# THE EFFECT OF THERMAL ENVIRONMENT ON *SALMONELLA* SHEDDING IN FINISHING PIGS

By

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## ABSTRACT

## THE EFFECT OF THERMAL ENVIRONMENT ON *SALMONELLA* SHEDDING IN FINISHING PIGS

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*Salmonella* species are one of the major causes of foodborne diseases in the US and worldwide. The objectives of this dissertation were to describe the shedding pattern of *Salmonella* in feces of naturally infected finishing pigs, to compare direct q–PCR detection of *Salmonella* in swine feces to the microbiological culture, to quantify the fecal concentration of *Salmonella* in naturally infected pigs, to evaluate the association between the environmental thermal parameters in the barn and *Salmonella* shedding in finishing pigs, and to estimate the proportion of total model variance attributable to cohort, pig and individual sample level effects when predicting *Salmonella* shedding in swine.

A 3 year longitudinal study was conducted on 3 sites of a multi-site farrow–to–finish production system. Individual fecal samples from 900 finishing pigs (8 collections per pig) were repeatedly collected from 18 cohorts (50 pigs per cohort). Fecal samples were collected every 2 weeks for 16 weeks. *Salmonella* was cultured from 453 (6.6%) of 6836 fecal samples. Individual fecal samples (positive (n=443), negative (n=1225) determined by microbiological culture) were submitted for q–PCR. Pen temperature and humidity were measured every 2 minutes during the study period. The thermal parameters of interest were: hourly average, minimum and maximum lagged temperatures, hourly temperature variation, temperature humidity index (THI) and cumulative number of hours/degree above and below the thermal of neutral zone at the pen level prior to fecal sampling for 6 time periods (12h, 24h, 48h, 72h, 1

week and 1 month). The pig–level incidence of *Salmonella* was 20.8% (187/899 pigs). *Salmonella* prevalence varied between both sites and cohorts within sites. The proportion of positive samples decreased over the finishing period from 12.9% to 2.8%. Intermittent detection of *Salmonella* was found in more than 50% of pigs that were positive at more than one collection. The finding that the majority of pigs shed intermittently has implications for surveillance and research study design when determining *Salmonella* status.

For culture positive samples, 15.4% (68/443) were detected by q–PCR, but only 3.4% (15/443) were within the q–PCR quantifiable range ($\geq 10^3$ CFU/g of feces). Of these latter samples, the concentration range was $1.06x10^3$–$1.73x10^6$ CFU/g feces. When high shedding was detected it was clustered within a single pig and its pen–mates. Direct q–PCR may be an alternative to traditional culture–dependent methods for detection of pigs with high fecal concentrations of *Salmonella*, but not for detection of pigs shedding low concentrations.

Multilevel logistic models using generalized linear models, with random intercepts at pig, pen and cohort levels to account for clustering were constructed. The outcome variable was *Salmonella* fecal status of the individual sample. Cold exposure (temperatures below the thermal neutral zone) and exposure to a THI >72 were both associated with risk of *Salmonella* shedding. Nursery *Salmonella* status, site, pig age and cohort mortality rate were also associated with *Salmonella* shedding. The largest proportion of model variance was associated with the individual fecal sample (44.7%) followed by cohort (24.1%) and pen (20.7%). Interventions that target the thermal environment may have an effect on reducing *Salmonella* shedding in swine and also improve pig well–being and production efficiency. Alternatively, thermal parameters may be used to identify groups of pigs at high risk for *Salmonella* shedding.

## DEDICATION

This thesis is dedicated to my brother, Jose A. A. Pires, who has been my ‘life coach’, for always being there for me and for his encouragements.

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## ABBREVIATIONS

| | |
|---|---|
| ACTH | Adrenocorticotropic hormone |
| AIAO | All–in All–out |
| AUC | Area under the curve |
| BP | base pairs |
| BPW | Buffered peptone water |
| CFU | Colony-forming unit |
| CNS | Central nervous system |
| Cq | Quantification cycle |
| DCPAH | Diagnostic Center for Population and Animal Health |
| ELISA | Enzyme-linked immunosorvent assay |
| ETEC | Enterotoxigenic *E .coli* |
| FSIS | Food Safety and Inspection Service |
| GN | Gram–negative |
| IQC | Integrated quality control |
| LCT | Lower critical temperature |
| LPS | Lipopolysaccharide |
| MPN | Most probably number |
| MRSV | Modified semisolid Rappaport–Vassiliadis |
| NAHMS | National Animal Health Monitoring System |
| OD | Optical density |
| PCR | Polymerase Chain Reaction |

| | |
|---|---|
| PRRSV | Porcine Reproductive and Respiratory Syndrome Virus |
| PRCV | Porcine Respiratory Coronavirus |
| QMRA | Quantitative microbial risk assessment |
| q–PCR | Quantitative real-time polymerase chain reaction |
| RH | Relative humidity |
| ROC | Receiver–operating characteristic curve |
| RV | Rappaport–Vassiliadis broth |
| SOP | Standard operating procedure |
| S/P | Proportion of absorbance between the sample and a positive control |
| SPF | Specific pathogen free program |
| $T_{db}$ | Dry bulb temperature |
| $T_{dp}$ | Dew point temperature |
| $T_{wb}$ | Wet bulb temperature |
| THI | Temperature humidity index |
| TNZ | Thermo neutral zone |
| TTB | Tetrathionate broth |
| UCT | Upper critical temperature |
| XTL4 | Xylose–Lysine–Tergitol–4 |
| ZAP | Zoonosis Action Plan |

# INTRODUCTION

## INTRODUCTION

Foodborne pathogens cause an estimated 9.4 million foodborne illnesses, 55,961 hospitalizations and 1,351deaths each year in the United States (Scallan et al., 2011). It has been well documented that *Salmonella* species are one of the major causes of foodborne diseases in the US and worldwide (Greig and Ravel, 2009; Anon, 2011; Henao et al., 2011; Scallan et al., 2011). In the US alone, it is estimated that 1,027 million nontyphoidal *Salmonella* human infections result in 19,336 hospitalizations and 378 deaths annually (Scallan et al., 2011) and costs $ 365 billion in direct medical expenditures annually (Anon, 2011). *Salmonella* is still one the most important bacteriological zoonotic hazards transmissible from pork to consumers (Fosse et al., 2009). A significant number of human cases of salmonellosis (1% to 25%) have been related to consumption of pork and pork products (Berends et al., 1998; Hald et al., 2006; Ravel et al., 2009; EFSA, 2010; Guo et al., 2011). Reduction of the *Salmonella* contamination of pork and pork products requires interventions at three stages: pre-harvest (farm), harvest (slaughter) and post-harvest (distribution systems and consumer) (Lo Fo Wong et al., 2002; Boyen et al., 2008). In the US there are two types of surveillance programs for *Salmonella* in swine, one at the slaughterhouse by the Food Safety and Inspection Service (FSIS) (sampling carcasses) and the other by the National Animal Health Monitoring System (NAHMS) (sampling on farm) (Bush et al., 2002; USDA-FSIS, 2010). No national *Salmonella* control program in swine production has been adopted in contrast to several European countries (Hautekiet et al., 2008; Abrahantes et al., 2009; Baptista et al., 2010; Snary et al., 2010; Merle et al., 2011). Therefore the strategies to reduce *Salmonella* at the farm are dependent on the individual producers' practices. Identification of effective control measures at the farm level might have better acceptance by swine producers if those measures have an impact on pig health and production as

well as food safety outcomes. In order to put in place on-farm control and intervention measures it is crucial to understand *Salmonella* infection dynamics in swine and identify risk factors which might be a target for interventions at the farm.

The dynamics of *Salmonella* infection in pigs and farms is complex. In last 20 years, a large body of literature has been published about cross-sectional studies which investigated mainly *Salmonella* prevalence and herd risk factors in swine. A limited number have assessed the fecal prevalence over time, with longitudinal studies showing high variability in *Salmonella* shedding at the farm, cohort and individual animal levels (Funk et al., 2001b; Beloeil et al., 2003; Kranker et al., 2003; Nollet et al., 2005; Rajic et al., 2005; Farzan et al., 2008; Dorr et al., 2009; Rostagno et al., 2012). However, few studies have investigated sources of variability of *Salmonella* prevalence in swine as well risk factors associated with each level of variability (farm, cohort, pen, pig and individual sample) (Funk et al., 2001a; Funk et al., 2007; Poljak et al., 2008). Organizational levels that explain the greatest amount of variation are considered the best for targeting interventions (Dohoo et al., 2001; Funk et al., 2007). The variability of the prevalence reported in those studies might be associated with factors related to introduction and maintenance of salmonellae in the herd environment; that is, transmission between pigs (or within herd) and persistence of *Salmonella* in the individual pig (Zheng et al., 2007; Hautekiet et al., 2008). In order to understand the transmission and persistence in the host and environment, one critical component to investigate is the shedding patterns and bacterial load in naturally infected pigs. A limited number of studies have quantified *Salmonella* concentration in the feces of naturally infected swine. These were either cross-sectional studies (Fravalo et al., 2003; Fablet et al., 2006; van Hoek et al., 2012) or estimates of pen-contamination in lairage (O'Connor et al., 2006; Boughton et al., 2007). Therefore, little is known regarding *Salmonella* concentration

shed by pigs and how it changes over time. Identification of quantification methods that allow enumeration of *Salmonella* in a large number of samples in an efficient time-cost and automated way (Malorny et al., 2008; Elizaquivel et al., 2011; Löfström et al., 2011) are warranted. Enumeration of bacterial load can be used to identify contamination pressure and to identify effective control measures to reduce contamination in swine herds (Fravalo et al., 2003). In addition, data are needed for quantitative microbial risk assessments and for modeling transmission patterns of *Salmonella* (Bollaerts et al., 2009; Lanzas et al., 2011).

