased. Mesa et al. (2003) reported that primiparous sows from genetic lines selected for larger litter sizes had more piglets with lighter birth weights, lighter placental weights, and 43% poorer placental efficiency (even when evaluated on a common litter size, 52% poorer) when

compared to randomly selected lines. Consequently, if large litters are characterized by reduced placental efficiency, poor placental efficiency is likely to be found associated with lighter birth weights and increased piglet mortality.

Interventions to Decrease Preweaning Mortality

Peripartum interventions can be implemented to improve birthweights and provide intensive management of neonates who have greater risk for mortality. Preweaning mortality occurs at a greater rate closer post-partum than later in the suckling period. Of the 16.8% preweaning mortality observed by Rootwelt et al. (2013), 78.5% died within the first 3 d post parturition. Consequently, preventive neonatal mortality strategies focused on giving low birth weight pigs a survival advantage within the period immediately following birth have great potential to improve preweaning mortality.

Kammersgaard et al. (2011) concluded that birth weight serves as the most critical determinant of neonates recuperating from hypothermia, likely via both direct and indirect means. Pedersen et al. (2011) observed piglets with lower body temperatures after birth had increased risk of mortality due to crushing, starvation, and disease. Moreover, lighter birth weight pigs also had a greater risk of mortality due to crushing and starvation. As reviewed by Kirkden et al. (2013), the incidence of piglet hypothermia and starvation can be reduced by implementing management practices that not only minimize health challenges and maximize sow milk production but also decrease farrowing duration and aid weak piglets via farrowing monitoring and interventions during the early post-natal period.

Other light birth weight offspring performance outcomes appear to be modulated by management practices. Surek et al. (2014) reported no correlation between birth weight and the preweaning ADG of heavy and light birth weight pigs when pigs were cross-fostered to

standardized litters of 11 piglets each and a birth weight CV of < 5%. It follows then that farm-specific management practices in the early neonatal period can affect the relationship of birth weight to preweaning mortality and subsequent pig performance.

Identification of At-Risk Piglets for Preweaning Mortality Based on Birth Weight

To facilitate strategic use of production resources, our goal was to identify "at-risk" piglets for preweaning mortality based on birth weight. Observations of 4,068 piglet birthweights and their corresponding preweaning survival rates across 4 different farms were compiled for meta-analysis (data from Jon. R. Bergstrom [Kansas State University, Manhattan, KS, personal communication] and Jourquin et al., 2015). All pigs were weighed individually within 18 h of birth. Average pre-wean mortality rates across the farms ranged from 9.6 to 15.2%. Our purpose was to identify a birthweight threshold for survival across different animal and farm specific influences on mortality.

A mixed effects logistic regression model was fit to estimate the probability of preweaning mortality based on birth weight. A random study effect was included to account for overall differences in mortality between the two studies. A piece-wise linear predictor was selected to best represent the drastic decrease in pre-wean mortality found as birth weights increase in the range of 0.5 to 1.0 kg and then the less extreme change in pre-wean mortality observed for changes in weight above 1.0 kg. The exact change point of the model was found by comparing the model fit for birth weights ranging from 0.5 kg to 2.0 kg based on maximizing the likelihood (Figure 1.1).

Results of the analysis indicated a 1.11 kg birth weight change point in the log odds of piglet preweaning mortality. Thus, every incremental change in birth weight below 1.11 kg had a

greater impact on mortality risk than incremental changes in birth weight above 1.11 kg. These differential relationships between mortality odds and birth weight above and below 1.11 kg are depicted in Figure 1.2 where the observed and modeled log odds of mortality for birth weight are plotted. This figure shows piglets weighing less than 1.11 kg are “at risk piglets” for poor preweaning survivability.

A linear predictor equation was used on the log scale of the preweaning mortality odds to estimate the associated preweaning mortality probability associated with every 50 g of piglet birth weight (Table 1.2). In addition, the percentage of piglets across the 4 farms which fell within each 50 g birth weight interval was calculated and piglets with a birth weight < 0.50 kg or > 2.3 kg were truncated into < 0.50 and > 2.30 weight categories, respectively. Across the 4 farms, 14.9% of the piglets had birth weights < 1.11 kg. Therefore, approximately 1 out of every 7 piglets born was within the weight range associated with increased risk for preweaning death.

In summary, individual piglet birth weight is strongly associated with a piglet’s risk of preweaning mortality. This relationship is not linear. Reducing the number of pigs with birth weights less than 1.11 kg would result in a substantial risk reduction for preweaning mortality relative to risk reduction made by improvements in birth weight of pigs weighing more than 1.11 kg at birth. A large percentage of the neonatal pig population falls below this weight threshold and therefore, interventions targeted at piglets below 1.11 kg have tremendous potential to improve piglet preweaning survivability. These pigs were determined to be “at risk” pigs using logistic regression analysis. Successful interventions may take the form of strategic neonatal intensive care or prenatal efforts to improve musculoskeletal development and birth weight. However, the latter approach of improving piglet birth weight may not only improve piglet

survivability outcomes, but also contribute to greater lifetime growth and productivity of the pig and profitability of the swine producer.

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Table 1.1. Piglet birth weight and associated preweaning mortality.

Piglet Birth Weight, kg Associated preweaning mortality, %							# of Piglets	Study
	≤ 1.0 40%			> 1.6 < 7%			14,950	Roehe et al., 1999
	< 1.0 17%[1]	> 1.0 3%[1]					12,041	Quiniou et al., 2002
	0.49 – 1.27 9.2%	1.271 – 1.54 6.4%	1.541 – 1.79 3.2%	1.791 – 2.75 2.7%			612	Panzardi et al., 2013
	0.90 32%		1.60 11%				745	Cabrera et al., 2012[2]
0.60 – 0.90 28.9 %	0.90 – 1.2 8.2%	1.2 – 1.5 3.8%	1.5 – 1.8 1.9%		1.8 – 2.1 3.7%	> 2.1 1.6%	2,004	Da Silva Duarte Furtado, 2012
< 0.68, 0.68 – 0.79 84.2, 67.6%[3]	0.79 – 0.91, 0.91 – 1.02 55.6, 46.9%[3]	1.02 – 1.13, 1.13 – 1.25 33.5, 27.9%[3]	1.25 – 1.36, 1.36 – 1.47 21.6, 21.2%[3]	1.47 – 1.59, 1.59 – 1.70 15.2, 14.0%[3]	1.70 – 1.81, 1.81 – 1.93 10.7, 11.4%[3]	1.93 – 2.04, > 2.04 9.7, 8.3%[3]	25,622	Kohler and Bierman, 2014
0.57 - 0.87 28.8%	0.88 – 1.04 2.9%	1.05 – 1.21, 1.22 – 1.38, 6.2, 4.4%	1.39 – 1.55, 1.56 – 1.72 20.4, 17.5%	1.73 – 1.89 21.6%	1.9 – 2.06 12.8%	2.07 – 2.85 13.7%	2,893	Smith et al., 2007
< 0.61, 0.61 – 0.8 85, 52%[5]	0.81 – 1.0 29%[5]	1.01 – 1.2 15%[5]	1.21 – 1.4, 1.41 – 1.6 11, 8%[5]	1.61 – 1.8 5%[5]	1.81 – 2.0, 2.01 – 2.2 5, 2%[5]	2.21 – 2.4, > 2.4 4, 3%[5]	12,041[4]	Quiniou et al., 2002
		1.10 – 1.15, 1.15 – 1.2 12.2, 15.9%[6]	1.2 – 1.25, 1.25 – 1.3 8.4, 9.0%[6]	1.3 – 1.35, 1.35 – 1.4 5.0, 1.3%[6]	1.4 – 1.45, 1.45 – 1.5 6.0, 3.4%[6]	> 1.5 0%[6]	600	Ferrari et al., 2014
	< 1.25 34.7%	1.25 – 1.433 12.6%	1.434 – 1.608 9.7%	1.609 – 1.787 7.8%	> 1.787 7.8%		508	Casellas et al., 2005

[1] % mortality to 24 h
[2] Piglets < 0.68 birth weight were excluded.
[3] % mortality to d 63
[4] Total born
[5] Survival calculated as number alive at weaning out ÷ number alive post cross-fostering within 48 h of birth.

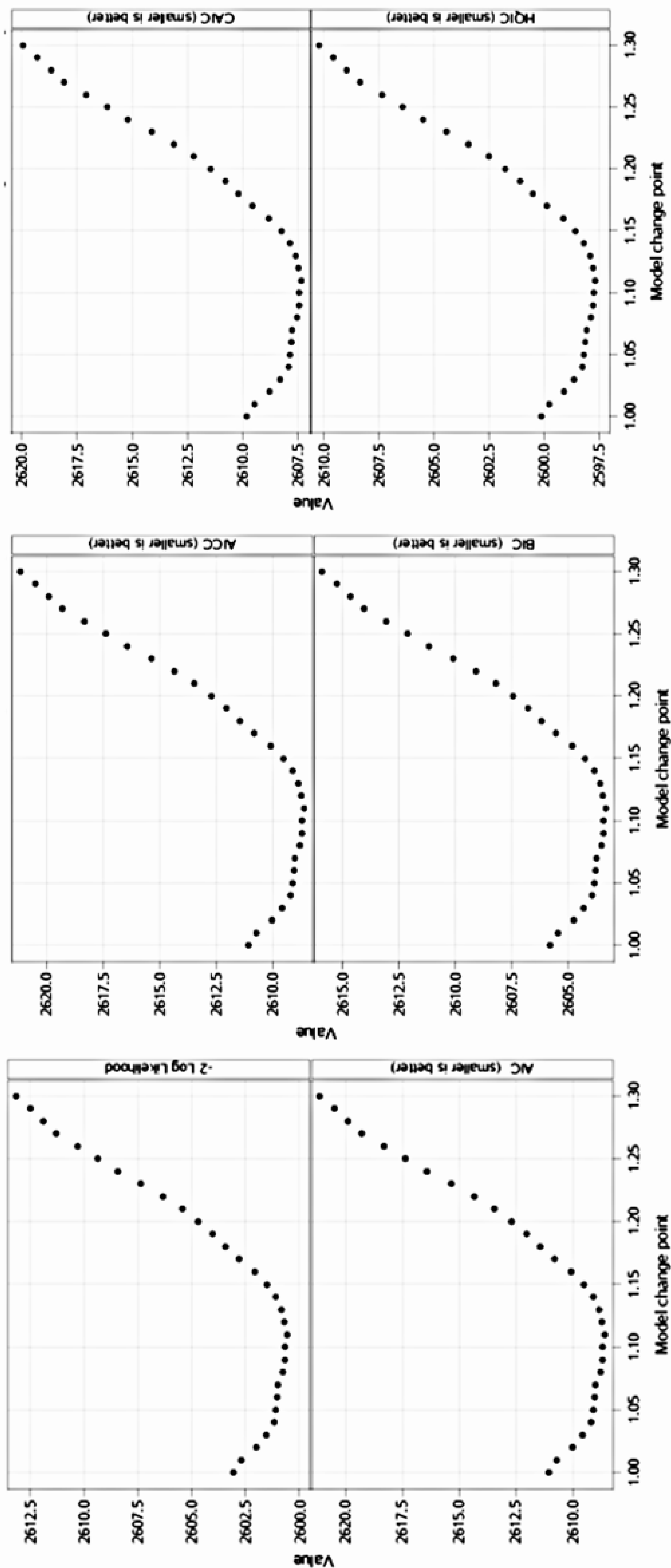

Figure 1.1. Goodness of fit assessment for change point in linearity.

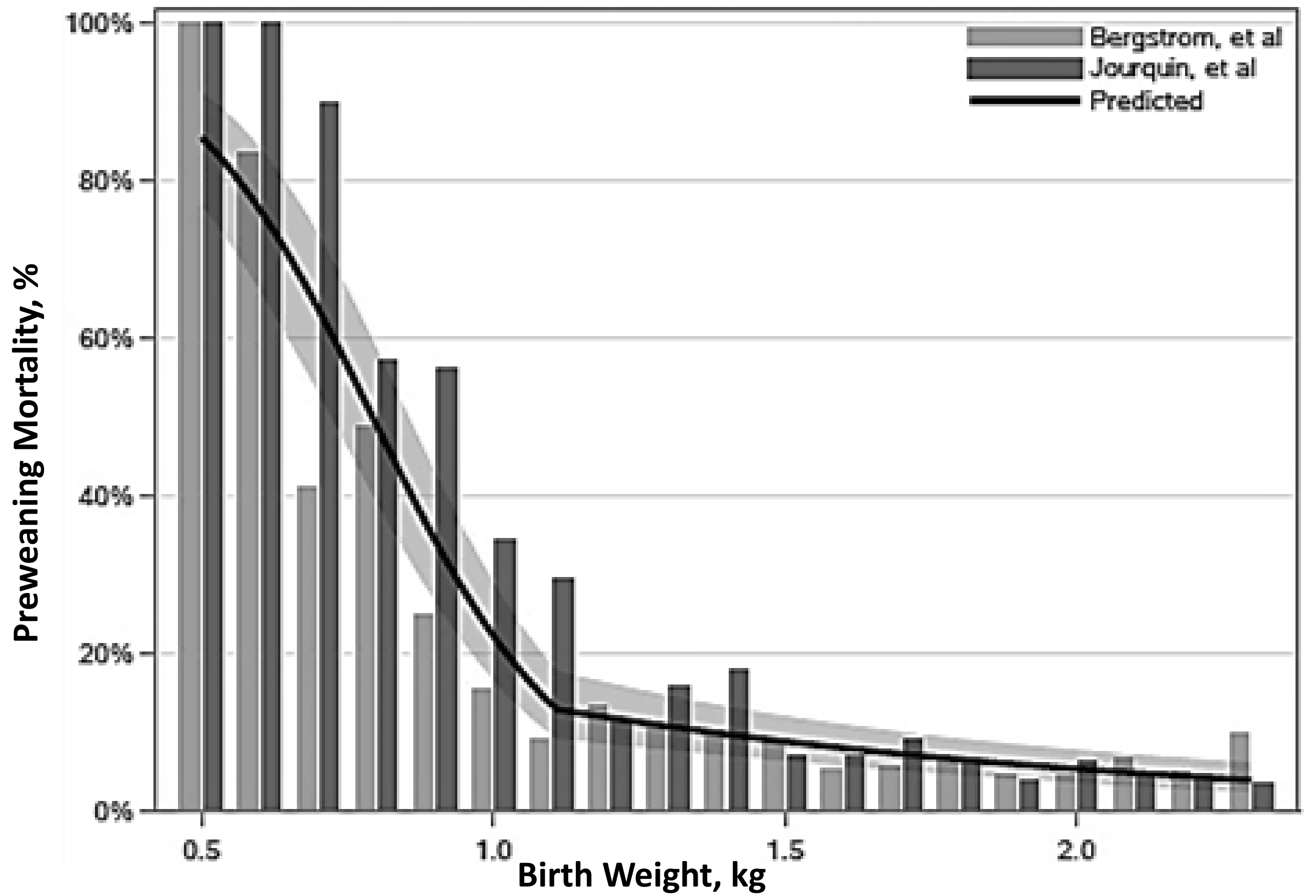

Figure 1.2. Predicted preweaning mortality by birth weight based on odds ratios of birth weight and mortality with 95% predicted interval (grey shaded area).

Table 1.2. Predicted preweaning mortality by birth weight.

Birth Weight, kg	Predicted Preweaning Mortality
0.50	85.4%
0.55	81.3%
0.60	76.2%
0.65	70.3%
0.70	63.7%
0.75	56.5%
0.80	49.0%
0.85	41.5%
0.90	34.4%
0.95	27.9%
1.00	22.3%
1.05	17.5%
1.10	13.6%
1.15	12.4%
1.20	11.8%
1.25	11.3%
1.30	10.7%
1.35	10.2%
1.40	9.7%
1.45	9.3%
1.50	8.8%
1.55	8.4%
1.60	8.0%
1.65	7.6%
1.70	7.2%
1.75	6.9%
1.80	6.5%
1.85	6.2%
1.90	5.9%
1.95	5.6%
2.00	5.3%
2.05	5.1%
2.10	4.8%
2.15	4.6%
2.20	4.3%
2.25	4.1%
2.30	3.9%
2.35	3.7%
2.40	3.5%
2.45	3.3%
2.50	3.2%
2.55	3.0%
2.60	2.9%
2.65	2.7%
2.70	2.6%
2.75	2.4%
2.80	2.3%

Chapter 2 - Myogenic and developmental characteristics of 60-day fetal pigs associated with intralitter relative crown-rump size.

ABSTRACT

Twelve gestating gilts (PIC 327 × 1050; initial BW 168 kg and age 7 mo) were used to quantify maternal serum IGF-1 concentrations from d 24 to d 60 of gestation as well as to evaluate the effect of d 60 intralitter fetal size on growth suppression, placental transport capacity, expression of myogenic growth and regulatory factors, and primary myogenesis. Gilts were synchronized and artificially inseminated at approximately 7 mo of age. Maternal serum was collected on d 24, 32, 39, 46, 53, and 60 of gestation. On d 60 of gestation, gilts were euthanized and fetal weights, measurements, and muscle samples were collected from male fetuses closest to the longest (LG), median (MD), and shortest (SM) crown-rump length sizes within each litter. Concentrations of IGF-1 in maternal serum decreased ($P < 0.05$) from d 24 to 60 of gestation. Fetal BW, liver weight, head width, and the ratio of head circumference to BW were smaller ($P < 0.05$) for SM fetuses compared to LG fetuses. Brain weight to liver weight ratio was larger ($P < 0.05$) in SM fetuses compared to MD and LG fetuses but no difference in placental gene expression of *Glut-1* was observed. The earlier maturing *Longissimus thoracis* had upregulated ($P = 0.046$) *IGF-2* mRNA, more ($P = 0.063$) primary myofibers of larger ($P = 0.002$) cross-sectional area, and a greater ($P = 0.025$) number of myonuclei per fiber than in the *Longissimus lumborum*. Despite larger fetuses having a larger ($P = 0.001$) *Longissimus* whole muscle area, no differences in primary myofiber development were evident among fetal sizes although *IGF-1*, *IGF-2*, and *MyoD* mRNA were upregulated ($P < 0.05$) in smaller fetuses. In summary, gestating gilt serum IGF-1 concentration declined in early to mid gestation. Small fetuses may have experienced nutrient restriction *in utero* as evidenced by brain sparing;

however, no differences in placental efficiency or primary myogenesis were observed among intralitter relative fetal sizes in mid gestation.

INTRODUCTION

Genetic selection for greater number of live pigs born per litter greatly increased present day litter sizes, but these larger litters are characterized by lighter average birth weights and negatively skewed birth weight distributions (Milligan et al., 2002). These lighter birth weights are attributed to limited uterine capacity of the dam. Uterine space is strongly correlated to prenatal conceptus survival (Chen and Dziuk, 1993) and by d 30 of gestation, intrauterine crowding has begun to retard fetal survival, growth, and development (Foxcroft et al., 2006). Yet not all organ growth is inhibited by prenatal undernutrition and placental insufficiency as evidenced by allometric brain growth, likely due to the prioritization of limited nutrients to sustained brain development (Ashworth et al, 2001; Vallet and Freking, 2006). Therefore, a large ratio of brain weight to other body organs and structures can be used as an indicator of piglets having experienced intrauterine growth restriction.

Birth weight is positively associated with myofiber number (Gondret et al., 2006; Oksbjerg et al, 2013). Compared to heavier birth weight pigs, light birth weight pigs take more days to reach market weight, have fatter carcasses, and fewer muscle fibers of larger cross-sectional area (Rehfeldt and Kuhn, 2006).

Fetal myogenesis is an intricate process with numerous regulatory factors controlling myoblast proliferation and differentiation. Repressed embryonic myoblast proliferation and accelerated differentiation will result in a decreased number of myofibers formed (Rehfeldt et al., 2011). Primary muscle fiber myogenesis in fetal pigs occurs between d 25 and 50 of gestation, while secondary muscle fiber myogenesis begins to occur around d 50 (Wigmore & Stickland,

1983). By d 90 of gestation, hyperplasia of both primary and secondary muscle fibers is complete (Oksbjerg et al., 2004), with subsequent muscle growth occurring via hypertrophy. Therefore, the potential for ultimate muscle mass is established prenatally in the pig and has numerous production implications.

Myogenic progenitor cells coexpress Pax3 and Pax7 transcription factors and give rise to embryonic and fetal myoblasts, or satellite cells (Biressi et al., 2007). Satellite cells are identifiable by expression of Pax7 transcription factor (Otto et al., 2009) and serve as a source of myonuclei for myofiber growth. The potential for postnatal muscle hypertrophy is partially dependent on number and proliferative rate of satellite cells per myofiber (Oksbjerg et al., 2013). Greater density of satellite cells has been observed in heavier birth weight mice as compared to the density in lighter birth weight littermates (Brown and Stickland, 1993).

The manipulation of the maternal growth hormone/somatotropic axis has been shown to mitigate some of the negative effects of intrauterine crowding on fetal myofiber number and birth weight (Rehfeldt and Kuhn, 2006). However, maternal insulin-like growth factors (IGF) do not cross the diffuse placenta in pigs so it has been proposed that maternal IGF might enhance nutrient transfer across the placenta thereby increasing fetal growth factors (Sterle et al., 1995). Quantification of unmodified endogenous concentrations of IGF-1 therefore can serve as a valuable reference for future research with the objective of modulating maternal levels.

The objectives of this experiment were to quantify maternal serum IGF-1 concentration in mid gestation, and to test the hypothesis that relatively small d 60 fetuses within each litter will 1) be characterized by suppressed growth, 2) have poorer placental transport capacity, 3) exhibit evidence of nutrient shunting to prioritized organs, 4) have decreased genetic expression

of myogenic growth and regulatory factors, and 5) possess fewer muscle fiber numbers with fewer nuclei per fiber.

MATERIALS AND METHODS

The protocol for this experiment was approved by the Kansas State University Institutional Animal Care and Use Committee. The study was conducted at the Kansas State University Swine Teaching and Research Center in Manhattan, KS.

Animal Management and Housing

Sexually mature gilts (n=12; PIC 327 × 1050; 168 kg initial BW and age 7 mo) across 2 breeding groups were used in this study. At approximately 185 d of age, group-penned gilts received daily exposure to mature boars and were observed for visual signs of estrous. After first recorded exhibition of estrus, gilts were moved to individual gestation stalls (2.88 m^2). On d 19 of their first recorded estrous cycle, gilts received 6.8 mL Matrix® (15 mg altrenogest; Intervet/Schering-Plough Animal Health, Millsboro, DE) top-dressed on their daily feed allowance. This continued for 14 consecutive d to synchronize estrus. Gilts were heat checked with mature boars twice daily starting approximately 3 d after cessation of Matrix® therapy. Gilts found to be in standing estrus were artificially inseminated (PIC 337 semen) up to 3 times at evening/morning intervals beginning 12 h after onset of estrus was first detected (onset of estrus = d 0). Pregnancy was confirmed via transcutaneous ultrasound on d 24 after first insemination.

Gilts gestated throughout the months of May, June and July (group 1), and July, August, and September (group 2). Gestating gilts were limit fed (2.2 kg d^{-1}) a standard non-medicated diet once daily that met or exceeded nutrient requirements for animals this size and stage of production (NRC, 2012).

Serum Collection

Gilts were restrained via snare approximately 4 h after feeding on d 24, 32, 39, 46, 53, and day 60 ± 4. Blood from the anterior vena cava/caudal jugular vein was collected into 10 mL glass vacuum tubes containing no additives (Covidien LP, Mansfield, MA). Whole blood was transported on ice to the K-State Muscle Biology Laboratory for further processing according to the methods of Tuck et al. (2009). Whole blood samples were incubated at room temperature for 30 to 45 min to allow for clot formation and centrifuged at 1,800 × g for 25 min at 4°C. Serum supernatant was transferred to sterile 1.7 mL microcentrifuge tubes and stored at -80°C until time of IGF-1 analysis.

Serum Processing and ELISA

A commercial IGF-1 (human) ELISA kit was used for the analysis of the serum IGF-1 (ADI-900-150; ENZO Life Sciences, Farmingdale, NY). Prior to analyzing study samples, the kit was validated for specificity and recovery. Percent recovery of human serum IGF-1 was specified as 95.9% at ≥ 1: 70 dilution, assay sensitivity was 34.2 pg mL^{-1}, and intra-assay coefficient of variation was 3.6-8.9%. Sow serum IGF-1 was extracted from binding proteins using a 1:5 ratio of sample to acidified ethanol (7:1 N HCl: 100% ethanol), centrifugation, and neutralization of the supernatant with a commercial neutralizing reagent (ADI-900-150; ENZO Life Sciences). The assay was performed according to manufacturer’s protocol and samples were run in triplicate. A spectrophotometer (Eon, BioTek Instruments, Winooski, VT) was used to detect the optical density of the colormetric signal at 450 nm. A pooled control sample was included on each plate as a plate to plate control. All samples’ concentrations fell within the standard curve range for parallelism (187 pg mL^{-1} to 6,000 pg mL^{-1}). The net optical density of

each of the standards was calculated and plotted against known concentrations. Sample concentrations of IGF-1 were calculated using the linear function.

Fetal Measurements and Sample Collection

On gestation d 59 ± 2, gilts were transported to the K-State Abattoir. On gestation d 60 ± 2, gilts were euthanized via exsanguination after electrical stunning. Reproductive tracts (ovaries, oviducts, uterine horns, uterine body, and cervix) were removed from the body. Each uterine horn was freed from surrounding connective tissue and dissected from the surrounding mesometrium. Within each horn, the fetal placental membranes were carefully separated from the uterine wall and the uterine wall was opened longitudinally from the ovarian end. The crown-rump length (poll to tail head) of each fetus was measured through the placental membranes. Due to the strong correlation between fetal crown-rump length and fetal BW (Knight et al., 1977), crown-rump length was employed to determine relative fetal sizes within each litter. Male fetuses having a crown-rump length closest to that of the longest, median, and shortest crown-rump lengths, within their respective litter, were identified as large (LG), median (MD), and small (SM) fetuses.

Tissue samples were taken from the middle of the allantochorion of each of the 3 fetuses after separating from the allantois. Care was taken to avoid collecting tissue containing large blood vessels. Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until further analysis for gene expression.

Fetal umbilical cords were severed approximately 1 cm from the fetus's abdomen and each fetus was individually weighed. The placental attachment length for each fetus was measured as the distance between each of the allantochorion's necrotic tips. The BW to attachment length ratio was calculated for each fetus.

Electronic digital calipers (Model 14-648-17; Fisher Scientific, Pittsburgh, PA) were used to measure the head length and width of each fetus. Head length was measured as the linear distance from the snout to the posterior point of the poll. Head width was measured as the linear distance immediately posterior to the eye socket and anterior to the base of the ear. The fetal head circumference was assessed by wrapping a string around the maximum circumference (poll to ventral surface of mandible). In a similar manner, the thoracic circumference just posterior to the shoulders of each fetus was determined. Ratios of head circumference:crown-rump length and of head circumference:BW were calculated for each fetus.

The whole left *Longissimus dorsi* was dissected from the fetus and weighed. The right *Longissimus dorsi* was segmented into the *Longissimus thoracis* and *Longissimus lumborum* and samples were collected for mRNA and immunohistochemical analysis. *Longissimus thoraci s* immunohistochemistry samples were taken immediately posterior to the point of the trapezius muscle, while mRNA samples were dissected from under the trapezius and anterior to the other sample. *Longissimus lumborum* immunohistochemistry samples were harvested at approximately the last rib and mRNA samples were collected posterior to this sample.

Muscle cross-sections which were to be used for immunohistochemistry were blotted on paper, the perimeter traced, blots scanned using a digital scanner (HP DeskJet 3050; Hewlett Packard, Palo Alto, CA), and whole muscle cross-sectional area was measured using Nikon NIS-Elements Basic (Nikon Instruments Inc, Melville, NY). The cross-section was then embedded in tissue embedding media (Fisher Scientific), frozen in liquid nitrogen cooled isopentane, and stored at -80°C. Portions of the muscle cross-sections to be used in mRNA analysis were deposited in individual tubes, snap-frozen, and stored at -80°C until further analysis.

The fetal skull was dissected and the complete brain removed and weighed. Similarly, the abdominal cavity of the fetus was opened and the liver was removed and weighed. The ratio of brain weight: liver weight was calculated for each fetus.

