ALTERNATIVE MEASURES OF PHYSIOLOGICAL STRESS IN NURSERY PIGS AND BROILER CHICKENS

by

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Dedicated to Esther, my wife and best friend

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ABSTRACT

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Farm animals face a variety of stressors during commercial production practices, and economic necessities and ethical considerations require mitigation strategies to help animals cope with stressors that might otherwise reduce animal performance or lead to morbidity. In swine production, arguably the most stressful period of a pig's life is the first several weeks following weaning (nursery period), where pigs must rapidly adapt to a host of environmental and physiological stressors. In broiler chickens, exposure to environmental temperatures above their comfort zone is a considerable stressor. Accordingly, several studies were conducted with the objective of developing alternative ways to measure physiological stress in nursery pigs and broiler chickens. These alternative methods may improve the ability of animal scientists to measure physiological stress and thereby aid in development of mitigation strategies. Nursery pigs frequently struggle with diarrhea and other intestinal diseases characterized by increases in intestinal permeability. Therefore, several studies were conducted to evaluate the use of non-metabolizable carbohydrates (lactulose and mannitol) as a non-invasive measure of intestinal health in weanling pigs. In Exp 2.1 and Exp 2.2, an aspirin-induced model of intestinal permeability was explored and the excretion pattern of lactulose and mannitol in urine over a 48 h urine collection period was determined. Aspirin at 15 mg/kg BW increased the excretion of lactulose over that of pigs given 0 or 30 mg/kg of aspirin, and therefore has potential to be used to induce intestinal permeability in nursery pigs. The excretion of lactulose and mannitol peaked at

4 h post-oral dose with a solution of lactulose and mannitol and was primarily complete by 8 h post-oral dose. In the few published reports of the lactulose and mannitol test of intestinal health in weanling pigs, the dose of lactulose and mannitol has varied considerably, raising questions as to the comparability of the results. Accordingly, in Exp 3.1, the impact of variation in the dose of lactulose and mannitol was explored, and pigs given the lowest dose at 0.2 g/kg BW lactulose and 0.02 g/kg BW mannitol had large numerical decreases in lactulose excretion warranting further investigation. Pigs in Exp 3.1 also demonstrated low urine recovery rates (50% successful urine collection averaged over the 3 urine collection time points) which were postulated to be due to the stresses associated with urine collection in metabolism cages combining with the stresses of weaning. Therefore, in Exp 3.2, an alternative urine collection procedure was developed that utilized a urine collection pad held in place by an elastic wrap. Results from Exp 3.2 with urine collected either by metabolism cages or via the urine collection pads indicated that the urine collection pad held promise as an alternative urine collection method that would not require the use of metabolism cages. Accordingly, the use of the collection pad was evaluated as part of a lactulose and mannitol test of intestinal health in group-housed nursery pigs in Exp 5.1. In brief, in Exp 5.1, pigs were weaned, transported for 12 h in a livestock trailer, and fed five treatment diets for 14 d post-weaning. The treatment diets were designed to evaluate the additive effects of L-glutamine and a combination of prebiotics and probiotics as potential antibiotic alternatives to aid in mitigating stress associated with weaning and transport. After two weeks of treatment diets, common diets were fed through market-weight. Urine collection pads were used on 40 pigs (8 pigs per treatment) on d 5 and d 12 post-weaning, with pigs allowed to remain in their familiar group pen during the urine collection period, and urine collection success rates averaged 84% across collection days. This improvement over that of Exp 3.1 may indicate that the use of a urine

collection pad may improve the ability to obtain urine from pigs shortly after weaning. Considerable variation in the excretion of lactulose and mannitol was still observed similarly to that seen in Exp 3.1 and precluded statistical differences among dietary treatments. An increase in urine collection length was postulated to be one potential way to reduce that variation. Additional responses to the nursery diets fed in Exp 5.1 are discussed in Chapters 4 and 5. In broiler chickens, measurement of physiological heat stress is limited by the existing techniques which have considerable disadvantages. Therefore, in Chapter 6 a simple surgical procedure is detailed that allows small data loggers to be placed into the abdominal cavity of anesthetized broiler chickens. After a period to allow the chickens to recover from the surgery, these data loggers can record the internal temperature of the chicken at user-defined intervals and have the ability to gather large amounts of internal body temperature data in a wide variety of research settings. A more traditional measure of physiological heat stress in chickens is measurement of the temperature of the cloaca with a thermometer. Therefore, to compare the two methods, values from implanted data loggers were compared to values obtained by measuring the temperature of the cloaca in Exp 6.1 and Exp 6.2, and in general, the two methods yielded similar results. Since surgery to implant data loggers is not always possible or practical, in Chapter 7 the development and evaluation of an equation to predict the internal temperature of broiler chickens was investigated. In Exp 7.1, broiler chickens were exposed to four ambient temperatures each day for four days. The surface temperature of the chicken's face was measured with a thermal imaging camera, while internal temperature was measured with data loggers as in Chapter 6. The resulting prediction equation contained the significant explanatory variables of the surface temperature of the face, the sex of the chicken, and the number of days of heat stress exposure. Accordingly, the accuracy of the prediction equation was evaluated in Exp 7.2, where chickens were exposed to the same 4 ambient temperatures and

temperatures of the face and the internal body measured as in Exp 7.1. The prediction equation developed in Exp 7.1 was then used with the inputs of the facial surface temperature, sex of the birds, and number of days of heat stress exposure from chickens in Exp 7.2 to calculate a predicted internal temperature. This predicted internal temperature was then compared to the internal temperature as measured with data loggers in Exp 7.2. While the accuracy varied by experimental day and ambient temperature, the predicted internal temperature averaged 0.32°C greater than the measured internal temperature. Therefore, while the prediction equation shows considerable promise as a non-invasive metric of physiological heat stress in broiler chickens, refinement of the equation may be required in future studies before internal temperature may be predicted with the accuracy desired in a research setting. In conclusion, the lactulose and mannitol urinary test of intestinal health requires more research before wide-spread use but has considerable promise as a non-invasive test. Surgical implantation of data loggers to measure internal temperature of broiler chickens enables precise measurement of physiological heat stress in broiler chickens, and further research may enable accurate prediction of internal body temperature of broiler chickens without requiring invasive measurement techniques.

CHAPTER 1. MEASURES OF PHYSIOLOGICAL STRESS IN NURSERY PIGS AND BROILER CHICKENS: A LITERATURE REVIEW

1.1 Physiological Stressors Faced by Nursery Pigs and Broiler Chickens During Commercial Production

Stress is an unavoidable component of life as all organisms face circumstances that challenge survival or well-being (Selye, 1976). Farm animals in production settings are no different and are presented with a variety of stressors including environmental, metabolic, disease, handling, social, and production-scenario specific stresses (Martínez-Miró et al., 2016). The ability to accurately measure the degree of physiological stress experienced by an animal is an essential step toward development of mitigation strategies in research settings as well as humane and profitable management of animals in commercial production.

1.1.1 Enteric Health in Nursery Pigs

Gastrointestinal "gut" health in animal production continues to be an ethereal concept with many discipline-specific definitions. However, more encompassing definitions of gut health have been proposed such as a "generalized condition of homeostasis in the GIT, with respect to its overall structure and function" (Pluske et al., 2018), and as "a steady state where the microbiome and the intestinal tract exist in symbiotic equilibrium and where the welfare and performance of the animal is not constrained by intestinal dysfunction" (Celi et al., 2017). From these definitions it becomes apparent that the discussion of gut health is not restricted to pathogenic events or disease as a variety of non-pathogenic events such as fasting can negatively impact gut health (Pluske et al., 2018). It also becomes clear from these definitions that the study of gut health in

farm animals is an important multi-disciplinary endeavor with economic, ethical, and human health implications.

1.1.1.1 Weaning

In the United States swine industry piglets are abruptly removed from sows at approximately three weeks of age (Turpin et al., 2017), while wild or feral swine, piglets will wean gradually until suckling ceases at 14 to 17 weeks of age (Jensen, 1986). The comparatively early weaning practiced commercially has advantages in that it allows the number of piglets born per sow per year to be maximized (Mahan, 1993), protects the welfare of the sow (e.g. unlike wild or feral swine, commercial sows typically have no way to escape from the demands of the litter; Jayaraman and Nyachoti, 2017), and may aid in preventing disease transfer from the sow to the piglets (Robert et al., 1999). Despite these advantages, standard weaning practices create what is arguably the most stressful period of a pig's life (Jayaraman and Nyachoti, 2017). Many of the stresses converge on the digestive tract (Moeser et al., 2017) as pigs must rapidly transition from a milk-based diet to consuming solid food (Spreeuwenberg et al., 2001). As a result, the immature GIT of pigs must rapidly adapt in digestion and absorptive function at weaning (Jayaraman and Nyachoti, 2017; Pluske et al., 2018). In addition, most pigs have low feed intake for at least 7 d post-weaning (de Lange et al., 2010) leading to weight loss and reduced nutrient supply to the GIT (Jayaraman and Nyachoti, 2017). Damage to the villi occurs as a result (Spreeuwenberg et al., 2001), with villus height (and therefore, brush-border digestive capacity and absorptive surface area) reaching a nadir 3 to 5 d post-weaning (Hampson, 1986). The immune and barrier functions of the GIT are also poorly developed post-weaning (Moeser et al., 2017), leading to potential colonization of the GIT by opportunistic pathogens such as enterotoxigenic *Escherichia coli*

(Everaert et al., 2017). In addition, increased entry of luminal bacteria and antigens into the body may trigger inflammation leading to further decreases in intestinal barrier function (Moeser et al., 2017). The combination of these events can lead to post-weaning diarrhea (Pluske et al., 2018), nutrient malabsorption (Celi et al., 2017), and poor growth performance (de Lange et al., 2010). Therefore, optimal management of pigs in the nursery period to aid in the prevention and recovery from the stresses of weaning is a key requirement for economical pork production.

1.1.1.2 Transportation

In modern swine production, weaned piglets are typically raised on a unit geographically isolated from the sow farm where they originated. This aids in disease control and promotes efficiency of production via specialization of the production phases (Sutherland et al., 2014). However, this necessitates transportation of the piglets immediately following weaning and thereby adds an additional stressor that piglets must face (Garcia et al., 2016). Transportation stresses may include handling during loading and unloading, mixing with unfamiliar piglets, crowding, delayed access to feed and water, vibration and transport noise, and thermal stresses (Lambooy, 1988; Wamnes et al., 2008; Sutherland et al., 2014). The effects of transportation are difficult to separate from the effects of weaning (Wamnes et al., 2006), as in many studies the two stressors are combined to simulate commercial practices. Indeed, the stresses of weaning and transport are believed to be additive (Berry and Lewis, 2001). Both the transport duration and ambient temperature have been shown to impact subsequent piglet performance (Berry and Lewis, 2001). Piglets were weaned and exposed to simulated transport for 0, 6, or 24 h at 20, 25, 30, and 35°C, and growth performance was subsequently measured over 14 d. Pigs transported for 24 h at either 20 or 35°C were found to have depressed growth on d 7 post-simulated transport, but by 14

d post-simulated transport there were no differences among treatments (Berry and Lewis, 2001). This finding could be explained by the observation that pigs that were transported in a similar study spent more than twice as much time eating over 3 d post-transport than control pigs that were not transported (Wamnes et al., 2008). It has been concluded that transport duration greater than 6 hours leads to greater stress than weaning alone (Sutherland et al., 2014), and that transport durations greater than 12 hours and transport in winter are particularly stressful for piglets (Wamnes et al., 2006). Therefore, it would appear that transport is a stressful event that healthy piglets are likely able to recover from within 7 to 14 d post-transport. However, it can be theorized (Sutherland et al., 2014) that this recovery time may be greater if the stressors of weaning and transport are combined with other stresses such as crowding, disease pressure, or thermal stress (Martínez-Miró et al., 2016).

1.1.2 Heat Stress in Broiler Chickens

Heat stress in poultry is of growing concern due to decreases in heat tolerance from modern strains of high-producing poultry coupled with projected increases in global environmental temperature (Deeb and Cahaner, 2002; Yahav et al., 2005; Nardone et al., 2010). Whether the result of seasonal heat waves or poultry production in tropical climates, heat stress has adverse impacts on the profitability of poultry production by decreasing growth performance, decreasing nutrient digestibility, increasing mortality, increasing disease susceptibility, and decreasing animal welfare (Lara and Rostagno, 2013).

Poultry, like other homeotherms, possess mechanisms by which they combat heat stress to maintain body temperature within stringent boundaries. As ambient temperature (T_a) increases, birds may seek to maintain their body temperature within their thermoneutral zone via behavioral

modifications such as seeking out a cooler environment or decreasing the thickness of feather cover via pilorelaxation (Dawson and Whittow, 2000). If the T_a increases to upper critical temperatures (which are poorly defined for modern broiler chickens), the bird must begin to increase heat loss via two primary mechanisms. The first is diversion of blood from organs deep within the body to surface tissues such as the skin, comb, and wattles. This occurs via vasodilation of peripheral blood vessels to allow core body heat to be lost to the environment (Wolfenson et al., 1981) and is under the control of hypothalamus-mediated input from peripheral and deep body thermal sensors (Dawson and Whittow, 2000). The second mechanism is evaporative cooling, either passively from the skin or actively from the respiratory tract (Menuam and Richards, 1975), with the later considered quantitatively more important (Dawson and Whittow, 2000). Under thermoneutral conditions heat is lost during respiration partly via evaporative cooling, and partly via heat exchange as cooler air is inhaled, warmed to body temperature in the anterior respiratory tract, and is exhaled after passing through air sacs and lungs (Menuam and Richards, 1975). Panting during heat stress enables substantial amounts of water from the anterior portion of the respiratory tract to transition from a liquid to a gas, during which there is the absorption of heat due to the phase change. As panting is an active process requiring energy, it will only increase as cooling via redirection of blood flow to the periphery and cutaneous evaporation become inadequate (Dawson and Whittow, 2000). Panting will increase in rate as required to maintain body temperature, but will eventually reach a maximum rate, at which point the bird is unable to regulate body temperature and further increases in T_a can result in death.

Commercial broilers experience heat stress when the rate of heat production exceeds the ability of the broilers to dissipate their body heat (Lara and Rostagno, 2013). Broilers nearing market weight are likely to be most sensitive to heat stress (Ryder et al., 2004) as larger body

weights, greater feed intake, and greater feather cover lead to greater heat production and reduced ability to dissipate heat (Deeb and Cahaner, 1999; Lin et al., 2006). Heat stress occurs seasonally in much of the United States and is a major economic burden to the broiler industry due to reduced growth and feed efficiency (Sahin et al., 2009), morbidity and mortality (Arjona et al., 1988), and reductions in carcass (Ryder et al., 2004) and meat quality (Zhang et al., 2012). In situations where heat stress levels exceed the ability of the chickens to cope, there are animal welfare as well as economic ramifications (Lara and Rostagno, 2013).

1.2 Invasive Measures of Physiological Stress in Nursery Pigs and Broiler Chickens

1.2.1 Invasive Measures of Enteric Health in Nursery Pigs

Attention to detail in the management of piglets in the nursery phase is critical to allow pigs to reach health and performance goals in the grow-finish phase. This is particularly true in the new paradigm of raising pigs from wean-to-finish without the inclusion of antibiotics in feed (de Lange et al., 2010). Attention to feeding behavior and nutrition, thermal comfort, biosecurity, and the management of disease are all essential (as reviewed by Jayaraman and Nyachoti, 2017). However, husbandry practices alone do not ensure good gut health in the nursery phase. For example, creep feeding has received a lot of attention as a potential way to improve feed intake at weaning. It would appear to make intuitive sense that pigs may transition to solid food better at weaning if they are regularly exposed to highly palatable feed before weaning. However, whether creep feeding actually improves gut health and piglet performance is still disputed, with only roughly half of the pigs offered creep feed actually consuming it (Pluske et al., 2018). Therefore, to maintain gut health and subsequent performance, producers continue to look for feed additives

and other nutritional products to replace antibiotics in the nursery phase diets. While there are many options to choose from, the efficacy of these alternative products in various production settings is not always easy to measure and thus may not be well documented (de Lange et al., 2010). As a result, producers often must conduct their own on-farm research studies and economic analysis before committing to a product, and variations in product performance by season, production site, and management practices are usually poorly understood and therefore difficult to manage (Pluske et al., 2018). Unfortunately, the ability to fill in these gaps is limited by existing methods available to measure a gut health response (Celi et al., 2017). One of the primary problems with existing methods to measure gut health is that euthanasia of animals is typically required to obtain intestinal tissue samples at each time point of interest. The loss of animals (and potentially experimental units) at each time point (e.g. Bosi et al., 2011) typically prevents repeated sampling leading to poor characterization of temporal effects (Berkeveld et al., 2008).

1.2.1.1 Histomorphology

Perhaps one of the most commonly used measures of gut health in weaning age pigs is histomorphology of sections of the small intestine. Pigs are euthanized at select time points, sections of small intestine are excised and preserved, thin sections of tissue are stained and mounted on slides, and measures such as villus height, crypt depth, and the ratio of villus height to crypt depth are measured under a light microscope (e.g. Xiao et al., 2012). This procedure is relatively simple, and translation of the results is straightforward. Weaning stress typically induces villous atrophy leading to shorter villi (and therefore reduced digestive and absorptive surface area) and crypt hyperplasia leading to longer crypts, and therefore a lesser villous height to crypt depth ratio (Jayaraman and Nyachoti, 2017). Depending on the stain used, other markers of gut health

may also be measured, including mast cell counts as a marker of inflammation, and goblet cell counts as an indicator of the protective mucus barrier (Moeser et al., 2017). Despite the advantages of histology, histological measures can be very subjective. For example, gross morphological measures of villi height and crypt depth is extremely subjective leading to considerable variation within and between individuals (Corazza et al., 1985).

1.2.1.2 Ussing Chamber

The primary method used to directly measure the permeability of the intestine of pigs is via Ussing chamber studies. Intestinal tissue from a freshly euthanized animal is obtained, stripped of the muscular layers, and the remaining tissue is clamped between two oxygenated electrolyte solutions (Bischoff et al., 2014). Electrical currents can then be applied to the tissue and the results are used to calculate measures of tight junction function (e.g. transepithelial electrical resistance) and active ion transport (e.g. short-circuit current) (Boudry, 2005). In addition, radio-labeled permeability markers such as mannitol (Peace et al., 2011) or fluorescently-labeled molecules (e.g. sodium fluorescein isothiocyanate; van der Meulen et al., 2010) can be added to the mucosal side of the tissue, and the concentration of the permeability marker on the serosal side of the tissue after incubation used as a marker of tissue permeability. Clarke (2009) provides a detailed guide to Ussing chamber methodology and data interpretation. While the Ussing chamber is a key tool in the development of our understanding of trans-epithelial transport of a variety of molecules, its use is limited by the need for specialized equipment and fresh intestinal tissue samples.

1.2.2 Invasive Measures of Heat Stress in Broiler Chickens

Accurate methods to measure the physiological strain broilers are experiencing during hot temperatures are required in order to study heat stress in broiler chickens and manage broiler chickens in the field. Conventionally used measures are limited and have a variety of disadvantages.

1.2.2.1 Cloacal Temperature

Measurement of the core body temperature of the bird is key to measuring physiological heat stress, as the bird is primarily attempting to regulate the temperature of critical internal organs such as the heart and liver (Dawson and Whittow, 2000). Core body temperature has traditionally been estimated in chickens via insertion of a thermometer into the cloaca of a restrained bird (Giloh et al., 2012). Cloacal temperature does appear to be a suitable proxy for core body temperature (De Basilio et al., 2003), but the procedure has several limitations. Birds must be handled to obtain cloacal temperatures, and the stress associated with handling causes a rapid increase in cloacal temperature of 0.2 to 0.3°C that can last up to 45 minutes after handling (Liljequist et al., 1994). This restricts the frequency with which cloacal temperatures can be obtained without handling stress impacting the measured temperature (Giloh et al., 2012). As a result, it is difficult to adequately characterize the temporal response of internal temperature to an event or treatment.

1.3 Non-Invasive Methods to Measure Enteric Health in Nursery Pigs and Heat Stress in Broiler Chickens

1.3.1 Non-Invasive Measures of Enteric Health in Nursery Pigs

As technologies continue to advance, new methods are developed to better study the natural world. This is also true in swine production, where advances in molecular techniques have enabled a much greater understanding of the mechanisms responsible for important issues such as disease (Tian et al., 2007) and meat quality (Wu et al., 2015). While advances have been made in the ability to measure gut health in weanling pigs, there is a still a need for the development of better ways to measure gut health in live animals (Celi et al., 2017) as well as refinement of existing technologies.

1.3.1.1 Fecal Scoring

Diarrhea (scours) is an attempt by the animal to eliminate luminal pathogenic bacteria or toxins and occurs in part due to increases in intestinal permeability (Tsai et al., 2017). Assessment of fecal consistency is therefore an indirect measure of gut health. Fecal scoring is typically conducted via a visual inspection of the pen floor on a daily basis over a specified period of interest and scoring of fecal consistency according to a predefined scale (e.g. Xiao et al., 2012). Color of feces may also be included in some scoring systems (e.g. Peace et al., 2011). In some cases, fecal scoring has been shown to yield similar results as intestinal morphology, with pigs that had lower incidences of diarrhea also having longer villi and shorter crypts indicating a healthier gut (Xiao et al., 2012). Fecal scoring has the advantage that measures may be conducted repeatedly over time on the same experimental unit to ascertain the temporal effect of a treatment without loss of

animals from the study. Fecal scoring has limitations in that it is subjective, requiring adherence to blinding of the individual doing the scoring (Peace et al., 2011). Fecal score is highly influenced by dietary components such as insoluble fiber (de Lange et al., 2010), and therefore is only suitable when comparisons are made within similar diets, and fecal score may not be reflective of feed intake (i.e. feces from a pig consuming a small amount of feed and losing weight may score better than feces from a pig that is eating more, and therefore having to adjust more rapidly to solid food, but is gaining weight).

1.3.1.2 Protein Abundance and Gene Expression

Molecular techniques that allow detection or quantification of the expression of genes or the presence of specific proteins in intestinal tissues has enabled substantial progress in understanding the etiology of poor gut health of nursery pigs as well as accessing the effectiveness of mitigation strategies. For example, the analysis of cytokine levels in intestinal mucosal tissue and immunohistochemistry analysis of intestinal tight junction proteins revealed that the beneficial effects of spray-dried porcine plasma when included in nursery diets may be mediated by improvements in tight junction function and a reduction in the inflammatory response (Peace et al., 2011). While a variety of methods have been used as technology has developed, protein content analysis via Western blotting (e.g. Huygelen et al., 2012), immunohistochemistry analysis for the abundance and tissue location of specific proteins (e.g. Moeser et al., 2007), and gene expression via the polymerase chain reaction (PCR; e.g. Roselli et al., 2007) are three molecular techniques that have seen extensive use in the study of gut health in nursery pigs in recent years. In brief, Western blot is a procedure where proteins are separated by size in a gel, transferred to a membrane, incubated with a series of antibodies to target a specific protein of interest, and the proteins

illuminated via a chromophore. The intensity of the signal provides a metric of the protein abundance (Mahmood and Yang, 2012). Immunohistochemistry also utilizes antibodies against specific proteins, but unlike the Western blot, allows the proteins to be identified in the context of the structure of a tissue. In brief, a preserved tissue is sliced thinly, incubated with a series of antibodies, labeled with fluorescent dyes, and visualized under a microscope at specific excitation wavelengths (Daneshtalab et al., 2010). The PCR procedure in general utilizes heat to separate the two strands of a DNA molecule (denaturation), binding of primers specific to the target sequence (annealing), and synthesis of new DNA strands containing the target sequence (elongation). By repeating this process many times (cycles), the target sequence is exponentially produced. Fluorescent dyes or probes allow quantification of the number of target sequences produced in a given number of cycles, and estimation of the original quantity of the target sequence in the tissue (Kubista et al., 2006). These techniques are powerful in that they enable the study of gut health from a variety of angles including absorptive capacity (e.g. abundance of intestinal phosphorus transporters via Western blot; Huber et al., 2015) barrier function (e.g. localization of intestinal tight junction proteins via immunohistochemistry; Peace et al., 2011), inflammatory status (e.g. gene expression of pro-inflammatory cytokines such as TNF-α via PCR; Pié et al., 2004). These techniques share the limitation that abundance of RNA or a protein does not always indicate functionality. For example, some tight junction proteins retract into the cell upon stimulation with pro-inflammatory cytokines leading to a loss of barrier functionality (Chiba et al., 2008) without changing the concentration of the tight junction protein in the tissue.

1.3.1.3 Endogenous Markers of Enteric Health

The use of biomarkers to measure gut health is limited, and the development of biomarkers of gut health has been identified as a pressing need (Celi et al., 2017). Validating biomarkers of gut health is not easy, for example, Verbeke et al. (2015) reviewed the many challenges and limitations of using microbial fermentation metabolites to measure the effect of prebiotics on gut health. However, several biomarkers have been investigated for use in weanling pigs. The level of circulating corticotrophin-releasing factor (CRF) in pigs has been correlated with measures of intestinal permeability after weaning (Moeser et al., 2007a), therefore, CRF levels in blood may be a potential biomarker of gut health. Intestinal fatty acid binding protein (IFAB) levels in plasma have received some interest as IFAB was shown to be rapidly released from the intestines into the blood following intestinal damage in pigs (Niewold et al., 2004). However, IFAB levels have been demonstrated to be a poor descriptor of gut health in pigs at weaning (Berkeveld et al., 2008; van der Meulen et al., 2010). Citrulline, a metabolite of metabolism of glutamine by the enterocyte, has been shown to correlate with small intestinal mass and surface area in humans (Jianfeng et al., 2005). In pigs plasma citrulline has been shown to decrease over time post-weaning (Berkeveld et al., 2008) similar to the well documented temporal post-weaning decrease in villi height (Hampson, 1986), and therefore may be a suitable biomarker for absorptive surface area in weanling pigs. Blood levels of lipopolysaccharide (LPS) are elevated in heat stress in pigs, likely due to decreased intestinal barrier function (Pearce et al., 2012). Plasma LPS in pigs has also mirrored permeability measures via Ussing chamber analysis (Kurundkar et al., 2010). Thus, plasma or serum LPS could be a potential biomarker for barrier function. Plasma carotenoid levels have been shown to be very responsive to intestinal damage from coccidiosis in chickens (Conway et al., 1993) but does not

appear to have been evaluated in weanling pigs. Other biomarkers in tissues, blood, and feces with potential for use in pigs are reviewed by Celi et al. (2018).

1.3.1.4 Exogenous Markers of Enteric Health

Non-metabolizable carbohydrates of various sizes are the primary exogenous markers of gut health. Molecules with larger size such as lactulose or cellobiose which are passively absorbed paracellularly are typically used to assess barrier function (i.e. paracellular permeability) of the intestines while smaller molecules such as mannitol or xylose which are passively absorbed transcellularly are typically used to assess absorptive surface area of the intestines (Wang et al., 2015). As used in human medicine (Sequeira et al., 2014a), a test of intestinal health typically includes an overnight fast followed by ingestion of an aqueous solution of a larger molecule (often lactulose) and a smaller molecule (often mannitol). Urine is then quantitatively collected for 4 to 6 hours in an attempt to primarily collect urine when the aqueous solution occupies the small intestine, concentrations of the marker molecules are measured in the urine, and total excretion of the marker molecules calculated from the urine volume collected. Greater excretion of the larger molecule would indicate a greater permeability of the intestine (i.e. poor barrier function) while lesser excretion of the smaller molecule would indicate an unhealthy intestine with lesser absorptive surface area. The ratio of excretion of the larger molecule to the excretion of the smaller molecule is believed to be the best measure of overall gut health as extraneous differences in excretion of the marker molecules are corrected for when taking the ratio (Mishra and Makharia, 2012).

The use of exogenously administered carbohydrates as a measure of gut health has seen limited use in weanling pigs (De Vos et al., 2012). Unfortunately, some of the studies that have

been conducted with pigs have relied on obtaining an aliquot of urine collected from the bladder of pigs after euthanasia, and have drawn conclusions from concentrations of lactulose and mannitol in the urine rather that total excretion (Bjornvad et al., 2008; Huygelen et al., 2012; Huygelen et al., 2014; De Vos et al., 2014). Total urine collection is required for reliability of results, as there is differential excretion of lactulose and mannitol over time (Sequeira et al., 2014b), as well as simple differences in hydration status among animals resulting in differences in urine concentration, can lead to misinterpretation of results.

Several studies have been conducted using total urine collection methods. In a piglet model of preterm human infants, lactulose and mannitol recovery from a 24 hour urine collection via bladder catheters was able to show differences in intestinal permeability between two feeding methods (Kansagra et al., 2003). The response to supplemental zinc post-weaning was measured by a urinary lactulose and mannitol test in pigs post-weaning (Zhang and Guo, 2009). Piglets were individually housed in metabolism crates, fasted overnight, and urine collected for 6 hours post-oral gavage of a solution of lactulose and mannitol. Zinc source impacted lactulose recovery and the ratio of lactulose to mannitol compared to the control diet indicating improved barrier function of pigs fed supplemental zinc (Zhang and Guo, 2009). In an experiment with similar methodology, pigs challenged with an enterotoxigenic Escherichia coli had higher lactulose to mannitol ratios compared to control pigs (Yang et al., 2014). Lactulose excretion in urine collected from male pigs via plastic pouches increased post-weaning; however, lactulose excretion was not correlated with bacterial translocation or permeability as measured via Ussing chambers (Wijtten et al., 2011). The impact of dietary lipids varying in oxidation damage was evaluated with weanling pigs fed diets containing thermally oxidized lipids for 4 weeks (Liu et al., 2014). Pigs were subsequently housed in metabolism crates and fed a meal containing lactulose and mannitol. Urine was

quantitatively collected for 6 hours. While no difference was detected in the lactulose to mannitol ratio among lipid sources or oxidation levels, diets containing supplemental lipids had lower lactulose to mannitol ratios compared to the control diet (Liu et al., 2014). Pigs housed in metabolism crates and fed high fiber diets supplemented with two carbohydrases post-weaning demonstrated reduced lactulose to mannitol ratios in urine collected over 12 hours from pigs fed one of the carbohydrases (Li et al., 2018).

While less common than urinary measurement, measurement of marker molecules in the blood can also be conducted. Mannitol levels over time were measured in plasma of pigs at weaning and compared to unweaned controls (Berkeveld et al., 2008). Mannitol levels were lower in the weaned pigs than the unweaned pigs, and for the weaned pigs reached a nadir at 4 d post-weaning similar to the nadir observed in villus height post-weaning (Hampson, 1986). Mannitol levels were also correlated with the severity of the growth check post-weaning (Berkeveld et al., 2008).

1.3.2 Non-Invasive Measures of Heat Stress in Broiler Chickens

1.3.2.1 Air Temperature

Ambient temperature is extensively used to estimate the physiological stress of broilers in research and in commercial production, as increases in ambient temperature will in general cause a reduction in the ability of broilers to dissipate heat. However, ambient temperature alone cannot accurately predict the physiological status of broilers, as other factors including relative humidity and ventilation rate (Liljequist et al., 1994; Giloh et al., 2012), and radiant heat exchange between chickens (Mitchell, 1985), all impact the ability of broilers to thermoregulate by altering the ability of broilers to dissipate body heat. Thermal imaging technology has clearly demonstrated the

limitations of relying on ambient temperature to predict bird comfort, with dramatic differences in surface temperature at similar ambient temperatures but with alterations in air flow (Czarick, 2007).

1.3.2.2 Behavioral Measures

Broilers display some behavioral indicators of heat stress that may be useful in characterizing the degree of heat stress experienced by the chickens. Broilers will increase panting rates in attempts to increase evaporative cooling from the respiratory tract (Menuam and Richards, 1975). Heat stressed chickens may also display ruffled feathers and wings held away from the body in attempts to more efficiently dissipate surface heat to the environment (Al-Ramamneh et al., 2016). In extreme cases, broilers will display heat prostration (Borges et al., 2004), where chickens are so exhausted by the attempts to cope with an extreme heat load that they prostrate on the floor with neck and wings extended. Death typically rapidly follows heat prostration (Reece et al., 1972).

1.3.2.3 Surface Temperature

Advances in thermal imaging technology have enabled non-invasive and high sensitivity measurement of surface temperatures of birds (McCafferty, 2013). In poultry research, this technology has seen increasing use in the last two decades. Broiler chicks reared from hatch to 7 d at 20, 25, or 35°C showed differences in surface temperature among ambient temperatures, with differences most pronounced in un-feathered regions such as the shank (Malheiros et al., 2000) due to the lack of insulation via the plumage. Detailed measures of surface temperature at 26 locations on the body of broiler chickens from hatch to market weight demonstrated that the highest and most consistent temperatures were on the sparsely feathered regions of the face of the

chicken, with facial surface temperature only decreasing 0.7°C from 3 to 37 d post-hatch compared to nearly a 10°C decrease in wing temperature over the same period (Cangar et al., 2008), again likely due to changes in insulation due to the growth of feathers over time. Indeed, facial temperature may be the best surface temperature estimate of core body temperature, as a strong correlation was demonstrated between cloacal temperature and facial surface temperature of male broilers ranging from 8 to 36 d post-hatch (Giloh et al., 2012). Thermal imaging may also be a useful tool for assessment of stress in chickens. Handling stress in laying hens caused a rapid 2°C decrease in comb temperature and more gradual elevations in eye and head temperatures over 20 minutes following the handling event (Edgar et al., 2013). While much of the aforementioned thermal imaging was conducted in research settings, application of thermal imaging in commercial poultry production also shows considerable promise. Measurement of flock surface temperatures, assessment of brooding design, and many other applications of thermal imaging in commercial poultry production have been discussed by Czarick (2007). In summary, the non-invasive nature of thermal imaging and the ease of obtaining measurements makes thermal imaging a versatile tool for both research and production settings (Giloh et al., 2012).

1.3.2.4 Implanted Thermal Sensors

Technological advances have enabled alternate methods to measure the internal temperature of chickens. These methods have overcome some of the limitations of manual measurement of cloacal temperatures in research settings. A limited number of automated cloacal measurement systems have been developed. A miniature radio transmitter was used to measure and transmit cloacal temperature of broilers subjected to hypothermia (Deaton and Reece, 1968). A harness and wired temperature probe system was developed to measure cloacal temperatures of individually

housed market weight broilers (Liljequist et al., 1994). Greater research emphasis has been placed on implanted data transmitters. The increase in internal temperature as a result of feed intake was measured in male broilers via surgically implanted radio transmitters (Wilson et al., 1989). A similar system, but with smaller transmitters, was used to obtain the circadian rhythm of internal temperature from a limited number of broiler chickens (Lacey et al., 2000). Implanted temperature transmitters were used to investigate the accuracy of manual measurement of cloacal temperature, changes in internal temperature with changing ambient temperature, and the impacts of thermal conditioning on later heat stress events (De Basilio et al., 2003). Implanted radio transmitters were used to document the circadian rhythm of internal temperature of restricted-fed and ad libitum-fed broilers (Savory et al., 2006). Implanted thermal sensors that logged body temperature to internal memory were used to measure the temporal febrile response to an LPS injection in individually housed laying hens (Lieboldt et al., 2016).

1.4 Research Gap

1.4.1 Enteric Health in Nursery Pigs

The use of exogenous markers of intestinal health such as lactulose and mannitol show promise for the measurement of gut health in nursery pigs (De Vos et al., 2012). However, in the limited number of studies that have used lactulose and mannitol to measure gut health the methodology has varied widely and has not always reflected the physiology of the weaned pig. Therefore, in order for the use of lactulose and mannitol as a measure of gut health to be comparable from one paper to the next and to ensure accurate results, standardization in methodology is required. The two key parameters that require investigation are the impacts of

varying the dose of lactulose and mannitol given, and the optimal time period following dosing to collect samples for measurement of lactulose and mannitol concentrations.

1.4.2 Heat Stress in Broiler Chickens

Measurement of physiological stress via thermal imaging by research scientists during heat stress research or producers during heat waves shows considerable promise (Czarick, 2007). However, broilers undergoing heat stress are primarily attempting to maintain a stable internal temperature, and thus internal temperature remains the gold standard of physiological stress during heat stress events (Dawson and Whittow, 2000). However, evidence that the cloacal temperature of broilers is correlated with facial surface temperature (Giloh et al., 2012) gives rise to the potential for a prediction equation to be developed that could enable estimation of internal temperature from facial surface temperature.

Where direct measurement of internal body temperature is desired in a research setting, automated systems of measuring cloacal or internal temperatures are desirable from data accuracy and labor standpoints (Liljequist et al., 1994). While the alternatives that have been developed are certainly a step in the right direction, there are limitations of the methods used. The majority of these systems require specialized equipment with technical requirements that preclude use in birds housed in industry relevant conditions. The use of self-contained data loggers by Lieboldt et al. (2016) overcomes many of these limitations, but due to surgical implantation, data loggers may preclude group housing due to risks of cannibalism (Kaesberg et al., 2018) and may require wire-floored housing to prevent incision site infections. This is particularly problematic in broiler chickens as industry relevant conditions include group housing in bedded conditions, and wire flooring is a known welfare concern for broilers (Wideman et al., 2012). Furthermore, surgery in

avians presents some unique challenges, primarily due to sensitivity of avians to anesthesia (Speer, 2016), which may discourage use of implanted data loggers. Therefore, there is a need for a simple surgical procedure for data logger implantation as well as investigation into the suitability of data logger use in group housed broiler chickens.

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CHAPTER 2. MEASUREMENT OF INTESTINAL HEALTH IN PIGS VIA A NON-INVASIVE ASSAY: METHOD DEVELOPMENT

2.1 Abstract

Two experiments were conducted to evaluate an aspirin-induced model of intestinal permeability in weanling pigs and to establish the temporal urinary excretion pattern of two markers of intestinal health, lactulose and mannitol, in nursery pigs. In Exp 2.1, 16 pigs (8 barrows and 8 gilts; 8.8 ± 0.39 kg BW; 14 d post-weaning) were given aspirin at 0, 5, 10, or 15 mg/kg BW at 24 and 1 h before receiving an oral dose of lactulose (0.28 g/kg BW) and mannitol (0.11 g/kg BW) in water. Blood was collected via jugular puncture at 0.5 and 1 h post-oral dose and serum was obtained. Serum was analyzed via a colorimetric kit and via HPLC. In Exp 2.2, 12 barrows (11.5 ± 0.58 kg BW; 18 d post-weaning) were given aspirin at 0 (Trt 1), 15 (Trt 2) or 30 (Trt 3) mg/kg BW at 24 and 1 h before receiving an oral dose of lactulose (0.5 g/kg BW) and mannitol (0.05 g/kg BW) in water. Urine was quantitatively collected at set intervals via metabolism cages for 48 h post-oral dose. Urochrome levels in urine were used to approximately equalize urine concentrations before analysis via HPLC. The experimental unit was the individual pig in both experiments. The colorimetric kit performance in Exp 2.1 was analyzed via Proc Reg and Proc Glimmix of SAS. In Exp 2.2, Proc Glimmix was used to analyze lactulose and mannitol excretion with urine collection time as a repeated measure where appropriate. In Exp 2.1, the performance of the colorimetric kit was improved by centrifugal-filtration of serum prior to analysis, but still lacked suitable accuracy. Analysis of serum via HPLC demonstrated that levels of lactulose and mannitol in serum were below the detection limit of the system (< 0.0135 mg/mL). In Exp 2.2, urinary lactulose excretion per hour was greater ($P < 0.01$) overall for Trt 2 (15 mg/kg BW aspirin; 482 ± 65.2 mg/h) than for Trt 3 (30 mg/kg BW aspirin; 186 ± 63.2 mg/h) with Trt 1 (0 mg/kg BW

aspirin; 289 ± 62.1 mg/h) being intermediate. Total lactulose excretion over the 48 h period was greater ($P < 0.01$) for 15 mg/kg BW aspirin (9220 ± 844.5 mg) than for 0 mg/kg BW aspirin (4854 ± 703.4 mg) and 30 mg/kg BW aspirin (3139 ± 703.4 mg), but neither total mannitol excretion ($P = 0.17$) nor the ratio of lactulose to mannitol ($P = 0.72$) were impacted by aspirin treatment. Time post-oral dose affected ($P < 0.01$) lactulose and mannitol excretion with peak lactulose (1373 ± 110.9 mg/h) and mannitol (1954 ± 474.1 mg/h) excretion occurring 4 h post-oral dose, with the majority of both molecules (86.2% and 88.9% of total excretion for lactulose and mannitol, respectively) being excreted by 8 h post-oral dose. Therefore, urine collection times greater than 8 h may not be required in lactulose and mannitol tests of intestinal health in nursery pigs, and aspirin at 15 mg/kg BW delivered 1 and 24 h before oral dosing with lactulose and mannitol may be an effective stimulus to induce increased intestinal permeability in nursery pigs.

Keywords: lactulose, mannitol, intestinal health, aspirin

2.2 Introduction

The typical methods used to obtain measures of intestinal health in nursery pigs include histological analysis of portions of the small intestine (Xiao et al., 2012), gene expression or protein concentrations throughout the small intestine (Huygelen et al., 2012), Ussing chamber analysis (Peace et al., 2011), or other methods on dissected or segregated segments of the small intestine (Wang et al., 2015). These methods typically require the euthanasia of pigs being sampled thereby removing sampled pigs from the experiment. Thus, additional numbers of pigs are used in studies of intestinal health to make up for pigs that are removed from the study via euthanasia for tissue collection at each time point when intestinal health is of interest. This is undesirable from an efficiency as well as an animal research use standpoint (Boo, 2005).

An improvement over these methods could be the use of a lactulose and mannitol oral dose test in pigs (De Vos et al., 2012). The lactulose and mannitol test is routinely used in human medicine to directly and quantitatively measure intestinal health (Sequeira et al., 2014a). In brief, the test consists of ingestion of a solution of two non-metabolizable sugars (e.g. lactulose and mannitol) and measurement of the appearance of the sugars in urine. Greater excretion of lactulose compared to mannitol indicates a reduction in intestinal health (Mishra and Makharia, 2012). This is a non-invasive and non-harmful test that potentially may be repeated on the same animal over time to measure temporal changes in intestinal health while at the same time collecting growth performance or other key data. Thus, use of a non-invasive measure of intestinal health would allow reduced animal numbers euthanized in research studies as well as greater characterization of the temporal response of intestinal health in pigs (Berkeveld et al., 2008).

The lactulose and mannitol test has seen some use in nursery pigs in recent years (e.g. Kansagra et al., 2003; Zhang and Guo, 2009), but the methods used vary substantially and appear to be modeled after the procedures used in humans rather than adapted for the physiology of nursery pigs. In particular, the temporal patterns of excretion for lactulose and mannitol for pigs are not known, therefore, the correct timing of sample collection is unknown (Sequeira et al., 2014b). As a result, development of standardized methodology specific for nursery pigs is important to ensure the accuracy and reproducibility of the test. A step towards development of these methods would be availability of a reproducible model of poor gut health in pigs. Aspirin and other non-steroidal anti-inflammatory drugs have been reported to reliably induce transitory permeability in the small intestines of humans (Lambert et al., 2012; Sequeira et al., 2014b). Therefore, our objectives were to create an aspirin-induced model of intestinal permeability in weanling pigs and to determine the temporal pattern of excretion of lactulose and mannitol in urine.

We hypothesized that aspirin at all levels greater than zero would lead to increased permeability of the small intestine and therefore greater absorption of lactulose from the small intestine. We evaluated this hypothesis in two experiments, the first (Exp 2.1) with serum levels of lactulose and mannitol as the response, and the second (Exp 2.2) with urinary levels of lactulose and mannitol as the response criteria.

2.3 Materials and Methods

2.3.1 Animals and Housing

All procedures involving live animals were approved by the Purdue University Animal Care and Use Committee (protocols #1707001599 and #1712001662) and animal husbandry practices were in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

In Exp 2.1, 24 mixed-sex pigs (12 barrows and 12 gilts) with an initial BW of 5.6 ± 0.10 kg were weaned at 18.2 ± 0.29 d of age and placed 6 pigs per pen into 4 nursery pens. Each pen measured 1.22 × 1.37 m, had fully slatted plastic floors, and was fitted with a 5-hole gravity-flow feeder and a nipple cup waterer in a mechanically ventilated room at the Purdue Animal Science and Education Center Swine Research Farm (West Lafayette, IN). Pigs were given *ad libitum* access to a medicated (carbadox, 55 ppm) commercially produced nursery phase I pelleted feed (23.5% CP, 1.60% SID Lys) for the first week post-weaning. For the second week post-weaning pigs were given *ad libitum* access to a medicated (carbadox, 55 ppm) commercially produced nursery phase II pelleted diet (23.3% CP, 1.54% SID Lys).

In Exp 2.2, twelve barrows were selected by BW (average BW = 11.5 ± 0.58 kg per pig) at 17 d post-weaning from a group of pigs that had been weaned, housed, and fed as described in

Exp 2.1. In addition, pigs in Exp 2.2 were maintained on nursery phase 2 diet from 14 to 21 d post-weaning to avoid a diet change prior to or during the experimental period that could potentially impact intestinal permeability or absorptive surface area. From 19 to 21 d post-weaning, pigs were individually housed in stainless steel metabolism cages to facilitate quantitative urine collection. Metabolism cages consisted of a main collection and living space of 72.4 cm wide x 86.4 cm long with a 58.4 cm wide by 38.1 cm long feeding area mounted on the front of the crate where a water nipple and feed cup were located. Pigs were provided with one piece of manipulatable plastic pipe as enrichment and maintained visual, olfactory, and auditory contact with the other pigs in the room.

2.3.2 Experimental Procedures

In Exp 2.1, pigs were individually weighed at d 7 and d 13 post-weaning, and 16 pigs (8 barrows and 8 gilts) were selected from the original 24 pigs to participate in the experiment on d 13 post-weaning (n = 4 pigs per treatment; average BW = 8.8 ± 0.39 kg per pig). The remaining 8 pigs had low ADG and were excluded from the experiment to eliminate pigs that had not transitioned well onto solid food. The eight excluded pigs were removed from the 4 nursery pens on d 13 post-weaning leaving 4 experimental pigs per pen. Fourteen d post-weaning at 1100 h pigs were orally given 0 (Trt A), 5 (Trt B), 10 (Trt C), or 15 (Trt D) mg/kg BW aspirin powder (Vedco Inc., St. Joseph, MO) contained in size 4 gelatin capsules (Empty Caps Company, Murrieta, CA) delivered with a pill gun (Bullseye Pill Gun, MAI Animal Health, Elmwood, WI). Pigs on Trt A (0 mg/kg BW of aspirin) each received a gelatin capsule containing powdered cellulose as a placebo. Pigs were fasted from 1800 h on d 14 post-weaning to 0600 h on d 15 post-weaning but retained access to water at all times. Feed was then returned to pigs for 1 h to allow pigs to consume a meal, and feeders were removed at 0700 h on d 15 post-weaning. Pigs were then given an

additional dose of aspirin at 1000 h as described for d 14 post-weaning and were individually weighed. Twenty-four h after the initial dose of aspirin (1100 h on d 15 post-weaning; time 0) pigs were orally dosed with an aqueous solution of lactulose (0.28 g/kg BW; Alfa Aesar, Thermo Fisher Scientific Chemicals, Inc., Ward Hill, MA) and D-mannitol (0.11 g/kg BW; Acros Organics, Fair Lawn, NJ) at 1.59 mL of solution per kg BW. The dose of lactulose and mannitol used was the average of the range of doses given to pigs in the published literature. The solution was delivered with a 4-inch ball-tipped livestock drenching needle (Agri-Pro Enterprises of Iowa Inc., Iowa Falls, IA) attached to a syringe.

In Exp 2.2, pigs were individually weighed on d 17 post-weaning, and 12 barrows were allotted to the experiment (average BW = 11.5 ± 0.58 kg per pig). Eighteen d post-weaning at 0730 h pigs were orally given 0 (Trt 1), 15 (Trt 2), or 30 (Trt 3) mg/kg BW aspirin powder (Vedco Inc., St. Joseph, MO) contained in size 2 gelatin capsules (Herb Affair, Palatine, IL) delivered with a pill gun (Bullseye Pill Gun, MAI Animal Health, Elmwood, WI). Pigs on Trt 1 (0 mg/kg BW of aspirin) each received a gelatin capsule containing powdered cellulose as a placebo. On d 19 post-weaning lights were turned on at 0300 h for 1 h to allow pigs to consume a meal, and then pigs were placed into metabolism cages without access to feed but with ad libitum access to water. Pigs were then given a second dose of aspirin at 0700 h as described for d 18 post-weaning and were individually weighed. Twenty-four h after the initial dose of aspirin (0800 h on d 19 post-weaning; time 0) pigs were orally dosed with an aqueous solution of lactulose (0.5 g/kg BW; Alfa Aesar, Thermo Fisher Scientific Chemicals, Inc., Ward Hill, MA) and D-mannitol (0.05 g/kg BW; Acros Organics, Fair Lawn, NJ) at 1.59 mL of solution per kg BW. The dose of lactulose and mannitol was chosen to match that of a previously described dose used for young pigs (Kansagra et al., 2003). The solution was delivered with a 4-inch ball-tipped livestock drenching needle (Agri-Pro

Enterprises of Iowa Inc., Iowa Falls, IA) attached to a syringe. Delivery of the oral dose to a pig took 5.2 min on average. Feed was returned to pigs at 4 h post-oral dose. Pigs were individually weighed at the termination of the experiment at 48 h post-oral dose.

2.3.3 Data Collection

In Exp 2.1 blood was collected into tubes (BD Vacutainer Serum Separation Tubes, 5.0 mL, Becton, Dickinson and Company, Franklin Lakes, NJ) from each pig via jugular venipuncture at 30 min and 1 h post-oral dose. After clotting, blood tubes were stored at 4°C until serum was separated by centrifugation at 1500 × g at 4°C. Serum was aliquoted and stored at -80°C.

In Exp 2.2 water intake and urine output were quantified from -4 to -0.5 h pre-oral dose, and from 0 to 2, 2 to 4, 4 to 6, 6 to 8, 8 to 12, 12 to 24, 24 to 36, and 36 to 48 h post-oral dose. Water intake for each time period was quantified by filling the water nipple reservoir to a set volume at the start of a period and measuring the volume of water required to bring the reservoir back to the set volume at the end of a period. To correct water intake for water spilled by pigs during drinking, a pre-weighed bucket was placed under the slatted floor of the metabolism cage under the water nipple and the volume of spilled water for each period was determined gravimetrically. Urine output was quantified gravimetrically via two sets of pre-weighed buckets. After each urine collection period an aliquot of urine (approximately 5 mL) was obtained and frozen at -20° C. Buckets were washed with a 10% bleach solution between each collection period to minimize bacteria growth.

2.3.4 Laboratory Analysis

2.3.4.1 Colorimetric Analysis of Lactulose and Mannitol in Serum in Exp 2.1

A commercially available colorimetric kit that was primarily designed to analyze lactulose and mannitol concentrations in urine (EnzyChrom Intestinal Permeability Assay Kit (EIPM-100), BioAssay Systems, Hayward, CA) was tested in three laboratory bench trials (Trial 1, Trial 2, and Trial 3) for suitability with serum according to the manufacturer's instructions for the analysis in urine. In Trial 1, pooled serum from pigs that had not received an oral dose of lactulose and mannitol was spiked with concentrated lactulose or mannitol by following the manufacturer's instructions for preparation of dilute standards but substituting serum for water in all dilution steps. This created a set of spiked serum samples with identical concentrations of lactulose or mannitol as the standards used to construct the standard curve (four concentrations). The spiked serum samples were then analyzed in tandem with standards for lactulose and mannitol using kit 1 and excess kit reagents were frozen at -20°C. In Trial 2, a new set of spiked serum was prepared as in Trial 1 and the remaining reagents from kit 1 were thawed at room temperature. The new spiked serum samples were then analyzed for lactulose in tandem with standards. In Trial 3, a second kit was used to evaluate pooled serum from pigs that had not received an oral dose of lactulose and mannitol. Serum received one of four treatments: Trt 1 was raw spiked serum as in Exp 1 and 2, Trt 2 was serum that was spiked after filtering through a spin filter designed to retain all molecules with a molecular weight greater than 3000 daltons (Amicon Ultra-0.5 Centrifugal Filter Device, Millipore Corporation, Billerica, MA) via centrifugation at 16,000 × g for 30 min at room temperature, Trt 3 was serum that was spiked before filtering through a spin filter, and Trt 4 was spiked serum that was heated at 56°C for 30 min in a water bath. The dilution factor for Trt 3 was determined by weighing the spin filter insert before and after centrifugation. The serum in all 4

treatments was spiked with lactulose or mannitol as in Exp 1 by substituting raw untreated serum (Trt 1, 3, and 4) or filtered serum (Trt 2) for water in all dilution steps. The same batches of standards and serum from the 4 treatments were then each assayed by 2 technicians working simultaneously using the second kit.

2.3.4.2 HPLC Analysis of Lactulose and Mannitol in Serum in Exp 2.1

A HPLC system comprised of an eluent degasser (Series 1050, Hewlett-Packard, Palo Alto, CA), an autoloader (SIL-9A, Shimadzu, Columbia, MD), a single-piston pump (LC-6A, Shimadzu, Columbia, MD), a column oven (CTO-6A, Shimadzu, Columbia, MD), a calcium-form resin-based ionic column (Aminex HPX-87C, 300 mm x 7.8 mm, Bio-Rad Laboratories, Hercules, CA) protected by a guard column (Micro-Guard Cation H Form, Bio-Rad Laboratories, Hercules, CA), and a refractive index detector (HP 1047A, Hewlett-Packard, Palo Alto, CA) were used for serum analysis. Ultrapure water was used as the eluent, eluent flow rate was 0.6 mL/min, column temperature was maintained at 85°C, injection volume was 20 µL, and the refractive index detector temperature was held at 40°C. The HPLC system and operating conditions were modeled after those of Catassi et al. (1991).

Stock solutions of lactulose (Alfa Aesar, Thermo Fisher Scientific Chemicals, Inc., Ward Hill, MA), D-mannitol (Acros Organics, Fair Lawn, NJ), and glucose (EMD Millipore Corporation, Billerica, MA) were prepared by dissolution of dry chemicals with ultrapure water. Standards ranging in concentration from 0.009 to 10 mg/mL were then created by dilution of stock solutions with ultrapure water. Serum was cleaned by spin filtration (Amicon Ultra-0.5 Centrifugal Filter Device, Millipore Corporation, Billerica, MA) via centrifugation of the serum through the filter membrane at 16,000 × g for 30 min at room temperature before injection.

2.3.4.3 HPLC Analysis of Lactulose and Mannitol in Urine in Exp 2.2

The same HPLC system described for Exp 2.1 was used to analyze lactulose and mannitol levels in urine with some modifications. The column was protected by a de-ashing guard column (Micro-Guard De-Ashing Cartridges, Bio-Rad Laboratories, Hercules, CA). Ultrapure water was used as the eluent, eluent flow rate was 0.6 mL/min, column temperature was maintained at 65° C, injection volume was 20 µL, and the refractive index detector temperature was held at 50°C. Stock solutions of lactulose (Alfa Aesar, Thermo Fisher Scientific Chemicals, Inc., Ward Hill, MA) and D-mannitol (Acros Organics, Fair Lawn, NJ) were prepared by dissolution of dry chemicals with ultrapure water. Standards ranging in concentration from 0.009 to 1.0 mg/mL were then created by dilution of stock solutions with ultrapure water. The area under the curve (AUC) for the standards analyzed in duplicates was linear through the concentrations evaluated. The standard curve generated for mannitol was $y = 7.8369x – 0.0426$ with an $R^2 = 0.999$, and the standard curve for lactulose was $y = 7.7351x – 0.0136$ with an $R^2 = 0.999$ where y is the AUC and x is the concentration of mannitol or lactulose in mg/mL.

The HPLC method was refined by developing a method to account for the observed variation in urine concentration over time. To do so, the urine concentrations were approximately equalized by using urochrome levels as an indicator of urine concentration. A spectral scan of representative urine samples yielded an optimal wavelength of 370 nm. The equation $C_1 \times V_1 = C_2 \times V_2$ was then used to determine the dilution required to equalize urine concentrations, where C_1 is the initial absorbance of a urine sample at 370 nm, V_1 is the initial volume of urine, C_2 is the desired final absorbance of the diluted urine sample, and V_2 is the final volume of diluted urine. The value of C_2 used was 0.19 absorbance units, as that concentration was experimentally determined to bring the concentration of analytes in pig urine within the optimal range of the HPLC

detector. The diluted urine was filtered through a 0.2 µm PTFE syringe filter (17 mm disk, Thermo Scientific, Waltham, MA) to remove particulates prior to injection. Urine collected shortly before the oral dose of lactulose and mannitol was also analyzed via HPLC to generate pre-oral dose chromatograms for each pig.

An Excel-based (Microsoft, Redmond, WA) integration tool was developed to enable subtraction of matrix peaks from the post-oral dose chromatograms to allow accurate and rapid integration of the lactulose and mannitol peaks. The program optimized the fit of the pre-oral dose urine chromatogram to the post-oral dose urine chromatogram for a given sample to allow the matrix peaks to be subtracted. This was done by programming the excel add-in "solver" to simultaneously alter the pre-oral dose chromatogram line via four separate parameters with the objective of minimizing the sum of squares between the adjusted pre-oral dose chromatogram and the original post-oral dose chromatogram over user-controlled sections of the chromatogram (excluding the lactulose and mannitol peak sections). The four-part parameter fit adjusts for small differences in urine concentration (α), linear changes in detector signal in $\pm$ y (zero adjustment; β) and $\pm$ x (time; δ) directions, and non-linear detector drift over time (γ) via the excel equation 'adjusted pre-oral dose line' = (OFFSET(signal1,δ,0,1,1)+β+γ*time1)*α where signal1 is the detector output for the original pre-oral dose urine sample at time1. The ratio of the sums of squares for the adjusted baseline and the number of time points used to calculate the sums of squares was used as a goodness of fit parameter. After optimizing the fit, the matrix peaks were then automatically subtracted, giving rise to a new post-oral dose chromatogram free of matrix peaks. Integration was then automatically completed on the adjusted post-oral dose chromatogram via a set of formulas that identify the lactulose and mannitol peaks, determines peak height, calculates peak start and stop time from a predetermined relationship between peak height and peak base

width, calculates the true baseline between the signal at the start and signal at the stop of the peak base, and sums the AUC down to the true baseline for each peak. Original and adjusted chromatograms as well as determined true baselines and final integrated peaks are then displayed graphically to allow a visual check of correct peak integration.

2.3.5 Calculations and Statistical Analysis

In Exp 2.2 absolute urinary excretion of mannitol or lactulose in mg was calculated as the concentration of analyzed mannitol or lactulose at a given time point multiplied by the urine output at a given time point. Excretion of mannitol or lactulose corrected for urine void interval was calculated by multiplying the concentration of mannitol or lactulose in urine at a given time point by urine output at a given time point, and then dividing by the number of hours since the last urine collection period where urine was actually obtained. This urine void interval correction was used to account for urine collection periods where no urine was voided and was applied to 4 pigs at 2 h, 2 pigs at 4 h, and 2 pigs at 6 h post-oral dose.

In both experiments, pigs were allotted to aspirin treatments in a randomized complete block design with initial BW as the blocking factor. The individual pig was considered the experimental unit. For Exp 2.1, Proc Reg of SAS 9.4 (SAS Institute, Carry, NC) was used to conduct regression analysis of absorbance data from the colorimetric lactulose and mannitol kits (expected concentration as the independent variable and with absorbance of standards or spiked serum as the dependent variable). Regression line slopes and intercepts for the colorimetric lactulose and mannitol kit data were compared using Proc Reg via general linear tests (test option) evaluating the null hypotheses that the slope of line1 was equal to the slope of line2, and that the intercept of line1 was equal to the intercept of line2 (Freund and Littell, 2000). Proc Glimmix of SAS 9.4 (SAS Institute, Carry, NC) was used to analyze lactulose recovery in bench trial 3, with serum treatment,

technician, and their interaction as fixed effects, and means separated with a Tukey's adjustment where appropriate. In Exp 2.2, data were analyzed via Proc Glimmix with aspirin treatment as a fixed effect and block as a random effect. Data collected over time were analyzed with repeated measures with the time unit as a fixed effect using the optimal covariance structure for each response variable as determined by goodness of fit criteria (Littell et al., 1998). A Kenward-Roger degrees of freedom correction was applied to all repeated measures analyses via the ddfm=kr option of the model statement (Kenward and Roger, 1997). Means were separated with a Tukey's adjustment where appropriate. Measures of uncertainty around means, slopes, and intercepts are presented as ± 1 SE unless otherwise stated. Significance was declared at $P < 0.05$, while a trend towards significance was considered at $0.05 \leq P < 0.10$.

2.4 Results

2.4.1 Experiment 2.1

Both the mannitol standards and mannitol spiked serum (n = 2 per mannitol concentration) in Trial 1 linearly increased in absorbance (y) as the concentration of mannitol (x) increased from 0 to 3 mM (0 to 0.547 mg/mL; Figure 2.1). The mannitol standards yielded the regression equation $y = 0.01 \pm 0.039 + 0.49 \pm 0.021x$ with an $R^2 = 0.99$, while the mannitol spiked serum had a regression equation of $y = -0.01 \pm 0.066 + 0.56 \pm 0.037x$ with an $R^2 = 0.97$. There was no difference between the slopes ($P = 0.13$) and intercepts ($P = 0.82$) of the regression lines for the standards and the spiked serum.

The lactulose standards and lactulose spiked serum (n = 3 per lactulose concentration) in Trial 1 also linearly increased in absorbance (y) as the concentration of lactulose (x) increased from 0 to 0.3 nM (0 to 0.103 mg/mL; Figure 2.2). The regression equation for the lactulose

standards in Trial 1 was y = 0.003 ± 0.0044 + 1.35 ± 0.024x with R^2 = 0.99 while the regression equation for the lactulose spiked serum in Trial 1 was y = -0.02 ± 0.009 + 1.09 ± 0.050x with R^2 = 0.99. The regression line for the spiked serum had reduced slope ($P < 0.01$; 19.26%) and intercept ($P = 0.01$, 0.02 units) as compared to the standard regression line.

A new batch of serum spiked with lactulose was prepared and used in Trial 2. The lactulose standards in Trial 2 (Figure 2.2) yielded a regression equation of y = 0.003 ± 0.0062 + 1.10 ± 0.034x with R^2 = 0.99 while the regression equation for the new lactulose spiked serum in Trial 2 was y = -0.02 ± 0.009 + 0.48 ± 0.049x with R^2 = 0.94. The regression line for the new lactulose spiked serum had reduced slope ($P < 0.01$; 56.36%) and intercept ($P = 0.02$; 0.02 units) as compared to the lactulose standard regression line from Trial 2. In addition, the lactulose standard line from Trial 2 had a reduction ($P < 0.01$; 18.52%) in slope but no change in intercept ($P = 0.45$) as compared to the standard line for lactulose from Trial 1. The regression line for the new lactulose spiked serum in Trial 2 had a similar intercept ($P = 0.71$) but a reduced slope ($P < 0.01$; 55%) compared to the regression line for the lactulose spiked serum from Trial 1.

The impact of four serum treatments on mannitol analysis via the colorimetric kit was evaluated in Trial 3 using standards (n = 3 per mannitol concentration) and treated serum (n = 2 per mannitol concentration; Figure 2.3). For simplicity, only the results from technician 1 are presented. The regression equation for the mannitol standards was y = -0.01 ± 0.015 + 0.54 ± 0.007x with R^2 = 0.99. The regression equation for serum Trt 1 was y = 0.10 ± 0.018 + 0.59 ± 0.009x with R^2 = 0.99. The regression equation for serum Trt 2 was y = 0.04 ± 0.021 + 0.48 ± 0.010x with R^2 = 0.99. The regression equation for serum Trt 3 was y = -0.11 ± 0.037 + 0.48 ± 0.018x with R^2 = 0.99. The regression equation for serum Trt 4 was y = 0.07 ± 0.078 + 0.59 ± 0.037x with R^2 = 0.98. The regression lines for all serum treatments except serum Trt 4 ($P = 0.06$)

had different ($P < 0.01$) slopes than the slope of the standards regression line. Serum Trt 1 and Trt 4 both had greater slopes by 9.3% than the standard line, while serum Trt 2 and Trt 3 both had reduced slopes by 11.1% as compared to the standard line slope. Serum Trt 1 and 3 had different intercepts ($P < 0.01$) than the intercept of the standards line with Trt 1 being 0.11 units greater, and Trt 3 being 0.10 units lesser. The intercept for serum Trt 2 ($P = 0.06$) and Trt 4 ($P = 0.15$) were not different than the intercept of the standards line.

The impact of the four serum treatments on lactulose analysis via the colorimetric kit was evaluated in Trial 3 with standards and treated serum (n = 3 per lactulose concentration) analyzed by technician 1 (Figure 2.4). The regression equation for the standards was $y = 0.001 \pm 0.0063 + 1.04 \pm 0.027x$ with $R^2 = 0.99$. The regression equation for serum Trt 1 was $y = -0.03 \pm 0.003 + 0.65 \pm 0.011x$ and with $R^2 = 0.99$ with a reduction in slope ($P < 0.01$; 37.50%) and intercept ($P = 0.01$; 0.03 units) as compared to the standards line. The regression equation for serum Trt 2 was $y = 0.05 \pm 0.014 + 1.02 \pm 0.059x$ with $R^2 = 0.99$ with no difference in slope ($P = 0.70$) but an increase in intercept ($P < 0.01$; 0.051 units) as compared to the standards line. The regression equation for serum Trt 3 was $y = 0.03 \pm 0.012 + 0.98 \pm 0.051x$ with $R^2 = 0.99$ with no difference in slope ($P = 0.26$) but an increase in intercept ($P = 0.03$; 0.031 units) as compared to the standards line. The regression equation for serum Trt 4 was $y = -0.02 \pm 0.009 + 0.61 \pm 0.038x$ with $R^2 = 0.98$ with a reduction in slope ($P < 0.01$; 41.35%) but no difference in intercept ($P = 0.26$) as compared to the standards line.