Seasonal patterns of foodborne diseases have been observed in temperate climates. For example, human illness caused by *Salmonella spp* rises in summer and decreases in the winter (Naumova et al., 2007). Seasonal variation of those foodborne diseases has been related with oscillations of several environmental factors (Naumova et al., 2007). Among those environmental factors, high ambient temperature has been consistently associated with human salmonellosis worldwide (Bentham and Langford, 2001; D'Souza et al., 2004; Fleury et al., 2006; Naumova et al., 2007). Overall, the association between the mean and highest temperature several weeks prior the onset of the human cases suggest that the temperature might affect the *Salmonella* dynamics at the farm level. Those effects might be to either create an environment favorable for the proliferation of bacteria in the environment and consequently increase the bacterial pressure and exposure to the livestock animals, or increase animal susceptibility to new infections or cause recurrence of existing infections. Unlike the human salmonellosis reports, there is no agreement regarding seasonal patterns of *Salmonella* prevalence/shedding in swine. On one hand some studies reported no seasonality (Benschop et al., 2008; Baptista et al., 2010) while others reported higher prevalence during different seasons, either by higher seroprevalence in winter and fall (Carstensen and Christensen, 1998; Christensen and Rudemo, 1998; Hald and

Andersen, 2001; Smith et al., 2010) or summer (Hautekiet et al., 2008) and higher fecal prevalence in winter and spring (Funk et al., 2001a). Season is characterized by environmental changes of temperature, humidity, precipitation and wind (Dowell, 2001; Naumova, 2006). Environmental factors such as temperature, rainfall, and sunshine have been associated with *Salmonella* prevalence in swine. Finishing pigs exposed to wide variations in daily high temperature were at greater risk of high *Salmonella* prevalence (Funk et al., 2001a). In addition, large differences in long-term averages in the monthly mean temperature, as well as high actual rainfall and hours of sunshine were associated with higher *Salmonella* seroprevalence in UK pigs (Smith et al., 2010). In both studies the environmental parameters were retrieved from the closest weather station. Therefore, the environmental parameters might not reflect the environment in the barns. Moreover, herds that had a controlled programmed temperature above the upper critical values of thermal neutral zone (TNZ) had a higher seroprevalence compared with herds with controlled programmed temperature within the TNZ (Hautekiet et al., 2008). A limitation of all of these studies is that they focused on investigation of risk factors at the herd level and were cross-sectional study designs. Nevertheless, these studies suggest that sub-optimal temperature and temperature variability appear to be an important factors associated with *Salmonella* infection in swine (Funk et al., 2001a; Funk and Gebreyes, 2004; Hautekiet et al., 2008; Smith et al., 2010). There is a lack of knowledge of risk factors at the pig-level and time-dependent risk factors, namely environmental thermal parameters within the barn and the association with *Salmonella* dynamics. Longitudinal studies with repeated sampling on farm, cohorts, and pigs are needed to investigate time − variant risk factors, such as environmental temperature (Funk et al., 2001b; Poljak et al., 2008). Interventions that target the thermal environment may have an effect on reducing the *Salmonella* shedding in swine and also improve

pig well – being and production efficiency. Identification of interventions for control of *Salmonella* in swine and simultaneously improve production performance increases the probability of adoption by swine producers. Moreover, thermal parameters may be used to identify groups of pigs at high risk for *Salmonella* shedding, which might require interventions either in pre-harvest or during harvest to reduce the risk of *Salmonella* contamination of swine carcasses.

The studies described in this thesis are components of a longitudinal study conducted on a multi-site farrow-to-finish production system located in the Midwestern U.S., from June of 2008 to August of 2011. The main goal of the study was to investigate the association between exposure to sub-optimal thermal parameters and *Salmonella* shedding in finishing pigs. The underlying hypothesis of this study is: There is an association between sub – optimal thermal parameters in the barn and *Salmonella* shedding in finishing pigs. The specific research objectives of this thesis are: 1) To describe the shedding pattern of *Salmonella* in feces of naturally infected finishing pigs; 2) To compare direct q-PCR detection of *Salmonella* in swine feces to the gold standard of microbiological culture; 3) To quantify the fecal concentration of *Salmonella* in naturally infected pigs; 4) To evaluate the association between the environmental thermal parameters in the barn and *Salmonella* shedding in finishing pigs; 5) To estimate the proportion of total model variance attributable to cohort, pig and individual sample level effects when predicting the *Salmonella* shedding in swine. This thesis is organized in the following four chapters: 1) Chapter 1, a literature review, which describes background information related to *Salmonella* in swine and provides justification for the study; 2) Chapter 2, a longitudinal study of *Salmonella* shedding in naturally infected finishing pigs, which describes *Salmonella* apparent prevalence in various units of observation (site, cohort, pig age and individual fecal sample) and

describes the *Salmonella* shedding pattern in feces of naturally infected finishing pigs; 3) Chapter 3, a study regarding the use of direct quantitative real-time PCR for enumeration of *Salmonella* in feces of naturally infected pigs; 4) Chapter 4, a multilevel analysis to evaluate the association between environmental thermal parameters and *Salmonella* shedding in finishing pigs. Chapters 2 – 4 in this thesis were each written and formatted as independent papers intended for publication in scientific journals, and as such some repetition is inevitable.

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# CHAPTER 1

## Literature Review

## *SALMONELLA* TAXONOMY

*Salmonella* was first isolated from pigs by Salmon and Smith, in 1886 and named *Salmonella* Choleraesuis because it was thought to be the cause of hog cholera (Fedorka-Cray et al., 2000). *Salmonella* is a genus of bacteria belonging to the family Enterobacteriaceae. The bacteria belonging to this genus are gram-negative straight rods, generally motile, facultative anaerobes, grow on nutrient agar, ferment glucose, often produce gas and oxidase negative (Grimont et al., 2000).

The genus *Salmonella* includes two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is divided in 6 subspecies: *S. enterica* supsp. *enterica* (I); *S. enterica* subsp. *salamae* (II); *S. enterica* subsp. *arizonae* (IIa), *S. enterica* subsp. *diarizonae* (IIIb); *S. enterica* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* (VI). *S. enterica* subsp. *enterica* affects warm-blooded animals, whereas the other 5 species are found in cold – blooded animals and in the environment (Brenner et al., 2000; Popoff et al., 2001; CDC, 2008). There are more than 2,500 serovars of *Salmonella enterica* identified to date. The serotypes are defined by the existence of somatic antigens (O, sugar and protein coats on the bacterial surface), flagellar antigens (H, flagellar proteins), and surface (Vi) antigens (Brenner et al., 2000; Grimont et al., 2000; Callaway et al., 2008; CDC, 2008). The majority of serovars belong to *S.enterica* susp. *enterica* (59%); among these the most common O antigen groups are A, B, C1, C2, D and E. These six O antigen groups are responsible for approximately 99% of human and warm-blooded animal infections (Brenner et al., 2000). Some serovars can infect a wide range of hosts while others are host-specific or host-restricted. For instance, *S.* Choleraesuis, a swine-specific serovar, is generally associated with severe systemic disease in swine. Other serovars, such as *S.* Typhimuirium and *S.* Enteritidis, can infect a broad range of unrelated hosts, including humans

(Fedorka-Cray et al., 2000; Barrow et al., 2010). Among the 2,500 *Salmonella enterica* serovars, only a few have been isolated from swine (Brenner et al., 2000; Boyen et al., 2008; Callaway et al., 2008). On other hand, the most common serovars isolated in swine (Bush et al., 2002; USDA-APHIS, 2009) are common to those found in human cases. Those serovars are *S.* Typhimurium, *S.* Heidelberg, *S.* Agona, and *S.* Infantis (Foley et al., 2008; Anon, 2010).

Salmonellae can be found in mammals, birds, reptiles, insects, rodents and environmental niches such as water, food, soil and contaminated environments. The natural habitat of *Salmonella* is considered the digestive tract of warm and cold-blooded animals. The presence of *Salmonella* in the environment results from contamination with fecal material of infected animals. The bacteria are ubiquitous, can multiply over a wide temperature range (7°C –45°C), and can persist for months to years in the environment (Murray, 2000; Griffith et al., 2006).

## IMPORTANCE OF *SALMONELLA* IN PUBLIC HEALTH

Foodborne pathogens cause an estimated 9.4 million foodborne illnesses, 55,961 hospitalizations and 1,351deaths each year in the United States (Scallan et al., 2011). Although the actual costs of foodborne disease in the US are not fully known, in 1996 it was estimated that costs stemming from medical costs and productivity losses ranged between $6.5 to $34.9 billion (based on a total population of 250 million) (Buzby and Roberts, 1996 ). *Salmonella*, *Campylobacte*r, *Listeria*, shiga toxin-producing *Escherichia coli, Shigella, Clostridium, Vibrio* and *Yersinia* are enteric pathogens commonly transmitted through food (Greig and Ravel, 2009; Vugia et al., 2009; Anon, 2011).

It has been well documented that *Salmonella* species are one of the major causes of foodborne diseases in the US and worldwide (Greig and Ravel, 2009; Anon, 2011; Henao et al., 2011; Scallan et al., 2011). In the US alone, it is estimated that 1,027 million nontyphoidal *Salmonella* human infections result in 19,336 hospitalizations and 378 deaths annually (Scallan et al., 2011) and cost $ 365 billion in direct medical expenditures annually (Anon, 2011). *Salmonella* typically causes mild-to moderate self-limited gastroenteritis, but serious disease resulting in death can also occur (Trevejo et al., 2003; Voetsch et al., 2004). Hospitalization and death rates are higher among young children, the elderly, immune compromised patients, males and certain ethnic groups (Trevejo et al., 2003; Cummings et al., 2010). Despite the Pathogen Reduction: Hazard Analysis and Critical Control Point (PRA, HACCP) program implemented in 1996 in US slaughterhouses to reduce *Salmonella* at harvest, the included measures have had little impact on the human incidence rate (Rose et al., 2002; Davies, 2011). The incidence of human salmonellosis in the US has not declined over the past 15 years; in fact, cases have increased about 10% in 2010 when compared with 2006 – 2008. The incidence rate in 2010 was not significantly different than the incidence prior to implementation of the HACCP Pathogen Reduction Act (Anon, 2011).

A wide range of *Salmonella* serovars have been isolated from human cases (CDC, 2008). According to the FoodNet's 2010 report, the three most common serovars representing a majority of the human salmonellosis infections (92%) included: *Salmonella enterica* Enteritidis (22%), Newport (14%) and Typhimurium (13%) (Anon, 2011). Those serovars are common to humans and livestock species (meat and poultry products). Livestock species are considered the reservoir of many serovars that can infect humans (Clothier et al., 2010; Anon, 2011). However, making the link between livestock species and human illness is not simple.

The attribution of foodborne human illness to specific sources is complex and traditionally relies on microbiological approaches, epidemiological approaches, intervention studies and/or expert opinion (Pires et al., 2009). The link between the cases in humans and infections in livestock or to specific food sources is not straightforward (Batz et al., 2005; Pires et al., 2009). A majority of the studies attribute human salmonellosis to consumption of contaminated food such as meat, eggs and fresh produce. Several studies have been done to estimate food or commodity attribution for human salmonellosis in North America and Europe (Greig and Ravel, 2009; Ravel et al., 2009; Guo et al., 2011). The most common sources of human salmonellosis outbreaks in Europe were eggs (32%) and meat and poultry meat products (15%) (Pires et al., 2010). Forty-two percent of cases have no known source (Pires et al., 2010). In the US, an estimate of relative contribution to domestically acquired sporadic human *Salmonella* infections was highest for chicken (48%) and ground beef (28%), followed by turkey (17%), egg products (6%), intact beef (1%) and pork (less than 1%) (Guo et al., 2011). In contrast, produce had the highest contribution (29%) to human salmonellosis in Canadian outbreaks, follow by poultry (15%), and meat other than poultry, pork and beef (15%) (Ravel et al., 2009).

*Salmonella* is still one of the most important bacteriological zoonotic hazards transmissible from pork to consumers (Fosse et al., 2008, 2009; Fosse et al., 2011). Despite pork being estimated to have a very low attribution rate for foodborne cases compared to other food vehicles (in the US), statistical models have predicted that every year approximately 100,000 human cases of salmonellosis associated with the consumption of pork resulting economic costs of about $ 80 million in the US (Miller et al., 2005). Contaminated pork is still considered an important hazard to public health (Boyen et al., 2008). In the US, *Salmonella* has been isolated in pork and pork products at the slaughterhouse (Carlson and Blaha, 2001; Bahnson et al., 2006a;

Gebreyes et al., 2006) and in retail pork (Duffy et al., 2001; Mollenkopf et al., 2011). Reduction of the *Salmonella* contamination of pork and pork products requires interventions at three levels: pre − harvest (farm), harvest (slaughter) and post − harvest (distribution systems and consumer) (Lo Fo Wong et al., 2002; Boyen et al., 2008). A stochastic cost-effectiveness study for controlling *Salmonella* in the pork production chain showed that interventions in the finishing and slaughtering stages are most cost-effective in reducing the prevalence of contaminated carcasses (van der Gaag et al., 2004).