Immunohistochemistry

From each fetal tissue sample, a 10-μm cryosection was affixed to a frost resistant slide to allow for immunodetection. The methods of Paulk et al. (2014) were used with few modifications. Sectioned muscle samples were incubated in 5% horse serum and 0.2% TritonX-100 in phosphate buffered saline (PBS; Fisher Scientific) for 30 min to bind non-specific antigen binding sites, washed 3 times with PBS for 5 min each, and subsequently incubated with primary antibodies diluted in blocking solution for 60 min.

Primary antibodies used were supernatant myosin heavy chain type 2A, IgG1 (SC-71; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), supernatant myosin heavy chain, slow, IgG2b (BA-D5; Developmental Studies Hybridoma Bank), Pax-7 (Developmental Studies Hybridoma Bank), and α-dystrophin (Thermo Scientific, Waltham, MA) diluted 1:10, 1:10, 1:10, and 1:500, respectively. After washing with PBS, cryosections were then incubated for 30 min with a secondary antibody solution simultaneously with 1:1000 Hoechst 33342 dye (Invitrogen; Carlsbad, CA) in blocking solution. The secondary antibodies were 1:1000 Alexa-Fluor 488 goat anti-mouse IgG1for SC-71 and Pax-7 (Invitrogen), Alexa-Fluor 633 goat anti-mouse IgG2b for BA-D5 (Invitrogen), and Alexa-Fluor 594 goat anti-rabbit heavy and light chains for α-dystrophin (Invitrogen). After incubation in secondary antibody solution for 30 min, cryosections were washed, covered with 9:1 glycerol/PBS solution, and cover-slipped.

A Nikon Eclipse TI-U inverted microscope equipped with a DS-QiMc digital camera (Nikon Instruments Inc.) was used to collect photomicrographs at 200× magnification. Two representative photomicrographs (> 100 primary fibers per sample) were captured, and were analyzed with NIS-Elements Imaging Software (Basic Research, 3.3; Nikon Instruments Inc.). Fibers that were positively stained for the BA-D5 antibody were categorized as primary muscle fibers and the fibers that positively stained for SC-71 were labelled as secondary fibers. Nuclei count was determined by all fiber associated nuclei that were Hoechst 33342 positive. Total primary fiber number of the whole muscle cross-sectional area was computed as the whole muscle area divided by average primary fiber cross-sectional area.

Real-time Quantitative PCR

The methods of Burnett et al. (2016) were utilized for the extraction of total RNA, cDNA synthesis, and quantitative PCR (qPCR) analysis. Briefly, nucleic acids were extracted from tissues by subjecting them to TRIzol® (Thermo Fisher Scientific, Waltham, MA) chloroform extraction. Nucleic acid pellets were purified by subjecting them to the PureLink™ RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA). Following elution, total RNA concentration and purity was assessed using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). All muscle tissue RNA extractions produced 260:280 nm absorbance ratios (A_{260}/A_{280}) greater than 1.9 and were deemed suitable for qPCR analysis. All extracted samples were stored at - 80°C until the time of qPCR analysis.

Complementary DNA was synthesized from 50 ng of total RNA using the High Capacity cDNA Archive kit (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Complementary DNA reactions were conducted twice for each sample. Real-time qPCR was conducted using a Realplex^2 S PCR System (Eppendorf North America, Hauppauge, NY). Gene

specific primers (Table 2.1), cDNA representing 1 ng of total RNA, and PerfeCTa Sybr FastMix SYBRGreen (Quanta Biosciences, Gaithersburg, MD) were used for the real-time reaction. For the muscle genes of interest, duplicate reactions were conducted under thermal cycling parameters consisting of an initial heating step of 50°C for 2 minutes, initial denaturing at 95°C for 10 minutes, and 50 cycles of denaturing at 95°C for 15 sec, annealing at the appropriate temperature for 30 seconds, and extension at 68°C for 20 sec. A final dissociation step was conducted and consisted of heating at 95°C for 15 sec and a 60 - 90°C temperature ramp conducted over 20 minutes. An internal control of pooled sample was included on each plate to ensure consistency across plates. Primers for each muscle gene of interest were validated by generating efficiency curves. Primers with efficiencies between 0.9 and 1.1 were considered acceptable for qPCR analysis.

For the muscle genes, the threshold cycle (Ct) was determined for each gene of interest and this value was then normalized to *Ribosomal protein L4* (*RPL4)* expression in the same sample to calculate the normalized expression (ΔCt) of the target gene. A pooled control sample representing samples from all fetal sizes and both muscles was used as the calibrator sample and the normalized expression (ΔCt) of each target gene in the calibrator sample was used to determine fold differences in relative gene expression using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Placental gene expression was calculated similarly using *18S rRNA* gene expression to normalize expression of the target gene; RNA obtained from swine testis tissue was used as the calibrator sample for calculation of fold differences.

Statistical Analysis

Maternal serum IGF-1 was analyzed as a completely randomized design with each gilt serving as an experimental unit to assess the effect of day of gestation on serum IGF-1

concentration. The MIXED procedure in SAS (v9.3, SAS Institute Inc., Cary, NC) was used to model day of gestation as a fixed effect with a random effect of gilt to account for repeated sampling of the gilts across days of gestation.

Fetal morphometrics and weights were analyzed as a randomized complete block design with a one way treatment structure and replicated over 3 sample collection days. Each fetus was considered an experimental unit and dam was considered a random block factor. The MIXED procedure in SAS was used to model fetal size as a fixed effect with random effects of sample collection day and dam nested within sample collection day. The random effects of fetal size × sample collection day and fetal size × dam within sample collection day were pooled into the residual.

Fetal muscle histology and gene expression was analyzed as a randomized complete block design with a split-plot, 3 × 2 treatment structure, and replication over 3 sample collection days. Hence, responses were modeled with fixed effects of fetal size on the fetus whole-plot experimental unit and muscle location on the *longissimus* split-plot experimental unit and with random effects of sample collection day, dam within sample collection day, and the interaction between fetal size × dam within sample collection day. The random interactions between fetal size × sample collection day and fetal size × dam within sample collection day were pooled into the whole-plot residual used to test the effect of fetal size. The random interactions between muscle location × sample collection day and muscle location × dam within sample collection day, as well as muscle location × fetal size × sample collection day and muscle location × fetal size × dam within sample collection day were pooled into the split-plot error term used to test the effect of muscle location and the interactions between fetal size and muscle location.

For all variables, differences between least squares means were computed using the PDIFF option in SAS. All results were considered statistically significant at $P \leq 0.05$; results with P-values > 0.05 and ≤ 0.10 were considered marginally significant.

RESULTS

Maternal Serum IGF-1 Concentration

Maternal serum IGF-1 concentrations decreased during the observed period of d 24 to d 60 of gestation. Serum IGF-1 decreased ($P < 0.05$) from d 24 gestation to d 32, from d 32 to 39, and from d 39 to 60 (Figure 2.1).

Fetal Morphometrics and Organ Weights

Fetal pigs were categorized as small (SM), medium (MD), or large (LG) size depending on their relative crown-rump length within litter. Consequently, the crown-rump length of SM fetuses was shorter ($P < 0.05$) than the crown-rump length of MD fetuses, which was shorter ($P < 0.05$) than the crown-rump length of LG fetuses (Table 2.2). Fetal BW followed a similar pattern as the BW of SM fetuses was less ($P < 0.05$) than the BW of LG fetuses, while the BW of MD fetuses was not different from either SM or LG. Individual placental attachment lengths did not differ ($P = 0.486$) across fetal sizes, nor were differences ($P = 0.403$) in the ratio of BW: attachment length observed across fetal sizes. Also, no difference ($P = 0.577$) in placental *Glut-1* mRNA expression was observed across fetal sizes.

No difference was observed between the thoracic circumferences of SM and MD fetuses although the thoracic circumference of LG fetuses was larger ($P < 0.05$) than either SM or MD fetuses. Fetal head dimensions of longer fetuses tended to be greater with marginally significant differences in head circumference ($P = 0.058$) and in head length ($P = 0.063$; Table 2.2). Head

width was wider ($P < 0.05$) for LG fetuses compared to SM fetuses, but the head width of MD fetuses was not different from either SM or LG. However, SM fetuses had a larger ($P < 0.05$) ratio of head circumference:crown-rump length than MD fetuses which in turn had a larger ($P < 0.05$) ratio than LG fetuses. Similarly, the ratio of head circumference:BW of SM fetuses was larger ($P < 0.05$) than the ratio of LG fetuses, and that of MD fetuses did not differ from that of either SM or LG fetuses.

Although head dimensions were marginally significantly greater for larger sized fetuses, no differences ($P = 0.380$) in brain weight were observed among the fetal sizes. In contrast, liver weights of LG fetuses were heavier ($P < 0.05$) than liver weights of SM fetuses and the liver weights of MD fetuses were not different from the liver weights of either LG or SM fetuses. Therefore, the ratio of brain weight:liver weight was greater ($P < 0.05$) for SM fetuses compared to that of MD and LG fetuses, whose brain weight:liver weight ratios were not different. No differences ($P = 0.119$) among the weights of the left *Longissimus dorsi* muscle due to fetal size were observed.

Fetal Muscle Histology

No fetal size × muscle location interactions were observed for the fetal histological properties aside from a marginally significant interaction ($P = 0.071$) for the number of fiber associated nuclei per primary muscle fiber (Table 2.3). The whole muscle cross-sectional area of the *Longissimus thoracis* was larger ($P = 0.001$) than the whole muscle cross-sectional area of the *Longissimus lumborum*. In addition, the whole muscle cross-sectional area of SM fetuses was less ($P < 0.05$) than that of MD and LG fetuses regardless of muscle location. No differences in the number ($P = 0.181$) or cross sectional area ($P = 0.114$) of primary muscle fibers due to fetal size were observed; however, independent of fetal size, there was a marginally significant greater

(*P* = 0.063) number of primary muscle fibers and greater (*P* = 0.002) primary fiber average cross-sectional area in the *Longissimus thoracis* than in the *Longissimus lumborum*. No differences in the number of secondary muscle fibers per primary muscle fiber were observed due to either fetal size (*P* = 0.359) or muscle location (*P* = 0.781).

More (*P* = 0.023) fiber associated nuclei per primary muscle fiber were present in the *Longissimus thoracis* than were observed in the *Longissimus* lumborum. Fetal size did not affect (*P* = 0.756) the number of fiber associated nuclei per primary muscle fiber, but the marginally significant interaction (*P* = 0.071) between muscle location and fetal size observed for the number of fiber associated nuclei per primary muscle fiber was the result of numerical increases in fiber associated nuclei in the *Longissimus thoracis* between SM, LG, and MD fetuses, respectively, but numerical increases in the *Longissimus lumborum* between the MD, LG, and SM fetuses, respectively. No differences (Size, *P* = 0.933; Muscle, *P* = 0.141) in the number of Pax7 positive nuclei per primary muscle fiber were observed due to either fetal size or muscle location.

Myogenic Gene Expression

No interactions (*P* > 0.10) between fetal size and muscle location were observed in myogenic gene expression (Table 2.4). Irrespective of fetal size, *IGF-2* gene expression was greater (*P* = 0.046) in the *Longissimus lumborum* than in the *Longissimus thoracis* but there was no observed difference (*P* = 0.544) in *IGF-2 receptor* gene expression between the two muscle locations. Across both muscle locations, SM fetus gene expression of *IGF-2* was greater (*P* < 0.05) than the expression in MD or LG fetuses which did not differ. Fetal size had a marginally significant effect (*P* = 0.103) on gene expression of *IGF-2 receptor* with expression least in LG fetuses.

Gene expression of *IGF-1* was greater ($P = 0.046$) in the *Longissimus lumborum* than in the *Longissimus thoracis* (Table 2.4). The *IGF-1* gene expression decreased with increasing fetal size with SM fetuses having greater ($P < 0.05$) expression than either MD or LG fetuses which did not differ. Gene expression of *IGF-1 receptor* did not appear to be affected (Size, $P = 0.326$; Muscle, $P = 0.466$) by either fetal size or muscle location.

Regardless of muscle location, *MyoD* gene expression decreased with increasing fetal size such that SM fetuses had greater ($P < 0.05$) expression than either MD or LG fetuses which did not differ. No differences in the gene expression of *MyoD* ($P = 0.487$), *Pref-1* ($P = 0.519$), *Myf5* ($P = 0.397$), *MyoG* ($P = 0.303$), *Pax7* ($P = 0.618$), *β-catenin* ($P = 0.219$), or *Myostatin* ($P = 0.515$) between the two muscle locations were observed. In addition, gene expression of *Pref-1*, *Myf5*, *MyoG*, *Pax7*, *β-catenin*, and *Myostatin* did not differ ($P = 0.145$, 0.201, 0.286, 0.337, 0.237, and 0.526, respectively) among fetal sizes.

DISCUSSION

Maternal IGF-I is unable to cross diffuse placental membranes and therefore cannot directly affect the pig fetus. However, increased maternal IGF-1 concentration promotes fetal growth, possibly increasing fetal IGF-1 production through enhanced nutrient transfer across the diffuse placenta (Hall et al., 1986; Sterle et al., 1995). Similar to our observations, Farmer et al. (2000) reported decreases of maternal IGF-1 from around 170 ng mL^{-1} at breeding to around 80 ng mL^{-1} by d 70 of gestation. These observations were obtained in similar aged but lighter weight gilts than those used in the present study which might explain the difference in values.

For the current study, relative fetal size classification was based on fetal crown-rump length. Knight et al. (1977) reported that the crown-rump length of fetuses was strongly correlated to their body weight (r = 0.92) from d 20 to d 100 gestation. Within the fetal subset

evaluated in this study, the BW of fetuses characterized as SM, MD, and LG increased with increasing crown-rump lengths at d 60 of gestation. Larger thoracic circumferences were observed in the largest fetuses compared to their smaller littermates, thus indicating that heart girth is reflective of fetal length in 60 d fetuses.

As seen in the present study, d 60 fetal liver weights increased with increasing fetal size. In contrast, Vallet and Freking (2006) observed that the relative weight of liver to fetal BW on d 45 was greater for larger fetuses so that the livers of small fetuses were a lesser proportion of BW than the proportion was for large fetuses. By d 65 however, the liver weight to BW percentage was almost proportional across all fetal sizes, implicating either the livers of the smaller fetuses had high impetus growth from d 45 or 65 or livers of larger fetuses experienced low impetus growth during this period. Yet Amdi et al. (2013) reported lighter liver weight relative to BW could still be observed in severely growth restricted neonates when compared to the ratio in normal birth weight pigs (Amdi et al., 2013).

The precocial nature of neonatal pigs presupposes substantial development of the neuroendocrine system and associated organs (Matthews, 2002), which are critical for regulation of fetal growth and development. Thus, fetal pigs may prioritize partitioning of limited nutrients to fetal brain development. In the present study, brain weight and the circumference and length of the head did not differ among the extremes of intralitter fetal sizes, but differences corresponding to fetal size were observed in head width. Yet as assessed by head circumference, the relative size of the head compared to BW or compared to crown-rump length decreased with increasing fetal size. Comparing the brain weight to liver weight ratios of different sized fetuses indicated that small fetuses within each litter had the greatest proportion of brain to liver organ

weight, thus suggesting that brain growth was prioritized over liver growth in nutrient-limited fetuses.

Vallet and Freking (2006) reported that the brain-sparing phenomenon was evident by d 45 of gestation and the weight of the brain relative to fetal BW increased with increasing fetal weight. As fetuses grew throughout gestation from d 45 to 85, the differences in the ratio of brain weight to BW for different sized fetuses increased in magnitude so that the differential relationship between brain weight and BW across fetal sizes was most pronounced by d 85 of gestation. At birth, Amdi et al. (2013) observed that the percentage of heart weight and brain weight relative to BW was still greater in severely growth restricted neonates than in normal birth weight pigs. Altogether, these observations indicate small fetuses prioritize brain growth over other body growth.

In the pig, maternal-fetal exchange occurs over the entire placental area in pigs (Fowden et al., 2006). Elongation of the spherical blastocyst occurs at d 11 - 12 of gestation (Geisert et al., 1982), while attachment begins by d 12 -13 and is completed by d 25 - 26 (Lawrence et al., 2012). This sequence of events has important ramifications as earlier elongating conceptuses establish claim to uterine space and placental area first and thus may experience less restricted placental growth and crowded growth conditions (Blomberg et al., 2005). In the present study, no appreciable differences were observed in the placental attachment lengths nor were any differences evident in the d 60 fetal body weight to attachment length ratios of different sized fetuses. It is possible that placental attachment length is not a consistently representative measurement of placental nutrient supply or function.

Placental function could be enhanced via several means such as morphological folding or degree of vascularization, but improved nutrient transport is widely proposed as a means of

compensational growth of crowded fetuses. Glucose is not synthesized in the fetal pig (Fowden et al., 1997). Therefore, glucose transporters of the *SLC2A* gene family located within the placental are critical for supplying glucose to the fetal pig. In the present study, no difference could be detected in the mRNA expression for *Glucose transporter-1* (*GLUT-1*) in the placental tissues of the SM, MD, and LG fetuses of each litter. Little research has examined relative expression of glucose transporters in pigs, but maternal growth hormone treatment increased *GLUT-1* expression across all fetuses while concurrently increasing fetal weight on d 50 (Tung et al., 2012). Expression of *GLUT-1* increases throughout gestation in the discoidal hemochorial placentas of rodents and in certain locations of that of humans (Yamaguchi et al., 1996; Baumann et al., 2002; Korgun et al., 2011) and in rats, *GLUT-1* gene expression is upregulated in the smallest placentas at the end of gestation when the nutrient demand for fetal growth is especially large (Coan et al., 2008). Although *GLUT-1* expression appears to be associated with greater fetal growth, there is not yet strong evidence that *GLUT-1* is upregulated in the placenta of growth-compromised pigs.

Despite no differences in the *Longissimus* muscle weight among the fetal sizes in the present study, the cross-sectional area of the *Longissimus* muscle increased with increasing fetal BW and intralitter size classification, thus implying observable differences in primary myogenesis by d 60 of gestation. In addition, significant myogenic differences attributable to anatomical location within the *Longissimus* muscle were observed in the d 60 fetus and these differences were consistent across fetal sizes. Parallel consideration of the effects of both fetal size and muscle location factors can be helpful for understanding the complex temporospatial growth and development of muscle.

Muscle location is an important consideration because the myogenic progenitor cells responsible for formation of the *Longissimus* muscle are derived from the epaxial dermomyotome (Ordahl and Le Douarin, 1992). Somites are formed from the embryonic mesoderm in a rostral to caudal direction (Sassoon et al., 1989) and within the somite, waves of precursor cells originating from the dermomyotome translocate to form the myotome and there elongate with succeeding cells displacing the earlier cells (Hollway and Currie, 2005). Myotome development continues sequentially with the proliferation of additional cells along all the edges of the dermomyotome, translocation of these cells to the myotome, intercalation among the existing myotomal cells, and elongation. Myocytes on the rostral and caudal edges of the somite elongate in one direction (Gros et al., 2004) so that the formation of the myotome structure occurs progressively in a rostral to caudal manner. As such, the myogenesis of the *Longissimus* muscle progresses so that the thoracis portion develops earlier than the lumborum portion.

In the current study, compared to the *Longissimus lumborum*, the *Longissimus thoracis* had greater whole muscle area, fiber number, fiber size, and fiber associated nuclei per fiber but less relative abundance of *IGF-1* mRNA and *IGF-2* mRNA. In addition, the mRNA abundance of both *IGF-1* and *IGF-2* decreased with increasing fetal size. Considered together, the observed effects of muscle location and fetal size on these various responses are consistent with each other and would imply that less developed muscle and less developed fetuses express a greater abundance of growth factor mRNA as a compensative mechanism for sustained growth and development.

Growth factors have important functions in myogenesis. Insulin-like growth factor 1 and IGF-2 stimulate myoblast differentiation *in vitro* (Ewton and Florini, 1981) and regulate both proliferation and differentiation of muscle cells in a very developmentally critical, time specific

manner (Oksbjerg et al., 2004). The prevailing theory submits that fetal pigs experience tissue-specific regulation of IGF-1 production during development and hepatically produced IGF's are not a primary source prenatally (Lee et al., 1993; Ramsay et al., 1994; Gerrard et al., 1998). As such, local production of growth factors in skeletal muscle is important for growth. *Insulin-like growth factor 2* mRNA abundance is highly expressed prenatally, peaks around d 59 of gestation, decreases to low expression postnatally, yet remains higher than that of IGF-1 from mid to late gestation (Lee et al. 1993; Peng et al., 1996; Gerrard et al., 1998; Fowden, 2003). Conversely, *IGF-1* gene expression increases during gestation and has maximum expression around birth (Gerrard et al., 1998) at which time *type 1 IGF receptor* mRNA also begins to decrease (Lee et al., 1993).

Growth factor gene expression in muscle tissue at the time of primary myogenesis in the fetal pig is largely uncharacterized but Tilley et al. (2007) investigated expression at the time of secondary myogenesis. Interestingly, when comparing small fetuses afflicted with compromised secondary myogenesis to average sized littermates, they did not observe differences in abundance of *IGF-1* and *IGF-2* mRNA, but did report greater mRNA abundances of *type 1 IGF receptor* on d 65 and d 100 and *type 2 IGF receptor* on d 100. Thus, growth-compromised fetuses mainly upregulated growth factor receptor genes while the small fetuses of the present study mostly upregulated growth factor gene expression earlier in development. This leads to speculation that throughout gestation, growth compromised fetuses may employ strategies to facilitate greater uptake of hepatically produced IGF's through increased receptor activity rather than attempting to increase local production.

The number of fiber-associated nuclei per primary fiber was greater in the earlier developing thoracis location as compared to the lumborum location yet did not differ across fetal

sizes. Combined with the fact that the thoracis location also had a marginally significant increase in primary fiber numbers, these observations are consistent with the theory that earlier development of the thoracis enables greater primary myogenesis. Possible mechanisms for this greater primary myogenesis include either a greater number of myocytes were present in the myotome of the thoracis location or this location experienced a greater degree of progenitor cell and/or myoblast proliferation along with increased myoblast differentiation.

The four myogenic regulatory (MRF) transcription factors responsible for orchestrating the commitment and differentiation of muscle precursor cells have distinct roles at specific developmental time points and locations. Myogenic factor 5 (Myf5) and Myogenic differentiation 1 (MyoD) facilitate dermomyotomal cell commitment to muscle cell type with Myf5 activation preceding that of MyoD in the epaxial cells (Sassoon et al., 1989; Borycki et al., 1999). Myogenin (MyoG) acts downstream of Myf5 and MyoD (Sassoon et al., 1989; Rawls et al., 1995) and is responsible for the exit of the cell cycle and terminal differentiation of committed cells to form myoblasts (Zhang et al., 1995) as is MRF4 (Hawke and Garry, 2001). Myogenin is also essential for myotube formation and fusion (Barnoy and Kosower, 2007).

Similar to the findings of the current study, Tilley et al. (2007) reported *Myogenin* gene expression did not differ between small and average sized fetuses at either d 65 or d 100 of gestation. Yet in the present study, *MyoD* mRNA was significantly downregulated across both anatomical locations as fetal size increased. Conversely, *Myf5* gene expression was relatively unaffected. By d 60, primary muscle fiber formation should be completed and the formation of the secondary fibers commencing. Presuming the primary myogenesis of the small fetuses was not prolonged, it would appear the smaller fetuses may be accelerating the commitment of progenitor cells to the myogenic lineage under the regulation of MyoD. Alternatively, if the

primary myogenesis of the small fetuses was prolonged as a consequence of nutritional deficiency and growth retardation, it would appear a sustained upregulation of *MyoD* may be a compensatory strategy for increasing the embryonic myoblast pool in small fetuses. However, a lack of concurrent upregulation in *Myogenin* for terminal differentiation of the myoblasts to myofiber formation seems inconsistent with this latter theory. Moreover, it appears that differential *MyoD* expression at d 60 is not associated with delayed or prolonged primary myogenesis considering that the *Longissimus lumborum* experienced later myogenic development as compared to the *Longissimus thoracis,* but elevated *MyoD* gene expression was not sustained in the *Longissimus lumborum*. Little research has examined the expression of *Myf5* across fetal pig sizes and the observations of the present analysis indicate *Myf5* and *Myogenin* genes are consistently expressed among fetal sizes at d 60, while *MyoD* is upregulated in smaller fetuses.

Despite the increase in *MyoD* gene expression in small fetuses, no differences in *Myostatin* mRNA abundance were observed. Myostatin functions as a negative regulator of muscle growth during myogenesis. *Myostatin* gene expression is upregulated during the formation of myofibers in the pig (Ji et al., 1998), possibly induced by MyoD (Deng et al., 2011) and may inhibit muscle development through the inhibition of myogenin (Theil et al., 2006). Based on the current findings, *Myostatin* does not appear to be upregulated in slower growing fetal pigs at d 60.

All progenitor cells originating from the dermomyotome co-express Pax 3 and Pax7 (Biressi et al., 2007). Pax3/7 positive cells are precursors to either embryonic and fetal myoblasts or to mononucleated satellite cells that express Pax7 and are morphologically identifiable after secondary myogenesis is complete (Seale et al., 2000; Biressi et al., 2007). The expression of

Pax7 inhibits the differentiating activity of MyoD while downregulation of Pax7 allows MyoD expression and the subsequent terminal differentiation of the myoblast via myogenin (Hawke and Garry, 2001; Olguín, 2011). In the present study, no differences in *Pax7* mRNA abundance or in the number of nuclei expressing *Pax7* in d 60 fetuses of different size or in *Longissimus* locations were observed. These results indicate that after formation of embryonic myoblasts on d 60, the pool of Pax7 positive progenitor cells available for secondary myogenesis and future satellite cells did not differ in number across fetal size or later developing muscle location.

Wnt signaling functions in numerous diverse developmental and metabolic pathways and has two types of signaling pathways: the "canonical Wnt" pathway which is a complex process requiring the cooperative action of β-catenin, or "noncanonical Wnt" pathways which occur independently of β-catenin (Song et al., 2014). Wnt /β-catenin mediated signaling has been shown to induce somite formation of the medial dermomyotome (Ikeya and Takada, 1998), induce commitment of somitic progenitor cells via activation of Myf5 expression (Borello et al., 2006) and indirect activation of MyoD expression although β-catenin independent activation of MyoD is possible (Brunelli et al., 2007), and has also been implicated in several additional myogenic regulatory mechanisms (Suzuki et al., 2015). The lack of differences in the expression of *β-catenin* mRNA in longissimus samples amongst fetal sizes is not altogether unsurprising considering no evidence for differences in *Myf5* expression were observed.

Both myocytes and adipocytes originate from the multipotent mesenchymal stem cells (Du et al., 2013). This common origin has led to speculation that myogenic activity can affect adipogenic outcomes. Preadipocytes are characterized by expression of preadipocyte factor-1 (Pref-1) which functions to prevent differentiation into adipocytes (Smas and Sul, 1996; Wang et al., 2006; O'Connell et al., 2011). Preadipocyte factor-1 reportedly is expressed in fetal skeletal

muscle (Friedrichsen et al., 2003) and elevated serum levels of Pref-1 have been observed in growth restricted human neonates (de Zegher et al., 2012). However, the *Pref-1* mRNA abundance did not differ among fetal sizes or muscle locations in the present study thus suggesting the *Pref-1* gene expression may not be affected by temporal developmental differences in *Longissimus* muscle or be differentially expressed among fetuses with or without upregulated *MyoD* gene expression.

CONCLUSION

Circulating maternal IGF-1 concentration decreases from early to mid gestation. During this time, fetuses within each litter experienced different growth rates but brain growth is prioritized. However, no differences in placental attachment lengths or upregulation of placental *GLUT1* were evident suggesting other measures of placental efficiency may have been employed by smaller pigs to counteract poor growth. Both *IGF-2* and *IGF-1*muscle mRNA are upregulated in less developed fetuses and muscle locations thus emphasizing the importance of these growth factors for fetal development. Myogenesis is more advanced in the anterior portion of the *Longissimus* muscle than in the posterior portion likely as a reflection of temporospatial differences in development yet myogenic regulatory factor gene expression did not significantly differ among the locations. In addition, differences in the myotocyte or myoblast number of the thoracis portion of the *Longisssimus* may contribute to greater nuclei per primary fiber at d 60 but the number of progenitor cells available for secondary myogenesis at the different locations or across fetal sizes was unchanged. Primary muscle fiber number or area was not significantly different among fetal sizes. *Myogenic differentiation 1* was the solely upregulated myogenic regulatory factor in smaller fetuses and may indicate a growth restricted fetuses have a greater reliance on progenitor cell commitment to the myogenic lineage. *Myostatin*, *β-catenin*, and

preadipocyte factor 1 gene expression do not appear to be differentially regulated in different sized fetuses or at developmentally different stages of the *Longissimus* muscle. Together, these observations provide valuable insight into early myogenic and developmental differences characterizing different sized pig fetuses by mid-gestation.