The impact of the four serum treatments on lactulose analysis via the colorimetric kit was further evaluated in Trial 3 with standards and treated serum (n = 3 per lactulose concentration) analyzed by technician 2 (Figure 2.5). The regression equation for the standards was $y = -0.002 \pm 0.0028 + 1.14 \pm 0.012x$ with $R^2 = 0.99$. The regression equation for serum Trt 1 was $y = -0.08 \pm$

0.005 + 0.73 ± 0.024x with R^2 = 0.99 with a reduction in slope (P < 0.01; 35.96%) and intercept (P < 0.01; 0.08 units) as compared to the standards line. The regression equation for serum Trt 2 was y = -0.05 ± 0.012 + 1.26 ± 0.053x with R^2 = 0.99 with an increase in slope (P < 0.01; 10.53%) and a reduction in intercept (P < 0.01; 0.05 units) as compared to the standards line. The regression equation for serum Trt 3 was y = -0.05 ± 0.015 + 1.08 ± 0.067x with R^2 = 0.98 with no difference in slope (P = 0.42) but a reduced (P < 0.01; 0.05 units) intercept as compared to the standards line. The regression equation for serum Trt 4 was y = -0.07 ± 0.011 + 0.60 ± 0.049x with R^2 = 0.95 with a reduction in slope (P < 0.01; 47.37%) and intercept (P < 0.01; 0.07 units) as compared with the standards line.

To evaluate the overall accuracy of the colorimetric kit for lactulose analysis, the calculated lactulose concentration of the serum from the 4 serum Trt were expressed as a percentage of the expected concentration for each of the three levels of lactulose tested (0.9, 1.8, and 3.0 mM of lactulose; Figure 2.6). Technician 2 (48.6 ± 5.99% recovery) had a reduction in lactulose recovery (P < 0.01) as compared with technician 1 (93.8 ± 5.99% recovery) regardless of serum treatment. Serum treatment impacted lactulose recovery (P < 0.01) with serum Trt 2 (105.5 ± 8.47%) and Trt 3 (121.7 ± 8.47%) being greater than serum Trt 1 (29.2 ± 8.47%) and Trt 4 (28.6% ± 8.47), with no differences seen between Trt 1 and 4 or between Trt 2 and 3, regardless of technician. There was no interaction between technician and serum treatment (P = 0.32).

A HPLC system was assembled and tested for its suitability to analyze lactulose and mannitol in serum. Serum from a pig that had not received lactulose or mannitol was first evaluated via the HPLC. The serum showed a very simple matrix with few matrix peaks (data not shown). One matrix peak which eluted at 10.352 min after injection was determined to be glucose via the use of a glucose standard, the identity of the remaining matrix peaks was not determined. Lactulose

and mannitol standards were analyzed and demonstrated lactulose and mannitol peaks eluting at 9.537 and 16.950 min post-injection, respectively. For serum samples obtained from pigs dosed in Exp 2.1, glucose was detectable for all serum samples, but neither lactulose nor mannitol peaks were detectable regardless of aspirin treatment level or time at which the blood sample was collected (data not shown). A representative chromatogram showing lactulose and mannitol standards at 5 mg/mL overlaid on a representative chromatogram of serum from a pig receiving aspirin at 10 mg/kg BW is shown in Figure 2.7. Further analysis of standards by the HPLC system demonstrated that lactulose and mannitol peaks were reliably detectable and quantifiable down to 0.0135 mg/mL (39.44 µM for lactulose, 74.09 µM for mannitol).

2.4.2 Experiment 2.2

Aspirin treatment did not impact initial or final pig BW or feed intake ($P \geq 0.86$; Table 2.1). Water intake over time was not impacted by aspirin treatment ($P = 0.95$; Table 2.1) but a main effect of time on water intake was observed ($P < 0.01$; Figure 2.8) with water intake at 2 (0.0 ± 0.14 g/h) and 4 (8.5 ± 8.11 g/h) h post-oral dose being lesser than all other time points. Water intake was greatest at 8 h post-oral dose (173.1 ± 21.63 g/h) and was greater ($P < 0.05$) than water intake at all other time points except 6 (99.3 ± 20.75 g/h) and 36 (118.8 ± 7.67 g/h) h post-oral dose. Water intake at 24 (70.1 ± 6.85 g/h) and 48 (48.5 ± 6.68 g/h) h post-oral dose was lesser ($P < 0.05$) than water intake at 8 (173.1 ± 21.63 g/h) and 36 (118.8 ± 7.67 g/h) h post-oral dose and greater ($P < 0.05$) than water intake at 2 (0.0 ± 0.14 g/h) and 4 (8.5 ± 8.11 g/h) h post-oral dose, but was not different than water intake at 6 (99.3 ± 20.75 g/h) and 12 (96.2 ± 9.80 g/h) h post-oral dose. There was no interaction between aspirin treatment and time post-oral dose for water intake ($P = 0.79$). Similarly, there was no impact of aspirin treatment, time post-oral dose, or their interaction on urine output per hour ($P \geq 0.18$; Table 2.1).

Data for mannitol and lactulose excretion are primarily not shown for the 24 to 48 h urine collection periods due to very low levels present by 24 h post-oral dose. Aspirin treatment did not impact absolute mannitol recovery ($P = 0.35$; Figure 2.9), but there was an effect of time ($P = 0.01$) with mannitol excretion at 4 h post-oral dose (5455 ± 1418.9 mg) being greater than all other time points (average of 234 ± 189.5 mg) except at 6 h post-oral dose (1262 ± 742.2 mg). There was no interaction between aspirin treatment and time post-oral dose for absolute mannitol excretion ($P = 0.36$). Similarly, there was no main effect of aspirin treatment on absolute lactulose recovery ($P = 0.32$; Figure 2.10), however, there was an interaction between aspirin treatment and time post-oral dose ($P < 0.01$) with Trt 2 at 4 h post-oral dose (7463 ± 1096.2 mg) being numerically more than double the other treatments at 4 h and greater than all other combinations except for Trt 2 at 6 h (3180 ± 1108.5 mg) post-oral dose. In addition, there was a main effect of time post-oral dose on absolute lactulose excretion ($P < 0.01$) with lactulose excretion at 4 h post-oral dose (4335 ± 604.8 mg) being greater than all other time points (average of 204 ± 124.2 mg) except at 6 h post-oral dose (1520 ± 690.9 mg).

Aspirin treatment did not impact mannitol recovery corrected for urine void interval ($P = 0.37$; Figure 2.11), but there was an effect of time ($P < 0.01$) with mannitol excretion per hour at 4 h post-oral dose (1954 ± 474.1 mg/h) being greater than all other time points (average of 138 ± 63.9 mg/h). Aspirin treatment impacted lactulose recovery corrected for urine void interval ($P < 0.01$; Figure 2.12) with Trt 2 (482 ± 65.2 mg/h) being greater than Trt 3 (186 ± 63.2 mg/h) with Trt 1 (289 ± 62.1 mg/h) being intermediate. Time post-oral dose impacted lactulose excretion ($P < 0.01$) with 4 h post-oral dose (1373 ± 110.9 mg/h) being greater than all other time points, 6 h post-oral dose (726 ± 123.0) being greater than 2 (150 ± 115.0 mg/h), 12 (65 ± 92.2 mg/h), 24 (0.8 ± 97.23 mg/h), 36 (0.0 ± 92.24 mg/h), and 48 (0.0 ± 92.24 mg/h) h post-oral dose, with 8 h post-

oral dose (236 ± 102.0 mg/h) being intermediate between 4 and 6 h post-oral dose. In addition, aspirin treatment interacted with time ($P < 0.01$) with lactulose excretion for Trt 2 at 4 h post-oral dose (1861 ± 225.9 mg/h) being similar to Trt 2 at 6 h (1501 ± 184.5 mg/h), Trt 1 at 4 h (1497 ± 159.8 mg/h), and Trt 3 at 4 h (761 ± 184.5 mg/h) post-oral dose but was greater than all other treatment by time combinations.

There was no impact of aspirin treatment ($P = 0.17$; Figure 2.13) on total mannitol excretion over the 48 h urine collection period with the average mannitol excretion being 6563 ± 2236.7 mg. Total lactulose recovery over the 48 h urine collection period varied by aspirin treatment ($P < 0.01$; Figure 2.14) with Trt 2 (9220 ± 844.5 mg) having greater lactulose excretion than Trt 1 (4854 ± 703.4 mg) and Trt 3 (3139 ± 703.4 mg). However, the ratio of total lactulose excretion to total mannitol excretion was not impacted by aspirin treatment ($P = 0.72$; Figure 2.15) and averaged 1.06 ± 0.369.

The duration of urinary excretion of lactulose post-oral dose before lactulose levels returned to baseline did not differ ($P = 0.88$; Table 2.1) by aspirin treatment and averaged 19.7 ± 4.51 h. Similarly, the duration of mannitol excretion was not impacted ($P = 0.97$) by aspirin treatment and averaged 15.2 ± 6.01 h.

2.5 Discussion

2.5.1 Experiment 2.1

Urinary lactulose and mannitol concentrations have been measured in a variety of ways including gas chromatography (Farhadi et al., 2006), HPLC with a cation-exchange column and refractive index detection (Sequeira et al., 2012), anion-exchange HPLC with pulsed-amperometric detection (Bao et al., 1996), liquid chromatography-tandem mass spectrometry (Lee

et al., 2014), and colorimetric methods (Behrens et al., 1984; Blood et al., 1991). Perhaps the most commonly reported method has been HPLC analysis via a cation-exchange column and a refractive index detector, likely due to the relative simplicity, sensitivity, and economy of the analysis as well as availability of suitable HPLC equipment (Catassi et al., 1991). The HPLC system used to analyze the pig serum from Exp 2.1 was modeled after that of Catassi et al. (1991) and analysis of standards demonstrated a quantification limit of 0.0135 mg/mL, similar to the detection limit of 0.01 mg/mL reported by Catassi et al. (1991). That the levels of mannitol and lactulose in the pig serum would be undetectable is not particularly surprising in retrospect, as serum levels of mannitol in nursery pigs was reported to be approximately 0.002 mg/mL (Berkeveld et al., 2008). In humans given an oral dose of lactulose and mannitol, serum mannitol was 0.03 mg/mL or less while serum lactulose was 0.428 µg/mL or less (Cox et al., 1999). While limited data exist on the levels of lactulose and mannitol in weaning-age pigs, it would appear from the present data and that of Berkeveld et al. (2008) that the HPLC analysis method of Catassi et al. (1991), despite its advantages, may not be sensitive enough for routine detection of lactulose and mannitol in pig serum or plasma. More specialized techniques such as derivatization and gas-chromatography analysis as done by Berkeveld et al. (2008) or ion chromatography with pulsed amperometric detection coupled with mass spectroscopy such as used by Kotnik et al. (2011) are likely required. Unfortunately, due to the labor of derivatization and the cost of specialized equipment, this could indicate that the use of a serum or plasma lactulose and mannitol test is not of practical use for routine measurement of gut health in nursery piglets. Given the limited research on serum lactulose and mannitol in pigs there is also the possibility that alternations in blood sampling time post-oral dose, or changes in intestinal permeability in alternate experimental designs could allow greater levels of lactulose and mannitol to appear in the blood than was found in the present study.

The physiology of the kidney likely explains why mannitol and lactulose are so low in pig serum. Blood that enters the nephron of the kidney is filtered in the glomerulus with water and many small molecules (e.g. glucose, amino acids, electrolytes) are filtered out of the blood (Hall, 2016). The filtrate enters the Bowman's capsule, which is the start of the renal tubule. Only molecules with receptors in the nephron (e.g. glucose, amino acids) or the ability to move osmotically (e.g. Na^+, water) are reabsorbed from the filtrate back into the blood. Everything else remaining in the filtrate continues its journey through the renal tubule and eventually makes it way to the bladder as urine (Hall, 2016). Neither lactulose nor mannitol are metabolized by the body (Mishra and Makharia, 2012) so there should not be receptors in the renal tubule for their reabsorption. When ingested and absorbed into the blood stream the body will treat lactulose and mannitol as waste products to be eliminated as quickly as possible in the urine rather than as a valuable commodity (e.g. glucose) to be conserved. Given that 10-12% of cardiac output in piglets is directed to the kidneys (Gruskin et al., 1970), if could be theorized that lactulose and mannitol were filtered out of the blood of the pigs at a rate that prevented accumulation of the molecules in the blood to detectable levels.

The mannitol results from the bench top Trial 1 and Trial 3 demonstrated the ability of the colorimetric kit to detect and quantify mannitol in porcine serum spiked with mannitol at the same level as contained in the standards. The reduction in slope observed for lactulose in spiked serum in Trial 1 as compared to the standard line was hypothesized to be due to an error in preparation of the spiked serum, however, the results from Trial 2 with a new spiked serum did not support that hypothesis. In addition, Trial 2 showed further reductions in performance as compared to Trial 1, most likely due to freezing and thawing of the kit reagents. It was hypothesized that an inhibitory compound present in serum was interfering with the lactulose analysis.

The four serum treatments supported this hypothesis with filtered serum having slopes and intercepts much closer to the standard line than those of unfiltered serum. While the exact compound(s) and mechanism(s) remains unknown, the large size of the molecule(s) (>3000 daltons) removed by the spin filter and the propensity of proteins to interact (Szklarczyk et al., 2015) would indicate that the inhibitor factor(s) are likely endogenous serum proteins. The heat treatment was postulated to denature interfering protein(s) in serum, but interestingly, the heat treatment was not able to remove the inhibitory factor(s). Regardless, given the large size of the molecules and the affinity of proteins to interact (Szklarczyk et al., 2015) it appears that the most likely explanation is that endogenously present protein(s) in serum inhibited the function of one or both of the two key enzymes in the lactulose analysis kit.

The consistent reduction in the recovery for technician 2 as compared to technician 1 would seem to indicate a factor consistently impacting results. However, given that each technician independently analyzed their own set of standards in tandem with the treated serum to construct separate standard curves, in theory, a consistent error with the serum, such as a pipetting error, should have also consistently impacted the standard curve to the same degree, and thus should not have impacted the percent recovery. Therefore, the cause of the differences observed between technicians for lactulose recovery is currently unknown.

Despite the ability of filtering to remove the inhibition of the lactulose analysis, the accuracy of the measurement was relatively poor with an average recovery of 121.68% when averaged over technician. When the effect of technician is included, the accuracy further decreases. For Trt 3 for example, technician 1 had 156.00% recovery while technician 2 had 87.36% recovery. Therefore, it was concluded that the analysis of lactulose via the colorimetric kit in spiked serum

that had been filtered to remove all molecules greater than 3000 daltons is possible, but lacked the accuracy desired in a research setting.

2.5.2 Experiment 2.2

The lack of differences in BW and FI among aspirin treatments indicates that aspirin at the levels given was well tolerated by pigs as anticipated (Patterson et al., 2007). The rapid increase in water intake from 4 to 8 h post-oral dose was consistent with the anecdotal observation that pigs were sleeping for the majority of the first 2 to 4 h post-oral dose, potentially in response to the stress of being restrained for approximately 5 min to receive the oral dose. The lag in water intake immediately following the oral dose may explain why some pigs had no urine output at 2, 4, and 6 h post-oral dose. However, the lag in water intake following the oral dose was not reflected in a significant change in urine output over time, potentially due to the large variation observed in urine output.

Initial HPLC analysis of urine using similar run conditions as Catassi et al. (1991) demonstrated that a large number of matrix peaks eluted at a similar time as lactulose and a single matrix peak eluted at a similar time as mannitol. Alterations in eluent flow rate and column temperature within the limitations of the system demonstrated that 0.6 mL/min flow rate and a column temperature of 65° C yielded the best separation with mannitol clearly separated from the matrix, and lactulose partially separated. Co-elution of lactulose and matrix peaks prevented accurate integration. Therefore, a two-step procedure was developed to allow the matrix peaks to be subtracted from the post-oral dose urine chromatograms to allow accurate lactulose integration. The first step was to approximately equalize the pre-oral dose and post-oral dose urine concentrations so that the matrix peaks to subtract would be of similar magnitude in both chromatograms. To do so, the use of urochrome as a marker of urine concentration was

investigated. Urochrome is the primary pigment in urine and is produced at a constant rate as a waste product from red blood cell recycling in the body (Ridley, 2018). Urine color has been validated as an approximate measure of hydration status in humans where dehydration results in a reduction in urine volume, and thus an increase in the concentration of urochrome (Kavouras et al., 2016). It was determined that the use of the absorbance of urine at 370 nm was a good estimator of urine concentration and could be used to approximately equalize urine concentrations before HPLC analysis. This had the additional benefit of bringing the concentration of all samples into optimal range for the HPLC detector. The second step required before subtraction of the matrix peaks was to optimize the fit between the matrix peaks in the pre-oral dose and post-oral dose chromatograms for each urine sample. This was accomplished with a spreadsheet-based program that corrected for any remaining differences in urine concentration as well as random variation in the chromatograms arising from small variations in HPLC system performance from one injection to the next. Subtraction of the matrix peaks after optimization by the program was found to be a suitable method to allow accurate integration of the lactulose peak and the resulting methodology was used for the analysis of all urine samples.

Multiple studies have been conducted in humans on the temporal excretion pattern of mannitol in urine after ingestion of a solution of mannitol. In general, excretion of mannitol in human urine peaks around 2 h post-ingestion and is primarily complete around 6 h post-ingestion (Sequeira et al., 2012; Sequeira et al., 2014a). In contrast, very limited data exists on the temporal pattern of mannitol excretion in pigs. Six-day-old piglets gavaged with mannitol excreted 82% of the total mannitol recovered over a 24 h urine collection period within 12 h of receiving the dose (Kansagra et al., 2003). In the present study, regardless of aspirin treatment, the maximal mannitol output for both absolute and per hour mannitol output occurred at 4 h post-oral dose, and excretion

of mannitol was primarily complete by 8 h post-oral dose with low levels detected in urine until an average of 15.2 h post-oral dose. The delayed peak in excretion of mannitol observed with the pigs (4 h) than observed in humans (2 h) may be a result of differences in study protocol between pigs and humans. Human studies typically having shorter urine collection periods and require research subjects to completely empty their bladders between periods (Sequeira et al., 2012), while in the pig study urine voiding was voluntary and bladders may not necessarily have been empty at the end of each collection period. As a result, urine may not have been voided as quickly by pigs as by humans, delaying the appearance of the mannitol peak.

The temporal excretion of lactulose in urine has been of particular interest in human research due to the fact that lactulose that is not absorbed in the small intestine is easily absorbed in the large intestine, which could thereby alter the lactulose to mannitol ratio (Sequeira et al., 2014b). In general, lactulose excretion peaks for the first time 1 to 3 h post-ingestion indicative of small intestinal absorption, with a second peak evident 5 to 6 h post-ingestion indicative of large intestinal absorption (Sequeira et al., 2012; Sequeira et al., 2014b). In the present study, regardless of aspirin treatment, lactulose recovery peaked at 4 h post-oral dose for absolute lactulose excretion, and the majority of lactulose was excreted by 8 h post-oral dose. Lactulose recovery per hour showed similar results except that the peak in lactulose excretion was more broad with maximal excretion occurring between 4 and 6 h post-oral dose. Small amounts of lactulose were detected in urine until 19.7 h post-oral dose. The bimodal distribution of lactulose excretion observed in some human studies was not apparent, potentially due to the limited frequency of urine collection from the pigs in this study. Additional research with pigs where urine may be collected more frequently is required to determine if the bimodal distribution of lactulose excretion also occurs in pigs.

Taken together, the temporal pattern of mannitol and lactulose excretion from pigs are similar, indicating that urine may be collected from a portion of the urine collection period used here without dramatic alternations in the lactulose to mannitol ratio. In addition, the vast majority of mannitol and lactulose were excreted by 8 h post-oral dose. Therefore, there does not appear to be a need to extend urine collection times to 12 or more hours (e.g. Kansagra et al., 2003) when measuring lactulose and mannitol in the urine of nursery pigs. Given that both mannitol and lactulose returned to baseline levels in less than 24 h, it would appear that a lactulose and mannitol test on an individual pig could be repeated a maximum of every 24 h if desired.

Aspirin is believed to interact with biological membranes (Zhou and Raphael, 2005; Lichtenberger et al., 2012) inducing increased permeability to some molecules (Lambert et al., 2012). Consistent with reports in humans (Sequeira et al., 2012), aspirin did not influence the excretion of mannitol in pig urine. Similarly, for absolute excretion of lactulose there was no main effect of aspirin treatment, likely due to the large variation observed in the lactulose output. When lactulose excretion was corrected for urine void interval, aspirin at 15 mg/kg BW had greater overall excretion than 30 mg/kg BW with 0 mg/kg BW being intermediate. The reduced lactulose excretion for aspirin at 30 mg/kg BW was surprising, as humans receiving 325, 650, and 975 mg of aspirin the night before and the morning of a study all had greater lactulose excretion than those receiving 0 mg of aspirin, and had numerical trends towards increasing lactulose excretion with increasing aspirin intake (Lambert et al., 2012). Similarly, human test subjects given two doses of 1.3 g of aspirin on the day of a lactulose and mannitol test all demonstrated increased excretion of lactulose compared to a prior test without taking aspirin (Hilsden et al., 1996). If it is assumed that the average BW of test subject in the experiment of Hilsden et al. (1996) was 90 kg, then the combined dose of 2.6 g of aspirin received on the test day would have been 29 mg/kg BW which

is similar to the 30 mg/kg BW given to pigs on the test day. Thus, it does not seem likely that the greater dose of aspirin received by pigs would cause a reduction in permeability compared to pigs given an intermediate dose of aspirin. However, data on the impacts of aspirin on intestinal permeability in weanling pigs is scarce, and additional investigation is required to determine the impacts of graded levels of aspirin as well as repeated doses of aspirin on intestinal permeability in weanling pigs.

The ratio of lactulose to mannitol is calculated to account for any systematic differences in gastric emptying time, kidney filtration rate, or other extraneous factors that could impact the absorption of lactulose from subject to subject (Mishra and Makharia, 2012). While the ratio of lactulose to mannitol should not be interpreted without considering the lactulose and mannitol excretion values as well (Sequeira et al., 2014a), it is generally believed to be the best indicator of the overall health of the small intestine. In the present study, the total recovery of lactulose over the 48 h collection period yielded a similar conclusion as the repeated measures analysis with aspirin at 15 mg/kg BW having greater total lactulose excretion than aspirin at 0 and 30 mg/kg BW. However, there was no difference in the ratio of lactulose to mannitol excretion among the treatments. This may be due to the considerable variation observed in the ratio of lactulose to mannitol from pig to pig within a treatment. Regardless, it appears that 15 mg/kg BW of aspirin given 24 and 1 h before oral-dosing with lactulose and mannitol may be an effective stimulus to induce a leaky gut in nursery pigs.

2.6 Conclusions

In Exp 2.1, pigs were dosed with aspirin and a solution of lactulose and mannitol and blood samples were collected. Experimentation with colorimetric kits demonstrated that an endogenous

component of serum with a molecular weight greater than 3000 daltons inhibited the color change when analyzing serum for lactulose, and that even when the inhibition was reduced, kits lacked sufficient accuracy for use in a research setting. A HPLC system capable of analyzing lactulose and mannitol in serum was assembled and demonstrated that the levels of lactulose and mannitol in the serum from experimental pigs were below the detection limit of the system. Therefore, it was concluded that while a serum lactulose and mannitol test initially held promise as a useful tool to measure intestinal permeability in pigs, the difficulty in measurement of the very low levels of lactulose and mannitol present in serum precluded additional investigation into its use.

In Exp 2.2 methods were developed to measure lactulose and mannitol in pig urine, and the temporal pattern of lactulose and mannitol excretion from weanling pigs was described. This temporal pattern will aid future research into the lactulose and mannitol test of intestinal permeability in pigs by helping to determine the optimal urine collection period, which appears to be 8 h or less. Additional work is required to ascertain if a bimodal distribution of lactulose excretion over time exists in pigs, and if so, determine when urine collection should cease to avoid the second peak. Aspirin treatment at 15 mg/kg BW successfully increased lactulose excretion, but additional research is required in pigs to elucidate the impacts of graded levels of aspirin dose on intestinal permeability in nursery pigs.

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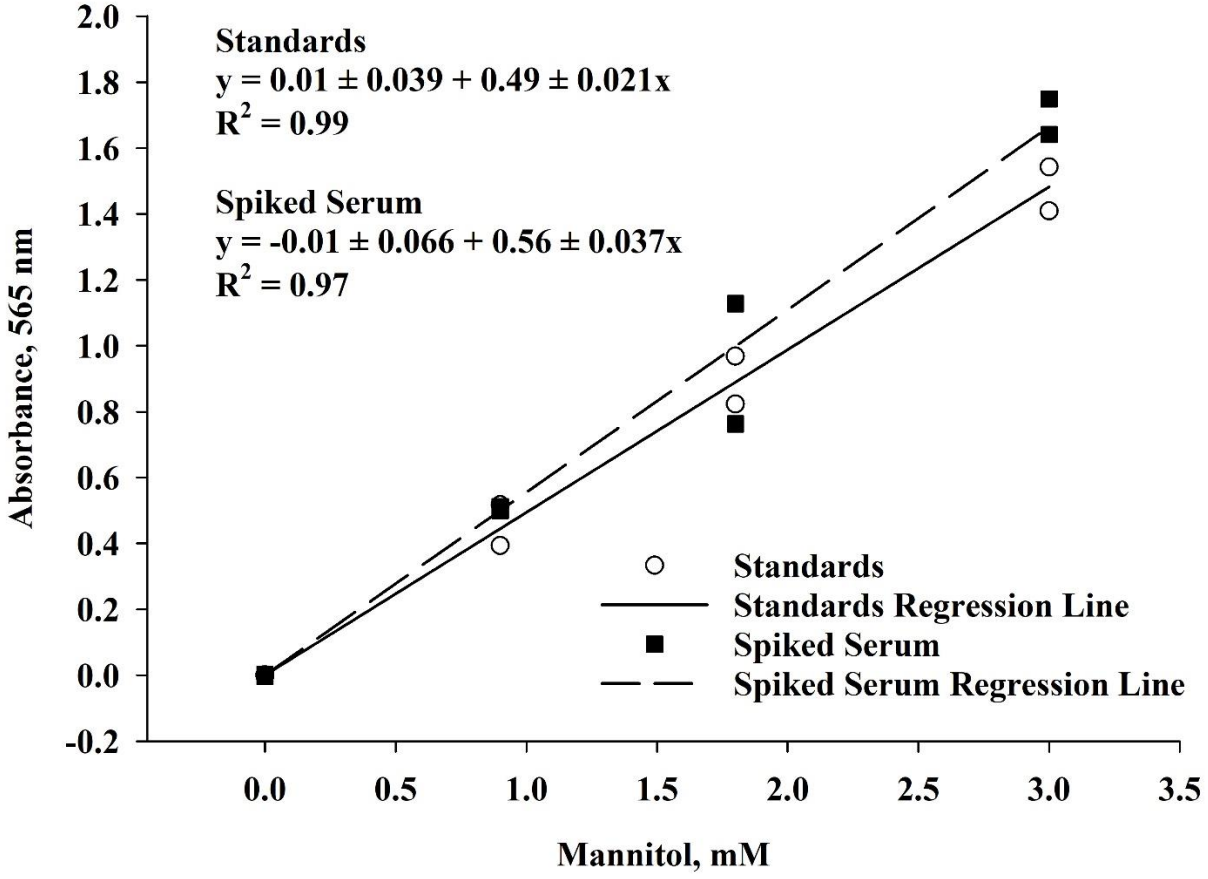

Figure 2.1. Absorbance of standards (n = 2 per mannitol concentration) and serum (n = 2 per mannitol concentration) spiked with the same concentrations of mannitol as the standards for bench Trial 1 in Exp 2.1.

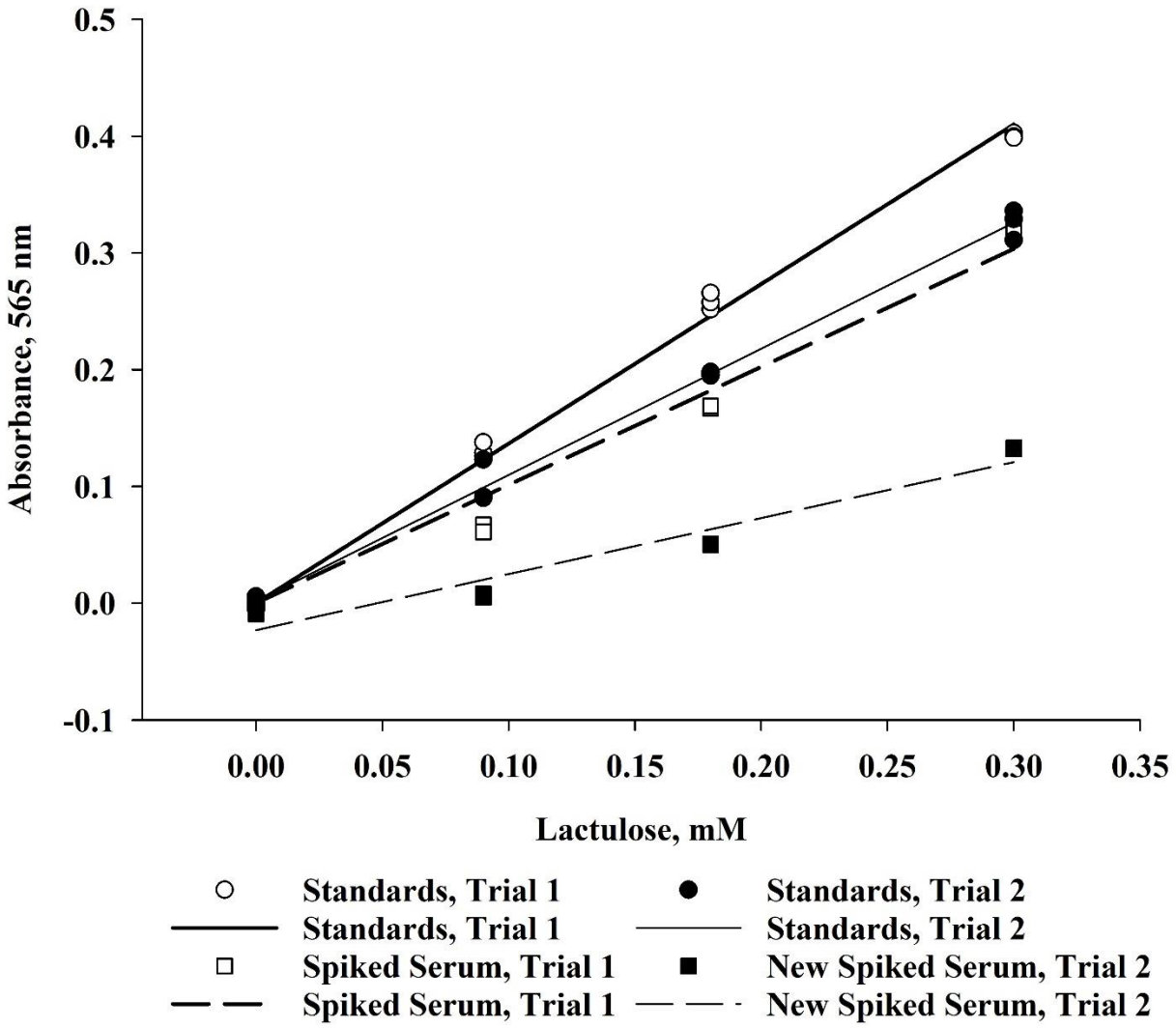

Figure 2.2. Absorbance of standards (n = 3 per lactulose concentration) and serum (n = 2 per lactulose concentration) spiked with the same concentrations of lactulose as the standards for bench Trial 1 and 2 in Exp 2.1. Symbols denote data points, lines represent regression lines. The regression equation for the standards in Trial 1 is y = 0.003 ± 0.0044 + 1.35 ± 0.024x with R^2 = 0.99. The regression equation for the spiked serum in Trial 1 is y = -0.02 ± 0.009 + 1.09 ± 0.050x with R^2 = 0.99. The regression equation for the standards in Trial 2 is y = 0.003 ± 0.0062 + 1.10 ± 0.034x with R^2 = 0.99. The regression equation for the new spiked serum in Trial 2 is y = -0.02 ± 0.009 + 0.48 ± 0.049x with R^2 = 0.94.

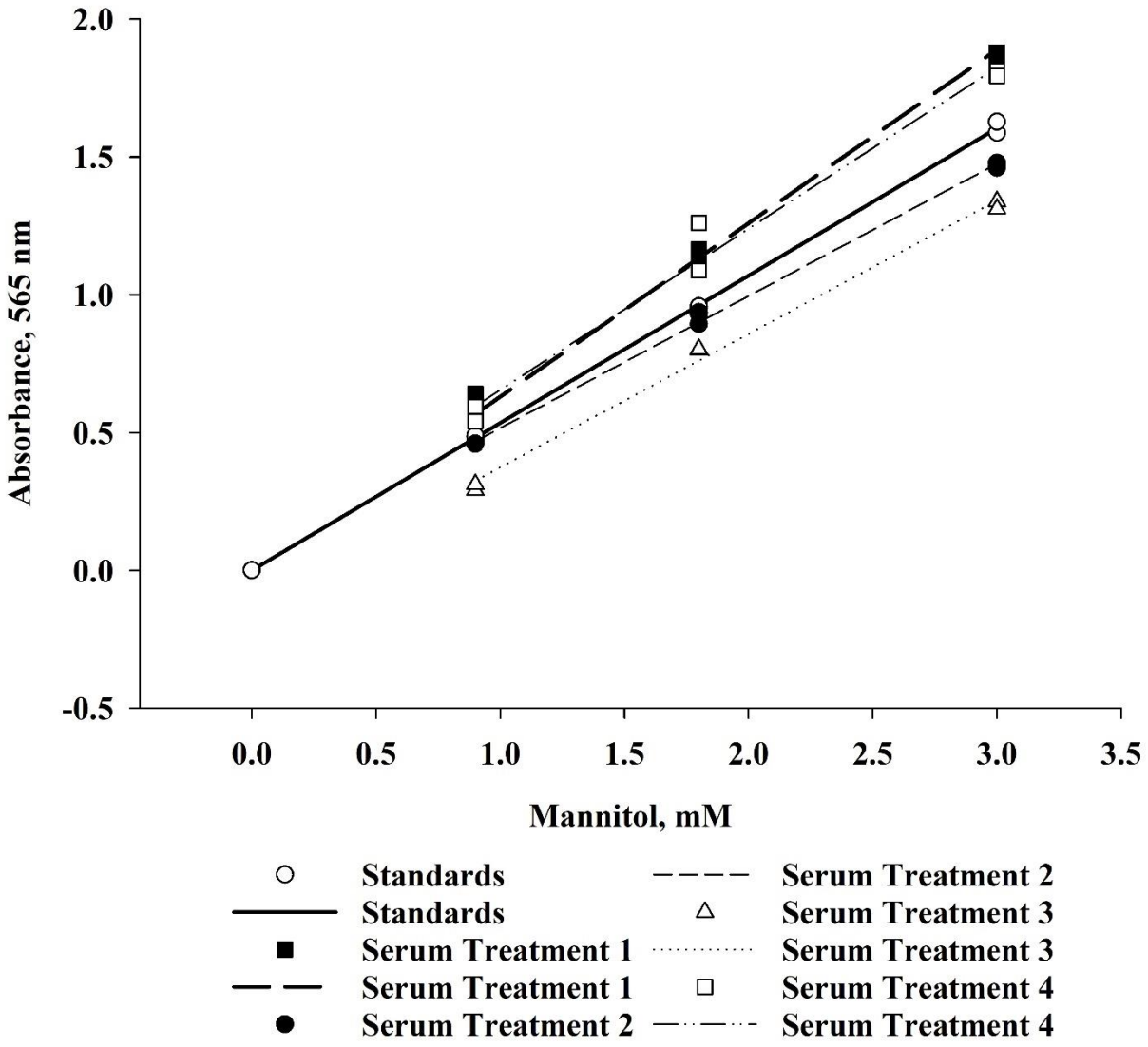

Figure 2.3. Absorbance of standards (n = 3 per mannitol concentration) and treated serum (n = 2 per mannitol concentration) spiked with the same concentrations of mannitol as the standards for bench Trial 3 in Exp 2.1. The serum treatments were: Trt 1, raw spiked serum; Trt 2, serum spin filtered to remove molecules greater than 3000 daltons then spiked after filtration; Trt 3, serum spiked before being spin filtered to remove molecules greater than 3000 daltons; Trt 4, serum spiked and then heated at 56°C for 30 min in a water bath. Symbols denote data points, lines represent regression lines. The regression equation for the standards is $y = -0.01 \pm 0.015 + 0.54 \pm 0.007x$ with $R^2 = 0.99$. The regression equation for serum Trt 1 is $y = 0.10 \pm 0.018 + 0.59 \pm 0.009x$ with $R^2 = 0.99$. The regression equation for serum Trt 2 is $y = 0.04 \pm 0.021 + 0.48 \pm 0.010x$ with $R^2 = 0.99$. The regression equation for serum Trt 3 is $y = -0.11 \pm 0.037 + 0.48 \pm 0.018x$ with $R^2 = 0.99$. The regression equation for serum Trt 4 is $y = 0.07 \pm 0.078 + 0.59 \pm 0.037x$ with $R^2 = 0.98$.

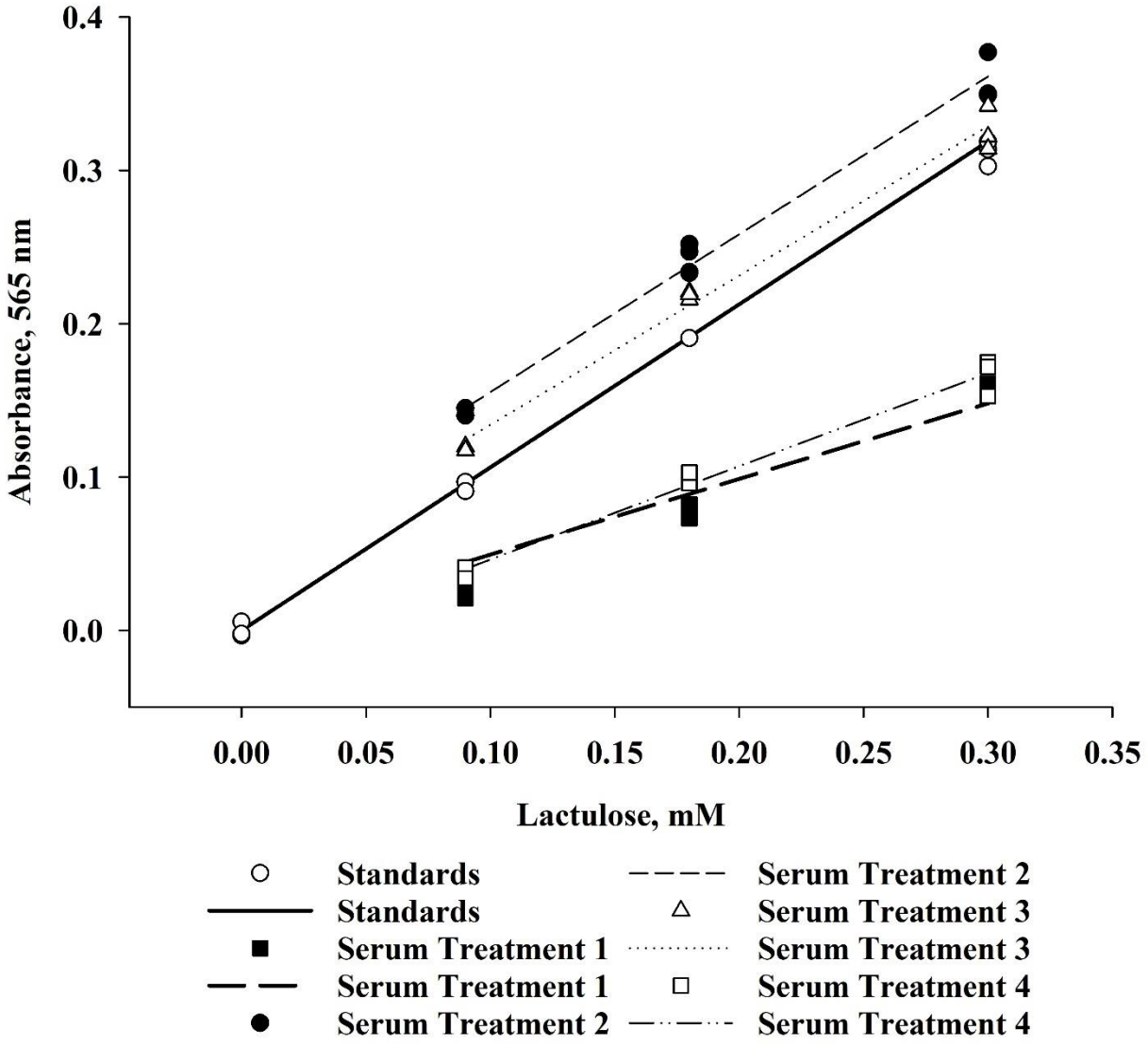

Figure 2.4. Absorbance of standards (n = 3 per lactulose concentration) and treated serum (n = 3 per lactulose concentration) spiked with the same concentrations of lactulose as the standards for bench Trial 3 as analyzed by technician 1 in Exp 2.1. The serum treatments were: Trt 1, raw spiked serum; Trt 2, serum spin filtered to remove molecules greater than 3000 daltons then spiked after filtration; Trt 3, serum spiked before being spin filtered to remove molecules greater than 3000 daltons; Trt 4, serum spiked and then heated at 56°C for 30 min in a water bath. Symbols denote data points, lines represent regression lines. The regression equation for the standards is y = 0.001 ± 0.0063 + 1.04 ± 0.027x with R^2 = 0.99. The regression equation for serum Trt 1 is y = -0.03 ± 0.003 + 0.65 ± 0.011x with R^2 = 0.99. The regression equation for serum Trt 2 is y = 0.05 ± 0.014 + 1.02 ± 0.059x with R^2 = 0.99. The regression equation for serum Trt 3 is y = 0.03 ± 0.012 + 0.98 ± 0.051x with R^2 = 0.99. The regression equation for serum Trt 4 is y = -0.02 ± 0.009 + 0.61 ± 0.038x with R^2 = 0.98.

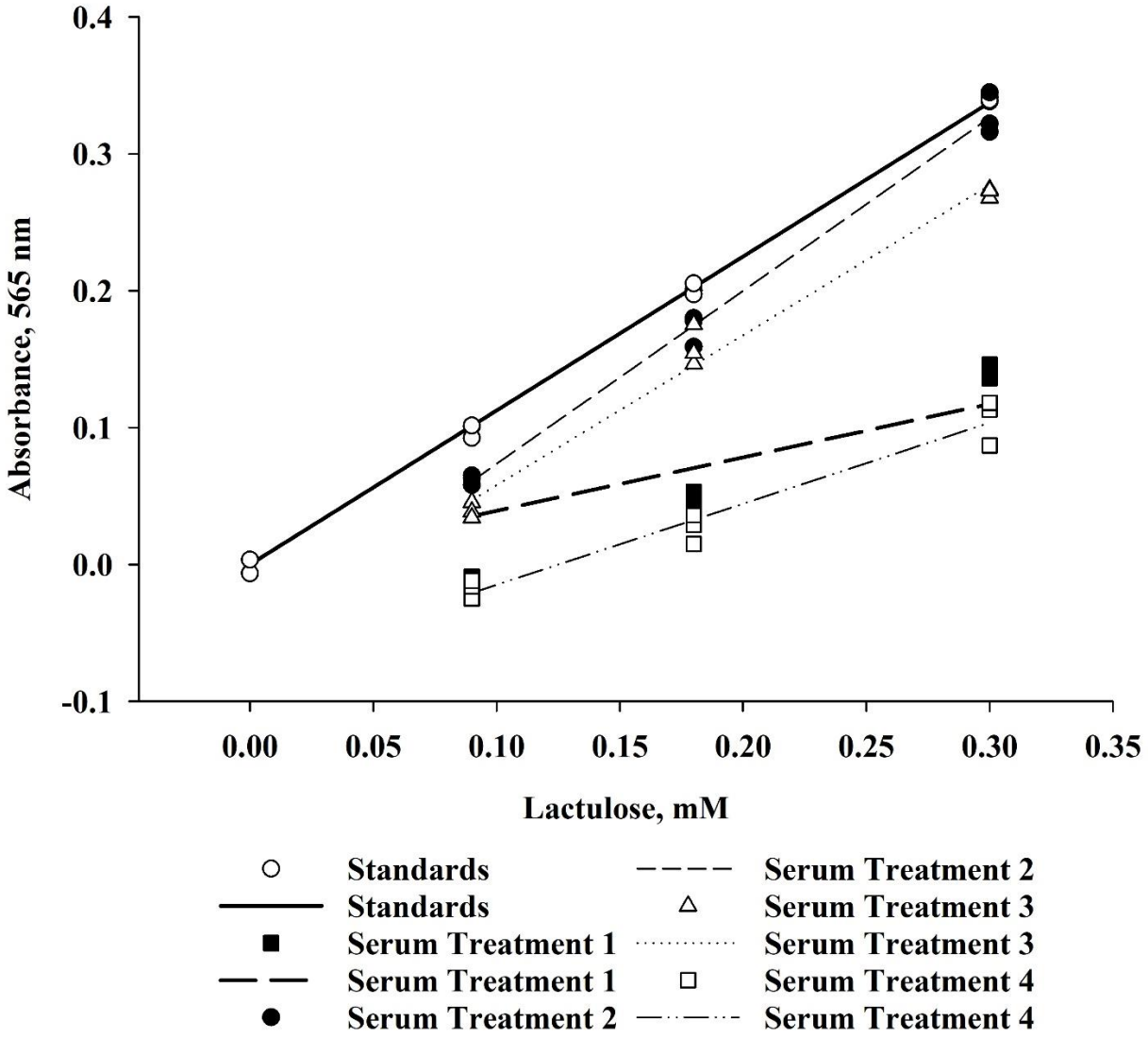

Figure 2.5. Absorbance of standards (n = 3 per lactulose concentration) and treated serum (n = 3 per lactulose concentration) spiked with the same concentrations of lactulose as the standards for bench Trial 3 as analyzed by technician 2 in Exp 2.1. The serum treatments were: Trt 1, raw spiked serum; Trt 2, serum spin filtered to remove molecules greater than 3000 daltons then spiked after filtration; Trt 3, serum spiked before being spin filtered to remove molecules greater than 3000 daltons; Trt 4, serum spiked and then heated at 56°C for 30 min in a water bath. Symbols denote data points, lines represent regression lines. The regression equation for the standards is y = -0.002 ± 0.0028 + 1.14 ± 0.012x with R^2 = 0.99. The regression equation for serum Trt 1 is y = -0.08 ± 0.005 + 0.73 ± 0.024x with R^2 = 0.99. The regression equation for serum Trt 2 is y = -0.05 ± 0.012 + 1.26 ± 0.053x with R^2 = 0.99. The regression equation for serum Trt 3 is y = -0.05 ± 0.015 + 1.08 ± 0.067x with R^2 = 0.98. The regression equation for serum Trt 4 is y = -0.07 ± 0.011 + 0.60 ± 0.049x with R^2 = 0.95.

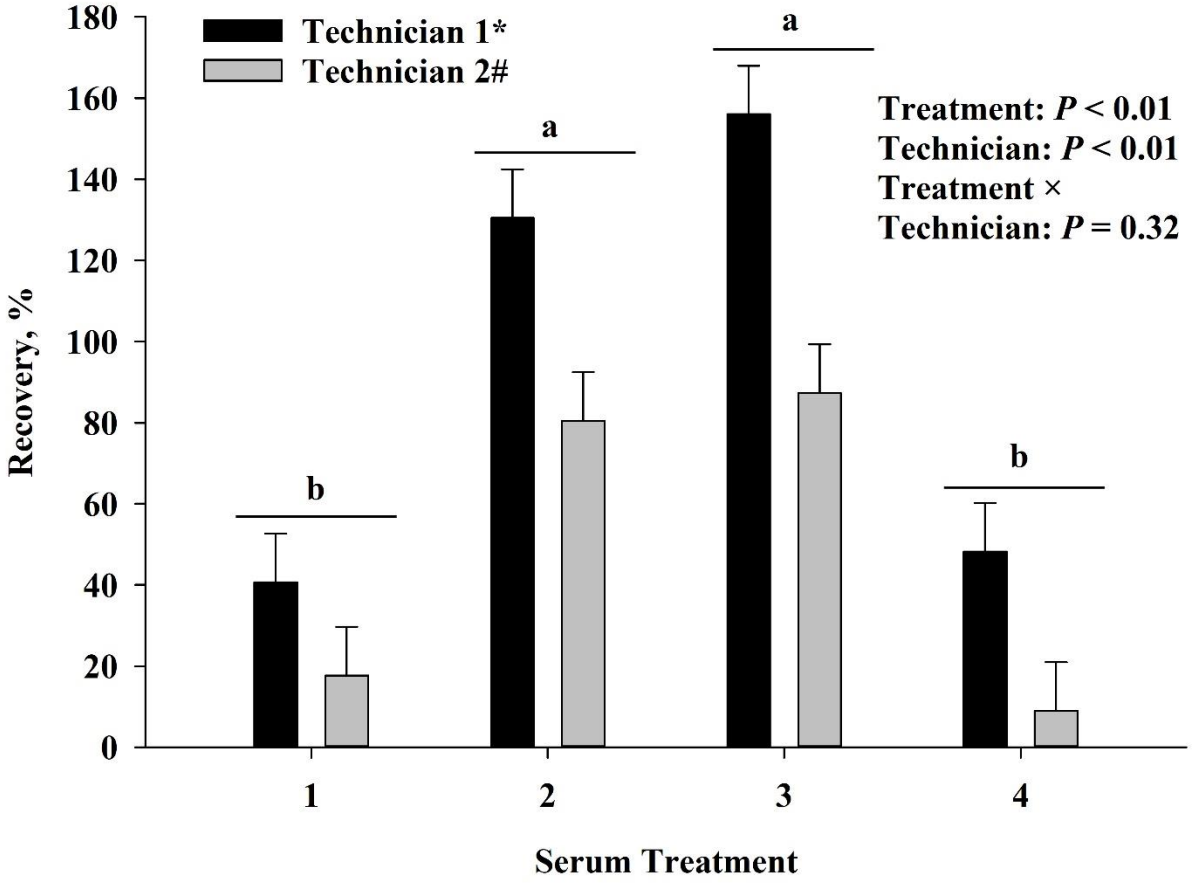

Figure 2.6. Recovery of lactulose from treated serum (n = 3) spiked with the same concentrations of lactulose as the standards for bench Trial 3 as simultaneously analyzed by two technicians in Exp 2.1. The serum treatments were: Trt 1, raw spiked serum; Trt 2, serum spin filtered to remove molecules greater than 3000 daltons then spiked after filtration; Trt 3, serum spiked before being spin filtered to remove molecules greater than 3000 daltons; Trt 4, serum spiked and then heated at 56°C for 30 min in a water bath. Values shown for each serum treatment are the least square means ± 1 SEM of lactulose recovery as a percentage of the expected value from serum spiked with 0.09, 0.18, and 0.30 mM of lactulose. Serum treatments lacking a common letter (a, b) differ ($P < 0.05$), technicians lacking a common symbol (*, #) differ ($P < 0.01$).

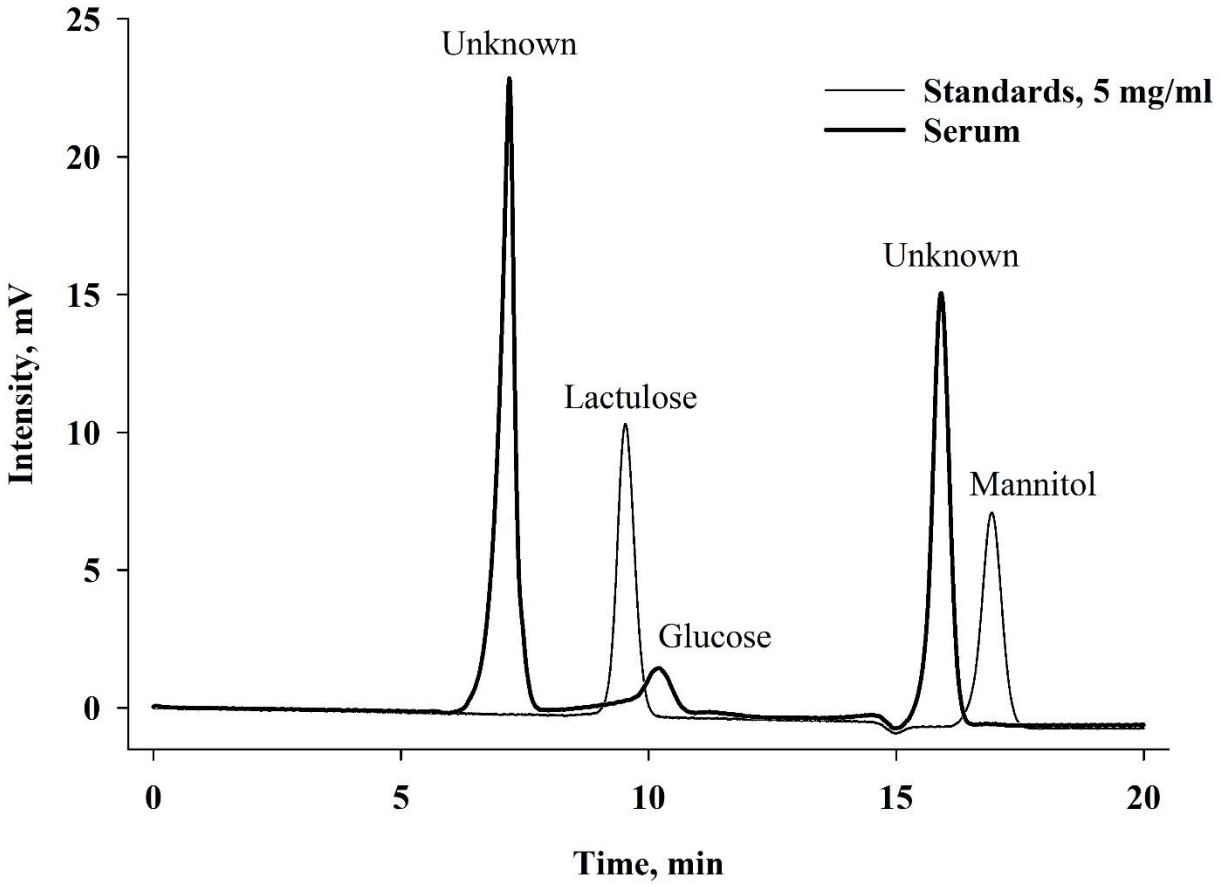

Figure 2.7. A representative chromatogram of lactulose and mannitol standards at 5 mg/mL in ultrapure water, and serum from a blood sample taken 1 h post-oral dose of lactulose and mannitol solution from a pig given 10 mg/kg BW aspirin in Exp 2.1. Lactulose and mannitol were undetectable in the serum chromatogram.

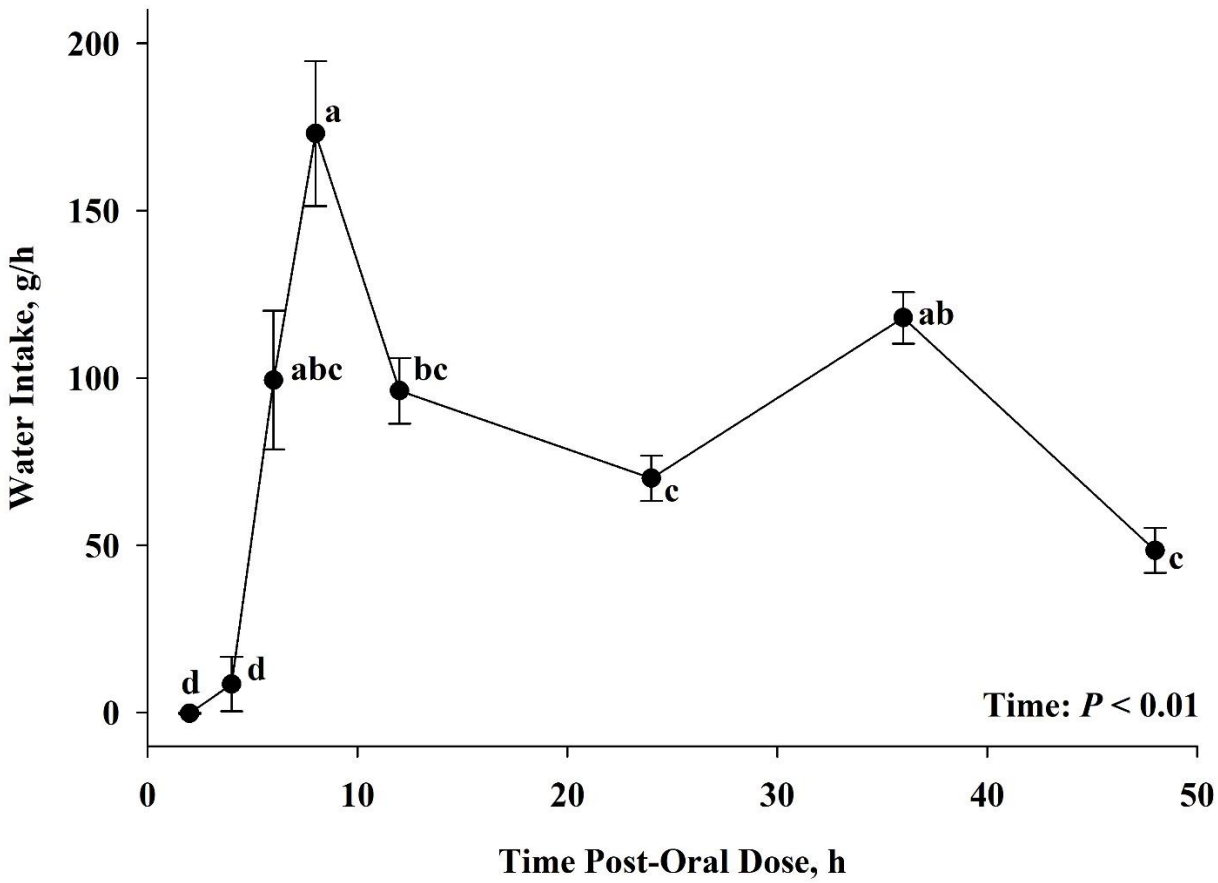

Figure 2.8. Water intake from pigs (n = 12) regardless of aspirin treatment over the 48 h urine collection period following oral dosing with a solution of lactulose and mannitol in water in Exp 2.2. Values not sharing a common letter (a, b, c) differ ($P < 0.05$). Error bars represent ± 1 SEM.

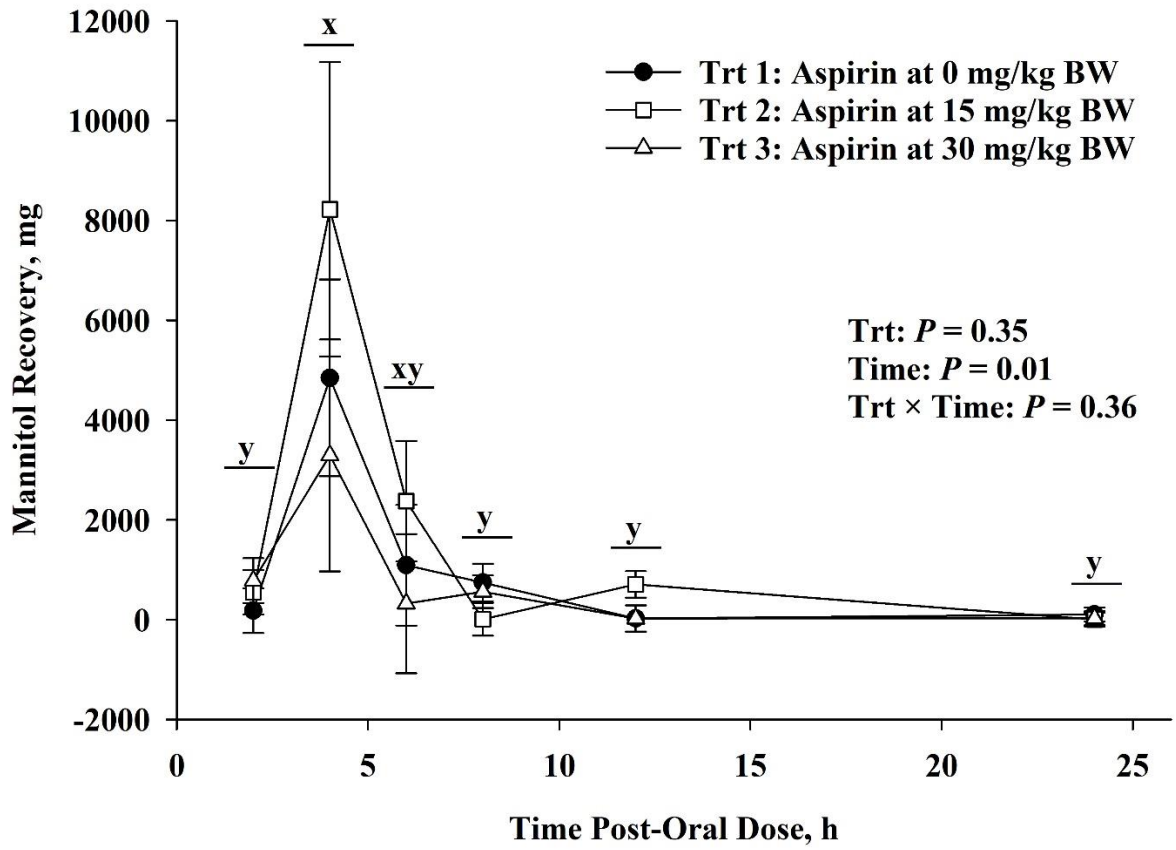

Figure 2.9. Absolute mannitol recovery in urine over time from pigs (n = 4 per treatment) receiving three levels of aspirin in Exp 2.2. Time points not sharing a common letter (x, y) differ ($P < 0.05$). Error bars represent ± 1 SEM.

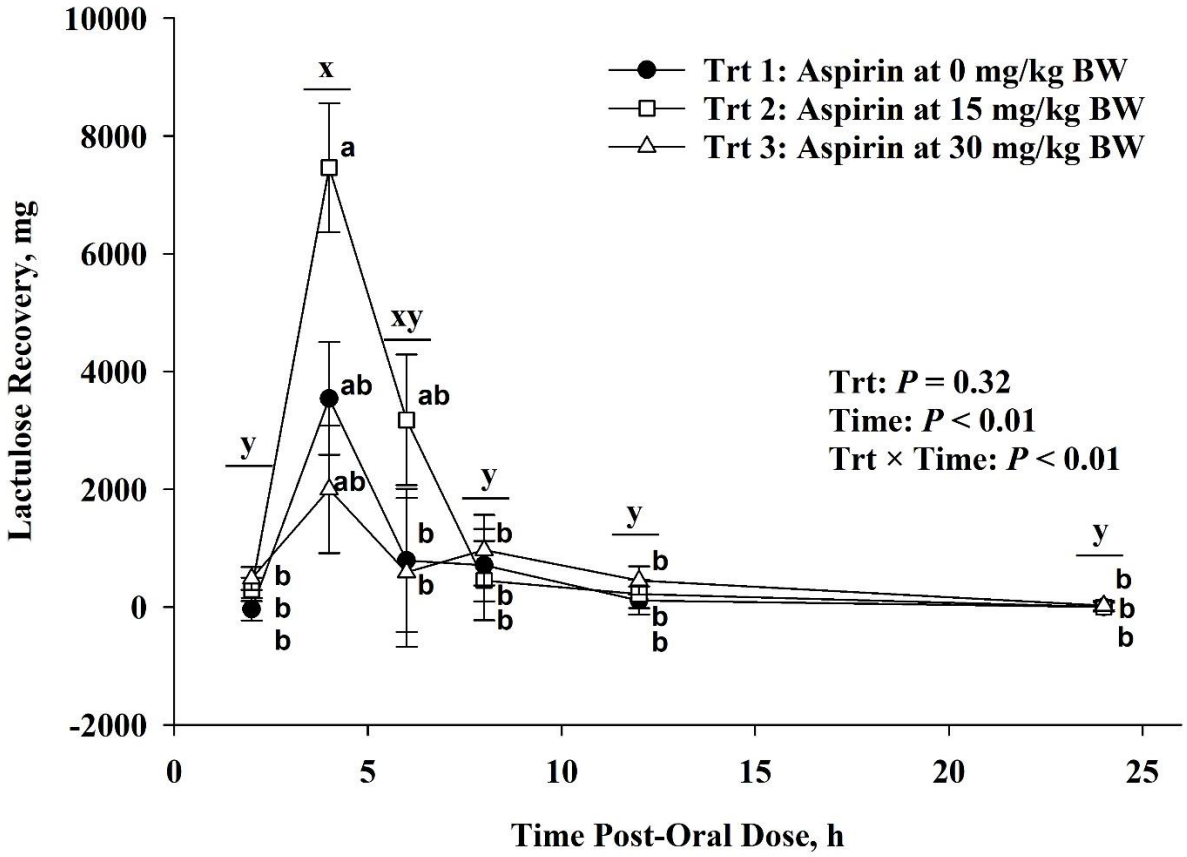

Figure 2.10. Absolute lactulose recovery in urine over time from pigs (n = 4 per treatment) receiving three levels of aspirin in Exp 2.2. Times not sharing a common letter (x, y) differ (P < 0.01). Treatment × Time values not sharing a common letter (a, b) differ (P < 0.05). Error bars represent ± 1 SEM.