## PATHOGENESIS OF *SALMONELLA* IN SWINE

### Clinical and subclinical syndromes

*Salmonella* infection in swine is mainly subclinical as pigs can be asymptomatic carriers. Swine can be infected by host-adapted serovars such as *S.* Choleraesuis var. Kunzendorf, and broad host-range serovars such as *S.* Typhimurium. Two clinical forms can be observed in swine: septicemia caused by *S.* Choleraesuis, and enterocolitis caused by *S.* Typhimurium (Reed et al., 1986; Fedorka-Cray et al., 2000; Barrow et al., 2010). Although other serovars can cause clinical disease, the top four serovars recovered from clinical cases submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) in 2003 and 2008 were: Typhimurium var. 5-, Choleraesuis var. Kunzendorf , Derby, Typhimurium and Heidelberg (Clothier et al., 2010). An observed decline of isolation of Choleraesuis var. Kunzendorf, and increased isolation of Typhimurium in US veterinary laboratories has been reported (Foley et al., 2008; Clothier et al., 2010).

Pigs can be infected with *Salmonella* at any age, but clinical cases due to *S.* Typhimurium are more common at 6 to 12 weeks old, while *S.* Choleraesuis var. Kunzendorf, can cause clinical disease at a wider range of ages (Fedorka-Cray et al., 2000). Clinical signs of the septicemic syndrome include: fever, depression, respiratory signs, cyanotic extremities and death. In the case of septicemic outbreaks the mortality is high and morbidity is in generally low. *Salmonella* enterocolitis is characterized by diarrhea, lethargy and fever. In this clinical presentation, morbidity can be high and mortality is low (Reed et al., 1986; Fedorka-Cray et al., 2000).

**Sources of infection**

In general, the major sources of infection are other infected pigs, since they are the main reservoir and environments are focally contaminated by pigs (Berends et al., 1996; Murray, 2000; Funk and Gebreyes, 2004; Griffith et al., 2006). The source of infection of host-adapted serovars (Choleraesuis var. Kunzendorf) is mostly due to infected pigs and environments contaminated by pigs. For other non-specific serovars (*S.* Derby, Typhimurium, Agona, etc), a diverse range of environments, vectors and fomites have been suggested as potential sources of infection (Berends et al., 1996; Murray, 2000; Funk and Gebreyes, 2004; Griffith et al., 2006). Vectors such as flies, rodents, cats, birds, and wild animals are potential vehicles and sources of *Salmonella* (Letellier et al., 1999; Barber et al., 2002; Funk and Gebreyes, 2004). In addition, *Salmonella* has been isolated from diverse fomites in barns such as dust, pens, floors, boots, feeders and waters (Berends et al., 1996; Letellier et al., 1999; Barber et al., 2002; Rajic et al., 2005). A contaminated environment poses a risk for long periods, because the organism can

remain viable and infective in several environments for more than 13 months (Murray, 2000; Gray and Fedorka-Cray, 2001; Griffith et al., 2006). Feed has also been identified as a source and risk factor (Berends et al., 1996; Funk and Gebreyes, 2004; Griffith et al., 2006). *Salmonella* has been isolated from feed at the farm, feed mill and from feed transportation vehicles in several studies, but not always was the same serovar identified in the feed and in the pigs consuming the feed (Berends et al., 1996; Funk and Gebreyes, 2004).

**Transmission, dose and serovars**

Transmission of *Salmonella* in swine can occur via direct contact (pig–to–pig, dam–to–offspring), or through indirect contact with vectors, fomites (Letellier et al., 1999; Barber et al., 2002; Rajic et al., 2005) or contaminated environments (contaminated environment–to–pig) (Fedorka-Cray et al., 1994; Loynachan and Harris, 2005; Griffith et al., 2006; Osterberg et al., 2010). The transmission occurs within a short period of time after pigs have been exposed to a contaminated environment (Fedorka-Cray et al., 1994; Hurd et al., 2001a). Pigs can become infected 30 minutes after exposure to a contaminated slurry with a minimum concentration of $10^3$ CFU (colony forming units) (Hurd et al., 2001a). The contact with a contaminated environment of least $10^3$ salmonellae per gram of feces leads to an acute infection of both alimentary and non-alimentary tissues (Hurd et al., 2001a; Loynachan and Harris, 2005).

The route of transmission of *Salmonella* between pigs is mainly feco − oral, but other routes such as nose–to–nose or airborne can occur (Fedorka-Cray et al., 1994; Fedorka-Cray et al., 1995; Proux et al., 2001; Oliveira et al., 2006; Oliveira et al., 2007). Pigs that were orally

challenged with $10^{10}$ CFU of *S.* Typhimurium show clinical signs after 48 h with shedding of *Salmonellae* that can infect other pigs either by commingling or contact with contaminated fecal material (Fedorka-Cray et al., 1994). Alternative routes of transmission have been tested experimentally. *Salmonella* Typhimurium was isolated in several gut and gut related tissues after intranasal challenge in esophagotomized pigs (Fedorka-Cray et al., 1995). The upper respiratory tract and lungs can be important sites for transmission and invasion of *Salmonella* (Fedorka-Cray et al., 1995; Proux et al., 2001; Oliveira et al., 2006; Oliveira et al., 2007). Airborne transmission is possible at short distances but may depend on the serovar (Oliveira et al., 2006; Oliveira et al., 2007).

The infectious dose of *Salmonella* is variable and dependent on transmission route and serovar. The majority of the experimental studies have used high doses of salmonellae, ranging from $10^{6}$ to $10^{10}$ CFU *S.* Typhimurium (Wood et al., 1989; Wood and Rose, 1992; Fedorka-Cray et al., 1994; Fedorka-Cray et al., 1995; Osterberg and Wallgren, 2008; Scherer et al., 2008) and $10^{7}$ to $10^{8}$ CFU *S.* Choleraesuis (Gray et al., 1995; Gray et al., 1996a; Anderson et al., 2000). There is a dose dependency for *Salmonella* to be infectious. Doses greater than $10^{3}$ CFU of *S.* Choleraesuis or *S.* Typhimurium are necessary to be able to induce acute *Salmonella* infection and fecal detection (Gray et al., 1996b; Loynachan and Harris, 2005; Osterberg and Wallgren, 2008). In addition, the challenge dose affects the length of fecal shedding and the persistence in tissues (Gray et al., 1996b; Osterberg and Wallgren, 2008; Osterberg et al., 2009). Pigs infected with higher doses shed for longer periods. Moreover, higher doses are required in order to lead to clinical disease and to create long-term carriers (Gray et al., 1996b; Osterberg

and Wallgren, 2008; Osterberg et al., 2009). The transmission of *Salmonella* might be serovar-dependent (van Winsen et al., 2001; Osterberg et al., 2010). For instance, the transmission between seeders (infected pigs) and sentinels (naïve pigs) of *S.* Goldcoast and *S.* Panama occur at a lower rate when compared with *S.* Typhimurium and *S.* Livingstone when inoculated with similar challenge (van Winsen et al., 2001).

*Salmonella* can be isolated in alimentary and non-alimentary tissues. The dissemination of *Salmonella* in pigs occurs rapidly after per–os or intranasal exposure. Several *Salmonella* serovars were isolated in tissues 3 hours after intranasal inoculation (Loynachan et al., 2004; Loynachan and Harris, 2005) and in 30 minutes after exposure to contaminated slurry (Hurd et al., 2001a). In terms of alimentary tissues the most common tissues from which *Salmonella* are isolated post–challenge are: tonsils, illeum, jejunum and cecum (Wood et al., 1989; Wood and Rose, 1992; Fedorka-Cray et al., 1994; Gray et al., 1995; Loynachan et al., 2004; Loynachan and Harris, 2005). The ileocecal/ileocolic and manibular lymph nodes and lungs are the most common non – alimentary tissues (Wood et al., 1989; Wood and Rose, 1992; Fedorka-Cray et al., 1994; Gray et al., 1995; Loynachan et al., 2004; Loynachan and Harris, 2005). In the initial stages of infection, tonsils are an important site (primary site) for colonization. *Salmonella* can persist in tonsils for a long period after the exposure (Wood et al., 1989; Wood and Rose, 1992; Fedorka-Cray et al., 1994; Fedorka-Cray et al., 1995). Isolation of *Salmonella* in cecal contents or rectal swabs is frequent in all stages post- infection. Experimentally infected pigs shed high levels of *Salmonella* in feces within a short period post-infection, declining with time. The duration of shedding has been reported to be as short as seven days and as long as 28 weeks (Wood and Rose, 1992; Scherer et al., 2008).

**Carriers and intermittent shedders**

Infection with *S.* Choleraesuis (Gray et al., 1995) or *S.* Typhimurium can result in long−term subclinical carriers (12 weeks to 28 weeks post−infection) (Wood et al., 1989; Wood and Rose, 1992; Fedorka-Cray et al., 1994; Scherer et al., 2008). The carrier status can persist up to 28 weeks in pigs orally challenged with *S.* Typhimurium (Wood et al., 1989). Persistently infected pigs have relatively low concentrations of *Salmonella* either in intestinal contents or lymph nodes (Wood and Rose, 1992). Weaned pigs orally challenged with *S.* Typhimurium can shed as high as $10^6$ CFU/g feces in the first 7 days post−infection. After 60 days post−infection the shedding rate decreased to levels less than 10 CFU /g and remaines intermittent during a five month study period (Scherer et al., 2008). In addition to a low concentration of bacteria shed, pigs also can become intermittent shedders with time (Osterberg and Wallgren, 2008; Scherer et al., 2008). Intermittent shedding has been observed in experimental infections with several different serovars, such as: *S.* Choleraesuis; *S.* Typhimurium; *S.* Derby, *S.* Cubana; *S.* Yoruba (Nielsen et al., 1995; Gray et al., 1996b; Osterberg and Wallgren, 2008; Osterberg et al., 2009), as well in observational studies (Beloeil et al., 2003; Kranker et al., 2003; Nollet et al., 2005a). Intermittent shedders are important not only at the farm as a source of transmission to pen mates (Kranker et al., 2003), during transportation to the slaughterhouse a reactivation of shedding when pigs are exposed to the stress of transportation and lairage can occur (Berends et al., 1996; Larsen et al., 2003; Nollet et al., 2005a).