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Table 2.1. Primer sequences, annealing temperatures, amplicon lengths, and efficiencies.

Porcine Genes	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	T_m, °C[1]	Amplicon Length	Efficiency
Ribosomal protein L4(RPL4)	AGGAGGCTGTTCTGCTTCTG	TCCAGGGATGTTTCTGAAGG	60.5	184	1.06
Insulin-like growth factor 2 (IGF-2)	ACCTCCCCATGTCAGGCTAGT	GGGAGATACAGACCAAGCCAAT	60.5	92	0.91
Insulin-like growth factor 2 receptor	GCCCCCCAGCAGGAATC	ACGTGACTTGGGAAATTGCAT	60.5	79	0.93
Insulin-like growth factor 1 (IGF-1)	TCTTCTACTTGGCCCTGTGCTT	CCAGCTCAGCCCCACAGA	60.5	80	0.92
Insulin-like growth factor 1 receptor	AACAACATTGCCTCGGAGCTA	TGGGAGTGGCGGATCTTC	60.5	80	0.92
Preadipocyte factor 1 (Pref-1)	AGGACGGCTGGGATGGA	CGAGGTTCGCGCAGGTT	61.9	87	1.11
Myogenic factor 5(Myf5)	TGGAAATCAGTTATAGGGAGTTTT	TTTGTGCTTACATTAAAAAGATGC	60.5	150	1.09
Myogenic differentiation 1 (MyoD)	ACTCAGACGCATCCAGCCC	AATCCATCATGCCGTCGG	60.5	50	0.96
Myogenin	AGTGAATGCAGTTCCCACAG	GAGGTGAGGGAGTGCAGATT	60.5	130	0.91
Paired Box 7 (Pax7)	CAACCACATCCGCCACAAGATAGT	AGAGGATCTTGGAGACACAGCCAT	60.5	106	0.9
Beta-Catenin	TCCTAGCTCGGGATGTTCACA	AGAGGACCCCTGCAGCTACTCT	64.6	87	1.11
Myostatin	GATTATCACGCTACGACGGA	GAAGCAGCATTTGGGTTTT	57.0	89	0.96

[1] T_m = melting temperature.

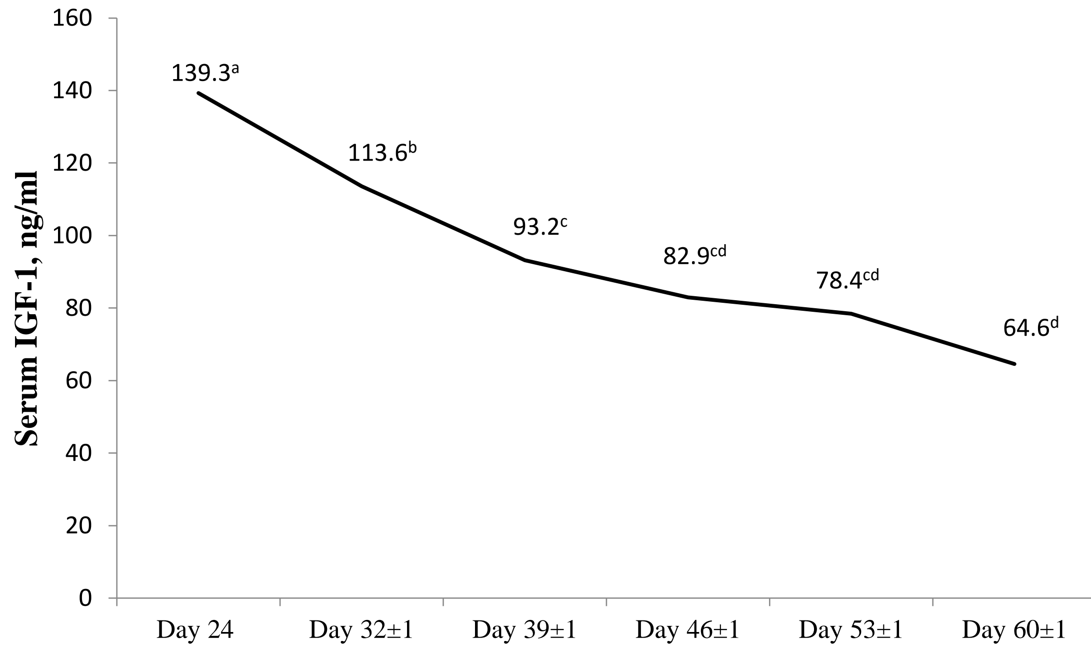

Figure 2.1. Serum IGF-1 concentration (ng ml^{-1}) decrease in nulliparous sows to d 60 of gestation. Means without a common superscript differ $P < 0.05$ (SEM = 8.87).

Table 2.2. Relationship between d 60 fetal crown-rump length and other measures of fetal growth and development.

60-d Fetal Size[1]:	SM	MD	LG		
n=	12	12	12	SEM	Size, *P* <
Response					
Crown-rump length, mm	117[a]	130[b]	139[c]	4.9	<0.001
Body weight, g	123.7[a]	135.0[ab]	149.0[b]	10.66	0.007
Attachment length, mm	144	143	159	13.0	0.486
Body weight:Attachment length	0.882	0.973	0.973	0.0549	0.403
Placental *Glut-1* mRNA fold difference	2.20	1.81	1.73	0.337	0.577
Thoracic circumference, mm	106[a]	108[a]	113[b]	4.2	0.020
Head circumference, mm	107	110	110	2.2	0.058
Head length, mm	45.2	45.9	46.5	1.07	0.063
Head width, mm	27.5[a]	28.4[ab]	28.9[b]	0.61	0.018
Head circumference:Crown-rump	0.918[a]	0.847[b]	0.793[c]	0.0174	<0.001
Head circumference:Body weight	0.909[a]	0.822[ab]	0.746[b]	0.0524	0.013
Brain weight, g	3.19	3.31	3.30	0.202	0.380
Liver weight, g	6.99[a]	8.26[ab]	8.80[b]	0.825	0.049
Brain weight:Liver weight	0.490[a]	0.415[b]	0.390[b]	0.0283	0.013
Left *Longissimus dorsi* weight, g	1.98	2.24	2.43	0.224	0.119

[1]Crown-rump length of fetus within litter: male fetus nearest the small (SM), median (MD), large (LG) fetus.

Table 2.3. Characteristics of fetal muscle as affected by d 60 fetal size.

Muscle[1]:	LT			LL				Probability, $P <$		
d-60 Fetal Size[2]:	SM	MD	LG	SM	MD	LG	SEM	Size	Muscle	Size × Musc
n=	12	12	12	12	12	12	---			
Item										
Whole muscle CS area, (mm^2) [3]	46	58	59	41	49	54	5.2	0.001	0.001	0.506
Calculated total 1° fiber in CS, count ($\times 10^5$)[4]	1.0	1.0	1.1	0.8	1.0	1.0	0.11	0.181	0.063	0.622
Avg CSA of 1° muscle fibers, (μm^2) [5]	490	600	559	457	488	498	29.7	0.114	0.002	0.284
2° fiber:1° fiber[6]	2.4	2.1	2.5	2.1	2.3	2.4	0.32	0.359	0.781	0.302
Fiber associated nuclei:1° fiber[7]	4.5	5.4	5.2	4.7	4.1	4.5	0.32	0.756	0.025	0.071
Pax7+ nuclei:1° fiber[8]	1.8	2.1	2.0	1.8	1.6	1.8	0.22	0.933	0.141	0.418

[1]Muscle either *Longissimus thoracis* (LT) or *Longissimus lumborum* (LL).
[2]Crown-rump length of fetus within litter: male fetus nearest the small (SM), median (MD), large (LG) fetus.
[3] Cross-sectional (CS) area of the whole muscle (mm^2).
[4]Total primary muscle fiber number is calculated as the whole muscle CS area divided by the average muscle fiber cross-sectional area of primary muscle fibers.
[5]Average cross-sectional area (μm^2) of a representative sample of primary muscle fibers (n > 100).
[6]The average number of secondary muscle fibers per primary muscle fiber.
[7]The average number of nuclei associated with each primary muscle fiber.
[8]The average number of Pax7+ nuclei associated with each primary muscle fiber.

Table 2.4. Effect of intralitter d 60 fetal size on expression of genes in fetal muscle.[1]

Muscle:[2]	*Longissimus thoracis*			*Longissimus lumborum*				$P <$ [4]	
60-d Fetal Size[3]:	SM	MD	LG	SM	MD	LG	SEM	Size	Muscle
n=	12	12	12	12	12	12			
Item									
IGF-2	0.428	0.418	0.353	0.598	0.407	0.407	0.0616	0.012	0.046
IGF-2R	0.424	0.516	0.315	0.524	0.445	0.381	0.0738	0.103	0.544
IGF-1	0.292	0.212	0.203	0.325	0.288	0.238	0.0339	0.017	0.046
IGF-1R	0.368	0.453	0.352	0.495	0.392	0.370	0.0704	0.326	0.466
Myf5	0.436	0.847	0.368	0.521	0.472	0.409	0.1971	0.201	0.397
MyoD	0.794	0.596	0.465	0.854	0.572	0.533	0.0729	0.001	0.487
MyoG	0.494	0.448	0.324	0.384	0.370	0.325	0.0758	0.286	0.303
Pax7	0.465	0.500	0.401	0.527	0.501	0.413	0.0786	0.337	0.618
Beta Catenin	0.017	0.019	0.015	0.018	0.015	0.014	0.0026	0.237	0.219
Myostatin	0.019	0.022	0.020	0.025	0.023	0.019	0.0043	0.526	0.515
Pref-1	0.632	0.586	0.508	0.679	0.613	0.536	0.0805	0.145	0.519

[1]mRNA expression normalized to *RPL4* reference gene; relative expression (RQ) compared to pool of control fetus samples.
[2]Muscle either *Longissimus thoracis* (LT) or *Longissimus lumborum* (LL).
[3]Crown-rump length of fetus within litter: male fetus nearest the small (SM), median (MD), large (LG) fetus.
[4]No fetal Size × Muscle location interactions were observed ($P \geq 0.107$).

Chapter 3 - Effect of 95-day fetal pig intralitter relative body weight on myogenic and developmental characteristics.

ABSTRACT

Five gestating gilts (PIC 337 × 1050; initial BW 168 kg and age 7 mo) were used to quantify maternal serum IGF-1 concentrations from d 24 to d 88 of gestation as well as to better understand the relationships among d 95 intralitter fetal size (small [SM], median [MD], or large [LG]) and measures of placental efficiency, secondary muscle fiber formation, circulating fetal IGF-1 concentrations on d 95, and fetal growth partitioning among organs and primal carcass sections. Gilts were synchronized and artificially inseminated at approximately 7 mo of age. Maternal serum was collected on d 24, 32, 39, 46, 53, 60, 67, 74, 81, and 88 of gestation. On d 95 of gestation, gilts were euthanized and fetal weights, measurements, and muscle and serum samples were collected. Concentrations of IGF-1 in maternal serum were greater ($P < 0.05$) on d 24 than on d 88 of gestation but little change was observed between d 53 and 88. Fetal BW, crown-rump length, head circumference, thoracic circumference, liver weight, brain weight to liver weight ratio, and weights carcass primal sections were less ($P < 0.05$) in SM fetuses compared to those of MD and LG fetuses, which did not differ. Serum IGF-1 concentration was greater ($P < 0.05$) in SM fetuses than in MD and LG fetuses, which did not differ. Weights of placental and ham primals were less ($P < 0.05$) in SM fetuses compared to LG fetuses. The later maturing *Longissimus lumborum* had more ($P < 0.05$) secondary and total muscle fibers, but smaller myofiber cross-sectional areas ($P < 0.05$), than the *Longissimus thoracis*. Despite heavier fetuses having larger ($P = 0.017$) whole muscle areas, no differences in myogenesis were evident among fetal sizes. In summary, the decline in maternal serum IGF-1 concentration plateaus in late gestation and SM fetuses surprisingly had greater circulating IGF-1 on d 95 than MD and

LG fetuses. Fetal BW differences were consistently reflected in carcass primal weight differences but brain growth was prioritized among SM fetuses. This suggests nutrient supply may have been limited. However, no evidence for differences in placental efficiency or secondary myogenesis was observed among intralitter relative fetal sizes in late gestation.

INTRODUCTION

Primary muscle fibers provide scaffolding for secondary myofiber formation with 16 to 20 secondary fibers formed around each primary fiber (Wigmore and Stickland, 1983). Hence, the number of secondary muscle fibers that are formed may be restricted by the degree of proliferation and differentiation of embryonic myoblasts and number of primary myofibers formed. Growth compromised piglets exhibit fewer secondary muscle fibers (Tilley et al., 2007; Bérard et al., 2010) and consequently fewer total fibers at birth.

Myogenic progenitor cells expressing Pax7 transcription factor are destined to become either embryonic or fetal myoblasts, or satellite cells (Biressi et al., 2007). Total lifetime muscle fiber number and hypertrophy potential can be evaluated by d 90 of gestation when muscle hyperplasia is completed in fetal pigs (Oksbjerg et al., 2004). By this stage, Pax7 positive nuclei that have not incorporated into myofibers will remain as quiescent satellite cells to contribute to future muscle hypertrophy and repair (Biressi et al., 2007; Zammit, 2008).

Compared to pigs with heavier birth weights, light birth weight pigs have slower growth rates and at market weight, have poorer lean percentages and less percentage of carcass weight in the ham and loin (Gondret et al., 2006). Whether this weight partitioning is reflected prenatally has not been determined.

Placental weights increase from d 30 to 100 of gestation, but the rate of change after d 60 is minimal and therefore the majority of fetal growth occurs after placental membranes have

fully developed (Bazer et al., 2012). As noted by Wise et al. (1997), the placentas of light fetuses weigh less than placentas of heavy fetuses throughout gestation. The ratio of placental weight to body weight is a simple measure of placental efficiency in pigs. However, the ratio does not have the same magnitude for small and large fetuses: the placental weight of lighter fetuses is a smaller percentage of fetal BW as compared to heavier fetuses (Vallet and Freking, 2007).

Fetal IGF-1 and IGF-2 induce myoblast proliferation and differentiation by upregulation of myogenin (Rehfeldt et al., 2011). Wise et al. (1997) reported fetal weight was positively associated with fetal serum IGF-1 concentrations with 92% of the variation in serum IGF-1 explained by fetal weight. However, IGF-1 is also produced in myofibers and the local concentration increases throughout gestation (Gerrard et al., 1998). There is evidence that regulation of IGF-1 mRNA expression is tissue specific (Ramsay et al., 1994).

Previous research has shown maternal circulating IGF-1 decreases from d 24 to 60 of gestation (Chapter 2). Similarly, Farmer et al. (2000) reported IGF-1 concentration decreases to d 70 of gestation with no further decrease to d 109 although estradiol and prolactin dramatically increase from d 70 to 109. Further quantification of maternal IGF-1 concentrations in late gestation is of interest because tissue specific suppression of IGF-1 by estradiol has been reported (Scheidegger et al., 2000).

The objectives of this experiment were to quantify maternal serum IGF-1 to d 88 of gestation, and to better understand the effect of d 95 fetal size differences on 1) measures of placental efficiency, 2) secondary muscle fiber formation and circulating IGF-1 concentration, and 3) how smaller fetuses partition growth among organs and primal carcass sections.

MATERIALS AND METHODS

The protocol for this experiment was approved by the Kansas State University Institutional Animal Care and Use Committee. The study was conducted at the Kansas State University Swine Teaching and Research Center in Manhattan, KS.

Animal Management and Housing

Gilts (PIC 337 × 1050) were initially group housed in pens where they had *ad libitum* access to water and a non-medicated diet which met or exceeded NRC (2012) recommendations. At approximately 175 d of age, gilts began receiving daily exposure to mature boars and were observed for visual signs of estrous. From this group, 5 sexually mature gilts (initially 168 kg BW and age 7 mo) were enrolled in the current study and individually housed in gestation stalls (2.88 m^2). Estrus was synchronized by administering 6.8 mL Matrix® (15 mg altrenogest) by top-dressing on each gilt's daily feed for 14 consecutive d. Beginning 3 d after last Matrix® administration, twice daily detections of estrus with mature boars began. Gilts were artificially inseminated (PIC 337 semen) up to 3 times at evening and morning intervals beginning 12 h after standing estrus was first detected (onset of estrus = d 0). Pregnancy was evaluated by transcutaneous ultrasound on d 24 to 26 of gestation. Gilts gestated throughout the months of October, November, December, and January. Animals were monitored daily and limit fed (2.3 kg day^{-1}) once daily a standard non-medicated diet that met or exceeded nutrient requirements (NRC, 2012).

Maternal Serum Collection, Processing, and ELISA

Gilts were restrained by a snout snare at approximately 4 h after feeding on d 24, 32, 39, 46, 53, 60, 67, 74, 81, and 88 of gestation for collection of blood from the anterior vena

cava/caudal jugular vein. Blood was collected into 10 ml red topped glass vacuum tubes containing no additives (Covidien LP, Mansfield, MA). Whole blood was transported on ice to the K-State Muscle Biology Laboratory for further processing according to the procedures outlined in Chapter 2. Concentration of the maternal serum IGF-1 was analysed using acommercial IGF-1 (human) ELISA kit (ADI-900-150; ENZO Life Sciences, Farmingdale, NY) according to the methods descrbed in Chapter 2.

Fetal Measurements and Sample Collection

Gilts were euthanized across 2 consecutive sample collection d. On gestation d 95 or 96, gilts were electrically stunned and exsanguinated. Entire reproductive tracts (ovaries, oviducts, gravid uterus, and cervix) were removed from the body. The uterine horns were freed from surrounding connective tissue and dissected from the mesometrium. The uterine wall was opened longitudinally from the ovarian end and gently pulled away from the fetal placental membranes. The crown-rump length of each fetus was measured (poll to tail head) without removing the fetal membranes. The attachment length of each placenta was measured as the length of the allantochorion between and not including the necrotic tips. The placenta of each fetus was uniquely identified as fetuses were removed from the placental and amniotic membranes. Umbilical cords were severed within 1 cm of the fetus. Individual placentas were then removed, blotted dry, and weighed. Fetuses were also individually weighed and 3 male fetuses with BW closest to those of the heaviest, median, and lightest BW's within each litter were identified as large (LG), median (MD), and small (SM) fetuses. Further measurements, weights, and *Longissimus* muscle samples were collected from this 3-fetus subset of each litter. Data collection was restricted to male fetuses to avoid potentially confounding results with differences

due to sex. The BW to attachment length and BW to placental weight ratios for each of the fetuses were calculated.

Electronic digital calipers (Model 14-648-17; Fisher Scientific, Pittsburgh, PA) were used to measure the head width of each fetus as the linear distance across the head immediately posterior to the eye. Fetal head circumference was assessed at the same anatomical location by wrapping a flexible cord around the maximum circumference (poll to mandible). Likewise, the thoracic circumference was determined by wrapping the cord around the thorax just posterior to the shoulders.

Fetal blood was collected by cardiac puncture from the 3-fetus subset using a 12 mL syringe and 18-gauge needle. Blood was transferred into red topped glass tubes containing no additives and kept on ice until processed for serum collection.

The *Longissimus dorsi* was segmented into the *Longissimus thoracis* and *Longissimus lumborum* and samples were collected for mRNA and immunohistochemical analysis. *Longissimus thoraci s* immunohistochemistry samples were taken immediately posterior to the point of the trapezius muscle, while mRNA samples were dissected from under the trapezius and anterior to the other sample. *Longissimus lumborum* immunohistochemistry samples were harvested at approximately the last rib and mRNA samples were collected posterior to this sample.

Muscle cross-sections which were to be used for immunohistochemistry were blotted on paper, the perimeter traced, blots scanned using a digital scanner (HP DeskJet 3050; Hewlett Packard, Palo Alto, CA), and whole muscle cross-sectional area was measured using Nikon NIS-Elements Basic (Nikon Instruments Inc, Melville, NY).

The cross-section was then embedded in tissue embedding media (Fisher Scientific), frozen in liquid nitrogen cooled isopentane, and stored at -80°C. Portions of the muscle cross-sections to be used in mRNA analysis were deposited in individual tubes, snap-frozen, and stored at -80°C until further analysis. The fetal skull was dissected and the complete brain removed and weighed. Similarly, the abdominal cavity of the fetus was opened and the liver was removed and weighed. The ratio of brain weight: liver weight was then calculated for each fetus.

Primal sections were fabricated from the fetal carcass according to anatomical reference points adapted from the IMPS Fresh Pork Series 400 (USDA, 2014). The leg (Item No. 401), whole shoulder (Item No. 403), skinned belly (Item No. 409), and loin (Item No. 410) were removed from each side of the fetal carcass. Each primal section was individually weighed then the weights of the respective primals from each side were summed to represent total shoulder, loin, belly, and ham primal weights for each fetus.

Fetal Sample Analyses

Serum Processing and Radioimmunoassay

Fetal serum IGF-I was analyzed in duplicate via radioimmunoassay (Echternkamp et al., 1990; Funston et al., 1995). Serum IGFBP were extracted from serum using a 1:17 ratio of sample to acidified ethanol (12.5% 2 N HCl: 87.5% absolute ethanol) (Daughaday et al., 1980). Extracted samples were centrifuged (12,000 x g at 4°C) to separate IGFBP. A portion of the resulting supernatant was removed and neutralized with 0.855 M Tris base, incubated for an additional 4 h at 4°C, then centrifuged at 12,000 x g at 4°C to remove any additional IGFBP. When samples of this extract, equivalent to the original serum sample, were subjected to Western ligand blot analysis and subsequent phosphorimagery, no detected binding of [125I]IGF-I to IGFBP was observed. Inhibition curves of the neutralized extracted serum ranging from 25 to

100 µL were parallel to the standard curve. Recombinant human IGF-I (GF-050; Austral Biological, San Ramon, CA, USA) was used as the standard and radioiodinated antigen. Antisera AFP 4892898 (National Hormone and Peptide Program, National Institutes of Diabetes, Digestive and Kidney Diseases, Bethesda, MD, USA) was used at a dilution of 1:62,500. Sensitivity of the assay was 16.9 pg per tube and intra-assay coefficient of variation was 8.2%.

Immunohistochemistry

A 10-µm cryosection from each fetal tissue sample was affixed to a frost resistant slide to allow for immunodetection. The immunohistochemistry methods outlined in Chapter 2 were used with no modifications. Whole muscle and fiber cross-sectional areas were measured using NIS-Elements Imaging Software (Basic Research, 3.3; Nikon Instruments Inc.). Fibers that stained positively for the BA-D5 antibody were categorized as primary muscle fibers while the fibers that stained positively for SC-71 were labelled as secondary fibers. Total nuceli count was determined by all fiber associated nuclei that were Hoechst 33342 positive. Total muscle fiber number was calculated as the whole muscle area divided by the average muscle fiber cross-sectional area of muscle fibers. Primary and secondary fiber numbers were calculated based on total fiber number and the relative percentage of each fiber type.

Statistical Analysis

Maternal serum IGF-1 was analyzed as a completely randomized design with each gilt serving as an experimental unit to assess the effect of day of gestation on serum IGF-1 concentration. The MIXED procedure in SAS (v9.3, SAS Institute Inc., Cary, NC) was used to model day of gestation as a fixed effect with a random effect of gilt to account for repeated sampling of the gilts across days of gestation.

Fetal serum IGF-1, morphometrics and weights were analyzed as a randomized complete block design with a one way treatment structure and replicated over 2 sample collection days. Each fetus was an experimental unit and dam was a random block factor. The MIXED procedure in SAS was used to model fetal size as a fixed effect with random effects of sample collection day and dam nested within sample collection day. The random effects of fetal size × sample collection day and fetal size × dam within sample collection day were pooled into the residual.

Fetal muscle histology was analyzed as a randomized complete block design with a split-plot, 3 × 2 treatment structure, and replication over 2 sample collection days. Hence, responses were modeled with fixed effects of fetal size on the fetus whole-plot experimental unit and muscle location on the *Longissimus* split-plot experimental unit and with random effects of sample collection day, dam within sample collection day, and the interaction between fetal size × dam within sample collection day. The random interactions between fetal size × sample collection day and fetal size × dam within sample collection day were pooled into the whole plot residual used to test the effect of fetal size. The random interactions between muscle location × sample collection day and muscle location × dam within sample collection day, as well as muscle location × fetal size × sample collection day and muscle location × fetal size × dam within sample collection day were pooled into the split-plot error term used to test the effect of muscle location and the interactions between fetal size and muscle location.

For all variables, differences between least squares means were computed using the PDIFF option in SAS. All results were considered statistically significant at $P \leq 0.05$; results with P-values > 0.05 and ≤ 0.10 were considered marginally significant.

RESULTS

Maternal and Fetal Serum IGF-1 Concentrations

Maternal serum IGF-1 concentrations decreased from d 24 of gestation to d 88 in a stepwise fashion. Concentrations of IGF-1were less ($P < 0.05$) on d 39 ± 1 than on d 32 ± 1; less ($P < 0.05$) on d 53 ± 1 than on d 24 ± 1, and less ($P < 0.05$) on d 88 ± 2 than levels on d 24 ± 1, 32 ± 1, 39 ± 1, and 46 ± 1 (Figure 3.1). On d 95 of gestation, the serum IGF-1 concentration of SM fetuses was greater ($P < 0.05$) than those of MD and LG fetuses (Table 3.1).

Fetal and Placental Morphometrics and Weights

The male fetuses evaluated in this study were classified into size categories based on BW proximity to the heaviest, lightest, and median BW fetuses of each litter. The BW and crown-rump length of SM fetuses were lighter and shorter ($P < 0.05$) than BW and crown-rump lengths of MD and LG fetuses (Table 3.1). The total primal weights of the fetuses corresponded to BW as the weights of shoulders, loins, and bellies of SM fetuses were lighter ($P < 0.05$) than those of MD and LG fetuses. The total weight of ham primals of SM fetuses was smaller ($P < 0.05$) than that of LG fetuses but not different from MD fetuses.

The thoracic and the head circumferences of SM fetuses were smaller ($P < 0.05$) than those of MD and LG fetuses (Table 3.1). Fetal size had a marginally significant effect ($P = 0.074$) on head width with S fetuses having the narrowest head widths. Brain weights were not different ($P = 0.163$) among fetal sizes, but liver weights of MD and LG fetuses were larger ($P < 0.05$) than that of SM fetuses. Thus, the brain weight:liver weight ratio of SM fetuses was greater ($P < 0.05$) than that of MD and LG fetuses.

No difference ($P = 0.131$) in placental attachment length across fetal sizes was observed (Table 3.1). Fetal size had a marginally significant effect ($P = 0.093$) on the ratio of body

weight:attachment length with MD, LG, and SM fetuses having decreasing ratios, respectively. Although attachment lengths were not different, the placental weights of LG fetuses were heavier ($P < 0.05$) than those of SM fetuses and those of MD fetuses were not different from the placental weights of either SM or LG fetuses. Consequently, no difference ($P = 0.538$) in BW:placental weight was observed for fetuses of the different size categories.

Fetal Muscle Histology

No interactions ($P \geq 0.206$) between fetal size and muscle location were observed in the fetal muscle histology results. The *Longissimus* whole muscle cross-sectional area was not different ($P = 0.176$) between the *Longissimus thoracis* and *Longissimus lumborum* (Table 3.2). However, the *Longissimus lumborum* was characterized by a greater number of total muscle fibers ($P = 0.009$) and total secondary muscle fibers ($P = 0.008$) than in the *Longissimus thoracis*. No difference ($P = 0.332$) in the number of primary muscle fibers was observed due to muscle location and consequently, there was a marginally significant greater ($P = 0.087$) number of secondary muscle fibers per primary muscle fiber in the *Longissimus lumborum* compared to in the *Longissimus thoracis*. However, the average cross-sectional area of muscle fibers in the *Longissimus thoracis* were larger than the average cross-sectional area of all muscle fibers ($P = 0.012$), primary muscle fibers ($P = 0.024$), and secondary muscle fibers ($P = 0.014$) in the *Longissimus lumborum.* No differences in the number of Pax7 positive nuclei per muscle fiber ($P = 0.953$) or myonuclei per muscle fiber ($P = 0.267$) were observed between the two muscle locations.