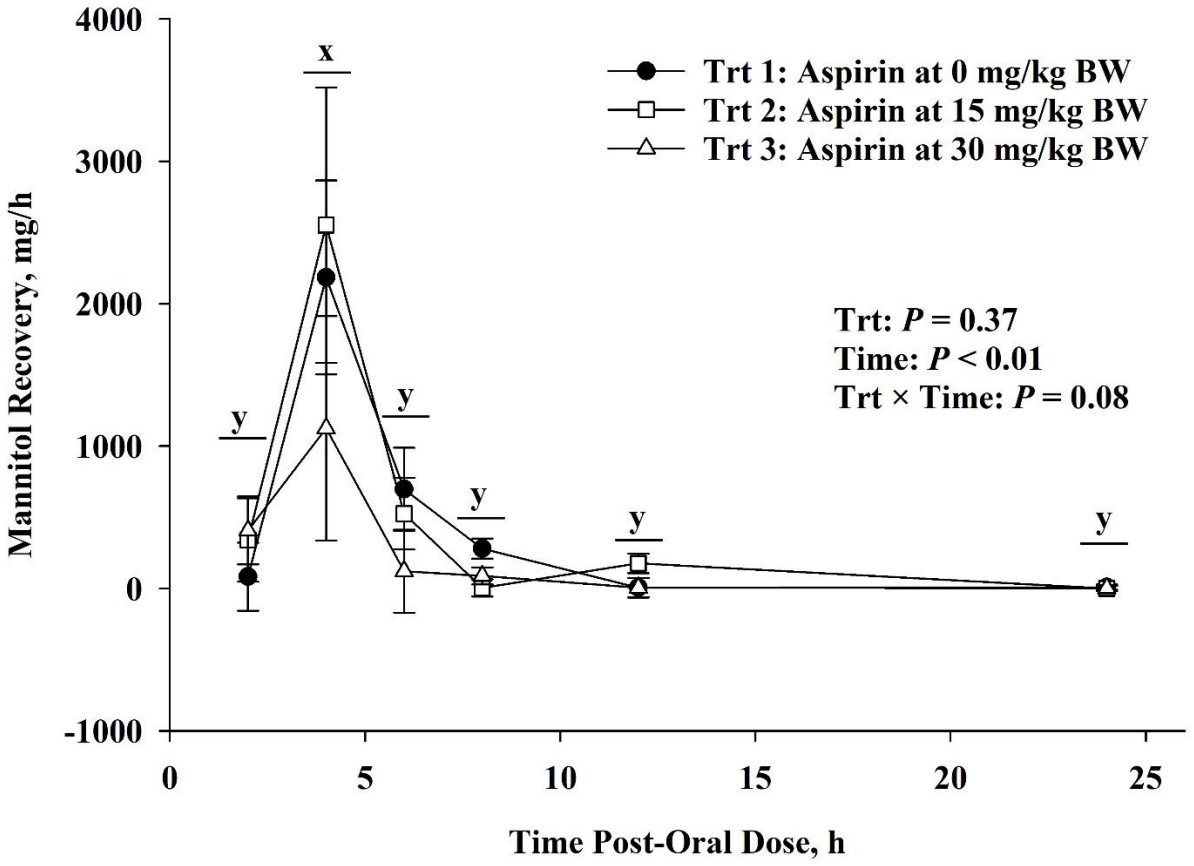

Figure 2.11. Mannitol recovery per hour in urine over time from pigs (n = 4 per treatment) receiving three levels of aspirin in Exp 2.2 corrected for urine void interval. Times not sharing a common letter (x, y) differ ($P < 0.05$). Error bars represent ± 1 SEM.

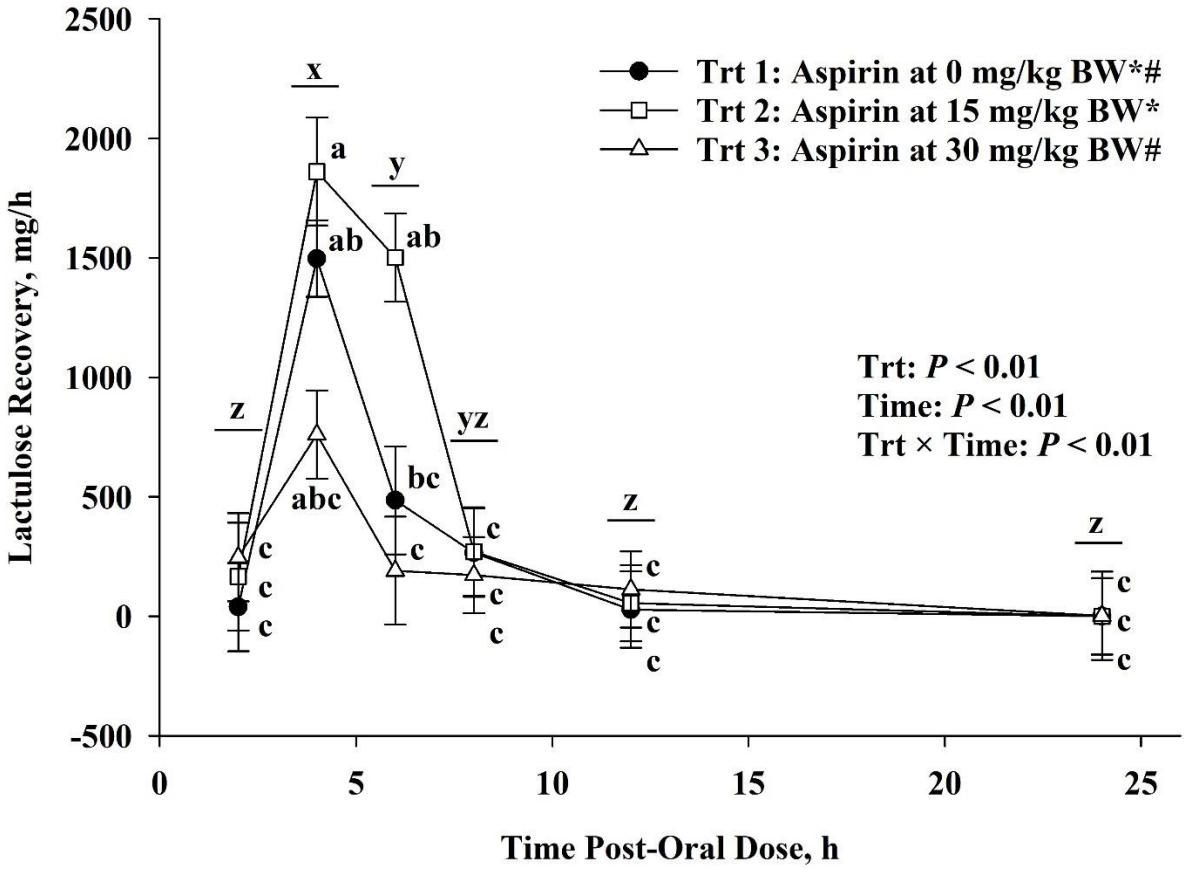

Figure 2.12. Lactulose recovery per hour in urine over time from pigs (n = 4 per treatment) receiving three levels of aspirin in Exp 2.2 corrected for urine void interval. Times not sharing a common letter (x, y, z) differ ($P < 0.05$). Treatment × Time values not sharing a common letter (a, b, c) differ ($P < 0.05$). Error bars represent ± 1 SEM. Aspirin treatments not sharing a common symbol (*, #) differ ($P < 0.05$).

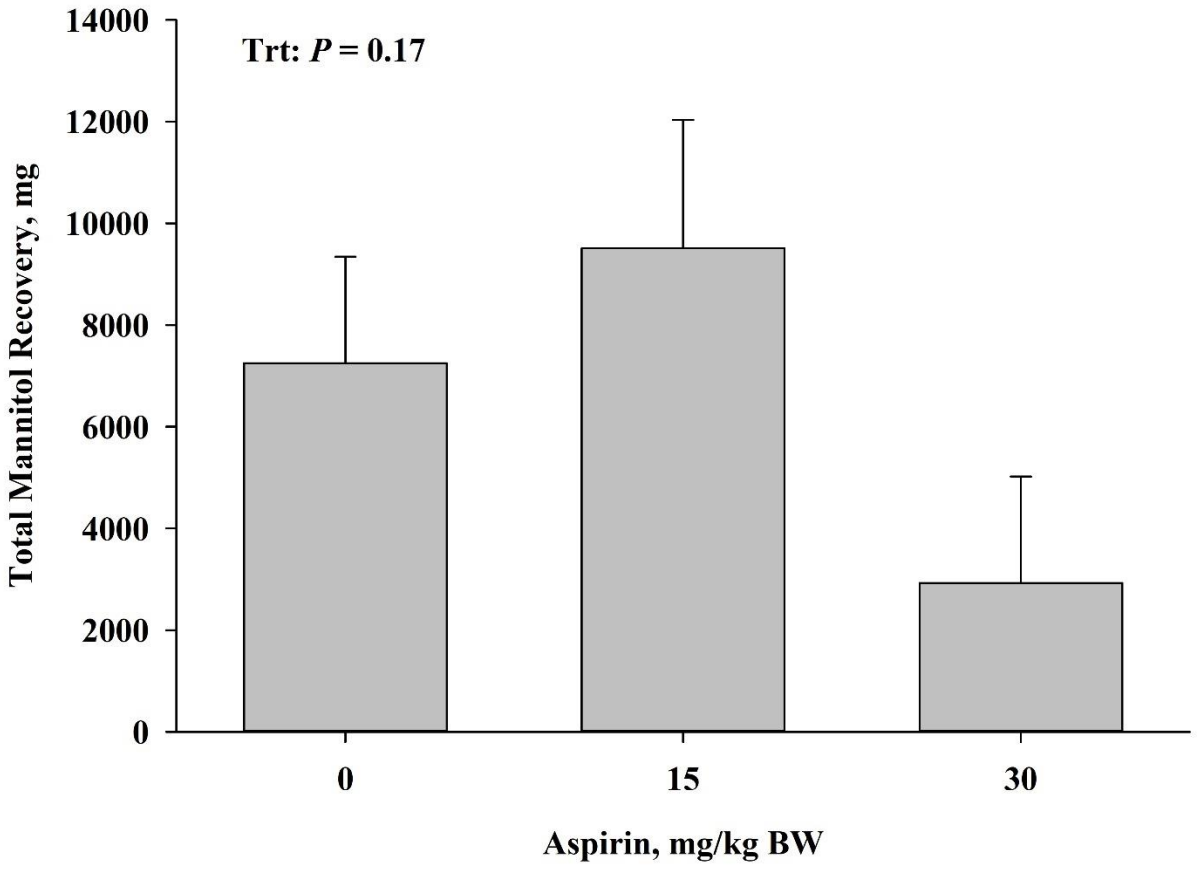

Figure 2.13. Total mannitol recovery in urine over a 48 h urine collection period from pigs (n = 4 per treatment) receiving aspirin at 0, 15, or 30 mg/kg BW in Exp 2.2. Error bars represent ± 1 SEM.

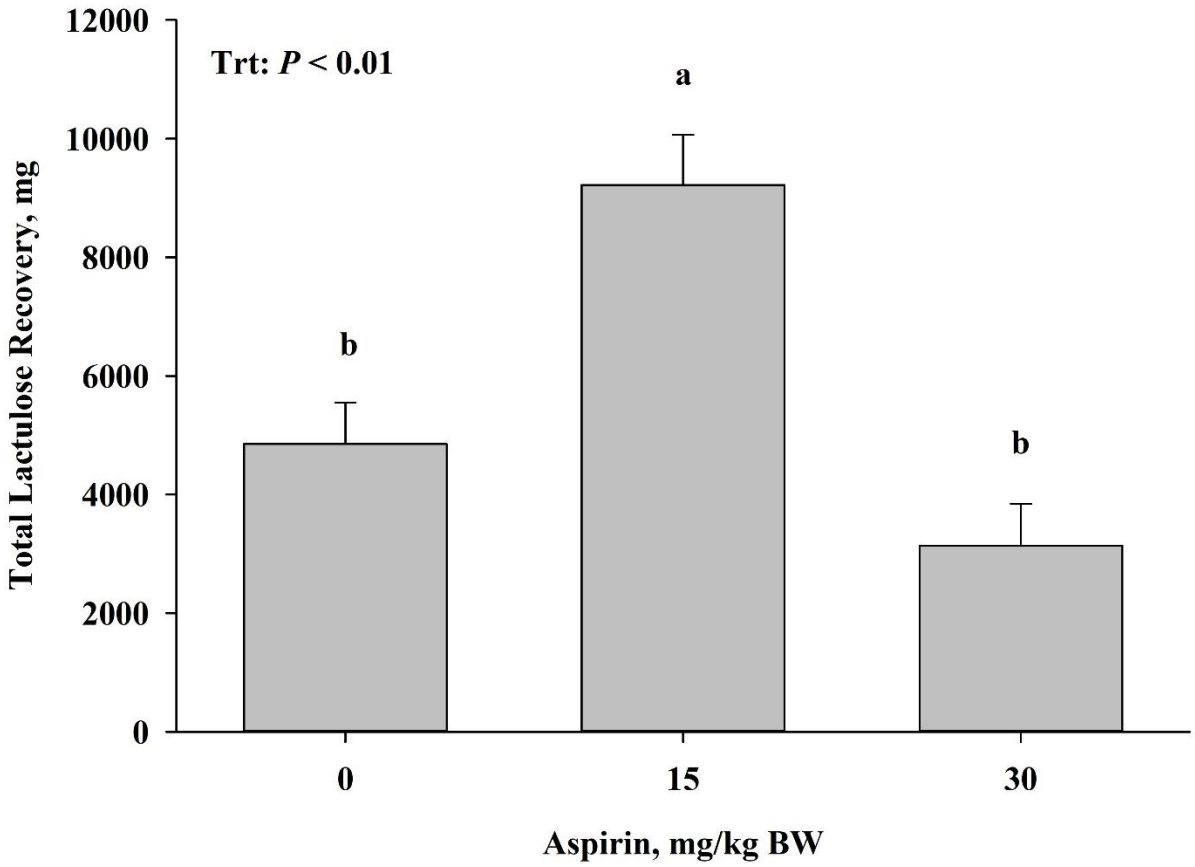

Figure 2.14. Total lactulose recovery in urine over a 48 h urine collection period from pigs (n = 4 per treatment) receiving aspirin at 0, 15, or 30 mg/kg BW in Exp 2.2. Aspirin treatments not sharing a common letter (a, b) differ ($P < 0.05$). Error bars represent ± 1 SEM.

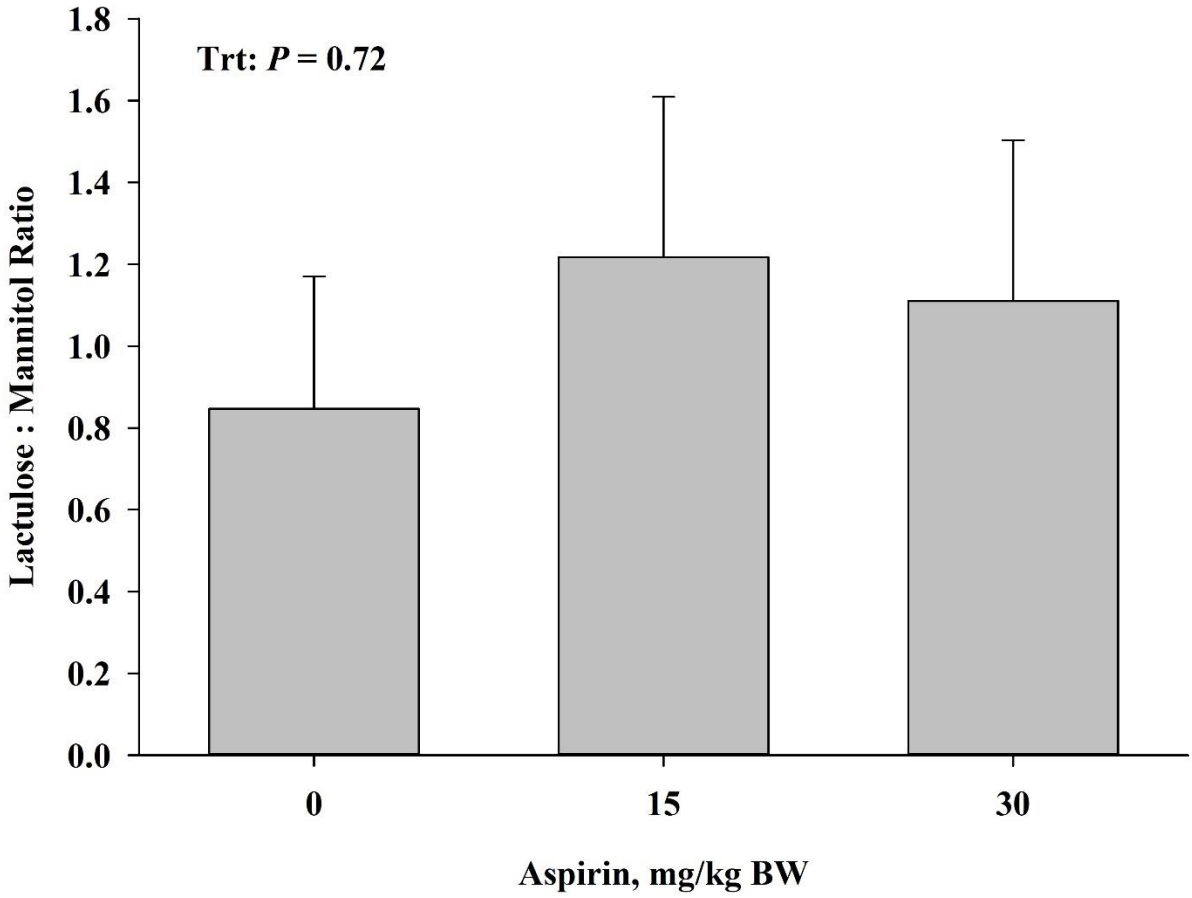

Figure 2.15. The ratio of total lactulose to total mannitol recovery in urine over a 48 h urine collection period from pigs (n = 4 per treatment) receiving aspirin at 0, 15, or 30 mg/kg BW in Exp 2.2. Error bars represent ± 1 SEM.

Table 2.1. Body weight, feed intake, water intake, urine output, and time that lactulose and mannitol levels returned to baseline for pigs given three levels of aspirin 24 and 1 h before being orally dosed with a solution of lactulose and mannitol[1] at time 0 followed by a 48 h quantitative urine collection period

	Aspirin, mg/kg BW				***P*-Value**		
Item	**0**	**15**	**30**	**SEM**	**Trt**	**Time**	**Trt × Time**
n	4	4	4	-	-	-	-
Intial BW, kg	11.4	11.6	11.5	0.58	0.86	-	-
Final BW, kg	12.3	12.2	12.4	0.80	0.90	-	-
FI, g	1170	1168	1222	102.1	0.88	-	-
Water intake, g/h							
-4 to -0.5 h	25.6	9.9	25.5	3.73	0.05	-	-
0 to 2 h[2]	0.0	0.0	0.0	17.67	0.95	< 0.01	0.79
2 to 4 h	24.5	0.0	1.6				
4 to 6 h	92.4	106.8	98.9				
6 to 8 h	165.0	199.0	155.3				
8 to 12 h	102.6	88.4	97.7				
12 to 24 h	61.2	72.3	76.8				
24 to 36 h	132.7	113.5	110.3				
36 to 48 h	32.9	56.5	56.0				
0 to 48 h[2]	77.0	80.7	79.6	9.33	0.93	-	-
Urine output, g/h							
-4 to -0.5 h	50.0	33.7	26.2	10.69	0.18	-	-
0 to 2 h[2]	8.6	10.8	12.9	7.18	0.25	0.32	0.94
2 to 4 h	38.3	29.1	20.9				
4 to 6 h	9.9	32.8	4.4				
6 to 8 h	27.4	32.3	19.1				
8 to 12 h	11.7	23.5	12.3				
12 to 24 h	9.8	18.1	16.3				
24 to 36 h	14.6	30.9	15.3				
36 to 48 h	13.8	20.2	18.7				
0 to 48 h[3]	14.0	23.6	16.0	4.58	0.29	-	-
Lactulose Tbase, h[4]	18.0	21.0	20.0	4.51	0.88	-	-
Mannitol Tbase, h[5]	14.0	16.0	15.5	6.01	0.97	-	-

[1]Pigs were given 0.5 g/kg BW lactulose and 0.05 g/kg BW mannitol.

[2]The SEM and *P*-values within this row are from a repeated measures analysis of all 8 time periods (period 0 to 2 h through period 36 to 48 h) with time period as the repeated measure and aspirin treatment as a fixed effect.

[3]The SEM and *P*-value within this row are from an analysis of the average response regardless of time period with aspirin treatment as a fixed effect.

[4]Time at which lactulose levels in urine returned to baseline after an oral dose of lactulose and mannitol.

[5]Time at which mannitol levels in urine returned to baseline after an oral dose of lactulose and mannitol.

CHAPTER 3. THE IMPACT OF THE LACTULOSE AND MANNITOL DOSE ON THE EXCRETION OF LACTULOSE AND MANNITOL OVER TIME AND DEVELOPMENT OF AN ALTERNATIVE METHOD FOR URINE COLLECTION IN NURSERY PIGS

3.1 Abstract

Variation in the dose of lactulose and mannitol given in tests of intestinal health in nursery pigs may prevent accurate and comparable results, and quantitative urine collection during lactulose and mannitol tests are challenging. Therefore, two studies were conducted with the objectives of determining the impact of variation in the dose of lactulose and mannitol on the excretion of lactulose and mannitol (Exp 3.1) and evaluating the use of a belly wrap as an alternative to metabolism cages for urine collection (Exp 3.2). In Exp 3.1, pigs were weaned and dosed with lactulose and mannitol 4 d post-weaning (n = 8 barrows; 5.7 ± 0.41 kg BW). Pigs received an oral dose of either Trt 1 (0.2 g/kg BW lactulose + 0.02 g/kg BW mannitol), Trt 2 (0.3 g/kg BW lactulose + 0.03 g/kg BW mannitol), Trt 3 (0.4 g/kg BW lactulose + 0.04 g/kg BW mannitol), or Trt 4 (0.5 g/kg BW lactulose + 0.05 g/kg BW mannitol) and urine was collected at 2, 4 and 6 h post-oral dose. Urinary lactulose and mannitol concentrations were determined via HPLC. In Exp 3.2, pigs (n = 6 barrows; 6.9 ± 0.05 kg BW; 9 d post-weaning) either received a belly wrap for urine collection (which consisted of absorbent cotton, a plastic moisture barrier, and elastic wrap), or had urine collected via metabolism crates. Urine was collected for 4 h and urine output was quantified gravimetrically. Data from both experiments were analyzed via Proc Glimmix of SAS with time-course data analyzed as repeated measures where appropriate. In Exp 3.1, urine collection from pigs 4 d post-weaning proved challenging, with urine output from 49% of pigs at 2 h, 47% of pigs at 4 h, and 53% of pigs at 6 h post-oral dose. Absolute mannitol excretion

increased ($P < 0.01$) over time with excretion at 2 h (50 ± 37.4 mg) being lesser than at 6 h (411 ± 93.5 mg), with 4 h (209 ± 68.5 mg) being intermediate. Absolute lactulose excretion was greater ($P < 0.01$) at 4 h (289 ± 95.6 mg) and 6 h (358 ± 69.2 mg) than at 2 h (37 ± 25.9 mg). Despite the lack of statistical differences among treatments, total lactulose recovery was remarkably stable for Trt 2 through Trt 4, indicating 0.3 to 0.5 g/kg BW lactulose may be an optimal range and warranting further research. In Exp 3.2, the urine collection method did not impact water intake or urine output ($P \geq 0.15$). Therefore, belly wraps may be a feasible alternative to metabolism crates for urine collection from nursery pigs.

Keywords: lactulose, mannitol, nursery pig, gut health, urine collection

3.2 Introduction

The absorption of non-metabolizable sugars such as lactulose and mannitol from the gastrointestinal tract can be used to measure intestinal health (Bjarnason et al., 1995). Greater absorption of the larger molecule lactulose indicates greater permeability (Sequeira et al., 2012), while greater absorption of the smaller molecule mannitol indicates greater absorptive surface area (Berkeveld et al., 2008). As extraneous factors such as stomach emptying time will impact both lactulose and mannitol recovery rates similarly (Mishra and Makharia, 2012), the ratio of lactulose to mannitol recovery may be a more robust metric of overall intestinal health than the recovery of the individual molecules. The ratio of lactulose to mannitol is therefore generally accepted as a metric of overall intestinal health, with increases in the ratio indicative of reduced intestinal health (Dastych et al., 2008; Mishra and Makharia, 2012). The use of the lactulose and mannitol test of intestinal health as used in humans (Sequeira et al., 2014a) has received some attention from researchers working with gut health in swine due to the non-invasive nature of the test (De Vos et

al., 2012). However, there is a dearth of research on how lactulose and mannitol studies of gut health should be conducted in swine. The published methods used with pigs appear to be loosely based on those of human studies (Kansagra et al., 2003; Wijtten et al., 2011), or with no justification given for the methods used (Li et al., 2018). As a result, there is considerable variation in how the studies are conducted (e.g. 24 h urine collection period of Kansagra et al. (2003) vs. 6 h urine collection period of Yang et al. (2014)), raising questions as to applicability of the test methodology to pigs, the accuracy of the results, and the comparability of results between published trials.

In the limited number of studies with lactulose and mannitol in pigs, the dose of lactulose has varied from 0.2 g/kg BW (Lindblom et al., 2017) to 0.75 g/kg BW (De Vos et al., 2014), and mannitol has varied from 0.04 g/kg BW (Lindblom et al., 2017) to 0.3 g/kg BW (De Vos et al., 2014). Within this range, the 0.5 g/kg BW lactulose and 0.05 g/kg BW mannitol of Kansagra et al. (2003) have seen the most consistent use, possibly due to its similarity to the dose frequently used in human studies (a fixed dose of 10 g lactulose and 5 g mannitol; Sequeira et al., 2014a). Despite this variation, there are no studies that the author is aware of that have investigated the impact of variation in the dose of lactulose and mannitol on the excretion and resulting ratio of lactulose and mannitol in pigs.

Furthermore, accurate quantification of lactulose and mannitol output in studies of intestinal health requires total urine collection (Sequeira et al., 2014a). Unfortunately, quantitative urine collection in animals presents unique challenges (Kurien et al., 2004), and in pigs the most common method used is the metabolism cage, where a screen under the flooring allows feces to be retained while urine passes through and is collected via a pan. While metabolism cages are an accepted method for urine collection in pigs (Adeola, 2001), they do have some disadvantages. To

obtain urine from an individual pig, pigs must be housed individually which is contrary to the social nature of pigs and therefore may impose stress on the animal (Ruis et al., 2001). Furthermore, the number of metabolism crates available limits the number of animals that may undergo urine collection at a given time, placing constraints on experimental design. Alternatives to metabolism crates that would overcome these limitations are few. Urinary catheters present technical challenges in swine (Kurien et al., 2004; Musk et al., 2015) and preclude group housing, while attempting to catch voided urine in attached bags or pouches also precludes group housing given the inquisitive and destructive nature of swine (Feddes and Fraser, 1994). Therefore, alternative urine collection procedures that would enable group housing of pigs are needed.

Accordingly, Exp 3.1 was designed with the objective of determining the impact of variation in lactulose and mannitol dose on the excretion of lactulose and mannitol, while Exp 3.2 was designed to evaluate the suitability of a belly wrap as a replacement urine collection technique for metabolism cages. It was hypothesized in Exp 3.1 that variation in the dose of lactulose and mannitol would impact the resulting excretion of lactulose or mannitol, while in Exp 3.2 it was hypothesized that pigs with the alternative belly wrap urine collection method would have reduced urine output as compared to urine collected by metabolism crates.

3.3 Materials and Methods

3.3.1 Animals and Housing

All procedures involving live animals were approved by the Purdue University Animal Care and Use Committee (protocols #1801001673 and #1801001678A002) and animal husbandry practices were in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

In Exp 3.1, a total of 32 barrows with an initial BW of 5.3 ± 0.15 kg were weaned at 19.1 ± 0.22 d of age and placed into 4 nursery pens (8 pigs per pen). The experiment was conducted in 3 repetitions. Pigs for repetition 1 (8 pigs total) were weaned 1 d before pigs on repetition 2 (12 pigs total) and repetition 3 (12 pigs total) to allow urine collection for each repetition to occur 4 d post-weaning (i.e. repetition 1 experiment occurred 1 d before the repetition 2 and repetition 3 experiments). Therefore, the average weaning ages were 18.0 ± 0.00 d in repetition 1, and 19.4 ± 0.25 d in repetitions 2 and 3. Each nursery pen measured 1.22 × 1.37 m, had fully slatted plastic floors, and was fitted with a 5-hole gravity-flow feeder and a nipple cup waterer in a mechanically ventilated room at the Purdue Animal Science and Education Center Swine Research Farm (West Lafayette, IN). Pigs were *ad libitum* fed a medicated (carbadox, 55 ppm) commercially produced nursery phase I (23.5% CP, 1.60% SID Lys) pelleted feed. During the experimental period starting 4 d post-weaning, all pigs were individually housed in metabolism cages to facilitate quantitative urine collection over a 10 h period. Metabolism cages consisted of a main collection and living space of 72.4 cm wide × 86.4 cm long with a 58.4 cm wide × 38.1 cm long feeding area mounted on the front of the crate where a water nipple and feeding bowl were located. Pigs were fasted during the 10 h period spent in the metabolism cages but retained *ad libitum* access to water. Pigs were provided with one piece of manipulatable plastic pipe as enrichment and maintained visual, olfactory, and auditory contact with the other pigs in the room. Pigs were returned to their nursery pens following the urine collection period.

In Exp 3.2, 12 barrows were selected by BW (pigs selected from the center of the weight distribution) at 9 d post-weaning (28.8 ± 0.30 d of age; 6.9 ± 0.05 kg BW) from the same group of pigs used in Exp 3.2. Pigs were individually housed in metabolism crates for 8 h on d 9 post-weaning as described for Exp 3.1. Pigs were fasted during the 8 h urine collection period but

retained *ad libitum* access to water. Pigs were returned to their nursery pens following the urine collection period.

3.3.2 Experimental Procedures

In Exp 3.1, pigs were individually weighed on d 3 post-weaning, and 32 barrows were allotted to the experiment (average BW = 5.7 ± 0.41 kg per pig) in 3 repetitions with 8 pigs in repetition 1, 12 pigs in repetition 2, and 12 pigs in repetition 3. Four d post-weaning pigs were individually placed in metabolism crates at 0700 h (repetition 1, d 1), 0200 h (repetition 2, d 2), or 1330 h (repetition 3, d 2). Four h after being placed in metabolism crates, pigs were weighed and orally dosed with varying amounts of an aqueous solution of lactulose (Alfa Aesar, Thermo Fisher Scientific Chemicals, Inc., Ward Hill, MA) and D-mannitol (Acros Organics, Fair Lawn, NJ). Pigs received either Trt 1 (0.2 g/kg BW lactulose + 0.02 g/kg BW mannitol), Trt 2 (0.3 g/kg BW lactulose + 0.03 g/kg BW mannitol), Trt 3 (0.4 g/kg BW lactulose + 0.04 g/kg BW mannitol), or Trt 4 (0.5 g/kg BW lactulose + 0.05 g/kg BW mannitol). The target dose was achieved by varying the volume of solution delivered, with Trt 1 receiving 0.634 mL/kg BW, Trt 2 receiving 0.951 mL/kg BW, Trt 3 receiving 1.268 mL/kg BW, and Trt 4 receiving 1.585 mL/kg BW. The solution of lactulose and mannitol had an osmolarity of 1095 milliosmoles/L and was delivered with a 4-inch ball-tipped livestock drenching needle (Agri-Pro Enterprises of Iowa Inc., Iowa Falls, IA) attached to a syringe. Delivery of the oral dose to a pig took 3.5 ± 0.16 min on average.

In Exp 3.2, pigs were individually weighed at d 9 post-weaning and 12 barrows (6.9 ± 0.05 kg BW) were selected for the experiment and individually placed in metabolism crates at 0640 h. Four h after being placed into metabolism crates pigs were either given a belly wrap to collect urine and returned to the metabolism cage or were sham wrapped (pigs experienced handling and restraint as if they were receiving a belly wrap) and urine was collected via the metabolism cage.

The belly wrap consisted of pre-weighed quantity of cotton balls (2.76 g/kg BW) placed around the sheath, several layers of plastic food wrap (ClingWrap, Glad Products Company, Oakland, CA) to act as a moisture barrier, and elastic wrap (Elastikon, Johnson and Johnson, New Brunswick, NJ) wrapped around the pig from the caudal point of the shoulder to the cranial point of the hip to secure the belly wrap in place. While most of the elastic wrap was in contact with the plastic wrap for easy removal, approximately 1.3 cm of elastic wrap was in contact with the skin at the front and end of the wrap to prevent rotation of the belly wrap. The average time required to apply the belly wrap was 3 ± 0.3 min per pig.

3.3.3 Data Collection

In Exp 3.1 water intake and urine output were quantified from -4 to -0.5 h pre-oral dose, and from 0 to 2, 2 to 4, and 4 to 6 h post-oral dose. Water intake for each time period was quantified by filling the water nipple reservoir to a set volume at the start of a period and measuring the volume of water required to bring the reservoir back to the set volume at the end of a period. To correct water intake for water spilled by pigs during drinking, a pre-weighed bucket was placed under the slatted floor of the metabolism cage under the water nipple and the volume of spilled water for each period was determined gravimetrically. Urine output was quantified gravimetrically via two sets of pre-weighed buckets. After each urine collection period an aliquot of urine (approximately 5 mL) was obtained and frozen at -20°C. Buckets were washed with a 10% bleach solution between each collection period to minimize bacteria growth.

In Exp 3.2, urine was collected from all pigs via metabolism crates from -4 to -0.5 h pre-oral dose, and then from 0 to 4 h post-oral dose via belly wraps or via metabolism cages. Belly wraps were removed and cotton balls weighed to determine urine output at the end of the urine

collection period. Water intake was quantified from -4 to -0.5 h pre-oral dose, and from 0 to 2, and 2 to 4 h post-oral dose as described for Exp 3.1.

3.3.4 Laboratory Analysis

A HPLC system comprised of an eluent degasser (Series 1050, Hewlett-Packard, Palo Alto, CA), an autoloader (SIL-9A, Shimadzu, Columbia, MD), a single-piston pump (LC-6A, Shimadzu, Columbia, MD), a column oven (CTO-6A, Shimadzu, Columbia, MD), a calcium-form resin-based ionic column (Aminex HPX-87C, 300 mm x 7.8 mm, Bio-Rad Laboratories, Hercules, CA) protected by a de-ashing guard column (Micro-Guard De-Ashing Cartridges, Bio-Rad Laboratories, Hercules, CA), and a refractive index detector (HP 1047A, Hewlett-Packard, Palo Alto, CA) were used for urine analysis in Exp 3.1 (Catassi et al., 1991). Ultrapure water was used as the eluent, eluent flow rate was 0.6 mL/minute, column temperature was maintained at 65°C, injection volume was 20 µL, and the refractive index detector temperature was held at 50°C.

Stock solutions of lactulose (Alfa Aesar, Thermo Fisher Scientific Chemicals, Inc., Ward Hill, MA) and D-mannitol (Acros Organics, Fair Lawn, NJ) were prepared by dissolution of dry chemicals with ultrapure water. Standards ranging in concentration from 0.009 to 1.0 mg/mL were then created by dilution of stock solutions with ultrapure water. The area under the curve (AUC) for the standards analyzed in duplicate was linear through the concentrations evaluated. The standard curve generated for mannitol was $y = 7.8369x – 0.0426$ with an $R^2 = 0.999$, and the standard curve for lactulose was $y = 7.7351x – 0.0136$ with an $R^2 = 0.999$ where y is the AUC and x is the concentration of mannitol or lactulose in mg/mL. Urine concentrations were approximately equalized by using urochrome levels as an indicator of urine concentration (Kavouras et al., 2016) and diluting with ultrapure water. Urine was filtered through a 0.2 µm PTFE syringe filter (17 mm disk, Thermo Scientific, Waltham, MA) prior to injection. Urine collected shortly before the oral

dose of lactulose and mannitol was also analyzed via HPLC to generate pre-oral dose chromatograms for each pig. An excel-based (Microsoft, Redmond, WA) integration tool was used to subtract pre-oral dose matrix peaks from the post-oral dose chromatograms to allow accurate and rapid integration of the lactulose and mannitol peaks.

3.3.5 Calculations and Statistical Analysis

In Exp 3.1 absolute urinary excretion of mannitol or lactulose in mg for each pigs was calculated as the concentration of analyzed mannitol or lactulose at a given time point multiplied by the urine output at a given time point. Excretion of mannitol or lactulose corrected for urine void interval was calculated for each pig by multiplying the concentration of mannitol or lactulose in urine at a given time point by urine output at a given time point, and then dividing by the number of hours since the last urine collection period where urine was obtained. This urine void interval correction was used to account for urine collection periods where no urine was voided. In repetition 1 this correction was applied to 2 pigs at 2 h and 4 pigs at 4 h post-oral dose. In repetition 2 this correction was applied to 6 pigs at 2 h and 5 pigs at 4 h post-oral dose. In repetition 3 this correction was applied to 4 pigs at 2 h, and 4 pigs at 4 h post-oral dose. In Exp 3.2, urine output for each pig with a belly wrap was calculated as the difference between the weight of cotton balls removed from the belly wrap and the dry weight of cotton balls.

In both experiments pigs were allotted to treatments in a randomized complete block design with initial BW as the blocking factor. The individual pig was considered the experimental unit. For Exp 3.1, data were analyzed with Proc Glimmix of SAS 9.4 (SAS Institute, Carry, NC) with treatment (4 doses of lactulose + mannitol) as a fixed effect, and with repetition and BW block as random effects. For Exp 3.2, data were analyzed with Proc Glimmix with urine collection method (metabolism cage vs belly wrap) as a fixed effect and BW block as a random effect. In both

experiments, data collected over time were analyzed with repeated measures with the time unit as a fixed effect using the optimal covariance structure for each response variable as determined by goodness of fit criteria (Littell et al., 1998). A Kenward-Roger degrees of freedom correction was applied to all repeated measures analyses via the ddfm=kr option of the model statement (Kenward and Roger, 1997). Means were separated with a Tukey's adjustment where appropriate. Results are presented as least square means ± 1 SEM unless otherwise stated. Significance was declared at $P < 0.05$, and a tendency towards significance was declared at $0.05 \leq P < 0.10$.

3.4 Results

3.4.1 Experiment 3.1

The percentage of pigs that urinated at a given time point fluctuated by repetition but averaged 90% at -4 to -0.5 h, 49% at 2 h, 47% at 4 h, and 53% at 6 h (Figure 3.1). The poorest rates of successful urine collection were 25% at 2 h post-oral dose for repetition 2, and 25% at 6 h post-oral dose for repetition 3. Of the post-oral dose urine collection, the greatest success rate was 83% at 6 h for repetition 2.

There was no impact of lactulose and mannitol dose, time period, or their interaction on water intake ($P \geq 0.22$; Table 3.1). Similarly, there was no impact of lactulose and mannitol dose or their interaction with time ($P \geq 0.26$; Table 3.1) for urine output, however, there was a main effect of time ($P = 0.02$) with urine output at 6 h (6.7 ± 1.32 g/h) being greater than urine output at 2 h (2.9 ± 1.30 g/h) and 4 h (3.0 ± 1.29 g/h) post-oral dose.

Absolute mannitol excretion was not impacted by lactulose and mannitol treatment or an interaction of treatment with time ($P \geq 0.93$; Figure 3.2), however, time post-oral dose affected ($P < 0.01$) mannitol excretion with excretion at 6 h (411 ± 93.5 mg) being greater than excretion at 2

h (50 ± 37.4 mg), with 4 h (209 ± 68.5 mg) being intermediate. Similarly, absolute lactulose recovery was not impacted by lactulose and mannitol dose (P = 0.13; Figure 3.3), but there was an effect of time (P < 0.01) with lactulose excretion at 6 h (358 ± 69.2 mg) and 4 h (289 ± 95.6 mg) being greater than 2 h (37 ± 25.9 mg) post-oral dose. In addition, there was a tendency (P = 0.07) for an interaction between lactulose and mannitol dose and time with absolute lactulose recovery for Trt 2 at 6 h tending to be greater than Trt 2, 3, and 4 at 2 h, and with Trt 3 at 6 h tending to be greater than Trt 1, 2, 3, and 4 at 2 h post-oral dose.

Mannitol excretion corrected for urine void interval was not impacted by lactulose and mannitol treatment or by an interaction of treatment with time ($P \geq 0.74$; Figure 3.4), however, time post-oral dose affected (P = 0.02) mannitol excretion per h with excretion at 6 h (122 ± 21.1 mg/h) being greater than excretion at 2 h (36 ± 21.1 mg/h), with 4 h (84 ± 21.8 mg/h) being intermediate. Lactulose excretion corrected for urine void interval was not affected by lactulose and mannitol dose (P = 0.38; Figure 3.5), but there was an impact of time (P < 0.01) with 4 h (112 ± 35.6 mg/h) and 6 h (112 ± 20.4 mg/h) being greater than 2 h (21 ± 9.2 mg/h). In addition, there was a tendency (P = 0.08) for lactulose and mannitol dose to interact with time, with Trt 3 at 6 h post-oral dose tending to be greater than Trt 2 and 3 at 2 h post-oral dose.

Total mannitol excretion over the 6 h urine collection period was not impacted by dose (P = 0.84; Figure 3.6) and averaged 384 ± 157.8 mg. There was no impact of lactulose and mannitol dose (P = 0.52; Figure 3.7) on total lactulose excretion, however, there was a numerical decrease for Trt 1 (174 ± 171.8 mg) as compared to the other three treatments (average of 448 ± 161.3 mg). Similarly, there was no impact of dose on the ratio of lactulose to mannitol (P = 0.45; Figure 3.8), but there was a numerical decrease for Trt 1 (0.26 ± 0.727) compared to the other treatments (average of 1.58 ± 0.641).

3.4.2 Experiment 3.2

There was no effect of urine collection method on water intake or urine output prior to application of the belly wrap ($P \geq 0.30$; Table 3.2). Similarly, there was no impact of urine collection method, time, or their interaction ($P \geq 0.17$) on water intake after application of the belly wrap, with water intake averaging 17 ± 11.0 g/h. Urine collection method did not impact urine output after the application of the belly wrap ($P = 0.15$).

3.5 Discussion

3.5.1 Experiment 3.1

In human studies of temporal lactulose and mannitol excretion, urine is voided at set intervals to ensure that subject's urinary bladders are empty at the end of each collection period (Sequeira et al., 2014b). Voluntary urine collection with pigs is more challenging (Wijtten et al., 2011). Compared to urine collection data from our lab with pigs at 2 to 3 weeks post-weaning (unpublished data), the ability to obtain urine from pigs at set intervals in the present study was relatively poor. Given the myriad of stresses pigs face at weaning (Jayaraman and Nyachoti, 2017), these pigs may have been less able to cope with additional stresses imposed by handling and restraint associated with the lactulose and mannitol test (Wijtten et al., 2011) and by social isolation (Ruis et al., 2001) than older pigs. The increase in urine output observed at 6 h in the present study could be an indication that pigs were starting to recover from these stresses. The successful urine collection rate of 49.7% (average of the success rates from 0 to 2 h, 2 to 4 h, and 4 to 6 h urine collection periods) obtained in the present study is reflective of results from a similar experiment, where, in a study of intestinal permeability in individually housed pigs, 57% of pigs at 4 d post-

weaning did not urinate over an 18 h urine collection period (Wijtten et al., 2011). Therefore, the cause of the low urine recovery in the present study may be a reflection of the combination of weaning stresses and experimental stresses as postulated by Wijtten et al. (2011). Indeed, Wijtten et al. (2011) reported dramatically improved urination rates from pigs at 12 d post-weaning, with only 10% of pigs failing to urinate over the 18 h collection period compared to 47% at 4 d post-weaning. Although the exact cause is unknown, difficulty in reliably obtaining urine from recently weaned pigs may be a substantial obstacle to use of the lactulose and mannitol test of intestinal permeability in recently weaned pigs. Reliable voiding of urine is crucial in the lactulose and mannitol test of intestinal permeability as variation in urine voiding will directly translate to variation in the calculated lactulose and mannitol output. The difficulty in reliably obtaining urine from newly weaned pigs is unfortunate, as the rapidly changing gut health in the immediate post-weaning period is of particular interest (Jayaraman and Nyachoti, 2017). While there are methods, such as bladder catheterization (Kansagra et al., 2003), that allow urine to be collected at set points, these methods present technical challenges and are invasive (Musk et al., 2015). Thus, development of methodology to minimize the stresses experienced by pigs during the lactulose and mannitol test to improve urine collection reliability may be required if the test is going to be practically useful for gut health research in nursery pigs.

In general, excretion of mannitol in human urine is typically greatest around 2 h post-ingestion of mannitol and is primarily complete around 6 h post-ingestion (Sequeira et al., 2012; Sequeira et al., 2014b). Lactulose excretion in human urine tends to be weakly bimodal, with a broad peak at 1 to 3 h post-ingestion representing small intestinal absorption, and a second broad peak around 5 to 6 h post-ingestion derived from large intestinal absorption (Sequeira et al., 2012; Sequeira et al., 2014b). In the present study, both absolute and urine void interval corrected

mannitol output increased over time without a defined peak in excretion while both absolute and urine void interval corrected lactulose output increased from 2 to 4 h and then was constant from 4 to 6 h post-oral dose. The lack of mannitol excretion peak at 2 to 4 h post-oral dose and the sustained excretion of lactulose from 4 to 6 h post-oral dose can be hypothesized to result from the difficulty in obtaining urine at each interval in the present study resulting in variable delays in urine voiding compared to human studies.

Despite large variation in the reported dose of lactulose and mannitol given to pigs, there are no studies that the author is aware of that have investigated the impact of variation in the dose of lactulose and mannitol on the excretion of lactulose and mannitol. In the present study, despite large numerical changes among treatments, the large variation from pig to pig within a treatment precludes statistical differences. As a result, it is not clear whether the results support or disprove our hypothesis that changes in the dose of lactulose and mannitol would impact the excretion of lactulose or mannitol. Additional research with alternate methods yielding less pig-to-pig variation is required to further explore this possibility.

3.5.2 Experiment 3.2

While most of the human urine samples needed for medical tests or experiments are easily obtained via free-catch methods (Ridley, 2018), alternative methods are needed for urine collection from human infants and toddlers. Alternative methods include urine collection pads placed within the child's diaper (Rao et al., 2004) or via plastic urine collection bags (Karacan et al., 2010). Although limited studies on alternative methods of urine collection in pigs have been conducted, Wijtten et al. (2011) utilized pouches taped and glued to the belly of male pigs to collect urine. The study demonstrated two problems: first, the percentage of pigs that urinated into the pouches over an 18 h period was less than 50% in some cases, and second, it is unlikely that this design

would be feasible in group housed animals. Therefore, in the present study, the use of a belly wrap containing a urine collection pad (absorbent cotton balls) was investigated for suitability in weanling pigs. It had been hypothesized that the belly wrap could cause inhibition of normal behaviors including drinking or urination that would lead to decreased urine output. However, the belly wrap did not impact water intake or urine output.

One reported limitation of alternative urine collection techniques for use in human infants and toddlers is loss of the collection device during urine collection (Karacan et al., 2010). Similar results were found in the present study. Even though pigs were individually housed, one pig was able to remove the belly wrap before the completion of the urine collection time leading to loss of urine collection data, while another pig shifted the belly wrap leading to some cotton balls escaping from the wrap towards the end of the collection period. Thus, the 1.3 cm of elastic wrap skin contact was not sufficient in all cases to secure the belly wrap. Use of belly wraps in group housed animals would likely require greater elastic wrap contact with the skin. An additional strategy could be placing the cotton balls within a cotton mesh bag or other device to aid in preventing loss of individual cotton balls in the event of alterations in the fit of the belly wrap over the urine collection period. Additional testing is required before it can be determined if the belly wrap is suitable for urine collection with group housed pigs.

The necessity of estimation of the correct volume of absorbent cotton is one limitation of the belly wrap. The volume of cotton balls was estimated from urine output data from pigs of similar ages from our laboratory (unpublished data). For quantitative collection, enough cotton must be present to absorb all urine that is produced. If insufficient cotton is present, urine may not be absorbed and thus could leak from the belly wrap. If too much cotton is present, there may not be enough urine present in an individual cotton ball to allow a urine sample to be expressed. Due to

the unexpected numerically increased urine output from pigs with belly wraps, urine volumes obtained were somewhat greater than anticipated, and cotton balls were approaching saturation when removed from pigs. Therefore, a cotton ball quantity somewhat greater than the 2.76 g/kg BW used here may be required to allow a margin of error.

3.6 Conclusions

In Exp 3.1, pigs had more variable urination rates than anticipated, likely leading to the substantial variation observed in the mannitol and lactulose excretion. Variable urination of pigs post-weaning may be one obstacle to the use of a lactulose and mannitol test of intestinal health in pigs and warrants research on how to improve voluntary urine voiding from newly weaned pigs. Additional research is required to elucidate potential impacts of variation in the dose of lactulose and mannitol given on lactulose and mannitol urinary excretion. If excretion does vary by dose, then a standard dose of lactulose and mannitol should be adopted to promote the accuracy of results and promote comparability among studies of gut health in nursery pigs.

In Exp 3.2, the use of belly wraps as an alternative method of urine collection was evaluated. Similarity in water intake and urine output between pigs with belly wraps and pigs without belly wraps indicate that belly wraps show promise as an alternative urine collection method to metabolism crates. Additional research is required to evaluate the performance of belly wraps in group housed pigs. If the latter is successful, belly wraps could provide a method to collect urine from pigs without the need to remove pigs from familiar environments or socially isolate them from conspecifics, thereby reducing the stress of the pig and potentially improving urine output.

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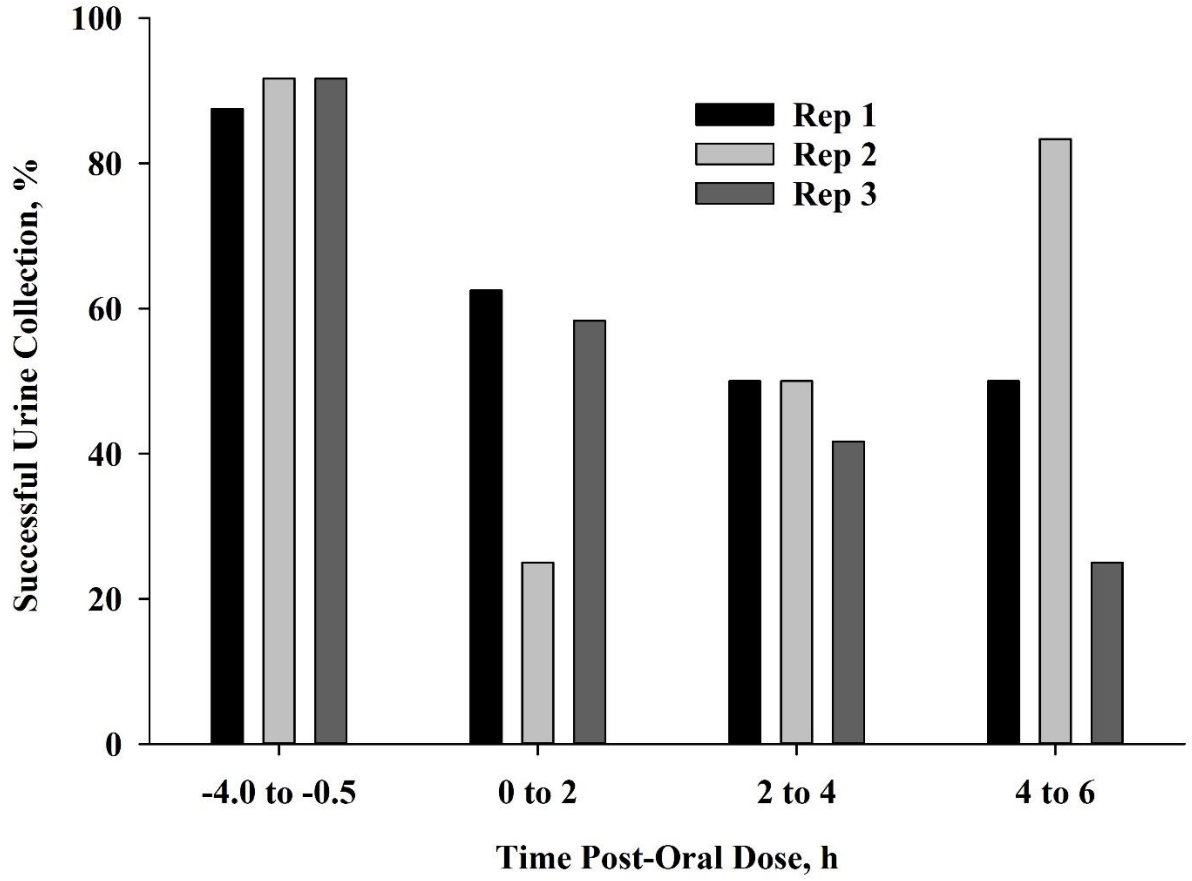

Figure 3.1. Percentage of pigs across three repetitions that urinated during a 3.5 h period prior to receiving an oral dose of lactulose and mannitol in water (time 0 h), and then from 0 to 2 h, 2 to 4 h, and 4 to 6 h post-oral dose in Exp 3.1

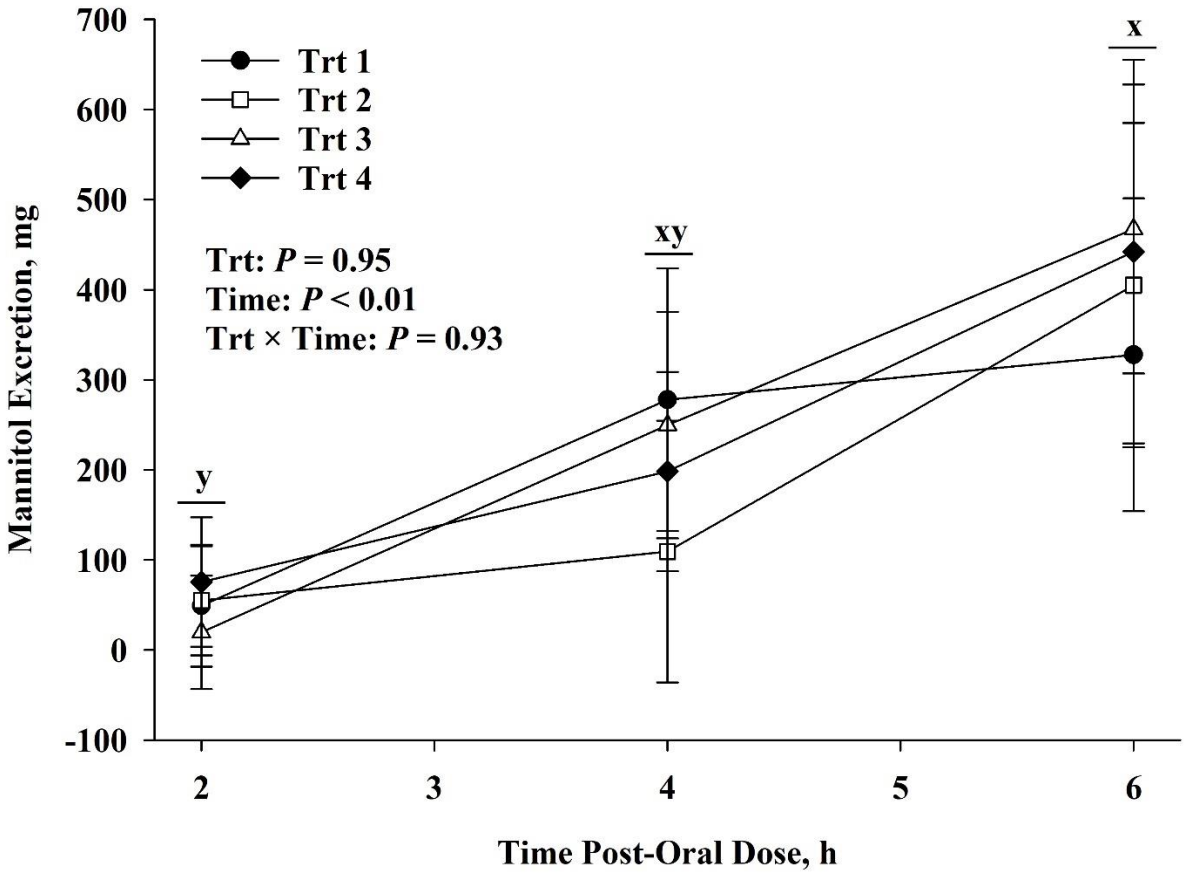

Figure 3.2. Absolute mannitol excretion at 2, 4, and 6 h post-oral dose from pigs (n = 8/trt; 4 d post-weaning) that received either Trt 1 (0.2 g/kg BW lactulose + 0.02 g/kg BW mannitol), Trt 2 (0.3 g/kg BW lactulose + 0.03 g/kg BW mannitol), Trt 3 (0.4 g/kg BW lactulose + 0.04 g/kg BW mannitol), or Trt 4 (0.5 g/kg BW lactulose + 0.05 g/kg BW mannitol) in Exp 3.1. Times not sharing a common letter (x, y) differ ($P < 0.05$). Error bars represent ± 1 SEM.

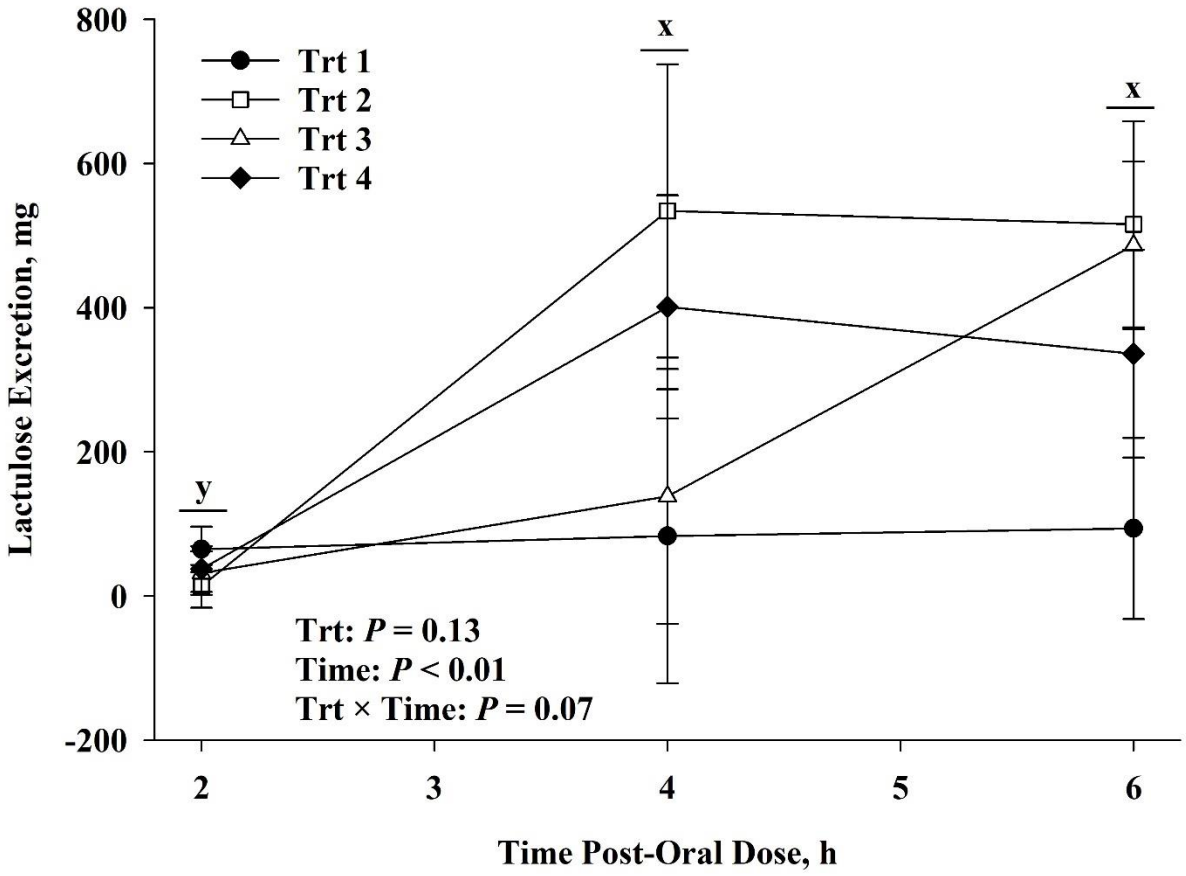

Figure 3.3. Absolute lactulose excretion at 2, 4, and 6 h post-oral dose from pigs (n = 8/trt; 4 d post-weaning) that received either Trt 1 (0.2 g/kg BW lactulose + 0.02 g/kg BW mannitol), Trt 2 (0.3 g/kg BW lactulose + 0.03 g/kg BW mannitol), Trt 3 (0.4 g/kg BW lactulose + 0.04 g/kg BW mannitol), or Trt 4 (0.5 g/kg BW lactulose + 0.05 g/kg BW mannitol) in Exp 3.1. Times not sharing a common letter (x, y) differ ($P < 0.05$). Error bars represent ± 1 SEM.

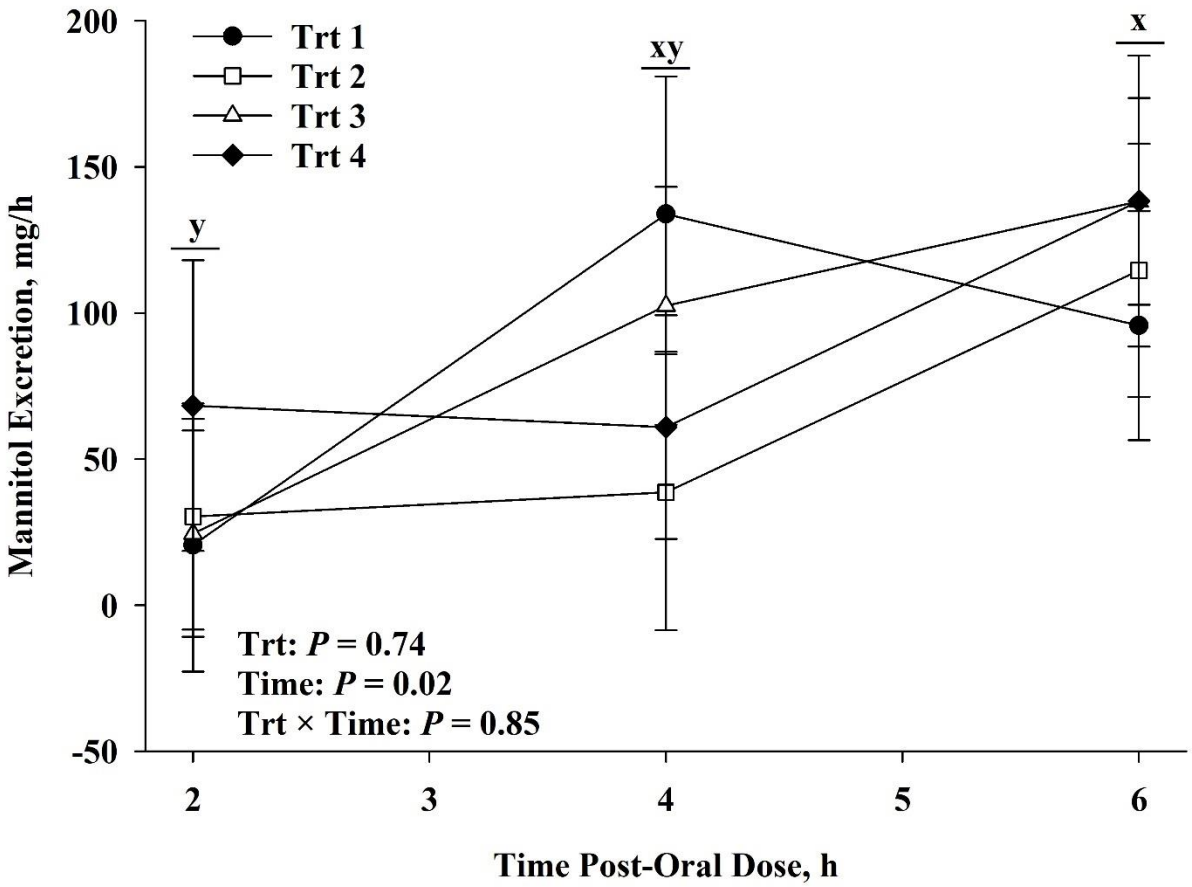

Figure 3.4. Urine void interval corrected mannitol excretion at 2, 4, and 6 h post-oral dose from pigs (n = 8; 4 d post-weaning) that received either Trt 1 (0.2 g/kg BW lactulose + 0.02 g/kg BW mannitol), Trt 2 (0.3 g/kg BW lactulose + 0.03 g/kg BW mannitol), Trt 3 (0.4 g/kg BW lactulose + 0.04 g/kg BW mannitol), or Trt 4 (0.5 g/kg BW lactulose + 0.05 g/kg BW mannitol) in Exp 3.1. Times not sharing a common letter (x, y) differ ($P < 0.05$). Error bars represent ± 1 SEM.

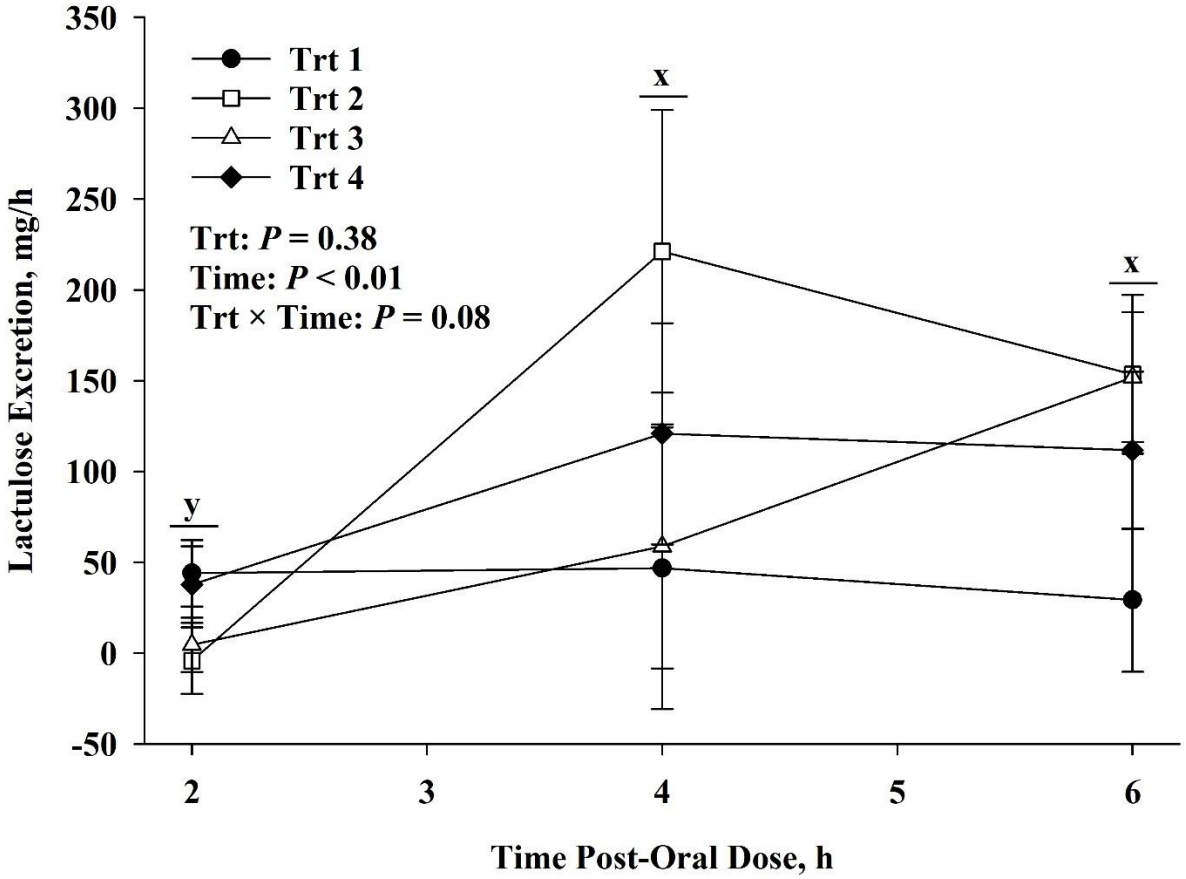

Figure 3.5. Urine void interval corrected lactulose excretion at 2, 4, and 6 h post-oral dose from pigs (n = 8/trt; 4 d post-weaning) that received either Trt 1 (0.2 g/kg BW lactulose + 0.02 g/kg BW mannitol), Trt 2 (0.3 g/kg BW lactulose + 0.03 g/kg BW mannitol), Trt 3 (0.4 g/kg BW lactulose + 0.04 g/kg BW mannitol), or Trt 4 (0.5 g/kg BW lactulose + 0.05 g/kg BW mannitol) in Exp 3.1. Times not sharing a common letter (x, y) differ ($P < 0.05$). Error bars represent ± 1 SEM.