**Seroconversion**

The host response to *Salmonella* involves both humoral (Gray et al., 1996b; Holt, 2000; Osterberg and Wallgren, 2008; Scherer et al., 2008; Osterberg et al., 2009) and cellular immune responses (Lumsden and Wilkie, 1992; Stabel et al., 1993; Gray et al., 1996b). Two types of humoral immunity are observed: mucosal and serological. Mucosal immunity includes secretion of IgA and the humoral production of IgM and IgG (Holt, 2000). The serological (antibody) response to *Salmonella* varies by the challenge dose, serovar, and time post–infection (Nielsen et al., 1995; Gray et al., 1996b; Osterberg and Wallgren, 2008; Scherer et al., 2008; Osterberg et al., 2009). In experimental studies, the onset of the serological response and peak seroprevalence occur approximately 7 to 14 days and 30 to 45 days post–infection, respectively (Nielsen et al., 1995; Gray et al., 1996b; Osterberg and Wallgren, 2008; Scherer et al., 2008; Osterberg et al., 2009). The IgM antibody response to the lipopolysaccharide (LPS) antigen of *S.* Choleraesuis surges in early post–infection, and disappears or remains at low titers after 7 weeks (Gray et al., 1996b). Conversely, serum IgG antibody to LPS antigen of *S.* Choleraesuis was detected later and remained at higher titers for longer periods up to 15 weeks post-infection (Gray et al., 1996b). Seroconversion is observed in a majority of the pigs experimentally infected with *S.* Derby and *S.* Typhimurium within 14–45 days post–infection (Osterberg and Wallgren, 2008; Scherer et al., 2008; Osterberg et al., 2009). However, lower titers or no detectable seroconversion was observed when pigs were exposed to lower doses of $10^3$ to $10^6$ (Osterberg and Wallgren, 2008; Osterberg et al., 2009). Anti-*Salmonella* Typhimurium IgG titers remained high for periods lasting longer than four months post–infection (Scherer et al., 2008). The presence of serum antibodies can be representative of exposure and not necessarily current

infection or active shedding. Pigs maintained high antibody titers even after being culture negative for *Salmonella* in fecal samples (Scherer et al., 2008). Pigs challenged with certain serovars do not seroconvert or also show low titers for a short period of time. Feed–related serovars showed less capacity for infection or invasion, and some do not induce seroconversion (Osterberg and Wallgren, 2008; Osterberg et al., 2009). For example, there was no seroconversion when pigs were challenged with the feed–related serovar *S*. Cubana (Osterberg et al., 2009). In naturally infected pigs, longer time periods between the peak prevalence in culture, the onset of serological response, and peak seroprevalence was observed (Kranker et al., 2003; Merialdi et al., 2008). The serological response was observed 30 days later than the peak of *Salmonella* shedding (around 90 days of age) (Kranker et al., 2003); that is the peak of seroprevalence was observed between 120 to 270 days of age (Kranker et al., 2003; Merialdi et al., 2008). The observed difference between experimental and observational studies under natural conditions can be explained as pigs can be infected at different points in time and also can be re–infected (Kranker et al., 2003).

## DIAGNOSIS OF SALMONELLA IN SWINE

The diagnostic tests available for pre–harvest *Salmonella* detection and prevalence estimation are based on: 1) culture of *Salmonella* in feces (Funk et al., 2001b; Wilkins et al., 2010b), 2) identification of *Salmonella* DNA (e.g. Polymerase Chain Reactions (PCR) techniques) (Wilkins et al., 2010b) and 3) detection of anti–*Salmonella* antibodies by serology (Alban et al., 2002; Baptista et al., 2009). At harvest, culture of carcass swabs (Gebreyes et al., 2006; Baptista et al., 2010b), lymph nodes (Carlson and Blaha, 2001; Hurd et al., 2004) and fecal

contents have been used (Hurd et al., 2004). The detection of antibodies in meat juice from the diaphragm has been used to assess *Salmonella* antibody concentration (O'Connor et al., 2006b; McKean and O'Connor, 2009; Baptista et al., 2010b).

**Serology**

Mix-ELISA assays targeting anti-LPS antibodies have been developed to detect antibodies to *Salmonella* serogroups O: 1,4,5,6,7,12 (Nielsen et al., 1995; Nielsen et al., 1998) and the serogroups O: 1,3,5,6,7,8,9,10,12 (Proux et al., 2000) in serum and muscle. Surveillance programs in swine based on serological analysis have been implemented in Denmark (Alban et al., 2002; Baptista et al., 2009; Baptista et al., 2010b), Germany (Merle et al., 2011), Belgium (Hautekiet et al., 2008) and the UK (Abrahantes et al., 2009; Snary et al., 2010). These serologic tests are applied either in serum from blood or meat juice. The diagnosis of *Salmonella* using the mix-Elisa assays are based on certain optical density (OD) cut-offs (e.g. 20%) (Denmark) (Alban et al., 2002; Baptista et al., 2009; Baptista et al., 2010b) or based on S/P ratio (a measure of relative proportion of absorbance between the sample and a positive control) (UK) (Abrahantes et al., 2009; Snary et al., 2010). Serological tests rely on the presence of antibodies and represent historical exposure to the bacterium. Serological testing of herds has been used to classify the herd at different risk levels in order to put in place reduction measures at both farm and slaughter (Alban et al., 2002; Baptista et al., 2009; Baptista et al., 2010b). Although a good correlation between the serological and bacteriological classification of herd is observed (Lo Fo Wong et al., 2004b; Rajic et al., 2007a), there is a poor correlation with individual *Salmonella* fecal prevalence (Funk et al., 2005; Rajic et al., 2007a).

### General *Salmonella* culture

In the literature there are numerous microbiological methods for isolation of *Salmonella* (Waltman, 2000). The detection of *Salmonella* organisms by culture of swine specimens depends on specimen type, microbiological culture method, sample aggregation (individual pig or pool pen) and serovar. Different protocols have been developed to isolate *Salmonella* from: carcass swabs (Sorensen et al., 2007), cecal contents (Skovgaard et al., 1985; Harvey et al., 2001; Champagne et al., 2005), feces (Davies et al., 2000; Funk et al., 2000; Hoorfar and Mortensen, 2000; Rostagno et al., 2005a; Love and Rostagno, 2008), rectal swabs (Haddock, 1970; Nietfeld et al., 1998), lymph nodes (Harvey et al., 2001) and environmental drag swabs (Zewde et al., 2009b). Advantages to the use of microbiological culture in comparison to other diagnostic tests are: 1) to identify viable bacteria, not DNA; 2) to quantify the bacteria load; 3) to facilitate further testing such as serotyping, genotyping, phage typing and susceptibility testing (Funk, 2003; Farzan et al., 2007).

### *Salmonella* fecal culture

Numerous studies have compared microbiological methods for the isolation of *Salmonella* in fecal material of naturally infected pigs (Skovgaard et al., 1985; Bager and Petersen, 1991; Hoorfar and Baggesen, 1998; Nietfeld et al., 1998; O'Carroll et al., 1999; Davies et al., 2000; Funk et al., 2000; Hoorfar and Mortensen, 2000; Harvey et al., 2001; Erdman and Harris, 2003; Jensen et al., 2003; Osumi et al., 2003; Pangloli et al., 2003; Korsak et al., 2004; Champagne et al., 2005; Rostagno et al., 2005a; Love and Rostagno, 2008). In general, the culture methods differ on: 1) type of pre–enrichment; 2) type of selective enrichment; 3)

enrichment incubation temperature; 4) enrichment incubation time; and 5) plating media. The pre–enrichment step permits the resuscitation of damaged or injured *Salmonella* organisms (Hoorfar and Baggesen, 1998; Waltman, 2000; Jensen et al., 2003). The pre–enrichment medium can be non–selective or selective. Several non-selective pre–enrichment broths have been used, with buffered peptone water (BPW) being one of the most frequently used as the first step of the enrichment process (Hoorfar and Baggesen, 1998; Davies et al., 2000; Funk et al., 2000; Hoorfar and Mortensen, 2000; Waltman, 2000; Erdman and Harris, 2003; Jensen et al., 2003; Korsak et al., 2004; Love and Rostagno, 2008). Enrichment media are necessary not only to revitalize damaged salmonellae but also to increase target bacteria numbers. Fecal samples have a wide variety of background bacteria that can inhibit *Salmonella* multiplication (Love and Rostagno, 2008). In addition, subclinical carrier pigs shed low concentrations of *Salmonella* (Fravalo et al., 2003; Fablet et al., 2006). Thus if the detection is the objective, selective enrichment media are required steps in microbiological fecal culture in order to selectively inhibit other bacteria while allowing *Salmonella* to multiply to levels to be detected in plating media. The three most common selective enrichments used in swine fecal culture protocols are: tetrathionate broth (TTB), Rappaport–Vassiliadis (RV) broth, and Gram–negative (GN) Hajna, either in combination with a pre–enrichment broth or double selective enrichment (Bager and Petersen, 1991; Davies et al., 2000; Funk et al., 2000; Hoorfar and Mortensen, 2000; Harvey et al., 2001; Erdman and Harris, 2003; Rostagno et al., 2005a; Love and Rostagno, 2008). The medium Xylose–Lysine–Tergitol–4 (XLT4) (Davies et al., 2000; Funk et al., 2000; Korsak et al., 2004; Rostagno et al., 2005a; Love and Rostagno, 2008) and modified Rappaport–Vassiliadis (MRSV) (Hoorfar and Baggesen, 1998; Hoorfar and Mortensen, 2000;

Jensen et al., 2003; Champagne et al., 2005) are the most frequent agars used to isolate *Salmonella* colonies following the growth in enrichments broths.

The temperature of pre–enrichment, enrichment, and plating is generally either 37°C or 42°C. The time for incubation varies between 24 hours or 48 hours depending on protocol (Davies et al., 2000; Funk et al., 2000; Rostagno et al., 2005a; Love and Rostagno, 2008). A consequence of the multiple enrichment step protocols for isolation of salmonellae is that it is both labor and cost intensive. Despite being an imperfect diagnostic test, fecal culture is considered the 'gold standard' for *Salmonella* diagnosis. The sensitivity of a diagnostic test is affected by analytical and diagnostic sensitivity (Saah and Hoover, 1997; Hurd et al., 2004). The diagnostic sensitivity is also affected by biological factors related to *Salmonella* infection (serovar, stage of infection), prevalence, targeted population, sampling strategy, and time of sampling (Greiner and Gardner, 2000; Hurd et al., 2004). Other factors that can affect culture sensitivity include: culture methods (Davies et al., 2000; Funk et al., 2000; Rostagno et al., 2005a; Love and Rostagno, 2008), fecal sample weight (Funk et al., 2000; Champagne et al., 2005); sample type (individual fecal versus pool) (Haddock, 1970; Davies et al., 2000; Rostagno et al., 2005a; Love and Rostagno, 2008; Arnold and Cook, 2009) and serovar (Osumi et al., 2003; Rostagno et al., 2005a).

Estimates of the relative sensitivity of fecal culture range from 6.5% to 95%, depending on culture method and parallel estimation of the sensitivity (Davies et al., 2000; Funk et al., 2000; Funk, 2003; Hurd et al., 2004; Rostagno et al., 2005a; Love and Rostagno, 2008). In general, independent of the fecal culture method the specificity is considered to be 100% (Funk, 2003; Champagne et al., 2005; Rostagno et al., 2005a). Conversely, a low specificity (10%) when swine fecal samples were enriched in RV and then plated on XLT4 was reported (Mejia et

al., 2005). These findings could be due to the fact that the enrichment was done in one unique selective broth which is diffferent than the methods used by the other authors.