The cross-sectional area of the *Longissimus* muscle increased with increasing fetal size so that the LG and MD fetuses had larger ($P < 0.05$) whole muscle cross-sectional areas than the SM fetuses (Table 3.2). However, fetal size did not affect ($P = 0.105$) muscle fiber average

cross-sectional areas in the small fetal subset of this study, nor were any differences in the total number of muscle fibers ($P = 0.318$), number of primary muscle fibers ($P = 0.817$), or number of secondary muscle fibers ($P = 0.273$) observed across the fetal sizes. In addition, the number of Pax7 positive nuclei per muscle fiber and the number of myonuclei per muscle fiber did not differ ($P = 0.474$ and 0.298, respectively) among fetal sizes.

DISCUSSION

Internal research has previously characterized the decline in endogenous gestating gilt IGF-1 concentration until d 60 of gestation (Chapter 2). Further quantification of maternal IGF-1 concentration later in the gestation period is of interest as estrogens increase in late gestation and tissue specific suppression of IGF-1 by estradiol has been reported in rat aortic smooth muscle (Scheidegger et al., 2000). In the current study, maternal circulating IGF-1 concentration decreased from d 24 to 88 of gestation in a stepwise fashion. This curious pattern is in contrast to the smooth decline previously observed among gestating gilts (Chapter 2). However, serum levels between consecutive sampling days significantly differed only between d 32 and d 39, so the fluctuations could be reflective simply of variation among the small number of gilts sampled in this study.

In the present study, little decline in circulating IGF-1 was observed from d 53 to 88. Likewise, Brown et al. (2007) observed a decrease in plasma IGF-1 of gestating gilts from d 0 to 40 of gestation then relatively constant levels to d 70 of gestation. In addition, Farmer et al. (2000) reported a similar pattern of declining IGF-1 from around 170 ng ml^{-1} at breeding to around 80 ng ml^{-1} by d 70 of gestation with no further decline evident by d 109. At the same time, estradiol levels on d 109 (561 pg ml^{-1}) were much greater than on d 70 (20 pg ml^{-1}) and circulating IGF-1 did not appear to have a concomitant decrease in either study. Notably,

circulating concentrations of IGF-1 were on average 35 ng ul^{-1} lower in the current study than levels reported by Farmer et al. (2000) and also less than levels observed in our previous study (Chapter 2). Between the studies, gilts had similar feed intake and age at breeding. However, the lesser amount of circulating IGF-1 in the gilts of the current study could have been due to the fact that these gilts were over 30 kg heavier at breeding and genetically a maternal x terminal line in contrast to the lighter, hyperprolific breeds studied by Farmer et al. (2000) but variability in gilt levels between our studies remains unexplained.

Fetal IGF-1 concentrations are less than IGF-2 prenatally, but increase postnatally (Lee et al., 1993) and low levels of the IGF’s are associated with retarded fetal growth in humans (Randhawa and Cohen, 2005). On d 95 of gestation, the circulating IGF-1 concentrations of SM fetuses were greater than levels of MD and LG fetuses. In contrast, Wise et al. (1997) reported serum IGF-1 concentrations on d 104 of gestation increased with increasing fetal weight ($R^2 = 0.92$), although Gondret et al. (2013) observed no difference between low and medium weight pigs on d 112 of gestation. The conflicting observations among the studies is unexplainable at this time. Regardless of fetal size however, bioavailability of the IGF’s can be decreased and their circulating half-life can simultaneously be extended through the action of the 6 IGF-binding proteins (IGFBP) which have important functions in mediating the IGF action (Allan et al., 2001; Monzavi, 2002). Fetal growth is thus orchestrated by the complex interactions between IGFBP’s, IGF’s, and IGF receptors so that quantification of circulating IGF-1 alone provides only limited insight to fetal growth regulation.

Fetal weight was significantly correlated with crown-rump length ($r = 0.92$) overall from d 20 to 100 of gestation (Knight et al., 1977) and in the present study, both BW and crown-rump length followed a similar pattern across fetal sizes where SM fetuses were both lighter and

shorter than MD and LG fetuses across litters. In addition, differences in thoracic circumference mirrored those of BW and crown-rump length. Interestingly, although fetuses were selected to represent the median and extreme fetal sizes within each litter, the LG and MD fetuses were of more similar size than MD and SM of each litter. Observations by Milligan et al. (2002) affirm this relationship as larger litters are associated with lighter birth weight pigs and a negatively skewed birth weight distribution.

Chen and Dziuk (1993) reported that growth retardation in fetuses was evident by d 40 of gestation. In general, the total primal weights of the fetuses from the study herein directly corresponded to fetal BW as the weights of shoulders, loins, and bellies of SM fetuses were lighter than those of the MD and LG fetuses which did not differ. Although relative carcass proportions were not calculated in the present study, the smaller proportions of loin and ham in the carcasses of lighter birthweight pigs reported by Gondret et al. (2006) may have developed postnatally due to factors established *in utero* but not phenotypically evident until later in life. Regardless, differences in BW appear to be reflected prenatally in retarded skeletal muscle growth across all carcass primals by d 95 of gestation.

The head circumferences of SM fetuses on d 95 were smaller than those of MD and LG fetuses and fetal size had a marginally significant effect on head width with SM fetuses having the narrowest head widths. In a previous study, no differences were observed in head circumference on d 60, but head widths decreased with fetal size (Chapter 2). Notwithstanding this difference between d 60 and d 95, the d 95 brain weights, liver weights, and ratios of brain weight to liver weight across fetal sizes were consistent with previous observations on d 60 (Chapter 2), where brain weight was conserved across fetal sizes, but liver weight reflected total fetal BW and therefore the ratio of brain weight to liver weight was greater in SM than in MD or

LG fetuses. These observations contradict those of Amdi et al. (2013) who reported lighter relative liver weights among severely growth restricted neonatal pigs and better align with the observations of Vallet and Freking (2006) who reported initial differences in relative liver weight due to fetal size, but found these differences had been rectified by mid-gestation. Therefore, it is possible that the fetuses of the present study could have experienced stunted liver growth earlier in gestation but had high impetus liver growth at some point prior to d 95, or the level of nutrient restriction simply was not extreme enough to affect liver weight. Because the liver weight mirrored total body weight, the ratio of brain to liver weight was greater in the SM fetuses than in the MD or LG fetuses and is suggestive of at least a small degree of nutrient restriction whereby small fetuses prioritized nutrients to brain development. Also, the head measurements appeared to better follow the pattern of total fetal BW across fetal sizes on d 95 than the d 60 weights mirrored each other. This phenomenon could be a reflection of the larger magnitude of the difference in fetal sizes later in gestation.

Knight et al. (1977) observed that placental length and width ($R^2 = 0.59$) and placental weight ($R^2 = 0.55$) were strongly associated with fetal weight and necessarily, average placental weight is negatively correlated with litter size (Wilson et al., 1999). Despite BW differences across the fetal size designations in the current study, placental attachment length did not differ nor was the ratio of fetal BW to attachment length different across fetal sizes. However, placental weights increased with increasing fetal size so that the ratio of BW to placental weight was not different between large versus small fetuses. Wise et al. (1997) similarly observed that placental weights of light fetuses were lighter than the placental weights of heavier fetuses (Wise et al., 1997). However, Mesa et al. (2003) reported that the relationship between placental weight and weight at birth is curvilinear such that birthweight increases with increasing placental weight

at a decreasing rate. Furthermore, Vallet et al. (2013) accounted for the fact that fetal weight gain is not directly proportional to changes in placental weight. They observed that the dependency of fetal weight on placental weight was not constant across fetal weights indicating some “sparing” mechanism may exist which enables placentas of smaller fetuses to be more efficient in nutrient transfer and facilitate relatively greater fetal growth (Vallet et al., 2013). Differences in placental function amongst the small number of fetal sizes in the current study may have been difficult to detect by solely comparing BW to placental weight ratios among fetal size designations because a change in placental weight is not proportional to a change in BW and the ratio of a given BW to a given placental weight changes across placental weights.

Wigmore and Stickland (1983) reported that the size of primary fibers reached their maximum size 18 d earlier in larger fetuses as compared to in small fetuses and this could be indicative of delayed primary myocyte and myofiber formation. Primary myofiber formation is completed by d 60 of gestation and after d 70, the size of primary myofibers begins to decrease. Yet regardless of fetal size and degree of primary myogenesis, the next wave of myoblast proliferation and differentiation for secondary myofiber formation begins by d 55 gestation. Research indicates that secondary myofiber formation in the epaxial muscle occurs due to fetal myoblasts traversing the dermomyotome then orienting themselves so as to attach to the primary myofiber scaffolding at which point the fetal myoblasts terminally differentiate and fuse with preexisting fibers or with other fetal myoblasts to form new myotubes (Hollway and Currie, 2005; Biressi et al., 2007). Myogenic cell hyperplasia and formation of secondary myofibers appears to be completed prenatally by d 95 of gestation (Wigmore and Stickland, 1983), thus requiring subsequent muscle growth to occur via hypertrophy of existing myofibers. The establishment of numerous myofibers is important in order to support the growth of lean muscle

mass through a greater abundance of moderately sized rather than (a fewer number of) extremely large individual muscle fiber areas. Large muscle fiber cross-sectional area is negatively correlated with undesirable pork quality characteristics such as drip loss and low pH (Rekiel et al., 2015).

In mature animals, muscle fiber diameters are reportedly larger in the *Longissimus lumborum* than in the *Longissimus thoracis* (Migdal et al., 2005) and quality parameters such as water holding capacity, color, and shear force are not consistent along the *Longissimus* muscle (van Oeckel and Warnants, 2003; Bertol et al., 2006). Some of these differences may arise due to the fact that myogenesis of the *Longissimus* muscle occurs so that the thoracis portion develops earlier than the lumborum portion. Specifically, previous research has indicated that the thoracis has a greater degree of primary myogenesis as evidenced by more primary muscle fiber numbers and larger cross-sectional areas than in the lumborum by d 60 of gestation (Chapter 2).

Yet in the current study, the *Longissimus lumborum* was characterized by a greater number of total muscle fibers by d 95 due to more total secondary muscle fibers than were in the *Longissimus thoracis.* Also, because identifiable primary muscle fiber number on d 95 did not differ between muscle locations, the *Longissimus lumborum* contained a marginally significant greater number of secondary muscle fibers per primary muscle fiber than were present in the *Longissimus thoracis*. However, the average cross-sectional areas of both primary and secondary muscle fibers in the *Longissimus thoracis* were larger than the average cross-sectional areas of primary and secondary muscle fibers in the *Longissimus lumborum* and this provides explanation for why the *Longissimus* whole muscle cross-sectional area was not different between the *Longissimus thoracis* and *Longissimus lumborum* locations.

Also, the inverse relationship between fiber number and cross-sectional area is reasonable considering the inconsonant processes of myoblast proliferation and differentiation: delayed terminal differentiation of fetal myoblasts will result in continued proliferation of cells and thereby a greater number of myogenic cells; in contrast, accelerated differentiation of myoblasts will curtail the proliferation of additional cells and differentiated myoblasts will more quickly undergo maturation and hypertrophic growth (Rehfeldt et al., 2011). Although the *Longissimus thoracis* experienced greater primary myogenesis, the current results suggest the *Longissimus lumborum* experienced a greater degree of fetal myoblast proliferation prior to terminal differentiation. Based on these findings along with the fact that no differences in Pax7 positive nuclei or nuclei per muscle fiber were observed, the *Longissimus lumborum* should have greater postnatal growth potential than the *Longissimus thoracis* among these pigs.

Quiescent muscle stem cells, referred to as satellite cells, serve as a source of myonuclei for muscle hypertrophy in the postnatal period (Zammit, 2008). These satellite cells residing between the sarcollema and basal lamina are identifiable by the expression of Pax7 which inhibits the differentiating activity of MyoD as downregulation of Pax7 allows MyoD expression to initiate terminal differentiation of these cells (Seale et al., 2000; Hawke and Garry, 2001; Olguín, 2011). By incorporating into pre-existing muscle fibers, myogenic satellite cells decrease the myonuclear domain and increase the myofiber's capacity for protein synthesis thereby supporting hypertrophy and repair of muscle fibers (Biressi et al., 2007). Pax7 positive nuclei which have not incorporated into myofibers by the end of secondary myogenesis remain as the muscle's future satellite cell pool. Also, more nuclei which have incorporated into each myofiber by 90 indicates greater potential for increased and more efficient muscle growth postnatally.

Research by Tilley et al. (2007) showed that on d 45, 65, and 100 of gestation small fetuses had fewer numbers of secondary muscle fibers per primary muscle fiber in the *Longissimus* muscle compared to average sized littermates. Likewise, Bérard et al. (2010) reported that pigs which had experienced crowding conditions *in utero* had less hyperplasia and fewer secondary muscle fibers, total muscle fibers, and fewer secondary fibers per primary fiber across various muscles. The observations of the current study failed to support these findings as no differences in total number of muscle fibers, number of primary muscle or secondary muscle fibers, or muscle fiber average cross-sectional area were observed across the fetal sizes in the small number of fetuses in this study. Granted, the *Longissimus* whole muscle cross-sectional area did increase with increasing fetal size possibly due to more connective tissue or adipocyte formation within the whole muscle. Yet no differences in the number of Pax7 positive nuclei per muscle fiber or nuclei per muscle fiber were observed among fetal sizes thus supporting the expectation that male fetuses across the BW range represented by the fetuses of this study have similar *Longissimus* muscle growth potential postnatally.

CONCLUSION

The declining maternal serum IGF-1 concentrations plateau in late gestation which may indicate estradiol has limited effects on circulating IGF-1 in gestating gilts. Surprisingly, SM fetuses had greater circulating IGF-1 concentration on d 95 than MD and LG fetuses. This finding warrants further investigation into potential differences in corresponding IGF-1 receptor and IGFBP levels in fetuses during late gestation. Differences in fetal growth appear to be consistently partitioned within the body except brain growth was prioritized among SM fetuses. This indicates that the nutrient supply of SM fetuses was limited; however, no evidence for

differences in placental efficiency or secondary myogenesis were observed among fetuses of different intralitter relative sizes in late gestation.

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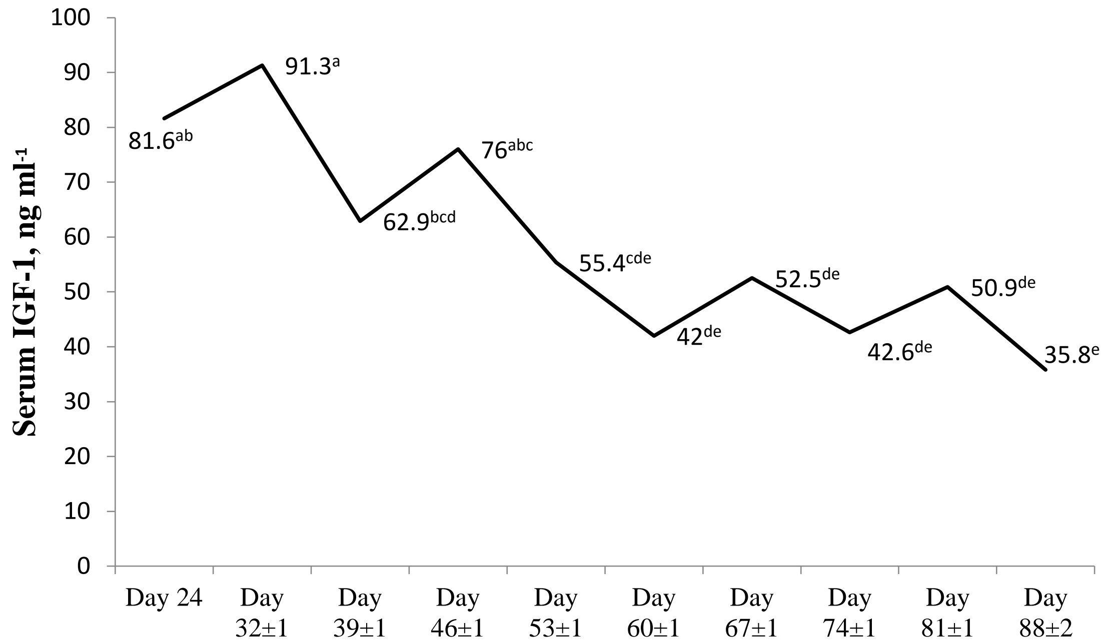

Figure 3.1. Serum IGF-1 level decrease in nulliparous sows to d 88 ± 2 gestation. Means without a common superscript differ, $P < 0.05$ (SEM = 10.45).

Table 3.1. Effect of relative size within litter on fetal serum IGF-1, growth and development.

95-d Fetal size:[1]	SM	MD	LG		
n=	5	5	5	SEM	Size, *P* <
Serum IGF-1, ng ml^{-1}	51.1[a]	36.7[b]	33.2[b]	6.02	0.018
Body weight, g	658.7[a]	892.0[b]	976.8[b]	62.14	0.002
Crown-rump length, mm	222[a]	245[b]	250[b]	6.9	0.016
Total shoulder primals, g	62.3[a]	93.9[b]	93.6[b]	7.35	0.009
Total loin primals, g	67.4[a]	88.2[b]	97.7[b]	7.50	0.012
Total belly primals, g	36.7[a]	52.0[b]	57.6[b]	4.72	0.005
Total ham primals, g	74.7[a]	95.7[ab]	114.0[b]	8.35	0.008
Thoracic circumference, mm	170[a]	192[b]	197[b]	6.9	0.011
Head circumference, mm	170[a]	183[b]	185[b]	4.4	0.013
Head width, mm	40.2	42.2	43.0	1.12	0.074
Brain weight, g	20.0	22.4	20.8	0.94	0.163
Liver weight, g	15.3[a]	23.0[b]	28.8[b]	2.16	0.004
Brain weight:Liver weight	1.38[a]	0.98[b]	0.74[b]	0.102	0.004
Attachment length, mm	237	243	304	39.0	0.131
Body weight:Attachment length	2.95	4.10	3.21	0.593	0.093
Placenta weight, g	189.5[a]	217.8[ab]	293.1[b]	42.08	0.040
Body weight:Placenta weight	3.54	4.31	3.54	0.677	0.538

[1]Male fetus closest to lightest (SM), median (MD), or heaviest (LG) body weight fetuses within litter.

Table 3.2. Fetal myogenesis corresponding to 95-d fetal intralitter relative size.

Muscle[1]:	LT			LL				Probablilty, $P <$	
d-95 Fetal Size[2]:	SM	MD	LG	SM	MD	LG	SEM	Size	Muscle
n=	5	5	5	5	5	5	---		
Item									
Whole muscle area, (mm^2) [3]	115	143	163	128	164	170	15.2	0.017	0.176
Average CSA, (μm^2) [4]	87	97	102	72	86	85	7.6	0.105	0.012
Average Primary CSA, (μm^2) [5]	159	155	161	127	140	138	11.7	0.840	0.024
Average Secondary CSA, (μm^2) [6]	84	94	98	70	83	82	7.6	0.109	0.014
Total fiber number (1×10^6)[7]	1.4	1.5	1.7	1.8	1.9	2.0	0.19	0.318	0.009
Total primary fiber number (1×10^4) [8]	8.1	6.3	8.1	9.1	9.1	7.7	1.47	0.817	0.332
Total secondary fiber number (1×10^6) [9]	1.3	1.4	1.6	1.7	1.8	2.0	0.18	0.273	0.008
2° fiber:1° fiber [10]	17	22	20	23	21	28	2.8	0.377	0.087
Pax7+ nuclei per muscle fiber[11]	0.13	0.19	0.21	0.18	0.16	0.20	0.035	0.474	0.953
Nuclei per muscle fiber[12]	1.4	1.1	0.92	1.2	0.84	0.68	0.304	0.298	0.267

[1]Muscle either *Longissimus thoracis* (LT) or *Longissimus lumborum* (LL); No fetal Size × Muscle location interactions were observed ($P \geq 0.206$).
[2] Male fetus nearest the small (SM), median (MD), large (LG) fetus of the litter.
[3] Cross-sectional area (mm^2) of the whole muscle.
[4]Average cross-sectional area (μm^2) of all muscle fibers.
[5]Average cross-sectional area (μm^2) of a representative sample of primary muscle fibers (n > 100)
[6]Average cross-sectional area (μm^2) of a representative sample of secondary muscle fibers (n > 100)
[7]Total muscle fiber number is calculated as the whole muscle area divided by the average muscle fiber cross-sectional area of muscle fibers.
[8]Total primary muscle fiber number is calculated as the whole muscle area divided by the average muscle fiber cross-sectional area of muscle fibers.
[9]Total secondary muscle fiber number is calculated based on total fiber number and the percentage of secondary muscle fibers.
[10]The average number of secondary muscle fibers per primary muscle fiber.
[11]The average number of Pax7+ nuclei associated with each muscle fiber.
[12]The average number of nuclei associated with each muscle fiber.

Chapter 4 - Effects of dietary chlortetracycline, *Origanum* essential oil, and pharmacological Zn and Cu on growth performance of nursery pigs.

ABSTRACT

Two 47-d experiments were conducted with 21-d old weaned pigs (PIC 1050, initially 6.1 kg) to determine the effects of feeding low or high doses of chlortetracycline (CTC) and antibiotic alternatives (Zn, Cu, and essential oil), alone or in combination, on growth performance. On d 5 postweaning, pens of 5 pigs were allotted to diet treatments with 8 (Exp. 1) or 7 (Exp. 2) replicate pens per treatment. In Exp. 1, treatments were fed from d 5 to 26 postweaning and arranged in a 2 × 3 factorial with main effects of added ZnO (0 vs. 2,500 ppm of Zn) and CTC (0, 55, or 441 ppm). In Exp. 2, treatments were fed from d 5 to 33 and structured in a 2 × 2 × 2 + 2 factorial with main effects of added $CuSO_4$ (0 vs. 125 ppm Cu), added ZnO (0 vs. 3,000 ppm Zn from d 5 to 12 and 2,000 ppm Zn from d 12 to 33), and Regano EX® (0 vs. 0.1% Regano EX® containing 5% *Origanum* oil; Ralco Animal Nutrition, Marshall, MN). The 2 additional treatments were subtherapeutic (55 ppm) and therapeutic (441 ppm) levels of CTC. Following the treatment period, a common diet without antimicrobial was fed until d 47. All diets contained 16.5 ppm Cu and either 110 ppm (Exp. 1) or 165 ppm (Exp. 2) Zn from the trace mineral premix. In Exp. 1, no ZnO × CTC interactions were observed. Feeding ZnO increased ($P < 0.05$) ADG, ADFI, and BW during the treatment period and increased ($P < 0.05$) ADG and ADFI overall (d 5 to 47). Pigs fed CTC had increased (linear, $P < 0.05$) ADG, ADFI, and BW during the treatment period and had marginally significant increases (linear, $P < 0.10$) in overall ADG and ADFI, but overall G:F tended (quadratic, $P = 0.070$) to increase then decrease as CTC increased. During the treatment period in Exp. 2, essential oil (EO) did not affect ADG or ADFI

whereas pharmacological levels of Cu, Zn, and CTC increased (*P* < 0.05) ADG with coinciding increases (*P* = 0.055, 0.006, and linear 0.079, respectively) in ADFI. Copper, Zn, and CTC did not affect G:F. Essential oil decreased (*P* = 0.009) G:F. Diet treatments had minimal carryover effects on subsequent nursery pig growth performance. Overall from d 5 to 47, Cu increased (*P* = 0.018) ADG, Zn increased (*P* < 0.05) ADG and ADFI, and EO tended to decrease (*P* = 0.086) G:F. In conclusion, increased dietary Cu, Zn, or CTC improved weanling pig performance while EO elicited no growth benefits. The benefits of added Zn from ZnO and CTC were additive and could be included together in diets to maximize growth performance of weaned pigs.

INTRODUCTION

Since feed-grade antibiotics became available in the 1950s, research has demonstrated that dietary inclusion of these antimicrobial agents improves growth rate and feed efficiency of nursery pigs. The broad-spectrum antibiotic chlortetracycline (CTC) has long been shown to improve the rate of gain and feed efficiency of pigs (NCR-89, 1984; Taylor and Rowell, 1957). Yet, alternative means of improving young pig growth performance via feed additives have been sought particularly with growing concern about antimicrobial resistance (Turner et al., 2001).

Feeding pharmacological levels of Zn from ZnO or Cu from $CuSO_4$ consistently improves feed intake and growth rate of nursery pigs (Pérez et al., 2011; Shelton et al., 2011). Essential oils have also been evaluated as alternatives to dietary antibiotics. The major constituents of oregano (*Origanum vulgare*) essential oil are the bioactive phenolic compounds carvacrol and thymol (Burt, 2004) which have antioxidant properties and antimicrobial action against both Gram positive and Gram negative bacteria (Windisch et al., 2008). Essential oils have been proposed to improve growth through establishment of a healthier gastrointestinal

microbiota with fewer pathogenic bacteria, less microbial fermentation, and thus an intestinal environment with enhanced digestive and absorptive capacity (Windisch et al., 2008).

Limited research has assessed whether pharmacological Zn influences the pigs' response to CTC and also whether dietary supplements commonly fed as antibiotic alternatives have interactive effects on weaned pig growth performance. Thus, 2 experiments were conducted to 1) evaluate whether a high level of dietary Zn fed in combination with low dosage or therapeutic levels of CTC have interactive effects on the performance of nursery pigs; and 2) compare the growth performance of pigs fed CTC with that of pigs fed pharmacological levels of Cu, pharmacological levels of Zn, and oregano essential oil, alone or in combination.

MATERIALS AND METHODS

General

The protocols for these experiments were approved by the Kansas State University Institutional Animal Care and Use Committee. The experiments were conducted at the K-State Segregated Early Weaning Facility in Manhattan, KS. Each pen (3.2 m^2) had metal tri-bar flooring, one 4-hole self-feeder, and a cup waterer to provide *ad libitum* access to feed and water. Pigs were weaned at approximately 21 d of age. To avoid any potentially confounded treatment responses due to postweaning lags in feed intake, all pigs were fed a common pelleted starter diet for the first 5 d after weaning. On d 5 postweaning, pens of 5 pigs each were randomly allotted to dietary treatments in a randomized complete block design with blocks based on location within barn.

The initial common diet contained no antimicrobial, no essential oil, nor any added Zn or Cu above that contained in the trace mineral premix (Table 4.1). The test ingredients were substituted for an equivalent amount of corn in the respective diets to form the experimental

treatments (Table 4.1). Diet samples were collected periodically throughout the study. Pooled samples of each diet were analyzed for DM (method 935.29; AOAC, 2012); CP (method 990.03; AOAC, 2012); crude fat using method 920.39a (AOAC, 2012) for preparation and ANKOM solvent extraction procedure (ANKOM, 2004) with ANKOM XT20 Fat Analyzer (Ankom Technology; Fairport, NY); CF using method 978.10 (AOAC, 2012) for preparation and ANKOM CF determination procedure (ANKOM, 2005) with ANKOM 2000 Fiber Analyzer (Ankom Technology; Fairport, NY); ash (method 942.05; AOAC, 2012); minerals and metals (in duplicate) with sample preparation according to method 968.08b (AOAC, 2012) and analysis using an iCAP 6500 series ICP Emission Spectrometer (Thermo Electron Corp., Marietta, OH) (Ward Laboratories, Inc., Kearney, NE; Table 4.2). Dietary ME and NE values were derived from feed ingredient energy values based on those in the NRC (2012).

Experiment 1

Animals and Management

A total of 240 nursery pigs (PIC 1050; initially 6.08 kg BW) were used in a 47-d study with 5 pigs per pen and 8 replications per treatment. Treatment diets were fed from d 5 to 26 postweaning at which time all pigs received a common diet and growth performance was monitored for an additional 3 wk to d 47 postweaning to assess potential carryover effects of dietary treatment. Average daily gain, ADFI, and G:F were determined by weighing pigs and measuring feed disappearance on d 5, 26, and 47.