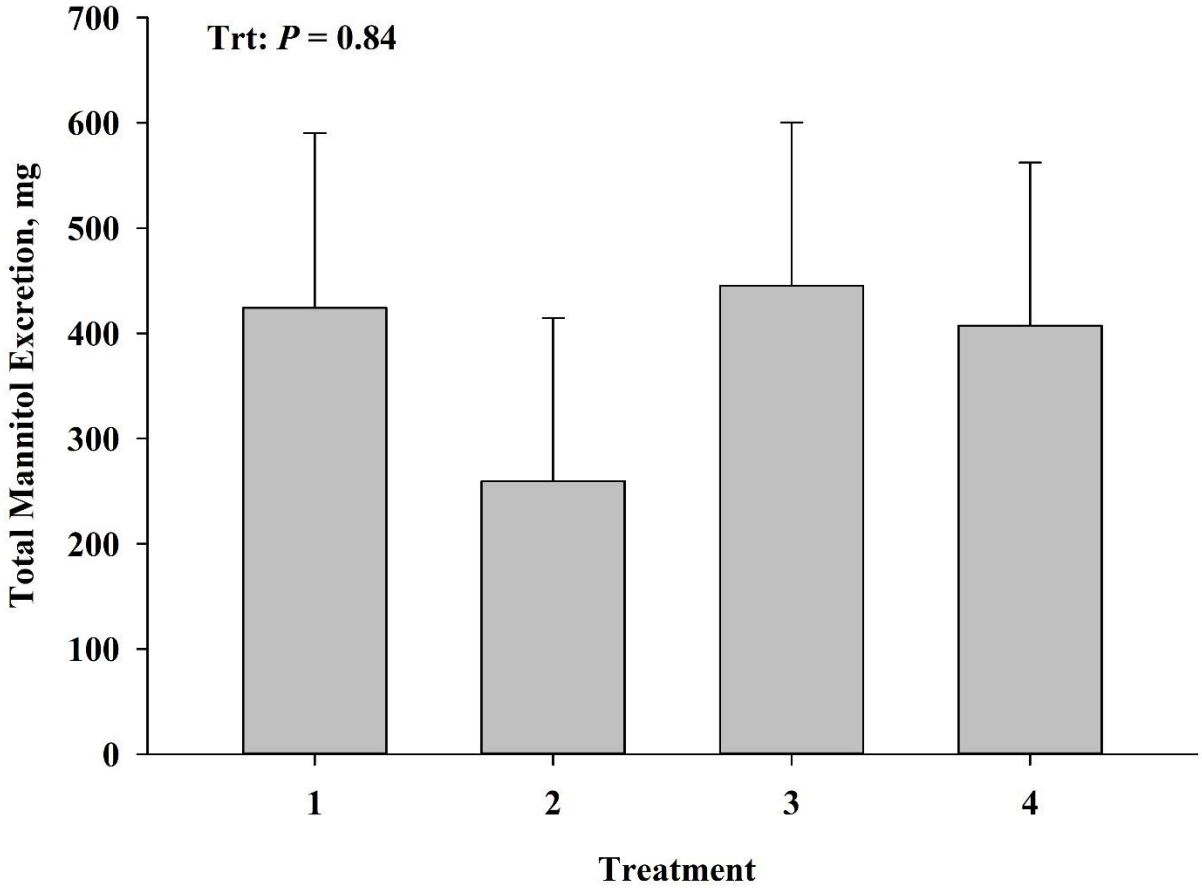

Figure 3.6. Total mannitol excretion over the 6 h urine collection period from pigs (n = 8/trt; 4 d post-weaning) that received either Trt 1 (0.2 g/kg BW lactulose + 0.02 g/kg BW mannitol), Trt 2 (0.3 g/kg BW lactulose + 0.03 g/kg BW mannitol), Trt 3 (0.4 g/kg BW lactulose + 0.04 g/kg BW mannitol), or Trt 4 (0.5 g/kg BW lactulose + 0.05 g/kg BW mannitol) in Exp 3.1. Error bars represent ± 1 SEM.

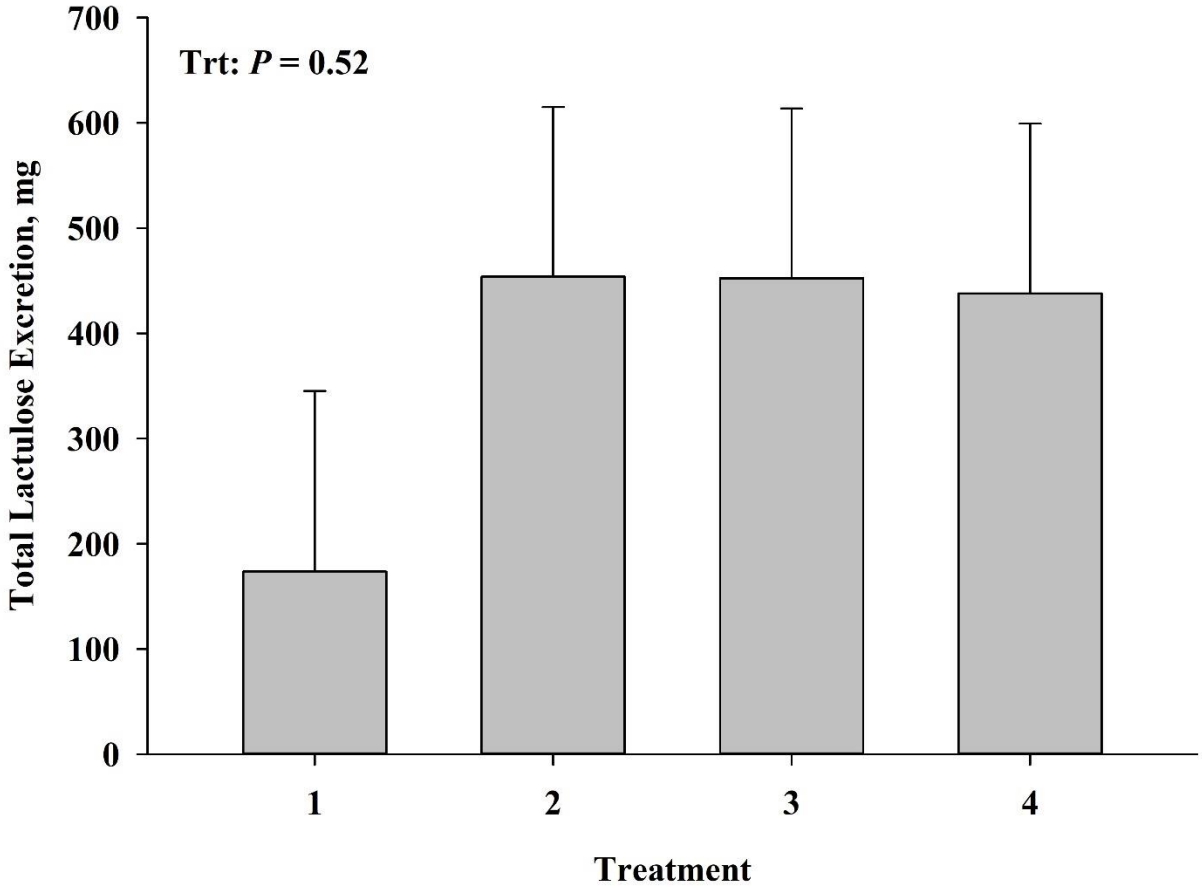

Figure 3.7. Total lactulose excretion over the 6 h urine collection period from pigs (n = 8/trt; 4 d post-weaning) that received either Trt 1 (0.2 g/kg BW lactulose + 0.02 g/kg BW mannitol), Trt 2 (0.3 g/kg BW lactulose + 0.03 g/kg BW mannitol), Trt 3 (0.4 g/kg BW lactulose + 0.04 g/kg BW mannitol), or Trt 4 (0.5 g/kg BW lactulose + 0.05 g/kg BW mannitol) in Exp 3.1. Error bars represent ± 1 SEM.

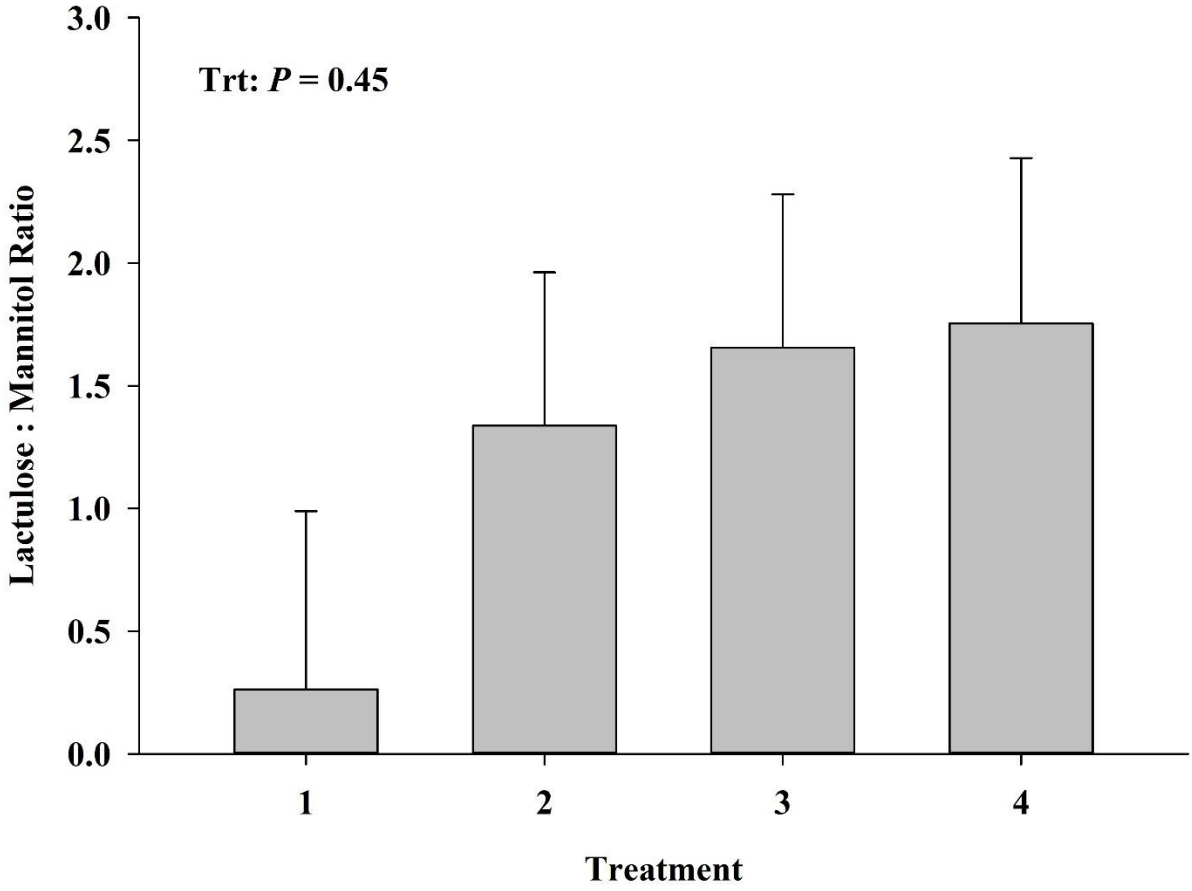

Figure 3.8. The ratio of total lactulose to total mannitol excretion over the 6 h urine collection period from pigs (n = 8/trt; 4 d post-weaning) that received either Trt 1 (0.2 g/kg BW lactulose + 0.02 g/kg BW mannitol), Trt 2 (0.3 g/kg BW lactulose + 0.03 g/kg BW mannitol), Trt 3 (0.4 g/kg BW lactulose + 0.04 g/kg BW mannitol), or Trt 4 (0.5 g/kg BW lactulose + 0.05 g/kg BW mannitol) in Exp 3.1. Error bars represent ± 1 SEM.

Table 3.1. Body weight, water intake, and urine output of pigs (n = 8 per treatment; 4 d post-weaning) given one of four quantities of a solution of lactulose and mannitol at time 0 h in Exp 3.1

	Treatment[1]					***P*-Value**		
Item	**1**	**2**	**3**	**4**	**SEM**	**Trt**	**Time**	**Trt × Time**
BW, kg	5.8	5.6	5.8	5.7	0.41	0.88	-	-
Water intake, g/h								
-4 to -0.5 h	5.2	14.8	8.2	10.8	5.04	0.22	-	-
0 to 2 h[2]	8.1	11.0	12.8	7.0	3.51	0.92	0.85	0.87
2 to 4 h	8.3	8.6	9.1	9.5				
4 to 6 h	11.6	8.3	11.2	8.9				
0 to 6 h[3]	9.3	9.2	10.9	8.4	2.56	0.94	-	-
Urine output, g/h								
-4 to -0.5 h	7.3	8.0	4.9	4.3	1.70	0.32	-	-
0 to 2 h[2]	2.8	2.4	4.0	2.5	2.29	0.26	0.02	0.29
2 to 4 h	4.6	2.9	1.8	2.6				
4 to 6 h	6.7	2.6	12.3	5.3				
0 to 6 h[3]	4.8	2.8	5.7	3.6	1.36	0.42	-	-

[1]Pigs received one of four treatments: Trt 1, 0.2 g/kg BW lactulose + 0.02 g/kg BW mannitol; Trt 2, 0.3 g/kg BW lactulose + 0.03 g/kg mannitol; Trt 3, 0.4 g/kg BW lactulose + 0.04 g/kg BW mannitol; Trt 4, 0.5 g/kg BW + 0.05 g/kg mannitol.

[2]The SEM and *P*-values within this row are from a repeated measures analysis of all three time periods (0 to 2 h, 2 to 4 h, and 4 to 6 h) with time period as the repeated measure and treatment as a fixed effect.

[3]The SEM and *P*-value within this row are from an analysis of the average response regardless of time period with treatment as a fixed effect.

Table 3.2. Water intake and urine output from pigs housed in metabolism cages with or without a belly wrap (applied at time 0 h) to collect urine in Exp 3.2

	Collection Method			***P*-Value**		
Item	**Belly Wrap[1]**	**Metabolism Cage**	**SEM**	**Method**	**Time**	**Method × Time**
n	5	6		-	-	-
Initial BW, kg	6.8	7.0	0.05	0.23	-	-
Water intake, g/h						
-4 to -0.5 h	1.0	20.0	11.65	0.30	-	-
0 to 2 h[2]	15.0	20.4	11.00	0.86	0.78	0.17
2 to 4 h	22.6	9.8				
0 to 4 h[3]	18.4	15.3	6.86	0.77	-	-
Urine output, g/h						
-4 to -0.5 h	3.6	6.3	2.47	0.42	-	-
0 to 4 h	22.2	11.8	4.09	0.15	-	-

[1]Belly wrap consisted of a weighed amount of cotton balls to absorb urine, a layer of plastic, and elastic adhesive wrap placed around the sheath of male pigs.
[2]The SEM and *P*-values within this row are from a repeated measures analysis of both time periods (0 to 2 h and 2 to 4 h) with time period as the repeated measure and urine collection method as a fixed effect.
[3]The SEM and *P*-value within this row are from an analysis of the average response regardless of time period with urine collection method as a fixed effect.

CHAPTER 4. HEALTH AND PRODUCTIVITY OF PIGS FED GLUTAMINE, SYNBIOTICS, OR BOTH, FOLLOWING WEANING AND TRANSPORT: WHOLE BODY MEASURES

4.1 Abstract

The additive effects of supplementing 0.2% L-glutamine and a synbiotic mixture on pig growth performance and health were investigated. Pigs were weaned (19.4 ± 0.23 d of age; N = 226, 5.9 ± 0.11 kg) and transported in a livestock trailer for 12 h. Pigs were placed into wean-to-finish pens (n = 8 pens/trt) and received diets consisting of a negative control (NC) with no dietary antibiotics, 0.2% supplementation of L-glutamine (GLN), supplementation of *Lactobacillus* + fructooligosaccharide + β-glucan (SYN), the combination of GLN and SYN additives (GLN+SYN), and a positive control (PC) containing dietary antibiotics. Treatment diets were fed for the first two weeks of the nursery period, and common antibiotic free diets were fed thereafter. Growth performance was monitored through the nursery (5 weeks) and grow-finish periods (~18 weeks). Blood, photographs of tear staining around the eyes of pigs, and therapeutic antibiotic injection rate data were collected throughout the nursery period. Data were analyzed via Proc Glimmix of SAS with time-course data analyzed as repeated measures where appropriate. For nursery growth performance in the dietary treatment phase, diet interacted with week (P = 0.02) with BW and ADG for PC pigs going from the least at week 1 to the greatest at week 2 and GLN pigs going from the greatest at week 1 to being intermediate in week 2. In the common diet period of the nursery, contrasts indicated an increase (P = 0.03; 13%) in ADFI for the PC over the NC, and a reduction (P = 0.03; 7%) in the gain to feed ratio for GLN pigs compared to the NC pigs. For the impact of nursery diet on subsequent performance during the grow-finish period, a reduction in gain to feed was observed in Phase 1 (end of the 5-week nursery period to d 26 of the

grow-finish period) for the SYN (*P* = 0.04; 5%) and the GLN+SYN (*P* = 0.03; 5%) treatments compared to the NC. In Phase 6 (d 111 to d 124 of the grow-finish period), ADG was improved for the GLN (*P* = 0.03; 16%) and SYN (*P* = 0.02; 17%) treatments over that of the NC. Pigs on the PC treatment consumed less feed (*P* = 0.02) than the SYN and NC treatments in grow-finish Phase 6. This led to greater feed efficiency (*P* = 0.02; 15%) for the PC and GLN treatments than the NC in grow-finish Phase 6. Therapeutic antibiotic use did not vary by treatment and was administered primarily for enteric challenges. Therapies for enteric challenges averaged 41 ± 6.5% in the first 14 d of the nursery period. Haptoglobin levels in plasma were measured as an indicator of stress or infection, and a contrast indicated a reduction (*P* = 0.01) in average nursery (d 2, 6, 13) haptoglobin for the PC (172 ± 107.5 µg/mL) vs. the NC (563 ± 106.7 µg/mL) diet. Dietary treatment did not significantly impact leukocyte differential counts at d 2, 6, 13, or 32 post-weaning, tear staining area at d 0, 7, 14, 21, 28, or 32 post-weaning, or pig behavior at d 2, 6 or 13 post-weaning. Therefore, dietary antibiotic inclusion improved some aspects of pig growth performance and welfare during the nursery period. Nursery period administration of dietary glutamine or synbiotics, but not their combination, improved some aspects of pig growth performance during Phase 6 of the grow-finish period.

Keywords: antibiotic alternative, pig, glutamine, synbiotic, growth performance

4.2 Introduction

As legislation and consumer pressure reduce the use of antibiotics in food production, swine producers must find consumer-accepted ways to help nursery pigs overcome the stresses encountered at weaning (de Lange et al., 2010). Weaning is considered the most stressful period in a pig's life, and these stresses can lead to reduced growth performance and increased morbidity

for several weeks after weaning (Jayaraman and Nyachoti, 2017). Antibiotics have traditionally been used to help pigs overcome these stresses, and antibiotic use in the nursery stage of pig production cannot simply be eliminated due to economic and welfare implications. A potential solution could be alternative products that consumers would view as more natural and that do not have potential to lead to antimicrobial resistance (Thacker, 2013). Accordingly, a plethora of feed additives with differing modes of action have been tested as replacements for antibiotics in the nursery phase of pig production (Pluske et al., 2018); however, many feed additives that have been explored are plagued by inconsistent results as compared to dietary antibiotics (de Lange et al., 2010). This could be attributed to the relatively poor understanding of the mode of action of many of these products, as well as a limited grasp on how these products perform in different production settings (Pluske et al., 2018).

L-glutamine, a conditionally essential amino-acid found in high levels in sow milk, is one potential feed additive with substantial research behind it (Wu, 2009). When fed to nursery pigs at levels of 0.5 to 1.5% of the diet, glutamine has been repeatedly shown to improve metrics of intestinal health and growth performance (Watford, 2015). Reduced levels of supplemental glutamine would improve the economics of glutamine supplementation, and there is some evidence that glutamine fed at 0.2% of the diet may still be effective at improving pig health and growth performance (Johnson and Lay, 2017). In addition, synbiotics (a combination of prebiotics and probiotics) have potential to reduce pathogen load in the intestine (Heo et al., 2013; Yang et al., 2014), thereby improving pig health and performance. Therefore, the study objective was to determine if combining dietary synbiotics (*Lactobacillus* + fructo-oligosaccharides + β-glucan) and 0.20% supplemental glutamine will result in a greater improvement in pig health and performance following weaning and transport when compared to dietary antibiotics

(chlortetracycline + tiamulin). Therefore, we hypothesize that administering glutamine and synbiotics after exposure to weaning and transport stress will have an additive effect to improve pig health and welfare over that of dietary antibiotics.

4.3 Materials and Methods

4.3.1 Animals and Diets

All procedures involving live animals were approved by the Purdue University Animal Care and Use Committee (protocol #1801001678A002) and animal husbandry practices were in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

Mixed-sex pigs (n = 226, 5.9 ± 0.11 kg; Duroc × (Landrace × Yorkshire) were individually weighed 1 d prior to weaning and allotted to 5 treatments in a randomized complete block design (n = 8 pens per treatment blocked by BW; 4 pens with 5 pigs and 4 pens with 6 pigs per pen) with sex ratios in pens maintained within BW blocks and balanced for ancestry. Pigs were weaned (19.4 ± 0.23 d of age) and transported for 12 h in May of 2018. Pigs were then placed into pens in a mechanically-ventilated wean-to-finish facility and grown to market weight. Barn average temperature and relative humidity (Hobo data logger, Onset, Bourne, MA) for the nursery period were: 29.5 ± 0.02°C and 35.7 ± 0.43% for week 1; 28.4 ± 0.03°C and 47.4 ± 0.45% for week 2; 26.7 ± 0.01°C and 53.9 ± 0.47% for week 3; 27.9 ± 0.07°C and 57.7 ± 0.71% for week 4; and 26.2 ± 0.05°C and 54.2 ± 0.77% in week 5, respectively. Barn average temperature and relative humidity for the grow-finish period were: 26.9 ± 0.05°C and 73.5 ± 0.23% for phase 1 (end of the 5-week nursery period to d 26 of the grow-finish period); 26.4 ± 0.05°C and 70.5 ± 0.32% for phase 2 (d 27 to d 47 of the grow-finish period); 25.4 ± 0.06°C and 71.2 ± 0.30% for phase 3 (d

48 to d 68 of the grow-finish period); 25.6 ± 0.08°C and 73.1 ± 0.34% for phase 4 (d 69 to d 89 of the grow-finish period); and 24.9 ± 0.08°C and 63.4 ± 0.38% in phase 5 (d 90 to d 110 of the grow-finish phase), respectively. Temperature and humidity data were not obtained for phase 6 (d 111 to d 124 of the grow-finish phase) due to data logger failure. Pens had fully slatted concrete floors, measured 1.8 meters × 2.4 meters, and contained a single-hole gravity feeder and a nipple waterer. One pig per pen was removed at 14 and 34 d post-weaning for tissue collection for a related project, leaving 3 (n = 4 pens per treatment) or 4 (n = 4 pens per treatment) pigs per pen for the grow-finish period.

Dietary treatments (Table 4.1) consisted of a negative control (NC) containing no dietary antibiotics, the NC plus 0.2% L-glutamine (GLN) (Ajinomoto North America Inc., Raleigh, NC), the NC plus a probiotic (*Lactobacillus*) and two prebiotics (β-glucan and fructooligosaccharide) (SYN), the NC plus 0.2% L-glutamine (GLN) in combination with the SYN additives (GLN+SYN), and a positive control (PC) containing antibiotics at formulated levels of 441 ppm chlortetracycline (Aureomycin, Zoetis, Parsippany, NJ) plus 38.6 ppm tiamulin (Denagard, Elanco Animal Health, Greenfield, IN). Dietary treatments were fed for nursery phase 1 (0 to 7 d post-weaning) and nursery phase 2 (7 to 14 d post-weaning), after which all pigs were fed common antibiotic free diets for the remainder of the experiment (Tables 4.1 and 4.3). All diets were formulated to meet or exceed the nutrient requirements of the pigs at a given phase (NRC, 2012).

4.3.2 Transportation

Pigs were weaned, herded up a ramp (2.13 m long, 11° incline) into a livestock trailer (2.35 × 7.32 m; Wilson Trailer Company, Sioux City, IA) bedded with wood shavings, and transported for 12 h over 819 km. Pig density in the trailer was 0.075 m^2 per pig. Two data loggers (Hobo; data logger, Onset, Bourne, MA) placed in the transport trailer obtained relative humidity (77.9 ±

0.44%) and ambient temperature (23.7 ± 0.10°C) readings in 1 min intervals for the duration of the transport. Pigs were not provided with feed or water during transport, and total transport time included loading of the trailer, transport, and unloading of the trailer. The transport route consisted of approximately 50% two-lane roads and 50% four-lane roads, with two stops to refuel the truck and change drivers during the transport. After arrival at the wean-to-finish facility, pigs were individually weighed and placed into pens. In addition, blood was collected into plasma tubes (lithium heparin, 6 mL, BD Vacutainer, Becton Dickinson and Company, Franklin Lakes, NJ) and serum tubes (BD Vacutainer Serum Separation Tubes, 5.0 mL, Becton Dickinson and Company, Franklin Lakes, NJ) from 6 sentinel pigs via jugular puncture immediately before and after transport to characterize the transport stress. An aliquot of whole blood from the lithium heparin tube was analyzed on site via a Vet Scan i-Stat1 machine with a CG8+ cartridge (model 300V; Abaxis Inc., Union City, CA) to quantify blood glucose and hematocrit, while the blood collected in serum tubes was allowed to clot, and then plasma and serum tubes were temporarily held at 4°C until further processing. The sentinel pigs were euthanized for tissue collection after the transport for a related experiment.

4.3.3 Nursery Phase

Pigs and feeders were individually weighed on d 7, 14, 21, 28, and 35 post-weaning to determine pig body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), and the gain to feed ratio. Therapeutic antibiotic injections given to pigs throughout the nursery period were recorded and classified according to the illness symptoms displayed. Categories included: enteric challenge (e.g. scours or loose watery stool), respiratory challenge (e.g. coughing or labored breathing), lameness (e.g. carrying a limb, difficulty walking, or swollen joints), un-thriftiness (e.g. BW loss, poor gain, loss of body condition, or rough hair coat), and all other

treatments (e.g. side paddling associated with *Streptococcus suis* infection, skin infection, and abscess). Decisions to treat individual pigs were primarily made by farm staff who were blinded to dietary treatments. Results of therapeutic treatments are reported as the percentage of pigs treated per pen (number of pigs per pen receiving ≥ 1 therapeutic antibiotic treatment divided by the total number of pigs in the pen) and as the number of therapies given per pig per pen (number of separate illness events requiring therapeutic antibiotic treatment in a pen divided by the total number of pigs in the pen). Blood was obtained via jugular puncture from 1 pig per pen immediately post-transport, and at d 2, 13, and 32 post-weaning. Blood was drawn from the same pig in each pen through d 13, at which point all blood draw pigs (1 pig per pen) were euthanized for tissue collection for a related experiment. Therefore, at d 32 post-weaning, blood was drawn from a different pig in each pen. Blood was collected into plasma blood tubes (EDTA, BD Vacutainer Tubes, 5.0 mL, Becton Dickinson and Company, Franklin Lakes, NJ). Blood tubes were briefly stored at 4°C until further processing. Pictures were taken of the right eye of the same two pigs per pen (1 barrow, 1 gilt) immediately post-transport, and at d 7, 14, 21, 28, and 32 post-weaning for quantification of tear staining as a non-invasive measure of stress. A laminated card with 1 cm × 1 cm squares was held next to the eye in each picture to calibrate the measurement software. In addition, pigs were video recorded from d 0 to 14 post-weaning for behavior analysis. Cameras (Panasonic WV-CP254H, Matsushita Electric Industrial Co. Ltd., Osaka, Japan) were mounted on the ceiling above the pens and video was recorded on a computer system (GeoVision VMS Software, GeoVision Inc., Tapei, Taiwan). One pig was removed from the study at the end of the first week due to weight loss and infection (PC dietary treatment), and one pig died for unknown reasons in the last week of the nursery period (NC dietary treatment).

4.3.4 Grow-Finish Period

After a 5-week nursery period, pigs were fed grow-finish diets and remained in their original wean-to-finish pens. Pigs and feeders were individually weighed on d 26 (end of phase 1), 47 (end of phase 2), 68 (end of phase 3), 89 (end of phase 4), 110 (end of phase 5), and 124 (end of phase 6) of the grow-finish period to obtain BW, ADG, ADFI, and gain to feed ratio. In addition, therapeutic antibiotic injection rate was recorded for the entire grow-finish period using the same criteria as in the nursery phase. Mortality during the grow-finish phase was primarily due to respiratory infections, and included 1 pig in phase 1 (PC dietary treatment), 1 pig in phase 2 (SYN dietary treatment), 1 pig in phase 4 (PC dietary treatment), and 2 pigs in phase 5 (GLN and PC dietary treatments). Four pigs were removed at the end of phase 5 due to hernias (NC and GLN+SYN dietary treatments) or other factors precluding marketing (NC and SYN dietary treatments).

4.3.5 Laboratory Analysis

Blood containing lithium heparin from sentinel pigs before and after transport as well as blood containing EDTA from 1 pig per pen collected at d 2, 6, 13, and 32 post-weaning were smeared on slides, stained (Hema 3 Stat Pack, Fisher Scientific, Pittsburgh, PA), and cover slipped for leucocyte differential count. In addition, serum from sentinel pigs and plasma from all other pigs at all blood draw time points was separated by centrifugation at 1500 × g at 4°C for 15 min. Serum or plasma were aliquoted and stored at -80°C until further analysis.

Serum from sentinel pigs before and after transport were analyzed for cortisol via a commercial radio-immune-assay (RIA) kit (ImmuChem Cortisol CT, MP Biomedicals LLC, Solon, OH). The intra-assay CV was 5.64%, and the inter-assay CV was 7.90%.

Blood smears were analyzed by one trained individual who was blinded to experimental treatments. Leukocytes were counted under 100X magnification and neutrophils, lymphocytes, monocytes, eosinophils, and basophils were identified according to nuclear and cytoplasmic staining and morphology. One hundred leukocytes were counted for each slide, and abundance for each cell type was expressed as a percentage of total cells counted. The neutrophil to lymphocyte ratio was then calculated.

Haptoglobin levels were determined in post-transport plasma, and in d 2, 6, and 13 post-weaning plasma. Haptoglobin was analyzed with a commercial kit (Porcine Haptoglobin ELISA Assay, ALPCO, Salem, NH) using a 1:10,000 dilution as per the manufacturer's instructions. The inter-plate CV was 19.25% and the intra-plate CV was 1.92%.

Tear staining area was quantified by one trained individual who was blinded to treatments. Briefly, images were analyzed using the tracing tool of ImageJ software (NIH, Bethesda, MD) to obtain the total area of tear staining for each image. The measurement scale was set from 1 cm × 1 cm squares on the laminated card present in each picture. Results were averaged across the two measured pigs per pen prior to analysis and are reported in cm^2 of tear staining.

Behavior analysis was completed by one trained individual who was blinded to treatments. Behaviors were analyzed using an instantaneous scan sample at 10 min intervals from 1000 h to 1600 h on d 2, 6, and 13 post-weaning. Behaviors analyzed were defined as: huddling, ≥ 3 pigs are lying down with ≥ 50% of a pig's body in contact with another pig; active, walking or interacting with pen-mates in a non-aggressive manner; resting, lying alone or loosely in groups with ≤ 50% contact between pigs; aggressive, engaged in agonistic interactions; eating, pig has nose in the feeder; drinking, pig has mouth on the waterer; non-visible, pig moves out of view and cannot be

observed. Results are presented as the average percentage of time spent for each behavior over the 6 h analyzed period each day.

4.3.6 Statistical Analysis

Pigs were allotted to dietary treatments in a randomized complete block design with initial BW as the blocking factor. Pen was considered the experimental unit. Data were primarily analyzed via Proc Glimmix of SAS 9.4 (SAS Institute, Carry, NC), with Proc Mixed of SAS 9.4 used to analyze for influential outliers via Cook's D test (Cook, 1977). Dietary treatment was considered a fixed effect while block was considered a random effect. Data where the interaction of time with the dietary treatments was of interest were analyzed with repeated measures with the time unit as a fixed effect, the subject as pen(treatment*block), and using the optimal covariance structure for each response variable as determined by the BIC goodness of fit criteria (Littell et al., 1998). A Kenward-Rogers degrees of freedom correction was applied to all repeated measures analysis via the ddfm=kr option of the model statement (Kenward and Roger, 1997). Nursery data were divided into the dietary treatment period (week 1 and 2) and the common diet period (weeks 3, 4, and 5) and separate repeated measures analyses were conducted for each period. Growth performance data from the grow-finish period were analyzed by phase. Means of significant effects or interactions were separated with a Tukey's adjustment. Single degree of freedom contrasts were used to analyze the effects of the NC vs. PC, NC vs. GLN, NC vs. SYN, and NC vs. GLN+SYN. All behavior data, leukocyte count data, and therapeutic injection rate data were log transformed prior to analysis to meet the assumptions of normality and homogeneity of variances but results are presented as arithmetic means for ease of interpretation. All other results are presented as least square means ± 1 SEM unless otherwise stated. Significance was declared at $P < 0.05$, while a tendency towards significance was considered at $0.05 \geq P < 0.10$.

4.4 Results

For descriptive measures of stress from sentinel pigs (Table 4.4; statistical analysis not conducted as the transportation event is an n = 1) there was a 10.5% increase in neutrophils and a 7.7% decrease in lymphocytes following transport that translated into a 0.3 unit increase in the neutrophil to lymphocyte ratio. Blood glucose was increased by 4.4 mg/dL, while hematocrit was decreased by 0.1% following transport. Cortisol increased by 9.86 µg/dL following transport.

Growth performance data from the nursery period are presented in Table 4.5 and contrasts of growth performance data (contrasts are described in section 4.3.6) are presented in Table 4.12. Dietary treatment interacted ($P = 0.04$) with time for BW during the treatment diet period (d 0 to 14), with the PC going from the least at week 1 to the greatest at week 2 and the GLN diet going from the greatest in week 1 to intermediate at week 2. In addition, an overall effect of week was observed for BW ($P < 0.01$) with week 1 (6.41 ± 0.637 kg) being lesser than week 2 (7.62 ± 0.640 kg). Similarly, for ADG for the dietary treatment period, diet interacted ($P = 0.02$) with time, with the PC going from the least at week 1 to the greatest at week 2 and the GLN diet going from the greatest in week 1 to intermediate at week 2. In addition, an overall effect of week was observed for ADG ($P < 0.01$) with week 1 (70 ± 6.1 g) being lesser than week 2 (172 ± 6.2 g). There was an overall effect of week ($P < 0.01$) for ADFI, with week 1 (99 ± 5.4 g) being lesser than week 2 (245 ± 8.6 g). In addition, there was a tendency ($P = 0.07$) for ADFI to be greater for the PC (0.11 kg increase) than for the SYN in the overall analysis of the day 14 to 35 data. There was a tendency ($P = 0.05$) for a diet by week interaction for gain to feed ratio, with the GLN diet most efficient in week 1 but second least efficient in week 2 (a decrease of 0.18), and with the PC being third least efficient in week 1 but most efficient in week 2 (an increase of 0.20). For BW in the common diet period, there was an impact of week ($P < 0.01$), with BW at week 3 (9.6 ± 0.67 kg) being lesser

than week 4 (12.3 ± 0.68 kg), which was lesser than week 5 (15.6 ± 0.71 kg). Similar results were detected for ADG in the common diet period with a main effect of week ($P < 0.01$) resulting in ADG being lesser at week 3 (0.28 ± 0.022 kg) which was lesser than week 4 (0.39 ± 0.021 kg) which was lesser than week 5 (0.48 ± 0.023 kg). In addition, a contrast demonstrated a trend ($P = 0.07$) for the PC to have greater ADG (12%) than the NC for the common diet period (d 14 to 35). There was an overall effect of week ($P < 0.01$) for ADFI in the common diet period, with week 3 (0.41 ± 0.030 kg) being lesser than week 4 (0.61 ± 0.031 kg) which was lesser than week 5 (0.81 ± 0.032 kg). In addition, a contrast demonstrated an increase ($P = 0.03$; 13%) in ADFI for the PC over the NC in the common diet period. Likewise, for the gain to feed ratio there was an effect of week ($P < 0.01$) with week 3 (0.67 ± 0.018), and 4 (0.65 ± 0.011) being greater than week 5 (0.60 ± 0.013). In addition, a contrast demonstrated a decrease ($P = 0.03$; 7%) in gain to feed ratio for GLN compared to the NC in the common diet period. For the analysis of the average response over the entire nursery period (d 0 to 35), contrasts indicated an increase in ADG ($P = 0.04$; 0.03 ± 0.016 kg) and ADFI ($P = 0.04$; 0.05 ± 0.025 kg) for the PC over the NC. No other significant or trending results for nursery growth performance were found.

Growth performance of pigs during the grow-finish period are presented in Table 4.6 and contrasts are presented in Table 4.12. For BW over the 6 phases, there was no impact of nursery diet ($P \geq 0.75$). A contrast for ADG in Phase 2 demonstrated a tendency ($P = 0.09$) for a reduction (7%) in ADG for the PC compared to the NC. A contrast for ADG in Phase 3 demonstrated a tendency ($P = 0.08$) for a reduction (5%) in ADG for the SYN compared to the NC. There was a tendency ($P = 0.06$) for ADG to be impacted by nursery diet in Phase 6; however, there was no tendency ($P > 0.10$) for differences among means when the Tukey's multiple comparison correction was applied. Contrasts for ADG in Phase 6 indicated an improvement for the GLN (*P*

= 0.03; 16%) and SYN (*P* = 0.02; 17%) treatments over the NC. Diet impacted ADFI in Phase 6 (*P* = 0.02), with pigs on the PC treatment consuming less feed (0.37 kg per day) than pigs in the SYN treatment, with all other diets being intermediate. A contrast also indicated that pigs fed the PC nursery diet had reduced (*P* = 0.02; 6%) ADFI in Phase 6 than pigs fed the NC. Contrasts for gain to feed ratio in Phase 1 indicated a trend (*P* = 0.07) for the PC to have reduced (5%) feed efficiency than the NC, as well as reduced feed efficiency for SYN (*P* = 0.04; 5%) and the GLN+SYN (*P* = 0.03; 5%) compared to the NC. There was a tendency (*P* = 0.08) for gain to feed ratio to be impacted by nursery diet in Phase 6, with contrasts indicating the PC and GLN having greater efficiency (*P* = 0.02; 15%) than the NC, and a tendency (*P* = 0.05; 12%) for SYN to have greater efficiency than the NC. No other significant or trending results for grow-finish performance were found.

Therapeutic injection data are presented in Table 4.7. For therapeutic injections for d 0 to 14 post-weaning the primary reason for therapies was enteric health with no impact of dietary treatment ($P \geq 0.56$) with an average of 41 ± 6.5% of nursery pigs receiving one or more therapies, and an average of 0.45 individual therapies given per pig per pen. Similarly, for therapeutic injections for d 14 to 35 post-weaning the primary reason for treatment was enteric health with no impact of dietary treatment ($P \geq 0.73$) with 1.6 ± 1.63% of nursery pigs per pen receiving a treatment and an average of 0.02 individual therapies given per pig per pen. There was a tendency (*P* = 0.08) for more pigs fed the NC to be treated for lameness during the d 14 to 35 period. The very low levels of therapies given for all other reasons at all other time points precludes additional meaningful comparisons.

Leukocyte differential data is presented in Table 4.8 and contrasts are presented in Table 4.12. For the blood collection days in the dietary treatment period, there was a tendency (*P* = 0.06)

for a diet by day interaction for percentage of neutrophils; however, there was no tendency (P > 0.10) for differences among means when the Tukey's multiple comparison correction was applied. Monocyte percentage during the dietary treatment period varied by day (P = 0.02), with d 13 (1.7 ± 0.22%) being greater than d 2 (0.9 ± 0.15%), with d 6 (1.3 ± 0.19%) being intermediate. Monocytes for the common diet phase (d 32 blood collection) tended (P = 0.07) to vary by diet, with pigs fed the SYN+GLN diet tending to have reduced numbers of monocytes (62% decrease) than pigs fed the GLN diet, with all others being intermediate. A contrast indicated a tendency (P = 0.09) for pigs fed the SYN+GLN diet to have reduced numbers of monocytes than pigs fed the NC diet. Results for basophils are not presented due to very few being present in the blood smears. No other significant or trending results for leukocyte data were found.

Results for plasma haptoglobin levels are presented in Table 4.9 with contrasts presented in Table 4.12. For haptoglobin over the 3 blood collection days, there was no impact of dietary treatment (P = 0.14), day (P = 0.30), or an interaction of treatment with day (P = 0.16). A contrast demonstrated a reduction (P = 0.01) in haptoglobin for the PC (172 ± 107.2 µg/mL) compared to the NC (563 ± 106.7 µg/mL). No other significant or trending results for plasma haptoglobin were found.

Tear staining data is presented in Table 4.10 and contrasts are presented in Table 4.12. For the treatment diet period (d 0 to 14) there was an impact of day (P < 0.01) with d 14 (0.42 ± 0.065 cm^2) being greater than d 7 (0.16 ± 0.018 cm^2) post-weaning. Contrasts for the treatment diet period indicated tendencies for the PC (P = 0.09; 0.21 ± 0.082 cm^2) and GLN (P = 0.06; 0.18 ± 0.082 cm^2) to have lesser tear staining than the NC (0.41 ± 0.082 cm^2). For the common diet period there was an effect of day (P < 0.01) with d 28 (0.53 ± 0.067 cm^2) and 32 (0.45 ± 0.054 cm^2) being greater than d 21 (0.32 ± 0.058 cm^2) post-weaning. Contrasts for the common diet period (d 14 to

35) indicated a tendency for GLN (P = 0.05; 0.34 ± 0.079 cm^2) to have lesser tear staining than the NC (0.52 ± 0.079 cm^2). No other significant or trending results for tear staining were found.

Data for behaviors during the nursery phase are presented in Table 4.11 and contrasts are presented in Table 4.12. For huddling behavior there was no impact of diet (P = 0.91), but there was a day effect (P < 0.01) with pigs at 2 (43 ± 3.5%) and 6 (46 ± 2.4%) d post-weaning huddling more than pigs at 13 d (29 ± 2.3%). For time spend active there was no effect of diet (P = 0.19), but there was a day effect (P < 0.01) with pigs at d 2 (23 ± 1.4%) and d 13 (23 ± 1.2%) being more active than at 6 d (18 ± 0.1%) post-weaning. For time spent resting there was no impact of dietary treatment (P = 0.14) but a day effect (P < 0.01) was observed with d 13 (24 ± 1.4%) being greater than d 2 (14 ± 1.2%) and d 6 (16 ± 1.4%). For aggressive behavior there was a tendency for a diet effect (P = 0.09) with SYN (1.8 ± 0.38%) tending to be greater than the GLN+SYN diet (0.7 ± 0.19%), with all others intermediate. A contrast indicated a tendency (P = 0.09) for the NC (1.1 ± 0.39%) pigs to be less aggressive than the SYN (1.8 ± 0.38%) pigs. In addition, there was an effect of day (P < 0.01) with pigs at 2 d (2.5 ± 0.38%) post-weaning being more aggressive than at 6 (0.5 ± 0.13%) or 13 (0.5 ± 0.15%) d. For eating behavior, there was no impact of diet (P = 0.62), but there was an effect of day (P < 0.01) with pigs at d 2 (1.9 ± 0.50%) eating the least, d 6 (9.2 ± 0.50%) eating more than at d 2, and d 13 (11.2 ± 0.59%) eating the most. A contrast indicated a tendency (P = 0.05) for NC pigs (15.9 ± 3.39%) to spend more time as non-visible than the SYN pigs (4.0 ± 1.24%). No other significant or trending results for behavior were found.

4.5 Discussion

The analyzed levels of antibiotics in the nursery diets were as expected, with no chlortetracycline present in any of the diets except the PC, with levels of chlortetracycline within

87% (Phase 1) and 93% (Phase 2) of formulated values, and tiamulin within 99% (Phase 1) and 95% (Phase 2) of formulated values. However, analyzed levels of supplemental glutamine (indirectly analyzed as total glutamic acid) in the diets yielded less straightforward results. For Phase 1 diets, GLN increased by 0.22% over the NC, while GLN+SYN increased by 0.21% over the NC, very close to the formulated 0.20% increase for those diets. However, the SYN diet, which was not formulated to contain elevated glutamine, had 0.10% more glutamine than the NC, and similarly, the PC diet had increased analyzed glutamine by 0.16% over that of the NC. For Phase 2 diets, GLN increased by 0.37% over the NC, while GLN+SYN increased by 0.32% over the NC, somewhat greater than the formulated 0.20% increase for those diets. However, the SYN diet, which was not formulated to contain elevated glutamine, was 0.33% increased over that of the NC, and similarly, the PC diet had increased analyzed glutamine by 0.25% over that of the NC. Diets were prepared by making a common basal diet and then adding treatment premixes to subdivisions of the basal diet for each treatment, therefore, differences in ingredient composition among diets is unlikely to explain the unexpected results for the glutamine analysis. While in theory contamination during feed mixing is possible, there was not enough glutamine present in the premixes for the glutamine diets to elevate the glutamine levels of all the diets to the observed extent. It is also possible that the complexity of the indirect analysis for glutamine led to analytical errors. In addition, the antibiotics and SYN additives were not analyzed alone for GLN, and there is the potential that these additives could have contained some GLN inherent to their production.

Weaning and transport are reported to be stressful for pigs (Sutherland et al., 2014). Physiological measures of increased stress reported in transported weanling pigs include increased cortisol levels (Sutherland et al., 2009), increased ratio of neutrophils to lymphocytes (Garcia et al., 2016), and increased hematocrit indicating dehydration (Berry and Lewis, 2001). The increase

in cortisol (699%) and the increase in the neutrophil to lymphocyte ratio (43%) observed post-transport in the present study indicate that pigs were stressed by the weaning and transport. Interestingly, no changes in hematocrit were observed, so dehydration does not appear to have been a significant factor in this transport.

Replacement products for dietary antibiotics in the nursery phase has been the topic of much research, with many alternatives evaluated. Unfortunately, one of the characteristics of the alternative products is that many yield less consistent results than dietary antibiotics (de Lange et al., 2010). This is in line with the results from the alternative products in the present study where, contrary to our hypothesis, the effects of the dietary treatment on growth performance during the nursery period were primarily limited to the PC over the NC. For the treatment diet phase, the interaction of diet with week for BW and ADG appears to be driven by the PC going from the least in week 1 to the greatest in week 2 and the GLN going from the best at week 1 to intermediate at week 2. This translated into a tendency for an interaction of diet by week for gain to feed ratio, with PC pigs shifting to be more efficient in week 2 than in week 1, while GLN and GLN+SYN pigs shifted to be less efficient in week 2 than in week 1. Similarly, in the common diet phase the PC had increased ADFI over that of the NC, while GLN had decreased gain to feed ratio than the NC. The improvements for the PC over the NC are as expected, however, the reduction in feed efficiency for the GLN pigs over the NC is less intuitive and appears to be driven by small increases in ADFI for GLN pigs that did not translate into improved ADG. The effects of week observed were as expected, with pigs eating more and growing faster as time progressed. Potential explanations for the inconsistency of antibiotic alternatives have been discussed (Heo et al., 2013; Pluske et al., 2018) and include complex interactions among management practices, pig microbiome, and pig immune status. In addition, the majority of literature reporting an impact of

supplemental GLN in nursery diets used levels of 0.5 to 1.5% GLN (Watford, 2015). A greater number of experimental units may be required to see an effect of lesser levels of glutamine supplementation on growth performance in future experiments.

While better nursery growth performance can be theorized to translate into better grow-finish growth performance, this is not always the case. In an experiment designed to test this, nursery pigs were offered simple or complex diets with or without antibiotic inclusion (Skinner et al., 2014). Treatment diets were fed over a 6-week nursery period, and diet did impact nursery performance with improvements in ADG for complex and antibiotic containing diets. However, pigs that performed poorly during the nursery (i.e. non-antibiotic fed pigs) compensated and had a greater rate of gain during the grow-finish period. At the end of the grow-finish period there were no differences in growth performance, carcass composition, or meat quality among treatments (Skinner et al., 2014). Similarly, the results from the present study indicated that improvements in ADG from PC pigs during the nursery period did not necessarily translate into improved performance during the grow-finish period. Gain to feed ratio was improved in Phase 1 for the NC over that of the SYN and GLN+SYN, which appears to have been driven by the combination of small increases in ADFI and small decreases in ADG for SYN and GLN+SYN over that of the NC. This improvement in efficiency for the NC may represent compensatory growth of pigs not given antibiotics during the nursery phase, similar to that observed by Skinner et al. (2014). After Phase 1 pigs performed very similarly until Phase 6, at which point treatments began to separate. Average daily gain was improved for GLN and SYN treatments over that of the NC, while ADFI was increased for the NC and SYN over that of the PC. This translated into the PC and the GLN treatments having improved gain to feed ratio over that of the NC. The reason for the separation among treatments in Phase 6 is unknown but may represent an interaction between nursery-diet-

induced nutritional imprinting (Lucas, 1998) and the complex physiological impacts of ractopamine (Mersmann, 1998) inclusion in the Phase 6 diet. Further research is required to investigate this potential interaction.

Individual injection of pigs with antibiotics to treat a disease does occur in commercial practices, however, individual therapies tend to be used less in commercial production than feed or water administered antibiotics (Rosengren et al., 2008). Individual injections were used in the present study to enable quantification of therapy by category and dietary treatment. The primary reason for therapeutic antibiotic use was enteric health challenges, with roughly 41% of pigs being treated for enteric health challenges during the first 14 d of the nursery period. This was independent of the diets and may simply reflect a weaning group with relatively poor enteric health. The enteric health challenges largely cleared up by d 14 with much lesser rates of therapies for enteric health from d 14 to 35. Therapies for reasons other than enteric challenge were quite low during the nursery period. Similarly, pigs were healthy during the grow-finish phase, with very few pigs receiving therapies.

The neutrophil to lymphocyte ratio is a marker of stress in pigs, with increases in the ratio indicative of increased stress (Widowski et al., 1989). In the present study, neutrophil to lymphocyte ratio was not impacted by the diet ($P = 0.42$) or the day post-weaning ($P = 0.87$) during the treatment diet phase. However, a marked decrease in the neutrophil to lymphocyte ratio at d 32 versus the treatment diet phase may indicate that by 32 d post-weaning pigs had recovered from the stresses of weaning and transport. This would be consistent with the belief that the first two weeks post-weaning are the most stressful portion of the nursery period (de Lange et al., 2010).

Haptoglobin is considered one of the major acute phase proteins in pigs, with elevated levels in response to stress or infection (Cray et al., 2009). The use of haptoglobin as an indicator of stress

is still relatively new in pigs, with some inconsistent results (Martínez-Miró et al., 2016). In the present study, contrasts demonstrated that the NC had reduced plasma haptoglobin as compared to the PC. This may indicate that the antibiotics in the PC diet reduced the stress (pathogenic or otherwise) experienced by pigs in the 2 to 13 d post-weaning period. The lack of a main effect of diet despite numerical differences is likely a result of the large variation in observed haptoglobin levels from pig to pig. Large variation in haptoglobin levels in healthy pigs has been observed previously, and was unable to be explained by age, BW, or sex (Sales et al., 2015). This apparently normal variation may make differences in haptoglobin among treatments difficult to detect, thereby limiting the usefulness of haptoglobin as an indicator of stress in pigs.

Excessive production of porphyrin leads to the appearance of red or brown “tear” staining around the eyes of pigs. This tear staining has been validated as a non-invasive measure of stress in pigs (DeBoer et al., 2015; Telkänranta et al., 2016), with greater tear staining indicating greater stress. In the present study, tear staining increased through d 21, and then remained relatively stable from d 28 to d 32. The increase in tear staining in the initial portion of the nursery period is similar to the increase seen over the 7-day experimental period of DeBoer et al. (2015) and may reflect the stresses associated with the immediate post-weaning period (Campbell et al., 2013). The trends for GLN and the PC to have decreased tear staining compared to the NC is interesting and warrants additional future investigation.

Animal behavior may be used as a non-invasive measure of health and welfare in farm animals (Duncan, 1987). While diet did not significantly impact behavior, there were mostly predictable results of day post-weaning. Pigs huddled more and rested less at 2 and 6 d post-weaning than at 13 d, spent more time eating at each successive day, and were more aggressive at 2 d post-weaning than at 6 or 13 d while establishing pen hierarchies. These results are likely

reflective of a gradual recovery from the stresses of weaning (Jayaraman and Nyachoti, 2017) over time. In addition, pigs were more active on d 2 and 13 than at d 6. This may be reflective of the peak in gut damage generally seen 3 to 5 d post-weaning (Hampson, 1986), as inactivity is a known sickness behavior (Johnson and von Borell, 1994).

4.6 Conclusions

Supplemental glutamine at 0.2% of the diet alone or in combination with a synbiotic mixture fed for the first 14 d post-weaning did not improve growth performance from weaning through grow-finish Phase 5. Nursery administration of glutamine or synbiotics, but not the combination, improved some aspects of growth performance in grow-finish Phase 6, potentially due to an interaction of nutritional imprinting and ractopamine administration. Measures of immune function and general measures of stress and welfare during the nursery phase were not significantly impacted by glutamine, synbiotics, or the combination of the two. Dietary antibiotics were effective in improving some aspects of nursery growth performance, but these improvements had limited impact on growth performance during the grow-finish phase.

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Table 4.1. Ingredient composition and calculated nutrient composition of nursery diets (as fed).

Item	**Phase 1**	**Phase 2**	**Phase 3**	**Phase 4**
Period fed, d post-weaning	0 - 7	7 - 14	14 - 21	21 - 35
Ingredient, %				
Corn	31.090	37.850	49.220	55.300
Soybean meal, 48% CP	13.900	17.950	20.800	22.810
DDGS, 7% fat	0.000	0.000	7.500	15.000
Monocalcium phosphate	0.380	0.490	0.070	0.510
Limestone	0.800	0.740	1.080	1.480
Salt	0.250	0.250	0.300	0.350
Soybean oil	5.000	5.000	3.000	0.000
Choice white grease	0.000	0.000	0.000	3.000
Lysine-HCl	0.070	0.200	0.340	0.480
DL-Methionine	0.220	0.225	0.160	0.125
L-Threonine	0.040	0.090	0.120	0.140
L-Tryptophan	0.010	0.015	0.025	0.030
Vitamin premix[1]	0.250	0.250	0.250	0.250
Trace mineral premix[2]	0.125	0.125	0.125	0.125
Selenium premix[3]	0.050	0.050	0.050	0.050
Dried whey	25.000	25.000	10.000	0.000
Lactose	5.000	0.000	0.000	0.000
Fish meal, menhaden	5.000	4.000	4.000	0.000
Plasma protein	6.500	2.500	0.000	0.000
Spray-dried blood meal	1.500	1.500	0.000	0.000
Soy protein concentrate	4.000	3.000	2.500	0.000
Zinc oxide	0.000	0.000	0.280	0.000
Copper sulfate	0.000	0.000	0.000	0.080
Banminth 48[4]	0.000	0.000	0.000	0.100
Phytase[5]	0.100	0.100	0.100	0.100
Clarifly, 0.67%[6]	0.140	0.090	0.080	0.070
Treatment premix[7]	0.575	0.575	0.000	0.000
Calculated Nutrient Composition				
ME, kcal/kg	3545	3520	3429	3407
CP, %	24.62	22.88	21.81	20.05
SID Lys, %	1.55	1.45	1.30	1.15
Ca, %	0.90	0.85	0.80	0.75
Available P, %	0.60	0.55	0.40	0.35

[1]Provided per kilogram of the diet: vitamin A, 6,614 IU; vitamin D3, 661 IU; vitamin E, 44 IU; vitamin K, 2.2 mg; riboflavin, 8.8 mg; pantothenic acid, 22 mg; niacin, 33 mg; B_{12}, 38.6 µg.

[2]Provided per kilogram of diet (available minerals): iron, 121 mg; zinc, 121 mg; manganese, 15 mg; copper, 11 mg; iodine, 0.46 mg.

[3]Provided 0.3 ppm Se.

[4]Banminth (Phibro Animal Health Corporation, Teaneck, NJ) provided 106 ppm pyrantel tartrate in the diet.

[5]Provided 600 FTU per kg of the diet.

[6]Clarifly (Central Life Sciences, Schaumburg, IL) provided 9.5 ppm (Phase 1), 6.1 ppm (Phase 2), 5.4 ppm (Phase 3) and 4.7 ppm (Phase 4) diflubenzuron in the diet.

[7]As a percent of the diet the treatment premixes consisted of: NC, 0.575% corn; GLN, 0.375% corn and 0.20% L-glutamine (Ajinomoto North America Inc., Raleigh, NC); SYN, 0.405% corn and 0.17% synbiotic mixture; GLN + SYN, 0.205% corn, 0.20% L-glutamine and 0.17% synbiotic mixture; PC, 0.40% Aureomycin (Zoetis, Parsippany, NJ; 110.3 g chlortetracycline/kg) and 0.175% Denagard (Elanco Animal Health, Greenfield, IN; 22.05 g tiamulin/kg).

Table 4.2. Analyzed composition of treatment period nursery diets on an as-fed basis[1]

Item	**NC**	**GLN**	**SYN**	**GLN + SYN**	**PC**
Phase 1[2]					
DM, %[3]	90.8	91.1	90.6	90.7	91.3
CP, %[3]	23.5	24.4	23.5	24.2	24.1
Chlortetracycline, ppm[4]	0.0	0.0	0.0	0.0	381.6
Tiamulin, ppm[5]	0.0	0.0	0.0	0.0	38.3
Total glutamic acid, %[6]	3.57	3.79	3.67	3.78	3.73
Total Lys, %[3]	1.56	1.60	1.60	1.58	1.63
Total Met, %[3]	0.52	0.53	0.51	0.52	0.52
Total Met + Cys, %[3]	0.90	0.94	0.91	0.91	0.93
Total Thr, %[3]	1.09	1.12	1.12	1.13	1.15
Total Trp, %[3]	0.32	0.32	0.31	0.32	0.33
Total Val, %[3]	1.17	1.17	1.19	1.19	1.22
Total Ile, %[3]	0.88	0.89	0.90	0.89	0.92
Total Arg, %[3]	1.25	1.26	1.23	1.25	1.31
Total His, %[3]	0.61	0.61	0.63	0.62	0.64
Total Phe + Tyr, %[3]	1.60	1.63	1.65	1.60	1.64
Phase 2[7]					
DM, %[3]	89.9	90.1	90.4	89.5	89.5
CP, %[3]	22.1	22.2	22.3	22.5	22.2
Chlortetracycline, ppm[4]	0.0	0.0	0.0	0.0	411.2
Tiamulin, ppm[5]	0.0	0.0	0.0	0.0	36.5
Total glutamic acid, %[6]	3.45	3.82	3.78	3.77	3.70
Total Lys, %[3]	1.46	1.42	1.51	1.43	1.49
Total Met, %[3]	0.48	0.47	0.48	0.47	0.48
Total Met + Cys, %[3]	0.80	0.81	0.82	0.82	0.83
Total Thr, %[3]	1.01	1.02	1.03	0.98	0.99
Total Trp, %[3]	0.28	0.28	0.30	0.30	0.28
Total Val, %[3]	1.04	1.06	1.09	1.05	1.08
Total Ile, %[3]	0.85	0.85	0.88	0.84	0.86
Total Arg, %[3]	1.19	1.23	1.28	1.22	1.24
Total His, %[3]	0.56	0.57	0.59	0.56	0.58
Total Phe + Tyr, %[3]	1.49	1.45	1.53	1.44	1.50

[1]Abbreviations: NC, negative control diet containing no dietary antibiotics; GLN, diet containing 0.2% L-glutamine; SYN, diet containing a mixture of prebiotics and probiotics; GLN + SYN, diet containing 0.2% L-glutamine and a mixture of prebiotics and probiotics; PC, positive control diet containing antibiotics (441 ppm chlortetracycline + 38.6 ppm tiamulin).
[2]Phase 1 was fed from 0 to 7 d post-weaning.
[3]Analyzed by Ajinomoto Heartland Inc., Chicago, IL.
[4]Analyzed by Zoetis, Parsippany, NJ.
[5]Analyzed by Elanco Animal Health, Greenfield, IN.
[6]Total Glu + Gln in the diet analyzed by Ajinomoto Heartland, Inc., Chicago, IL.
[7]Phase 2 was fed from 7 to 14 d post-weaning.

Table 4.3. Ingredient composition and calculated nutrient composition of grow-finish diets (as fed).

Item	**Phase 1**	**Phase 2**	**Phase 3**	**Phase 4**	**Phase 5**	**Phase 6**
Time period diets were fed, d	26	21	21	21	21	14
Ingredient, %						
Corn	59.200	60.890	61.505	64.640	73.640	67.913
DDGS, 7% fat	20.000	25.000	30.000	30.000	22.500	15.000
Soybean meal, 48% CP	15.500	10.000	4.650	1.760	0.550	11.450
Choice white grease	2.000	1.000	1.000	1.000	1.000	2.500
Limestone	1.540	1.520	1.450	1.340	1.230	1.360
Selenium premix[1]	0.050	0.050	0.050	0.050	0.025	0.050
Salt	0.350	0.350	0.300	0.300	0.250	0.300
Monocalcium phosphate	0.200	0.050	0.000	0.000	0.000	0.100
Vitamin premix[2]	0.150	0.140	0.130	0.120	0.100	0.125
Trace-mineral premix[3]	0.100	0.090	0.080	0.070	0.050	0.080
Availa Zn 120[4]	0.000	0.000	0.000	0.000	0.000	0.042
L-Threonine	0.130	0.120	0.115	0.095	0.085	0.155
Lysine-HCl	0.500	0.520	0.540	0.500	0.440	0.450
L-Tryptophan	0.030	0.035	0.045	0.045	0.040	0.030
DL-Methionine	0.080	0.045	0.015	0.000	0.000	0.095
Clarifly[5]	0.070	0.090	0.070	0.080	0.090	0.100
Phytase[6]	0.100	0.100	0.050	0.000	0.000	0.100
Paylean 2.25[7]	0.000	0.000	0.000	0.000	0.000	0.150
Calculated Nutrient Composition						
ME, kcal/kg	3363	3329	3342	3347	3362	3405
CP, %	18.6	16.8	15.7	14.5	12.6	15.4
SID Lys, %	1.00	0.90	0.80	0.70	0.60	0.85
Ca, %	0.70	0.65	0.60	0.55	0.50	0.60
Available P, %	0.30	0.29	0.27	0.23	0.18	0.25

[1]Provided 0.3 ppm Se at 0.05% inclusion and 0.15 ppm Se at 0.025% inclusion.

[2]For each 0.01% premix inclusion in the diet the following vitamins were provided per kilogram of the diet: vitamin A, 265 IU; vitamin D_3, 26.5 IU; vitamin E, 1.76 IU; vitamin K, 0.09 mg; riboflavin, 0.33 mg; pantothenic acid, 0.88 mg; niacin, 1.32 mg; B_{12}, 1.54 µg.

[3]For each 0.01% premix inclusion in the diet the following available minerals were provided per kilogram of the diet: iron, 9.7 mg; zinc, 9.7 mg; manganese, 1.2 mg; copper, 0.90 mg; iodine, 0.037 mg.

[4]Zinpro Corporation, Eden Prairie, MN.

[5]Clarifly (Central Life Sciences, Schaumburg, IL) provided 0.67 ppm diflubenzuron in the diet for every 0.01% inclusion in the diet.

[6]Provided 600 FTU per kg of the diet.

[7]Paylean (Elanco Animal Health, Greenfield, IN) provided 7.5 ppm ractopamine HCl in the diet.