**Fecal culture protocols and test performance**

Two culture methods for *Salmonella* that are routinely performed in epidemiological studies were compared by Davies et al. (2000). Method 1 used a non−selective pre−enrichment (BPW, buffered peptone water), followed by RV enrichment. Method 2 used selective enrichment in TTB or GN Hajna broth followed by RV enrichment. The total proportions of positive samples were identical in both methods. However, when considering only samples with identical weight the selective enrichment broths TTB (74%) and GN Hajna (48%) had a higher proportion of positive samples compared with BPW (23%). The relative sensitivity of the tests evaluated in this study ranged from 55 to 74 % (Davies et al., 2000). The lack of a difference between the non−selective and selective methods could be due to weight differences between fecal samples that directly affects the sensitivity of culture. Funk *et al.* (2000) compared rectal swabs, 1 g, 10 g and 25 g fecal samples using the same culture method 1 (pre-enrichment in BPW, followed by enrichment in RV and XLT4 media). Relative sensitivity was significantly higher in 25g samples (78.3%) when compared with 10 g (52.2%), 1g (21.2%) and rectal swabs (8.7%) (Funk et al., 2000). Identical findings were reported by Champagne et *al.* (2005) with higher detection of *Salmonella* in 10 g samples than 1 g with a MSRV protocol (Champagne et al., 2005). On the other hand, the effect of sample weight on sensitivity is greater when the number of clusters of organisms is low. The sensitivity increases as the number of the clusters increases, in other words the sensitivity increases with homogeneity of the distribution of the

organisms in a sample (Cannon and Nicholls, 2002). Funk et *al.* (2000) suggested that a non–homogenous distribution of *Salmonella* in swine feces could explain why an isolation of bacteria in 1 g samples but not in 10 g or 25 g samples (Funk et al., 2000). Although differences in the sensitivity of stomached and non–stomached feces was not statistically significant (Funk et al., 2000), stomaching of enrichment broths is a common practice of many culture protocols (O'Connor et al., 2006a; Poljak et al., 2008).

Recently, Love and Rostagno (2008) compared five culture protocols for isolation of *Salmonella enterica* from fecal samples of naturally infected swine, representing the most common methods reported in epidemiologic studies of *Salmonella* in swine. The five culture methods had different combinations of non–selective enrichment broth (BPW); selective enrichment broth (GN, RV, TTB) and selective /differential agar plates (XLT4, MSRV); incubation temperature ($37^{o}$C or $42^{o}$C) and time (24h, 48h). None of the methods identified all positive samples, when compared with the'standard test' as a sample being dectected posive by at least one of the methods. Based on highest relative sensitivity (91.3%), the recommended culture method was inoculation of 10 g of feces into 100 ml of TTB with incubation at $37^{\circ}$C for 48 h, a subculture of 10 ml of the TTB into 100 ml RV broth with incubation at $37^{\circ}$C for 24 h, and then inoculation on XLT4 agar plates with 10 µl of RV broth and incubated 24 h at $37^{\circ}$C (Love and Rostagno, 2008). Nevertheless, when the objective of the study is to determine the diversity and serovar distribution in a population, a parallel culture methodology should be considered, since culture methods have differential ability to isolate *Salmonella* serovars (Bager and Petersen, 1991; Osumi et al., 2003; Rostagno et al., 2005a). Even at the sample level there

may be challenges in identifying all serovars present if only one culture method is used. This may of particular importance when pooled fecal sampling is used. Because pooled samples are a combination of 2 or more individual samples it may be more likely that more than one serovar is present (Rostagno et al., 2005a). However, even individual pigs can shed multiple serovars at once (O'Carroll et al., 1999; Funk et al., 2000, 2001b). Although higher serovar diversity is expected in pooled samples than in individual samples, use of one culture method might miss the identification of certain serotypes in pigs infected with multiple serovars. In addition to multiple culture methods, it has been suggested to select multiple colonies per plate in order to increase the probability of detecting more than one serovar within the same sample (Funk, 2003; Rostagno et al., 2005a).

The sensitivity of fecal culture can be increased by delayed selective enrichment, which consists of keeping samples in enrichment broth (RV) at room temperature for several days (4–8 days), followed by inoculation in RV (Nietfeld et al., 1998; O'Carroll et al., 1999; Davies et al., 2000). This additional step can increase the sensitivity by up to 25% (Davies et al., 2000). In epidemiological studies the culture of fecal samples is often delayed for several hours or days after collection. The storage of samples for 6 days at $-4^{\circ}$C did not affect the sensitivity of culture methods of swine fecal samples; however the detection of *Salmonella* decreased when samples were frozen at $-15^{\circ}$C for 14 days (O'Carroll et al., 1999).

In summary, the sensitivity of culture methods can be improved by using multiple specific enrichment broths (Davies et al., 2000; Rostagno et al., 2005a; Love and Rostagno, 2008) , increasing weight of the sample (Funk et al., 2000), and using delayed secondary enrichment (Davies et al., 2000).

**Consequences of imperfect sensitivity of fecal culture**

The sensitivity of a diagnostic test is not only affected by the test itself and sampling characteristics but also by the biological characteristics of the disease or infection/colonization within host. Pigs can be asymptomatic carriers of *Salmonella*. An intermittent pattern of *Salmonella* shedding has been reported in the literature, in both experimentally infected (Nielsen et al., 1995; Gray et al., 1996b; Osterberg and Wallgren, 2008; Scherer et al., 2008; Osterberg et al., 2009) and naturally infected pigs (Funk et al., 2001b; Kranker et al., 2003). Pig status may change over time, depending upon the time of sampling (Funk et al., 2001b; Funk, 2003). Obtaining multiple fecal samples from individuals can increase the sensitivity of the test (Funk, 2003; Thurmond and Johnson, 2004) for period prevalence estimates.

In summary, some of the strategies to increase the sensitivity of the culture methods based on sample and culture methods are: sample weight (Funk et al., 2000), parallel culture, double enrichment (Davies et al., 2000; Funk, 2003; Rostagno et al., 2005a; Love and Rostagno, 2008), delayed selective methods (O'Carroll et al., 1999; Davies et al., 2000), homogeneity of the sample (Cannon and Nicholls, 2002) and culture of multiple samples per pig (Funk et al., 2001b; Funk, 2003; Rostagno et al., 2005a). In a meta-analysis done by Sanchez et al., (2007), the diagnostic procedure was among the top 3 predictors that most affected the estimation of *Salmonella* prevalence either at the farm–level or pig–level (Sanchez et al., 2007).

**Pooled versus individual fecal samples**

Microbiological analysis of pooled fecal samples has been widely used in epidemiologic studies of *Salmonella* in swine. Pooled fecal samples at the farm have been used to classify herd

status, estimate within herd prevalence (Stege et al., 2000a; Stege et al., 2000b; Lo Fo Wong et al., 2003; Rajic et al., 2005; Farzan et al., 2006; García-Feliz et al., 2007; Farzan et al., 2008a; Farzan et al., 2008b; Wilkins et al., 2010a) and investigate on-farm risk factors for *Salmonella* infection in swine (van der Wolf et al., 1999; Mejia et al., 2006; Rajic et al., 2007b; Poljak et al., 2008; Wilkins et al., 2010a). Compared to individual sampling, pooling of individual fecal samples to assess herd or pen prevalence and risk factors offers a cost–effective and practical methodology (Funk, 2003). Two strategies have been used for pooling fecal samples. These include pooling individual fecal samples of five or more pigs (Funk et al., 2000) or about five grams of composite fecal material collected in five or more different locations on the pen floor (Rajic et al., 2005; Farzan et al., 2008a; Wilkins et al., 2010a).

Pooled fecal sampling offers several advantages compared to individual sampling: 1) there is no need for restraining pigs, thus the stress of handling is minimized (Arnold et al., 2005; Arnold and Cook, 2009); 2) the required personnel at the farm is reduced; 3) the number of individuals that can be represented at the same time increases (Christensen and Gardner, 2000; Arnold et al., 2005; Arnold and Cook, 2009); 4) fewer samples are submitted to the laboratory, reducing costs and burden (Christensen and Gardner, 2000; Arnold and Cook, 2009) ; 5) the diversity of serovars detected within a farm increases (Rostagno et al., 2005a); and 6) the probability of detecting an intermittent shedder in a pen increases (Arnold et al., 2005; Arnold et al., 2009).

These benefits are evident in studies comparing pooled of composite and individual fecal sampling. When pooled fecal samples were compared with individual samples, the proportion of pooled fecal samples that were positive was higher than the proportion of individual samples (Farzan et al., 2008a; Arnold and Cook, 2009; Wilkins et al., 2010a). The pool sensitivity

increases with the number of positive samples in the pool; pools of 5, 10 and 20 grams are more sensitive than individual sampling, and pools of 20 have the highest sensitivity for farm– or pen–level detection (Arnold et al., 2005). Moreover, the increase in sensitivity with pooled sampling is greater when the prevalence is low (Christensen and Gardner, 2000).

In general the pooled sample sensitivity and specificity can be affected by several factors such as dilution, concentration of the analyte (bacteria) and sampling probabilities that each can consequently affect the herd sensitivity (Christensen and Gardner, 2000). The sensitivity of pooled fecal sampling specifically regarding *Salmonella* can be affected by the concentration of *Salmonella* in pig feces, sample weight of individual samples, dilution effect if the prevalence is low and clustering of pigs within pens (Arnold et al., 2005; Arnold and Cook, 2009). The sensitivity of pooled sampling is greatly reduced due to dilution effect as the proportion of negative individual samples in each pool increases (Enoe et al., 2003; Arnold et al., 2005; Arnold and Cook, 2009). The effect of mixing negative samples with positive samples might also be greater in the presence of clustering of the organisms and small number of clusters (i.e. lack of homogeneity) (Cannon and Nicholls, 2002). In addition, a greater proportion of competing micro–organisms and inhibitory substances in the negative material relative to the positive material in pools can further decrease the pool sensitivity (Baggesen et al., 2007). In contrast, Funk et al., (2000) showed no difference between stomached and non–stomached samples, which theorically should increase homogeneity, for detection of *Salmonella* in individual fecal samples (Funk et al., 2000); however, this may not be consistent with results for pooled samples. On the other hand, there is a decreased sensitivity of pooled fecal samples when clustering within pens is taken into account (Arnold and Cook, 2009). This should be taken into account in epidemiologic studies with pen sampling of the herd occurs, since pooled

samples can be either collected representing a single pen, or a composite of samples from multiple pens.

Overall, pooled fecal sampling offers a good alternative to determine herd− or pen− prevalence, but in some situations it would not be indicated. If the goal of the study is to investigate the individual dynamics of *Salmonella* infection and risk factors at the pig level, individual sampling is preferred to the pool sampling. In addition, pooled fecal samples might not be representative of individual pigs. Serovars and phage types at the pen and farm level were reported to be different when individual pig samples were compared with pooled pen samples in a Canadian study (Farzan et al., 2008a). In addition, pooled samples obtained from pen floors may not represent active shedders, as there could be residual contamination in the pen environment. In terms of feasibility, weight /or volume of the pooled samples might also be a constraint for laboratory processing (Christensen and Gardner, 2000) .