Diet Composition

The 6 dietary treatments were arranged in a 2 × 3 factorial with main effects of added Zn from ZnO (0 vs. 2,500 ppm of added Zn) and CTC (0, 55, or 441 ppm). United States Food and Drug Administration regulations (Code of Federal Regulations) prohibit the continuous feeding

of therapeutic levels of CTC longer than 14 d. Thus, on study d 15, the feeders from pens assigned to the 441 ppm CTC diets were emptied and pigs were fed the control diet with or without the 2,500 ppm of added Zn. The normal treatment diet containing CTC at 441 ppm was then re-added to the feeders on d 16 and fed for the remainder of the 21-d period. Treatment diets were corn-soybean meal-based and contained 10% dried whey, 1.25% fish meal, and 1.25% blood cells. From d 26 to 47, a common corn-soybean meal–based diet with no added ZnO and no CTC was fed to all pigs to evaluate any carryover effects from the treatment diets. All diets contained 110 ppm of Zn from the trace mineral premix. As determined by analysis, the common diet fed for the first 5 d postweaning contained 166 ppm Zn and 29 ppm Cu whereas the common diet fed after the treatment period contained 160 ppm Zn and 21 ppm Cu.

Experiment 2

Animals and Management

A total of 350 nursery pigs (PIC 1050; initially 6.05 kg BW) were used in a 47-d study with 5 pigs per pen and 7 replications per treatment. Weaned pigs exhibited clinical signs of influenza infection upon entry into the barn. Pigs with clinical signs for which injectable treatment was deemed necessary were removed from the study which contributed to an elevated 4% removal rate during the study. Removal rate was not influenced by dietary treatment. Treatment diets were fed from d 5 to 33 postweaning at which time all pigs received a common diet and growth performance was monitored for an additional 2 wk to d 47 postweaning in order to assess potential carryover effects of dietary treatment. Average daily gain, ADFI, and G:F were determined by weighing pigs and measuring feed disappearance on d 5, 33, and 47.

Diet Composition

The basal diet formulations used in Exp. 1 were used for Exp. 2. The 10 dietary treatments fed from d 5 to 33 were structured as a 2 × 2 × 2 + 2 factorial with main effects of added Cu from copper sulfate (CuSO4; 0 vs. 125 ppm Cu), added Zn from zinc oxide (ZnO; 0 vs. 3,000 ppm Zn from d 5 to 12 and 2,000 ppm Zn from d 12 to 33), or Regano EX® (0 vs. 0.1% Regano EX® containing *Origanum* oil; Ralco Animal Nutrition, Marshall, MN). The 2 additional treatments were CTC at sub-therapeutic (55 ppm) or therapeutic (441 ppm) levels. All diets contained 16.5 ppm Cu and 165 ppm of Zn from the trace mineral premix. As determined by analysis, the Phase 1 common diet contained 114 ppm Zn and 24 ppm Cu, whereas the Phase 3 common diet contained 144 ppm Zn and 24 ppm Cu.

Similar to Exp. 1, in order to comply with United States Food and Drug Administration regulations, on d 19 of the study the feeders from pens assigned to the 441 ppm CTC diet were emptied and pigs were fed the control diet for 1 d. The normal treatment diet containing CTC at 441 ppm was then re-added on d 20 and fed for the remainder of the 28-d period (d 5 to 33). From d 33 to 47, a common corn-soybean meal–based diet without any antimicrobial, essential oil, or pharmacological levels of Cu or Zn was fed to all pigs to evaluate any carryover effects from the treatment diets.

Statistical Analysis

For each experiment, growth data were analyzed as a randomized complete block design with pen as the experimental unit. The PROC MIXED procedure of SAS (v9.3, SAS Institute Inc., Cary, NC) was used to model diet treatment as a fixed effect and barn location nested within barn as a random effect. The main effects of Zn, Cu, CTC, and essential oil, as well as any interactions, were tested using *a priori* orthogonal CONTRAST statements. Within the CTC treatments, linear and quadratic contrasts were used. Results were considered statistically

significant at $P \leq 0.05$; results with P-values > 0.05 and ≤ 0.10 were considered marginally significant.

In Exp. 2, analysis of studentized residual values revealed a geographic cluster of four pens, each on a different treatment (essential oil, Cu+Zn, Cu+essential oil, Cu+Zn+essential oil), which had ADG or feed efficiency observations greater than 3 standard deviations from the mean. Taking this as evidence for data outliers, these pens were removed from the dataset used for analysis.

RESULTS

In Exp. 2, analyzed Zn concentrations were consistently less than calculated concentrations for all diets containing added ZnO (Table 4.2). Although analyzed Ca levels were consistent across treatment diets within each experiment, analyzed Ca levels were greater than formulated levels (0.8 %; Table 1) across all Phase 2 experimental diets and analyzed levels in Exp. 1 diets were greater than those of diets in Exp. 2 (Table 4.2). Further investigation and analysis failed to identify a single explanatory cause for the differences between calculated and reported analyzed Zn and Ca levels. However, high pharmacological levels of Zn were clearly achieved in the respective experimental diets. Analyzed levels of all other nutrients were similar to calculated levels and Cu concentrations of diets containing added $CuSO_4$ in Exp. 2 were within the Association of American Feed Control Officials acceptable analytical variation range for Cu (AOAC, 2000).

Within each experiment, the growth rates of pigs did not differ during the first 5 d when a common starter diet was fed.

Experiment 1

In Exp. 1, no ZnO × CTC interactions were observed for any response criteria in any period (Table 4.3). During the 21 d treatment period, added Zn increased ($P < 0.001$) ADG, ADFI, and BW on d 26 but did not affect G:F. Similarly, increasing CTC increased ($P \leq 0.017$) ADG, ADFI, and BW. Increasing CTC also resulted in a marginally significant improvement (quadratic, $P = 0.083$) in caloric efficiency with pigs fed CTC at 55 ppm having the best G:F. Except for a small decrease ($P = 0.025$) in the G:F of pigs previously fed pharmacological Zn, no differences were observed in the post-treatment period (d 26 to 47) growth rates or feed intakes of pigs that had previously received ZnO or CTC in their diets. Nevertheless, the improvements in ADG and ADFI from feeding pharmacological Zn from ZnO during the treatment period were maintained over the post-treatment period as evidenced by greater ($P < 0.05$) overall ADG and ADFI from d 5 to 47. The respective improvements in ADG and ADFI due to feeding CTC during the treatment period remained marginally significant (linear, $P = 0.062$ and 0.058, respectively) overall from d 5 to 47. Also, the overall feed and caloric efficiency of pigs fed CTC had marginally significant quadratic improvements (quadratic, $P < 0.10$) as 55 ppm of CTC was added to the diet with no further improvement at the 441 ppm level.

Experiment 2

During the d 5 to 33 treatment period, increasing CTC level linearly increased ($P = 0.028$) ADG and induced marginally significant increases (linear, $P = 0.079$) in ADFI which resulted in marginally heavier (linear, $P = 0.074$) BW on d 33 (Tables 4.4 and 4.5). When the pigs ceased consuming CTC and were fed the common diet from d 33 to 47, pigs previously fed CTC had a marginally significant linear reduction ($P = 0.095$) in ADG compared to pigs which had not previously been fed CTC. Consequently, increasing levels of CTC did not linearly affect

overall ADG or ADFI from d 5 to 47 and although CTC had failed to affect G:F during either the treatment period or the succeeding common period, CTC induced marginally significant improvements (quadratic, $P = 0.093$) in overall G:F and caloric efficiency with pigs fed 55 ppm CTC having the best feed efficiency.

During the treatment period, there was a marginally significant 3-way interaction between essential oil, Cu, and Zn (Cu × EO × Zn, $P = 0.098$). Adding essential oil to the control diet numerically reduced ADG whereas it had no impact on growth rate when added to diets containing pharmacological levels of Zn and Cu. Pharmacologic Cu and Zn each increased ($P < 0.01$) ADG, resulting in greater ($P < 0.05$) BW on d 33 at the end of the treatment period. No interactions between treatment ingredients were observed for feed intake; pharmacologic Zn increased ($P = 0.006$) ADFI, pharmacologic Cu induced a marginally significant improvement ($P = 0.055$) in ADFI, and essential oil did not affect feed intake during the treatment period. Despite the concomitant increases in both ADG and ADFI, a marginally significant improvement in caloric efficiency ($P = 0.089$ for ME and 0.084 for NE) was observed due to pharmacologic Zn. The main effect of essential oil was worsened ($P = 0.009$) G:F during the treatment period and a Cu (regardless of Zn inclusion) × essential oil interaction ($P = 0.024$) was observed due to the numeric improvements in G:F induced by Cu being eliminated when essential oil was also fed in combination. These interactive (Cu × EO, $P = 0.025$) and main effects ($P = 0.015$) of essential oil were also observed when considering efficiency on a caloric basis for both ME and NE during the treatment period.

During the 14 d common period from d 33 to 47, no differences were observed in growth performance due to previous diet treatment except for a marginally significant interaction between previous Cu and Zn treatments whereby numeric decreases in common period ADG of

pigs previously fed either Cu or Zn were non-additive so that pigs previously fed both Cu and Zn had better (Cu × Zn, $P = 0.095$) ADG than pigs previously fed the minerals individually, although the interactive ADG was still numerically lower than that of control pigs which had not previous received Cu or Zn. No interactions between treatment ingredients were observed on overall growth performance from d 5 to 47. Feeding pharmacological Zn for 28 d increased ($P < 0.05$) overall ADG and ADFI over the entire 42 d experiment which resulted in greater ending BW on d 47 compared to that of pigs not fed added Zn. Similarly, pigs that received pharmacologic Cu for 28 d had greater ($P = 0.018$) overall ADG and marginally significant improvements ($P = 0.099$) in ending d 47 BW compared to that of pigs not receiving added Cu. In contrast, dietary inclusion of essential oil for 28 d did not affect pig gain rate or feed intake but resulted in a marginally significant decrease ($P = 0.086$) in overall G:F from d 5 to 47 compared to that of pigs not receiving the essential oil.

DISCUSSION

In the present study, feeding pharmacological levels of 2,500 Zn from ZnO for 21 d or at 3,000 ppm for 7 d then 2,000 ppm for another 21 d increased rates of gain with coinciding increases in feed intake but did not affect feed efficiency in either experiment. A meta-analysis by Sales (2013) provides a summation of evidence in the literature that feeding pharmacological concentrations of Zn from ZnO to weaned pigs improves rate of gain, feed intake, and efficiency of gain. While pharmacologic Zn from ZnO is capable of inducing efficiency improvements, this efficiency response is more variable than the consistently observed improvements in gain and feed intake. In contrast to the meta-analysis of Sales (2013), no evidence for improved efficiency of gain due to Zn supplied above the pigs' physiological requirement was observed in the present study. Similar findings were reported by Woodworth et al. (2005) when 3,000 ppm Zn was fed

for the first 10 d postweaning then 2,000 ppm Zn was fed from d 10 to 20. They found efficiency was initially improved from d 0 to 10 but no improvement in efficiency due to pharmacological Zn could be detected from d 0 to 20.

In the present study, increased feed intake resulted in a linear improvement in BW gain as dietary inclusion of CTC increased from 0 to 55 ppm to 441 ppm for 21 or 28 d. In modern multisite production systems, dietary antibiotics remain efficacious in improving weaned pig growth but appear to have limited application for improving efficiency of gain (Dritz et al., 2002). Marginal evidence for CTC improving overall feed and caloric efficiencies in Exp. 1 indicated the greatest improvements were obtained when 55 ppm of CTC was added to the diet, with no further improvement at 441 ppm. This observation is not unexpected when considering that CTC levels greater than 55 ppm are indicated for the control or prevention of clinical disease while the lower dosage is indicated for production benefit. Chlortetracycline appeared to elicit a greater improvement in growth rate and feed intake in Exp. 1 than in Exp. 2 in which there was no difference in overall growth performance of pigs due to feeding increasing levels of CTC. In Exp. 1, pigs fed 441 ppm CTC had an 8% improvement in ADG compared to that of control pigs while pigs in Exp. 2 had a 5% improvement. Summarizing a large number of studies, Dritz et al. (2002) reported a 5% improvement in ADG due to feeding anitibiotic amongst nursery pigs in commercial environments yet cautioned that antibiotic growth responses are smaller when baseline growth performance is high. Several factors could have contributed to the variation in CTC response in the present experiments, such as a greater number of replicate pens fed CTC in Exp. 1 than in Exp. 2, or initial BW of pigs (6.45 kg in Exp. 1 and 6.55 kg in Exp. 2).

No interactions between pharmacologic Zn from ZnO and CTC were observed, indicating that the effects of each are additive in nature. Similar effects of pharamacological Zn and other

broad-spectrum antibiotics fed in combination have been reported. Hill et al. (2001) observed additive improvements in growth rate, feed intake, and efficiency of gain when a subtherapeutic level of carbadox was fed with up to 3,000 ppm Zn from ZnO in weaned pig diets. Furthermore, Woodworth et al. (2005) reported additive improvements in rate of gain due to feeding a combination of neomycin and tetracycline with a pharmacological level of Zn for 20 d. In addition, they reported improved feed intake due to Zn but no interactive effect of Zn and concomitant feeding of the antibiotics on feed intake.

Early research established that supplementing basal diets with graded levels of 0 to 500 ppm Cu from $CuSO_4$ induced non-linear responses in weaned pig growth performance with maximum growth rates, feed intake, and efficiency of gain achieved when feeding approximately 250 ppm Cu (Roof and Mahan, 1982; Cromwell et al., 1989). However, 75 to 80% of this maximum response was realized by supplementation with a more moderate level of 125 ppm Cu Cromwell et al., 1989) and Stahly et al. (1980) reported that rate of gain and feed intake was maximized at 125 ppm Cu when diets included antibiotics. In the present study, feeding Cu above the nursery pigs' physiological requirement at level 125 ppm for 28 d improved rate of gain with a marginally significant increase in feed intake but no significant improvement in G:F. When Shelton et al. (2011) fed 125 ppm Cu, improvements in efficiency of gain were observed in addition to increased rate of gain and voluntary feed intake compared to those of pigs not fed additional Cu (Shelton et al, 2011). It is possible that a greater response to Cu may have occurred in the present study if a greater concentration of Cu had been fed.

Including 0.1% *Origanum* essential oil supplement containing approximately 5% oil into weaned pig diets for 28 d failed to affect daily gain or feed intake but resulted in poorer feed and caloric efficiencies in the present study. The interactive effect of pharmacologic Cu and

Origanum essential oil on feed efficiency suggests that *Origanum* essential oil had an antagonistic effect on the numeric improvement in gain efficiency induced by Cu. Previous research in weaned pigs has shown that oregano essential oil supplied at equivalent concentrations to that fed in the present study, or at 2x greater or lesser concentrations, for 28 d did not elicit any improvements in growth rate, feed efficiency, or feed intake (Neill et al., 2006). Moreover, no improvements in any growth performance parameters were observed due to the feeding of the combined oil extracts of oregano (5% carvacrol), cinnamon (3% cinnamaldehyde), and Mexican pepper (2% capsicum oleoresin) when included in the diet at up to 300 ppm (Manzanilla et al., 2004). Little research has demonstrated detrimental effects of oregano essential oil although Jugl-Chizzola et al. (2006) did report that weaned pigs had a lesser preference for feed containing oregano herbs (supplying 0.002 or 0.02% essential oil) than for unsupplemented feed and Zhang et al. (2012) reported decreased G:F during the initial 2 wk period of feeding oregano essential oil plant extract to weaned pigs.

Yet in pigs afflicted with proliferative enteropathy, *Origanum vulgaris* successfully improved rate of gain over that of pigs receiving no essential oil nor antibiotic therapy, although no differences were observed in feed intake (Papatsiros et al., 2009). Also, oregano essential oil has been shown to be as effective as a feed-grade antibiotic in inducing improved rate of gain and feed conversion ratios in poultry (Mathlouthi et al., 2010). However, compared to pharmacologic Zn or Cu, there is a scarcity of literature supporting the supposition that oregano plant essential oil improves growth performance in non-disease challenged weaned pigs. Li et al. (2012) did report that feeding an essential oil blend containing thymol and cinnamaldehyde improved ADG and decreased *E. coli* in the cecum, colon, and rectum of weaned pigs. Yet, as summarized by Zeng et al. (2015), there are currently few studies which would support positive

effects of carvacrol and thymol fed in combination on nursery pig growth. Until additional research can determine more specific *in vivo* applications for the active compounds of oregano essential oil and appropriate dose and feeding strategies, the role of *Origanum* essential oil in stimulating weaned pig growth performance appears limited.

In the present study, lack of interaction among pharmacological Zn, Cu, and *Origanum* essential oil for feed intake indicates any individual improvements due to Zn and Cu are additive. Few studies have evaluated any interactive effects of essential oil with other growth-promoting agents although no interactions were observed between oregano essential oil and feed-grade antibiotics when both were fed together (Neill et al., 2006).

The effects of feed antibiotic levels and pharmacologic Zn or Cu in weaned pig diets are reported as additive in several studies. Additive effects of up to 3,000 ppm Zn from ZnO and sub-therapeutic doses of a feed-grade antibiotic on weaned pig growth performance have been reported (Hill et al., 2001; Woodworth et al., 2005).The effects of 250 ppm Cu from $CuSO_4$ were non-additive when fed together with feed-grade antibiotics in the diet of grow-finish pigs (Ribeiro de Lima et al., 1981) but in weaned pigs, additive effects have been observed (Stahly et al., 1980; Edmonds et al., 1985) even with 125 ppm Cu (Roof and Mahan, 1982).

Additivity of pharmacological levels of Cu and Zn has been demonstrated less consistently, possibly due to interactions between the minerals competing for absorption at the gut level. Some studies have demonstrated non-additive effects between pharmacological levels of 250 ppm Cu from $CuSO_4$ and 3,000 ppm Zn from ZnO (Smith et al., 1997; Hill et al., 2000) while others have demonstrated additive growth responses when feeding 125 ppm Cu in combination with Zn (3,000 ppm for 14 d postweaning and 2,000 ppm from d 14 to 42

postweaning; Shelton et al., 2011) or when feeding Cu at either 250 ppm from $CuSO_4$ or 100 ppm from an organic Cu source in combination with 3,000 ppm Zn (Pérez et al., 2011).

Few studies monitor for potential treatment carryover effects into the immediate post-treatment period. Minimal carryover effects from any of the dietary treatments on subsequent nursery pig growth performance were observed in this study. The observed overall gain response of pigs previously fed both pharmacologic Cu and Zn for 28 d provides marginal evidence that previous feeding of Cu plus Zn may not be as detrimental to rate of gain after supplementation of the pharmacologic levels of Cu and Zn has ceased compared to gain of pigs which were previously fed each mineral without the other. Bunch et al. (1963) observed 250 ppm CuSO4 improved ADG from weaning to 57 kg BW but when no $CuSO_4$ was subsequently fed from 57 to 91 kg BW the pigs which had previously received the added Cu had a better rate of gain compared to pigs which had not previously received $CuSO_4$.

In Exp. 1 only, a small depression in feed efficiency was observed in pigs following cessation of pharmacologic Zn supplementation in the previous 21 d but no carryover effect of the Zn on efficiency was observed in experiment 2. Woodworth et al. (2005) fed an antibiotic combination of neomycin and oxytetracycline for 21 d and reported that pigs previously receiving the antimicrobial exhibited decreased feed efficiency in the 7 d following cessation of the antimicrobial feeding. Numerically, but non-significantly, depressed feed efficiency was also observed in pigs previously fed pharmacologic Zn compared to pigs which had not received previous Zn treatment. Together, these observations suggest that modulated or depressed rates of gain or efficiency of gain following a period of stimulated growth are not unprecedented. Moreover, no further improvements in the growth performance of the pigs in these studies were observed after the antibiotics and added minerals were removed from the diets. Similar results

were reported by Bosi et al. (2011) when considering the later growth performance of weaned pigs which had experienced improved ADG, ADFI, and G:F due to antibiotic feeding compared to that of pigs which had not been fed antiobiotics in the preceding 21 d period; no differences were observed in the pigs' growth performance during the 7 d common period following cessation of antibiotic feeding.

CONCLUSION

This study illustrates the value of feeding pharmacological concentrations of Zn, Cu, and CTC to newly weaned pigs to promote growth but no improvement due to *Origanum* essential oil was observed. Although there were no improvements in feed efficiency due to Cu or Zn, the inclusion of an essential oil worsened feed and caloric efficiency. There were minimal carryover effects from the dietary treatments on subsequent nursery pig growth performance. Also, this study agrees with previous research findings with the data collectively suggesting that the benefits of feeding CTC and pharmacological levels of Zn are additive for nursery pigs. Furthermore, most effects of Zn, Cu, and essential oil on piglet growth performance appear to occur independently. In conclusion, pharmacological levels of Cu, Zn, or CTC improved weanling pig performance while *Origanum* essential oil elicited no growth performance benefits.

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Table 4.1. Diet composition (as-fed basis)[1]

	Phase 1 common diet (d 0 to 5)	Phase 2 experimental diets (d 5 to 26 or 33)	Phase 3 common diet (d 26 or 33 to 47)
Ingredient, %			
Corn	37.54	54.73	63.83
Soybean meal (47.7% CP)	19.86	29.53	32.86
Spray-dried blood cells	1.25	1.25	---
Spray-dried animal plasma	4.00	---	---
Corn DDGS[2], 6 - 9% oil	5.00	---	---
Select menhaden fish meal	1.25	1.25	---
Spray-dried whey	25.00	10.00	---
Choice white grease	3.00	---	---
Monocalcium phosphate	0.90	0.80	1.00
Limestone	1.00	1.10	1.03
Salt	0.30	0.30	0.35
L-Lys HCL	0.225	0.300	0.300
DL-Met	0.150	0.175	0.115
L-Thr	0.085	0.150	0.115
Trace mineral premix[3]	0.150	0.150	0.150
Vitamin premix[4]	0.250	0.250	0.250
Choline chloride, 60%	0.035	---	---
Phytase[5]	---	0.015[4]	0.015[4]
$CuSO_4$, ZnO, Regano EX®, CTC-50 additives[6]	---	0 to 0.965	---
Total	100.00	100.00	100.000
Calculated analysis			
Standardized ileal digestible (SID) amino acids, %			
Lys	1.40	1.35	1.22
Ile:Lys	56	58	63
Leu:Lys	128	125	129
Met:Lys	32	35	33
Met & Cys:Lys	57	58	57
Thr:Lys	63	64	63
Trp:Lys	19	18	19
Val:Lys	71	69	69
Total Lys, %	1.57	1.50	1.37
CP, %	22.2	22.2	21.4
ME, kcal/kg	3,470	3,291	3,272
NE, kcal/kg	2,599	2,429	2,410
SID Lys:ME, g/Mcal	4.0	4.1	3.7
Ca, %	0.85	0.80	0.70
P, %	0.73	0.63	0.61
Available P, %	0.51	0.44[7]	0.39[7]

[1] Common Phase 1 diet was fed from d 0 to 5 after weaning, experimental Phase 2 diets were fed from d 5 to 26 (Exp. 1) or 33 (Exp. 2), and the common Phase 3 diet was fed from d 26 (Exp. 1) or 33 (Exp. 2) to 47.
[2] Distillers dried grains with solubles.
[3] Provided per kg of diet: 40 mg Mn from manganese oxide, 165 mg Fe from iron sulfate, 165 mg Zn from zinc sulfate, 16.5 mg Cu from copper sulfate, 0.30 mg I from calcium iodate, and 0.30 mg Se from sodium selenite.
[4] Provided per kg of diet: 11,023 IU vitamin A, 1,653 IU vitamin D_3, 44 IU vitamin E, 4.4 mg vitamin K, 8.3 mg riboflavin, 27.6 mg pantothenic acid, 50 mg niacin, and 0.04 mg vitamin B_{12}.
[5] Diets in Exp. 1 contained 0.0125% Phytase 600 (Phyzyme; Danisco Animal Nutrition, St Louis, MO), providing 750.7 phytase units (FTU)/kg and an estimated release of 0.12% available P. Diets in Exp. 2 contained 0.015% HiPhos 2700 (DSM Nutritional Products, Inc., Parsippany, NJ), providing 406.3 phytase units (FTU)/kg and an estimated release of 0.10% available P.
[6] Exp.1 treatment diets contained added ZnO at 0 or 0.347% and CTC-50 at 0, 0.05, or 0.4%. Exp. 2 treatment diets contained zinc oxide added at 0 or 0.415% from d 5 to 12 and at 0 or 0.28% from d 12 to 33, copper sulfate added at either 0 or 0.05%, Regano EX® (Ralco Animal Nutrition, Marshall, MN) containing approximately 5% essential oil added at either 0 or 0.1%, and CTC-50 added at 0, 0.05, or 0.4%. Additions of treatment ingredients were made in place of an equivalent amount of corn in respective experimental diets.
[7] Available P (%) levels were calculated as 0.47 and 0.41% for Exp. 1 Phase 2 and 3 diets, respectively.

Table 4.2. Analyzed dietary composition and mineral concentrations of Phase 2 treatment diets (as-fed basis)[1]

	Phase 2 treatment diets[2]							Analyzed composition	
Diets	DM, %	CP, %	CF, %	Fat, %	Ash, %	Ca, %	P, %	Zn, ppm	Cu, ppm
Experiment 1									
0 CTC	90.78	23.0	2.0	2.2	7.18	1.52	0.62	148	53
55 CTC	90.59	22.5	1.5	2.1	6.27	1.21	0.65	317	24
441 CTC	90.40	23.7	1.7	2.4	5.59	0.93	0.60	186	22
0 CTC + Zn	90.79	21.9	1.5	2.0	6.09	1.12	0.60	2,918	23
55 CTC + Zn	90.77	22.4	1.7	2.3	6.45	1.12	0.64	2,946	20
441 CTC + Zn	90.79	22.3	1.4	1.8	6.57	1.19	0.59	2,823	27
Experiment 2									
Control	90.96	23.0	1.8	2.5	5.00	0.86	0.67	140	16
Cu	90.27	23.2	1.9	2.6	5.01	0.89	0.64	115	109
Zn[4]									
d 5 to 12	90.32	22.9	1.8	2.7	5.31	0.88	0.63	2,110	20
d 12 to 33	90.41	22.9	1.7	2.7	5.49	0.92	0.74	1,632	25
Essential oil (EO)	90.18	22.8	1.9	2.7	5.00	0.96	0.69	177	25
Cu + Zn[4]									
d 5 to 12	90.34	22.7	1.7	2.7	5.65	0.96	0.70	2,254	166
d 12 to 33	90.45	23.0	1.7	2.6	5.45	0.98	0.68	1,778	135
Cu + EO	89.92	22.4	1.7	2.8	5.00	1.03	0.69	385	161
Zn + EO[4]									
d 5 to 12	89.66	23.2	1.9	2.8	5.36	0.96	0.66	2,166	19
d 12 to 33	90.55	22.9	1.6	2.9	5.63	0.98	0.66	1,780	21
Cu + Zn + EO[4]									
d 5 to 12	90.44	22.6	1.8	2.7	5.56	0.96	0.66	2,181	120
d 12 to 33	90.14	22.5	1.9	2.9	5.32	0.93	0.65	1,701	137
55 CTC	90.75	23.2	2.0	2.8	5.02	0.90	0.64	219	22
441 CTC	90.26	22.9	1.9	2.9	5.06	0.93	0.66	205	22

[1] Analysis was performed by Ward Laboratories, Inc. (Kearney, NE) on pooled diet samples. All diets were formulated to contain 16.5 ppm Cu and 110 (Exp. 1) or 165 (Exp. 2) ppm of Zn from the trace mineral premix.

[2] Phase 2 treatment diets were fed from d 5 to 26 (Exp. 1) or to d 33 (Exp. 2), whereas a Phase 1 common was fed to all pigs from d 0 to 5 and a Phase 3 common diet was fed to all pigs from d 26 (Exp. 1) or 33 (Exp. 2) to d 47.

[3] From Regano EX ®(Ralco Animal Nutrition, Marshall, MN).

[4] In Exp. 2 only, pharmacologic Zn diet treatments had an addition of 3,000 ppm Zn from added ZnO from d 5 to 12 and an addition of 2,000 ppm Zn from added ZnO from d 12 to 33.