Table 4.4. Descriptive measures from sentinel pigs (N = 6) taken before and after a 12 h transport

Item	**Pre-Transport**	**Post-Transport**	**SEM**
Leukocyte count			
Neutrophils, %	35.2	45.7	7.00
Lymphocytes, %	61.0	53.3	6.76
Neutrophil : lymphocyte ratio	0.7	1.0	0.24
Monocytes, %	1.5	0.8	0.51
Eosinophils, %	2.3	0.2	0.42
Blood parameters			
Glucose, mg/dL	122.3	126.7	4.85
Hematocrit, %	26.4	26.3	1.48
Cortisol, µg/dL	1.4	11.3	1.23

Table 4.5. Growth performance, feed intake, and feed efficiency of pigs fed five treatment diets from d 0 to d 14 of the nursery phase and common diets from d 14 to 35 of the nursery phase[1]

							***P*-Value**		
Item	**NC**	**GLN**	**SYN**	**GLN + SYN**	**PC**	**SEM**	**Diet**	**Week**	**Diet × Week**
Post-transport									
BW, kg	5.92	5.90	5.94	5.91	5.94	0.647	0.61	-	-
Treatment Diet Period									
BW, kg									
Week 1[2]	6.35[a]	6.50[a]	6.41[a]	6.45[a]	6.34[a]	0.649	0.79	< 0.01	0.04
Week 2	7.51[b]	7.62[b]	7.55[b]	7.51[b]	7.90[b]				
ADG, g									
Week 1[2]	61[c]	86[bc]	67[c]	78[c]	57[c]	15.5	0.50	< 0.01	0.02
Week 2	166[a]	160[a]	163[a]	151[ab]	221[a]				
ADFI, g									
Week 1[2]	97	100	107	101	92	13.0	0.93	< 0.01	0.21
Week 2	235	240	240	242	267				
Gain:Feed									
Week 1[2]	0.61	0.84	0.61	0.77	0.63	0.068	0.53	0.96	0.05[3]
Week 2	0.70	0.66	0.67	0.62[z]	0.83[y]				
Common Diet Period									
BW, kg									
Week 3[2]	9.47	9.24	9.41	9.51	10.26	0.818	0.38	< 0.01	0.69
Week 4	12.15	12.07	11.91	12.29	13.14				
Week 5	15.32	15.11	15.22	15.60	16.69				
ADG, kg									
Week 3[2]	0.28	0.23	0.26	0.28	0.33	0.031	0.12	< 0.01	0.35
Week 4	0.38	0.40	0.36	0.40	0.41				
Week 5	0.47	0.45	0.49	0.49	0.52				
ADFI, kg									
Week 3[2]	0.40	0.38	0.39	0.42	0.46	0.039	0.14	< 0.01	0.51
Week 4	0.60	0.60	0.57	0.63	0.66				
Week 5	0.77	0.83	0.79	0.82	0.86				
Gain:Feed									
Week 3[2]	0.69	0.60	0.66	0.68	0.72	0.031	0.18	< 0.01	0.42
Week 4	0.65	0.67	0.64	0.64	0.63				
Week 5	0.61	0.54	0.61	0.60	0.61				
Overall Analysis[4]									
ADG									
Day 0 to 14, g	113	123	115	114	138	10.6	0.44	-	-
Day 14 to 35, kg	0.38	0.36	0.37	0.39	0.42	0.025	0.12	-	-
Day 0 to 35, kg	0.26	0.25	0.25	0.26	0.29	0.016	0.12	-	-

Table 4.5 is continued on the next page

Table 4.5 continued

Item	**NC**	**GLN**	**SYN**	**GLN + SYN**	**PC**	**SEM**	***P*-Value**		
							Diet	**Week**	**Diet × Week**
ADFI									
Day 0 to 14, g	166	170	173	172	179	12.0	0.91	-	-
Day 14 to 35, kg	0.72[yz]	0.74[yz]	0.70[z]	0.76[yz]	0.81[y]	0.045	0.07	-	-
Day 0 to 35, kg	0.39	0.41	0.40	0.42	0.44	0.025	0.22	-	-
Gain:Feed									
Day 0 to 14	0.68	0.72	0.63	0.67	0.78	0.042	0.19	-	-
Day 14 to 35	0.52	0.49	0.52	0.51	0.52	0.015	0.41	-	-
Day 0 to 35	0.65	0.62	0.64	0.64	0.67	0.014	0.16	-	-

[a-c]Treatment × week lsmeans for a response parameter not sharing a common letter are different ($P < 0.05$).

[y-z]Treatment × week lsmeans for repeated measures data or treatment lsmeans for overall analysis data not sharing a common letter are tending to be different ($0.05 \leq P < 0.10$).

[1]Abbreviations: NC, negative control diet containing no dietary antibiotics; GLN, diet containing 0.2% L-glutamine; SYN, diet containing a mixture of prebiotics and probiotics; GLN + SYN, diet containing 0.2% L-glutamine and a mixture of prebiotics and probiotics; PC, positive control diet containing antibiotics (441 ppm chlortetracycline + 38.6 ppm tiamulin); BW, body weight; ADG, average daily gain; ADFI, average daily feed intake.

[2]The SEM and *P*-values within this row are from a repeated measures analysis of the associated weeks with week as the repeated measure and diet as a fixed effect.

[3]Due to variation in the SE of the interaction lsmeans, GLN+SYN at week 2 tends to be different ($0.05 \geq P < 0.10$) than the PC at week 2, but neither GLN+SYN at week 2 nor the PC at week 2 tend to be different ($P > 0.10$) than any of the remaining week by diet lsmeans.

[4]Analysis of the average response over the time period specified with diet as a fixed effect.

Table 4.6. Growth performance, feed intake, and feed efficiency of pigs during the grow-finish period that were fed five treatment diets from d 1 to d 14 of the nursery period and common diets from d 15 through market-weight[1]

Item	**NC**	**GLN**	**SYN**	**GLN+SYN**	**PC**	**SEM**	***P*-Value**
BW, kg							
Phase 1[2]	33.9	32.6	33.1	33.4	34.4	1.99	0.75
Phase 2[3]	53.0	51.9	52.4	53.1	52.7	2.14	0.94
Phase 3[4]	73.4	71.9	72.3	73.5	72.7	2.55	0.90
Phase 4[5]	91.8	90.0	90.9	91.9	91.5	2.86	0.91
Phase 5[6]	111.8	109.8	110.5	111.0	111.3	3.48	0.94
Phase 6[7]	124.8	127.2	127.9	127.0	126.8	4.17	0.92
ADG, kg							
Phase 1[2]	0.71	0.69	0.67	0.69	0.69	0.032	0.87
Phase 2[3]	0.91	0.92	0.90	0.93	0.85	0.027	0.16
Phase 3[4]	1.00	0.95	0.95	0.97	0.95	0.029	0.39
Phase 4[5]	0.88	0.86	0.89	0.88	0.86	0.026	0.90
Phase 5[6]	0.95	0.94	0.91	0.91	0.91	0.048	0.85
Phase 6[7]	1.07	1.24	1.25	1.09	1.14	0.061	0.06[8]
Phase 1 – 6[9]	0.92	0.93	0.93	0.91	0.90	0.025	0.43
ADFI, kg							
Phase 1[2]	1.27	1.27	1.28	1.30	1.30	0.064	0.98
Phase 2[3]	2.10	2.00	2.06	2.07	2.04	0.071	0.59
Phase 3[4]	2.53	2.46	2.45	2.52	2.44	0.071	0.64
Phase 4[5]	2.73	2.66	2.77	2.71	2.73	0.090	0.86
Phase 5[6]	2.96	3.02	3.03	3.02	3.02	0.102	0.97
Phase 6[7]	3.16[ab]	3.23[ab]	3.35[a]	3.13[ab]	2.98[b]	0.130	0.02
Phase 1 – 6[9]	2.44	2.44	2.49	2.46	2.42	0.072	0.89
Gain:Feed							
Phase 1[2]	0.56	0.54	0.53	0.53	0.53	0.010	0.17
Phase 2[3]	0.44	0.46	0.44	0.45	0.42	0.014	0.17
Phase 3[4]	0.40	0.39	0.39	0.39	0.39	0.009	0.92
Phase 4[5]	0.32	0.33	0.32	0.32	0.31	0.011	0.94
Phase 5[6]	0.32	0.31	0.30	0.30	0.30	0.013	0.58
Phase 6[7]	0.33	0.38	0.37	0.35	0.38	0.017	0.08[8]
Phase 1 – 6[9]	0.39	0.40	0.39	0.39	0.39	0.005	0.50

[a-b]Values within a row not sharing a common letter are different ($P < 0.05$).

[1]Abbreviations: NC, negative control diet containing no dietary antibiotics; GLN, diet containing 0.2% L-glutamine; SYN, diet containing a mixture of prebiotics and probiotics; GLN + SYN, diet containing 0.2% L-glutamine and a mixture of prebiotics and probiotics; PC, positive control diet containing antibiotics (441 ppm chlortetracycline + 38.6 ppm tiamulin); BW, body weight; ADG, average daily gain; ADFI, average daily feed intake.

[2]Phase 1 was fed from the end of the 5-week nursery period to d 26 of the grow-finish period .

[3]Phase 2 was fed from d 27 to d 47 of the grow-finish period.

[4]Phase 3 was fed from d 48 to d 68 of the grow-finish period.

[5]Phase 4 was fed from d 69 to d 89 of the grow-finish period.

[6]Phase 5 was fed from d 90 to d 110 of the grow-finish period.

[7]Phase 6 was fed from d 111 to d 124 of the grow-finish period.

[8]No trending differences ($P > 0.10$) among means were found after applying a Tukey's mean separation adjustment with $\alpha = 0.10$.

[9]The SEM and *P*-value within this row are from an analysis of the average response over the 6 phases with diet as a fixed effect.

Table 4.7. Percentage of pigs treated with injectable antibiotics and number of individual injectable antibiotic therapies given per pig during the dietary treatment phase (d 0 to 14), the common diet phase (d 14 to 35), and during the grow-finish phase[1]

Item	**NC**	**GLN**	**SYN**	**GLN + SYN**	**PC**	**SEM**	***P*-Value**
Pigs treated/pen, %[2]							
Day 0 – 14 post-weaning							
Enteric[3]	38.3	43.3	43.3	43.8	35.8	6.48	0.64
Lame[4]	2.1	2.1	2.1	0.0	6.7	1.90	0.31
Unthrifty[5]	0.0	0.0	0.0	2.1	0.0	0.42	0.42
Respiratory[6]	0.0	0.0	0.0	0.0	0.0	0.00	NA[7]
Other[8]	0.0	2.1	2.1	0.0	0.0	0.83	0.58
Day 14 – 35 post-weaning							
Enteric[3]	0.0	3.1	2.5	2.5	0.0	1.63	0.76
Lame[4]	5.0	0.0	0.0	0.0	0.0	0.65	0.08[9]
Unthrifty[5]	2.5	0.0	0.0	0.0	0.0	0.50	0.42
Respiratory[6]	2.5	3.1	0.0	3.1	0.0	1.75	0.76
Other[8]	0.0	0.0	0.0	0.0	0.0	0.00	NA[7]
Grow-finish period							
Enteric[3]	0.0	0.0	0.0	0.0	0.0	0.00	NA[7]
Lame[4]	0.0	0.0	0.0	0.0	0.0	0.00	NA[7]
Unthrifty[5]	3.1	0.0	4.2	3.1	0.0	2.08	0.76
Respiratory[6]	0.0	6.3	0.0	0.0	10.4	2.65	0.27
Other[8]	3.1	0.0	0.0	0.0	6.3	1.88	0.58
Therapy number/pig/pen[10]							
Day 0 – 14, nursery							
Enteric[3]	0.38	0.53	0.50	0.44	0.40	0.068	0.56
Lame[4]	0.04	0.02	0.02	0.00	0.07	0.023	0.44
Unthrifty[5]	0.00	0.00	0.00	0.02	0.00	0.004	0.42
Respiratory[6]	0.00	0.00	0.00	0.00	0.00	0.000	NA[7]
Other[8]	0.00	0.02	0.02	0.02	0.00	0.013	0.76
Day 14 – 35, nursery							
Enteric[3]	0.00	0.03	0.03	0.05	0.00	0.021	0.73
Lame[4]	0.05	0.00	0.00	0.00	0.00	0.007	0.08[9]
Unthrifty[5]	0.03	0.00	0.00	0.00	0.00	0.005	0.42
Respiratory[6]	0.03	0.03	0.00	0.03	0.00	0.018	0.76
Other[8]	0.00	0.00	0.00	0.00	0.00	0.000	NA[7]
Grow-finish period							
Enteric[3]	0.00	0.00	0.00	0.00	0.00	0.000	NA[7]
Lame[4]	0.00	0.00	0.00	0.00	0.06	0.013	NA[7]
Unthrifty[5]	0.03	000	0.04	0.03	0.00	0.021	0.75
Respiratory[6]	0.00	0.06	0.00	0.00	0.10	0.026	0.30
Other[8]	0.03	0.00	0.00	0.00	0.06	0.019	0.56

[1]Abbreviations: NC, negative control diet containing no dietary antibiotics; GLN, diet containing 0.2% L-glutamine; SYN, diet containing a mixture of prebiotics and probiotics; GLN + SYN, diet containing 0.2% L-glutamine and a mixture of prebiotics and probiotics; PC, positive control diet containing antibiotics (441 ppm chlortetracycline + 38.6 ppm tiamulin).
[2]Number of pigs receiving ≥ 1 therapeutic antibiotic treatment as a percentage of the number of pigs in the pen.
[3]Treatment with therapeutic antibiotics for enteric challenge.
[4]Treatment with therapeutic antibiotics for lameness.
[5]Treatment with therapeutic antibiotics for unthriftiness.
[6]Treatment with therapeutic antibiotics for respiratory challenge.
[7]NA, insufficient pigs within a category received therapeutic antibiotic injections precluding a meaningful statistical analysis.
Table 4.7 is continued on the next page

Table 4.7 continued

[8]Treatment with therapeutic antibiotics for all other conditions.
[9]No trending differences ($P > 0.10$) among means were found after applying a Tukey's mean separation adjustment with $\alpha = 0.10$.
[10]Number of separate illness events requiring therapeutic antibiotic treatment per pig per pen.

Table 4.8. Leukocyte differential from pigs that were fed five treatment diets from d 0 to d 14 of the nursery phase and common diets from d 14 to d 35 of the nursery phase[1]

							***P*-Value**		
Item	**NC**	**GLN**	**SYN**	**GLN + SYN**	**PC**	**SEM**	**Diet**	**Day**	**Diet × Day**
Neutrophils, %									
Day 2[2]	54.5	43.1	52.3	46.4	48.5	4.71	0.52	0.23	0.06[3]
Day 6	54.4	55.8	52.0	48.1	51.6				
Day 13	45.7	47.8	63.0	45.3	57.5				
Day 32[4]	31.9	33.9	34.7	28.3	31.0	3.55	0.85	-	-
Lymphocytes, %									
Day 2[2]	43.6	55.6	46.3	51.0	49.3	4.67	0.42	0.93	0.10
Day 6	43.3	42.3	46.1	49.5	46.9				
Day 13	52.1	49.1	35.6	52.6	40.4				
Day 32[4]	65.9	61.4	63.0	69.9	65.5	3.80	0.60	-	-
Neutrophil: lymphocyte ratio									
Day 2[2]	2.2	0.8	1.7	1.1	1.2	0.28	0.42	0.87	0.11
Day 6	1.6	1.5	1.2	1.2	1.3				
Day 13	1.0	1.0	1.8	0.9	1.5				
Day 32[4]	0.6	0.6	0.6	0.4	0.5	0.09	0.72	-	-
Monocytes, %									
Day 2[2]	1.1	0.6	0.9	0.7	1.1	0.41	0.95	0.02	0.62
Day 6	1.5	1.3	0.9	1.5	1.3				
Day 13	1.5	2.1	1.6	1.9	1.1				
Day 32[4]	2.5[yz]	3.3[y]	3.0[yz]	1.3[z]	2.6[yz]	0.64	0.07	-	-
Eosinophils									
Day 2[2]	0.8	0.6	0.6	1.9	1.1	0.35	0.46	0.61	0.19
Day 6	0.8	0.8	0.9	0.9	0.4				
Day 13	0.8	1.0	0.3	0.6	1.3				
Day 32[4]	0.5	1.4	0.3	0.8	1.3	0.41	0.33	-	-

[y-z] Values within a row not sharing a common letter tend to differ ($0.05 \leq P < 0.10$).

[1]Abbreviations: NC, negative control diet containing no dietary antibiotics; GLN, diet containing 0.2% L-glutamine; SYN, diet containing a mixture of prebiotics and probiotics; GLN + SYN, diet containing 0.2% L-glutamine and a mixture of prebiotics and probiotics; PC, positive control diet containing antibiotics (441 ppm chlortetracycline + 38.6 ppm tiamulin).

[2]The SEM and *P*-values within this row are from a repeated measures analysis of the 3 blood collection days that occurred during the dietary treatment phase (d 2, 6, and 13) with blood collection day as the repeated measure and diet as a fixed effect.

[3]No trending differences ($P > 0.10$) among means were found after applying a Tukey's mean separation adjustment with $\alpha = 0.10$.

[4]The SEM and *P*-value within this row are from an analysis of the 1 blood collection day that occurred after the dietary treatment phase (d 32) with diet as a fixed effect.

Table 4.9. Plasma haptoglobin levels from one pig per pen from pigs that were fed five treatment diets from d 0 to d 14 of the nursery phase and common diets from d 14 to d 35 of the nursery phase[1]

							***P*-Value**		
Haptoglobin, µg/mL	**NC**	**GLN**	**SYN**	**GLN + SYN**	**PC**	**SEM**	**Diet**	**Day**	**Diet × Day**
Post-transport	496	502	486	152	377	171.3	0.49	-	-
Day 2[2]	381	422	229	243	271	133.4	0.14	0.30	0.16
Day 6	705	456	422	345	84				
Day 13	602	257	380	461	162				

[1]Abbreviations: NC, negative control diet containing no dietary antibiotics; GLN, diet containing 0.2% L-glutamine; SYN, diet containing a mixture of prebiotics and probiotics; GLN + SYN, diet containing 0.2% L-glutamine and a mixture of prebiotics and probiotics; PC, positive control diet containing antibiotics (441 ppm chlortetracycline + 38.6 ppm tiamulin).

[2]The SEM and *P*-values within this row are from a repeated measures analysis of all 3 blood collection days that occurred from d 2 to d 13 post-weaning with blood collection day as the repeated measure and diet as a fixed effect.

Table 4.10. Tear staining of pigs fed five treatment diets from d 0 to d 14 of the nursery phase and common diets from d 14 to 35 of the nursery phase[1, 2]

							***P*-Value**		
Tear stain area, cm²	**NC**	**GLN**	**SYN**	**GLN + SYN**	**PC**	**SEM**	**Diet**	**Day**	**Diet × Day**
Post-transport	0.09	0.07	0.07	0.07	0.09	0.015	0.74	-	-
Treatment Diet Period									
Day 7[3]	0.19	0.13	0.20	0.16	0.13	0.093	0.17	< 0.01	0.31
Day 14	0.63	0.24	0.60	0.33	0.29				
Common Diet Period									
Day 21[3]	0.38	0.29	0.21	0.33	0.40	0.092	0.18	< 0.01	0.61
Day 28	0.60	0.43	0.46	0.49	0.65				
Day 32	0.57	0.29	0.44	0.43	0.48				

[1]Photos were taken of the right eye of the same two pigs per pen each measurement day. Results are presented as the average tear staining area per pig.

[2]Abbreviations: NC, negative control diet containing no dietary antibiotics; GLN, diet containing 0.2% L-glutamine; SYN, diet containing a mixture of prebiotics and probiotics; GLN + SYN, diet containing 0.2% L-glutamine and a mixture of prebiotics and probiotics; PC, positive control diet containing antibiotics (441 ppm chlortetracycline + 38.6 ppm tiamulin).

[3]The SEM and *P*-values within this row are from a repeated measures analysis of the indicated tear stain measurement days with day as the repeated measure and diet as a fixed effect.

Table 4.11. Behavior as a percentage of time of pigs fed five treatment diets from d 0 to d 14 of the nursery phase and common diets from d 14 to 35 of the nursery phase [1, 2]

							***P*-Value**		
Item[3]	**NC**	**GLN**	**SYN**	**GLN + SYN**	**PC**	**SEM**	**Diet**	**Day**	**Diet × Day**
Huddling, %									
Day 2[4]	42.2	36.3	43.1	47.2	46.7	5.87	0.91	< 0.01	0.56
Day 6	43.7	49.2	44.1	47.3	47.0				
Day 13	22.0	38.1	33.8	29.2	23.8				
Active, %									
Day 2[4]	21.3	23.5	26.7	24.1	19.1	2.41	0.19	< 0.01	0.58
Day 6	17.9	15.9	20.4	19.6	13.8				
Day 13	26.4	19.6	24.0	23.8	22.1				
Resting, %									
Day 2[4]	14.0	11.5	17.2	11.5	14.9	3.01	0.14	< 0.01	0.98
Day 6	13.4	14.1	18.6	16.1	17.2				
Day 13	22.8	21.7	25.4	22.3	27.9				
Aggressive, %									
Day 2[4]	2.3	1.5	3.9	1.4	3.3	0.45	0.09	< 0.01	0.28
Day 6	0.7	0.3	0.7	0.4	0.4				
Day 13	0.4	0.9	0.7	0.3	0.1				
Eating, %									
Day 2[4]	2.4	3.3	2.0	1.8	0.2	1.11	0.62	< 0.01	0.25
Day 6	7.8	9.5	11.1	8.5	8.7				
Day 13	11.6	9.5	11.9	10.0	12.8				
Drinking, %									
Day 2[4]	1.4	1.5	1.3	1.5	1.1	0.37	0.78	0.16	0.36
Day 6	0.6	1.3	1.0	0.7	1.4				
Day 13	1.5	1.3	1.9	1.6	0.6				
Non-visible, %									
Day 2[4]	16.5	22.5	5.7	12.6	15.0	5.04	0.32	0.40	0.86
Day 6	15.9	9.8	4.1	7.5	11.4				
Day 13	15.3	8.9	2.3	12.7	12.6				

[1]Behavior was analyzed via a scan sample at 10 min intervals from 1000 h to 1600 h on d 2, 6, and 13 post-weaning. Values presented are averages over the 6 h period for each day.

[2]Abbreviations: NC, negative control diet containing no dietary antibiotics; GLN, diet containing 0.2% L-glutamine; SYN, diet containing a mixture of prebiotics and probiotics; GLN + SYN, diet containing 0.2% L-glutamine and a mixture of prebiotics and probiotics; PC, positive control diet containing antibiotics (441 ppm chlortetracycline + 38.6 ppm tiamulin).

[3]Behaviors defined as: Huddling, ≥3 pigs are lying down with ≥ 50% of a pig's body in contact with another pig; Active, walking or interacting with pen-mates in a non-aggressive manner; Resting, lying alone or loosely in groups with ≤ 50% contact between pigs; Aggressive, engaged in agonistic interactions; Eating, piglet has nose in the feeder; Drinking, piglet has mouth on waterer; Non-visible, piglet moves out of view and cannot be observed.

[4]The SEM and *P*-values within this row are from a repeated measures analysis of all 3 d of behavior analysis with day as the repeated measure and diet as a fixed effect.

Table 4.12. Responses where at least one contrast resulted in a *P*-value of < 0.10[1, 2]

	***P*-Values**			
Item	**NC vs. PC**	**NC vs. GLN**	**NC vs. SYN**	**NC vs. GLN+SYN**
Nursery growth performance				
ADG, d 14 to 35	0.07	0.52	0.76	0.60
ADG, d 0 to 35	0.04	0.83	0.86	0.64
ADFI, d 14 to 35	0.03	0.57	0.96	0.28
ADFI, d 0 to 35	0.04	0.55	0.90	0.30
Gain:Feed, d 14 to 35	0.97	0.03	0.53	0.53
Grow-finish growth performance				
ADG, Phase 2[4]	0.09	0.76	0.85	0.48
ADG, Phase 3[5]	0.12	0.13	0.08	0.33
ADG, Phase 6[6]	0.34	0.03	0.02	0.78
ADFI, Phase 6[6]	0.02	0.87	0.41	0.28
Gain:Feed, Phase 1[3]	0.07	0.15	0.04	0.03
Gain:Feed, Phase 6[6]	0.02	0.02	0.05	0.37
Monocytes, d 32	0.37	0.32	0.48	0.09
Plasma haptoglobin, d 2, 6, and 13	0.01	0.22	0.14	0.15
Tear staining				
Day 7 to 14	0.09	0.06	0.96	0.17
Day 21 to 32	0.91	0.05	0.10	0.25
Behavior[7]				
Aggressive	0.67	0.63	0.09	0.33
Non-visible	0.60	0.63	0.05	0.38

[1]Abbreviations: NC, negative control diet containing no dietary antibiotics; GLN, diet containing 0.2% L-glutamine; SYN, diet containing a mixture of prebiotics and probiotics; GLN + SYN, diet containing 0.2% L-glutamine and a mixture of prebiotics and probiotics; PC, positive control diet containing antibiotics (441 ppm chlortetracycline + 38.6 ppm tiamulin); ADG, average daily gain; ADFI, average daily feed intake.
[2]Treatment diets were fed from d 1 to 14 post-weaning, and then common diets were fed until 35 d post-weaning.
[3]Phase 1 was fed from the end of the 5-week nursery period to d 26 of the grow-finish period.
[4]Phase 2 was fed from d 27 to d 47 of the grow-finish period.
[5]Phase 3 was fed from d 48 to d 68 of the grow-finish period.
[6]Phase 6 was fed from d 111 to d 124 of the grow-finish period.
[7]Behaviors defined as: Aggressive, engaged in agonistic interactions; Non-visible, piglet moves out of view and cannot be observed.

CHAPTER 5. HEALTH AND PRODUCTIVITY OF PIGS FED GLUTAMINE, SYNBIOTICS, OR BOTH FOLLOWING WEANING AND TRANSPORT: INTESTINAL MEASURES

5.1 Abstract

The additive effects of supplementing 0.2% L-glutamine and a synbiotic mixture on pig intestinal health, and use of an alternative urine collection method for a lactulose and mannitol test of intestinal health in group-housed pigs, were investigated. Pigs were weaned (19.4 ± 0.23 d of age; N = 226, 5.9 ± 0.11 kg) and transported in a livestock trailer for 12 h. Pigs were placed into wean-to-finish pens (n = 8 pens/trt) and received diets consisting of a negative control (NC) with no dietary antibiotics, 0.2% supplementation of L-glutamine (GLN), supplementation of *Lactobacillus* + fructooligosaccharide + β-glucan (SYN), the combination of GLN and SYN additives (GLN+SYN), and a positive control (PC) containing dietary antibiotics. Treatment diets were fed for the first two weeks of the nursery period, and common antibiotic free diets were fed thereafter. Urine was collected via urine collection pads at d 5 and d 12 post-weaning, while intestinal tissues were harvested at d 14 and 34 post-weaning and analyzed for measures of intestinal health. Data were analyzed via Proc Glimmix of SAS with time-course data analyzed as repeated measures where appropriate. Urine collection pads showed promise as a feasible alternative to potentially more stressful urine collection techniques, with successful urine collection from 84% of pigs on average. Lactulose excretion was greater (P = 0.02) at d 12 (458 ± 49.4 mg/pig) than at d 5 (267 ± 56.4 mg/pig). For the villi height to crypt depth ratio in the jejunum, there was an interaction of dietary treatment and tissue collection d (P = 0.03), with SYN and the PC having the lowest ratio at d 14 but the greatest ratio at d 34 post-weaning. Greater numbers of goblet cells per mm of villi height (P = 0.02) were found for the SYN diet (68% increase) than the

NC at d 34 in the jejunum. In addition, diet interacted with tissue collection day (P = 0.02) for mast cell density in the ileum, with GLN and GLN+SYN being least at d 14 but greatest at d 34. Gene expression of pro-inflammatory cytokines decreased from d 14 to d 34 post-weaning, but few effects of dietary treatment on gene expression were observed. In conclusion, supplementation of glutamine or synbiotics in the diets of nursery pigs improved some metrics of intestinal health, and the use of urine collection pads appears to be a viable alternative urine collection method in group-housed nursery pigs.

Keywords: urine collection, intestinal permeability, antibiotic alternative, nursery pig

5.2 Introduction

Weaning is considered the most stressful period in a pig's life, and these stresses can lead to economically important reductions in growth performance as well as increases in morbidity for several weeks after weaning (Jayaraman and Nyachoti, 2017). Antibiotics have traditionally been used to help pigs overcome these stresses, but as legislation and consumer pressure reduce the use of antibiotics in food production, swine producers must find consumer-accepted ways to help nursery pigs overcome the stresses encountered at weaning (de Lange et al., 2010). A potential solution could be alternative products that consumers would view as more natural and that do not have potential to lead to antimicrobial resistance (Thacker, 2013). Many feed additives with differing modes of action have been developed and tested as replacements for antibiotics in the nursery phase of pig production (Pluske et al., 2018). Unfortunately, many feed additives that have been explored are plagued by inconsistent results as compared to dietary antibiotics (de Lange et al., 2010). While research is still on-going to attempt to understand this inconsistency, it is currently believed to result from the relatively poor understanding of the mode of action of many

of these products, as well as a limited information on how these products perform in various production settings (Pluske et al., 2018).

Improved methods to measure the effectiveness of alternative products could aid the development of more dependable antibiotic alternatives. The absorption of non-metabolizable sugars, such as lactulose and mannitol, from the gastrointestinal tract can be used as a measure of intestinal health (Bjarnason et al., 1995), and has received some attention for use in swine due to the non-invasive nature of the test (De Vos et al., 2012). However, quantitative urine collection is required for accuracy of the test, which presents unique challenges in animals (Kurien et al., 2004). In pigs the most common method used is the metabolism cage, where a screen under the flooring allows feces to be retained while urine passes through and is collected via a pan (Adeola, 2001). Urine collection via metabolism cages typically includes individual housing of pigs which is contrary to the social nature of pigs and therefore may impose stress on the animal (Ruis et al., 2001). This stress may be particularly problematic when attempting to collect urine from pigs in the immediate post-weaning period that are already coping with the stresses of weaning (Jayaraman and Nyachoti, 2017). Indeed, attempts to collect urine from individually housed pigs 4 d post-weaning had a 43% success rate over an 18 h urine collection period (Wijtten et al., 2011). Alternatives to metabolism crates that would overcome these limitations are few. Urinary catheters present technical challenges in swine (Kurien et al., 2004; Musk et al., 2015) and preclude group housing, while attempting to catch voided urine in attached bags or pouches (Paulson and Cottrell, 1984) also precludes group housing given the inquisitive and destructive nature of swine (Feddes and Fraser, 1994). One potential solution could be a urine collection pad that our laboratory has previously developed and tested for urine collection in individually housed pigs (unpublished data). However, it is unknown if this urine collection pad will be effective in group-housed pigs.

L-glutamine, a conditionally essential amino-acid found in high levels in sow milk, is one potential feed additive (Wu, 2009). When fed to nursery pigs at levels of 0.5 to 1.5% of the diet, glutamine has been repeatedly shown to improve metrics of intestinal health and growth performance (Watford, 2015). Reduced levels of supplemental glutamine would improve the economics of glutamine supplementation, and there is some evidence that glutamine fed at 0.2% of the diet may still be effective at improving pig health and growth performance (Johnson and Lay, 2017). In addition, synbiotics (a combination of prebiotics and probiotics) have potential to reduce pathogen load in the intestine (Heo et al., 2013; Yang et al., 2014), thereby improving pig health and performance. Therefore, the study objectives are to: (1) evaluate the use of a urine collection pad to quantitatively collect urine as part of a lactulose and mannitol test of intestinal health in group-housed pigs, and (2) determine if combining dietary synbiotics (*Lactobacillus* + fructo-oligosaccharides + β-glucan) and 0.20% supplemental glutamine will result in a greater improvement in pig intestinal health following weaning and transport when compared to dietary antibiotics (chlortetracycline + tiamulin). We hypothesize that urine collection pads will be an effective replacement for metabolism crates, and that administering glutamine and synbiotics after exposure to weaning and transport stress will have an additive effect to improve piglet health over that of dietary antibiotics.

5.3 Materials and Methods

5.3.1 Animals and Diets

All procedures involving live animals were approved by the Purdue University Animal Care and Use Committee (protocol #1801001678A002) and animal husbandry practices were in

accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

Mixed-sex pigs (n = 226, 5.9 ± 0.11 kg; Duroc × (Landrace × Yorkshire) were individually weighed 1 d prior to weaning and allotted to 5 treatments in a randomized complete block design (n = 8 pens per treatment blocked by BW; 4 pens with 5 pigs and 4 pens with 6 pigs per pen) with sex ratios in pens maintained within BW blocks and balanced for ancestry. Pigs were weaned (19.4 ± 0.23 d of age) and transported for 12 h in May of 2018. Pigs were then placed into pens in a mechanically-ventilated wean-to-finish facility and grown to market weight. Barn average temperature and relative humidity (Hobo data logger, Onset, Bourne, MA) for the nursery period were: 29.5 ± 0.02°C and 35.7 ± 0.43% for week 1; 28.4 ± 0.03°C and 47.4 ± 0.45% for week 2; 26.7 ± 0.01°C and 53.9 ± 0.47% for week 3; 27.9 ± 0.07°C and 57.7 ± 0.71% for week 4; and 26.2 ± 0.05°C and 54.2 ± 0.77% in week 5, respectively. Pens had fully slatted concrete floors, measured meters 1.8 × 2.4 meters, and contained a single-hole gravity feeder and a nipple waterer. One pig per pen was removed at d 14 and 34 post-weaning for tissue collection.

Dietary treatments (Table 5.1) consisted of a negative control (NC) containing no dietary antibiotics, the NC plus 0.2% L-glutamine (GLN) (Ajinomoto North America Inc., Raleigh, NC), the NC plus a probiotic (*Lactobacillus*) and two prebiotics (β-glucan and fructo-oligosaccharide) (SYN), the combination of the GLN and SYN additives (GLN+SYN), and a positive control (PC) containing antibiotics at formulated levels of 441 ppm chlortetracycline (Aureomycin, Zoetis, Parsippany, NJ) plus 38.6 ppm tiamulin (Denagard, Elanco Animal Health, Greenfield, IN). Dietary treatments were fed for nursery phase 1 (0 to 7 d post-weaning) and nursery phase 2 (7 to 14 d post-weaning), after which all pigs were fed common diets for the remainder of the

experiment (Table 5.1). All diets were formulated to meet or exceed the nutrient requirements of the pigs at a given phase (NRC, 2012).

5.3.2 Transportation

Pigs were weaned, herded up a ramp (2.13 m long, 11° incline) into a livestock trailer (2.35 × 7.32 m; Wilson Trailer Company, Sioux City, IA) bedded with wood shavings, and transported for 12 h over 819 km. Pig density in the trailer was 0.075 m^2 per pig. Two data loggers (Hobo data logger, Onset, Bourne, MA) placed in the transport trailer obtained relative humidity (77.9 ± 0.44%) and ambient temperature (23.7 ± 0.10°C) readings in 1 min intervals for the duration of the transport. Pigs were not provided with feed or water during transport, and total transport time included loading of the trailer, transport, and unloading of the trailer. The transport route consisted of approximately 50% two-lane roads and 50% four-lane roads, with two stops to refuel the transport truck and change drivers during the transport. After arrival at the wean-to-finish facility, pigs were individually weighed, placed into pens, and given access to treatment diets.

5.3.3 Experimental Procedures

At d 5 and 12 post-weaning, 1 pig per pen was tested for intestinal health via a lactulose and mannitol test. Pigs were individually weighed 1 d before the test so that urine collection pads could be assembled for each pig. On the day of the test, feeders were removed from pens at 0600 h, baseline urine was collected for 3 h starting at 0700 h, then pigs were gavaged (Levin-style 14-French × 41 cm long feeding tube; Cardinal Health Inc., Dublin, OH) with an aqueous solution (3.47 mL/kg BW; osmolarity of 500 mOsm) of lactulose (0.5 g/kg BW; Alfa Aesar, Thermo Fisher Scientific Chemicals, Inc., Ward Hill, MA) and D-mannitol (0.05 g/kg BW; Acros Organics, Fair Lawn, NJ), and urine was collected for a 3 h period post-gavage, after which feeders were returned.

Water was freely available via a water nipple in each pen during both urine collection periods. Urine was collected by placing a pre-weighed urine collection pad (cotton balls wrapped with cheesecloth; 2.1 g of cotton/kg BW) on the sheath of barrows, several layers of plastic food wrap (ClingWrap, Glad Products Company, Oakland, CA) to act as a moisture barrier, and elastic wrap (Elastikon, Johnson and Johnson, New Brunswick, NJ) wrapped around the pig from the caudal point of the shoulder to the cranial point of the hip to secure the belly wrap in place. While most of the elastic wrap was in contact with the plastic wrap for easy removal, approximately 5 cm of elastic wrap was in contact with the skin at the front and end of the wrap to prevent rotation of the belly wrap. Pigs were placed back in their original pens for the duration of the urine collection periods. Belly wraps were removed at the end of each urine collection period, and urine collection pads weighed to determine urine output. An aliquot of urine was then collected by squeezing individual cotton balls until 5 mL of urine had been collected. Urine was then stored at 4°C until further analysis. The process of removing the baseline urine collection pad, oral gavage, and application of the post-gavage urine collection pad took an average of 2.7 min per pig on d 5, and 2.5 min per pig on d 12.

On d 14 and 34, intestinal tissues were collected from 1 pig per pen. Pigs were euthanized by CO_2 gas asphyxiation followed by exsanguination (AVMA, 2013). A 15 cm section of jejunum was removed 1.2 meters (d 14) or 1.8 meters (d 34) from the pyloric sphincter, and a 15 cm section of ileum was removed immediately prior to the ileo-cecal junction. Intestinal sections were rinsed with PBS to remove digesta, and a 7 cm section of jejunum and ileum were placed into 10% neutral-buffered formalin until further processing for histology. The remaining 7 cm section was opened longitudinally, mucosa was obtained by gently scraping the luminal surface with a glass slide, snap frozen in liquid nitrogen, and stored at -80°C until further processing.

5.3.4 Laboratory Analysis

A HPLC system comprised of an eluent degasser (Series 1050, Hewlett-Packard, Palo Alto, CA), an autoloader (SIL-9A, Shimadzu, Columbia, MD), a single-piston pump (LC-6A, Shimadzu, Columbia, MD), a column oven (CTO-6A, Shimadzu, Columbia, MD), a calcium-form resin-based ionic column (Aminex HPX-87C, 300 mm x 7.8 mm, Bio-Rad Laboratories, Hercules, CA) protected by a de-ashing guard column (Micro-Guard De-Ashing Cartridges, Bio-Rad Laboratories, Hercules, CA), and a refractive index detector (HP 1047A, Hewlett-Packard, Palo Alto, CA) were used for urine analysis (Catassi et al., 1991). Ultrapure water was used as the eluent, eluent flow rate was 0.6 mL/minute, column temperature was maintained at 65°C, injection volume was 20 µL, and the refractive index detector temperature was held at 50°C.

Stock solutions of lactulose (Alfa Aesar, Thermo Fisher Scientific Chemicals, Inc., Ward Hill, MA, and D-mannitol (Acros Organics, Fair Lawn, NJ) were prepared by dissolution of dry chemicals with ultrapure water. Standards ranging in concentration from 0.009 to 1.0 mg/mL were then created by dilution of stock solutions with ultrapure water. The area under the curve (AUC) for the standards analyzed in duplicate was linear through the concentrations evaluated. The standard curve generated for mannitol was $y = 7.8369x – 0.0426$ with an $R^2 = 0.999$, and the standard curve for lactulose was $y = 7.7351x – 0.0136$ with an $R^2 = 0.999$ where y is the AUC and x is the concentration of mannitol or lactulose in mg/mL. Urine concentrations were approximately equalized by using urochrome levels (absorbance at 370 nm) as an indicator of urine concentration and diluting with ultrapure water. Urine was filtered through a 0.2 µm PTFE syringe filter (17 mm disk, Thermo Scientific, Waltham, MA) prior to injection. Baseline urine collected shortly before the oral gavage of lactulose and mannitol was also analyzed via HPLC to generate pre-oral dose chromatograms for each pig. An excel-based (Microsoft, Redmond, WA) integration tool was used

to subtract matrix peaks from the post-oral dose chromatograms to allow accurate and rapid integration of the lactulose and mannitol peaks.

Jejunum and ileum sections were processed by the Purdue Histology Research Laboratory using an Alcian Blue-Giemsa combination stain that allowed mast cells, goblet cells, and gross morphology to be easily visualized. In brief, cross sections of preserved tissues were placed in paraffin blocks and sections cut at 5 µm. Sections were deparaffinized, hydrated in water, and placed on slides. Slides were stained in Alcian Blue pH 2.5 stain for 20 min, and were then washed in running tap water for 5 min. Slides were then placed in preheated alkaline alcohol for 20 min, washed in running tap water for 5 min, and then immediately placed in methanol for 2 min. The Giemsa staining solution was preheated to 60°C and filtered into coplin jars containing the slides. Slides were stained for 2 min at 60°C, and then rinsed quickly in distilled water. Slides were dipped in 1% acetic acid, rinsed quickly in distilled water, and then in three changes of methanol. Tissues were cleared in xylene and protected with cover slips.

For histological measures, correct identification of mast cells, goblet cells, and the location of the crypt/villi interface were verified by a pathologist prior to making histological measurements. All histology measures were made by one trained individual who was blinded to dietary treatments. Villi height was defined as the distance from the tip of the villus to the start of the crypt, and crypt depth as the distance from the start of the crypt to its termination in the submucosa. Only intact villi with an associated crypt were measured. To avoid selection bias (e.g. selection of large prominent villi only), a random location on the slide was selected at 4 or 10X magnification depending on villi length, and the first 6 villi meeting the selection criteria were imaged (MoticamBTW, Motic, Hong Kong, China) for later analysis. ImageJ software (NIH, Bethesda, MD) was used to measure villi height and crypt depth from the obtained images. ImageJ was

calibrated prior to image analysis using images of a slide containing a 1 mm scale bar taken at the same magnification as images of villi and crypts. A total of 6 villi and corresponding crypts for each tissue type for each pig were measured. The number of goblet cells present on each measured villi were also counted. Goblet cell count for each villi was divided by the corresponding villi height to obtain goblet cell count per mm of villi (Horn et al., 2009). Mast cells in the submucosa were counted at 40X in 9 fields of view (one field of view measured 47.27 μm^2) per pig. To avoid selection bias (e.g. only selecting areas containing large numbers of mast cells), a random section of submucosa was selected, and mast cells were counted in 3 immediately adjacent fields of view. This process was repeated 3 times to obtain mast cell counts in 9 fields of view for each tissue type for each pig. Results are presented as mast cells per mm^2 (Horn et al., 2014). All histology measures were averaged per pig prior to data analysis.

For gene expression, mRNA was extracted from intestinal mucosal scrapings using a commercial kit (RNeasy Mini Kit, QIAGEN, Germantown, MD). Using another kit (Invitrogen TaqMan Reverse Transcription Reagents, ThermoFisher Scientific, Carlsbad, CA) cDNA was made via a thermocycler. Real-time PCR was then used to determine gene expression using Invitrogen TaqMan Universal PCR Master Mix (ThermoFisher Scientific, Carlsbad, CA) and Invitrogen TaqMan Gene Expression Assays combined primer and probe sets (ThermoFisher Scientific, Carlsbad, CA) for the following genes (primer and probe sets identified with assay ID): tumor necrosis factor-α (TNF-α; assay ID Ss03391318_g1), interleukin-8 (IL-8; assay ID Ss03392437_m1, zonula occludens-1 (ZO-1; assay ID Ss03373514_m1), glucagon-like peptide 2 receptor (GLP2R; assay ID Ss04322851_m1), claudin-1 (CL-1; assay ID Ss04246284_s1) and 18S ribosomal RNA (18S; assay ID Hs03003631_g1). Results were quantified by the standard curve

method, and data are expressed as the relative abundance of the genes of interest to the reference gene (18S).

5.3.5 Statistical Analysis

Pigs were allotted to dietary treatments in a randomized complete block design with initial BW as the blocking factor. Pen was considered the experimental unit. Data were primarily analyzed via Proc Glimmix of SAS 9.4 (SAS Institute, Carry, NC), with Proc Mixed of SAS 9.4 used to analyze for influential outliers via Cook's D test (Cook, 1977). Dietary treatment was considered a fixed effect while block was considered a random effect. Data collected over time were analyzed with repeated measures as appropriate with the time unit as a fixed effect, the subject as pen(treatment*block), and using the optimal covariance structure for each response variable as determined by the BIC goodness of fit criteria (Littell et al., 1998). A Kenward-Rogers degrees of freedom correction was applied to all repeated measures analysis via the ddfm=kr option of the model statement (Kenward and Roger, 1997). Means of significant main effects or interactions were separated with a Tukey's adjustment. Single degree of freedom contrasts were used to analyze the NC vs. PC, NC vs. GLN, NC vs. SYN, and NC vs. GLN+SYN. Results are presented as least square means ± 1 SEM unless otherwise stated. Significance was declared at $P < 0.05$, while a tendency towards significance was considered at $0.05 \geq P < 0.10$.

5.4 Results

On d 5 post-weaning, 22.5% of pigs did not urinate in the 3 h post-gavage urine collection period, while 10% did not urinated post-gavage on d 12 post-weaning (Figure 5.1). Of the d 5 post-weaning urine, 22.5% of pigs had not urinate during the baseline urine collection, and an additional

12.5% of pigs had urine that was unable to be integrated accurately. Therefore, on d 5, 77.5% of pigs urinated post-gavage, but usable urine was obtained from 42.5% of pigs. Of the d 12 post-weaning urine, 12.5% of pigs did not urinate during the baseline urine collection, and an additional 12.5% of pigs had urine that was unable to be integrated accurately. Therefore, on d 12, 90% of pigs urinated post-gavage, but usable urine was obtained from 65% of pigs.

Urinary lactulose and mannitol excretion data are presented in Table 5.3 with contrasts presented in Table 5.8. Pre-gavage urine output was greater ($P = 0.03$) for d 12 (41.1 ± 5.66 g/pig) than for d 5 (23.1 ± 6.32 g/pig) while post-gavage urine output was not impacted by day and averaged 35.6 ± 13.35 g/pig. Lactulose output was not impacted by dietary treatment ($P = 0.21$) but was impacted by day ($P = 0.02$), with greater excretion at d 12 (458 ± 49.4 mg/pig) than at d 5 (267 ± 56.4 mg/pig) post-weaning. In addition, a contrast indicated a tendency ($P = 0.08$) for lactulose output to be greater for the NC (470 ± 99.7 mg) than for the PC (217 ± 94.0 mg). There was a diet by day interaction ($P < 0.01$) for mannitol excretion, with mannitol excretion increasing from d 5 to d 12 for the NC, GLN, and SYN treatments, but with mannitol excretion decreasing from d 5 to d 12 for the GLN+SYN and PC treatments. The ratio of lactulose to mannitol was not impacted by diet, day, or their interaction ($P \geq 0.74$) and averaged 3.6 ± 1.53.

Histology measures analyzed as repeated measures are presented in Table 5.4 with contrasts presented in Table 5.8. There was an interaction of diet and day for villi height in the jejunum ($P = 0.04$), primarily because the PC was the shortest at d 14, but was the longest at d 34 (205.8 µm increase), but with the NC only increasing by 59.9 µm and the GLN+SYN only increasing 44.8 µm over the same period. There was also a main effect of day ($P < 0.01$), with villi being longer at d 34 (438 ± 12.6 µm) than at d 14 (316 ± 13.9 µm). Similarly, crypt depth in the jejunum was greater ($P < 0.01$) at d 34 (379 ± 7.3 µm) than at d 14 (347 ± 8.1 µm). For the villi height to crypt

depth ratio in the jejunum, there was an interaction of treatment with day (P = 0.03), with SYN and the PC having the lowest ratio at d 14 but the greatest ratio at d 34 and with the NC having the second highest ratio at day 14 but the lowest ratio at day 35. In addition, d 34 had greater (P < 0.01; 1.19 ± 0.044) villi height to crypt depth ratio in the jejunum than d 14 (0.92 ± 0.049). For goblet cells per villi in the jejunum, there was a tendency (P = 0.08) for an interaction of day and treatment, with the NC being greatest at d 14 but decreasing to be the least at d 34, and with goblet cell numbers more than doubling for the SYN from d 14 to d 34. There were more (P < 0.01) goblet cells per villi at d 34 (3.4 ± 0.29) than at d 14 (2.2 ± 0.32) in the jejunum. When villi length was accounted for, there was no impact of diet, day, or their interaction ($P \geq 0.33$) on goblet cells per mm of villi length in the jejunum. The GLN treatment had greater (P = 0.02; 264 ± 17 cells/mm^2) numbers of mast cells in the jejunum than the GLN+SYN treatment (200 ± 17 cells/mm^2). In addition, a contrast indicated a tendency (P = 0.09) for density of mast cells in the jejunum for the GLN diet (264 ± 16.9 cells/mm^2) than for the NC (231 ± 16.9 cells/mm^2).

Villi height in the ileum was impacted by day (P < 0.01), with villi at d 34 (400 ± 9.1 µm) being longer than at d 14 (291 ± 12.4 µm). Similarly, crypt depth in the ileum was affected by day (P < 0.01), with crypts longer at d 34 (306 ± 4.7 µm) than at d 14 (248 ± 6.3 µm). As a result, there was no impact of day on the ratio of villi height to crypt depth in the ileum. A contrast demonstrated a tendency (P = 0.08) for goblet cell count per villi in the ileum to be increased for the PC (8.0 ± 0.56) over that of the NC (6.6 ± 0.56). Also, goblet cell count per villi in the ileum was greater (P < 0.01) for d 34 (8.8 ± 0.50) than for d 14 (6.0 ± 0.39), but when villi height was accounted for, there was no impact of diet, day, or their interaction ($P \geq 0.21$) in goblet cells per mm of villi length in the ileum. There was an interaction (P = 0.02) of diet and day for mast cells in the ileum, with GLN and GLN+SYN having the lowest density of mast cells at d 14, but then having the

greatest density at d 34. A contrast of the repeated measures data indicated greater (P = 0.03) density of mast cells in the ileum for the GLN diet (604 ± 38.8 cells/mm^2) than for the NC (504 ± 38.8 cells/mm^2). In addition, mast cell density was greater (P < 0.01) on d 34 (649 ± 30.0 cells/mm^2) than at d 14 (437 ± 30.0 cells/mm^2).

Histology results separated by day are presented in Table 5.5 with contrasts presented in Table 5.8. For d 14, there was a tendency for mast cell counts to be impacted by diet (P = 0.09), but with no tendencies ($P \geq 0.10$) among means. As indicated by a contrast, crypt depth was greater (P = 0.04; 16%) at d 14 in the jejunum for the SYN treatment compared to the NC. Contrasts indicated tendencies for villi height to crypt depth ratio at d 14 in the jejunum to be reduced for the SYN (P = 0.08; 26%) and PC (P = 0.07; 25%) treatments compared to the NC. A contrast indicated a tendency (P = 0.07) for goblet cell count per villi at d 14 in the jejunum to be greater (88%) for the NC than the PC. A contrast indicated a tendency (P = 0.05) for mast cell counts at d 14 in the jejunum to be decreased (29%) for the GLN+SYN compared to the NC. A contrast indicated an increase (P = 0.03; 21%) in villi height for the PC over the NC at d 34 in the jejunum. Contrasts indicated tendencies (P = 0.08) for increases in villi height to crypt depth ratio at d 34 in the jejunum for SYN (25%) and the PC (25%) over that of the NC. For d 34 in the jejunum, there was a tendency (P = 0.06) for goblet cell count/villi to be increased for the SYN treatment compared to the NC, with all other treatments being intermediate. Similarly, for d 34 in the jejunum, there was a tendency (P = 0.07) for goblet cell count/mm of villi height to be increased for the SYN treatment compared to the PC, with all other treatments being intermediate. Contrasts indicated increases in goblet cell count per villi (P < 0.01; 96%) and goblet cell count per mm of villi (P = 0.02; 68%) for SYN compared to the NC at d 34 in the jejunum. Mast cell counts at d 34 in the ileum were impacted by diet (P = 0.01) with GLN having greater mast cell density than the

NC and the SYN treatments, with the GLN+SYN and PC treatments being intermediate. A contrast also identified mast cell counts to be increased ($P < 0.01$; 37%) for GLN over that of the NC at d 34 in the ileum.

Gene expression data from intestinal tissues analyzed over time are presented in Table 5.6, with contrasts presented in Table 5.8. There was no main effect of dietary treatment ($P \geq 0.16$) or an interaction of diet and time ($P \geq 18$) for any genes analyzed. Expression of TNF-α in the jejunum was greater ($P < 0.01$) at d 14 (0.28 ± 0.019) than at d 34 (0.19 ± 0.012). In addition, a contrast demonstrated a tendency ($P = 0.09$) for reduced expression of TNF-α in the GLN+SYN treatment (0.21 ± 0.024) than the NC (0.27 ± 0.024) in the jejunum. Similar results for TNF-α in the ileum were found with greater ($P < 0.01$) expression at d 14 (1.00 ± 0.090) than at d 34 (0.38 ± 0.048). Expression of IL-8 in the jejunum was greater ($P < 0.01$) at d 14 (1.31 ± 0.133) than at d 34 (0.75 ± 0.079) with similar results in the ileum with greater ($P = 0.03$) expression at d 14 (0.53 ± 0.042) than at d 34 (0.39 ± 0.035). Expression of CL-1 in the jejunum was impacted by day ($P < 0.01$), with greater expression at d 34 (1.10 ± 0.088) than at d 14 (0.77 ± 0.088). In addition, contrasts indicated a reduction in expression of CL-1 for GLN ($P = 0.03$; 0.74 ± 0.131) and a tendency for a reduction for the PC ($P = 0.09$; 0.82 ± 0.131) over that of the NC (1.13 ± 0.131) in the jejunum. Expression of GLP2R in the jejunum was impacted by day ($P < 0.01$), with greater expression at d 34 (0.39 ± 0.031) than at d 14 (0.28 ± 0.019), while the opposite was seen in the ileum with greater ($P < 0.01$) expression of GLP2R at d 14 (0.58 ± 0.046) than at d 34 (0.30 ± 0.024). There was a tendency ($P = 0.05$) for ZO-1 expression in the ileum to be greater for d 14 (1.45 ± 0.07) than for d 34 (1.25 ± 0.07). In addition, a contrast indicated reduced ($P = 0.03$) expression of ZO-1 in the ileum for SYN (1.16 ± 0.10) compared to the NC (1.48 ± 0.10). No other significant or trending results were found for gene expression over time.

Gene expression of intestinal tissues analyzed per tissue collection day are presented in Table 5.7, and contrasts are presented in Table 5.8. For d 14 there was no main effect of dietary treatment on gene expression in the jejunum ($P \geq 0.74$) or the ileum ($P \geq 0.11$). Contrasts for gene expression of ZO-1 in the ileum at d 14 indicated a reduction in gene expression for the SYN ($P = 0.01$; 36%) and a tendency for a reduction for GLN+SYN ($P = 0.07$; 23%) compared to the NC. For d 34 there was a tendency ($P = 0.09$) for TNF-α expression in the jejunum to be greater for GLN (0.24 ± 0.024) than for the PC (0.15 ± 0.024), with all other treatments intermediate. Contrasts for d 34 gene expression in the jejunum indicated tendencies for reduced expression for the PC compared to the NC for TNF-α ($P = 0.09$; 29%) ZO-1 ($P = 0.09$; 26%), and CL-1 ($P = 0.08$; 31%), as well as reduced expression of CL-1 for GLN ($P = 0.02$; 42%) than the NC. For d 34 there was no main effect of dietary treatment on gene expression in the ileum ($P \geq 0.61$). No other significant or trending results were found for gene expression separated by time point.

5.5 Discussion

The use of the lactulose and mannitol test of intestinal health as used in humans (Sequeira et al., 2014a) has received some attention from researchers working with gut health in swine due to the non-invasive nature of the test (De Vos et al., 2012). However, quantitative urine collection in animals presents unique challenges (Kurien et al., 2004). Urine collection methods in pigs typically require individual housing of animal which is contrary to the social nature of pigs and therefore may impose stress on the animal (Ruis et al., 2001). This stress may be particularly problematic when attempting to collect urine from pigs in the immediate post-weaning period that are already coping with the stresses of weaning (Jayaraman and Nyachoti, 2017). Indeed, attempts to collect urine from individually housed pigs 4 d post-weaning had a 43% success rate over an 18 h urine

collection period (Wijtten et al., 2011). One potential solution could be a urine collection pad that our laboratory has previously developed and tested for urine collection in individually housed pigs (unpublished data). This urine collection pad was tested for use in group-housed pigs in the present study. The 78% urine collection success rate at d 5 and the 90% success rate at d 12 over the 3 h post-gavage urine collection period would indicate that the belly wrap urine collection method may be a viable alternative to metabolism crates for nursery pigs in the immediate post-weaning period. It is postulated that allowing pigs to remain in their original pens with their conspecifics reduced the stress (Ruis et al., 2001) associated with the urine collection period and improved the urine collection success rate over that seen with similar age pigs (Wijtten et al., 2011). Over the two urine collection days, two pigs were able to remove the wrap on d 5, with pen-mates finding the end of the elastic wrap and rapidly unwinding it. An improvement could be to use a small amount of quickly drying adhesive on the end of the last wrap to preclude this problem in future studies. Therefore, the use of a urine collection pad affixed with a belly wrap may be useful in future studies requiring quantitative urine collection in group-housed nursery pigs.

Despite the success of the belly wraps to obtain urine in group-housed pigs, there was still considerable variation in urine output from pig to pig, with 1 SEM for post-gavage urine output being 37.5% of the average post-gavage urine volume across days and dietary treatments. This may be an inherent characteristic of voluntary urine voiding from animals (Bannink et al., 1999); however, this variation limits the usefulness of the urinary lactulose and mannitol test of intestinal health in nursery pigs. Variation in urine output during the time period when lactulose and mannitol are being absorbed from the intestines will directly translate into variation in the resulting excretion of lactulose and mannitol, making differences among treatments difficult to detect. One solution to this problem is to collect urine for a longer time period to ensure that all lactulose and mannitol

that are absorbed are eventually excreted and accounted for (e.g. Kansagra et al. (2003), Wang et al. (2015)). However, in humans it is known that the large intestine will readily absorb lactulose (Sequeira et al., 2014b), and therefore lactulose and mannitol tests in humans are timed to terminate urine collection before the ingested dose of lactulose and mannitol reaches the large intestine (Sequeira et al., 2014a). Accordingly, the urine collection period in this study was estimated to primarily occur before the delivered dose of lactulose and mannitol reached the large intestine of the pigs. However, the substantial variation observed in urine output (and thus lactulose and mannitol excretion) even with the use of belly wraps in a group-housed setting may be a bigger problem than the undesirable absorption of lactulose in the large intestine. However, in pigs it is unknown how much of the lactulose will escape fermentation in the cecum and be available for absorption in the large intestine. Thus, while additional research is required, a longer urine collection time may result in more uniform excretion of the absorbed lactulose and mannitol, and lesser variation in lactulose and mannitol recovery (e.g. Wang et al. (2015). To enable the lactulose and mannitol test of intestinal health to be practically useful in nursery pigs, additional investigation into how to minimize extraneous variation in excretion of lactulose and mannitol is required.

Greater absorption of the larger molecule lactulose indicates greater permeability (Sequeira et al., 2012), while greater absorption of the smaller molecule mannitol indicates greater absorptive surface area (Berkeveld et al., 2008). The ratio of lactulose to mannitol is therefore a metric of overall intestinal health (Dastych et al., 2008; Mishra and Makharia, 2012). The increase in lactulose excretion from d 5 to d 12 was not expected, as it was anticipated that the intestines of pigs would be starting to recover from the stresses of weaning by d 12 (de Lange et al., 2010). Increases in the surface area of the intestines for pigs at d 12 could be postulated to create a larger

area for lactulose to permeate, but this hypothesis is not supported by the mannitol results, which did not significantly change from d 5 to d 12 but rather demonstrated an interaction of day and treatment, with GLN increasing and GLN+SYN decreasing in mannitol excretion from d 5 to d 12. However, transient decreases in permeability of the jejunum immediately following weaning has been observed in pigs via Ussing chambers, with greater permeability at 8 d post-weaning then at 2 d post-weaning (Boudry et al., 2004). In addition, barrier function of pigs has been reported to be compromised through 14 d post-weaning (Hu et al., 2013). Therefore, it is possible that the increased lactulose excretion at d 12 is indicative of increasing permeability from d 5 through d 12.

Villi height and crypt depth are reliable indicators of intestinal health in pigs, with consistent decreases in villi height and increases in crypt depth evident rapidly post-weaning (Heo et al., 2013). This may be due to transient decreases in luminal nutrients as pigs learn how to eat solid food post-weaning, and is generally believed to translate into reduced digestion and absorption of nutrients (Pluske et al., 1996). In the present study, the interaction of day and treatment observed for villi height in the jejunum appears to be driven by the PC, and to a lesser degree the SYN treatment, dramatically increasing in height from d 14 to d 34, with only modest increases for the NC over the same time period. With crypt depth remaining relatively stable, this translated into improvements in the villi height to crypt depth ratio from d 14 to d 34 for the SYN and PC, while the NC remained unchanged. While the exact cause of this interaction is unknown, these interactions may be caused by delayed impacts of the SYN and PC that resulted in improved intestinal health in the common diet period. While villi height and crypt depth in the ileum were predictably impacted by time post-weaning (Boudry et al., 2004), there was less impact of dietary treatment in the ileum than in the jejunum.

Mucins produced by goblet cells in the intestines act as an important defense against luminal bacteria, with decreases in mucus layer thickness resulting in intestinal disease (Kim and Ho, 2010). While goblet cell count per villi was impacted by day in both the jejunum and ileum, there was no effect of day in goblet cell density per mm of villi height. Thus, the differences between days detected in goblet cells per villi are likely a reflection of differences in villi height between days, and not goblet cell density. This underscores the importance of expressing goblet cell density as a function of villi height (Horn et al., 2009). Interestingly, the SYN treatment, but not the GLN+SYN treatment, had nearly twice as many goblet cells per mm of villi than the NC or PC at d 34 in the jejunum. A known potential mode of action of probiotics is stimulation of mucin production (Sherman et al., 2009), but why this would be present at d 34 rather than at d 14, and why increased goblet cell density was not observed with the combination of synbiotics and glutamine, remains to be elucidated.

Mast cells are components of the innate immune system that are able to rapidly respond to pathogens to protect the host (Abraham and St. John, 2010). However, mast cells are a double-edged sword, as this rapid response may actually be a negative in pigs at weaning, with mast cell activation and hyperplasia believed to be key players in weaning-associated decreases in intestinal health (Moeser et al., 2017). In the present study, greater mast cell density in the jejunum was observed for GLN than for the GLN+SYN treatment in the repeated measures analysis. Likewise, the GLN treatment had greater density of mast cells in the ileum at d 34 than the NC or the SYN treatment. In addition, the interaction of day and treatment for mast cells in the ileum appears to be driven by marked increases in mast cell density from d 14 to 34 for the GLN and GLN+SYN treatments, with more modest increases for the other treatments. Therefore, glutamine supplementation alone, or in combination with synbiotics in some cases, appears to have resulted

in greater mast cell density, particularly during the common diet period of the nursery. Therefore, an alternate explanation could be that mast cell numbers were somewhat reduced in the presence of glutamine during the treatment phase and rebounded once glutamine was absent in the common diet phase. As activation status of mast cells was not measured in this study, it is unclear if this increase in mast cells represents a more robust innate immune system (Abraham and St. John, 2010), or if it represents hyperplasia and mast cell activation that will lead to decreases in intestinal health (Smith et al., 2010).

Tight junction proteins are key regulators of paracellular permeability in the intestines and alterations in density or function can compromise intestinal barrier function (Günzel and Yu, 2013). Dietary glutamine when supplemented at 1% has shown some ability to improve the abundance of some tight-junction proteins in pigs 1 week post-weaning (Wang et al., 2015). Surprisingly, upregulation of CL-1 for the NC over that of the GLN diet was observed in the jejunum both in the repeated measures analysis and at d 34. Similarly, upregulation of ZO-1 was observed for the NC over that of the SYN diet in the ileum both in the repeated measures analysis and at d 14. The cause for this upregulation of tight junction proteins in the intestines of the NC pigs is currently unknown.

Pro-inflammatory cytokines such as TNF-α and IL-8 play important roles in immune function and inflammation (Watkins et al., 1995). Weaning induces increased expression of many pro-inflammatory cytokines in pigs, most of which return to pre-weaning levels within 8 d of weaning (Pié et al., 2004). In the present study, expression of TNF-α and IL-8 were both reduced at d 34 compared to d 14 regardless of tissue type. This is consistent with the expected decrease over time of pro-inflammatory cytokines post-weaning (Hu et al., 2013).

Glucagon-like peptide 2 (GLP2) is a epithelial-specific factor responsible for stimulating growth of the intestines (Deng et al., 2016), among other functions (Connor et al., 2016), and is produced in response to nutrient ingestion (Burrin et al., 2003). Levels of GLP2 in serum are reported to increase with increased food intake in pigs, with receptor (GLP2R) expression in intestinal tissues inversely related (Petersen et al., 2003). Interestingly, GLP2R levels in the jejunum and ileum were differentially impacted by time, with greater levels of GLP2R expression at d 34 in the jejunum, and greater levels of GLP2R expression at d 14 in the ileum. The reason for the tissue differences over time is currently unknown.

5.6 Conclusions

The use of a belly wrap to quantitatively collect urine in group-housed nursery pigs was validated in the present study and presents a potentially low-stress alternative to metabolism crates. Increased excretion of lactulose was observed at d 12 compared with d 5 post-weaning, potentially indicating increased intestinal permeability at d 12 than at d 5. Dietary synbiotics and antibiotics improved some histological measures of intestinal health in the common diet period, while glutamine supplementation appeared to increase intestinal mast cell density, primarily in the common diet period. There was limited impact of dietary treatment on gene expression at 14 and 34 d post-weaning. Dietary inclusion of glutamine or synbiotics may improve some metrics of pig intestinal health post-weaning, but requires further research.