**Quantification of *Salmonella***

Quantitative methods for enumeration of *Salmonella* in pigs and pork products are required for quantitative microbial risk assessments (QMRA), necessary to investigate source attribution and traceability of foodborne pathogens responsible for human illness (Bollaerts et al., 2009; Boone et al., 2009; Smid et al., 2011). The quantitative data used in such models are based mainly on expert opinion due a lack of empirical data (Bollaerts et al., 2009; Boone et al., 2009; Smid et al., 2011). The majority of QMRA models focus on measures of frequency (prevalence at the farm, lairage and microbial carcass contamination) (Miller et al., 2005; Hurd et al., 2008; Bollaerts et al., 2009; Delhalle et al., 2009; Bollaerts et al., 2010). Data in

pre–harvest are needed in order to identify possible high shedding pigs either at the farm or at lairage (Hurd et al., 2001c; Hurd et al., 2002; Rostagno et al., 2003). In addition, during lairage pigs are exposed to temporal and spatial variations in concentrations of *Salmonella* in the pen environment (O'Connor et al., 2006a; Boughton et al., 2007). Quantitative data pre–harvest can be used to identify animals or environments that can be a potential source of *Salmonella*.

Quantification of bacterial load can be used to identify the contamination pressure in different stages of pre–harvest and harvest; to implement and to test the effectiveness of control at the farm or slaughter (Fravalo et al., 2003; Boughton et al., 2007). Furthermore, quantitative data on numbers of organisms being shed by pigs are necessary to better model the transmission of *Salmonella* in pre–harvest settings (Lanzas et al., 2011). There is a lack of quantitative data either during pre–harvest or harvest, that might contribute to the large level of uncertainty in mathematical models. Current data on the quantity of *Salmonella* shed by pigs in feces are mainly based on experimentally infected pigs (Wood and Rose, 1992; Fedorka-Cray et al., 1994; Gray et al., 1996a; Osterberg and Wallgren, 2008; Scherer et al., 2008; Osterberg et al., 2009).

**Techniques available to quantify: MPN, MRSV, real–time PCR**

Methods to quantify *Salmonella* can be divided into culture–dependent and culture–independent approaches (Malorny et al., 2008). Traditionally, quantification of *Salmonella* in fecal and carcass samples has been based on two culture–dependent methodologies: most-probable number (MPN) and direct plating (Malorny et al., 2008).

The MPN is based on culture of three– to five– tube replicates that are prepared from 10–fold serial dilutions and tested using horizontal culture methods (Malorny et al., 2008;

Jasson et al., 2010). MPN has been used to quantify the *Salmonella* load in fecal material of experimentally infected pigs (Wood and Rose, 1992; Fedorka-Cray et al., 1994; Gray et al., 1996a), contaminated slurry (Hurd et al., 2001b) and contaminated lairage environments (O'Connor et al., 2006a; Boughton et al., 2007). The MPN is best used in the presence of low bacterial concentrations (<50 CFU/g for food samples) and allow for enrichement (Malorny et al., 2008; Jasson et al., 2010; Krämer et al., 2011). This type of methodology is time consuming, labor intensive and costly; therefore it can be an impediment to use in studies with large numbers of samples. In the case of samples with high concentrations of bacteria ($10^2$ to $10^3$ CFU/g or more) quantities can be more readily estimated by direct plating. However, the ability to quantify bacteria using direct plating depends also on the sample matrix and background flora (Malorny et al., 2008; Jasson et al., 2010; Krämer et al., 2011).

Modified culture methods have been developed to quantify *Salmonella* in pig feces and on pork carcasses; for example a semi−quantitative approach based on modified semisolid Rappaport−Vassiliadis (MRSV) (Jensen et al., 2003; Osterberg and Wallgren, 2008; Osterberg et al., 2009) and mini−MSRV MPN technique (Fravalo et al., 2003; Fablet et al., 2006; Krämer et al., 2011) have been described. In the MRSV method, samples are serially diluted in enrichment media, followed by plating on semi−solid MSRV agar plates. The semi−quantitative result is recorded using scores (Osterberg and Wallgren, 2008; Osterberg et al., 2009). The mini−MSRV method is based on miniaturization of dilution, pre−enrichment and selective enrichment on MSRV steps in a 12 micro−well plate. The automation of the method and the minimal amount of media used are advantages of this method compared to the traditional MPN (Fravalo et al., 2003; Fablet et al., 2006; Krämer et al., 2011).

Overall, quantitative methods based on culture are time consuming (3–7 days), labor intensive and costly. The detection limit is variable, depending upon on the method. In general the limit of detection ranges between 1.8 to $10^2$ CFU/g depending on the laboratory (Malorny et al., 2008; Jasson et al., 2010). For these reasons, quantitative methods should not be used to detect *Salmonella* positive samples, rather only to quantify. This is important considering that asymptomatic carrier pigs shed *Salmonella* in feces in low concentrations (Osterberg and Wallgren, 2008; Scherer et al., 2008; Osterberg et al., 2009), and these might not be detectable by traditional quantitative culture methods.

**Quantitative real–time PCR**

Culture–independent methods have been developed in order to reduce the cost and time of processing (Malorny et al., 2008; Jasson et al., 2010; Krämer et al., 2010; Löfström et al., 2011). Among the culture–independent methods, quantitative real–time polymerase chain reaction (PCR) methods have been used to quantify *Salmonella* in food matrices, pig feces and pig carcasses (Abley et al., 2005; Malorny et al., 2008; Park et al., 2008; Jasson et al., 2010; Krämer et al., 2010; Löfström et al., 2011).

PCR is based on amplification of a specific DNA sequence during a short period of time (Heid et al., 1996; Levin, 2009). Real–time PCR quantifies gene copy numbers by measuring the accumulation of a specific or non-specific fluorescent probe. The fluorescent signal generated by the probe is directly proportional to the amount of PCR product generated. Quantification is based on the exponential increase of the initial DNA amount and determined

by the number of PCR cycles performed to threshold values (Heid et al., 1996; Malorny et al., 2008).

The genes targeted in real−time PCR pig samples differ by study and matrix. Real−time PCR protocols, targeting the ttrRSBCA locus (Krämer et al., 2011; Löfström et al., 2011) and *invA* gene (Abley et al., 2005; Guy et al., 2006) have been developed to quantify *Salmonella* in pig carcass and fecal samples. The *invA* gene is located on *Salmonella* Pathogenecity Island 1 (SPI1) and is fundamental for epithelial invasion (Galan, 1996). The *invA* gene is a unique sequence common to 626 *Salmonella* strains (Rahn et al., 1992). Hoorfar et *al*. (2000) developed a 5' nuclease TaqMan assay for identification of *Salmonella enterica*; the PCR oligonucleotides primers and probe had target the *invA* gene, in order to amplify a DNA sequence of 119 base pairs (bp). The *Salmonella* probe was labeled with 6-carboxyfluorescein (FAM) (reporter dye) and 6-carboxytetramethylrhodamine (TAMRA) (quencher dye). This TaqMan assay was demonstrated to identify 110 *Salmonella* strains (Hoorfar et al., 2000).

**Enrichment and no enrichment prior to real−time PCR**

A pre−enrichment step before the PCR assay is part of several real−time PCR protocols for either detection or quantification of *Salmonella* on pig carcasses (Bohaychuk et al., 2007; Malorny et al., 2008; Krämer et al., 2010) or feces (Malorny and Hoorfar, 2005; Wilkins et al., 2010b).

The theoretical analytical sensitivity of PCR is one positive microbial cell (live or dead) per PCR reaction (e.g. 1 genome/PCR). Due to sample preparation (e.g. PCR inhibition) and small−volume (approximately 10µl extracted DNA) used in this molecular technique's

diagnostic sensitivity is $10^3$–$10^4$ cells /ml or gram of sample (Malorny and Hoorfar, 2005; Malorny et al., 2008; Jasson et al., 2010). Enrichment of the samples is required when the levels of *Salmonella* are low in order to raise the concentration to a level approximately $10^3$ to $10^4$ cells per ml of enriched broth (Malorny and Hoorfar, 2005). In addition, the enrichment should inhibit the growth of background flora and recover or revitalize stressed or damaged salmonellae (Malorny and Hoorfar, 2005; Malorny et al., 2008; Krämer et al., 2011). The selection of enrichment broth can affect the detection limit of the assay; thus, enrichment protocols should be standardized to better compare results (Malorny and Hoorfar, 2005; Malorny et al., 2008). Malorny et al., (2008) recommend a non–selective broth (e.g. BPW) as pre–enrichment when the concentration is less than 500 cells per gram or ml either in food or environmental samples (Malorny et al., 2008). Lately, Kramer et al. (2011) developed a combined enrichment and real–time PCR method for enumeration of salmonellae from pig carcasses, that allows enumeration of low numbers of *Salmonella* in cork borer samples with a short time period of enrichment (8h) (Krämer et al., 2011). On the other hand, the use of an enrichment step in quantitative PCR prevents the ability to relate the real numbers of organisms in the analyzed sample and the enumeration by PCR (Jasson et al., 2010); however, relative comparisons within study are still possible.

Several studies described direct quantification in carcasses (pig, beef and chicken) (Guy et al., 2006; Wolffs et al., 2006; Wolffs et al., 2007; Löfström et al., 2011), food samples (Fukushima et al., 2007; Cheng et al., 2009; Elizaquivel et al., 2011) and fecal material (horses and pigs) (Abley et al., 2005; Harris et al., 2007; Pusterla et al., 2010) using real–time PCR. The quantification limit varies, depending on matrix, assay and pre−processing.

Separation and DNA extraction of the target cells for quantitative analysis without enrichment can be processed either by a single or two–step approach prior to the real–time PCR (Malorny et al., 2008). In single–step protocols, DNA extraction is processed in a lytic buffer solution (Malorny et al., 2008). In a two–step approach, DNA extraction is preceded by methods such as filtration (Wolffs et al., 2006; Fukushima et al., 2007), flotation (Fukushima et al., 2007; Wolffs et al., 2007; Löfström et al., 2011), sedimentation (Fukushima et al., 2007) and pre–treatment of the matrix with ethidium monoazide (Guy et al., 2006). These pre–extraction steps are applied in order to separate, concentrate or detect viable bacteria cells before the DNA extraction itself (Malorny et al., 2008). The quantification performance of the PCR can be affected by type of DNA extraction protocol such as, protocol efficiency, the nature of matrixes, and presence of PCR inhibitors (Jensen and Hoorfar, 2002; Malorny and Hoorfar, 2005; Klerks et al., 2006; Levin, 2009). In on study the QIAamp® DNA stool kit showed better performance, calculated as Ct (threshold cycle) value of real–time PCR in pig fecal samples spiked with *S.* Typhimurium; on the other hand for pork samples Charge Switch® gDNA minibacteria was more suitable (Lettini et al., 2011).

**Detection limit of real–time PCR**

Few studies have enumerated *Salmonella* in swine fecal samples using real-time PCR without enrichment. Only two studies have reported the use of real–time PCR without enrichment in swine fecal material. Abley et al. (2005) evaluated the performance of a real–time PCR assay on pig fecal samples spiked with *Salmonella enteritidis* (ATCC 13076).