Table 4.3. Effects of ZnO and CTC on nursery pig growth performance (Exp. 1)[1,2]

ZnO, ppm:	0			2500				Probability, $P <$				
								ZnO × CTC			CTC	
CTC, ppm:	0	55	441	0	55	441	SEM	Linear	Quadratic	ZnO	Linear	Quadratic
BW, kg												
d 5	6.46	6.46	6.43	6.45	6.46	6.44	0.077	0.923	0.974	0.981	0.751	0.912
d 26	13.91	14.45	14.57	14.78	14.80	15.24	0.203	0.986	0.175	<0.001	0.011	0.251
d 47	28.90	29.81	29.61	29.66	29.86	30.04	0.425	0.952	0.409	0.240	0.387	0.239
d 5 to 26												
ADG, kg	0.35	0.38	0.39	0.40	0.40	0.42	0.008	0.894	0.121	<0.001	0.002	0.208
ADFI, kg	0.50	0.51	0.53	0.55	0.54	0.57	0.012	0.826	0.399	<0.001	0.017	0.914
G:F	0.71	0.74	0.73	0.73	0.73	0.73	0.013	0.646	0.362	0.567	0.436	0.119
ME caloric efficiency[3]	4,684	4,466	4,482	4,534	4,472	4,460	78.2	0.611	0.315	0.359	0.207	0.083
NE caloric efficiency[3]	3,456	3,295	3,306	3,345	3,299	3,289	57.7	0.613	0.315	0.350	0.200	0.083
d 26 to 47												
ADG, kg	0.71	0.73	0.72	0.71	0.72	0.74	0.015	0.262	0.648	0.978	0.449	0.405
ADFI, kg	1.15	1.16	1.17	1.16	1.17	1.21	0.024	0.500	0.845	0.273	0.155	0.757
G:F	0.62	0.63	0.61	0.61	0.61	0.61	0.007	0.424	0.635	0.025	0.155	0.289
d 5 to 47												
ADG, kg	0.53	0.55	0.55	0.55	0.56	0.58	0.009	0.416	0.322	0.045	0.062	0.244
ADFI, kg	0.83	0.84	0.85	0.86	0.86	0.89	0.016	0.555	0.665	0.022	0.058	0.830
G:F	0.65	0.66	0.65	0.65	0.65	0.65	0.006	0.743	0.329	0.235	0.639	0.070
ME caloric efficiency[3]	5,066	4,947	5,044	5,072	5,033	5,055	50.5	0.732	0.360	0.364	0.754	0.083
NE caloric efficiency[3]	3,732	3,644	3,716	3,736	3,707	3,723	37.2	0.730	0.361	0.368	0.762	0.083

[1] A total of 240 nursery pigs (PIC 1050, initially 21 d of age and 6.08 kg BW) were used in a 47 d study with 5 pigs per pen and 8 pens per treatment.
[2] Experimental treatment diets were fed from d 5 to d 26, and a common diet was fed to all pigs from d 26 to 47.
[3] Caloric efficiency is expressed as kcal per kg of live weight gain.

Table 4.4. Effects of added dietary Cu, Zn, essential oil, and chlortetracycline (CTC) on nursery pig growth performance (Exp. 2)[1,2]

Added Cu[3]:	-	+	-	-	+	+	-	+	-	-	
Added Zn[4]:	-	-	+	-	+	-	+	+	-	-	
Essential oil[5]:	-	-	-	+	-	+	+	+	-	-	
CTC, ppm:	0	-	-	-	-	-	-	-	55	441	SEM
Initial d 5 BW, kg	6.57	6.56	6.56	6.53	6.61	6.69	6.56	6.62	6.53	6.55	0.084
d 5 to 33											
ADG, kg	0.44	0.46	0.47	0.42	0.49	0.47	0.47	0.48	0.43	0.46	0.012
ADFI, kg	0.56	0.58	0.61	0.55	0.61	0.62	0.61	0.62	0.55	0.59	0.018
G:F	0.77	0.79	0.77	0.76	0.81	0.76	0.78	0.76	0.79	0.78	0.012
BW on d 33, kg	18.94	19.61	19.82	19.08	20.47	19.91	19.85	19.96	18.88	19.64	0.361
d 33 to 47											
ADG, kg	0.72	0.69	0.70	0.70	0.72	0.68	0.69	0.71	0.72	0.69	0.016
ADFI, kg	1.17	1.13	1.15	1.13	1.16	1.12	1.13	1.14	1.12	1.12	0.028
G:F	0.62	0.62	0.61	0.62	0.62	0.61	0.61	0.62	0.64	0.61	0.113
BW on d 47, kg	29.02	29.32	29.68	28.83	30.56	29.50	29.47	29.89	28.90	29.28	0.503
d 5 to 47											
ADG, kg	0.53	0.54	0.55	0.51	0.57	0.54	0.55	0.55	0.53	0.54	0.011
ADFI, kg	0.76	0.76	0.79	0.73	0.79	0.79	0.78	0.79	0.74	0.77	0.019
G:F	0.70	0.71	0.69	0.69	0.72	0.69	0.70	0.70	0.72	0.70	0.009
Caloric efficiency[6]											
d 5 to 33											
ME	4,262	4,163	4,253	4,338	4,049	4,304	4,196	4,293	4,165	4,193	65.2
NE	3,145	3,071	3,137	3,200	2,986	3,175	3,095	3,166	3,073	3,092	48.1
d 5 to 47											
ME	4,726	4,652	4,721	4,754	4,553	4,752	4,704	4,702	4,590	4,666	61.5
NE	3,483	3,429	3,479	3,504	3,355	3,502	3,467	3,465	3,383	3,439	45.3

[1] A total of 350 nursery pigs (PIC 1050, initially 6.05 kg BW) were used in a 47-d study with 5 pigs per pen and 7 replicate pens per treatment except for 4 treatments (essential oil, Cu+Zn, Cu+essential oil, Cu+Zn+essential oil), which had 6 replicate pens each.

[2] Experimental treatment diets were fed from d 5 to d 33. All diets contained 16.5 ppm Cu and 165 ppm of Zn from the trace mineral premix.

[3] Cu from $CuSO_4$ was added to treatment diets at either 0 or 125 ppm.

[4] Pharmacological Zn diet treatments had an addition of 3,000 ppm Zn from added ZnO from d 5 to 12 and an addition of 2,000 ppm Zn from added ZnO from d 12 to 33.

[5] Regano EX®(Ralco Animal Nutrition, Marshall, MN) was added to treatment diets at either 0 or 0.1%.

[6] Caloric efficiency is expressed as kcal per kg of live weight gain.

Table 4.5. Probability (*P* <) for effects of added Cu, Zn, essential oil, and chlortetracycline (CTC) on nursery pig growth performance (Exp. 2)[1, 2]

	Probability, *P* <								
								CTC	
	Cu	Zn	Essential oil (EO)	Cu × Zn	Cu × EO	Zn × EO	Cu × Zn × EO	Linear	Quadratic
BW, kg									
d 5	0.250	0.976	0.685	0.830	0.442	0.710	0.519	1.000	0.723
d 33	0.022	0.009	0.965	0.437	0.689	0.331	0.463	0.074	0.739
d 47	0.099	0.034	0.514	0.796	0.945	0.516	0.544	0.590	0.808
d 5 to 33									
ADG, g	0.003	<0.001	0.605	0.120	0.822	0.707	0.098	0.028	0.755
ADFI, g	0.055	0.006	0.444	0.173	0.182	0.798	0.444	0.079	0.392
G:F	0.150	0.186	0.009	0.958	0.024	0.886	0.137	0.833	0.265
ME caloric efficiency[3]	0.138	0.089	0.015	0.870	0.025	0.858	0.144	0.645	0.226
NE caloric efficiency[3]	0.137	0.084	0.015	0.870	0.025	0.858	0.144	0.631	0.226
d 33 to 47									
ADG, g	0.928	0.608	0.136	0.095	0.692	0.965	0.782	0.101	0.987
ADFI, g	0.675	0.696	0.355	0.347	0.668	0.978	0.675	0.377	0.222
G:F	0.659	0.972	0.488	0.321	0.930	0.958	0.845	0.340	0.135
d 5 to 47									
ADG, g	0.018	0.001	0.207	0.573	0.621	0.825	0.111	0.422	0.771
ADFI, g	0.225	0.025	0.818	0.425	0.225	0.942	0.304	0.499	0.240
G:F	0.141	0.293	0.086	0.549	0.145	0.972	0.549	0.973	0.093
ME caloric efficiency[3]	0.131	0.207	0.111	0.560	0.147	0.976	0.561	0.937	0.084
NE caloric efficiency[3]	0.131	0.202	0.111	0.560	0.147	0.977	0.560	0.930	0.084

[1]A total of 350 nursery pigs (PIC 1050; initially 6.05 kg BW) were used in a 47-d study with 5 pigs per pen and 7 replicate pens per treatment except for 4 treatments (essential oil, Cu+Zn, Cu+essential oil, Cu+Zn+essential oil), which had 6 replicate pens each.
[2] Experimental treatment diets were fed from d 5 to d 33. All diets contained 16.5 ppm Cu and 165 ppm of Zn from the trace mineral premix.
[3] Caloric efficiency is expressed as kcal per kg of live weight gain.

Chapter 5 - Effects of dietary Cu, Zn, and ractopamine-HCl on finishing pig growth performance, carcass characteristics, and antimicrobial susceptibility of enteric bacteria.

ABSTRACT

A total of 480 pigs (PIC 327 ±1050; initially 48.7 kg) were used to determine the interactive effects of supplemental Cu, Zn, and ractopamine HCl (RAC) on finishing pig growth performance, carcass characteristics, and antimicrobial susceptibility of enteric bacteria. Treatments were arranged in a 2×2×2 factorial with main effects of added Cu (CuSO4; 0 vs. 125 ppm Cu), Zn (ZnO; 0 vs. 150 ppm Zn), and RAC (0 vs. 10 ppm during the last 28 d prior to marketing; Paylean®; Elanco Animal Health, Greenfield, IN). All diets contained 11 ppm Cu and 73 ppm Zn from the trace mineral premix. Pens of pigs were balanced and blocked on initial BW then randomly allotted to 1 of the 4 mineral treatment diets. At 28 d prior to marketing, pens within each block and mineral treatment were randomly assigned to receive either 0 or 10 ppm RAC in addition to the mineral treatment. Adding either Cu or Zn alone did not improve ADG or ADFI yet resulted in numerical improvements in overall G:F and caloric efficiencies but improvements were not additive (Cu×Zn, P=0.057, 0.068 and 0.064 for G:F and caloric efficiency on a ME and NE basis, respectively). Ractopamine improved ($P < 0.001$) overall ADG, G:F, and caloric efficiency thereby increasing final BW by 3% with no change in ADFI. Ractopamine also increased ($P < 0.001$) HCW, percentage carcass yield, HCW G:F, loin depth, and percent fat-free lean and decreased ($P = 0.014$) backfat. Adding Zn or Cu alone to diets containing RAC numerically improved percent yield and HCW G:F, but this effect was absent when the Cu or Zn was added to the control diet or when Cu and Zn were fed in combination in RAC diets (Cu×Zn×RAC, $P = 0.011$ and 0.018 for yield and HCW G:F, respectively). Fecal

samples were collected on d 0 and at the conclusion of the finishing period (d 90) for bacterial isolation and antimicrobial susceptibility determinations according to CLSI MIC breakpoints. *Enterococcus* spp. and *E. coli* isolates displayed varying levels of resistance to certain antibiotics prior to initiation of treatments on d 0. Resistance to most antibiotics decreased ($P < 0.05$) over time or was stable for those that had a low baseline percentage of resistance. Neither Zn nor RAC adversely affected antimicrobial resistance but extended feeding of 125 ppm Cu throughout the finishing period appeared to antagonize any time-associated decrease in enterococcal resistance to tetracycline, tylosin, and quinupristin/dalfopristin.

INTRODUCTION

Ractopamine HCl, a β-adrenergic agonist, increases synthesis and accretion of skeletal muscle protein (Bergen et al., 1989) and has lypolytic ability (Mills et al., 2003). When included in late finishing pig diets, ractopamine dramatically improves rate and efficiency of gain, and carcass weight, leanness, and cut yields without negatively impacting pork quality (Watkins et al., 1990; Stoller et al., 2003; Kutzler et al., 2011).

Considerable research has been conducted to determine the supporting nutrient requirements for the pig to realize its maximum lean growth potential due to ractopamine. Much emphasis has been placed on identifying amino acids requirements (Boyd et al., 2001; Apple et al., 2004; Webster et al., 2007; Frantz et al., 2009) and assessing the effects of dietary energy density (Apple et al., 2004; Hinson et al., 2011) on pig response to ractopamine. In addition, limited research has been directed toward the effects of mineral supplementation on the response to ractopamine with some research proposing additional improvements in the response to ractopamine with supplemental Zn in the diet (Patience et al., 2011); however, the response has

been mixed (Paulk et al., 2015). Copper also has been shown to improve growth and feed intake of finishing pigs (Coble et al., 2014).

Bacterial antibiotic resistance remains a paramount public health concern yet limited research has been undertaken to determine the impact of lower doses of heavy metals and β-agonists on the ecology of antimicrobial resistant bacteria in finishing pigs. Microorganisms are sensitive to high levels of minerals such as Cu and Zn (Porcheron et al., 2013). In enterococci, the plasmid-borne transferable copper gene (*tcrB*) that encodes resistance to Cu is associated with the prevalence of certain antibiotic resistance genes in enteric bacteria (Amachawadi et al., 2013) while a gene conferring zinc resistance (*czrC*) has been associated with decreased antibiotic susceptibility (Cavaco et al., 2010). Furthermore, evidence that feeding high levels of Cu increases copper resistance in young pigs (Amachawadi et al., 2011) suggests Cu and Zn feeding will co-select for mineral and antibiotic resistance in livestock.

Endogenous catecholamines use the adrenergic receptors to induce physiological changes (Liang et al., 1985; Frishman, 2003). In addition, bacterial growth and plasmid transfer are upregulated by endogenous catecholamines such as adrenaline and epinephrine (Peterson et al., 2011) thereby possibly expediting the propagation of antibiotic resistant bacteria. However, there is some indication that α-blockers and β-blockers mitigate these effects of catecholamines on bacteria (Peterson et al., 2011). Consequently, there is speculation that adrenergic agonists such as ractopamine HCl could actually acquiesce the progression of antibacterial resistance.

The objective of this study was to determine the interactive effects of supplemental Cu, Zn, and ractopamine HCl on finishing pig growth performance, carcass characteristics, and antimicrobial susceptibility of enteric bacteria.

MATERIALS AND METHODS

Growth Performance

The protocol for this experiment was approved by the Kansas State University Institutional Animal Care and Use Committee. The study was conducted at the Kansas State University Swine Teaching and Research Center in Manhattan, KS. Pigs were housed in an environmentally controlled tunnel-ventilated barn and reared on completely slatted concrete flooring over deep pits for manure storage. Each pen was equipped with a 2-hole stainless steel dry self-feeder (Farmweld, Teutopolis, IL) and a cup waterer to provide pigs with ad libitum access to feed and water. Feed delivery to each individual pen was accomplished and recorded via a robotic feeding system (FeedPro; Feedlogic Corp., Wilmar, MN).

A total of 480 pigs (PIC 327 × 1050; initially 48.7 kg) from 2 finishing groups were used for this study. Prior to placement on experimental finisher diets, the pigs did not receive any chlortetracycline in their feed or water to avoid potential confounding study impacts due to disturbances to the intestinal microbiome. However, the pigs did receive dietary neomycin and oxytetracycline antibiotics immediately post-weaning. Individual pig treatments were recorded.

All finishing diets were a corn-soybean meal based diet fed in meal-form which contained a trace mineral (TM) premix providing 73 ppm zinc and 11 ppm Cu to the diet (Table 5.1). The diets were formulated to be fed in 4 phases (36 to 57 kg, 57 to 79 kg, 79 to 100 kg, and 100 to 132 kg) during the finishing period and were prepared at the K-State O.H Kruse Feed Technology Innovation Center.

Dietary treatments were arranged in a 2 × 2 × 2 factorial with main effects of added copper sulfate (CuSO4; 0 vs. 125 ppm Cu), added zinc oxide (ZnO; 0 vs. 150 ppm Zn) and ractopamine HCl (0 vs. 10 ppm during the last 28 d prior to marketing; Paylean®; Elanco

Animal Health, Greenfield, IN). The dietary treatments were as follows: (1) Control, (2) Control + 125 ppm Cu, (3) Control + 150 ppm Zn, (4) Control + 125 ppm Cu + 150 ppm Zn, (5) Control + 10 ppm ractopamine HCl during final 28 d only, (6) Control + 125 ppm Cu + 10 ppm ractopamine HCl during final 28 d only, (7) Control + 150 ppm Zn + 10 ppm ractopamine HCl during final 28 d only, and (8) Control + 125 ppm Cu + 150 ppm Zn + 10 ppm ractopamine HCl during final 28 d only.

The finishing period of the first pig group spanned from January to April while pigs in the second finishing group were housed in a different room from March to June. Upon entry into the finisher, pigs were randomly allotted to pens of either 7 (group 1) or 8 (group 2) pigs per pen. Pens contained 4 gilts and either 3 (group 1) or 4 (group 2) barrows each. Pen space was maintained at 0.929 m^2/pig across both groups by adjusting pen size according to the number of animals per pen.

The study design was structured as a randomized complete bock design with a split-plot and replicated over 2 finishing groups with 32 pens each. At the beginning of the study, 32 pens of pigs were arranged into 4 weight blocks per group based on similar pen initial average BW. Two pens per weight block were then randomly allotted to 1 of the 4 mineral treatment diets (negative control, +125 ppm Cu, +150 ppm Zn, or +125 ppm Cu with +150 ppm Zn) and balanced on initial pen average BW across blocks. At 28 d prior to marketing, pens within each block and mineral treatment diet were randomly assigned to receive either 0 or 10 ppm ractopamine HCl in addition to their mineral treatment and all diets were formulated to contain 0.90% standardized ileal digestible (SID) lysine. Ractopamine HCl treatment assignments were balanced across blocks on current pen average BW at the time of allotment to ractopamine HCl

treatments. Hence for the final 28 d of the finishing period, each of the four weight blocks in each group contained 1 pen per each of the 8 diet treatments.

Feed samples from each batch of feed were taken from feeders. Samples were pooled within each phase to form a composite diet sample that was subsequently analyzed for Ca, P, Cu, and Zn (method 985.01; AOAC, 2000) in duplicate with modifications of ashing 0.35 g sample for 1 h at 535°C, digestion in open crucible for 20 min in 15% nitric acid on hotplate, sample dilution to 50 ml and analyzed on ICP (Perkin Elmer 3300 XL and 5300 DV ICP; Perkin Elmer, Shelton, CT); DM (method 930.15; AOAC., 2000); CP (method 990.03; AOAC, 2000); crude fat (method 2003.05; AOAC, 2006); CF (method 978.10; AOAC, 2000); ash (method 942.05; AOAC, 2000) with modifications of 1.5 g sample, 4 h ash time, and hot weight (Cumberland Valley Analytical Services, Hagerstown, MD; Table 5.2). Across both finishing groups, a composite sample of each of the 4 diets containing Paylean® was assayed for ractopamine HCl concentration by a commercial laboratory (Covance Laboratories, Greenfield, IN) using an HPLC feed assay for ractopamine that was developed and validated by Elanco Animal Health (method B04372 FDA revision 5, December 2003 – Turberg, unpublished) as part of the New Animal Drug Application for Paylean® in the United States (see Table 5.2).

Pigs and feeders were weighed approximately every 3 wk to determine ADG, ADFI, F/G and both ME and NE caloric efficiency (kcal energy per kg diet × total diet intake per pen ÷ total pen weight gain) on a pen basis. Dietary ME and NE values were derived from feed ingredient energy values based on those in the NRC (2012).

At the conclusion of the 90-d (group 1) or 83-d (group 2) experimental period, all pigs were individually weighed and tattooed with a unique identifier. Pigs were transported to a commercial harvesting facility (Triumph Foods LLC, St. Joseph, MO) and held in lairage

overnight prior to processing and carcass data collection. Carcass characteristics measured at the plant included HCW immediately after evisceration, and backfat and loin depths via an optical probe.

Percent carcass yield was calculated by dividing individual HCW obtained at the packing plant by the corresponding individual final live weight obtained at the farm. An average percentage carcass yield for each pen was then calculated by averaging the observed yields of pigs for each pen. Pen average HCW was calculated by multiplying the pen average percent yield by the pen average final live weight. Percentage lean was calculated by dividing the standardized fat-free lean (SFFL; NPPC, 2000) by individual HCW according to the following equation:

SFFL, % = 100 × [15.31 – (331.277 × backfat depth, in.) + (3.813 × loin muscle depth, in.) + (0.51 × HCW, lb)] ÷ HCW, lb

Initial carcass weight on study d 0 was assumed to equal 75% of the initial pen average live BW, thus HCW gain on a pen basis was calculated using the formula: final pen average HCW, kg – (0.75 × initial pen average BW on d 0). Subsequently, HCW ADG was calculated by dividing the average individual HCW gain of each pen by the number of study days. Similarly, HCW F/G was calculated for each pen by dividing the average daily feed intake per pig (overall ADFI) by average daily HCW gain.

Fecal samples from 5 randomly selected pigs per pen were collected into individual Whirl-Pak (Nasco, Ft. Atkinson, WI) bags on d 0 and again on d 90 from the first group of pigs (32 pens). Samples were transported to the Molecular Epidemiology and Microbial Ecology laboratory at Kansas State University for bacterial isolation and antimicrobial susceptibility analysis.

Microbiological Procedures

Bacterial Isolation and Species Identification

Fecal samples were stored at 4°C prior to processing. Approximately 1 g of feces from each of the 5 samples per pen was suspended in 9 mL phosphate-buffered saline. Fifty μL (per agar) of the fecal suspension was then spread-plated onto both a M-*Enterococcus* agar and a MacConkey agar for the isolation of *Enterococcus* spp. and *Escherichia coli*, respectively, from each fecal sample. Unless otherwise specified, all the culture media was obtained from Difco (Becton Dickinson, Sparks, MD). M-*Enterococcus* plates were incubated at 42°C and MacConkey plates at 37°C, for 24 h.

Two putative colonies (pin-point red, pink, or metallic red) were selected from each M-*Enterococcus* agar and 2 distinct lactose fermenting colonies were picked from each MacConkey agar; each of these colonies was individually streaked onto a blood agar plate (Remel, Lenexa, KS) and incubated at 37°C for 24 h. Preliminary genus confirmation of each of the enterococcal isolates was done by esculin hydrolysis. Indole test was done to confirm each of the *E. coli* isolates. The 2 confirmed *E. coli* and 2 confirmed *Enterococcus* isolates per original fecal sample were preserved using cryo-protect beads (Cryocare®, Key Scientific Products, Round Rock, TX) and stored at -80°C for future use.

Antimicrobial Susceptibility of Enterococcus and E. coli Isolates

The microbroth dilution method as outlined by the Clinical and Laboratory Standards Institute (CLSI, 2013) was used on 1 *E. coli* and 1 *Enterococcus* spp. bacterial isolate per original fecal sample to determine the minimal inhibitory concentrations (MIC) of several antibiotics. For both *E. coli* and *Enterococcus* spp., bacterial isolate preserved in a cryo-protect bead was streaked onto a blood agar plate and incubated at 37°C for 24 h. Individual colonies

were selected and suspended in demineralized water (Trek Diagnostic Systems, Cleveland, OH) and turbidity was adjusted to 0.5 McFarland turbidity standards. Then, 10 μL of the bacterial inoculum was added to Mueller-Hinton broth and vortexed to mix. A Sensititre® automated inoculation delivery system (Trek Diagnostics Systems) was used to dispense 100 μL of the broth into National Antimicrobial Resistance Monitoring System (NARMS) panel plates designed for Gram-positive (CMV3AGPF, Trek Diagnostic Systems; Table 5.3) and Gram-negative (CMV3AGNF, Trek Diagnostic Systems; Table 5.4) bacteria. *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922 (American Type Culture Collection, Manassas, VA) strains were included as quality controls for *Enterococcus* and *E. coli* susceptibility testing, respectively.

Plates were incubated at 37°C for 18 h then bacterial growth was assessed using Sensititer ARIS® and Vizion™ systems (Trek Diagnostic Systems). Clinical and Laboratory Standards Institute (CLSI, 2013) guidelines were used to classify each bacterial isolate as resistant or non-resistant (intermediate and susceptible) according to the breakpoints established for each antimicrobial.

Statistical Analysis

Growth and carcass data were analyzed as a randomized complete block design with a 2 × 2 × 2 treatment structure and replicated over 2 groups. Pen was the experimental unit. The MIXED procedure in SAS (v9.3, SAS Institute Inc., Cary, NC) was used to model diet treatment as a fixed effect with random effects of group and initial weight block nested within group. The main effects of Cu, Zn, and ractopamine HCl, as well as their interactions, were tested using a priori orthogonal CONTRAST statements. Hot carcass weight was used as a covariate in the analyses of backfat, loin depth, and percentage lean. The antibiotic susceptibility data was

analyzed as a binary distribution using the SAS GLIMMIX procedure and a logit link function to model the data as a repeated measure with pen as the experimental unit and 5 sample observations per pen per day. Sample nested within pen was modeled as a random effect to account for clustering within pen. Treatment main effects of Cu, Zn, and ractopamine HCl, day of sampling (baseline or d 90), and dietary main effect within day were evaluated for antibiotics having a non-zero resistance variance component for these fixed effects. For all data, the Kenward Roger method was used to compute denominator degrees of freedom for tests of fixed effects. Results were considered statistically significant at $P \leq 0.05$; results with P-values > 0.05 and ≤ 0.10 were considered marginally significant.

RESULTS

Analyzed CP levels were consistently greater than calculated levels for all diets, possibly as a reflection of higher protein levels in feedstuffs compared to nutrient book values used in the diet formulation (see Tables 5.1 and 5.2). Analyzed Cu and Zn levels reflected the addition of 125 ppm Cu and 150 ppm Zn to respective treatment diets. Analyzed concentrations were at the upper end of the Association of American Feed Control Officials acceptable analytical variation range for the minerals (AOAC, 2000). Analyzed levels of all other nutrients were similar to calculated levels.

Growth Performance

No significant Cu × Zn × ractopamine HCl (Rac) interactions were observed in the growth performance responses measured in this study. There was no difference among pig BW on d 0 except the pigs which would eventually receive ractopamine HCl later in the finishing period were initially slightly heavier ($P = 0.012$) than those pigs which would not receive ractopamine HCl (Table 5.5). Prior to the final 28 d of the finishing period, no effects of Cu or

Zn on ADG or ADFI were observed. However, a significant interaction (Cu × Zn, $P < 0.05$) between Cu and Zn was observed for feed and caloric efficiency where pigs fed diets with either added Cu or Zn alone had numeric improvement in efficiency, but no improvement when both minerals were added together. On d 62 (d 55 for group 2), there was still no difference among the BW of pigs fed 125 ppm added Cu and/or 150 ppm added Zn nor was there any difference between the BW of pigs which would begin at that time to receive 10 ppm ractopamine HCl for the next 28 d compared to the BW of pigs which would not receive ractopamine HCl.

On the final d of the study (d 90 and 83 for groups 1 and 2, respectively), pigs receiving ractopamine HCl for the previous 28 d had heavier ($P < 0.001$) BW than pigs which had not received ractopamine HCl (Table 5.5). The heavier final BW of pigs receiving ractopamine HCl was the result of greater ($P < 0.001$) ADG in the final 28 d before harvest which consequently improved ($P < 0.001$) their G:F and caloric efficiency on both a ME and NE basis compared to that of pigs not fed ractopamine HCl. In contrast, no effect of added Cu and/or Zn was observed on the ADG or G:F of pigs during the final 28 d of the finishing period. There were no effects of the minerals or ractopamine HCl on ADFI in the final 28 d of the finishing period aside from a marginally significant effect of pigs fed Zn and ractopamine HCl in combination having less (Zn × Rac, $P = 0.084$) ADFI than would have been expected considering added Zn numerically increased ADFI and ractopamine HCl numerically decreased ADFI when each was fed not in combination with the other.