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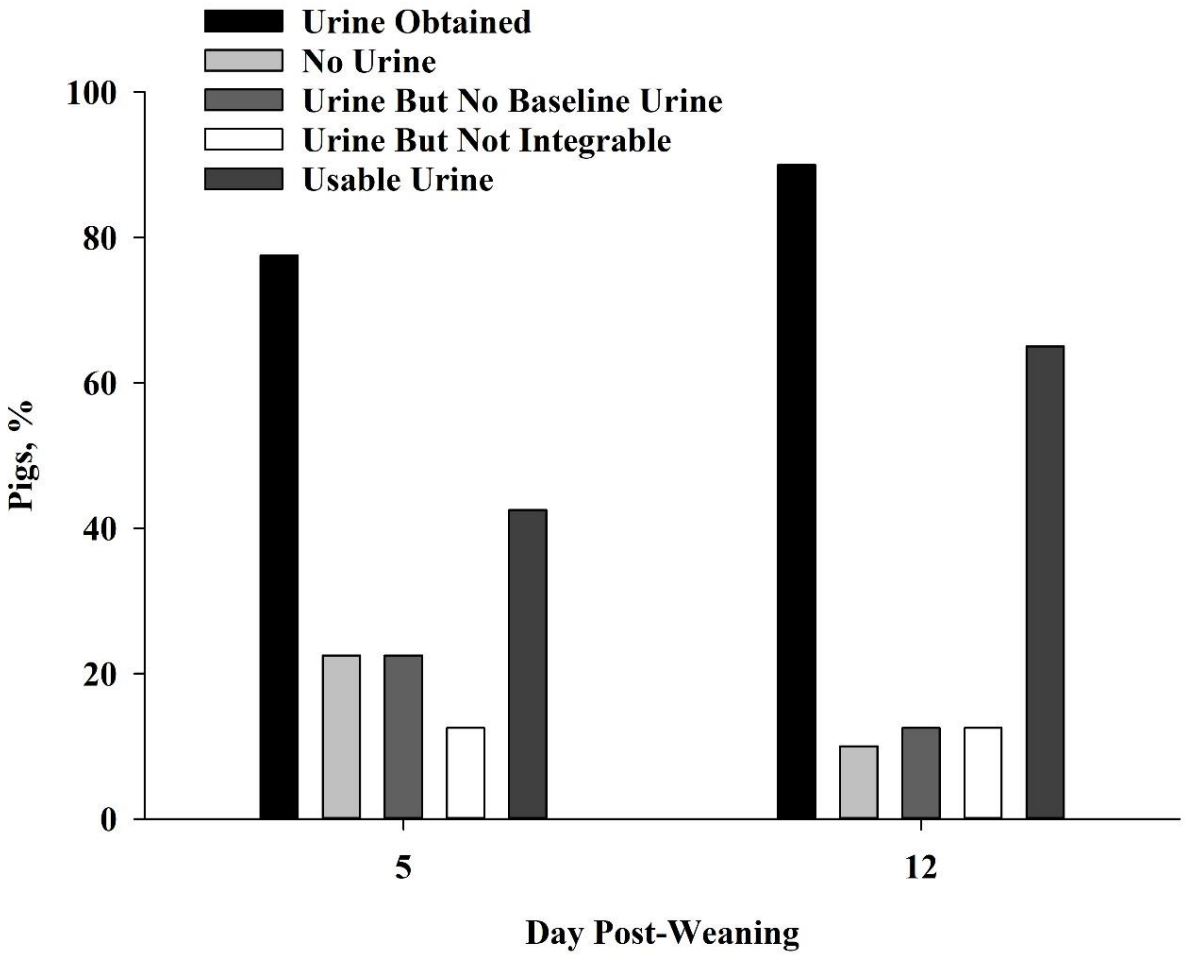

Figure 5.1. Percentage of pigs that urinated, failed to urinate post-gavage or pre-gavage, or had urine that was unable to be accurately analyzed for lactulose and mannitol concentrations, leaving the percentage of urines for each collection day that were used to measure intestinal health of pigs fed five treatment diets from d 1 to 14 of the nursery period.

Table 5.1. Ingredient composition and calculated nutrient composition of nursery diets

Item	**Phase 1**	**Phase 2**	**Phase 3**	**Phase 4**
Period fed, d post-weaning	0 - 7	7 - 14	14 - 21	21 - 35
Ingredient, %				
Corn	31.090	37.850	49.220	55.300
Soybean meal, 48% CP	13.900	17.950	20.800	22.810
DDGS, 7% fat	0.000	0.000	7.500	15.000
Monocalcium phosphate	0.380	0.490	0.070	0.510
Limestone	0.800	0.740	1.080	1.480
Salt	0.250	0.250	0.300	0.350
Soybean oil	5.000	5.000	3.000	0.000
Choice white grease	0.000	0.000	0.000	3.000
Lysine-HCl	0.070	0.200	0.340	0.480
DL-Methionine	0.220	0.225	0.160	0.125
L-Threonine	0.040	0.090	0.120	0.140
L-Tryptophan	0.010	0.015	0.025	0.030
Vitamin premix[1]	0.250	0.250	0.250	0.250
Trace mineral premix[2]	0.125	0.125	0.125	0.125
Selenium premix[3]	0.050	0.050	0.050	0.050
Dried whey	25.000	25.000	10.000	0.000
Lactose	5.000	0.000	0.000	0.000
Fish meal, menhaden	5.000	4.000	4.000	0.000
Plasma protein	6.500	2.500	0.000	0.000
Spray-dried blood meal	1.500	1.500	0.000	0.000
Soy protein concentrate	4.000	3.000	2.500	0.000
Zinc oxide	0.000	0.000	0.280	0.000
Copper sulfate	0.000	0.000	0.000	0.080
Banminth 48[4]	0.000	0.000	0.000	0.100
Phytase[5]	0.100	0.100	0.100	0.100
Clarifly, 0.67%[6]	0.140	0.090	0.080	0.070
Treatment premix[7]	0.575	0.575	0.000	0.000
Calculated Nutrient Composition				
ME, kcal/kg	3545	3520	3429	3407
CP, %	24.62	22.88	21.81	20.05
SID Lys, %	1.55	1.45	1.30	1.15
Ca, %	0.90	0.85	0.80	0.75
Available P, %	0.60	0.55	0.40	0.35

[1]Provided per kilogram of the diet: vitamin A, 6,614 IU; vitamin D3, 661 IU; vitamin E, 44 IU; vitamin K, 2.2 mg; riboflavin, 8.8 mg; pantothenic acid, 22 mg; niacin, 33 mg; B_{12}, 38.6 µg.

[2]Provided per kilogram of diet (available minerals): iron, 121 mg; zinc, 121 mg; manganese, 15 mg; copper, 11 mg; iodine, 0.46 mg.

[3]Provided 0.3 ppm Se.

[4]Banminth (Phibro Animal Health Corporation, Teaneck, NJ) provided 106 ppm pyrantel tartrate in the diet.

[5]Provided 600 FTU per kg of the diet.

[6]Clarifly (Central Life Sciences, Schaumburg, IL) provided 9.5 ppm (Phase 1), 6.1 ppm (Phase 2), 5.4 ppm (Phase 3) and 4.7 ppm (Phase 4) diflubenzuron in the diet.

[7]As a percent of the diet the treatment premixes consisted of: NC, 0.575% corn; GLN, 0.375% corn and 0.20% L-glutamine (Ajinomoto North America Inc., Raleigh, NC); SYN, 0.405% corn and 0.17% synbiotic mixture; GLN + SYN, 0.205% corn, 0.20% L-glutamine and 0.17% synbiotic mixture; PC, 0.40% Aureomycin (Zoetis, Parsippany, NJ; 110.3 g chlortetracycline/kg) and 0.175% Denagard (Elanco Animal Health, Greenfield, IN; 22.05 g tiamulin/kg).

Table 5.2. Analyzed composition of treatment phase nursery diets on an as-fed basis[1]

Item	**NC**	**GLN**	**SYN**	**GLN + SYN**	**PC**
Phase 1[2]					
DM, %[3]	90.8	91.1	90.6	90.7	91.3
CP, %[3]	23.5	24.4	23.5	24.2	24.1
Chlortetracycline, ppm[4]	0.0	0.0	0.0	0.0	381.6
Tiamulin, ppm[5]	0.0	0.0	0.0	0.0	38.3
Total glutamic acid, %[6]	3.57	3.79	3.67	3.78	3.73
Total Lys, %[3]	1.56	1.60	1.60	1.58	1.63
Total Met, %[3]	0.52	0.53	0.51	0.52	0.52
Total Met + Cys, %[3]	0.90	0.94	0.91	0.91	0.93
Total Thr, %[3]	1.09	1.12	1.12	1.13	1.15
Total Trp, %[3]	0.32	0.32	0.31	0.32	0.33
Total Val, %[3]	1.17	1.17	1.19	1.19	1.22
Total Ile, %[3]	0.88	0.89	0.90	0.89	0.92
Total Arg, %[3]	1.25	1.26	1.23	1.25	1.31
Total His, %[3]	0.61	0.61	0.63	0.62	0.64
Total Phe + Tyr, %[3]	1.60	1.63	1.65	1.60	1.64
Phase 2[7]					
DM, %[3]	89.9	90.1	90.4	89.5	89.5
CP, %[3]	22.1	22.2	22.3	22.5	22.2
Chlortetracycline, ppm[4]	0.0	0.0	0.0	0.0	411.2
Tiamulin, ppm[5]	0.0	0.0	0.0	0.0	36.5
Total glutamic acid, %[6]	3.45	3.82	3.78	3.77	3.70
Total Lys, %[3]	1.46	1.42	1.51	1.43	1.49
Total Met, %[3]	0.48	0.47	0.48	0.47	0.48
Total Met + Cys, %[3]	0.80	0.81	0.82	0.82	0.83
Total Thr, %[3]	1.01	1.02	1.03	0.98	0.99
Total Trp, %[3]	0.28	0.28	0.30	0.30	0.28
Total Val, %[3]	1.04	1.06	1.09	1.05	1.08
Total Ile, %[3]	0.85	0.85	0.88	0.84	0.86
Total Arg, %[3]	1.19	1.23	1.28	1.22	1.24
Total His, %[3]	0.56	0.57	0.59	0.56	0.58
Total Phe + Tyr, %[3]	1.49	1.45	1.53	1.44	1.50

[1]Abbreviations: NC, negative control diet containing no dietary antibiotics; GLN, diet containing 0.2% L-glutamine; SYN, diet containing a mixture of prebiotics and probiotics; GLN + SYN, diet containing 0.2% L-glutamine and a mixture of prebiotics and probiotics; PC, positive control diet containing antibiotics (441 ppm chlortetracycline + 38.6 ppm tiamulin).
[2]Phase 1 was fed from 0 to 7 d post-weaning.
[3]Analyzed by Ajinomoto Heartland Inc., Chicago, IL.
[4]Analyzed by Zoetis, Parsippany, NJ.
[5]Analyzed by Elanco Animal Health, Greenfield, IN.
[6]Total Glu + Gln in the diet analyzed by Ajinomoto Heartland, Inc., Chicago, IL.
[7]Phase 2 was fed from 7 to 14 d post-weaning.

Table 5.3. Urinary lactulose and mannitol excretion on d 5 and d 12 post-weaning from barrows fed five treatment diets from d 0 to d 14 of the nursery phase[1]

							***P*-Value**		
Item	**NC**	**GLN**	**SYN**	**GLN + SYN**	**PC**	**SEM**	**Diet**	**Day**	**Diet × Day**
Pigs per treatment[2]									
Day 5	4	4	4	3	2	-	-	-	-
Day 12	2	6	4	6	6	-	-	-	-
Pre-gavage urine output, g/pig[3]									
Day 5[4]	21.5	40.7	33.5	7.1	21.6	13.17	0.14	0.03	0.50
Day 12	27.6	52.4	60.8	38.6	26.0				
Post-gavage urine output, g/pig[5]									
Day 5[4]	33.3	46.4	47.4	14.5	15.0	13.35	0.42	0.30	0.56
Day 12	30.0	49.2	40.2	35.3	44.6				
Lactulose, mg/pig									
Day 5[4]	357.6	313.4	229.4	227.5	209.4	115.69	0.21	0.02	0.71
Day 12	581.4	637.2	477.0	368.7	224.8				
Mannitol, mg/pig									
Day 5[4]	115.2[ab]	152.7[ab]	98.0[ab]	229.6[ab]	117.3[ab]	62.48	0.16	0.22	< 0.01
Day 12	173.7[ab]	383.1[a]	178.1[ab]	97.8[ab]	84.7[b]				
Lactulose : mannitol ratio									
Day 5[4]	3.1	2.5	2.9	4.6	4.5	1.53	0.74	0.84	0.82
Day 12	5.1	2.3	4.1	4.4	2.7				

[a-b]Values for a response criterion regardless of day not having a common letter differ ($P < 0.05$).

[1]Abbreviations: NC, negative control diet containing no dietary antibiotics; GLN, diet containing 0.2% L-glutamine; SYN, diet containing a mixture of prebiotics and probiotics; GLN + SYN, diet containing 0.2% L-glutamine and a mixture of prebiotics and probiotics; PC, positive control diet containing antibiotics (441 ppm chlortetracycline + 38.6 ppm tiamulin).

[2]Number of pigs per treatment that yielded usable data that was used in the data analysis.

[3]Urine collected over a 3 h period prior to oral gavage with a solution of lactulose and mannitol in water. Results are shown for pigs with successful urine collections post-oral gavage.

[4]The SEM and *P*-values within this row are from a repeated measures analysis of both urine collection days with day post-weaning as the repeated measure and diet as a fixed effect.

[5]Urine collected over a 3 h period after oral gavage with a solution of lactulose and mannitol in water. Results are shown for pigs with successful urine collections post-oral gavage.

Table 5.4. Histological measures of intestinal health analyzed as repeated measures over time of pigs fed five treatment diets from d 0 to d 14 of the nursery phase and common diets from d 14 to 35 of the nursery phase[1]

	Day 14					Day 34						*P*-Value		
Item	**NC**	**GLN**	**SYN**	**GLN + SYN**	**PC**	**NC**	**GLN**	**SYN**	**GLN + SYN**	**PC**	**SEM**	**Trt**	**Day**	**Trt × Day**
Jejunum														
Villi height, µm	340[bcd]	295[dc]	295[dc]	374[abcd]	277[d]	400[abcd]	432[abc]	457[ab]	419[abc]	482[a]	29.5	0.85	< 0.01	0.04
Crypt depth, µm	324	345	377	340	352	386	399	357	378	374	17.2	0.91	< 0.01	0.12
Villi height : crypt depth ratio	1.05[abc]	0.90[abc]	0.79[bc]	1.09[abc]	0.79[c]	1.05[abc]	1.14[abc]	1.31[a]	1.13[abc]	1.31[ab]	0.103	0.94	< 0.01	0.03
Goblet cell count/villi	3.1[yz]	1.9[z]	2.1[z]	2.1[z]	1.6[z]	2.6[z]	3.3[yz]	5.1[y]	3.1[yz]	2.9[yz]	0.63	0.28	< 0.01	0.08
Goblet cell count/mm	9.2	6.6	7.0	7.2	5.6	6.8	7.5	11.4	7.4	6.3	1.62	0.36	0.46	0.33
Mast cell count/mm^2	238	254	212	168	252	224	273	280	231	201	25.6	0.02	0.32	0.16
Ileum														
Villi height, µm	280	260	307	282	327	382	413	398	394	415	24.0	0.58	< 0.01	0.54
Crypt depth, µm	253	267	258	223	237	304	314	296	303	312	11.4	0.19	< 0.01	0.32
Villi height : crypt depth ratio	1.12	1.03	1.20	1.32	1.39	1.26	1.33	1.35	1.32	1.33	0.111	0.48	0.13	0.53
Goblet cell count/villi	5.3	5.8	5.9	5.7	7.2	7.9	8.9	9.0	9.6	8.8	1.00	0.47	< 0.01	0.92
Goblet cell count/mm	18.7	21.4	19.6	19.3	22.3	20.8	21.9	22.2	25.1	21.7	2.46	0.84	0.21	0.76
Mast cell count/mm^2	426[bcd]	410[cd]	485[bcd]	363[d]	500[bcd]	582[bc]	797[a]	603[abc]	635[ab]	627[ab]	50.2	0.13	< 0.01	0.02

[a-d]Values within a row not sharing a common letter are different ($P < 0.05$).

[1]Abbreviations: NC, negative control diet containing no dietary antibiotics; GLN, diet containing 0.2% L-glutamine; SYN, diet containing a mixture of prebiotics and probiotics; GLN + SYN, diet containing 0.2% L-glutamine and a mixture of prebiotics and probiotics; PC, positive control diet containing antibiotics (441 ppm chlortetracycline + 38.6 ppm tiamulin).

Table 5.5. Histology separated by day for pigs fed five treatment diets from d 0 to d 14 of the nursery phase and common diets from d 14 to 35 of the nursery phase[1]

Item	**NC**	**GLN**	**SYN**	**GLN + SYN**	**PC**	**SEM**	***P*-Value**
Day 14							
Jejunum							
Villi height, µm	340	294	294	375	277	33.0	0.29
Crypt depth, µm	326	342	379	343	354	17.9	0.30
Villi height : crypt depth ratio	1.05	0.90	0.78	1.09	0.79	0.101	0.13
Goblet cell count/villi	3.2	1.9	2.1	2.2	1.7	0.57	0.40
Goblet cell count/mm	9.2	6.6	7.0	7.1	5.6	1.76	0.65
Mast cell count/mm^2	238	254	212	168	252	25.5	0.09[3]
Ileum							
Villi height, µm	280	260	307	282	327	27.6	0.54
Crypt depth, µm	253	267	258	223	237	13.4	0.16
Villi height : crypt depth ratio	1.12	1.03	1.20	1.32	1.39	0.140	0.46
Goblet cell count/villi	5.3	5.8	5.9	5.7	7.2	0.88	0.59
Goblet cell count/mm	18.7	21.4	19.6	19.3	22.3	2.17	0.68
Mast cell count/mm^2	426	410	485	363	500	53.9	0.23
Day 34							
Jejunum							
Villi height, µm	400	431	457	419	482	26.4	0.20
Crypt depth, µm	385	399	357	376	374	16.3	0.48
Villi height : crypt depth ratio	1.05	1.14	1.31	1.13	1.31	0.104	0.27
Goblet cell count/villi	2.6^{z}	3.3^{yz}	5.1^{y}	3.1^{yz}	2.9^{yz}	0.66	0.06
Goblet cell count/mm	6.8^{yz}	7.5^{yz}	11.4^{y}	7.4^{yz}	6.3^{z}	1.49	0.07
Mast cell count/mm^2	224	273	280	231	201	25.7	0.10
Ileum							
Villi height, µm	382	413	398	394	415	20.2	0.77
Crypt depth, µm	304	314	296	303	312	9.3	0.64
Villi height : crypt depth ratio	1.26	1.33	1.35	1.32	1.33	0.082	0.96
Goblet cell count/villi	7.9	8.9	9.0	9.6	8.8	1.12	0.87
Goblet cell count/mm	20.8	21.9	22.2	25.1	21.7	2.76	0.84
Mast cell count/mm^2	582^{b}	797^{a}	604^{b}	635^{ab}	627^{ab}	46.3	0.01

$^{a\text{-}b}$Values within a row not sharing a common letter are different ($P < 0.01$).

$^{y\text{-}z}$Values within a row not sharing a common letter tend to differ ($0.05 \leq P < 0.10$).

[1]Abbreviations: NC, negative control diet containing no dietary antibiotics; GLN, diet containing 0.2% L-glutamine; SYN, diet containing a mixture of prebiotics and probiotics; GLN + SYN, diet containing 0.2% L-glutamine and a mixture of prebiotics and probiotics; PC, positive control diet containing antibiotics (441 ppm chlortetracycline + 38.6 ppm tiamulin).

[2]No trending differences ($P > 0.10$) among means were found after applying a Tukey's mean separation adjustment with $\alpha = 0.10$.

Table 5.6. Gene expression of intestinal tissues analyzed as repeated measures over time of pigs fed five treatment diets from d 0 to d 14 of the nursery phase and common diets from d 14 to 35 of the nursery phase[1, 2]

	Day 14					**Day 34**						***P*-Value**		
Item	**NC**	**GLN**	**SYN**	**GLN + SYN**	**PC**	**NC**	**GLN**	**SYN**	**GLN + SYN**	**PC**	**SEM**	**Trt**	**Day**	**Trt × Day**
Jejunum														
TNF-α	0.33	0.26	0.27	0.26	0.30	0.21	0.24	0.18	0.17	0.15	0.033	0.41	< 0.01	0.44
IL-8	1.27	1.16	1.47	1.18	1.47	0.84	0.83	0.77	0.67	0.62	0.224	0.87	< 0.01	0.86
GLP2R	0.27	0.26	0.30	0.28	0.28	0.43	0.49	0.28	0.38	0.35	0.056	0.76	< 0.01	0.18
ZO-1	0.85	0.93	1.06	1.06	0.99	1.07	0.97	0.86	0.86	0.79	0.143	0.97	0.48	0.51
Cl-1	0.87	0.67	0.84	0.81	0.68	1.39	0.81	1.31	1.06	0.96	0.182	0.16	< 0.01	0.81
Ileum														
TNF-α	1.17	1.07	0.80	1.20	0.76	0.40	0.45	0.31	0.39	0.37	0.154	0.29	< 0.01	0.71
IL-8	0.60	0.55	0.51	0.46	0.54	0.40	0.41	0.40	0.37	0.37	0.086	0.80	0.03	0.98
GLP2R	0.58	0.58	0.67	0.47	0.59	0.27	0.29	0.28	0.30	0.35	0.071	0.76	< 0.01	0.64
ZO-1	1.79	1.49	1.14	1.38	1.48	1.17	1.37	1.19	1.37	1.14	0.173	0.19	0.05	0.23
Cl-1	1.97	1.30	1.51	1.44	1.28	1.36	1.66	1.70	2.27	1.51	0.390	0.79	0.43	0.48

[1]Abbreviations: NC, negative control diet containing no dietary antibiotics; GLN, diet containing 0.2% L-glutamine; SYN, diet containing a mixture of prebiotics and probiotics; GLN + SYN, diet containing 0.2% L-glutamine and a mixture of prebiotics and probiotics; PC, positive control diet containing antibiotics (441 ppm chlortetracycline + 38.6 ppm tiamulin); TNF-α, tumor necrosis factor α; IL-8, interleukin 8; GLP2R, glucagon like peptide 2 receptor; ZO-1, zonula occludens 1; Cl-1, claudin 1.

[2]Results were quantified by the standard curve method, and data are expressed as the relative abundance of the genes of interest to the reference gene (18s).

Table 5.7. Gene expression of intestinal tissues separated by day for pigs fed five treatment diets from d 0 to d 14 of the nursery phase and common diets from d 14 to 35 of the nursery phase[1, 2]

Item	**NC**	**GLN**	**SYN**	**GLN + SYN**	**PC**	**SEM**	***P*-Value**
Day 14							
Jejunum							
TNF-α	0.33	0.26	0.27	0.26	0.30	0.041	0.74
IL-8	1.27	1.16	1.47	1.18	1.47	0.286	0.90
GLP2R	0.27	0.26	0.30	0.28	0.28	0.043	0.95
ZO-1	0.85	0.93	1.06	1.06	0.99	0.170	0.76
CL-1	0.87	0.67	0.84	0.81	0.68	0.167	0.89
Ileum							
TNF-α	1.17	1.07	0.82	1.20	0.76	0.200	0.44
IL-8	0.60	0.55	0.51	0.46	0.54	0.094	0.87
GLP2R	0.58	0.58	0.67	0.47	0.59	0.099	0.73
ZO-1	1.79	1.49	1.14	1.38	1.48	0.158	0.11
CL-1	1.97	1.30	1.51	1.44	1.28	0.338	0.68
Day 34							
Jejunum							
TNF-α	0.21^{yz}	0.24^{y}	0.18^{yz}	0.17^{yz}	0.15^{z}	0.025	0.09
IL-8	0.84	0.83	0.77	0.67	0.62	0.160	0.81
GLP2R	0.43	0.49	0.30	0.38	0.35	0.070	0.31
ZO-1	1.07	0.97	0.86	0.86	0.79	0.118	0.45
CL-1	1.39	0.81	1.31	1.06	0.96	0.195	0.11
Ileum							
TNF-α	0.40	0.45	0.31	0.39	0.37	0.108	0.93
IL-8	0.40	0.41	0.40	0.37	0.37	0.079	0.99
GLP2R	0.27	0.29	0.28	0.30	0.35	0.043	0.67
ZO-1	1.17	1.37	1.19	1.37	1.14	0.144	0.71
CL-1	1.36	1.66	1.70	2.27	1.51	0.430	0.61

[y-z]Values within a row not sharing a common letter tend to differ ($0.05 \leq P < 0.10$).

[1]Abbreviations: NC, negative control diet containing no dietary antibiotics; GLN, diet containing 0.2% L-glutamine; SYN, diet containing a mixture of prebiotics and probiotics; GLN + SYN, diet containing 0.2% L-glutamine and a mixture of prebiotics and probiotics; PC, positive control diet containing antibiotics (441 ppm chlortetracycline + 38.6 ppm tiamulin); TNF-α, tumor necrosis factor α; IL-8, interleukin 8; GLP2R, glucagon like peptide 2 receptor; ZO-1, zonula occludens 1; Cl-1, claudin 1.

[2]Results were quantified by the standard curve method, and data are expressed as the relative abundance of the genes of interest to the reference gene (18s).

Table 5.8. Responses where at least one contrast resulted in a *P*-value of < 0.10[1, 2]

	***P*-Values**			
Item	**NC vs. PC**	**NC vs. GLN**	**NC vs. SYN**	**NC vs. GLN+SYN**
Lactulose excretion, mg	0.08	0.96	0.38	0.20
Jejunum histology				
Day 14 to 34				
Mast cell count/mm^2	0.81	0.09	0.43	0.10
Day 14				
Crypt depth	0.23	0.50	0.04	0.48
Villi height : crypt depth ratio	0.07	0.30	0.08	0.79
Goblet cell count/villi	0.07	0.13	0.20	0.23
Mast cell count/mm^2	0.67	0.62	0.44	0.05
Day 34				
Villi height	0.03	0.39	0.13	0.61
Villi height : crypt depth ratio	0.08	0.53	0.08	0.56
Goblet cell count/villi	0.73	0.43	< 0.01	0.56
Goblet cell count/mm	0.81	0.68	0.02	0.74
Ileum histology				
Day 14 to 34				
Goblet cell count/villi	0.08	0.33	0.28	0.17
Mast cell count/mm^2	0.20	0.03	0.38	0.91
Day 34				
Mast cell count/mm^2	0.46	< 0.01	0.72	0.39
Jejunum gene expression				
Day 14 to 34				
TNF-α	0.17	0.55	0.18	0.09
Cl-1	0.09	0.03	0.74	0.27
Day 34				
TNF-α	0.09	0.38	0.38	0.20
ZO-1	0.09	0.55	0.21	0.20
Cl-1	0.08	0.02	0.72	0.17
Ileum gene expression				
Day 14 to 34				
ZO-1	0.22	0.72	0.03	0.44
Day 14				
ZO-1	0.17	0.19	0.01	0.07

[1]Abbreviations: NC, negative control diet containing no dietary antibiotics; GLN, diet containing 0.2% L-glutamine; SYN, diet containing a mixture of prebiotics and probiotics; GLN + SYN, diet containing 0.2% L-glutamine and a mixture of prebiotics and probiotics; PC, positive control diet containing antibiotics (441 ppm chlortetracycline + 38.6 ppm tiamulin); TNF-α, tumor necrosis factor α, Cl-1, claudin 1; ZO-1, zonula occludens 1.

[2]Treatment diets were fed from d 1 to 14 post-weaning, and then common diets were fed until 35 d post-weaning. One pig per pen was removed at 14 and 34 d post-weaning for tissue collection.

CHAPTER 6. A SIMPLE SURGICAL PROCEDURE TO MEASURE THE DEEP BODY TEMPERATURE OF BROILER CHICKENS VIA IMPLANTED DATA LOGGERS

6.1 Abstract

Internal body temperature is a key response variable under heat stress conditions, but measurement methods for poultry are few and have considerable drawbacks. Two experiments (Exp 6.1 and Exp 6.2) were conducted with the objective to use surgically implanted data loggers to measure internal body temperature in 6 week old mixed sex Ross 708 broiler chickens housed in bedded floor pens and exposed to 4 ambient temperature (T_a) periods: thermoneutral (TN; 22.3 ± 0.08°C for 1 h), MILD (26.5 ± 0.08°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), HOT (34.9 ± 0.12°C for 40 min) each day over 4 consecutive days. The resulting body temperature values from the implanted data loggers ($T_{abdominal}$) were compared to body temperatures obtained from the same birds by insertion of a thermometer into the cloaca ($T_{cloacal}$) of 4 birds in Exp 6.1, and 9 birds in Exp 6.2. Surgery to implant data loggers was conducted on d 34 post-hatch via a simple procedure using isoflurane gas anesthetic. No morbidity or mortality occurred during the surgery or during the one-week recovery period in either experiment. Data were analyzed with Proc Glimmix of SAS 9.4 in a mixed model with the measurement method ($T_{cloacal}$ vs. $T_{abdominal}$), sex of the chicken, T_a period (TN, MILD, MOD, HOT), measurement day (d 1 through 4), and their interactions as fixed effects. In Exp 6.1 no difference (P = 0.95) was detected between $T_{abdominal}$ (41.6 ± 0.08°C) and $T_{cloacal}$ (41.6 ± 0.08°C). Similarly, in Exp 6.2 no difference (P = 0.52) was detected between $T_{abdominal}$ (41.4 ± 0.13°C) and $T_{cloacal}$ (41.4 ± 0.13°C). However, in Exp 6.1 measurement method interacted with day (P = 0.02) with $T_{cloacal}$ varying more by day than $T_{abdominal}$. One individual obtained all $T_{cloacal}$ values in Exp 6.2 in an attempt to limit variation over time, but

a day by measurement method interaction was still observed ($P < 0.01$) in Exp 6.2. Therefore, surgically implanted data loggers appear to be a viable alternative to measurement of internal body temperature via a cloacal thermometer as data loggers may be less susceptible to extraneous sources of variation. Implanted data loggers have potential to be a valuable tool to accurately and precisely measure internal body temperature of chickens in a wide variety of research settings.

Key words: body temperature, broiler chicken, data logger, heat stress, surgery

6.2 Introduction

While considerable effort has been exerted towards characterizing the negative impacts of heat stress (HS) on domestic chickens and developing mitigation strategies (Lara and Rostagno, 2013), there has been less emphasis on the methodology available to quantify the degree of HS that chickens are experiencing in laboratory and production settings. During a thermal challenge the bird is primarily attempting to regulate its internal body temperature within a narrow range to maintain optimal function of critical internal organs, thus internal body temperature is considered the best measure of thermal stress (Dawson and Whittow, 2000). However, methods to measure internal body temperature in poultry species present a variety of challenges and the most commonly reported method is insertion of a thermometer into the cloaca (Giloh et al., 2012). However, this requires bird handling and temporary restraint that can increase heart rate and body temperature (Liljequist et al., 1994; Cabanac and Aizawa, 2000), disrupt normal behavior, and limits the number of measurements that can be practically obtained in a given time period (Giloh et al., 2012).

Limitations of manually taking cloacal temperature (**$T_{cloacal}$**) have been previously recognized and systems have been designed to automatically measure body temperature via data

transmitters surgically implanted in the body (Wilson et al., 1989; Lacey et al., 2000; De Basilio et al., 2003; Savory et al., 2006), or through the use of wired (Liljequist et al., 1994) and wireless (Deaton and Reece, 1968) temperature probes maintained in the cloaca. Unfortunately, these methods require specialized apparatus and methods that may place limitations on bird movement, preclude group housing of birds, or present other challenges that preclude routine use in many research settings. Therefore, a potential alternative may be small, self-contained temperature sensors that directly log temperature readings on internal memory rather than transmitting data to an external receiver. These data loggers have advantages over other methods to automatically obtain body temperature in that they do not require wires connected to the bird, there is no necessity of maintaining implanted birds within a certain range of data receivers, on-site computers and software to record transmitted data are not required, and there is no customization of the data loggers required by the user. This technology has been used to obtain internal body temperatures in larger livestock species via insertion into the vagina (Polsky et al., 2016), the digestive tract (Goumon et al., 2013), or being surgically implanted into the animal (Johnson et al., 2015). However, use of these data loggers to measure body temperature in poultry has been limited. Several studies have used market age broiler chickens that swallowed data loggers shortly before slaughter (Watts et al., 2011; Dadgar et al., 2012; Rajaei-Sharifabadi et al., 2017) but the data loggers are typically too large to pass through the digestive tract and have potential to negatively impact feed intake. Therefore, a potential way to harness the advantages of the data loggers without compromising long-term feed intake could be to surgically place sterile data loggers into the abdominal cavity, which would allow direct measurement of internal body temperature for an extended period.

Results from surgical implantation of data loggers in laying hens (Lieboldt et al., 2016) indicates this is likely feasible in broiler chickens. However, while Lieboldt et al. (2016) reported no infections or other complications due to the surgery, the hens were individually housed in wire-floored cages post-surgery. Wire flooring is advantageous to keep incision sites clean during recovery, but wire flooring is known to negatively impact broiler health and welfare (Wideman et al., 2012). Thus, it is unclear if implantation of data loggers into the abdominal cavity of broilers housed in bedded floor pens would be complicated by incision site or intra-peritoneal infections.

Given that the majority of the internal body temperature data for poultry species in the literature originates from cloacal measurement via a thermometer it is important to know if there are differences between body temperature as measured via a thermometer inserted into the cloaca or via data loggers placed in the abdominal cavity that would preclude meaningful comparison of internal temperature values found in the literature and those derived from implanted data loggers. Previously, internal body temperature measurement method (cloacal thermometer versus implanted data transmitter) was found to significantly interact with ambient temperature (T_a) as measured in broilers (De Basilio et al., 2003). To our knowledge, there has been no reported comparison between body temperature values derived from intraperitoneally implanted data loggers and from cloacal temperature measurement.

Therefore, two experiments (Exp 6.1 and Exp 6.2) were conducted to determine if intraperitoneally-placed data loggers are a viable alternative to measurement of $T_{cloacal}$ to obtain internal body temperature in broiler chickens housed in commercially relevant conditions. We hypothesized that there will be no difference between body temperatures obtained from implanted data loggers or from thermometers inserted into the cloaca.

6.3 Materials and Methods

All procedures involving the use of live animals were approved by the Purdue University Animal Care and Use Committee (protocols #1705001580 and #1807001772) and animal husbandry practices were in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

6.3.1 Birds and Husbandry

In Exp 6.1, mixed sex broiler chicks (Ross 708; n = 15 males and 15 females) were obtained from a commercial hatchery (Hoover's Hatchery, Rudd, IA) at hatch and placed into floor pens (1.2 × 1.2 m) bedded with pine shavings in a heated room. Chickens were initially placed 15 per pen into two pens and were further split into three pens at d 21 to maintain acceptable stocking density. Heat lamps provided supplemental heat until d 13 post-hatch. Chicks had *ad libitum* access to water via bell-style drinkers and feed was provided *ad libitum* in mash form. Diets were based on corn and soybean meal and were formulated to meet or exceed breeder nutrient recommendations (Aviagen, 2014). The starter diet was fed from hatch to 22 d post-hatch and contained 3090 kcal/kg AME_n, 23% CP, 9.6 g/kg total Ca, 4.8 g/kg non-phytate P, 7.2 g/kg total Met and 14.4 g/kg total Lys (calculated values). The grower diet was fed from 23 to 49 d post-hatch and contained 3185 kcal/kg AME_n, 19.5% CP, 7.8 g/kg total Ca, 3.9 g/kg non-phytate P, 5.8 g/kg total Met and 11.5 g/kg total Lys (calculated values). The T_a and relative humidity of the room were: 25.2 ± 0.02°C and 65.2 ± 0.31% for d 1 to 7 post-hatch; 25.7 ± 0.02°C and 61.4 ± 0.20% for d 8 to 14 post-hatch; 24.9 ± 0.03°C and 78.1 ± 0.27% for d 15 to 21 post-hatch; 23.7 ± 0.03°C and 66.9 ± 0.28% for d 22 to 28 post-hatch; and 21.6 ± 0.07°C and 68.4 ± 0.43% for d 29

to 33 post-hatch. Lighting was maintained on a 23:1, 20:4, and 17:7 light:dark cycle from d 0 to 7, 8 to 30, and 31 to 49 post-hatch, respectively.

In Exp 6.2, mixed sex broiler chicks (Ross 708; n = 15 males and 15 females) were obtained from a commercial hatchery (Townline Hatchery, Zeeland, MI) at hatch and placed into floor pens (1.2 × 1.2 m) bedded with pine shavings in a heated room. Chickens were initially placed 15 per pen into two pens and were further split into three pens at d 21 to maintain acceptable stocking density. Heat lamps provided supplemental heat during the brooding period. Chicks had *ad libitum* access to water via bell-style drinkers and feed was provided *ad libitum* in mash form. Diets were the same and fed on the same schedule as in Exp 6.1. The T_a and relative humidity of the room were: 31.5 ± 0.04°C and 51.5 ± 0.34% for d 1 to 7 post-hatch; 27.6 ± 0.03°C and 62.1 ± 0.24% for d 8 to 14 post-hatch; 26.5 ± 0.02°C and 55.6 ± 0.25% for d 15 to 21 post-hatch; 25.9 ± 0.02°C and 57.3 ± 0.17% for d 22 to 28 post-hatch, 25.6 ± 0.01°C and 56.7 ± 0.34% for d 29 to 35 post-hatch; and 25.3 ± 0.02°C and 37.9 ± 0.20% for d 36 to 40 post-hatch. Lighting was maintained on a 23:1, 20:4, and 17:7 light:dark cycle from d 0 to 7, 8 to 30, and 31 to 44 post-hatch, respectively.

Chickens in both experiments were group housed pre-surgery, post-surgery, and during the experimental temperature cycle days in the same floor pens that the chickens had occupied since hatch. Litter was not removed from pens until the completion of each experiment, rather, clean shavings were added to the pens periodically. Feed and water were available at all times following surgery through the end of each experiment.

6.3.2 Data Logger Implantation

In Exp 6.1, at d 33 post-hatch, chickens were individually identified with numbered leg bands and 10 birds of each sex (n = 20 birds) that were closest to the median BW (2.2 ± 0.06 kg for males, 1.8 ± 0.04 kg for females) were selected and fasted overnight. On d 34 post-hatch water

was removed for 2 h before surgery. Chickens were weighed individually and injected with midazolam HCl (0.5 mg/kg BW; 5 mg/mL, Akorn Inc., Lake Forest, IL) and buprenorphine HCl (0.05 mg/kg BW; 0.3 mg/mL, Par Pharmaceuticals Companies Inc., Spring Valley, NJ) intramuscularly 15 min prior to anesthesia. Chickens were then held to prevent movement and their heads were placed into a small cone anesthesia mask (8.9 cm maximum inner diameter, 7.0 cm deep; #32393B4, Smiths Medical, Dublin, OH). Laboratory tape was placed around the seal of the mask to reduce the diameter as required. Anesthesia was induced via isoflurane (IsoFlo, Zoetis Inc., Kalamazoo, MI) inhalation with the vaporizer (Isotec, Smiths Medical, Dublin, OH) set to 5% isoflurane and with oxygen flow at 2.0 L/min. Anesthesia was maintained at 1.5 to 3% isoflurane and oxygen flow at 2.0 L/min with manual positive pressure artificial respiration 2 to 4 times per minute. Uncuffed tracheal tubes of 3, 4, and 5 mm diameter (Midmark Animal Health, Versailles, OH) were available in the event of respiratory distress but no chickens required intubation. Anesthetized chickens were placed in dorsal recumbency, and the feathers plucked from the surgical site (abdominal wall on the right side of the bird, ventral to the caudal tip of the sternum). Laboratory tape was used to hold the right leg up towards the front of the bird to expose the surgical site and was also used to keep feathers from surrounding areas from contacting the surgical site (Figure 6.1). The surgical site was then sterilized by alternating rounds of a povidone-iodine solution (Betadine, Purdue Products LP., Stamford, CT) and 70% ethanol. Additional analgesic was provided at the incision site by a subcutaneous injection of 4 mg per kg BW of lidocaine HCl (20 mg/mL, Vedco Inc., St. Joseph, MO). Adequate depth of anesthesia was evaluated via a periodic comb pinch. Using sterile technique, a 2.5 cm incision was made in the skin using a scalpel, and through the muscle layers and peritoneum using iris scissors. Two sterile data loggers (iButton, accuracy of 0.1°C; DS1921H High-Resolution Thermochron Device, Maxim Integrated Products

Inc., San Jose, CA; 17.4 mm long by 6.9 mm wide, weight of 3.3 g) were placed into the abdominal cavity to monitor abdominal temperature ($T_{abdominal}$) in 10 min intervals 24 h per day. Two data loggers were used to preclude data loss in the event of a data logger malfunction. Data loggers in Exp 6.1 were sterilized by autoclaving, while data loggers in Exp 6.2 were cold sterilized by submersion in a disinfectant (CIDEX Activated Dialdehyde Solution, Johnson and Johnson, New Brunswick, NJ) prior to placement in the chickens. The incision was closed with 3-0 absorbable suture (PDS II, Ethicon US LLC., Bridgewater, NJ) with a reverse cutting 24 mm circle needle via a continuous stitch (muscle layers) and interrupted stitches (skin). An antibiotic ointment (Neosporin, Johnson and Johnson, New Brunswick, NJ) was then applied to the incision site, and 20 mg per kg BW of a broad-spectrum antibiotic (Liquamycin LA-200, Zoetis Inc., Kalamazoo, MI) was injected intramuscularly. The average surgery duration (start of anesthesia to completion of surgery) was 20 ± 1 min. Chickens were placed under heat lamps until they regained consciousness and then were returned to their pens. An additional injection of buprenorphine HCl (0.05 mg/kg BW) and application of antibiotic ointment to the incision site were administered 12 h post-surgery.

In Exp 6.2, chickens were individually identified with numbered leg bands at d 33 post-hatch, and 25 chickens (12 males, 2.1 ± 0.11 kg BW; 13 females, 1.9 ± 0.10 kg BW) were fasted overnight. On d 34 post-hatch water was removed for 2 h before surgery, and the 25 chickens underwent surgery to receive data loggers as described for Exp 6.1. The average surgery time from initiation of anesthesia to completion of the surgery was 16 ± 0.6 min in Exp 6.2.

6.3.3 Temperature Cycle and Internal Body Temperature Measurement

In both experiments, chickens were given a surgery recovery period of 1 week and were then exposed to a T_a cycle (Figure 6.2) each day on d 42, 43, 44, and 45 post-hatch. For Exp 6.1,

each T_a cycle started at 0700 h each day and consisted of a thermoneutral period (TN; 22.5 ± 0.12°C), MILD HS period (MILD; 26.3 ± 0.12°C), moderate HS period (MOD; 30.1 ± 0.12°C), and severe HS period (HOT; 34.5 ± 0.15°C). In Exp 6.2, each T_a cycle consisted of a thermoneutral period (TN; 22.0 ± 0.04°C), MILD HS period (MILD; 26.6 ± 0.04°C), moderate HS period (MOD; 30.1 ± 0.12°C), and severe HS period (HOT; 35.2 ± 0.08°C). During each T_a cycle, TN, MILD, and MOD were maintained for 1 h, while HOT was maintained for 40 min. There was a 1 h acclimation period between each T_a period to allow chickens to adjust to the increased T_a (Figure 6.2). All chickens were returned to TN conditions at the conclusion of the HOT period each day. Ambient temperature was controlled via thermostats (T631A Farm-O-Stat Airswitch Controller, Honeywell, Charlotte, NC) connected to forced-air heaters.

In Exp 6.1, $T_{cloacal}$ was measured on each bird once at the end of each of the 4 periods (approximately 2 h between measurements) on each T_a cycle day using a lubricated thermometer (accuracy ± 0.2°C; ReliOn model #144-MT-118-BF, Mabis Healthcare Inc., Waukegan, IL) inserted approximately 2.5 cm into the cloaca of 4 chickens (2 chickens/sex) and the $T_{abdominal}$ was simultaneously measured via implanted data loggers (calibrated in a water bath) which recorded each temperature reading with an associated date and time stamp. Cloacal temperatures were obtained by several different technicians throughout the 4 T_a cycle days. The remaining 16 birds that had data logger implants were involved in a separate experiment (unpublished data). All birds were euthanized on d 49 post-hatch via carbon-dioxide asphyxiation, and then data loggers were retrieved from the abdominal cavity and the data were downloaded (OneWireViewer, Maxim Integrated Products Inc., San Jose, CA).

In Exp 6.2, $T_{cloacal}$ was measured on each bird every 15 min throughout the 4 periods on each T_a cycle day using a lubricated thermometer (accuracy ± 0.2°C; Cooper Atkins model #

TM99A, Middlefield, CT) inserted exactly 2.5 cm into the cloaca (depth determined by a marking on the probe at 2.5 cm) of 9 chickens (4 males, 5 females) and the $T_{abdominal}$ was simultaneously measured via implanted data loggers (calibrated in a water bath) which recorded each temperature reading with an associated date and time stamp. Cloacal temperatures were obtained by one technician (with a second technician aiding with bird handling and restraint) using a set procedure throughout the 4 T_a cycle days. The technique used each day was to insert the thermometer probe 2.5 to 3 cm into the cloaca, ensuring the probe was in contact with cloacal tissue and not excreta, and recording the temperature once a stable value was displayed for 10 sec. The remaining 16 birds that had data logger implants were involved in a separate experiment (unpublished data). All birds were euthanized on d 45 post-hatch via carbon-dioxide asphyxiation, and then data loggers were retrieved from the abdominal cavity and the data were downloaded (OneWireViewer, Maxim Integrated Products Inc., San Jose, CA). It was anecdotally noted in Exp 6.2 that many data loggers were found near the cloaca of the bird during retrieval from the abdominal cavity.

6.3.4 Statistical Analysis

Both experiments were allotted as completely randomized designs with the individual chicken as the experimental unit. Data were primarily analyzed with Proc Glimmix of SAS 9.4 (SAS Institute, Cary, NC) with influential outliers analyzed with Proc Mixed of SAS 9.4 via a Cook's D test (Cook, 1977). The assumptions of normality and homogeneity of variances were verified via examination of residual plots. In addition, prior to analysis, the strength of the linear relationship between $T_{abdominal}$ and $T_{cloacal}$ values were evaluated via regressing one response against the other, with an ideal slope being 1. In Exp 6.1 the slope was found to be 0.87 while in Exp 6.2 the slope was found to be 0.96. The statistical model included the fixed effects of the T_a cycle day (1 through 4), the T_a period (TN, MILD, MOD, HOT), sex of the chicken, body

temperature measurement method ($T_{abdominal}$ vs. $T_{cloacal}$) and their interactions. The optimal model (as determined by a BIC goodness of fit parameter) also contained the random factors of chicken(sex), day*chicken(sex), period*chicken(sex), day*period*chicken(sex), measurement method*chicken(sex), measurement method*day*chicken(sex), and measurement method*period*chicken(sex). A Kenward-Rogers degrees of freedom adjustment was included via the ddfm=kr option of the model statement (Kenward and Roger, 1997). Means of significant interactions of the measurement method with other factors were separated with the slicediff option of the LSmeans statement to compare the effect of the measurement method at each level of the other factor. In all other cases a Tukey's multiple comparison test was used in pair-wise comparisons of least square means. Statistical significance was declared at a $P < 0.05$. Values are presented as least squares means ± 1 SEM.

6.4 Results

6.4.1 Surgery Outcomes

There was no mortality during surgery or during the 1 week recovery period. Incisions were monitored for signs of infection (inflammation, redness, necrosis) during the recovery period and none were observed. Chickens gained weight during the recovery period in Exp 6.1 with the pre-surgery BW of males and females being 2.2 ± 0.06 and 1.8 ± 0.04 kg, respectively, and the BW at the end of the recovery period for males and females being 3.1 ± 0.15 and 2.5 ± 0.05 kg, respectively. Similarly, in Exp 6.2 the pre-surgery BW of males and females was 2.1 ± 0.10 and 1.9 ± 0.10 kg, respectively, and the BW at the end of the recovery period for males and females was 2.9 ± 0.11 and 2.4 ± 0.07 kg, respectively. Incision sites and the abdominal cavities were inspected during data logger retrieval and no signs of infection (inflammation, redness, necrosis,

thickened or opaque air sack membranes) were observed in either experiment. In addition, there was no indication of picking at the incision site from pen-mates in either experiment.

In Exp 6.1, multiple data loggers were not functional upon retrieval from the chickens. As a result, usable data logger data was obtained from one data logger per chicken from 16 of the 20 chickens that underwent surgery. This failure was believed to be due to battery failure caused by the heat of autoclaving, and accordingly in Exp 6.2 data loggers were cold sterilized. In Exp 6.2, one data logger demonstrated inconsistent readings and was not used in the analysis. For all other chickens both data loggers performed appropriately and the results for the two implanted data loggers were averaged for each chicken prior to analysis. Therefore, usable data was obtained from all 25 chickens that underwent surgery. Cold sterilization techniques are recommended for all future uses of surgically implanted data loggers.

6.4.2 Body Temperatures

In Exp 6.1, there was no main effect of body temperature measurement method (P = 0.95; $T_{abdominal}$ = 41.6 ± 0.08°C, $T_{cloacal}$ = 41.6 ± 0.08°C). There was an interaction (P = 0.03) of T_a cycle day, T_a period, and measurement method (Figure 6.3) but with no significant difference between measurement methods at any of the day by T_a period combinations. The body temperature measurement method varied by T_a cycle day (P = 0.02) with the difference between the $T_{cloacal}$ and the $T_{abdominal}$ values being 0.02°C on d 1, 0.22°C on d 2, -0.15°C on d 3, and -0.06°C on d 4 (Figure 6.4), but with no significant differences between the measurement methods at any time point. Body temperature was reduced (P < 0.01) as days progressed, with d 1 (41.9 ± 0.07°C) and d 2 (41.7 ± 0.06°C) being greater than d 3 (41.4 ± 0.07°C) and d 4 (41.4 ± 0.07°C), regardless of body temperature measurement method. In addition, regardless of measurement method, body temperature was reduced (P < 0.01) during TN (41.0 ± 0.11°C) compared to HOT (42.2 ± 0.11°C),

with MILD (41.4 ± 0.11°C) and MOD (41.8 ± 0.11°C) being intermediate. Overall, females had greater body temperature (P = 0.04; 41.9 ± 0.08°C) than males (41.3 ± 0.08°C). No other significant differences were detected with any comparison for Exp 6.1.

In Exp 6.2, there was no main effect of body temperature measurement method (P = 0.52; $T_{abdominal}$ = 41.4 ± 0.13°C, $T_{cloacal}$ = 41.4 ± 0.13°C). There was an interaction (P < 0.01) of T_a cycle day, T_a period, and measurement method (Figure 6.4), with the data logger (40.7 ± 0.14°C) being greater than the thermometer (40.4 ± 0.14°C) at d 1 in TN conditions but with no additional differences between measurement methods at any other day and period combinations. The body temperature measurement method varied by T_a cycle day (P < 0.01) with the difference between the $T_{cloacal}$ and the $T_{abdominal}$ values being -0.07°C on d 1, 0.16°C on d 2, 0.05°C on d 3, and 0.18°C on d 4 (Figure 6.6), but with no significant differences between the measurement methods at any time point. Body temperature measurement method interacted with T_a cycle day (P = 0.04), with the difference between the $T_{cloacal}$ and the $T_{abdominal}$ values being 0.01°C in TN, 0.16°C in MILD, 0.06°C in MOD, and 0.08°C in HOT conditions (Figure 6.7), but with no significant differences between the measurement methods at any T_a period. There was an interaction (P < 0.01) of T_a cycle day and T_a period for body temperature (Figure 6.8), with d 4 in HOT conditions being lesser than HOT at d 1, 2, or 3; d 4 in MOD conditions being lesser than MOD at d 1, 2, or 3; d 4 at MILD being lesser than MILD at d 1 and 2, with d 3 being intermediate; and with d 1 in TN conditions being lesser than TN at d 2, 3, or 4. Body temperature was impacted by day (P < 0.01), with d 2 (41.5 ± 0.12°C) being greater than d 1 (41.4 ± 0.12°C) and d 4 (41.3 ± 0.12°C), but not different than d 3 (41.5 ± 0.12°C), and with d 1 and d 3 being greater than d 4, regardless of body temperature measurement method. In addition, regardless of measurement method, body temperature was increased (P < 0.01) by each increase in T_a with TN (40.7 ± 0.12°C) being lesser

than MILD (41.2 ± 0.12°C), which was lesser than MOD (41.6 ± 0.12°C), which was lesser than HOT (42.1 ± 0.12°C). There was no impact of sex of the chicken ($P = 0.98$) or an interaction of sex with any other factor ($P \geq 0.11$). No other significant differences were detected with any comparison for Exp 6.2.

6.5 Discussion

While abdominal surgery is not common in chickens, it has been performed for purposes such as removal of the ceca in birds used in metabolism studies (Kim et al., 2011), caponization (Chen et al., 2005), and the implantation of devices to collect physiological data (Lieboldt et al., 2016). While many aspects of abdominal surgery in chickens use surgical skills common across species, anesthesia in chickens or other avian species diverges from that of mammals (Fedde, 1978). Birds have unique respiratory anatomy and physiology and faster metabolic rates than mammals making avian species more sensitive to anesthesia level than mammals (Speer, 2016). As a result, birds can move from a surgically optimal anesthetic level to dangerously deep anesthetic levels more rapidly than most mammals (Abou-Madi, 2001). This makes the use of injectable anesthetic agents problematic due to the potential for overdose of the anesthetic (Fedde, 1978; Gunkel and Lafortune, 2005), and as a result inhaled anesthetics are generally preferred for avian anesthesia (Abou-Madi, 2001). Therefore, in the present studies, inhaled isoflurane was used to induce and maintain anesthesia, which enabled depth of anesthesia to be modified throughout the surgical procedure and allowed birds to rapidly regain consciousness after the surgery (Speer, 2016). Buprenorphine and midazolam were injected intramuscularly prior to induction of anesthesia as an analgesic and preanesthetic aid, respectively. Midazolam, along with other benzodiazepines, is known to aid in smooth induction and recovery from anesthesia (Gunkel and

Lafortune, 2005). Although intubation was not required by any of the chickens in these studies, previous experience of the authors has shown that chickens, similar to other avians, may experience respiratory distress during anesthesia (Abou-Madi, 2001), and thus intubation supplies were prepared. Even though anesthesia in avians is more challenging than in mammals (Speer, 2016), the anesthetic protocol described herein allowed data loggers to be successfully implanted into 45 broiler chickens without morbidity or mortality.

Perhaps the most commonly reported reason for abdominal surgery in chickens is removal of the ceca in chickens used in amino acid digestibility studies (Payne et al., 1971; Kessler et al., 1981; Kim et al., 2011). Cecectomized chickens are typically laying strains such as Single Comb White Leghorns and are housed individually in wire-floored cages post-surgery to facilitate excreta collection (e.g. Chen et al., 2015). Housing individually in wire-floored cages post-surgery rather than in more industry relevant housing likely reduces the potential for incision site infection and prevents cannibalism by pen-mates (Kaesberg et al., 2018). Housing broilers in wire-floored pens during the recovery period is contraindicated as wire flooring is known to compromise broiler health and welfare (Wideman et al., 2012). In the present studies, broilers group housed in bedded floor pens did not develop incision site infections and showed no interest in the incision sites of their pen mates. The injection of a broad-spectrum antibiotic immediately post-surgery likely contributed to the lack of infection, while the antibiotic ointment application over the incision immediately post-surgery and again 12 h later likely helped create a barrier against incision site microbial invasion. The lack of interest in the incision sites by pen-mates may be due to the docile nature of *ad libitum* fed broiler chickens as compared to laying strain chickens (Mench, 1988). Thus, other strains or types of poultry (e.g. Single Comb White Leghorns) may not tolerate group housing well during the surgery recovery period due to a greater propensity towards feather

picking and cannibalism than broilers (Kaesberg et al., 2018). The lack of post-surgery complications observed in the present studies indicates that specialized housing, such as wire-floored cages, may not be required during recovery from abdominal surgery in broiler chickens. This is advantageous as the detrimental impacts of wire-flooring on broiler health and welfare can be avoided, and broilers can be housed in industry-relevant conditions.

Accurate measurement of internal body temperature is vital to understanding the physiological status of birds during a thermal challenge (Lacey et al., 2000). The measurement of internal body temperature in poultry is most commonly done through taking $T_{cloacal}$ with a thermometer; however, there are a number of limitations to this method, largely due to the stress of handing and restraint (Liljequist et al., 1994; Edgar et al., 2013). Similar limitations of taking rectal temperatures have been reported for laboratory rats (Dangarembizi et al., 2017). Although several systems have been devised to aid in overcoming the limitations of manual measurement of $T_{cloacal}$ in poultry, few have compared body temperature between the alternative measurement method and manual measurement of $T_{cloacal}$. In 3 week old male broilers, De Basilio et al. (2003) found an average reduction of 0.4°C in body temperature measured with a cloacal thermometer than from data transmitters surgically placed in the abdominal cavity. The same study found an interaction between body temperature measurement method and T_a with greater differences between body temperature measurement methods at greater T_a. In the present experiments, neither Exp 6.1 nor Exp 6.2 showed a main effect of measurement method. However, Exp 6.2 demonstrated an interaction of measurement method and T_a similar to De Basilio et al. (2003), except the interaction appears to have been driven by small increases in $T_{cloacal}$ at all T_a conditions except TN in the present study. Small differences in $T_{cloacal}$ measurement procedure may be the cause of these inconsistencies.

One advantage of implanted data loggers may be removal of extraneous sources of variability. More consistent body temperature values were obtained from broilers via implanted data transmitters than by measurement of $T_{cloacal}$ with a thermometer (De Basilio et al., 2003). Similarly, it was noted in rats that rectal temperatures taken with a thermometer were more variable and fluctuated above and below that of an implanted data logger over time (Dangarembizi et al., 2017). In Exp 6.1, fluctuations in $T_{cloacal}$ over time between and within days were observed and led to an interaction between measurement method, T_a cycle day, and T_a period, as well as an interaction between measurement method and T_a cycle day. While the $T_{abdominal}$ data show a relatively uniform curvilinear decrease over the 4 days consistent with the expected acclimation of the chickens to the T_a cycles over time (May et al., 1987; Yahav et al., 1997), the $T_{cloacal}$ data show a less uniform pattern, particularly on T_a cycle d 2 and 3, giving rise to the interaction between body temperature measurement method and T_a cycle day. It was hypothesized that the cause of the interactions may be that different technicians obtained $T_{cloacal}$ values on different days and on different T_a periods in Exp 6.1. There is inherent subjectivity of obtaining $T_{cloacal}$ values given that the depth of thermometer insertion and the amount of time an individual waits for the thermometer reading to stabilize may vary (De Basilio et al., 2003), which could lead to the observed less-consistent pattern of the $T_{cloacal}$ values as compared to the $T_{abdominal}$ values over time. Accordingly, in Exp 6.2, one technician obtained all cloacal measures over the 4 measurement days using a set procedure. Surprisingly, in Exp 6.2 there was an interaction between measurement method, T_a cycle day, and T_a period, as well as an interaction between measurement method and T_a cycle day, with $T_{cloacal}$ fluctuating more by day than $T_{abdominal}$. Therefore, even when variation due to technician obtaining the measurement was removed, other extraneous factors appear to have impacted $T_{cloacal}$ within and between measurement days. Regardless of the source of variation, it

can be concluded that measurement of internal body temperature via implanted data loggers or by cloacal thermometers primarily yields similar results, but $T_{cloacal}$ may vary somewhat over time. Therefore, one advantage of the implanted data loggers may be removal of some extraneous sources of variability, especially when frequent measures of internal body temperature are desired (Dangarembizi et al., 2017).

The vast majority of studies that report measurement of internal body temperature in broilers did so only in males (e.g. May et al., 1987; Yalçin et al., 2008; Giloh et al., 2012) or results were not separated by sex (Al-Rukibat et al., 2017), or experimental design precludes meaningful comparison between sexes (Wilson et al., 1989). A significant difference in body temperature of 0.15°C between the sexes was reported for 7 week old broilers, but data were not reported by sex nor was it reported in which sex body temperature was greater (Deeb and Cahaner, 1999). It was hypothesized that part of the body temperature sex difference observed in Exp 6.1 (females 0.53°C greater than males) was due to the increased feather cover and decreased comb and wattle size of females compared to males at 6 weeks post-hatch, which would hinder heat dissipation in females (Deeb and Cahaner, 1999; Al-Ramamneh et al., 2016). However, results from Exp 6.2 with a larger number of birds of each sex do not support that hypothesis, with nearly identical body temperatures for males and females. More research is needed to explore the potential sex differences in body temperatures of modern broiler chickens and elucidate how sex differences in thermoregulation may contribute to HS susceptibility.

Animals exposed to HS conditions are known to acclimate to the HS over time thereby becoming less susceptible to the negative effects of HS (Moraes et al., 2003). In chickens, this acclimation can be rapid, with 7 week old male broilers showing reductions in internal body temperature compared to controls after only 3 d of cyclic HS (May et al., 1987), and heat stressed

laying hens having reduced internal body temperature by the second day of HS exposure (Fujita et al., 1990). The results from Exp 6.1 demonstrated that acclimation to the T_a cycle primarily occurred over the first 3 T_a cycle days, with a reduction in body temperature of 0.23 and 0.34°C from T_a cycle d 1 to d 2, and T_a cycle d 2 to d 3, respectively, with little change between T_a cycle d 3 and 4. This rapid decrease in body temperature over time is consistent with the concept that chickens that survive the initial exposure to HS conditions (Reece et al., 1972) demonstrate rapid acclimation (May et al., 1987; Fujita et al., 1990). Surprisingly, this same acclimation pattern was not observed in Exp 6.2, where little change in body temperature was observed until d 4, where body temperature decreased noticeably for MOD and HOT T_a periods at d 4 compared to the previous days, leading to the interaction of day and T_a period. While the exact cause of this lack of acclimation to the HS is unknown, it may be due to a febrile effect of the frequent handling to obtain $T_{cloacal}$ measures in Exp 6.2. Chickens are known to have a rapid elevation in body temperature following handling (Cabanac and Aizawa, 2000) that can persist for up to 45 min after the handling event (Liljequist et al., 1994). Therefore, small decreases in body temperature over time due to acclimation to the HS may have been masked by increases in body temperature due to handling in Exp 6.2. Accordingly, one advantage of the use of implanted data loggers is that chickens do not have to be handled, thereby precluding handling-associated changes in body temperature.

6.6 Conclusions

The lack of complications during or following intra-abdominal surgery to implant data loggers indicates that neither the surgery to implant data loggers nor the presence of the data loggers in the abdominal cavity obviously impaired the livability or welfare of broiler chickens

group housed in bedded floor pens. Furthermore, surgical implantation of data loggers in broiler chickens appears to be a viable alternative to measurement of body temperature by taking $T_{cloacal}$ with a thermometer. Advantages of implanted data loggers include minimizing extraneous sources of variation in measured internal body temperatures, chickens do not need to be handled or restrained thus preventing a stress-induced elevation of body temperature that might otherwise prevent accurate results, the response of body temperature to an event or experimental treatment may be recorded with greater frequency, and thus greater detail, than can be achieved with cloacal measurement, and extensive amounts of body temperature data may be collected over weeks, or even months in mature laying hens, with minimal effort. The use of implanted data loggers to measure body temperature in broiler chickens via the methods described herein will aid future investigations into understanding the physiology of HS in broilers and the developing of mitigation strategies by increasing the ability of research scientists to accurately and easily obtain detailed internal body temperature data in broilers.

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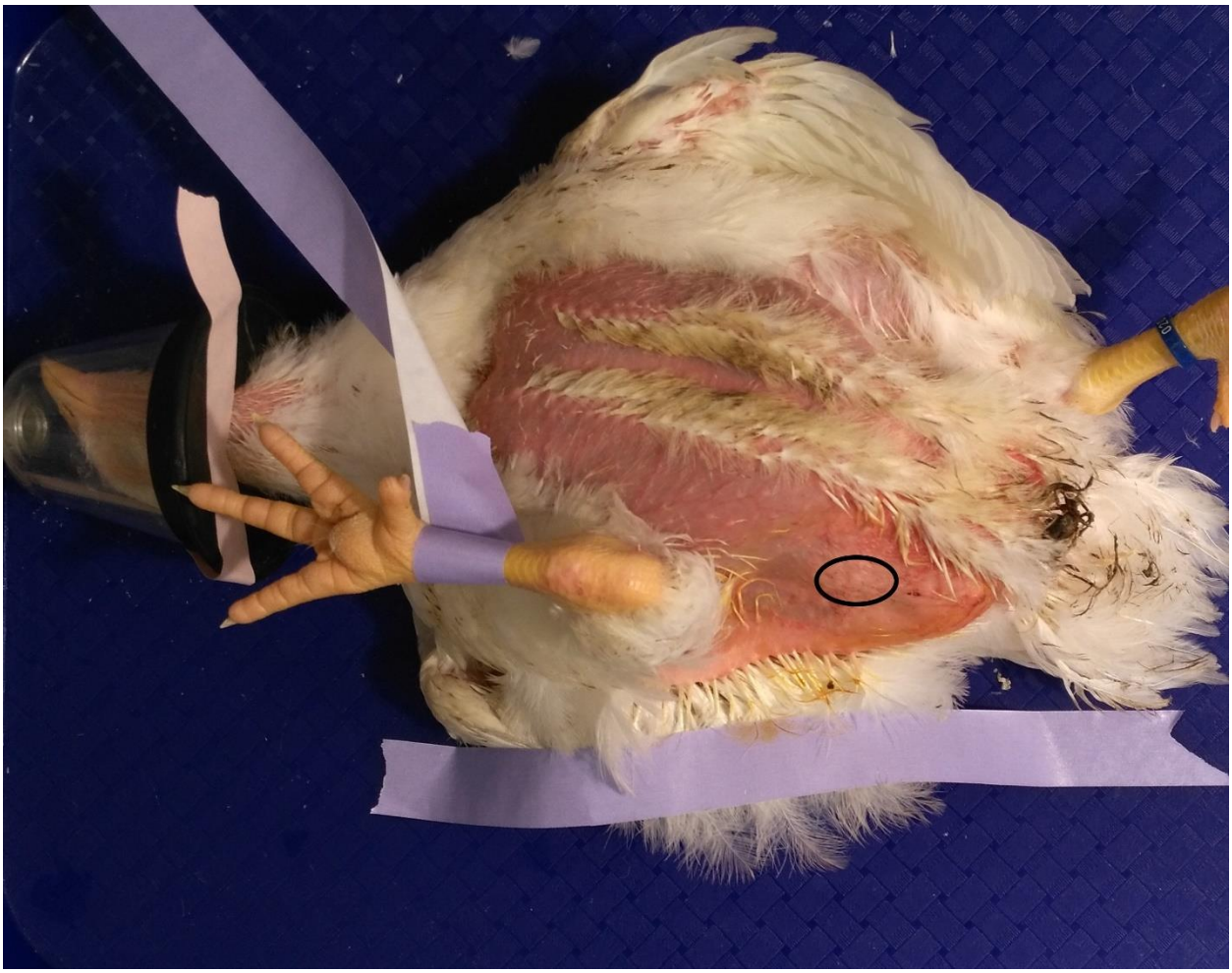

Figure 6.1. A chicken prepared for surgery except for application of a surgical drape. The surgical site is indicated by the black oval. Laboratory tape was used to keep feathers from contacting the surgical site, as well as to position the right leg of the chicken away from the surgical site.