*Salmonellae* were inoculated in negative fecal samples with final concentrations ranging from $10^1$ to $10^8$ CFU/gram. This assay could sporadically detect concentrations as low as 10 copies per gram and quantify at concentrations greater than $10^3$ copies per gram of feces (Abley et al., 2005). A higher limit for quantification was obtained by other authors (Harris et al., 2007). *Salmonella* free fecal samples were spiked with ten–fold dilutions from $10^1$ to $10^8$ CFU. The assay could quantify *Salmonella* as low as $10^4$ CFU in feces. The estimated concentration by real–time PCR was within one $\log_{10}$ when compared to MPN. In addition, the limit of detection of real–time PCR for artificially contaminated concrete (using a hydrosponge to sample the concrete) was $10^4$ CFU/ hydrosponge (Harris et al., 2007).

In summary, quantitative real-time PCR is an alternative to the traditional quantitative culture-dependent method. It allows enumeration of *Salmonella* in a large number and variety of samples in an efficient time–cost and automated method (Malorny et al., 2008; Elizaquivel et al., 2011; Löfström et al., 2011). However, it is limited due a small volume of target sample used in the PCR assay. The lower limit for quantification without any enrichment is approximately $10^2$ to $10^4$ cells per gram (or milliliter) and can depend on the matrix and protocol (Abley et al., 2005; Harris et al., 2007; Malorny et al., 2008; Jasson et al., 2010). This limitation should be considered in decisions to apply this methodology for numeration of *Salmonella* in specific matrices as food (Malorny et al., 2008; Jasson et al., 2010), environmental samples from lairage (Harris et al., 2007) or feces from naturally infected pigs (Fravalo et al., 2003; Fablet et al., 2006), or any sample type where expected concentrations are

below the detection limit. In addition, to those factors previously mentioned, other factors can affect the performance of real–time PCR. These includes the standard curve setup, the type of sampling and sampling technique, homogenization of the sample, and the type of cells in samples (dead or viable versus stressed) (Malorny et al., 2008).

In natural settings, the majority of pigs shed low concentrations of bacteria, below the quantitative limit of q–PCR. In a quantitative study using the mini MSRV–MPN technique , 86% of swine fecal samples had less than 200 organisms per gram (Fravalo et al., 2003). Using the same technique, concentrations of 2.4 to 350 MPN per gram of feces were reported in pooled fecal samples of finishing pigs in a study on French farms (Fablet et al., 2006). Quantitative studies at lairage have reported variable and relatively low bacterial loads, with median pen floor concentrations ranging from 1.8–11.5 organisms/100cm$^2$ (Boughton et al., 2007) and 457–1071 organisms/ml of slurry (O'Connor et al., 2006a). However, the differences found between lairage and individual pigs are not directly comparable because of different sampling methodologies and the likely cumulative contamination of lairage. In experimental studies pigs shed low concentrations within a few days after challenge that decrease over time to levels that are not detectable (Scherer et al., 2008).

## EPIDEMIOLOGY OF SALMONELLA IN SWINE

### Prevalence of *Salmonella* in swine in the US

*Salmonella* is wide spread in livestock production systems. In a multi−state study (Tennessee, North Carolina, Alabama, California and Washington) swine production systems had the highest proportion of *Salmonella* positive samples (57.3%), followed by dairy (17.9%), poultry (16.2%) and beef cattle (8.5%) (Rodriguez et al., 2006).

In the US, there are two types of surveillance programs for *Salmonella* in swine, one at the slaughterhouse by the Food Safety and Inspection Service (FSIS) (sampling carcasses) and the other by the National Animal Health Monitoring System (NAHMS) (sampling on farm). The prevalence of *Salmonella* at the slaughterhouse is variable, depending on the study and year, with a relative stable proportion of positive carcasses around 2.5 % the last three years (USDA-FSIS, 2010). The proportion of *Salmonella* positive hog market carcasses reported by FSIS has decreased from 6.25% in 2000 to 2.28% in 2009 (USDA-FSIS, 2010). At the farm level, based on the 2006 NAHMS survey, the proportion of positive farms was 52.6% and the proportion of positive fecal samples was 7.2%. This is an increase relative to the 2000 NAHMS data (34.7% and 6.6%, respectively) (Bush et al., 2002; USDA-APHIS, 2009). The top five serovars in the 2000 and 2006 NAHMS surveys were: *S.* Derby, *S.* Typhimurium var. Copenhagen , *S.* Agona , *S.* Anatum and *S.* Heidelberg (Bush et al., 2002; USDA-APHIS, 2009).

In the US, no national *Salmonella* control program in swine production has been adopted in contrast to several European countries (Hautekiet et al., 2008; Abrahantes et al., 2009; Baptista et al., 2010b; Snary et al., 2010; Merle et al., 2011). Several epidemiological studies have been conducted to estimate prevalence and risk factors involving a smaller number of states

and herds. Prevalence estimates have been based either on culture of lymph nodes (Carlson and Blaha, 2001; Gebreyes et al., 2004; Bahnson et al., 2005; Bahnson et al., 2006a; Bahnson et al., 2006b), fecal culture of individual pigs (Davies et al., 1997b; Davies et al., 1998; Funk et al., 2001a; Funk et al., 2001b; Barber et al., 2002; Gebreyes et al., 2004; Hurd et al., 2004; Bahnson et al., 2006b; Gebreyes et al., 2006; Dorr et al., 2009; Wang et al., 2010; Rostagno et al., 2011), or serology (serum or meat juice) (Funk et al., 2005; O'Connor et al., 2006b; Gebreyes et al., 2008; McKean and O'Connor, 2009). There is a high variability in the prevalence estimates; the herd prevalence ranges from 64% to 100% , and the individual fecal prevalence can be as low as 0% and high as 84% (Davies et al., 1997b; Davies et al., 1998; Carlson and Blaha, 2001; Funk et al., 2001a; Funk et al., 2001b; Barber et al., 2002; Gebreyes et al., 2004; Hurd et al., 2004; Bahnson et al., 2005; Funk et al., 2005; Bahnson et al., 2006a; Bahnson et al., 2006c; Gebreyes et al., 2006; O'Connor et al., 2006b; Gebreyes et al., 2008; Dorr et al., 2009; McKean and O'Connor, 2009; Wang et al., 2010; Rostagno et al., 2011). Generalization of results among the studies is challenging due to differences in study design (cross-sectional versus longitudinal), sampling strategy, targeted population (production stage), and diagnostic test (culture of feces versus lymph nodes, serology).

Prevalence estimates based on bacteriological culture of the tissues or fecal material at slaughter may (Bahnson et al., 2005; Bahnson et al., 2006b) or may not (Gebreyes et al., 2004) be representative of the infection status at the farm. Numerous studies have reported higher prevalence of *Salmonella* at slaughter (cecal contents or lymph nodes) compared to fecal sampling at the farm (Hurd et al., 2001c; Hurd et al., 2002; Gebreyes et al., 2004; Dorr et al., 2009). In addition, isolation of different serovars at slaughter as compared to those isolated at the farm suggest new infections acquired between the farm and slaughter (Hurd et al., 2001c;

Gebreyes et al., 2004; Bahnson et al., 2005; Dorr et al., 2009). Despite the difference of sampling between farm and slaughter; this discrepancy suggests that transport and lairage might result in increased shedding of bacteria and acquisition of new infections (Hurd et al., 2001c; Hurd et al., 2002; Gebreyes et al., 2004; Dorr et al., 2009). Therefore, *Salmonella* positive samples taken at slaughter might be a result of exposure or activation of infections at the farm, transportation and slaughter. Knowing pigs harboring *Salmonella* are the main reservoir and consequently being the source of infection for non-infected pigs either at farm, transportation or lairage.

Based on epidemiological studies, the within-herd fecal prevalence on finishing swine farms in North America ranges from 2% to 84% and the proportion of positive samples ranges from 2% to 38% (Davies et al., 1997b; Funk et al., 2001b; Bush et al., 2002; Hurd et al., 2004; Rajic et al., 2005; Bahnson et al., 2006b; Gebreyes et al., 2006; Rajic et al., 2007b; Farzan et al., 2008a; Poljak et al., 2008; Dorr et al., 2009; Wang et al., 2010; Wilkins et al., 2010a; Rostagno et al., 2011). The most commonly isolated serovars in finishing fecal samples are: *S.* Typhimurium, *S.* Typhimurium var Copenhagen, *S.* Derby, *S.* Agona, *S.* Mbandaka, *S.* Infantis, *S.* Muenster (Davies et al., 1997b; Davies et al., 1998; Funk et al., 2001b; Gebreyes et al., 2004; Rajic et al., 2005; Farzan et al., 2008a; Farzan et al., 2008b; Dorr et al., 2009; Wang et al., 2010; Wilkins et al., 2010a). Several reasons can explain this variability in prevalence estimates such as: 1) differences in swine production systems between countries, states or regions, 2) study design (cross-sectional versus longitudinal), 2) type of sampling (individual versus pooled), 3) type of diagnostic test culture methods), 4) time of sampling, and 5) intermittent shedding.

## Cross–sectional versus longitudinal studies

### Importance of longitudinal studies

Most of the epidemiological studies that have provided estimates of prevalence and risk factors of salmonellosis in swine have been cross-sectional. Cross-sectional studies are 'snapshots' of the population status with the respect to disease or exposure variables, in which all the information refers to the same point in time. The estimation of prevalence depends upon incidence and duration of the disease (Dohoo et al., 2010). In infectious diseases with asymptomatic carriers and intermittent shedding, such as salmonellosis, prevalence is the most common measure of disease frequency described in epidemiologic studies. This is because it is often not possible to distinguish a new infection from recurrence of a previous infection. In addition, due to the use of imperfect diagnostic tests, such as fecal culture, with low to moderate sensitivity, apparent prevalence is the frequency measure described in the majority of the studies. Few studies have reported the true prevalence, adjusted based on assumptions of diagnostic test performance (sensitivity and specificity) (Funk et al., 2000) or herd sensitivity (Farzan et al., 2008b). Therefore, considering the characteristic chronic/intermittent shedding and the use of an imperfect test, one point–in–time estimate may not be adequate to determine farm or pig *Salmonella* status. Epidemiologic studies based on point–estimates of the prevalence may result in misclassification of both farms and pigs (Funk et al., 2001b; Rajic et al., 2005; Rajic et al., 2007a; Farzan et al., 2008b). Cross–sectional studies are not the best to assess the time–variant risk factors associated with diseases (Dohoo et al., 2010). Longitudinal studies allow investigation of time−variant risk factors associated with diseases.

A number of studies have been conducted longitudinally either at the farm level (van der Wolf et al., 2001a; Lo Fo Wong et al., 2004b; Rajic et al., 2005; Rajic et al., 2007a; Farzan et al., 2008b; Rostagno et al., 2011), at the cohort level (Merialdi et al., 2008; Dorr et al., 2009; Vigo et al., 2009) or at the pig level (Funk et al., 2001b; Beloeil et al., 2003; Kranker et al., 2003; Nollet et al., 2005a; Merialdi et al., 2008; Vigo et al., 2009). For the purpose of this review, longitudinal studies at the farm level are defined as repeated sampling of the farm by sampling different groups of pigs; cohort–level represents repeated sampling of the same group of pigs; and pig–level sampling represents repeated sampling of the pigs individually.