No effects of added Cu, Zn, and/or ractopamine HCl were observed on overall ADFI (Table 5.5). Overall ADG, G:F, and caloric efficiency on both a ME and NE basis were improved ($P < 0.001$) due to feeding ractopamine HCl in the last 28 d of the finishing period. Conversely, there were no main effects of added Cu or Zn on overall growth performance.

Feeding Cu or Zn alone numerically improved overall G:F and overall caloric efficiency, but had no influence on overall efficiencies when both were added to the diet (Cu × Zn, $P = 0.057$, 0.068, and 0.064 for G:F, ME, and NE, respectively).

Carcass Characteristics

The added Cu and Zn had minimal effects on growth performance and congruently had minimal effects on carcass characteristics. No differences in pen average HCW or HCW ADG due to the added minerals were observed. However, the finishing growth performance improvements induced by feeding ractopamine HCl during the last 28 d prior to marketing resulted in increased ($P < 0.001$) HCW and HCW ADG (Table 5.7).

Feeding ractopamine HCl also increased ($P < 0.001$) percentage carcass yield and the magnitude of the increase was numerically greater when either Cu or Zn was added to the diet containing ractopamine HCl; however, the minerals did not provide any carcass yield benefit when both were fed together with ractopamine HCl (Cu × Zn × Rac, $P = 0.011$; Table 5.7). No main effects of Cu or Zn on percentage carcass yield were observed. Efficiency of carcass gain followed a similar pattern to percent carcass yield with no main effect due to added Cu or Zn and with a Cu × Zn × Rac interaction ($P = 0.018$). Again, ractopamine HCl improved ($P < 0.001$) HCW G:F with further numeric improvement when either Cu or Zn was added with ractopamine HCl, but no improvement due to the minerals was observed when both Cu and Zn were included in diets together with ractopamine HCl.

When compared on a common HCW basis, pigs fed ractopamine HCl had less ($P = 0.014$) backfat and greater ($P < 0.001$) loin depth and percentage of fat-free lean compared to pigs not fed ractopamine HCl. In contrast, no differences in backfat, loin depth, or fat-free lean were observed due to added Cu or Zn.

Antimicrobial Resistance

Antibiotic susceptibility results are shown in Tables 5.9 through 5.12. For each antibiotic, the percentage resistant for each of the 8 dietary treatments are listed in Tables 5.9 and 5.11. As can be seen from these interactive means, some treatment combinations were associated with 0 or 100% resistance to certain antibiotics within sampling day which prevented calculation of variance components and test statistics for statistical analysis of some of the antimicrobial responses. However, evaluation of the diet treatment main effects rather than interactive effects resulted in model convergence for some of these antibiotics and the results are displayed in Tables 5.10 and 5.12.

E. coli Resistance

None of the fecal *Escherichia coli* (*E. coli*) isolates were categorized as resistant to ciprofloxacin, sulfisoxazole, and trimethoprim/sulfamethoxazole at either d 0 (baseline) or d 90 (Table 5.9). None of the isolates grown in the presence of nalidixic acid were resistant except for 1 isolate (5% resistant) on d 0 in 1 pen subsequently fed the Cu and ractopamine treatment diet; all other baseline as well as d 90 isolates were categorized as nonresistant. All baseline *E. coli* isolates were resistant to Ampicillin (AMP) but by d 90, the percentage decreased to 85% of isolates from pigs which had received the control diet, 90% for the Cu and Zn diet, and 95% for the Zn and ractopamine diet while 100% resistance was still observed for isolates from pigs fed all other treatments.

For *E. coli* isolates, the percentage resistant to amoxicillin/clavulanic acid 2:1 ratio, cefoxitin , ceftiofur, ceftriaxone, gentamicin, streptomycin, and tetracycline decreased ($P < 0.05$) from d 0 to d 90 (Table 5.10). Ractopamine HCl did not affect the percentage of antibiotic resistant *E. coli* isolates. Zinc did not adversely affect antibiotic resistance. Pigs fed Zn had a

greater rate (Zn within Day, $P < 0.05$) of decreasing percent resistant to streptomycin compared to that in pigs fed the diets without added zinc. This resulted in a significant decrease ($P < 0.05$) in percent of *E. coli* resistant to *E. coli* from baseline to d 90 for pigs fed Zn but only a numerical decrease for those fed diets without Zn. Because *E. coli* isolates from all pigs fed diet treatments containing Zn were 100% susceptible to gentamicin on d 90, the corresponding variance components were zero and no statistics computed (Table 5.9). However, feeding diets containing Zn resulted in a larger numerical decrease in resistance to gentamicin compared to diets without Zn.

Copper treatment did not affect antimicrobial resistance over time aside from a marginally significant effect ($P = 0.069$) on resistance to ceftiofur and ceftriaxone. *E. coli* isolates had a similar pattern of resistance to both ceftiofur and ceftriaxone where isolates from pigs subsequently fed Cu had greater percent resistance on d 0 than from pigs not fed Cu; however, resistance decreased by d 90 among pigs not fed Cu but did not decrease among pigs fed Cu so that by d 90 the resistance was not different between pigs that had been fed Cu versus those which had not been fed Cu.

Enterococcus Resistance

No resistant isolates were detected among fecal *Enterococcus* spp. isolates grown in the presence of nitrofurantoin and vancomycin (Table 5.11). On d 0 prior to initiation of diet treatments, enterococcal isolates displayed a low level of resistance (≤ 10%) to chloramphenicol, gentamicin, linezolid, penicillin, and tigecycline; by d 90, no resistant isolates to these antibiotics were observed across all diet treatments.

Across all treatments, estimated percentage of enterococcal isolates that were resistance to erythromycin, lincomycin, and quinupristin/dalfopristin decreased ($P < 0.05$) from d 0 to d 90

while resistance to tetracycline increased ($P < 0.05$) throughout the finishing period (Table 5.12). Neither Zn nor ractopamine HCl treatments affected antibiotic susceptibility of the enterococcal isolates.

Cu did not significantly affect the percentage of enterococcal isolates resistant to daptomycin, erythromycin, kanamycin, lincomycin, or streptomycin over time (Table 5.12). However, percentage of resistance to quinupristin/dalfopristin and tylosin tartrate was affected ($P = 0.023$) by Cu treatment over time. Resistance to quinupristin/dalfopristin and tylosin decreased ($P < 0.05$) from d 0 to d 90 among isolates from pigs not fed added Cu while percent of resistant isolates from pigs fed added Cu did not differ from d 0 to d 90. Consequently, the percentage of quinupristin/dalfopristin resistant isolates from pigs fed Cu was greater ($P < 0.05$) on d 90 than that of isolates from pigs which were not fed Cu. Cu treatment also affected ($P = 0.003$) the percentage of enterococcal isolates resistant to tetracycline over time as baseline isolates from pigs subsequently fed added Cu were initially more ($P < 0.05$) susceptible to tetracycline than isolates from pigs which would not be fed added Cu; however, resistance to tetracycline among isolates from pigs fed added Cu increased so that by d 90 there was no difference in *Enterococcus* spp. susceptibility to tetracycline among isolates from pigs fed added Cu compared to isolates of pigs which had not.

There were no ciprofloxacin resistant enterococcal isolates detected on d 90 from pigs fed diets without added Cu so the corresponding variance components were zero and no statistics computed (Table 5.11). However, baseline resistance across all future treatments initially ranged from 0 to 15% and over time, isolates from pigs fed diets containing added Cu had a smaller numerical decrease in resistance to ciprofloxacin compared to isolates from pigs fed diets without added Cu.

DISCUSSION

The combined or lone supplementation of 125 ppm Cu and 150 ppm Zn above trace mineral premix levels failed to improve gain, feed intake, efficiency, or carcass characteristics. The growth benefits of feeding added Cu and Zn to weaned pigs in the nursery phase are well established and are seemingly driven largely by increased feed intake although efficiency of gain can also be improved (Sales, 2013; Ma et al., 2015). In contrast, the response to supplementation later in the growth period, such as the finishing period of our study, is less consistent.

In a series of experiments conducted by the NCR-42 Committee on Swine Nutrition (1974), the response to copper was highly variable but overall failed to improve gain or efficiency over the growing-finishing period. In contrast, Davis et al. (2002) reported improved gain and efficiency with 175 ppm Cu. Several studies have demonstrated that a decrease in or complete elimination of any trace mineral supplementation including Cu and Zn to a typical corn-soybean meal based diet during all or part of the finishing period did not significantly worsen growth performance or carcass characteristics (Creech et al., 2004; Shelton et al., 2004; Ma et al., 2012; Gowanlock et al., 2013). It should be noted that some of these studies included antibiotics in the diets which may have bolstered performance in the absence of trace mineral supplementation.

The pigs in the current study were enrolled in this finishing period study at a relatively heavy weight (49 kg) and had high levels of feed intake across all treatments. Moreover, this study was conducted in a research setting conducive to high growth performance. Together, these factors possibly precluded any potential for mineral supplementation to improve the growth responses of these pigs.

Prior to the final 28 d of the finishing period, a significant interaction between Cu and Zn was observed for feed and caloric efficiency where pigs fed diets with either added Cu or Zn alone had numeric improvement in efficiency, but no improvement when both minerals were added together. In the final 28 d of finishing period, no evidence for interactive effects between the minerals was observed possibly indicating the combined level of heavy metal minerals in the smaller BW pigs was detrimental to efficient gain. Accordingly, although supplementation of both copper and zinc at levels well above the pig's physiological requirements improve weaned pig performance (Pérez et al., 2011; Shelton et al., 2011), extended supplementation of minerals may lead to poor performance. Yet Kline et al. (1972) reported no main or interactive effects of up to 500 ppm Cu and up to 300 ppm Zn on feed efficiency when fed from 17.3 to 90.8 kg.

In the present study, ractopamine HCl fed at 10 ppm during the final 28 d of the finishing period improved late finishing growth rate, final BW, HCW, and percentage carcass yield without increasing feed intake thereby causing greater caloric and feed efficiency. Notably, the pigs of the study herein had a heavy average BW of 136 kg which corresponded to an average HCW of 101 kg. The effects of ractopamine HCl on late finishing pig growth performance have been extensively studied but few reports have documented the effects of ractopamine on finished pigs of this BW. Apple et al. (2007) conducted a meta-analysis across a wide range of genotypes and environments and reported that ractopamine minimally affects feed intake when fed at 5 to 10 ppm but can decrease feed intake slightly when considered over a large number of studies. In addition, pigs fed 10 ppm exhibited respective improvements over controls in daily gain, feed efficiency, and HCW of 11.8%, 13.3%, 3.1%, and dressing percent ranged from 0.7 to 2.2%. Accordingly, pigs of the present study demonstrated no difference in feed intake and similar magnitudes of improvement in daily gain, feed efficiency, HCW, and dressing percent of 17.5%,

15.3%, 4.5%, and 1.5%, respectively. Moreover, Crome et al. (1996) compared the effects of 28 d of ractopamine supplementation from 68 to 107 kg or from 85 to 125 kg BW and reported no interactions between ractopamine inclusion and weight range indicating that the effects of ractopamine are consistent across pigs at different growth stages.

In the current study, a marginally significant interaction between Zn and ractopamine was observed due to pigs fed Zn and ractopamine HCl in combination having less feed intake than would have been expected considering added Zn numerically increased ADFI and ractopamine HCl numerically decreased ADFI when each was fed not in combination with the other. Patience et al. (2011) proposed that organic Zn supplementation throughout all or the later part of the finishing period may assuage decreased feed intake attributable to ractopamine, based on observations of decreased feed intake for inorganic Zn supplemented pigs fed ractopamine compared to pigs supplemented with organic Zn and fed ractopamine whose feed intake did not differ from that of pigs fed inorganic Zn but no ractopamine. Together, these observations seemingly suggest inorganic Zn supplementation throughout the finishing period does not mitigate negative effects of ractopamine on feed intake.

When compared on a common HCW basis, pigs of the study herein that were fed ractopamine HCl had less backfat, greater loin depth and percentage of fat-free lean compared to pigs not fed ractopamine HCl. Despite consistent improvements in loin depth and predicted fat-free lean percentage, decreased back fat depth is a less consistent response although a meta-analysis demonstrated small but significant effects of 5 and 10 ppm ractopamine on decreasing back fat depth (Apple et al., 2007). Fernández-Dueñas et al. (2008) observed that feeding 5.0 or 7.4 ppm ractopamine increased HCW's and primal yield from carcass in heavy pigs (HCW of 90 to 94 kg) but failed to decrease back fat depth, or improve loin depth or percent lean. However,

the carcass characteristics were not compared on a common HCW basis which could have masked potential effects of ractopamine at these heavy weights.

In the current study, the magnitude of the increase in percent yield due to ractopamine was numerically greater for pigs fed either added Cu or Zn throughout the entire finishing period; however, the minerals did not provide any carcass yield benefit when both were fed together and ractopamine HCl was also fed. No other significant interactions between ractopamine and the minerals were observed in the growth and carcass responses. These observations indicate that 150 ppm Zn supplemented throughout the entire finishing period did not improve growth and carcass responses when fed with or without ractopamine.

In contrast, several studies have suggested that additional improvements to the increased feed efficiency realized through ractopamine feeding can be achieved with concurrent Zn supplementation. This supposition is biologically based on the fact that Zn mediates insulin-like growth factor-1 (IGF-1) induced cell proliferation and growth (MacDonald, 2000; Salgueiro et al., 2002) and may be a key nutrient to support the lean gain of pigs fed ractopamine.

Fry et al. (2013) reported a marginally significant improvement in feed efficiency when Zn was supplemented in a late finishing diet containing ractopamine and also observed that an organic source of Zn tended to improve carcass yield more than an inorganic Zn source when both sources were supplemented to diets containing ractopamine. Similarly, Rambo et al. (2012) reported an organic source of Zn tended to improve performance over that of Zn from an inorganic source when fed in a late finisher diet containing ractopamine and when Zn was supplemented throughout the entire finisher period and the final phase diets contained ractopamine, Patience et al. (2011) found rate of gain was greater when organic Zn versus inorganic Zn was supplemented. Most recently, Paulk et al. (2015) reported inconsistent effects

of Zn and ractopamine where in one experiment, late finishing diets containing ractopamine with increasing supplementation of Zn from ZnO linearly improved feed efficiency. In a follow up study comparing supplemented Zn from inorganic and organic sources, no response to Zn from either source was observed in the presence of ractopamine. However, none of these studies assessed the effects of Zn supplementation independently of ractopamine and it remains unclear whether the pigs responded positively to Zn because of a greater requirement induced by ractopamine or because of an underlying insufficiency.

Conversely, when Gowanlock et al. (2013) fed diets without any trace mineral supplementation for the entire finishing period or supplemented just Zn from an organic source, no significant differences in growth performance (gain, feed intake, feed efficiency) or in carcass characteristics (HCW, back fat depth, loin muscle area) were observed due to a lack of Zn supplementation despite the inclusion of 10 ppm ractopamine for 21 d prior to marketing. Also, the current study demonstrates that supplemented Zn from ZnO throughout the entire finishing period did not improve pig performance or carcass characteristics nor augment the pigs' response to ractopamine as evidenced by a lack of an interaction between the two compounds. In sum, the response to Zn supplementation is highly variable and not consistently associated with duration of supplementation in the finisher or whether the Zn is supplied from an organic or inorganic source. Some pigs demonstrate a need for supplemented Zn in the late finisher diet but there is a lack of strong evidence that this requirement is directly predicated upon ractopamine HCl inclusion in the diet.

Several genomic or plasmid borne genes have been implicated for antibiotic resistance of bacteria while phenotypic resistance is conferred through multiple and often complex mechanisms. Although antibiotic resistant genes are ubiquitously present among both human and

livestock waste streams (Agga et al., 2015), it is important to understand what dietary factors, if any, may contribute to increased antibiotic resistance among fecal bacteria of finishing swine.

Feeding 150 ppm Zn above basal premix trace mineral level throughout the entire finishing period did not adversely affect *E. coli* or *Enterococcus* susceptibility to antibiotics but Zn actually decreased *E. coli* resistance to streptomycin and possibly to gentamicin. Curiously, both streptomycin and gentamicin belong to the aminoglycoside drug class which primarily targets the 16S rRNA thereby interfering with ribosomal function (Davies, 1971). This aminoglycosidic action is predominantly thwarted through bacterial production of enzymes which chemically modify the antibiotic or protect 16S rRNA and these mechanisms are encoded by transferable plasmid borne genes (Yamane et al., 2005) which are not restricted to within species transfer (Shaw et al., 1993). The observations of the current study indicate Zn may downregulate aminoglycoside resistance among *E. coli* through some mechanistic role in these processes.

Although methicillin resistant *Staphylococcus aureus* (MRSA) has a higher phenotypic resistance to ZnCl and a greater prevalence of the Zn resistant (*czrC*) gene compared to methicillin susceptible *Staphylococcus aureus* (MSSA) (Aarestrup et al., 2010; Cavaco et al., 2011), it is unclear whether Zn and methicillin experience co-selection (Yazdankhah et al., 2014). MRSA and MSSA isolates showed similar resistance to erythromycin, penicillin, and tetracycline despite the difference in Zn susceptibility (Aarestrup et al, 2010). In contrast to the results of the current study, Bednorz et al. (2013) reported pharmacological Zn from ZnO increased multi drug resistance among Gram negative bacteria possibly through co-selection or through increased rate of plasmid assimilation. Vahjen et al. (2015) reported that pharmacological levels of Zn from ZnO increased tetracycline (*tet*A) and sulfonamide (*sul*1)

resistance genotypes. However, Jacob et al. (2010) observed no effect of feeding cattle 300 ppm Zn on expression of *erm*(B) and *tet*(M) genes which are associated with macrolide and tetracycline resistance, respectively. Furthermore, feeding Zn at this level did not affect isolated *E. coli* resistance to erythromycin, penicillin, or tylosin nor affect enterococcal isolate resistance to chloramphenicol, ciprofloxacin, gentamicin, linezolid, penicillin, streptomycin, or vancomycin in cattle. Thus, extended feeding of a concentration of 150 ppm Zn in finishing pig diets may not elicit the antimicrobial resistance responses which higher concentrations appear to stimulate.

Supplementation of 125 ppm Cu above basal premix trace mineral level throughout the finishing period increased *Enterococcus* resistance to tetracycline and antagonized the decrease in *Enterococcus* resistance to the streptogramin antibiotics quinupristin/dalfopristin and a macrolide drug, tylosin tartrate, over time. All 3 of these drug classes are protein synthesis inhibitors and interfere with the normal ribosomal function in bacteria. Transferable copper resistance gene (*tcrB*) carrying plasmids have been observed to also carry genes conveying resistance to macrolides and tetracyclines (Hasman and Aarestrup, 2002; Hasman et al., 2006; Amachawadi et al., 2011, 2013) indicating Cu co-selects for greater Cu tolerance and multiple drug resistance. Along with tetracycline, phenotypic erythromycin resistance has been associated with Cu resistant enterococci (Silveira et al., 2014). As such, Cu would have been expected to also increase resistance to erythromycin, another macrolide drug, in addition to tylosin tartrate but this effect was not observed in the present study. However, 100 ppm Cu in cattle did not affect genotypic resistance to macrolides and tetracycline (Jacob et al., 2010). Hence, the phenotypic expression of bacterial resistance to all macrolide drugs may not be observable at a dose of 125 ppm in finishing pigs.

Supplementation of 125 ppm Cu antagonized the decrease in *E. coli* resistance to the beta lactam subgroup 3rd generation cephalosporins ceftiofur and ceftriaxone. Agga et al. (2014) reported association between ceftiofur and tetracycline resistance of *E. coli* in young pigs as well as between bla_{CMY-2} and *tetA* genes encoding for their resistance, respectively. In the current study, Cu significantly increased tetracycline resistance among *Enterococcus,* but not *E. coli* isolates, possibly because Gram negative bacteria are less sensitive to high concentrations of Cu than Gram positive bacteria (Aarestrup and Hasman, 2004). Thus, Cu may increase Gram negative bacterial resistance to cephalosporins apart from co-selection with tetracycline.

Feeding pigs 10 ppm ractopamine HCl for 28 d prior to marketing did not affect the susceptibility of fecal *E. coli* or *Enterococcus* to antibiotics. This novel observation suggests ractopamine feeding to pigs according to typical production practice does not pose a significant risk to increasing bacterial resistance to a wide range of antibiotics. Limited research has investigated the roles of α and β adrenergic receptors in mediating the bacterial response to catecholamines. Although both the α and β receptors appear to be involved, the α adrenergic receptor may have a dominant role in facilitating bacterial growth, conjugative gene transfer, and increased bacterial virulence in response to catecholamines (Peterson et al., 2011). As such, β adrenergic agonists such as ractopamine HCl may have limited ability to in influence antimicrobial resistance.

In the present study, *Enterococcus* and especially *E. coli* resistance to a number of antibiotics decreased throughout the finishing period regardless of dietary treatment. Similar observations have led to the postulation that the gastrointestinal microbiota of older animals is less vulnerable to population with multiple antibiotic resistant bacteria compared to younger animals (Langlois et al., 1986; Dewulf et al., 2007; Berge et al., 2010). This phenomenon could

also be an artifact of greater antimicrobial use among younger livestock inducing microbial drug resistance early in life then decreasing selection pressure over time causing these resistant microbes to have poorer relative competitiveness and survival (Dewulf et al., 2007).

In the bacterial population of the present study, resistance to tetracycline was high among both *E. coli* and *Enterococcus* isolates and *E. coli* isolates also showed a high level of resistance to AMP. This low bacterial susceptibility to tetracycline and ampicillin may be reflective of these drugs' use in upstream production within the sow herd. Tetracycline resistance is conferred through *tet* and *otr* genes (Roberts, 2011) and subtherapeutic use of tetracycline has been shown to induce tetracycline resistant genotypes as well as phenotypes (Funk et al., 2006; Dewulf et al., 2007; Agga et al., 2014). In addition, tetracycline resistance is associated with ampicillin drug resistance among Gram negative bacteria (Funk et al., 2006; Dewulf et al., 2007). Curiously, in the present study, the ampicillin resistance remained high over time but tetracycline resistance decreased throughout the finishing period among *E. coli* isolates while tetracycline resistance concomitantly increased among *Enterococcus* isolates. However, tetracycline resistance was initially greater in baseline *E. coli* isolates than in *Enterococcus* baseline isolates and both exhibited a similar, intermediate, level of resistance by d 90. Genetic exchange between Gram negative and Gram positive bacteria can occur so that plasmid mediated transfer of a common tetracycline resistance gene(s) from *E. coli* to *Enterococcus* would not be impossible (Courvalin, 1994; Roberts, 2011).

CONCLUSION

In closing, supplementation of 125 ppm Cu or 150 ppm Zn above basal premix TM levels in diets containing ractopamine HCl did not improve finishing pig growth performance of pigs with high feed intake levels as observed in this study. Inclusion of 10 ppm ractopamine HCl in

the diet for 28 d prior to marketing dramatically improved carcass leanness as well as the feed and caloric efficiencies of pigs. Ractopamine HCl did not adversely affect antimicrobial resistance among fecal bacterial isolates. Extended feeding of 125 ppm Cu throughout the finishing period resulted in less bacterial susceptibility to some antibiotics while there were no adverse effects of feeding 150 ppm of added Zn noted on antimicrobial resistance. In general with the exception of tetracycline, resistance to most antibiotics decreased over time or was stable for antibiotics that had a low percentage of resistance at baseline.

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Table 5.1. Diet composition (as-fed basis)[1]

Item	Phase 1	Phase 2	Phase 3	Phase 4[2]
Ingredient, %				
Corn[3]	72.54	78.02	80.66	76.55
Soybean meal (47.7% CP)	25.00	19.70	17.20	21.20
Limestone	0.90	0.90	0.90	0.90
Monocalcium phosphate	0.60	0.50	0.40	0.40
Sodium chloride	0.35	0.35	0.35	0.35
L-Lys HCl	0.28	0.25	0.23	0.25
DL-Met	0.05	0.02	0.00	0.04
L-Thr	0.08	0.05	0.05	0.10
Trace mineral premix[4]	0.10	0.10	0.10	0.10
Vitamin premix[5]	0.10	0.10	0.10	0.10
Phytase[6]	0.02	0.02	0.02	0.02
$CuSO_4$, ZnO, ractopamine HCl additives[7]	0 – 0.07	0 – 0.07	0 – 0.07	0 – 0.27
Total	100.00	100.00	100.00	100.00
Calculated analysis				
Standardized ileal digestible (SID) amino acids, %				
Lys	1.01	0.86	0.78	0.90
Ile:Lys	64	64	66	65
Leuc:Lys	139	149	157	147
Met:Lys	30	29	29	31
Met & Cys:Lys	56	57	57	58
Thr:Lys	62	62	64	67
Trp:Lys	18.3	18.1	18.2	18.3
Val:Lys	70	73	75	72
Total Lys, %	1.14	0.97	0.89	1.02
CP, %	18.3	16.1	15.1	16.8
ME, kcal/kg	3,303	3,311	3,316	3,314
NE, kcal/kg	2,474	2,507	2,524	2,500
SID Lys:ME, g/Mcal	3.06	2.60	2.35	2.71
SID Lys:NE, g/Mcal	4.08	3.43	3.09	3.59
Ca, %	0.55	0.51	0.49	0.50
P, %	0.50	0.45	0.42	0.44
Available P, %	0.30	0.27	0.24	0.25

[1] All diets were fed in meal form and formulated to be fed in 4 phases from 36 to 57, 57 to 79, 79 to 100, and 100 to 132 kg BW.
[2] Phase 4 diets were fed for the final 28 d prior to slaughter.
[3] Corn levels represent control level prior to addition of treatment diet ingredients which replaced an equivalent amount of corn in respective experimental diets.
[4] Provided per kg of diet: 27 mg Mn from manganese oxide, 110 mg Fe from iron sulfate, 110 mg Zn from zinc sulfate, 11 mg Cu from copper sulfate, 0.20 mg I from calcium iodate, and 0.20 mg Se from sodium selenite.
[5] Provided per kg of diet: 4,409 IU vitamin A, 661 IU vitamin D_3, 18 IU vitamin E, 1.8 mg vitamin K, 3.3 mg riboflavin, 11.0 mg pantothenic acid, 19.8 mg niacin, and 0.02 mg vitamin B_{12}.
[6] HiPhos 2700 (DSM Nutritional Products, Inc., Parsippany, NJ), providing 540 phytase units (FTU)/kg and an estimated release of 0.10% available P.
[7] The 8 dietary treatments contained $CuSO_4$ at either 0 or 0.05%, ZnO at either 0 or 0.021%, and Paylean® (Elanco Animal Health, IN) in the final 28 d of the finishing period at either 0 or 0.2%.

Table 5.2. Analyzed dietary concentrations (as-fed basis)[1]

	Analyzed Dietary Composition[2]							Analyzed Concentrations[3]		
Diets	DM, %	CP, %	CF, %	Ether extract, %	Ash, %	Ca, %	P, %	Cu, ppm[4]	Zn, ppm[5]	Ractopamine HCl, ppm[6]
Phase 1										
Control	87.70	20.6	2.9	2.85	5.26	0.74	0.58	25	117	---
Cu	87.70	20.4	2.6	2.97	5.20	0.74	0.57	196	116	---
Zn	87.65	20.3	2.6	2.77	5.15	0.76	0.60	26	298	---
Cu+Zn	87.70	20.2	2.8	3.00	5.44	0.77	0.57	187	299	---
Phase 2										
Control	87.75	18.0	2.5	3.22	5.10	0.80	0.53	24	140	---
Cu	87.65	18.3	2.6	3.26	5.43	0.85	0.55	179	126	---
Zn	87.45	18.2	2.7	3.28	5.38	0.71	0.52	44	279	---
Cu+Zn	87.70	17.7	2.5	3.63	5.07	0.71	0.52	182	307	---
Phase 3										
Control	87.40	16.9	2.6	3.23	4.36	0.67	0.48	25	138	---
Cu	87.55	17.0	2.6	3.37	4.88	0.72	0.50	181	133	---
Zn	86.35	17.1	2.3	3.28	4.75	0.75	0.52	49	276	---
Cu+Zn	81.85	16.8	2.6	3.26	5.13	0.77	0.50	175	309	---
Phase 4										
Control	75.30	19.1	3.0	3.71	5.14	0.71	0.52	39	129	---
Cu	88.15	18.8	2.8	3.45	4.95	0.74	0.51	182	106	---
Zn	87.75	18.8	2.5	3.57	5.19	0.78	0.52	31	320	---
Cu+Zn	87.65	18.7	2.7	3.97	4.86	0.74	0.53	191	297	---
Control+Rac	87.55	18.7	2.5	3.57	5.05	0.75	0.51	29	127	9.3
Cu+Rac	87.50	18.6	2.7	3.67	5.12	0.77	0.52	197	138	9.7
Zn+Rac	88.55	19.1	2.8	3.48	5.29	0.76	0.54	33	250	8.0
Cu+Zn+Rac	87.85	18.8	2.7	4.00	5.20	0.74	0.52	200	340	9.3

[1] Phase 1, 2, 3, and 4 diets fed in meal form from approximately 48 to 68, 68 to 91, 91 to 109, and 109 to 136 kg BW, respectively.
[2] Analysis performed by Cumberland Valley Analytical Services (Hagerstown, MD) on pooled diet samples within each dietary phase; results represent the average of both finishing groups.
[3] Mineral analysis was performed by Cumberland Valley Analytical Services (Hagerstown, MD) using ICP spectrometry; means represent the average of 2 to 4 duplicate feed samples within each dietary phase and each finishing group which were subsequently analyzed in duplicate.
[4] Cu from $CuSO_4$ was added at 125 ppm to diets containing 11 ppm Cu from the trace mineral premix.
[5] Zn from ZnO was added at 150 ppm to diets containing 73 ppm Zn from the trace mineral premix.
[6] Analysis was performed by Covance Laboratories (Greenfield, IN) on a composite sample of each of the 4 diets containing ractopamine HCl across both finishing groups.