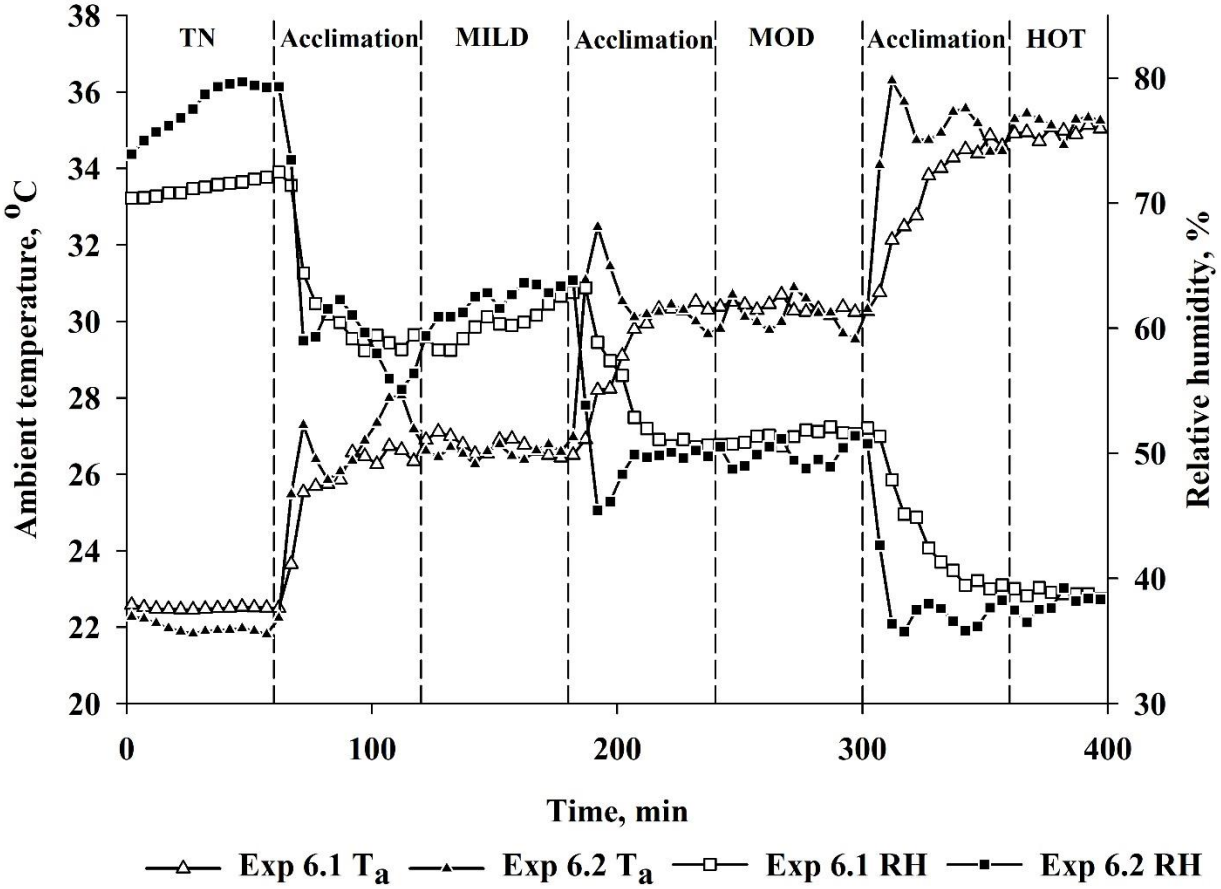

Figure 6.2. Ambient temperature (T_a) and relative humidity (RH) during the temperature cycle that mixed-sex 6 week old broiler chickens were exposed to for 4 consecutive days in Exp 6.1 and Exp 6.2. Values shown for each experiment are averages of the 4 days. The T_a cycle consisted of thermoneutral (TN; 22.3 ± 0.08°C for 1 h), MILD (26.5 ± 0.08°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), HOT (34.9 ± 0.12°C for 40 min) conditions with 1 h of acclimation between each period.

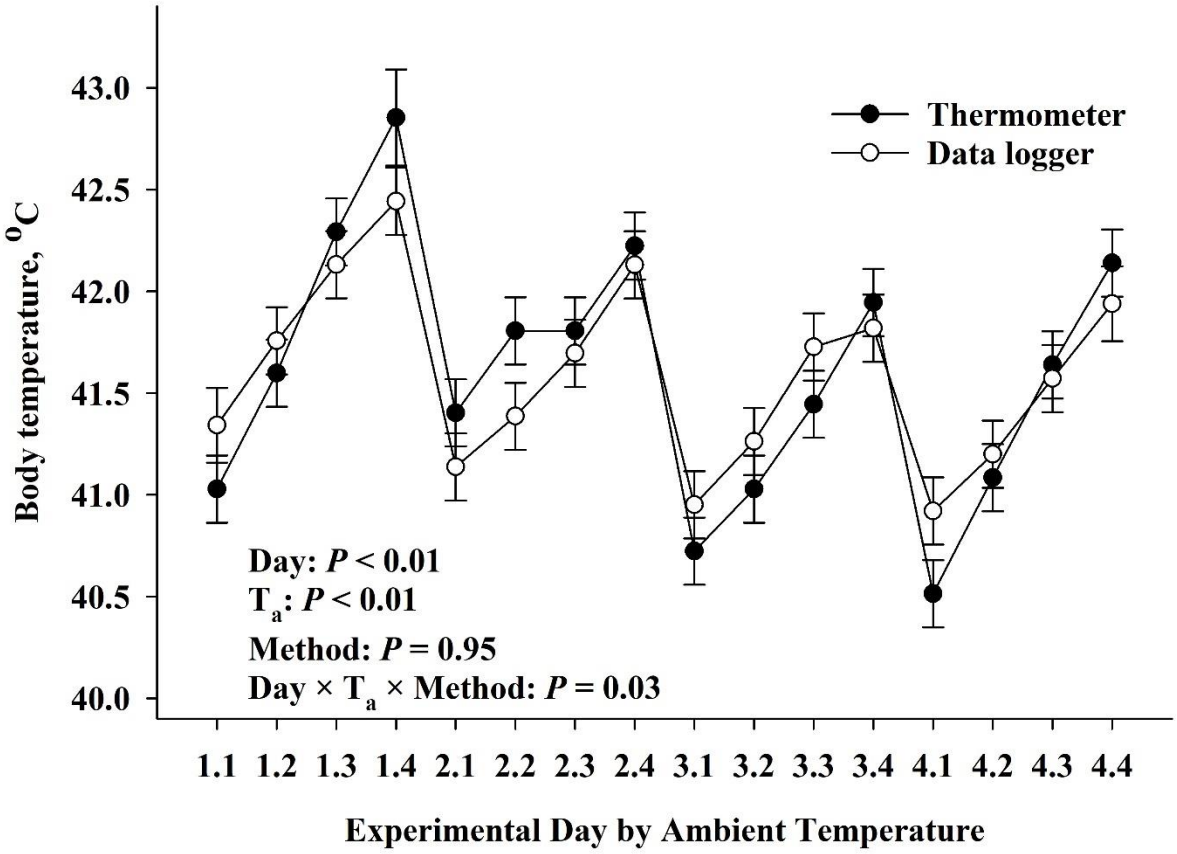

Figure 6.3. The interaction of experimental day, ambient temperature (T_a) period, and internal body temperature measurement method in 6 week old broiler chickens (n = 2 males and 2 females) in Exp 6.1. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN(1); 22.5 ± 0.12°C for 1 h), MILD(2) (26.3 ± 0.12°C for 1 h), moderate (MOD(3); 30.1 ± 0.12°C for 1 h), and HOT(4) (34.5 ± 0.15°C for 40 min) with 1 h of acclimation between each T_a period. Body temperature was measured via 2 methods: a data logger surgically placed in the abdominal cavity and by a thermometer inserted into the cloaca. Body temperature was measured by both methods 4 times per day at 2 h intervals over 4 days starting at 0800 h each day. Error bars indicate ± 1 SEM. Experimental day by T_a period combinations on the X axis are denoted by experimental day by T_a period values. There were no differences ($P > 0.05$) between measurement methods at any experimental day by T_a combination.

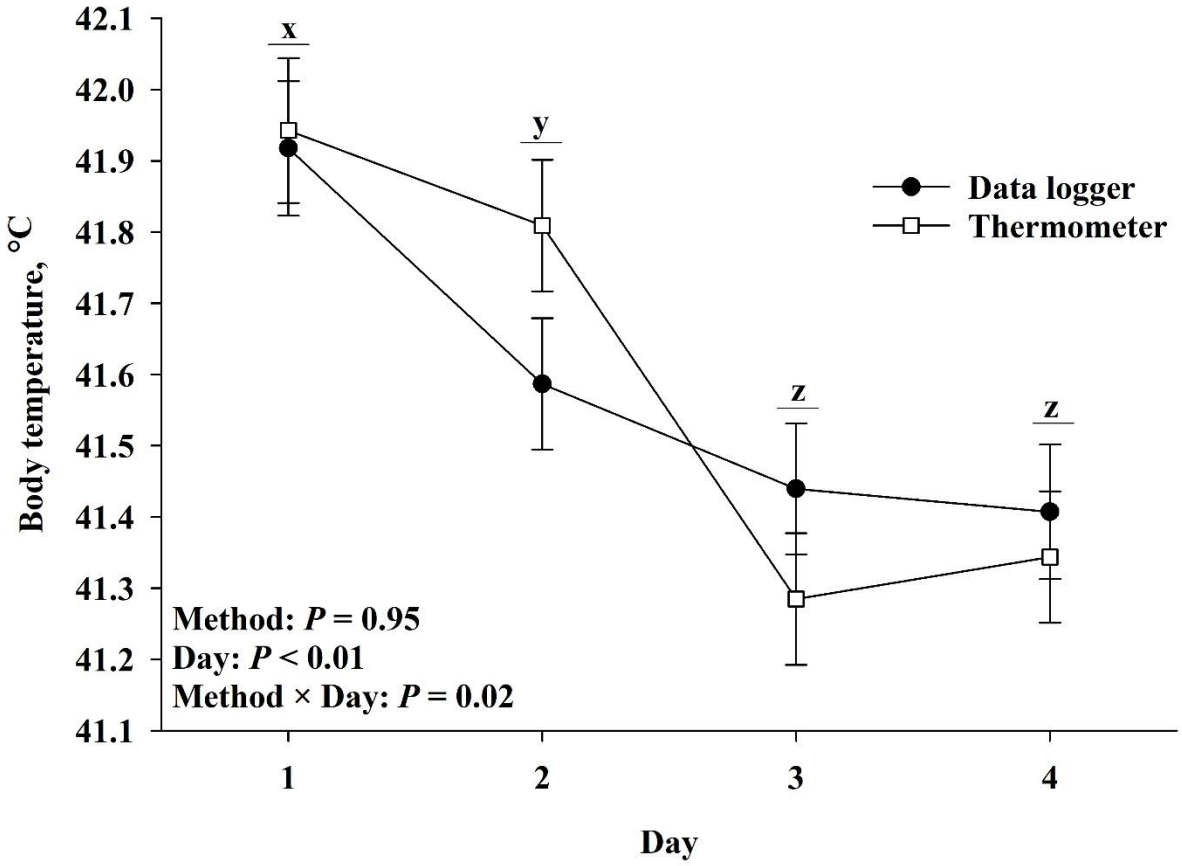

Figure 6.4. Internal body temperature of mixed sex 6 week old broiler chickens (n = 2 males and 2 females) as measured via 2 methods in Exp 6.1: a data logger surgically placed in the abdominal cavity and by a thermometer inserted into the cloaca. Body temperature was measured 4 times per day at 2 h intervals over 4 days starting at 0800h each day. Error bars indicate ± 1 SEM. Days lacking a common letter (x, y, z) differ ($P < 0.05$). There were no differences ($P > 0.05$) between measurement methods at any experimental day.

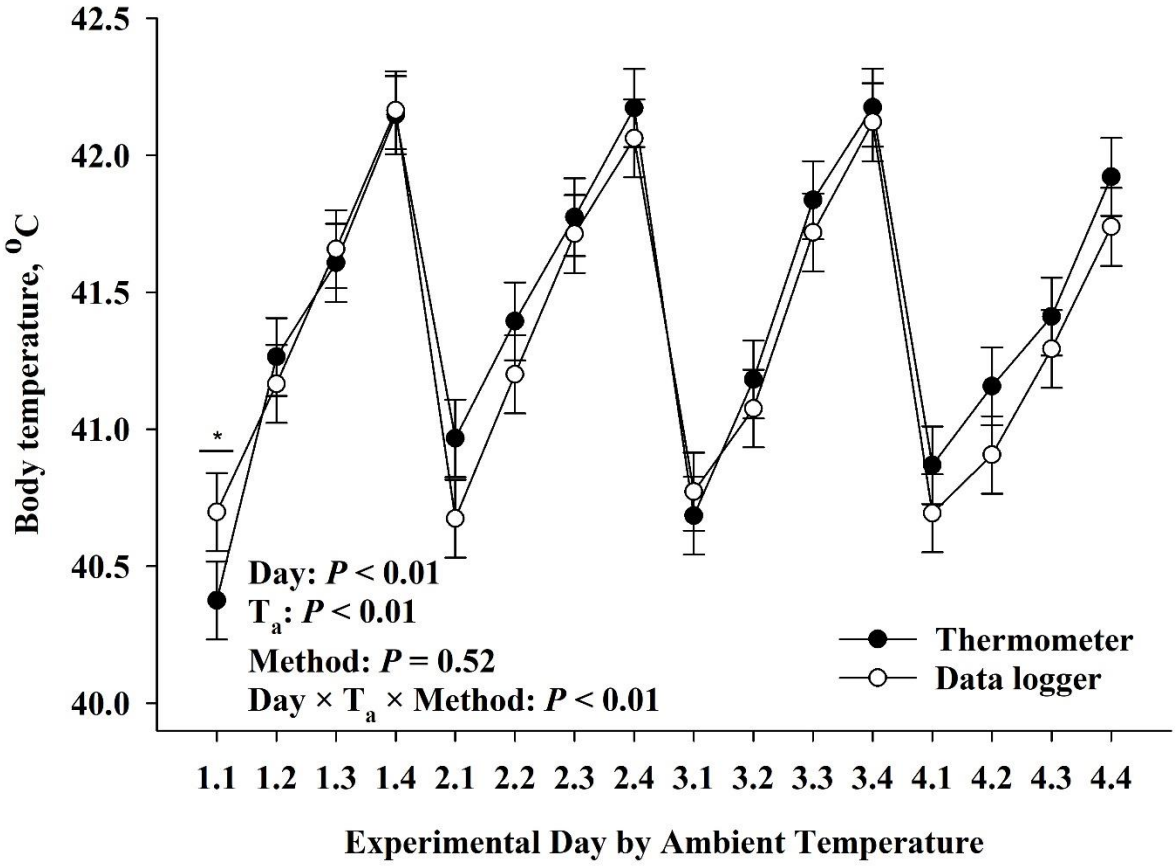

Figure 6.5. The interaction of experimental day, ambient temperature (T_a) period, and internal body temperature measurement method in 6 week old broiler chickens (n = 4 males and 5 females) in Exp 6.2. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN(1); 22.0 ± 0.04°C for 1 h), MILD(2) (26.6 ± 0.04°C for 1 h), moderate (MOD(3); 30.1 ± 0.12°C for 1 h), and HOT(4) (35.0 ± 0.08°C for 40 min) with 1 h of acclimation between each T_a period. Body temperature was measured via 2 methods: a data logger surgically placed in the abdominal cavity and by a thermometer inserted into the cloaca. Body temperature was measured by both methods at 15 min intervals within each T_a period over 4 days. Error bars indicate ± 1 SEM and a * indicates a difference ($P < 0.05$) between measurement methods at a given day by T_a period combination on the X axis.

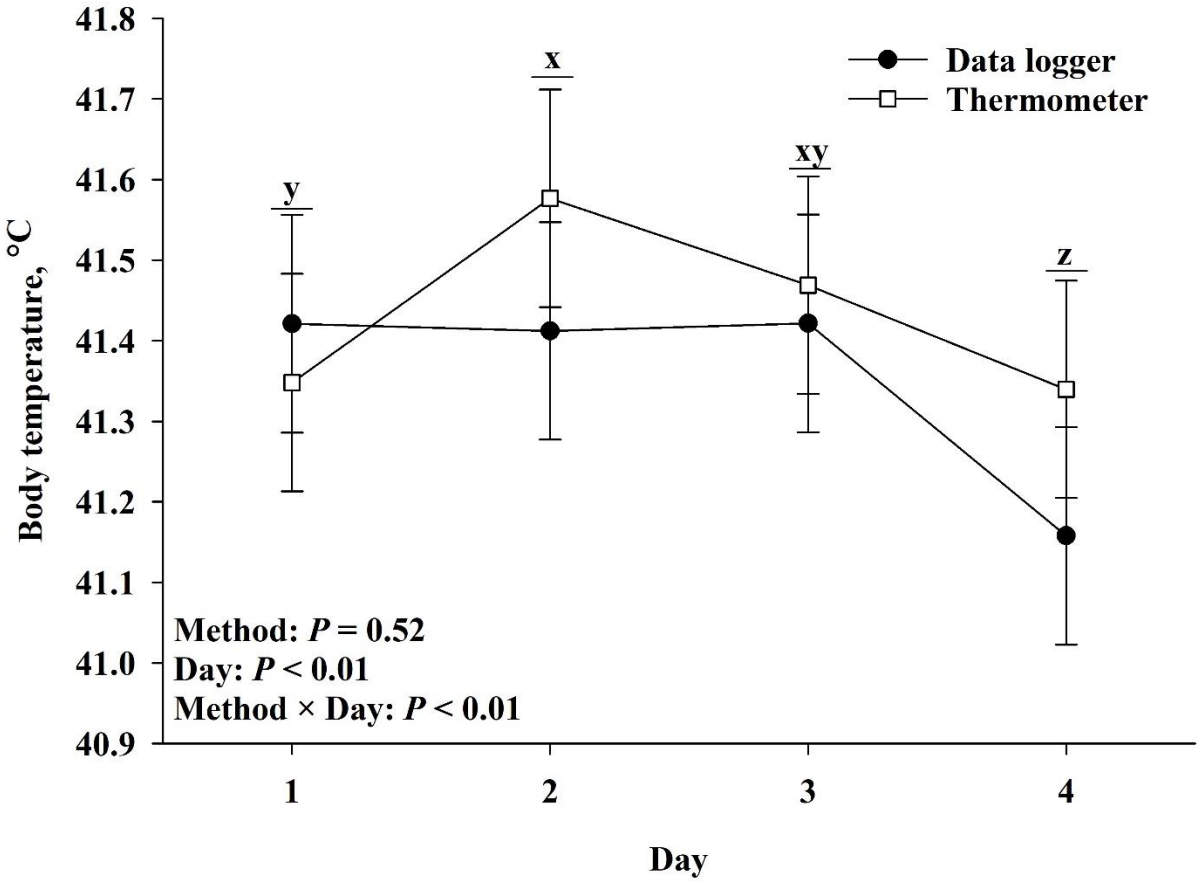

Figure 6.6. The interaction of measurement method and experimental day in Exp 6.2. The internal body temperature of mixed sex 6 week old broiler chickens (n = 4 males and 5 females) was measured via 2 methods: a data logger surgically placed in the abdominal cavity and by a thermometer inserted into the cloaca. Body temperature was measured by both methods at 15 min intervals within each T_a period over 4 days. Error bars indicate ± 1 SEM. Days lacking a common letter (x, y, z) differ ($P < 0.05$). There were no differences ($P > 0.05$) between measurement methods at any experimental day.

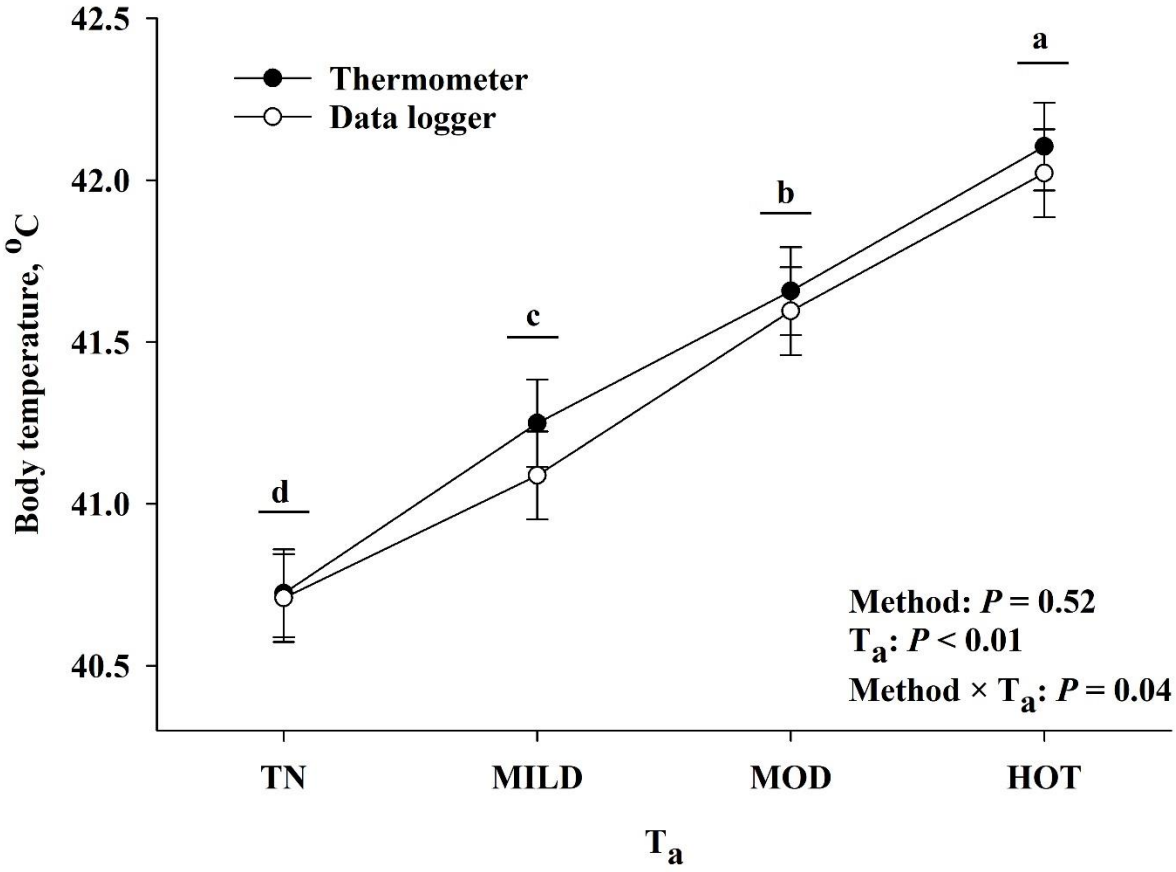

Figure 6.7. Measurement method and ambient temperature (T_a) cycle interacted in Exp 6.2. The internal body temperature of mixed sex 6 week old broiler chickens (n = 4 males and 5 females) was measured via 2 methods: a data logger surgically placed in the abdominal cavity and by a thermometer inserted into the cloaca. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN; 22.0 ± 0.04°C for 1 h), MILD (26.6 ± 0.04°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), and HOT (35.0 ± 0.08°C for 40 min) with 1 h of acclimation between each T_a period. Body temperature was measured by both methods at 15 min intervals within each T_a period over 4 days. Error bars indicate ± 1 SEM. Ambient temperature periods lacking a common letter (a, b, c, d) differ ($P < 0.05$). There were no differences ($P > 0.05$) between measurement methods at any T_a period.

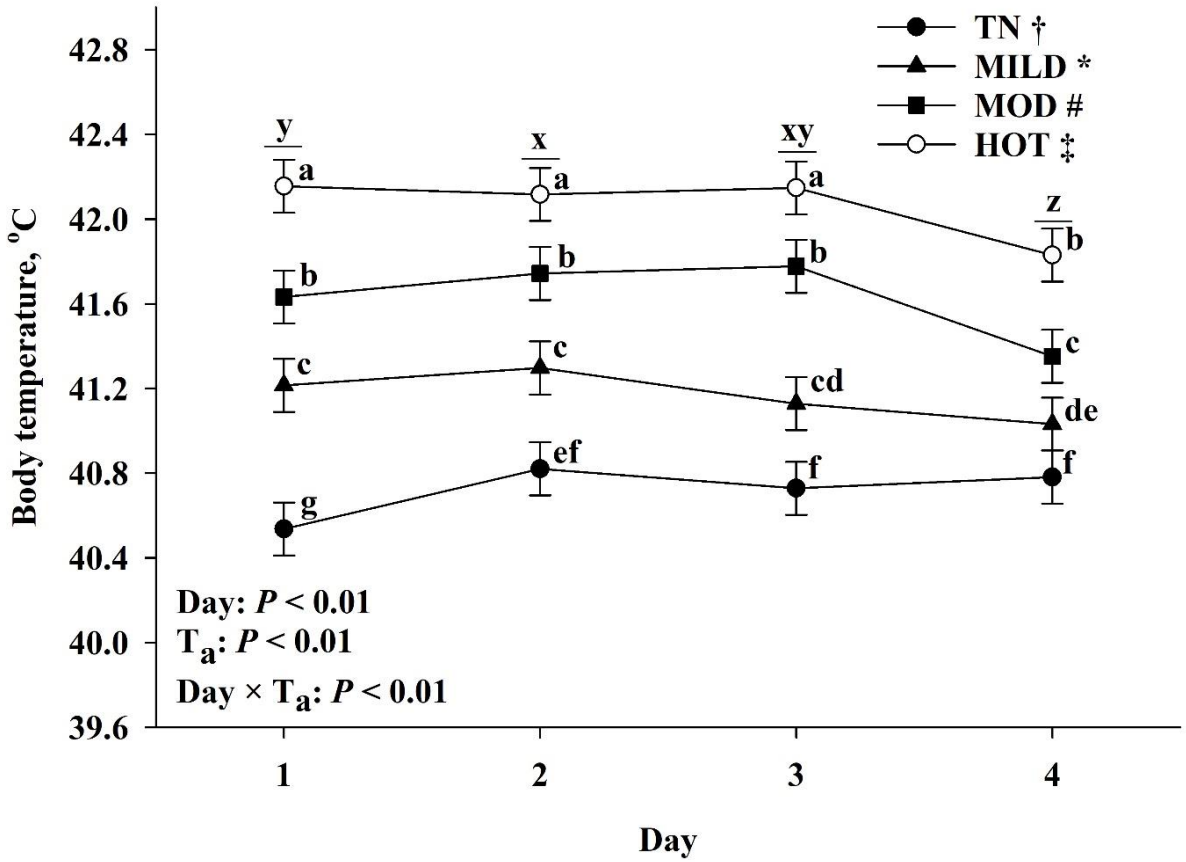

Figure 6.8. The interaction of measurement day and ambient temperature (T_a) cycle in Exp 6.2. The internal body temperature of mixed sex 6 week old broiler chickens (n = 4 males and 5 females) was measured via 2 methods: a data logger surgically placed in the abdominal cavity and by a thermometer inserted into the cloaca. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN; 22.0 ± 0.04°C for 1 h), MILD (26.6 ± 0.04°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), and HOT (35.0 ± 0.08°C for 40 min) with 1 h of acclimation between each T_a period. Body temperature was measured by both methods at 15 min intervals within each T_a period over 4 days. Error bars indicate ± 1 SEM. Days lacking a common letter (x, y, z) differ ($P < 0.05$), day × T_a values lacking a common letter (a, b, c, d, e, f, g) differ ($P < 0.05$), and T_a periods lacking a common symbol (‡ > # > * > †) differ ($P < 0.05$).

CHAPTER 7. DEVELOPMENT AND EVALUATION OF AN EQUATION TO PREDICT INTERNAL BODY TEMPERATURE IN BROILER CHICKENS

7.1 Abstract

Internal body temperature is a key response variable under heat stress conditions, but measurement methods for poultry are few and have considerable drawbacks. Two experiments (Exp 7.1 and Exp 7.2) were conducted with the objective to develop and evaluate an equation to predict internal body temperature from facial skin temperature in 6 week old mixed sex Ross 708 broiler chickens housed in bedded floor pens and exposed to 4 ambient temperature (T_a) periods: thermoneutral (TN; 22.3 ± 0.08°C for 1 h), MILD (26.5 ± 0.08°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), HOT (34.9 ± 0.12°C for 40 min) each day over 4 consecutive days. Internal body temperature from surgically implanted data loggers ($T_{abdominal}$), and facial skin temperature (T_{face}) via a thermal imaging camera, were obtained at 10 min intervals during each T_a period each day from 12 chickens in Exp 7.1, and 16 chickens from Exp 7.2. Surgery to implant data loggers was conducted on d 34 post-hatch via a simple procedure using isoflurane gas anesthetic. Data from Exp 7.1 were analyzed with Proc Reg of SAS 9.4 to develop an equation to predict $T_{abdominal}$, and T_{face}, sex of the chicken, and number of days of heat stress exposure were found to be explanatory parameters ($P < 0.01$). Data from Exp 7.2 were then used in the equation from Exp 7.1 to obtain predicted internal body temperature values ($T_{predicted}$). The relationship of $T_{predicted}$ to $T_{abdominal}$ was analyzed via Proc Glimmix of SAS 9.4 in a mixed model with the measurement method ($T_{predicted}$ vs. $T_{abdominal}$), sex of the chicken, T_a period (TN, MILD, MOD, HOT), measurement day (d 1 through 4), and their interactions as fixed effects. Values for $T_{predicted}$ (41.7 ± 0.07°C) were greater ($P < 0.01$) than for $T_{abdominal}$ (41.4 ± 0.07°C). An analysis conducted on the

$T_{difference}$ ($T_{predicted}$ minus $T_{abdominal}$) indicated that variation due to the number of heat cycle days ($P < 0.01$) and T_a period were not fully accounted for in the prediction equation. Potential explanations for this discrepancy between $T_{abdominal}$ and $T_{predicted}$ are differences in acclimation and physiological heat stress levels between experiments. This initial attempt to produce and validate an equation to predict internal body temperature of broilers demonstrates the potential of the method, and inclusion of additional explanatory variables in the equation in future studies may allow refinement towards a more accurate and robust prediction equation.

Key words: body temperature, broiler chicken, skin temperature, heat stress, thermal imaging

7.2 Introduction

Heat stress in poultry is of growing concern due to decreases in heat tolerance from modern strains of high-producing poultry coupled with projected increases in environmental temperature due to climate change (Deeb and Cahaner, 2002; Nardone et al., 2010). While considerable effort has been exerted towards characterizing the negative impacts of heat stress and developing mitigation strategies, there are limited technologies available to measure the physiological temperature of birds in the laboratory and in production settings. Ambient temperature is relied on heavily, and while it certainly is an important factor, does not always adequately reflect the physiological status of the bird (Czarick, 2007; Giloh et al., 2012). Given that the internal temperature of critical organs is what the bird is attempting to tightly regulate, this is the most illustrative measure of the state of heat stress in a bird (Dawson and Whittow, 2000).

The primary method used in the literature to measure the internal temperature of poultry is taking cloacal temperature readings by inserting a thermometer a short distance into the cloaca (e.g. Giloh et al., 2012). This requires handling of birds and temporary restraint that can lead to

increases in heart rate and T_{core} (Liljequist et al., 1994; Cabanac and Aizawa, 2000), disruption of normal behavior, and limits the number of readings that can practically be obtained in a given time period. A viable alternative could be the use of thermal imaging via infra-red cameras to measure surface temperature of birds (McCafferty, 2013), as this has been reported to be a viable non-invasive option that more accurately reflects the temperature status of the bird than ambient temperature (Giloh et al., 2012) and if measured via thermal imaging does not require handling of the bird. However, skin or surface temperature is likely to be lower and more variable than internal temperature, and thus may not be a suitable replacement for internal body temperature measurements during heat stress research. However, the facial skin, eye, or comb have been found to yield the highest and most stable surface temperature readings in broilers (Cangar et al., 2008; Edgar et al., 2013). Indeed, thermal image skin temperature of the facial area (T_{face}) has been shown to be highly correlated with internal body temperature in broiler chickens (Giloh et al., 2012). Therefore, the potential exists to harness the non-invasive nature of thermal imaging while simultaneously obtaining a better estimate of the physiological thermal status of the chicken by using an equation to predict the internal temperature with the inputs of T_{face} and potentially other explanatory variables. Thus, our objective in Exp 7.1 was to determine the relationship between T_{face} and internal temperature in market-age broiler chickens and develop a prediction equation to account for changes in this relationship due to bird sex, ambient temperature, and number of days of heat stress exposure in Exp 7.1, while our objective was to test this equation in Exp 7.2. We hypothesized that the prediction equation would reliably estimate internal body temperature.

7.3 Materials and Methods

All procedures involving the use of live animals were approved by the Purdue University Animal Care and Use Committee (protocols #1705001580 and #1807001772) and animal husbandry practices were in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

7.3.1 Birds and Husbandry

In Exp 7.1, mixed sex broiler chicks (Ross 708; n = 15 males and 15 females) were obtained from a commercial hatchery (Hoover's Hatchery, Rudd, IA) at hatch and placed into floor pens (1.2 × 1.2 m) bedded with fresh pine shavings in a heated room. Chickens were initially placed 15 per pen into two pens and were further split into three pens at d 21 to maintain acceptable stocking density. Heat lamps provided supplemental heat until d 13 post-hatch. Except for a brief period prior to surgery, chickens had *ad libitum* access to water via bell-style drinkers and feed was provided *ad libitum* in mash form from a gravity-flow feeder. Diets were based on corn and soybean meal and were formulated to meet or exceed breeder nutrient recommendations (Aviagen, 2014). The starter diet was fed from hatch to d 22 post-hatch and contained 3090 kcal/kg AME_n, 23% CP, 9.6 g/kg total Ca, 4.8 g/kg non-phytate P, 7.2 g/kg total Met and 14.4 g/kg total Lys (calculated values). The grower diet was fed from d 23 to 49 post-hatch and contained 3185 kcal/kg AME_n, 19.5% CP, 7.8 g/kg total Ca, 3.9 g/kg non-phytate P, 5.8 g/kg total Met and 11.5 g/kg total Lys (calculated values). The T_a and relative humidity of the room were: 25.2 ± 0.02°C and 65.2 ± 0.31% for d 1 to 7 post-hatch; 25.7 ± 0.02°C and 61.4 ± 0.20% for d 8 to 14 post-hatch; 24.9 ± 0.03°C and 78.1 ± 0.27% for d 15 to 21 post-hatch; 23.7 ± 0.03°C and 66.9 ± 0.28% for d 22 to 28 post-hatch; and 21.6 ± 0.07°C and 68.4 ± 0.43% for d 29 to 33 post-hatch. Lighting was maintained

on a 23:1, 20:4, and 17:7 light:dark cycle from d 0 to 7, 8 to 30, and 31 to 49 post-hatch, respectively.

In Exp 7.2, mixed sex broiler chicks (Ross 708; n = 15 males and 15 females) were obtained from a commercial hatchery (Townline Hatchery, Zeeland, MI) at hatch and placed into floor pens bedded with fresh pine shavings in a heated room. Chickens were initially placed 15 per pen into two pens and were further split into three pens at d 21 to maintain acceptable stocking density. Heat lamps provided supplemental heat during the brooding period. Except for a brief period prior to surgery, chickens had *ad libitum* access to water via bell-style drinkers and feed was provided *ad libitum* in mash form from a gravity-flow feeder. Diets were the same and fed on the same schedule as in Exp 6.1. The T_a and relative humidity of the room were: 31.5 ± 0.04°C and 51.5 ± 0.34% for d 1 to 7 post-hatch; 27.6 ± 0.03°C and 62.1 ± 0.24% for d 8 to 14 post-hatch; 26.5 ± 0.02°C and 55.6 ± 0.25% for d 15 to 21 post-hatch; 25.9 ± 0.02°C and 57.3 ± 0.17% for d 22 to 28 post-hatch, 25.6 ± 0.01°C and 56.7 ± 0.34% for d 29 to 35 post-hatch; and 25.3 ± 0.02°C and 37.9 ± 0.20% for d 36 to 40 post-hatch. Lighting was maintained on a 23:1, 20:4, and 17:7 light:dark cycle from d 0 to 7, 8 to 30, and 31 to 44 post-hatch, respectively.

Chickens in both experiments were group housed pre-surgery, post-surgery, and during the experimental temperature cycle days in the same floor pens that the chickens had occupied since hatch. Litter was not removed from pens until the completion of each experiment, rather, clean shavings were added to the pens periodically. Feed and water were available at all times following surgery through the end of each experiment.

7.3.2 Data Logger Implantation

In Exp 7.1, at d 33 post-hatch, chickens were individually identified with numbered leg bands and 10 birds of each sex (n = 20 birds) that were closest to the median BW (2.2 ± 0.06 kg for males, 1.8 ± 0.04 kg for females) were selected and fasted overnight. On d 34 post-hatch, water was removed for 2 h before surgery. Chickens were weighed individually and injected with midazolam HCl (0.5 mg/kg BW; 5 mg/mL, Akorn Inc., Lake Forest, IL) and buprenorphine HCl (0.05 mg/kg BW; 0.3 mg/mL, Par Pharmaceuticals Companies Inc., Spring Valley, NJ) intramuscularly 15 min prior to anesthesia. Chickens were then held to prevent movement and their heads were placed into a small cone anesthesia mask (8.9 cm maximum inner diameter, 7.0 cm deep; #32393B4, Smiths Medical, Dublin, OH). Laboratory tape was placed around the seal of the mask to reduce the diameter as required. Anesthesia was induced via isoflurane (IsoFlo, Zoetis Inc., Kalamazoo, MI) inhalation with the vaporizer (Isotec, Smiths Medical, Dublin, OH) set to 5% isoflurane and with oxygen flow at 2.0 L/min. Anesthesia was maintained at 1.5 to 3% isoflurane and oxygen flow at 2.0 L/min with manual positive pressure artificial respiration 2 to 4 times per minute. Uncuffed tracheal tubes of 3, 4, and 5 mm diameter (Midmark Animal Health, Versailles, OH) were available in the event of respiratory distress but no chickens required intubation. Anesthetized chickens were placed in dorsal recumbency, and the feathers plucked from the surgical site (abdominal wall on the right side of the bird, ventral to the caudal tip of the sternum). Laboratory tape was used to hold the right leg up towards the front of the bird to expose the surgical site and was also used to keep feathers from surrounding areas from contacting the surgical site. The surgical site was then sterilized by alternating rounds of a povidone-iodine solution (Betadine, Purdue Products LP., Stamford, CT) and 70% ethanol. Additional analgesic was provided at the incision site by a subcutaneous injection of 4 mg per kg BW of lidocaine HCl (20 mg/mL, Vedco

Inc., St. Joseph, MO). Adequate depth of anesthesia was evaluated via a periodic comb pinch. Using sterile technique, a 2.5 cm incision was made in the skin using a scalpel, and through the muscle layers and peritoneum using iris scissors. Two sterile data loggers (iButton, accuracy of 0.1°C; DS1921H High-Resolution Thermochron Device, Maxim Integrated Products Inc., San Jose, CA; 17.4 mm long by 6.9 mm wide, weight of 3.3 g) were placed into the abdominal cavity to monitor abdominal temperature ($T_{abdominal}$) in 10 min intervals 24 h per day. Two data loggers were used to preclude data loss in the event of a data logger malfunction. Data loggers in Exp 7.1 were sterilized by autoclaving, while data loggers in Exp 7.2 were cold sterilized by submersion in a disinfectant (CIDEX Activated Dialdehyde Solution, Johnson and Johnson, New Brunswick, NJ) prior to placement in the chickens. The incision was closed with 3-0 absorbable suture (PDS II, Ethicon US LLC., Bridgewater, NJ) with a reverse cutting 24 mm circle needle via a continuous stitch (muscle layers) and interrupted stitches (skin). An antibiotic ointment (Neosporin, Johnson and Johnson, New Brunswick, NJ) was then applied to the incision site, and 20 mg per kg BW of a broad-spectrum antibiotic (Liquamycin LA-200, Zoetis Inc., Kalamazoo, MI) was injected intramuscularly. The average surgery duration (start of anesthesia to completion of surgery) was 20 ± 1 min. Chickens were placed under heat lamps until they regained consciousness and then were returned to their pens. An additional injection of buprenorphine HCl (0.05 mg/kg BW) and application of antibiotic ointment to the incision site were administered 12 h post-surgery.

In Exp 7.2, chickens were individually identified with numbered leg bands at d 33 post-hatch, and 25 chickens (12 males, 2.1 ± 0.11 kg BW; 13 females, 1.9 ± 0.10 kg BW) were fasted overnight. On d 34 post-hatch water was removed for 2 h before surgery, and the 25 chickens underwent surgery to receive data loggers as described for Exp 7.1. The average surgery time from initiation of anesthesia to completion of the surgery was 16 ± 0.6 min in Exp 7.2.

7.3.3 Temperature Cycle and Body Temperature Measurement

In both experiments, chickens were given a surgery recovery period of 1 week and were then exposed to a T_a cycle (Figure 7.1) each day on d 42, 43, 44, and 45 post-hatch. For Exp 7.1, each T_a cycle started at 0700 h each day and consisted of a thermoneutral period (TN; 22.5 ± 0.12°C), MILD HS period (MILD; 26.3 ± 0.12°C), moderate HS period (MOD; 30.1 ± 0.12°C), and severe HS period (HOT; 34.5 ± 0.15°C). In Exp 7.2, each T_a cycle consisted of a thermoneutral period (TN; 22.0 ± 0.04°C), MILD HS period (MILD; 26.6 ± 0.04°C), moderate HS period (MOD; 30.1 ± 0.12°C), and severe HS period (HOT; 35.2 ± 0.08°C). During each T_a cycle, TN, MILD, and MOD were maintained for 1 h, while HOT was maintained for 40 min. There was a 1 h acclimation period between each T_a period to allow chickens to adjust to the increased T_a (Figure 7.1). All chickens were returned to TN conditions at the conclusion of the HOT period each day. Ambient temperature was controlled via thermostats (T631A Farm-O-Stat Airswitch Controller, Honeywell, Charlotte, NC) connected to forced-air heaters.

In both experiments, thermal images (accuracy ± 0.1°C; model T440, FLIR Systems, Wilsonville, OR) were taken every 10 min for 12 chickens (6 males and 6 females) in Exp 7.1, and 16 chickens (8 males and 8 females) in Exp 7.2, during each T_a period each day while $T_{abdominal}$ was simultaneously measured via the implanted data loggers. Thermal images were taken to include the face, comb, and wattles of chickens. Thermal images were later analyzed via a software program (FLIR Tools, FLIR Systems, Wilsonville, OR) and the maximum surface temperature (T_{face}) detected by the software was recorded for each image. Birds were euthanized on d 49 (Exp 7.1) or d 44 (Exp 7.2) post-hatch via carbon-dioxide asphyxiation, and then data loggers were retrieved from the abdominal cavity and the data were downloaded (OneWireViewer, Maxim Integrated Products Inc., San Jose, CA). Two male chickens died following the HOT period on d

1 in Exp 7.1, therefore, 4 male chickens and 6 female chickens were used in d 2, 3, and 4 of Exp 7.1. There was no mortality in Exp 7.2.

7.3.4 Statistical Analysis

Both experiments were allotted as completely randomized designs with the individual chicken as the experimental unit. Data collected from each chicken within each T_a period each day were averaged over the T_a period prior to analysis. Data were analyzed with Proc Glimmix or Proc Reg of SAS 9.4 (SAS Institute, Cary, NC) with influential outliers analyzed via a Cook's D test of Proc Reg (Cook, 1977). The assumptions of normality and homogeneity of variances were verified via examination of residual plots. Regression analysis was conducted to develop prediction equations for experiments with the dependent variable as $T_{abdominal}$, and the independent variables of T_{face}, sex of the chicken (0 for males and 1 for females), and the number of days of heat stress exposure (1 through 4). For the mixed model analysis of $T_{abdominal}$, T_{face}, and the ratio of $T_{abdominal}$: T_{face} in both experiments, and the difference ($T_{difference}$) between internal body temperature predicted using an equation (**$T_{predicted}$**) and $T_{abdominal}$ (e.g. statistical analysis with $T_{difference}$ as the dependent variable where $T_{difference} = T_{predicted} - T_{abdomional}$) in Exp 7.2, the statistical model included the fixed effects of the T_a cycle d (1 through 4), the T_a period (TN, MILD, MOD, HOT), sex of the chicken, and their interactions. The model also contained the random factors of chicken(sex), day*chicken(sex), and period*chicken(sex). For analysis of the accuracy of the prediction equation in Exp 7.2, the model was similar with the addition of the fixed effect of the method used to obtain internal body temperature ($T_{abdominal}$ vs. $T_{predicted}$) and its interaction with the other fixed effects, and the addition of the random effects of day*period*chicken(sex), chicken(sex)*method, chicken(sex)*day*method, chicken(sex)*period*method. A Kenward-Rogers degrees of freedom adjustment was included via the ddfm=kr option of the model statement (Kenward and Roger,

1997) for all mixed model analysis. Means of significant interactions of interest were separated with the slicediff option of the LSmeans statement to compare the effect of the factor of interest at each level of the other factor. In all other cases a Tukey's multiple comparison test was used in pair-wise comparisons of least square means. Statistical significance was declared at a $P < 0.05$. Values are presented as least squares means. Measures of uncertainty of means and regression equation components are presented as ± 1 SE.

7.4 Results

7.4.1 Surgery Outcomes

There was no mortality during surgery or during the 1 week recovery period. Incisions were monitored for signs of infection (inflammation, redness, necrosis) during the recovery period and none were observed. Chickens gained weight during the recovery period in Exp 7.1 with the pre-surgery BW of males and females being 2.2 ± 0.06 and 1.8 ± 0.04 kg, respectively, and the BW at the end of the recovery period for males and females being 3.1 ± 0.15 and 2.5 ± 0.05 kg, respectively. Similarly, in Exp 7.2 the pre-surgery BW of males and females was 2.1 ± 0.10 and 1.9 ± 0.10 kg, respectively, and the BW at the end of the recovery period for males and females was 2.9 ± 0.11 and 2.4 ± 0.07 kg, respectively. Incision sites and the abdominal cavities were inspected during data logger retrieval and no signs of infection (inflammation, redness, necrosis, thickened or opaque air sack membranes) were observed in either experiment. In addition, there was no indication of picking at the incision site from pen-mates in either experiment.

In Exp 7.1, multiple data loggers were not functional upon retrieval from the chickens. As a result, usable data logger data was obtained from one data logger per chicken from 16 of the 20 chickens that underwent surgery. This failure was believed to be due to battery failure caused by

the heat of autoclaving, and accordingly in Exp 7.2 data loggers were cold sterilized. In Exp 7.2, one data logger demonstrated inconsistent readings and was not used in the analysis. For all other chickens both data loggers performed appropriately and the results for the two implanted data loggers were averaged for each chicken prior to analysis. Therefore, usable data was obtained from all 25 chickens that underwent surgery. Cold sterilization techniques are recommended for all future uses of surgically implanted data loggers.

7.4.2 Exp 7.1

An initial examination of the data demonstrated a linear relationship between $T_{abdominal}$ and T_{face}, with an $R^2 = 0.84$ (Figure 7.2). Further analysis was conducted to understand the impact of the fixed effects on $T_{abdominal}$, T_{face}, and their ratio, prior to use of the data for development of an equation to predict $T_{abdominal}$. Day impacted $T_{abdominal}$ values ($P < 0.01$) with d 3 (42.15 ± 0.100°C) being lesser than d 1 (42.27 ± 0.099°C) with d 2 (42.19 ± 0.100°C) being intermediate, and with d 4 (41.95 ± 0.100°C) being lesser than all other days (Figure 7.3). There was also an effect of T_a period on $T_{abdominal}$ values ($P < 0.01$) with $T_{abdominal}$ being greater at each increase in T_a with TN at 41.3 ± 0.11°C, MILD at 41.8 ± 0.11°C, MOD at 42.4 ± 0.11°C, and HOT at 43.1 ± 0.11°C. In addition, there was interaction of chicken sex and day ($P < 0.01$), with $T_{abdominal}$ being reduced for females at d 4 (42.07 ± 0.140°C) compared to d 1 (42.33 ± 0.140°C), d 2 (42.36 ± 0.140°C), and d 3 (42.35 ± 0.140°C), but with $T_{abdominal}$ for males being reduced at d 2 (42.02 ± 0.144°C), d 3 (41.96 ± 0.144°C), and d 4 (41.84 ± 0.145°C) compared to d 1 (42.22 ± 0.140°C), and with d 4 being reduced compared to d 2, with d 3 being intermediate (Figure 7.4).

There was an effect of T_a period ($P < 0.01$) on T_{face}, with T_{face} being greater at each increase in T_a with TN at 39.97 ± 0.090°C, MILD at 40.78 ± 0.090°C, MOD at 41.65 ± 0.090°C, and HOT at 42.90 ± 0.092°C. In addition, a day by T_a period interaction impacted ($P < 0.01$) T_{face}, with T_{face}

at the HOT T_a being reduced at d 3 (42.84 ± 0.112°C) compared to d 1 (43.17 ± 0.100°C) with d 2 (42.96 ± 0.112°C) being intermediate, and with d 4 (42.64 ± 0.113°C) being reduced compared to d 1 and d 2, with d 3 being intermediate, but with no significant change by day for the other T_a periods (Figure 7.5).

Day impacted the ratio of $T_{abdominal}$ to T_{face} (P = 0.01), with the ratio at d 1 (1.02 ± 0.002) being greater than the ratio at d 4 (1.02 ± 0.002), with d 2 (1.02 ± 0.002) and d 3 (1.02 ± 0.002) being intermediate. There was also an effect of T_a period (P < 0.01) on the ratio of $T_{abdominal}$ to T_{face}, with the ratio being lesser at each increase in T_a with TN at 1.032 ± 0.0024, MILD at 1.024 ± 0.0024°C, MOD at 1.018 ± 0.0024°C, and HOT at 1.005 ± 0.0024. In addition, an interaction of day by T_a period (P < 0.01) impacted the ratio of $T_{abdominal}$ to T_{face}, with the ratio at the TN T_a being reduced at d 3 (1.030 ± 0.0027) and d 4 (1.029 ± 0.0027) compared to d 1 (1.038 ± 0.0026), with d 2 (1.032 ± 0.0027) being intermediate, and with the ratio at the MILD T_a being reduced at d 2 (1.023 ± 0.0027), 3 (1.022 ± 0.0027), and 4 (1.021 ± 0.0027) compared to d 1 (1.029 ± 0.0026), but with no significant change by day for the other T_a periods (Figure 7.6).

Accordingly, a regression equation was developed to encompass these sources of variation and T_{face}, sex of the chicken, and T_a cycle day were all found to be significant (P < 0.01) factors. Therefore, the regression equation was determined to be $y = 15.92 \pm 0.791 + 0.64 \pm 0.019(x_1) + 0.33 \pm 0.044(x_2) - 0.08 \pm 0.019(x_3)$, where y is $T_{abdominal}$ in °C, x_1 is T_{face} in °C, x_2 is sex of the chicken (0 for males, 1 for females), and x_3 is T_a day (1 to 4) and with R^2 = 0.88.

7.4.3 Exp 7.2

An initial examination of the data demonstrated a linear relationship between $T_{abdominal}$ and T_{face}, with an R^2 = 0.70 (Figure 7.7). Further analysis was conducted to understand the impact of the fixed effects on $T_{abdominal}$, T_{face}, and their ratio, prior to use of the data for evaluation of the

prediction equation developed in Exp 7.1. Day impacted $T_{abdominal}$ values ($P < 0.01$) with d 1 (41.07 ± 0.097°C) being lesser than d 2 (41.51 ± 0.097°C), d 3 (41.50 ± 0.097°C), and d 4 (41.36 ± 0.097°C) (Figure 7.8). There was also an effect of T_a period on $T_{abdominal}$ values ($P < 0.01$) with $T_{abdominal}$ being greater at each increase in T_a with TN at 40.77 ± 0.092°C, MILD at 41.11 ± 0.092°C, MOD at 41.51 ± 0.092°C, and HOT at 42.05 ± 0.092°C. In addition, a day by T_a period interaction ($P < 0.01$) impacted $T_{abdominal}$. For the HOT T_a, $T_{abdominal}$ was greatest at d 3 (42.35 ± 0.104°C), with d 2 (42.14 ± 0.104°C) and d 4 (42.11 ± 0.104°C) greater than d 1 (41.60 ± 0.104°C). For the MOD T_a, d 3 (41.77 ± 0.104°C) was greater than d 1 (41.24 ± 0.104°C) and d 4 (41.43 ± 0.104°C) with d 2 (41.62 ± 0.104°C) being similar to d 3 and d 4 but greater than d 1. For the MILD T_a, d 2 (41.33 ± 0.104°C) was greater than d 1 (40.93 ± 0.104°C), d 3 (41.13 ± 0.104°C), and d 4 (41.06 ± 0.104°C). For the TN T_a d 2 (40.96 ± 0.104°C) was greater than d 1 (40.53 ± 0.104°C) and d 3 (40.76 ± 0.104°C), with d 4 (40.84 ± 0.104°C) being intermediate, and d 3 and 4 being greater than d 1 (Figure 7.9).

Day impacted T_{face} values ($P < 0.01$) with d 1 (40.18 ± 0.061°C) being lesser than all other days, and d 3 (40.88 ± 0.061°C) being greater than d 4 (40.71 ± 0.061°C), with d 2 (40.80 ± 0.061°C) being intermediate (Figure 7.10). There was an effect of T_a period ($P < 0.01$) on T_{face}, with T_{face} being greater at each increase in T_a with TN at 39.46 ± 0.065°C, MILD at 40.32 ± 0.065°C, MOD at 40.88 ± 0.065°C, and HOT at 41.90 ± 0.065°C. In addition, a day by T_a period interaction impacted ($P < 0.01$) T_{face} (Figure 7.11). For the HOT T_a, T_{face} was greater at d 3 (42.19 ± 0.078°C) than d 4 (41.89 ± 0.078°C), with d 2 (42.13 ± 0.078°C) being intermediate, and d 1 (41.39 ± 0.078°C) being lesser than all the other days. For the MOD T_a, d 3 (41.27 ± 0.078°C) was greatest, and with d 2 (41.00 ± 0.078°C) and d 4 (40.89 ± 0.078°C) being greater than d 1 (40.36 ± 0.078°C). For the MILD T_a, d 2 (40.47 ± 0.078°C) and d 3 (40.51 ± 0.078°C) were greater than

d 1 (40.04 ± 0.078°C) and d 4 (40.26 ± 0.078°C), and with d 4 being greater than d 1. For the TN T_a, d 4 (39.79 ± 0.078°C) was greatest, and d 2 (39.60 ± 0.078°C) and d 3 (39.54 ± 0.078°C) were greater than d 1 (38.91 ± 0.078°C).

Day impacted the ratio of $T_{abdominal}$ to T_{face} (P = 0.01), with the ratio at d 1 (1.023 ± 0.0016) being greater than the ratio at d 2 (1.018 ± 0.0016), d 3 (1.016 ± 0.0016) and d 4 (1.016 ± 0.0016). Sex also impacted the ratio (P = 0.01) with females (1.023 ± 0.0022) being greater than males (1.013 ± 0.0022). There was also an effect of T_a period (P < 0.01) on the ratio of $T_{abdominal}$ to T_{face}, with the ratio being lesser at each increase in T_a with TN at 1.033 ± 0.0016, MILD at 1.020 ± 0.0016, MOD at 1.016 ± 0.0016, and HOT at 1.004 ± 0.0016. In addition, an interaction of day by T_a period (P < 0.01) impacted the ratio of $T_{abdominal}$ to T_{face} (Figure 7.12). For the HOT T_a, the ratio was greater at d 1 (1.005 ± 0.0015) and d 4 (1.005 ± 0.0015) than d 2 (1.000 ± 0.0015), with d 3 (1.004 ± 0.0015) being intermediate. For the MOD T_a, d 1 (1.022 ± 0.0015) was greater than d 2 (1.015 ± 0.0015), d 3 (1.012 ± 0.0015), and d 4 (1.013 ± 0.0015). For the MILD T_a, d 3 (1.015 ± 0.0015) was lesser than d 1 (1.022 ± 0.0015), d 2 (1.021 ± 0.0015), and d 4 (1.020 ± 0.0015). For the TN T_a, d 1 (1.042 ± 0.0015) was greatest, and d 2 (1.035 ± 0.0015) and d 3 (1.031 ± 0.0015) were greater than d 4 (1.027 ± 0.0015).

The regression equation developed in Exp 7.1 was used to calculate the predicted body temperature ($T_{predicted}$) using the inputs of T_{face}, chicken sex, and day of T_a exposure. The resulting $T_{predicted}$ values were then compared to the $T_{abdominal}$ values for each chicken at each day and T_a period combination to ascertain if there was a difference between the $T_{predicted}$ and $T_{abdominal}$ values. Values for $T_{predicted}$ (41.68 ± 0.068°C) were greater (P < 0.01) than values for $T_{abdominal}$ (41.36 ± 0.068°C). In addition, day, T_a period, and method of determining body temperature (measurement vs. prediction) interacted (P < 0.01; Figure 7.13), with $T_{predicted}$ being greater than $T_{abdominal}$ at all

day by T_a period combinations except d 2 at TN. In addition, method interacted with day (P < 0.01), and with T_a period (P < 0.01; data not presented). To aid in understand these results, an analysis was conducted on the residual values of $T_{predicted}$ minus $T_{abdominal}$ ($T_{difference}$). Significant factors in the model would indicate sources of variation incompletely accounted for in the prediction equation. Day was significant (P < 0.01), with the discrepancy between $T_{predicted}$ and $T_{abdominal}$ greater at d 1 (0.43 ± 0.073°C), than at d 2 (0.31 ± 0.073°C), d 3 (0.29 ± 0.073°C), and d 4 (0.24 ± 0.073°C). Period also accounted for some variation in $T_{difference}$ (P < 0.01), with the difference at HOT (0.43 ± 0.070°C) being greater than at MOD (0.32 ± 0.070°C) and at TN (0.16 ± 0.070°C), with MILD (0.37 ± 0.070°C) being similar to HOT and MOD, and with TN being lesser than all other T_a periods. In addition, day and T_a period interacted (P < 0.01; Figure 7.14), with $T_{difference}$ for HOT at d 1 (0.67 ± 0.079°C) and d 2 (0.53 ± 0.079°C) being greater than at d 3 (0.28 ± 0.079°C) and d 4 (0.25 ± 0.079°C), and with $T_{difference}$ for MILD being greater at d 1 (0.50 ± 0.079°C) and d 3 (0.43 ± 0.079°C) than at d 2 (0.29 ± 0.079°C) and d 4 (0.26 ± 0.079°C).

An additional regression equation was developed for Exp 7.2 data and T_{face} (P < 0.01) and sex of the chicken (P < 0.01), but not T_a cycle day (P = 0.52), were found to be significant predictors of $T_{abdominal}$. Therefore, the Exp 7.2 regression equation was determined to be $y = 17.71 \pm 0.839 + 0.58 \pm 0.021(x_1) + 0.36 \pm 0.040(x_2)$, where y is $T_{abdominal}$ in °C, x_1 is T_{face} in °C, and x_2 is sex of the chicken (0 for males, 1 for females), with an $R^2 = 0.77$. In addition, a visual comparison of the data points from Exp 7.1 and Exp 7.2 is presented in Figure 7.15.

7.5 Discussion

As evidenced by the three-way interaction of day, T_a period, and method of determining body temperature (measurement vs. prediction), the greatest discrepancy between the

measurement methods occurred at d 1 and 2. In addition, the interaction of day and T_a period for $T_{difference}$ demonstrated similar effects of day, with the difference between $T_{predicted}$ and $T_{abdominal}$ being least at d 4. Also, the interaction of day and T_a period for $T_{difference}$ showed that TN consistently had the least difference, while HOT had the greatest difference, especially at d 1 and d 2. Out of the three components of the prediction equation from Exp 7.1, the effect of sex was the most similar between the two experiments, and with the effect of sex being non-significant in the analysis of $T_{differences}$, it would appear that the effect of sex in Exp 7.2 was adequately described by the Exp 7.1 equation. Therefore, at a minimum, the equation components of day and T_a period were unable to adequately account for the variation seen in the response observed in Exp 7.2.

Chickens, like other homeotherms, possess the ability to adapt to heat stress via mechanisms such as alterations in blood flow, thereby enabling chickens to control their body temperature more effectively in the face of repeated heat stress events (Yahav et al., 1997; Dawson and Whittow, 2000). In the present studies, adaptation to the repeated heat stress events differed between studies and may have impacted the accuracy of the $T_{predicted}$ values. The day effect from the equation in Exp 7.1 was as expected, with a decrease of 0.08°C per day. This decrease was reflective of the uniform decrease in $T_{abdominal}$ over time seen in Exp 7.1, consistent with the expected acclimation of the chickens to the applied heat stress each day. In contrast, the day effect from Exp 7.2 was puzzling, with $T_{abdominal}$ increasing by 0.43°C from d 1 to d 2, and then remaining fairly stable through d 4. A somewhat similar pattern was observed in the day by T_a period for $T_{abdominal}$ in Exp 7.2, with MOD and HOT increasing from d 1 to a peak at d 3, before starting to decrease on d 4, and with TN and MILD both being greatest at d 2. The day effect on T_{face} and the day by T_a period interaction for T_{face} in Exp 7.2 demonstrated very similar patterns as for $T_{abdominal}$ in Exp 7.2. This would indicate that although the effect of day observed is unexpected, it does appear to be real as

opposed to a measurement error, as $T_{abdominal}$ and T_{face} are independent measurement methods. In addition, the changes observed in T_{face} over time for all of the T_a periods in Exp 7.2 are in contrast to T_{face} in Exp 7.1, where T_{face} was uniform over time and did not vary by day except for the HOT T_a period, which demonstrated a uniform decrease over the 4 days consistent with acclimation to heat stress. The lack of a uniform acclimation over time in Exp 7.2 for both $T_{abdominal}$ and T_{face} unlike that observed for Exp 7.1 likely explains why day was not a significant predictor of variation in the Exp 7.2 regression model, as well as why day did account for significant variation in the analysis of the differences. Therefore, dis-similar responses of chickens over the 4 days between the two experiments is likely one reason for the discrepancies observed between the $T_{abdominal}$ values and the $T_{predicted}$ values in Exp 7.2.

Relative humidity, ambient temperature, and air flow rate are all major factors influencing thermoregulation in chickens (Giloh et al., 2012; Lara and Rostagno, 2013). Despite the similarity in room ambient temperature and relative humidity between the experiments during the 1 h of data collection at each T_a, the effect of T_a period on $T_{abdominal}$ and T_{face} was somewhat different between experiments. Chickens in Exp 7.2 had lesser $T_{abdominal}$ than in Exp 7.1, with decreases of 0.50, 0.64, 0.90, and 1.09°C at TN, MILD, MOD, and HOT, respectively. Similarly, chickens in Exp 7.2 had lesser T_{face} than in Exp 7.1, with decreases of 0.51, 0.46, 0.77, and 1.00°C at TN, MILD, MOD, and HOT, respectively. Therefore, it would appear that chickens in Exp 7.2 did not experience the same degree of physiological heat stress as chickens in Exp 7.1, with differences more pronounced at elevated T_a. This difference in the observed level of physiological heat stress may be an explanation for the observed differences in the day effect between the experiments, with birds in Exp 7.1 demonstrating more acclimation due to experiencing greater physiological heat stress. While the exact cause of the differences observed in $T_{abdominal}$ and T_{face} between experiments is

unknown, one possibility is that air flow differences could have been a factor. Air-flow rate is known to have a large impact on the ability of chickens to thermoregulate (Mitchell, 1985), leading to commercial practices such as tunnel-ventilation (Lott et al., 1998). In Exp 7.1 air-flow rate was limited, with circulation in the room provided by one fan (approximately 51 cm in diameter) directed at the ceiling-mounted forced-air heater to aid in uniform heat distribution. In Exp 7.2, circulation within the room was similar, except that a larger fan (approximately 107 cm diameter) was used to circulate the air in the room. While air flow was not measured, small differences in air flow between the studies may have impacted the degree of physiological stress the chickens experienced. Therefore, it is possible that inclusion of air flow in the prediction equation in future studies may enable a more robust prediction equation.

The relationship between internal body temperature and facial skin temperature in broilers has been reported to be strong, with 83% of the variation in facial temperature being explained by internal body temperature (Giloh et al., 2012). Similarly, in Exp 7.1 T_{face} accounted for 84% of the variation in $T_{abdominal}$, while in Exp 7.2 T_{face} accounted for somewhat less of the variation, with

70% of the variation in $T_{abdominal}$ being explained by T_{face}. Possibly as a result, the relationship between $T_{abdominal}$ and T_{face} varied between experiments as reflected by differences in the ratio of $T_{abdominal}$ to T_{face} observed between experiments. In Exp 7.1, the ratio was constant for HOT and MOD T_a periods over the 4 days, while TN and MILD demonstrated a uniform decrease over the first two days. In Exp 7.2, the ratio for TN uniformly decreased over time, while the ratio for the other three T_a periods varied by day in a less uniform pattern. While the source of the variation in the relationship between $T_{abdominal}$ and T_{face} in Exp 7.2 is unknown, it may be why T_{face} had less explanatory value for $T_{abdominal}$ in Exp 7.2 than Exp 7.1, and may have contributed to the discrepancies observed between $T_{abdominal}$ and $T_{predicted}$ in Exp 7.2.

Given that the vast majority of studies that report measurement of internal body temperature in broilers did so only in males (e.g. May et al., 1987; Yalçin et al., 2008; Giloh et al., 2012), the consistent effect of sex observed between experiments is interesting, with females displaying elevated $T_{abdominal}$ over that of males. This may reflect sex differences in thermoregulation, possibly due to the increased feather cover and decreased comb and wattle size of females compared to males at 6 weeks post-hatch which would hinder heat dissipation in females (Deeb and Cahaner, 1999; Al-Ramamneh et al., 2016). Further research is warranted to explore the differences in thermoregulation between the sexes in broilers and the potential impact on heat stress susceptibility in commercial production.

7.6 Conclusions

The results of these two studies show that the use of a prediction equation to estimate the degree of physiological thermal stress experienced by broiler chickens holds promise. A prediction equation would allow the many advantages of thermal imaging to be realized, while obtaining a better estimate of physiological thermal stress than T_{face} alone can provide. The discrepancies observed between $T_{abdominal}$ and $T_{predicted}$ may indicate that additional factors are needed to encompass the variability in $T_{abdominal}$ across various settings, and further emphasize that ambient temperature and relative humidity alone are not adequate predictors of physiological heat stress in broiler chickens. Expansion of the prediction equation to include other factors such as air flow rate may enable refinement of the prediction equation that would enable better estimation of $T_{abdominal}$ from T_{face} in future studies.

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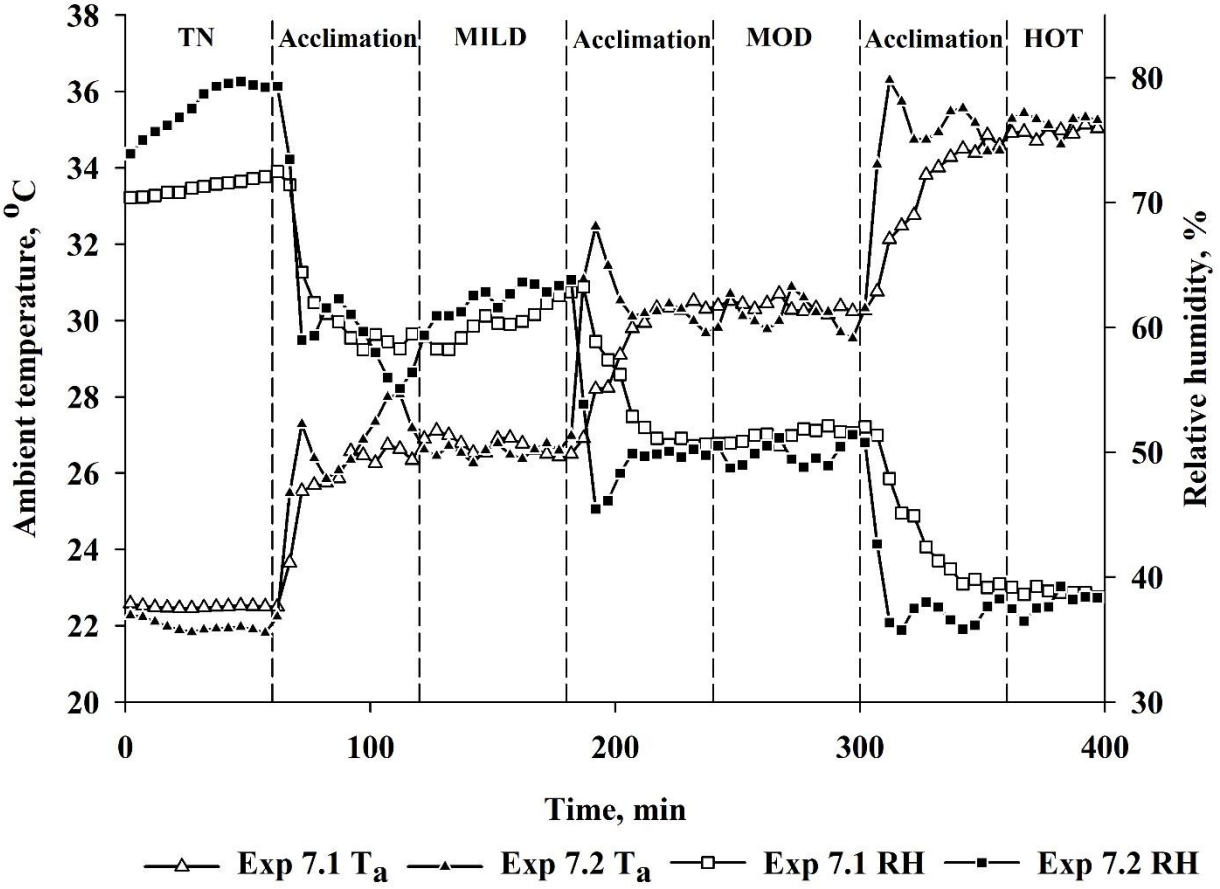

Figure 7.1. Ambient temperature (T_a) and relative humidity (RH) during the temperature cycle that mixed-sex 6 week old broiler chickens were exposed to for 4 consecutive days in Exp 7.1 and Exp 7.2. Values shown for each experiment are averages of the 4 days. The T_a cycle consisted of thermoneutral (TN; 22.3 ± 0.08°C for 1 h), MILD (26.5 ± 0.08°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), HOT (34.9 ± 0.12°C for 40 min) conditions with 1 h of acclimation between each period.

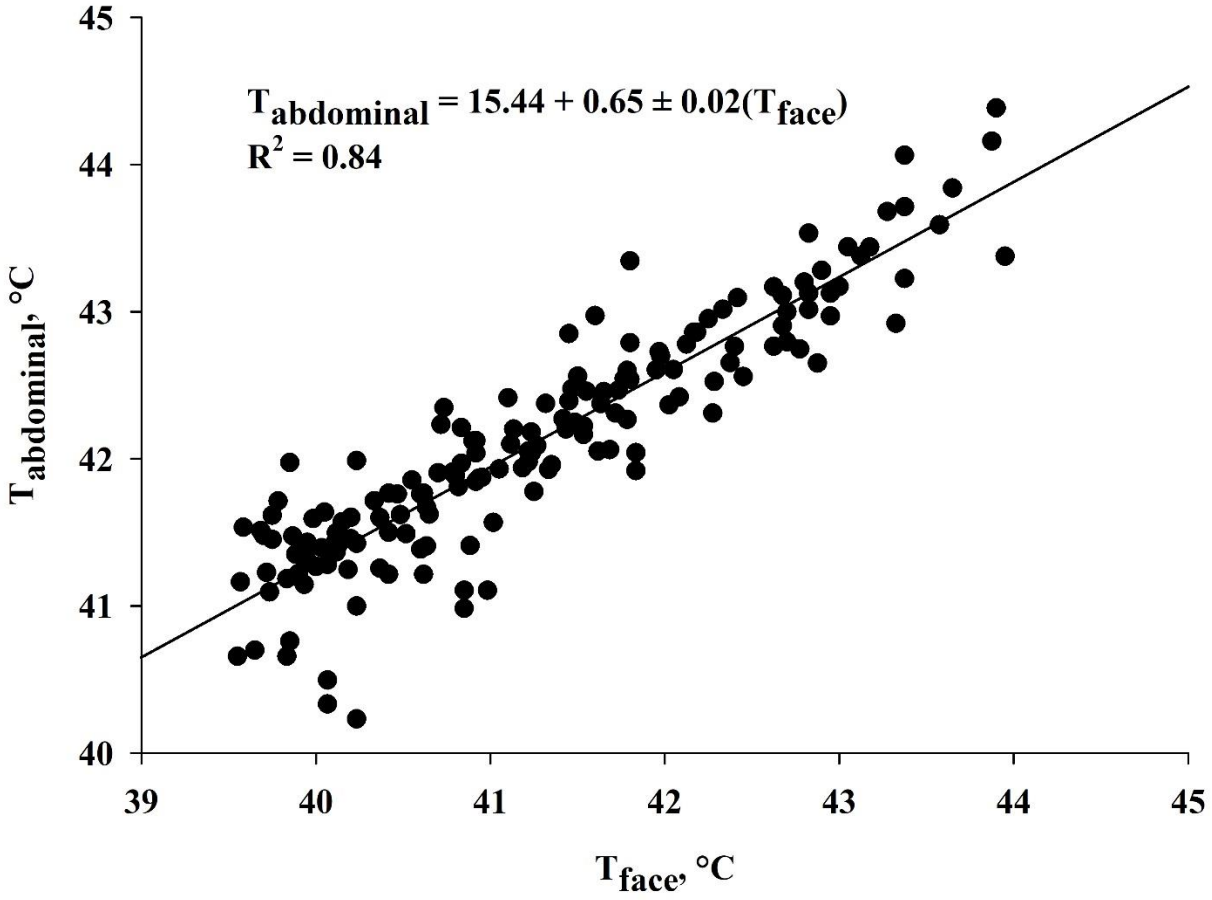

Figure 7.2. The overall relationship between internal body temperature ($T_{abdominal}$) and maximum surface face temperature (T_{face}) in 6 week old broiler chickens (n = 6 males and 6 females) in Exp 7.1. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN; 22.5 ± 0.12°C for 1 h), MILD (26.3 ± 0.12°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), and HOT (34.5 ± 0.15°C for 40 min) with 1 h of acclimation between each T_a period. Measurements of $T_{abdominal}$ and T_{face} were taken every 10 min during each T_a period each day. Each data point represents the average within each T_a period for each chicken at each day.

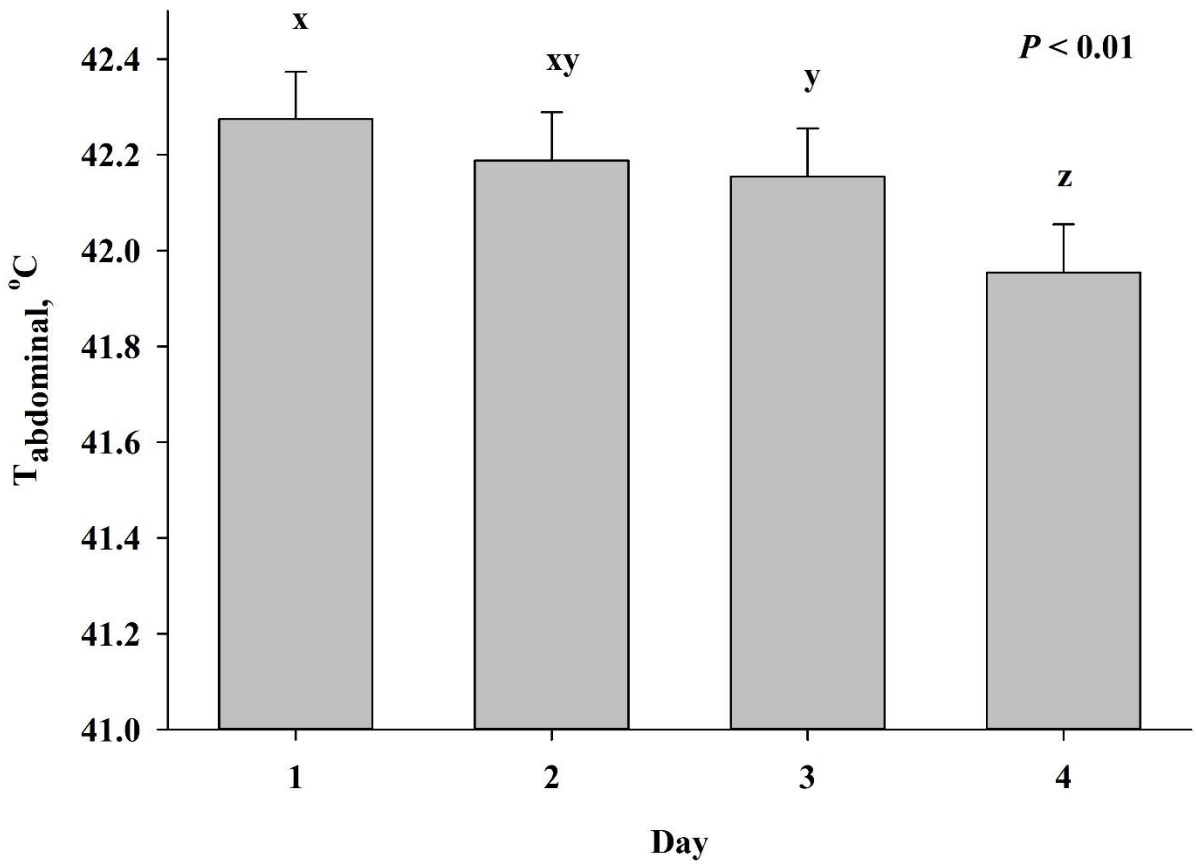

Figure 7.3. The effect of experimental day on internal body temperature ($T_{abdominal}$) in 6 week old broiler chickens (n = 6 males and 6 females) in Exp 7.1. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN; 22.5 ± 0.12°C for 1 h), MILD (26.3 ± 0.12°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), and HOT (34.5 ± 0.15°C for 40 min) with 1 h of acclimation between each T_a period. Measurements of $T_{abdominal}$ were taken every 10 min during each T_a period each day and averaged within each T_a period each day prior to analysis. Days lacking a common letter (x, y, z) differ ($P < 0.05$). Error bars indicate ± 1 SEM.

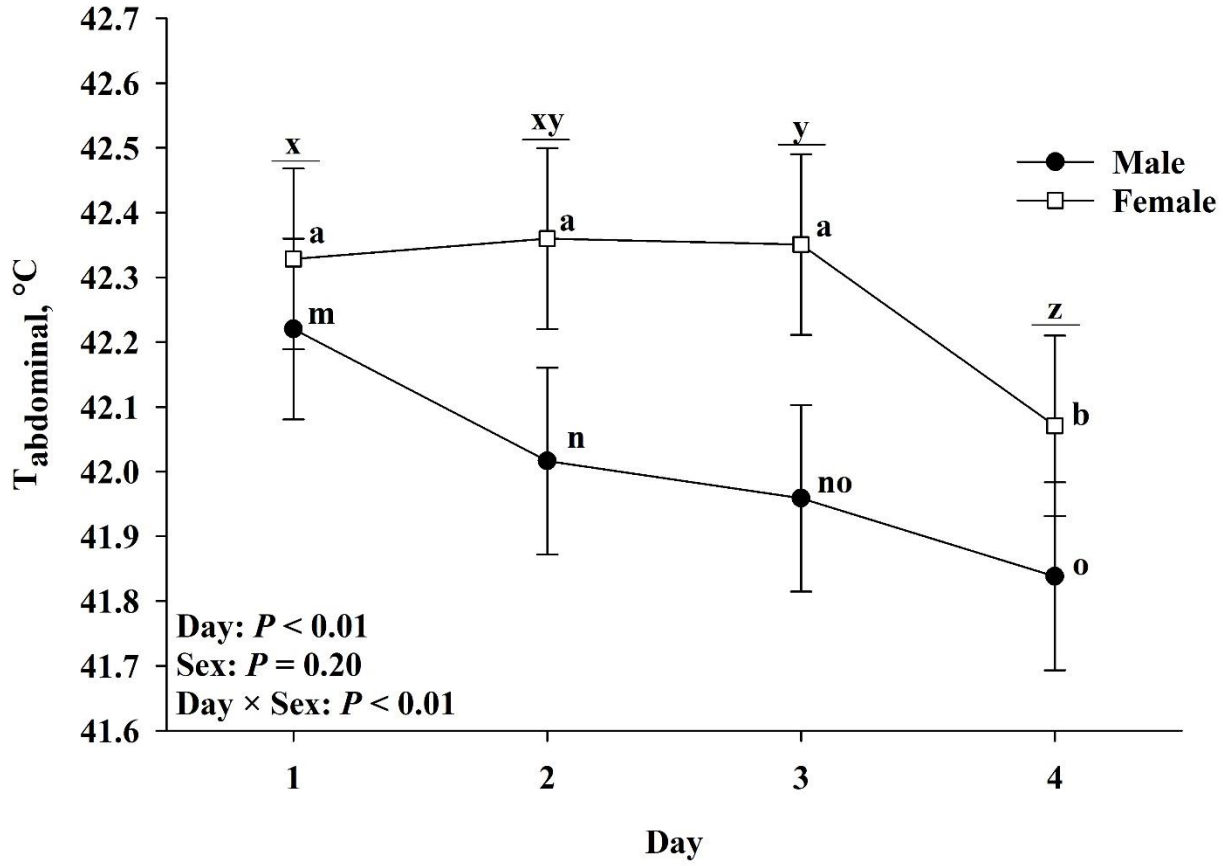

Figure 7.4. Internal body temperature ($T_{abdominal}$) over the 4 experimental days varied by sex of the chicken in Exp 7.1. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN; 22.5 ± 0.12°C for 1 h), MILD (26.3 ± 0.12°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), and HOT (34.5 ± 0.15°C for 40 min) with 1 h of acclimation between each T_a period. Measurements of $T_{abdominal}$ were taken every 10 min during each T_a period each day and averaged within each T_a period each day prior to analysis. Days lacking a common letter (x, y, z) differ ($P < 0.05$). Values within a sex lacking a common letter (a, b, for females; m, n, o, for males) differ ($P < 0.05$). Error bars indicate ± 1 SEM.

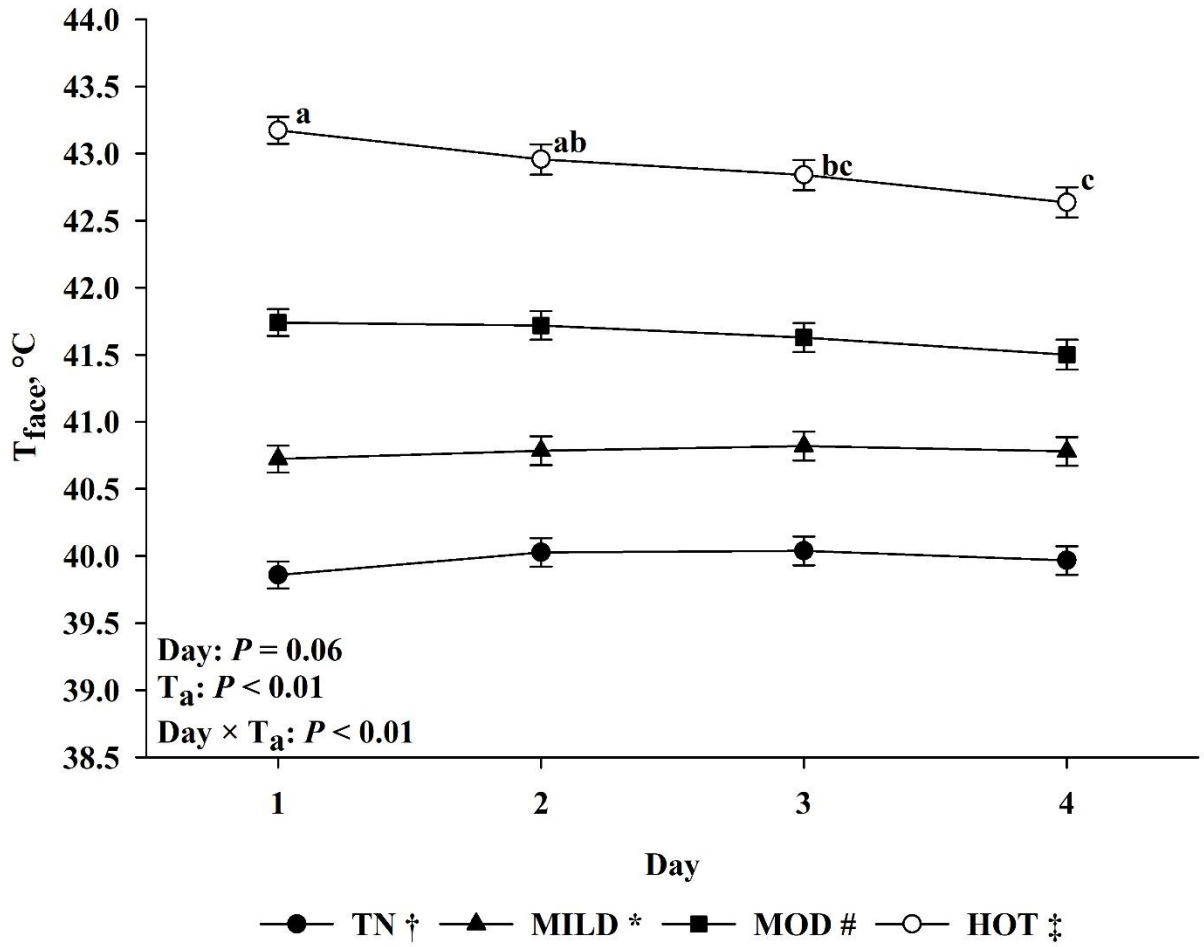

Figure 7.5. Facial surface temperature (T_{face}) of chickens was impacted by a day by ambient temperature (T_a) period interaction in Exp 7.1. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN; 22.5 ± 0.12°C for 1 h), MILD (26.3 ± 0.12°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), and HOT (34.5 ± 0.15°C for 40 min) with 1 h of acclimation between each T_a period. Measurements of T_{face} were taken every 10 min during each T_a period each day and averaged within each T_a period each day prior to analysis. Values within a T_a period lacking a common letter (a, b, c) differ ($P < 0.05$). Error bars indicate ± 1 SEM.

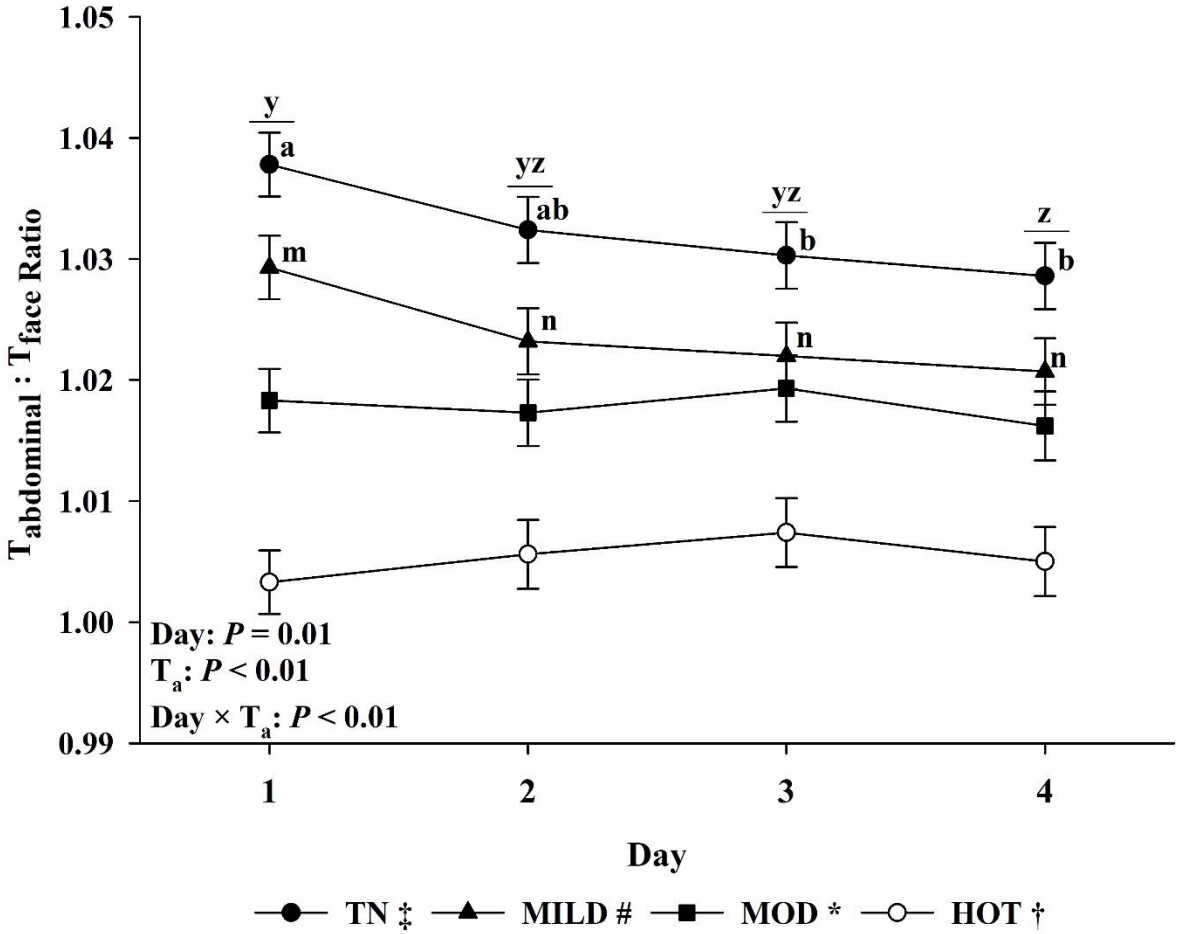

Figure 7.6. The ratio of internal body temperature ($T_{abdominal}$) to facial surface temperature (T_{face}) of chickens was impacted by a day by ambient temperature (T_a) period interaction in Exp 7.1. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN; 22.5 ± 0.12°C for 1 h), MILD (26.3 ± 0.12°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), and HOT (34.5 ± 0.15°C for 40 min) with 1 h of acclimation between each T_a period. Measurements of $T_{abdominal}$ and T_{face} were taken every 10 min during each T_a period each day and averaged within each T_a period each day prior to analysis. Days lacking a common letter (y, z) differ (P < 0.05). Values within a T_a period lacking a common letter (a, b, for TN; m, n, for MILD) differ (P < 0.05). Ambient temperature periods lacking a common symbol (‡ > # > * > †) differ (P < 0.05). Error bars indicate ± 1 SEM.

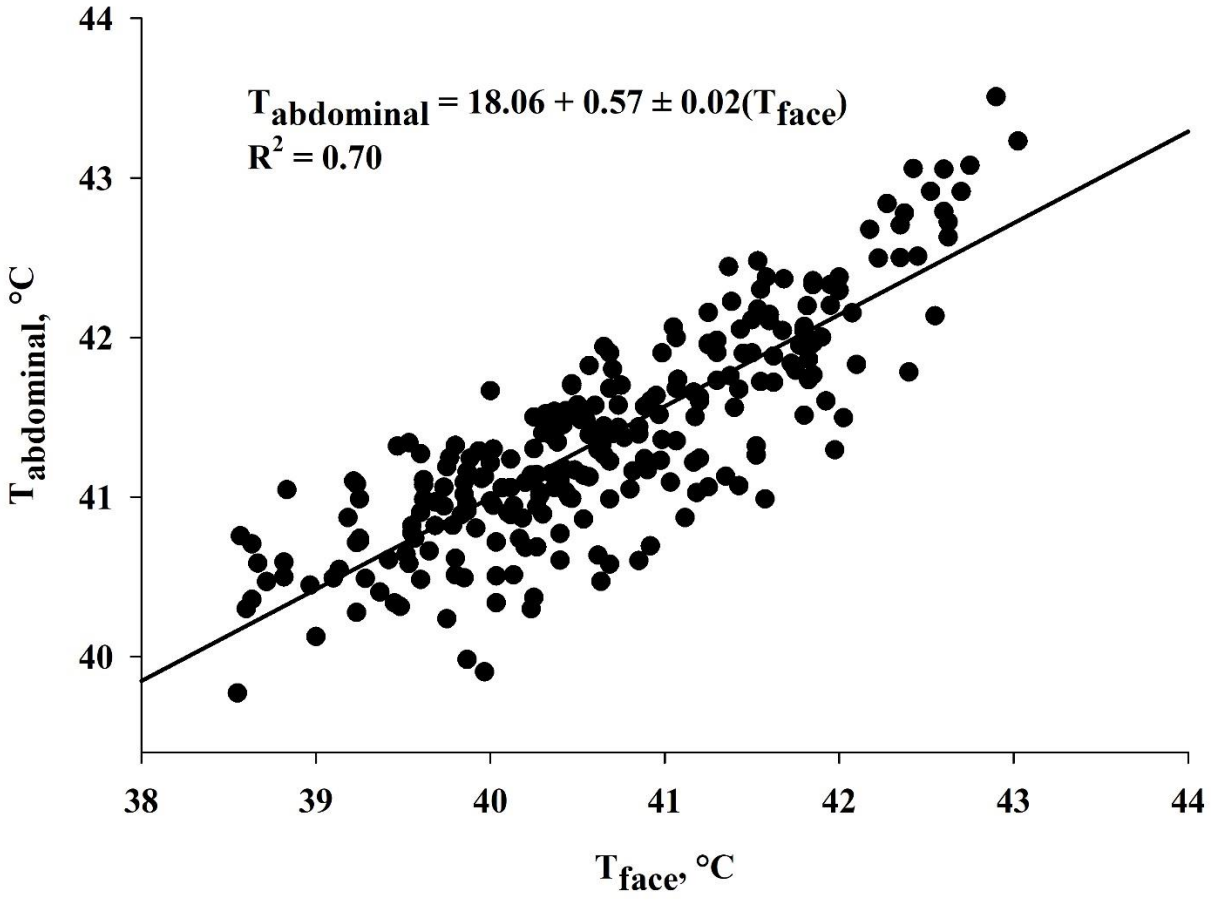

Figure 7.7. The overall relationship between internal body temperature ($T_{abdominal}$) and surface face temperature (T_{face}) in 6 week old broiler chickens (n = 8 males and 8 females) in Exp 7.2. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN; 22.0 ± 0.04°C for 1 h), MILD (26.6 ± 0.04°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), and HOT (35.0 ± 0.08°C for 40 min) with 1 h of acclimation between each T_a period. Measurements were taken every 10 min during each T_a period each day. Each data point represents the average within each T_a period for each chicken at each day.

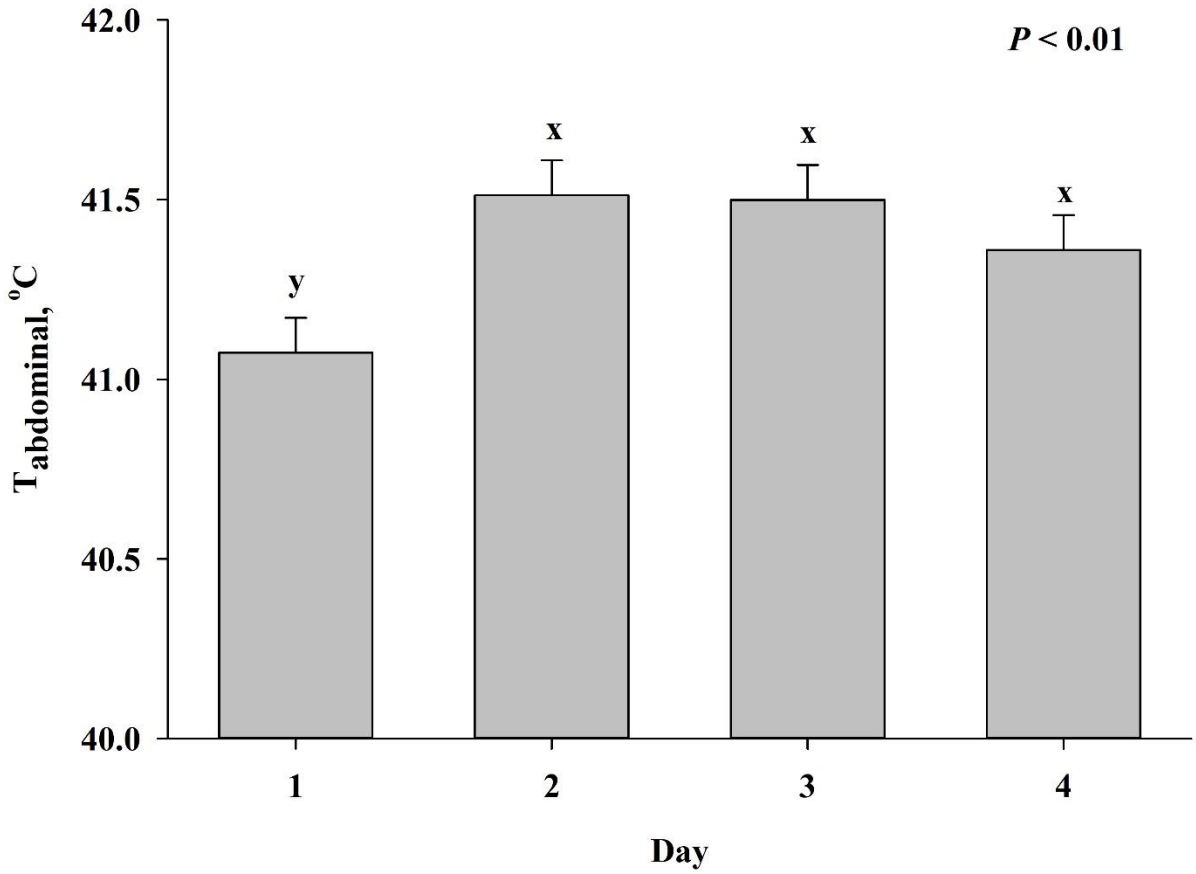

Figure 7.8. The effect of experimental day on internal body temperature ($T_{abdominal}$) in 6 week old broiler chickens (n = 8 males and 8 females) in Exp 7.2. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN; 22.0 ± 0.04°C for 1 h), MILD (26.6 ± 0.04°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), and HOT (35.0 ± 0.08°C for 40 min) with 1 h of acclimation between each T_a period. Measurements of $T_{abdominal}$ were taken every 10 min during each T_a period each day and averaged within each T_a period each day prior to analysis. Days lacking a common letter (x, y) differ ($P < 0.05$). Error bars indicate ± 1 SEM.

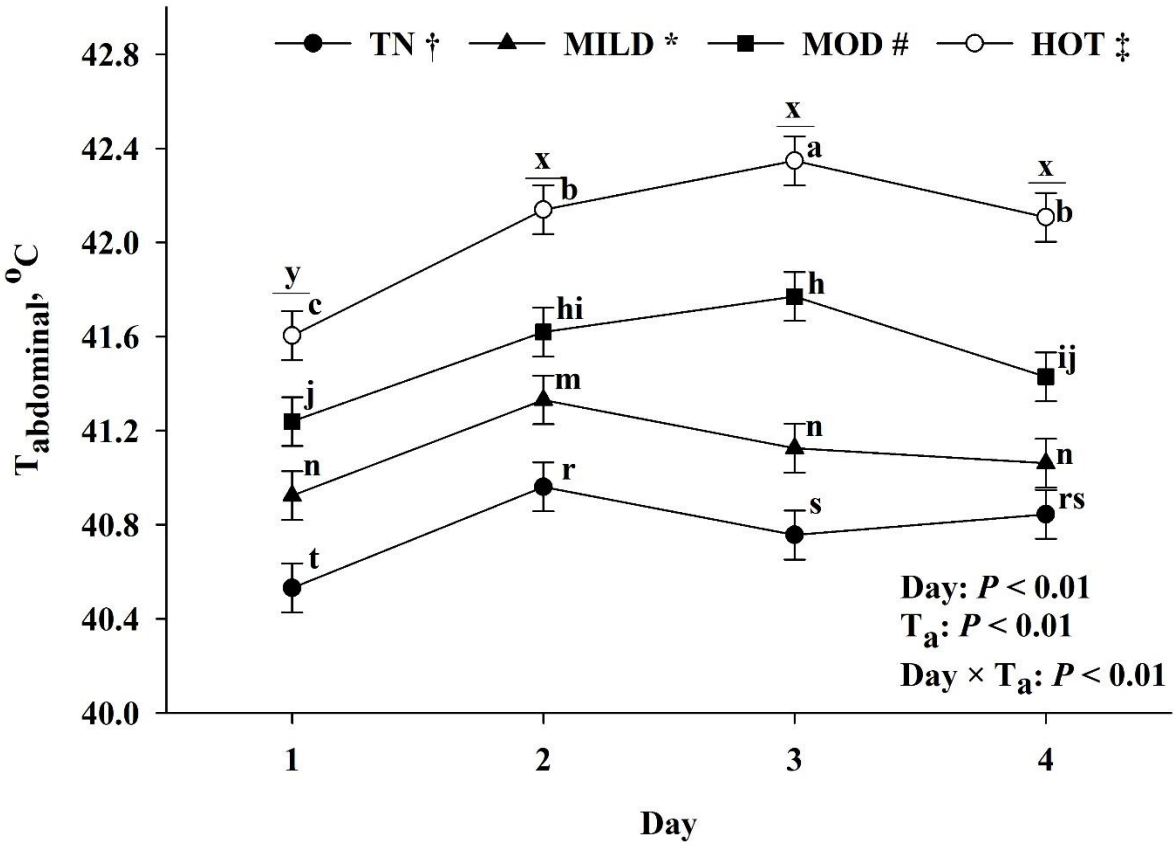

Figure 7.9. Internal body temperature ($T_{abdominal}$) was impacted by a day by ambient temperature (T_a) period interaction in 6 week old broiler chickens (n = 8 males and 8 females) in Exp 7.2. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN; 22.0 ± 0.04°C for 1 h), MILD (26.6 ± 0.04°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), and HOT (35.0 ± 0.08°C for 40 min) with 1 h of acclimation between each T_a period. Measurements of $T_{abdominal}$ were taken every 10 min during each T_a period each day and averaged within each T_a period each day prior to analysis. Days lacking a common letter (x, y) differ ($P < 0.05$). Values within a T_a period lacking a common letter (a, b, c, for HOT; h, i, j, for MOD, m, n, for MILD, and r, s, t, for TN) differ ($P < 0.05$). Ambient temperature periods lacking a common symbol (‡ > # > * > †) differ ($P < 0.05$). Error bars indicate ± 1 SEM.

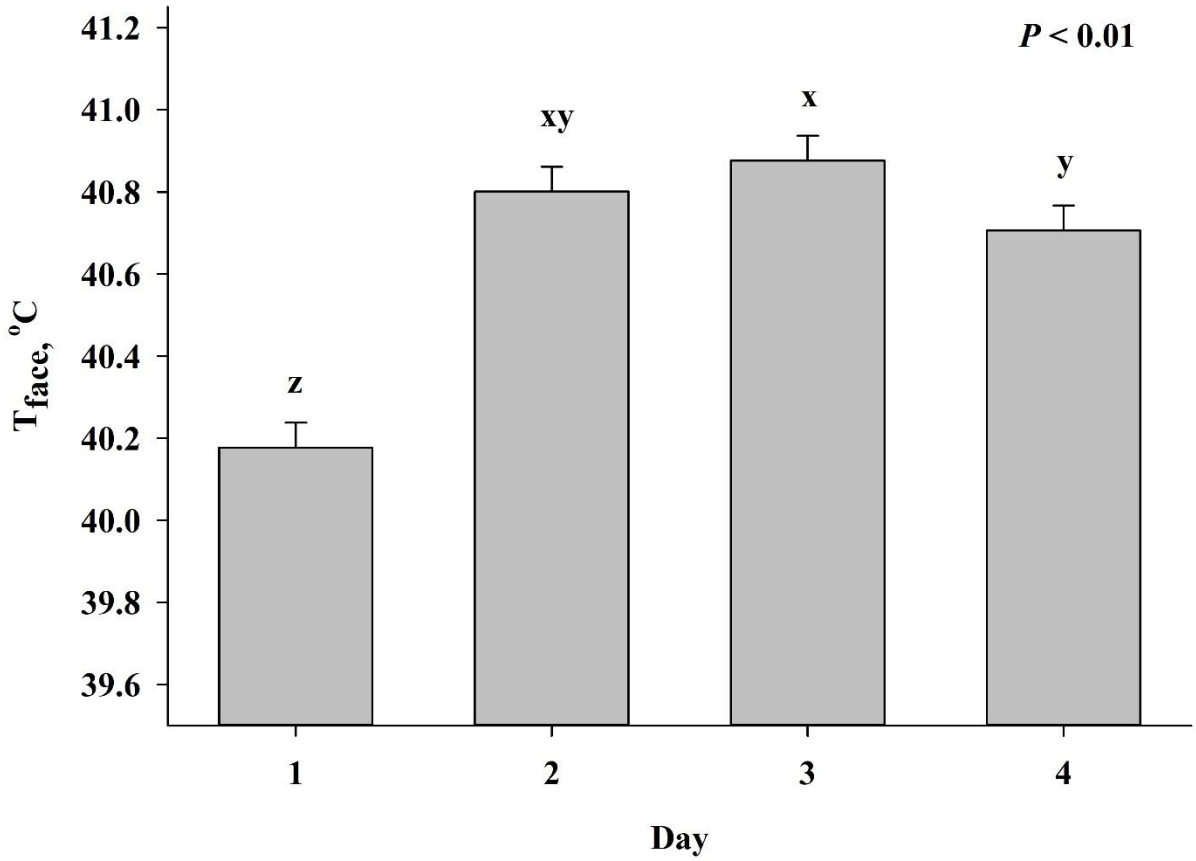

Figure 7.10. The effect of experimental day on maximum facial surface temperature (T_{face}) in 6 week old broiler chickens (n = 8 males and 8 females) in Exp 7.2. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN; 22.0 ± 0.04°C for 1 h), MILD (26.6 ± 0.04°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), and HOT (35.0 ± 0.08°C for 40 min) with 1 h of acclimation between each T_a period. Measurements of T_{face} were taken every 10 min during each T_a period each day and averaged within each T_a period each day prior to analysis. Days lacking a common letter (x, y, z) differ ($P < 0.05$). Error bars indicate ± 1 SEM.

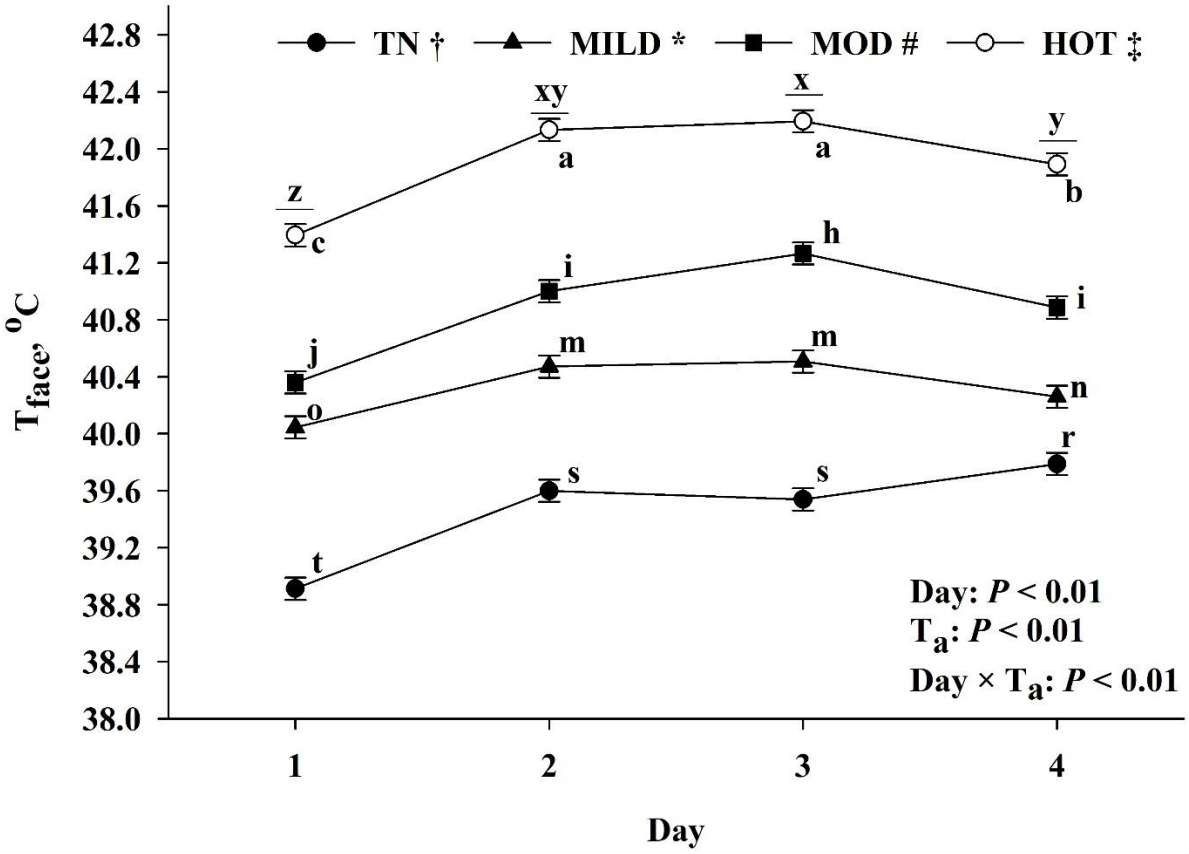

Figure 7.11. Maximum facial surface temperature (T_{face}) was impacted by a day by ambient temperature (T_a) period interaction in 6 week old broiler chickens (n = 8 males and 8 females) in Exp 7.2. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN; 22.0 ± 0.04°C for 1 h), MILD (26.6 ± 0.04°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), and HOT (35.0 ± 0.08°C for 40 min) with 1 h of acclimation between each T_a period. Measurements of T_{face} were taken every 10 min during each T_a period each day and averaged within each T_a period each day prior to analysis. Days lacking a common letter (x, y, z) differ ($P < 0.05$). Values within a T_a period lacking a common letter (a, b, c, for HOT; h, i, j, for MOD, m, n, o, for MILD, and r, s, t, for TN) differ ($P < 0.05$). Ambient temperature periods lacking a common symbol (‡ > # > * > †) differ ($P < 0.05$). Error bars indicate ± 1 SEM.

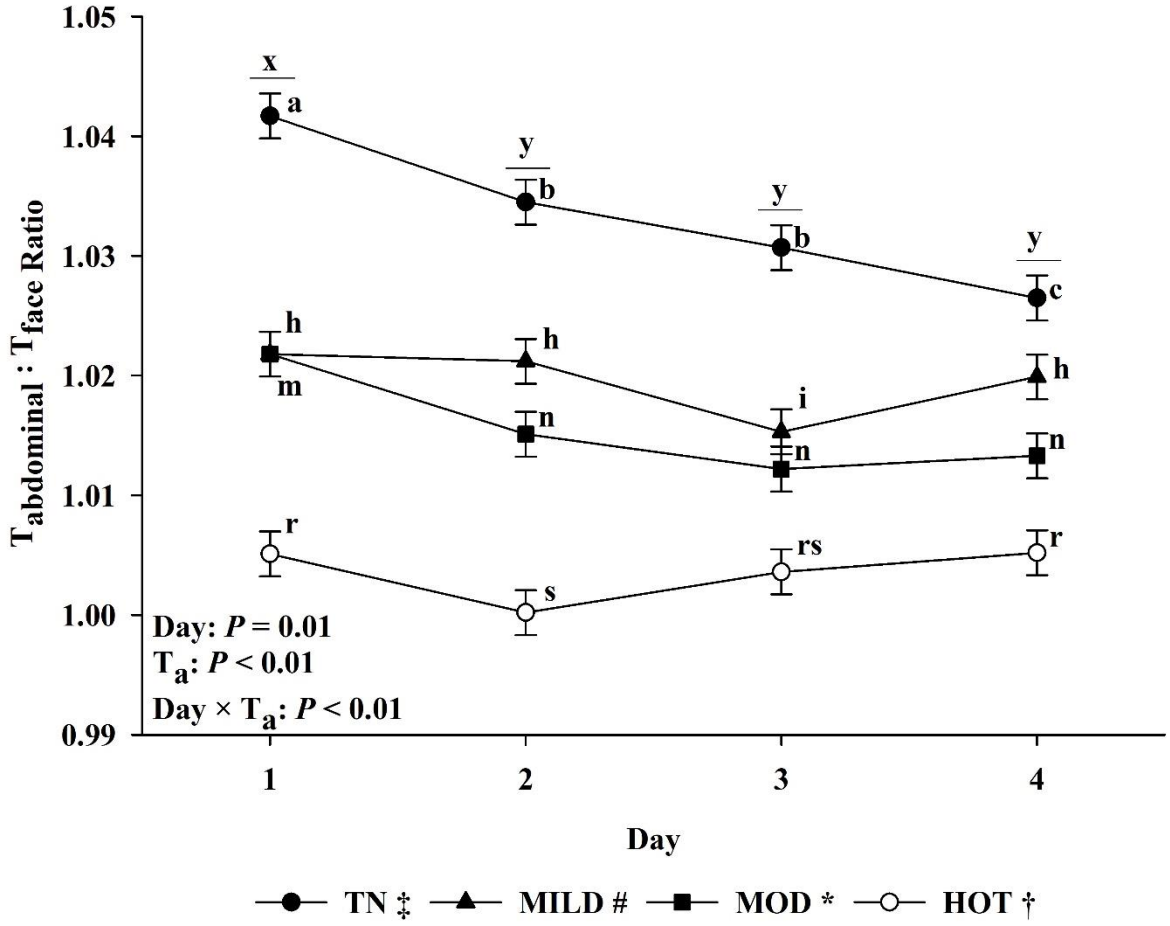

Figure 7.12. The ratio of internal body temperature ($T_{abdominal}$) to maximum facial surface temperature (T_{face}) was impacted by a day by ambient temperature (T_a) period interaction in 6 week old broiler chickens (n = 8 males and 8 females) in Exp 7.2. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN; 22.0 ± 0.04°C for 1 h), MILD (26.6 ± 0.04°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), and HOT (35.0 ± 0.08°C for 40 min) with 1 h of acclimation between each T_a period. Measurements of T_{face} were taken every 10 min during each T_a period each day and averaged within each T_a period each day prior to analysis. Days lacking a common letter (x, y) differ ($P < 0.05$). Values within a T_a period lacking a common letter (a, b, c, for TN; h, i, for MILD, m, n, for MOD, and r, s, for TN) differ ($P < 0.05$). Ambient temperature periods lacking a common symbol (‡ > # > * > †) differ ($P < 0.05$). Error bars indicate ± 1 SEM.

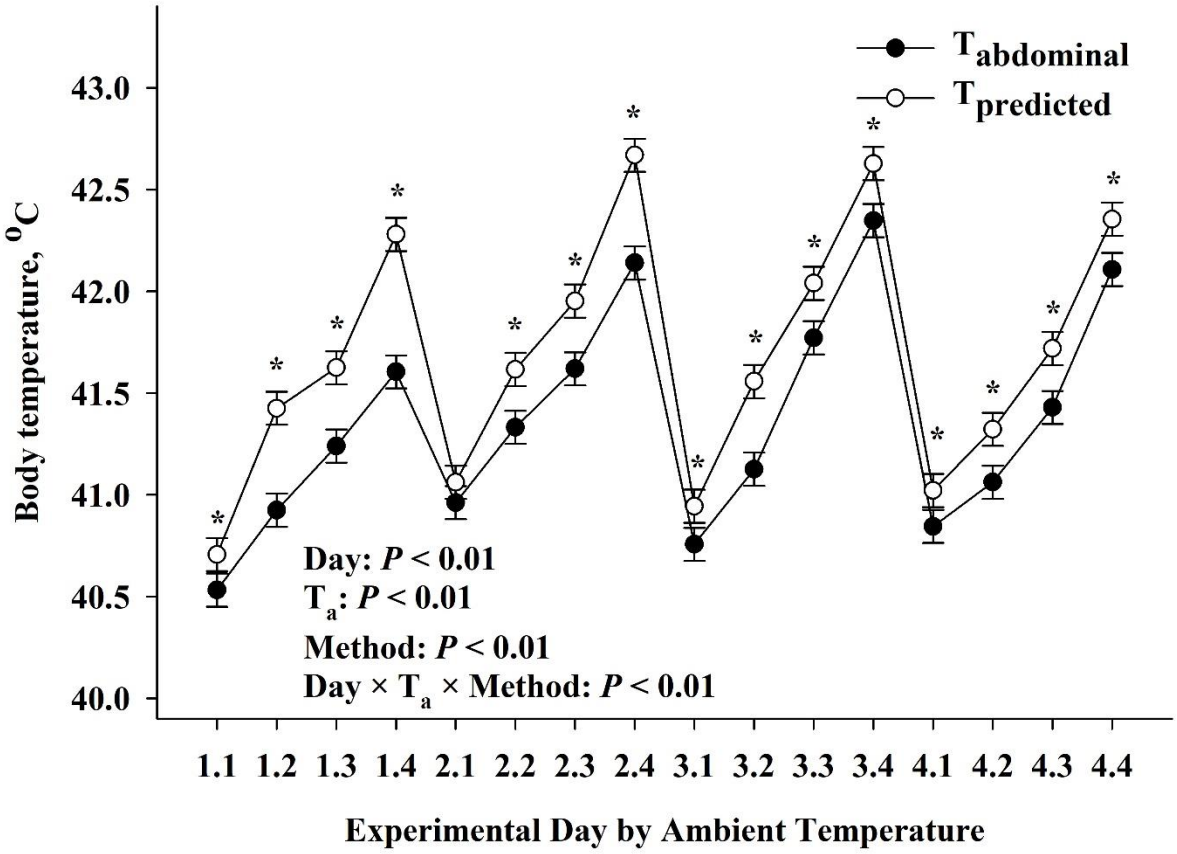

Figure 7.13. The interaction of experimental day, ambient temperature (T_a) period, and internal body temperature determination method (measured via implanted data loggers ($T_{abdominal}$) vs. predicted with an equation ($T_{predicted}$)) in in 6 week old broiler chickens (n = 8 males and 8 females) in Exp 7.2. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN(1); 22.0 ± 0.04°C for 1 h), MILD(2) (26.6 ± 0.04°C for 1 h), moderate (MOD(3); 30.1 ± 0.12°C for 1 h), and HOT(4) (35.0 ± 0.08°C for 40 min) with 1 h of acclimation between each T_a period. Measurements of $T_{abdominal}$ were taken every 10 min during each T_a period each day and averaged within each T_a period each day. Error bars indicate ± 1 SEM and a * indicates a difference ($P < 0.05$) between measurement methods at a given day by T_a period combination.

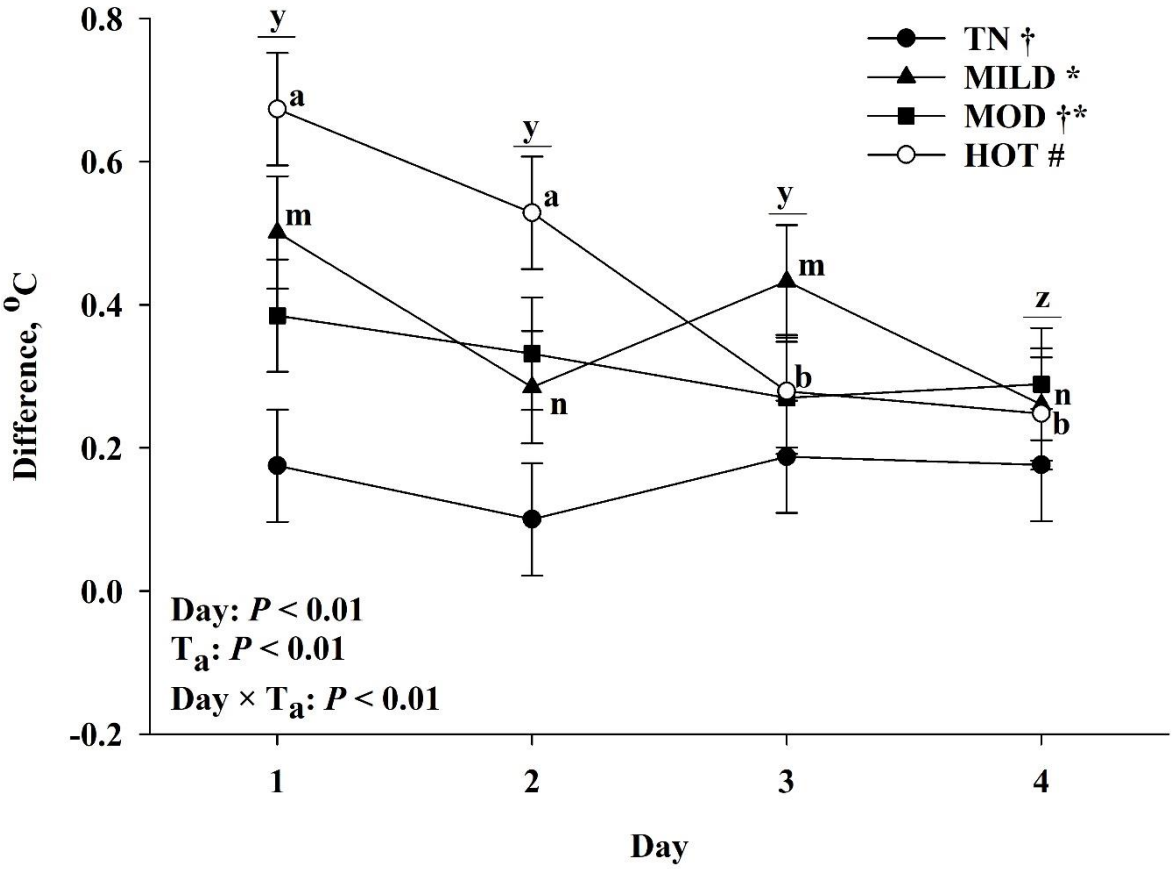

Figure 7.14. The difference ($T_{difference}$) of internal body temperature predicted from an equation ($T_{predicted}$) and internal body temperature determined by implanted data loggers ($T_{abdominal}$) was impacted by a day by ambient temperature (T_a) period interaction in 6 week old broiler chickens (n = 8 males and 8 females) in Exp 7.2. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN; 22.0 ± 0.04°C for 1 h), MILD (26.6 ± 0.04°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), and HOT (35.0 ± 0.08°C for 40 min) with 1 h of acclimation between each T_a period. Measurements of $T_{abdominal}$ were taken every 10 min during each T_a period each day and averaged within each T_a period each day prior to analysis. Days lacking a common letter (y, z) differ ($P < 0.05$). Values within a T_a period lacking a common letter (a, b, for HOT; m, n, for MILD) differ ($P < 0.05$). Ambient temperature periods lacking a common symbol (# > * > †) differ ($P < 0.05$). Error bars indicate ± 1 SEM.

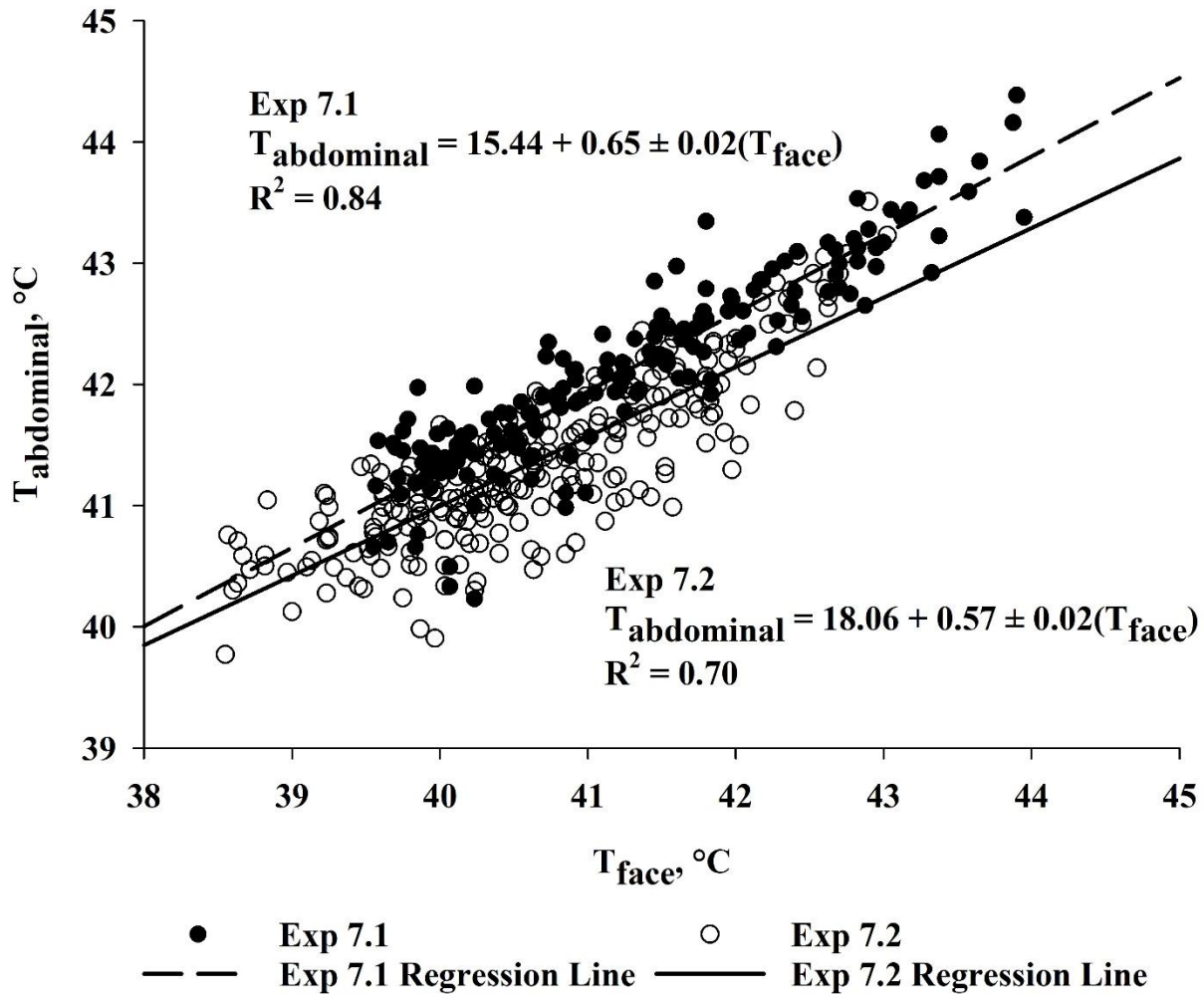

Figure 7.15. Comparison of the overall relationship between internal body temperature ($T_{abdominal}$) and maximum surface face temperature (T_{face}) in 6 week old broiler chickens in Exp 7.1 and Exp 7.2. Chickens were exposed each day for 4 days to a T_a cycle consisting of thermoneutral (TN; 22.3 ± 0.08°C for 1 h), MILD (26.5 ± 0.08°C for 1 h), moderate (MOD; 30.1 ± 0.12°C for 1 h), HOT (34.8 ± 0.12°C for 40 min) with 1 h of acclimation between each T_a period. Measurements of $T_{abdominal}$ and T_{face} were taken every 10 min during each T_a period each day. Each data point represents the average within each T_a period for each chicken on each day.

CHAPTER 8. FUTURE DIRECTIONS

8.1 Lactulose and Mannitol Test of Intestinal Health in Nursery Pigs

Aspirin has received attention in human health as a reproducible stimulus to increase intestinal permeability (Sequeira et al., 2012). Experiments 2.1 and 2.2 are the first known experiments to investigate the use of aspirin to induce intestinal permeability in weanling pigs. While Exp 2.2 produced some evidence that aspirin induced increased intestinal permeability at 15 mg/kg BW given 24 and 1 h before the test of intestinal permeability, results with 30 mg/kg BW of aspirin were less clear. As a result, additional research is required to elucidate the dose-dependent response of aspirin on intestinal permeability in weanling pigs. Selection of an optimal dose of aspirin to induce permeability in weanling pigs may allow a standardized model of intestinal permeability to be developed in pigs that could aid research in intestinal health in weanling pigs. Research to develop an aspirin model of intestinal permeability in slightly older pigs may reduce variation in urine output and therefore achieve clearer results than found in the present studies.

The temporal urinary excretion profile of lactulose and mannitol in studies of intestinal health is key to determining the optimal urine collection period (Sequeira et al., 2014a). Experiments 2.2 and 3.1 are the first known experiments to determine this excretion profile in pigs. While this is an important step in the progression of the development of a lactulose and mannitol test of intestinal health in the pig, additional research is required to determine the best urine collection period. While human lactulose and mannitol studies of intestinal health focus on terminating urine collection before the ingested dose of lactulose and mannitol reaches the large intestine to increase the accuracy of the test (Sequeira et al., 2014b), results from Exp 3.1 and Exp

5.1 using carefully timed urine collection periods indicate this may not be of primary concern in pigs. Given the large variation in quantitative voluntary urine collection from pigs in Exp 3.1 and Exp 5.1, it is likely that attempts to terminate urine collection before urinary excretion of lactulose and mannitol are complete leads to considerable variation in lactulose and mannitol excretion. Therefore, the loss of accuracy due to absorption of lactulose in the large intestine of pigs in longer urine collection periods may be minor compared to the loss of accuracy associated with shorter urine collection times. Use of re-entrant ileo-cecal cannulated pigs may be one way to study the impact of large intestinal absorption of lactulose to elucidate these trade-offs. Therefore, additional research is required to ascertain how to minimize extraneous sources of variation in the urinary output of lactulose and mannitol during the urine collection period in order for meaningful results to be obtained.

Published methods used in the lactulose and mannitol test of intestinal health in nursery pigs vary considerably, and typically are not supported by research in pigs but rather appear to stem from methods used in human literature. One factor that varies widely among published reports is the dose of lactulose and mannitol given to pigs, yet there is a dearth of research investigating the impacts of variation in dose given on excretion of lactulose and mannitol. Accordingly, Exp 3.1 investigated the impact of variation of lactulose and mannitol dose on lactulose and mannitol excretion patterns. Results were inconclusive with considerable pig-to-pig variation in lactulose and mannitol output. However, for experiments to be comparable, a standardized dose should be developed with the constant factors of a set dosed volume of liquid per kg of BW, a fixed osmolarity of solution that avoids extreme hyper-osmolarity (Bjarnason et al., 1995), and a constant g/kg BW dosage rate of lactulose and mannitol at a fixed ratio. A considerable amount of research is required before this standardized dose can be developed, but results from the lactulose

and mannitol test of intestinal health will be of unknown accuracy and repeatability until a standardized dose specific to the physiology of the nursery pig is developed.

8.2 Measurement or Estimation of Physiological Heat Stress in Broiler Chickens

Research to understand the physiology of heat stress in chickens as well as to develop and evaluate mitigation strategies are hampered by the limited methods to accurately obtain detailed internal temperature data in chickens. The simple surgical method presented in Chapter 6 to implant data loggers to measure internal body temperature represents a substantial step forward with the potential for unparalleled generation of detailed internal body temperature data in chickens. However, surgical methods are invasive, require some specialized knowledge, and may not be a viable option for some research groups due to institutional animal care and use restrictions. In addition, the surgical method is not practical for commercial producers, leaving measurement of ambient temperature and relative humidity as primary metrics of physiological heat stress in production settings. Therefore, the use of a prediction equation, as generated in Exp 7.1, to estimate internal body temperature from easily measured predictor variables is advantageous in some settings. While surface temperature of the face of broilers was shown to account for a substantial portion of the variation in internal body temperatures of broilers in Exp 7.1 and 7.2 and some published research (Giloh et al., 2012), a prediction equation with the inputs of facial surface temperature, sex of the bird, and number of days of heat stress exposure lacked the desired accuracy in Chapter 7. Therefore, future directions would be to refine the prediction equation to account for the impacts of other variables, such as air flow velocity and relative humidity, in addition to those investigated in Exp 7.1 and Exp 7.2.

8.3 Conclusions

Animal scientists grapple with the responsibility to feed the growing human population nutritious, safe, and affordable animal protein products in an economical manner without compromising the welfare of farmed animals. This is a considerable challenge, yet technological advances hold the keys to success. Development and adoption of superior methods to measure physiological stress in farm animals will aid in development of mitigation strategies to reduce the impact of negative and excessive stressors. These strategies will enable the food animal industry to meet the challenge of feeding the world in an efficient manner while maintaining high standards of animal wellbeing.

8.4 Literature Cited

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