Table 5.3. Resistance breakpoints and evaluated concentrations for antimicrobials of NARMS gram positive bacteria panel (CMV3AGPF).

Antimicrobial	WHO Classification[1]	Concentration (μg/ml)	Breakpoint (μg/ml)[2]
Chloramphenicol	Highly important	2 - 32	≥ 32
Ciprofloxacin	Critically important	0.12 - 4	≥ 4
Daptomycin	Critically important	0.25 – 16	N/A[3]
Erythromycin	Critically important	0.25 – 8	≥ 8
Gentamicin	Critically important	128 – 1024	> 500
Kanamycin	Critically important	128 – 1024	≥ 1024
Lincomycin	Highly important	1 – 8	≥ 8
Linezolid	Critically important	0.5 – 8	≥ 8
Nitrofurantoin	Important	2 – 64	≥ 128
Penicillin	Critically important	0.25 – 16	≥ 16
Quinupristin/Dalfopristin	Highly important	0.5 – 32	≥ 4
Streptomycin	Critically important	512 – 2048	> 1000
Tetracycline	Highly important	1 – 32	≥ 16
Tigecycline	Critically important	0.015 – 0.5	N/A[4]
Tylosin tartrate	Critically important	0.25 - 32	≥ 32
Vancomycin	Critically important	0.25 - 32	≥ 32

[1] World Health Organization categorization of antimicrobials according to importance for human medicine.
[2] Breakpoints established by Clinical and Laboratory Standards Institute (CLSI, 2013).
[3] A susceptibility breakpoint of ≤ 4 μg/ml for daptomycin exists but no resistant breakpoint has been established. In this study, isolates with a MIC ≥ 8 μg/ml were categorized as resistant.
[4] A susceptibility breakpoint of ≤ 0.25 μg/ml for tigecycline exists but no resistant breakpoint has been established. In this study, isolates with a MIC ≥ 0.5 μg/ml were categorized as resistant.

Table 5.4. Resistance breakpoints and evaluated concentrations for antimicrobials of NARMS gram negative bacteria panel (CMV3AGNF).

Antimicrobial	WHO Classification[1]	Concentration (μg/ml)	Breakpoint (μg/ml)[2]
Amoxicillin/Clavulanic acid 2:1 ratio	Critically important	1/0.5 – 32/16	≥ 32/16
Ampicillin	Critically important	1 - 32	≥ 32
Azithromycin	Critically important	0.12 - 16	N/A[3]
Cefoxitin	Highly important	0.5 - 32	≥ 32
Ceftiofur	Critically important	0.12 - 8	≥ 8
Ceftriaxone	Critically important	0.25 - 64	≥ 4
Chloramphenicol	Highly important	2 - 32	≥ 32
Ciprofloxacin	Critically important	0.015 – 4	≥ 1
Gentamicin	Critically important	0.25 – 16	≥ 16
Nalidixic Acid	Critically important	0.5 – 32	≥ 32
Streptomycin	Critically important	32 - 64	≥ 64
Sulfisoxazole	Highly important	16 - 256	≥ 512
Tetracycline	Highly important	4 – 32	≥ 16
Trimethoprim/Sulfamethoxazole	Highly important	0.12/2.4 – 4/76	≥ 4/76

[1] World Health Organization categorization of antimicrobials according to importance for human medicine.
[2] Breakpoints established by Clinical and Laboratory Standards Institute (CLSI, 2013).
[3] NARMS has not established breakpoints for azithromycin interpretation thus there is no CLSI resistant breakpoint.

Table 5.5. Effects of added Cu, Zn, and ractopamine HCl on carcass characteristics of finishing pigs[1]

[2]Added Cu, 125 ppm:	-	+	-	+	-	+	-	+	
[3]Added Zn, 150 ppm:	-	-	+	+	-	-	+	+	
[4]Ractopamine HCl, 10 ppm:	-	-	-	-	+	+	+	+	SEM
BW, kg									
d 0	48.2	48.6	48.5	48.6	49.4	48.8	48.8	48.9	0.57
d 62[5]	108.5	108.8	109.2	108.9	108.7	108.7	109.0	109.0	2.59
d 90[6]	134.5	133.8	135.0	134.1	138.4	138.1	138.6	138.3	4.29
d 0 to 62[5]									
ADG, kg	1.03	1.03	1.03	1.03	1.02	1.03	1.03	1.03	0.024
ADFI, kg	2.62	2.61	2.61	2.63	2.63	2.61	2.60	2.65	0.038
G:F	0.39	0.39	0.40	0.39	0.39	0.39	0.40	0.39	0.013
ME caloric efficiency[7]	8,428	8,395	8,376	8,432	8,590	8,442	8,370	8,537	269.2
NE caloric efficiency[7]	6,375	6,346	6,334	6,376	6,498	6,381	6,329	6,456	200.9
d 62 to 90[5,6]									
ADG, kg	0.88	0.90	0.92	0.90	1.06	1.06	1.07	1.05	0.056
ADFI, kg	2.78	2.78	2.88	2.80	2.86	2.79	2.80	2.76	0.101
G:F	0.32	0.32	0.32	0.32	0.37	0.38	0.38	0.38	0.008
ME caloric efficiency[7]	10,483	10,325	10,403	10,309	8,939	8,739	8,689	8,733	229.4
NE caloric efficiency[7]	7,909	7,795	7,849	7,776	6,746	6,594	6,557	6,589	173.1
Overall (d 0 to 90)[6]									
ADG, kg	0.98	0.99	0.99	0.99	1.03	1.03	1.04	1.03	0.011
ADFI, kg	2.67	2.67	2.70	2.68	2.70	2.67	2.67	2.68	0.053
G:F	0.37	0.37	0.37	0.37	0.38	0.39	0.39	0.38	0.006
ME caloric efficiency[7]	8,996	8,957	8,973	8,986	8,697	8,535	8,472	8,595	134.5
NE caloric efficiency[7]	6,799	6,768	6,780	6,790	6,573	6,448	6,402	6,495	100.1

[1] A total of 480 pigs (PIC 327 × 1050; initially 48.7 kg) were used in a 90-d (group 1) or 83-d (group 2) study with 7 (group 1) or 8 (group 2) pigs per pen and 8 replications per treatment.
[2] Cu from $CuSO_4$ was added to treatment diets at either 0 or 125 ppm. All diets contained 11 ppm Cu from the trace mineral premix.
[3] Zn from ZnO was added to treatment diets at either 0 or 150 ppm. All diets contained 73 ppm Zn from the trace mineral premix.
[4] Ractopamine HCl (Paylean®; Elanco Animal Health, Greenfield, IN) was added to treatment diets at either 0 or 10 ppm during the final 28 d prior to marketing.
[5] d 62 (group 1) corresponds to d 55 of group 2 and marks the beginning of the 28 d period of ractopamine HCl treatments in addition to the mineral combination treatments.
[6] d 90 (group 1) corresponds to d 83 of group 2 and is the final day of the study.
[7] Caloric efficiency is expressed as kcal per kg of live weight gain.

Table 5.6. Statistical analysis of added Cu, Zn, and ractopamine HCl on finishing pig growth performance[1]

	Probability, $P <$						
	Cu	Zn	Ractopamine HCl	Cu × Zn	Cu × Rac	Zn × Rac	Cu × Zn × Rac
BW, kg							
d 0	0.908	0.797	0.012	0.729	0.269	0.287	0.231
d 62[2]	0.982	0.516	0.994	0.801	0.963	0.917	0.781
d 90[3]	0.392	0.676	<0.001	0.920	0.737	0.883	0.959
d 0 to 62[2]							
ADG, kg	0.829	0.478	0.448	0.704	0.823	0.686	0.733
ADFI, kg	0.685	0.810	0.711	0.283	0.838	0.958	0.636
G:F	0.789	0.446	0.121	0.041	0.949	0.643	0.226
ME caloric efficiency[4]	0.831	0.476	0.122	0.045	0.983	0.575	0.249
NE caloric efficiency[4]	0.874	0.483	0.124	0.039	0.984	0.576	0.250
d 62 to 90[2,3]							
ADG, kg	0.565	0.540	<0.001	0.440	0.738	0.440	0.828
ADFI, kg	0.125	0.834	0.773	0.635	0.735	0.084	0.396
G:F	0.424	0.363	<0.001	0.444	0.983	0.489	0.525
ME caloric efficiency[4]	0.338	0.408	<0.001	0.467	0.819	0.705	0.670
NE caloric efficiency[4]	0.339	0.396	<0.001	0.480	0.835	0.721	0.654
Overall (d 0 to 90[3])							
ADG, kg	0.818	0.379	<0.001	0.456	0.993	0.872	0.833
ADFI, kg	0.694	0.778	0.920	0.595	0.966	0.435	0.480
G:F	0.806	0.344	<0.001	0.057	0.975	0.332	0.142
ME caloric efficiency[4]	0.722	0.377	<0.001	0.068	0.942	0.349	0.205
NE caloric efficiency[4]	0.693	0.374	<0.001	0.064	0.933	0.359	0.201

[1] A total of 480 pigs (PIC 327 × 1050; initially 48.7 kg) were used in a 90-d (group 1) or 83-d (group 2) study with 7 (group 1) or 8 (group 2) pigs per pen and 8 replications per treatment.
[2] d 62 (group 1) corresponds to d 55 of group 2 and marks the beginning of the 28 d period of ractopamine HCl treatments in addition to the mineral combination treatments.
[3] d 90 (group 1) corresponds to d 83 of group 2 and is the final day of the study.
[4] Caloric efficiency is expressed as kcal per kg of live weight gain.

Table 5.7. Effects of added Cu, Zn, and ractopamine HCl on finishing pig growth performance[1]

[2]Added Cu, 125 ppm:	-	+	-	+	-	+	-	+	
[3]Added Zn, 150 ppm:	-	-	+	+	-	-	+	+	
[4]Ractopamine HCl, 10 ppm:	-	-	-	-	+	+	+	+	SEM
Carcass characteristics									
HCW, kg	99.3	98.4	99.4	99.0	103.3	103.9	103.9	103.1	4.32
Carcass yield, %	73.8	73.5	73.6	73.8	74.6	75.2	74.9	74.5	0.86
Backfat, mm[5]	19.2	19.3	18.7	19.3	18.2	18.0	18.1	17.8	0.89
Loin depth, cm[5]	6.55	6.48	6.48	6.55	6.77	6.92	6.85	6.90	0.22
Fat-free lean, %[5,6]	51.5	51.7	51.6	51.5	52.6	52.8	52.6	53.1	0.30
Carcass performance									
HCW ADG, kg	0.73	0.72	0.73	0.72	0.77	0.78	0.78	0.77	0.020
HCW G:F	0.27	0.27	0.27	0.27	0.28	0.29	0.29	0.29	0.003

[1] A total of 480 pigs (PIC 327 × 1050; initially 48.7 kg) were used in a 90-d (group 1) or 83-d (group 2) study with 7 (group 1) or 8 (group 2) pigs per pen and 8 replications per treatment.
[2] Cu from $CuSO_4$ was added to treatment diets at either 0 or 125 ppm. All diets contained 11 ppm Cu from the trace mineral premix.
[3] Zn from ZnO was added to treatment diets at either 0 or 150 ppm. All diets contained 73 ppm Zn from the trace mineral premix.
[4] Ractopamine HCl (Paylean®; Elanco Animal Health, Greenfield, IN) was added to treatment diets at either 0 or 10 ppm during the final 28 d prior to marketing.
[5] Adjusted for individual HCW using HCW as a covariate.
[6] SFFL (NPPC. 2000. Procedures for Estimating Pork Carcass Composition. Natl. Pork Prod. Council, Des Moines, IA.) ÷ HCW.

Table 5.8. Statistical analysis of added Cu, Zn, and ractopamine HCl on carcass characteristics of finishing pigs[1]

	Probability, $P <$						
	Cu	Zn	Ractopamine HCl	Cu × Zn	Cu × Rac	Zn × Rac	Cu × Zn × Rac
Carcass characteristics							
HCW, kg	0.494	0.829	<0.001	0.675	0.630	0.644	0.391
Carcass yield, %	0.710	0.638	<0.001	0.360	0.655	0.380	0.011
Backfat, mm[2]	0.809	0.505	0.014	0.721	0.376	0.790	0.734
Loin depth, cm[2]	0.465	0.826	<0.001	0.874	0.455	0.848	0.387
Fat-free lean, %[2,3]	0.334	0.829	<0.001	0.911	0.550	0.589	0.587
Carcass performance							
HCW ADG, kg	0.517	0.776	<0.001	0.582	0.419	0.855	0.197
HCW G:F	0.743	0.962	<0.001	0.197	0.320	0.479	0.018

[1] A total of 480 pigs (PIC 327 × 1050; initially 48.7 kg) were used in a 90-d (group 1) or 83-d (group 2) study with 7 (group 1) or 8 (group 2) pigs per pen and 8 replications per treatment.
[2] Adjusted for individual HCW using HCW as a covariate.
[3] SFFL (NPPC. 2000. Procedures for Estimating Pork Carcass Composition. Natl. Pork Prod. Council, Des Moines, IA.) ÷ HCW.

Table 5.9. Effects of added Cu, Zn, and ractopamine HCl on percentage fecal *E. coli* antimicrobial resistance according to NARMS established breakpoints[1]

[2]Added Cu, 125 ppm:	-	+	-	+	-	+	-	+
[3]Added Zn, 150 ppm:	-	-	+	+	-	-	+	+
[4]Ractopamine HCl, 10 ppm:	-	-	-	-	+	+	+	+
Amoxicillin/clavulanic acid 2:1 ratio								
Baseline	35	10	30	15	15	20	35	10
Day 90	5	15	5	10	5	0	15	5
Ampicillin								
Baseline	100	100	100	100	100	100	100	100
Day 90	85	100	100	90	100	100	95	100
Cefoxitin								
Baseline	35	10	30	15	15	20	35	10
Day 90	5	15	5	10	5	0	15	5
Ceftiofur								
Baseline	35	10	30	15	15	20	35	10
Day 90	5	25	10	10	5	5	15	10
Ceftriaxone								
Baseline	35	10	30	15	15	20	35	10
Day 90	5	25	10	10	5	5	15	10
Chloramphenicol								
Baseline	25	0	20	10	15	10	5	15
Day 90	5	5	10	10	15	10	0	10
Ciprofloxacin								
Baseline	0	0	0	0	0	0	0	0
Day 90	0	0	0	0	0	0	0	0
Gentamicin								
Baseline	25	15	30	25	15	10	25	20
Day 90	5	5	0	0	10	0	0	0
Nalidixic Acid								
Baseline	0	0	0	0	0	5	0	0
Day 90	0	0	0	0	0	0	0	0
Streptomycin								
Baseline	35	20	35	25	15	20	30	30
Day 90	10	20	5	0	15	5	5	0
Sulfisoxazole								
Baseline	0	0	0	0	0	0	0	0
Day 90	0	0	0	0	0	0	0	0
Tetracycline								
Baseline	100	80	70	90	80	80	95	90
Day 90	60	80	70	80	85	75	70	75
Trimethoprim/sulfamethoxazole								
Baseline	0	0	0	0	0	0	0	0
Day 90	0	0	0	0	0	0	0	0

[1] Values represent the percentage resistant of 20 *E. coli* isolates per sampling day (d 0 baseline or d 90); 5 random fecal samples were collected per pen per day and 1 *E. coli* isolate per fecal sample was assessed. There were a total of 224 pigs (PIC 327 × 1050; initially 49 kg) housed with 7 (group 1) pigs per pen and 4 replicate pens per treatment.
[2] Cu from $CuSO_4$ was added to treatment diets at either 0 or 125 ppm. All diets contained 11 ppm Cu from the trace mineral premix.
[3] Zn from ZnO was added to treatment diets at either 0 or 150 ppm. All diets contained 73 ppm Zn from the trace mineral premix.
[4] Ractopamine HCl (Paylean®; Elanco Animal Health, Greenfield, IN) was added to treatment diets at either 0 or 10 ppm during the final 28 d prior to the end of the study (d 62 to 90).

Table 5.10. Main effects of added Cu, Zn, and ractopamine HCl on percentage fecal *E. coli* antimicrobial resistance according to NARMS established breakpoints[1]

	[2]Cu			Cu (day)	[3]Zn			Zn (day)	[4]Ractopamine HCl:			Rac (day)
	-	+	SEM	*P*, <	-	+	SEM	*P*, <	-	+	SEM	*P*, <
Amoxicillin/clavulanic acid 2:1 ratio [d]												
Baseline	28.8	13.8	5.61	0.253	20.0	22.5	5.19	0.791	22.5	20.0	5.13	0.788
Day 90	7.5	7.5			6.3	8.8			8.8	6.3		
Cefoxitin [d]												
Baseline	28.8	13.8	5.61	0.253	20.0	22.5	5.19	0.791	22.5	20.0	5.13	0.788
Day 90	7.5	7.5			6.3	8.8			8.8	6.3		
Ceftiofur [d]												
Baseline	28.8	13.8	5.48	0.069	20.0	22.5	5.08	0.980	22.5	20.0	5.08	0.726
Day 90	8.8	12.5			10.0	11.3			12.5	8.8		
Ceftriaxone [d]												
Baseline	28.8	13.8	5.48	0.069	20.0	22.5	5.08	0.980	22.5	20.0	5.08	0.726
Day 90	8.8	12.5			10.0	11.3			12.5	8.8		
Chloramphenicol												
Baseline	16.3	8.8	3.77	0.216	12.5	12.5	3.41	0.810	13.8	11.3	3.52	0.566
Day 90	7.5	8.8			8.8	7.5			7.5	8.8		
Gentamicin [d]												
Baseline	23.8	17.5	5.09	0.576	16.3	25.0	---	---	23.8	17.5	5.07	0.741
Day 90	3.8	1.3			5.0	0			2.5	2.5		
Streptomycin [d]												
Baseline	28.8	23.8	5.94	0.900	22.5[ab]	30.0[a]	5.58	0.030	28.8	23.8	5.81	0.898
Day 90	8.8	6.3			12.5[b]	2.5[c]			8.8	6.3		
Tetracycline [d]												
Baseline	86.3	85.0	6.38	0.558	85.0	86.3	6.19	0.819	85.0	86.3	6.27	0.896
Day 90	71.3	77.5			75.0	73.8			72.5	76.3		

[1] Values represent the percentage resistant among 80 *E. coli* isolates for determination of treatment main effects within day. Five random fecal samples were collected per pen per day (d 0 baseline or d 90) and 1 *E. coli* isolate per fecal sample was assessed. There were a total of 224 pigs (PIC 327 × 1050; initially 49 kg) housed with 7 (group 1) pigs per pen and 4 replicate pens per treatment.

[2] Cu from $CuSO_4$ was added to treatment diets at either 0 or 125 ppm. All diets contained 11 ppm Cu from the trace mineral premix.

[3] Zn from ZnO was added to treatment diets at either 0 or 150 ppm. All diets contained 73 ppm Zn from the trace mineral premix.

[4] Ractopamine HCl (Paylean®; Elanco Animal Health, Greenfield, IN) was added to treatment diets at either 0 or 10 ppm during the final 28 d prior to the end of the study (d 62 to 90).

[a,b,c] Means within main effect and antibiotic lacking common superscripts differ, $P < 0.05$.

[d] Percentage of resistant isolates decreased (Day, $P < 0.05$) between d 0 (baseline) and d 90.

Table 5.11. **Effects of added Cu, Zn, and ractopamine HCl on percentage fecal *Enterococcus* spp. antimicrobial resistance according to NARMS established breakpoints**[1]

[2]Added Cu, 125 ppm:	-	+	-	+	-	+	-	+
[3]Added Zn, 150 ppm:	-	-	+	+	-	-	+	+
[4]Ractopamine HCl, 10 ppm:	-	-	-	-	+	+	+	+
Chloramphenicol								
Baseline	0	10	0	5	0	0	10	0
Day 90	0	0	0	0	0	0	0	0
Ciprofloxacin								
Baseline	10	15	5	5	10	15	0	0
Day 90	0	5	0	10	0	5	0	0
Daptomycin [5]								
Baseline	5	0	5	15	5	5	10	10
Day 90	10	0	15	10	5	10	15	5
Erythromycin								
Baseline	80	80	80	85	75	80	65	80
Day 90	15	30	15	25	5	30	20	5
Gentamicin								
Baseline	0	5	5	0	0	0	0	0
Day 90	0	0	0	0	0	0	0	0
Kanamycin								
Baseline	10	5	20	20	5	0	5	15
Day 90	0	0	10	10	0	10	0	0
Lincomycin								
Baseline	100	100	95	95	100	95	95	95
Day 90	95	70	85	60	90	80	90	70
Linezolid								
Baseline	0	0	0	5	0	0	5	0
Day 90	0	0	0	0	0	0	0	0
Nitrofurantoin								
Baseline	0	0	0	0	0	0	0	0
Day 90	0	0	0	0	0	0	0	0
Penicillin								
Baseline	0	5	0	5	10	5	5	0
Day 90	0	0	0	0	0	0	0	0
Quinupristin/dalfopristin								
Baseline	40	45	55	40	65	40	50	50
Day 90	5	20	10	35	10	25	25	40
Streptomycin								
Baseline	10	5	5	15	0	0	5	15
Day 90	5	5	10	5	0	15	0	0
Tetracycline								
Baseline	75	70	80	40	75	55	70	40
Day 90	60	90	65	80	70	65	85	80
Tigecycline [6]								
Baseline	0	0	5	0	0	0	0	0
Day 90	0	0	0	0	0	0	0	0
Tylosin tartrate								
Baseline	30	20	45	20	15	5	25	25
Day 90	15	30	10	30	5	30	20	5
Vancomycin								
Baseline	0	0	0	0	0	0	0	0
Day 90	0	0	0	0	0	0	0	0

[1] Values represent the percentage resistant of 20 *Enterococcus* spp. isolates per sampling day (d 0 baseline or d 90); 5 random fecal samples were collected per pen per day and 1 enterococcal isolate per fecal sample was assessed. There were a total of 224 pigs (PIC 327 × 1050; initially 49 kg) housed with 7 (group 1) pigs per pen and 4 replicate pens per treatment.
[2] Cu from $CuSO_4$ was added to treatment diets at either 0 or 125 ppm. All diets contained 11 ppm Cu from the trace mineral premix.
[3] Zn from ZnO was added to treatment diets at either 0 or 150 ppm. All diets contained 73 ppm Zn from the trace mineral premix.
[4] Ractopamine HCl (Paylean®; Elanco Animal Health, Greenfield, IN) was added to treatment diets at either 0 or 10 ppm during the final 28 d prior to the end of the study (d 62 to 90).
[5] A susceptibility breakpoint of ≤ 4 μg/ml for daptomycin exists but no resistant breakpoint has been established. In this study, isolates with a MIC ≥ 8 μg/ml were categorized as resistant.
[6] A susceptibility breakpoint of ≤ 0.25 μg/ml for tigecycline exists but no resistant breakpoint has been established. In this study, isolates with a MIC ≥ 0.5 μg/ml were categorized as resistant.

Table 5.12. Main effects of added Cu, Zn, and ractopamine HCl on percentage fecal *Enterococcus* spp. antimicrobial resistance according to NARMS established breakpoints[1]

	[2]Cu				[3]Zn				[4]Ractopamine HCl			
	-	+	SEM	Cu (day) *P*, <	-	+	SEM	Zn (day) *P*, <	-	+	SEM	Rac (day) *P*, <
Ciprofloxacin												
Baseline	6.3	8.8	---	---	12.5	2.5	4.39	0.265	8.8	6.3	3.95	0.645
Day 90	0	5.0			2.5	2.5			3.8	1.3		
Daptomycin [5]												
Baseline	6.3	7.5	3.58	0.338	3.75	10.0	3.50	0.654	6.3	7.5	3.29	0.824
Day 90	11.3	6.3			6.25	11.3			8.8	8.8		
Erythromycin [d]												
Baseline	75.0	81.3	5.44	0.719	78.8	77.5	5.47	0.785	81.3	75.0	5.50	0.930
Day 90	13.8	22.5			20.0	16.3			21.3	15.0		
Kanamycin												
Baseline	10.0	10.0	3.95	0.555	5.0	15.0	4.26	0.668	13.8	6.3	4.25	0.895
Day 90	2.5	5.0			2.5	5.0			5.0	2.5		
Lincomycin [d]												
Baseline	97.5	96.3	5.11	0.367	98.8	95.0	4.81	0.435	97.5	96.3	4.77	0.480
Day 90	90.0	70.0			83.8	76.3			77.5	82.5		
Quinupristin/dalfopristin [d]												
Baseline	52.5[a]	43.8[ab]	6.61	0.023	47.5	48.8	6.64	0.243	45.0	51.3	6.73	0.741
Day 90	12.5[c]	30.0[b]			15.0	27.5			17.5	25.0		
Streptomycin												
Baseline	5.0	8.8	3.55	0.955	3.8	10.0	3.74	0.169	8.8	5.0	3.63	0.956
Day 90	3.8	6.3			6.3	3.8			6.3	3.8		
Tetracycline [d]												
Baseline	75.0[a]	51.3[b]	5.43	0.003	68.8	57.5	5.69	0.113	66.3	60.0	5.76	0.518
Day 90	70.0[a]	78.8[a]			71.3	77.5			73.8	75.0		
Tylosin tartrate												
Baseline	28.8[a]	17.5[ab]	5.34	0.023	17.5	28.8	5.61	0.157	28.8	17.5	5.52	0.725
Day 90	12.5[bc]	23.8[ab]			20.0	16.3			21.3	15.0		

[1] Values represent the percentage resistant among 80 *Enterococcus* spp. isolates for determination of treatment main effects within day. Five random fecal samples were collected per pen per day (d 0 baseline or d 90) and 1 enterococcal isolate per fecal sample was assessed. There were a total of 224 pigs (PIC 327 × 1050; initially 49 kg) housed with 7 (group 1) pigs per pen and 4 replicate pens per treatment.

[2] Cu from $CuSO_4$ was added to treatment diets at either 0 or 125 ppm. All diets contained 11 ppm Cu from the trace mineral premix.

[3] Zn from ZnO was added to treatment diets at either 0 or 150 ppm. All diets contained 73 ppm Zn from the trace mineral premix.

[4] Ractopamine HCl (Paylean®; Elanco Animal Health, Greenfield, IN) was added to treatment diets at either 0 or 10 ppm during the final 28 d prior to the end of the study (d 62 to 90).

[5] A susceptibility breakpoint of ≤ 4 μg/ml for daptomycin exists but no resistant breakpoint has been established. In this study, isolates with a MIC ≥ 8 μg/ml were categorized as resistant.

[a,b,c] Means within main effect and antibiotic lacking common superscripts differ, $P < 0.05$.

[d] Percentage of resistant isolates differed (Day, $P < 0.05$) between d 0 (baseline) and d 90.