Longitudinal studies at the farm level based on seroprevalence (Lo Fo Wong et al., 2004b; Rajic et al., 2007a) or fecal prevalence (Rajic et al., 2005; Rajic et al., 2007a; Farzan et al., 2008b) have shown high variability of farm status over time. Raijic et al. (2005) conducted a large scale longitudinal study in ninety Alberta swine finishing farms where pooled fecal and environment samples were collected three times over a five month period. The authors reported that 63.3% of finishing farms had no *Salmonella*–positive samples on one visit but had one or more positive samples on other visits (Rajic et al., 2005). A significant variability over time in farm status was presented when those farms were categorized based on seroprevalence (Rajic et al., 2007a). In a five–year study conducted by Farzan et al. (2008) in 113 Ontario swine farms, there was variability of fecal prevalence and isolated serovars during the study period (Farzan et al., 2008b). A limitation of this study was that different sampling strategies, type of sampling (individual versus pools) and culture protocols were used across the years, which may in part contribute to the variability in the results (Farzan et al., 2008b). Therefore, classifying farms as *Salmonella* positive or *Salmonella* negative based on a single sampling and using diagnostic tests of poor sensitivity is likeky to lead to misclassification of the true farm status (Funk et al.,

2001b; Rajic et al., 2005; Rajic et al., 2007a).

**Longitudinal studies at the pig level**

Longitudinal studies at the pig level have reported time variability of fecal shedding associated with cohort or batch of pigs (Funk et al., 2001b; Beloeil et al., 2003; Kranker et al., 2003; Nollet et al., 2005a). Funk et al. (2001) conducted a longitudinal study in 2 multi–site production systems, where individual fecal samples were collected from sows (gestation and pre-wean) and pigs (piglets pre–weaned to finishing, sampled six times) in five cohorts of pigs. There was high variability of *Salmonella* prevalence and serovar distribution within cohorts over time, and among cohorts within systems (Funk et al., 2001b). Finishing pigs were sampled 3 times approximately monthly; there was no uniform trend for prevalence during this phase, as some cohorts decreased and others increased over the finishing phase (Funk et al., 2001b). In contrast, according to one Belgium farrow–to–finish farm study conducted by Beloil et al. (2003), the individual fecal and environmental prevalence (pen swabs) were higher in the first third of the finishing phase (Beloeil et al., 2003). In another longitudinal study by Kranker et al. (2003), in three Danish farrow-to-finish herds, the overall fecal prevalence reached a peak at 60 days of age (nursery) and decreased over time during the finishing phase. However, a marked variation was observed between herds and cohorts (Kranker et al., 2003). Nollet et al. (2005) conducted a study in 3 cohorts in 3 farrow–to finish herds in Belgium, pigs were followed–up from weaning through the finishing phase (sampled between 5 to 6 times during the finishing phase). Increasing prevalence was observed when the pigs were moved to the finishing unit in one herd, and in the other herd two peaks in prevalence occurred after the pigs were moved to

the growing and finishing unit, although the prevalence subsequently decreased during the finishing phase (Nollet et al., 2005a). Similarly, Vigo et al. (2009) observed increased *Salmonella* shedding when pigs were placed in finishing units (Vigo et al., 2009). It was suggested by the authors that the increased prevalence after moving to a new facility could be due to the stress caused by transportation, comingling with new pigs, changes in feed type and exposure to residual contamination at the new location (Nollet et al., 2005a; Vigo et al., 2009).

The distribution of serovars varies among the studies. The Kranker et al. (2003) study took place on 3 farms known to be infected with *S.* Typhimurium (Kranker et al., 2003), while in the Beloeil et al. (2003) study two serovars (*S.* Typhimuirium and *S.* Brabdenburg) were isolated during finishing phase (Beloeil et al., 2003). On the other hand, a wider diversity of serotypes were found by Funk et al. (2001) and Nollet et al. (2005), different serovars were identified within same system, cohort, pen or pig (Funk et al., 2001b; Nollet et al., 2005b). The diverse serovars found in those studies can be indicative of multiple infections on the farm or introduction of new strains.

Although temporal variability of *Salmonella* shedding is generally observed, there is an inconsistency of prevalence trend along the finishing period. Some studies have reported a decrease in fecal prevalence (Beloeil et al., 2003; Kranker et al., 2003; Nollet et al., 2005b; Vigo et al., 2009; Molla et al., 2010). Other studies demonstrated both increases and decreases, depending on cohort (Funk et al., 2001b), or an increase as the pig got older (Dorr et al., 2009). It is unclear what factors contribute to such differences. Poljak et al. (2008) suggested that the association between the pig weight and *Salmonella* shedding can be explained due to correlation between pig weight, age and health status. Management factors such as changes in feed and in−feed antibiotics, stress caused by transport, comingling pigs and stocking density, variation of

environmental temperature in barn and housing conditions might affect the *Salmonella* shedding of finishing pigs (Nollet et al., 2005a; Funk et al., 2007; Hautekiet et al., 2008; Poljak et al., 2008; Vigo et al., 2009). Indeed, the variation in prevalence throught the finishing phase might be due to management factors that have not been explored during the majority of the epidemiologic studies.

There are major study design differences that can affect the estimation of prevalence and isolated serovars of the previous studies; these includes number of farms, type of farm (multi−site versus farrow−to−finishing), sampling frequency (monthly versus weekly), type of sampling (10 gram feces versus rectal swabs) and serovars present (one versus several serovars). Nevertheless, all of the studies found a high variability of *Salmonella* shedding over time either at the cohort or pig level. Longitudinal studies with repeated sampling of farm, cohorts, and pigs are needed to investigate the potential time−variant risk factors (Funk et al., 2001b; Poljak et al., 2008). Furthermore, longitudinal studies at the individual level based on bacteriological fecal culture should be performed in order to investigate the dynamics of *Salmonella* infection (e.g. age, duration of infection, and disease transmission patterns) in swine over time. The dynamics of *Salmonella* infection is likely to be complex. The duration of shedding in naturally infected pigs has been estimated to be between 7 to 101 days, with a mean time 18 or 26 days, depending on the assumptions and based on monthly sampling (Kranker et al., 2003). Another study showed that the majority of pigs were detected to be shedding once based on weekly samplings (Beloeil et al., 2003). Thus, monthly sampling may underestimate new infections or status of individual pigs.

**Herd risk factors**

Numerous studies worldwide have focused on evaluation of fecal shedding and risk factors during the finishing phase (Davies et al., 1997a; Davies et al., 1997b; Davies et al., 1998; van der Wolf et al., 1999; Stege et al., 2000a; Funk et al., 2001a; Funk et al., 2001b; Lo Fo Wong et al., 2003; Beloeil et al., 2004; Gebreyes et al., 2004; Hurd et al., 2004; Rajic et al., 2005; Bahnson et al., 2006b; Gebreyes et al., 2006; García-Feliz et al., 2007; Rajic et al., 2007b; Farzan et al., 2008a; Farzan et al., 2008b; Magistrali et al., 2008; Poljak et al., 2008; Dorr et al., 2009; Garcia-Feliz et al., 2009; Lomonaco et al., 2009; Wang et al., 2010; Wilkins et al., 2010a; Rostagno et al., 2011).

The aforementioned studies were focused on assessing the farm level prevalence in a large number of finishing farms. Differences in management of the farms might contribute to variation of *Salmonella* prevalence. In a cross–sectional study on 80 farms in Ontario done by Polkjak et al. (2008), the farm–level variance contributed the largest proportion to the total variance of *Salmonella* shedding of pigs, followed by the pen (Poljak et al., 2008). In this study the majority of the variables associated with *Salmonella* shedding were farm related variables (e.g. feed; disinfection, closed barn, number of pigs); no time–dependent variables were assessed. Thus, the level of highest variability might change to another level when variables at other levels are accounted for in multilevel analysis. For example, Funk et al. (2007) reported that the individual pig fecal sample was the level which contributed the most to the odds of fecal sample being *Salmonella* positive when compared pigs treated with and without subtherapeutic chlortetracycline (Funk et al., 2007).

The herd level risk factors associated with *Salmonella* prevalence in pig herds are tipically related to introduction of the pathogen to the herd, transmission among pigs (or within herd), and the survival of *Salmonell*a in the individual pig (Zheng et al., 2007; Hautekiet et al., 2008). The introduction and maintenance of the pathogen in the herd environment involves factors associated with biosecurity and herd management such as type of production, hygiene and presence of vectors (domestic and wildlife animals). The transmission among the pigs and survival of *Salmonella* in the host includes herd and health management factors such as feed type, concomitant diseases and use of antibiotics (Funk and Gebreyes, 2004; Zheng et al., 2007; Hautekiet et al., 2008; Fosse et al., 2009).

Biosecurity measures include a set of practices to avoid the introduction of new infections, to prevent the spread of diseases and mitigate the persistence of pathogens on farm (Twomey et al., 2010). Those measures include among others: all–in all–out flow management, cleaning and disinfection, personnel hygiene, access to the herd by visitors, and contact and presence of domestic animals and wildlife (Funk and Gebreyes, 2004; Fosse et al., 2009; Baptista et al., 2010a; Twomey et al., 2010). Recently, two studies, one in Portugal and the other in the U.K., investigated the association between biosecurity measures and *Salmonella* infection. Herds with poor biosecurity measures were more likely to be *Salmonella* positive compared with herds with good biosecurity, suggesting that multiple biosecurity measures should be applied simultaneously in order to prevent the introduction and spread of *Salmonella* (Baptista et al., 2010a; Twomey et al., 2010). Nevertheless, farmers might be less receptive to implement those procedures to control foodborne zoonoses, particularly if the cost is high (Fraser et al., 2010).

All–in all–out flow management has been associated with decreased *Salmonella* prevalence on swine farms (Lo Fo Wong et al., 2004a; Farzan et al., 2006; Hautekiet et al., 2008; Cardinale et al., 2010). This type of production system decreases the risk of cross–contamination because it allows segregation of age groups and 'down' time between batches for cleaning and disinfection (Funk and Gebreyes, 2004). In a recent review by Fosse et al. (2009), clean downtime ('empty and clean') periods of less than three, six and seven days in fattening, farrowing and post–weaning stages, respectively, were associated with higher risk of presence of *Salmonella* (Fosse et al., 2009).

Although it is generally accepted that cleaning and disinfection of the facilities and equipment decreases *Salmonella* environmental contamination; those practices have been inconsistently associated with a decrease in *Salmonella* prevalence on swine farms (Funk and Gebreyes, 2004; Fosse et al., 2009). On one hand, a higher risk for *Salmonella* (Cardinale et al., 2010) and other enteric diseases (Pearce, 1999) in the absence of disinfection of the farrowing rooms or between batches of pigs, respectively, was observed. In addition, disinfection between batches was associated with lower seroprevalence in Belgium market pigs (Hautekiet et al., 2008). On the other hand, a study of 80 Ontario farms found that the increased frequency of disinfection and washing with cold water were positively associated with *Salmonella* positivity measured by bacteriological culture at the farm and pen level (Poljak et al., 2008). Identical findings were reported by van der Wolf et al. (2001); the omission of disinfection of the rooms after pressure washing, was associated with lower *Salmonella* seroprevalence (van der Wolf et al., 2001b). In contrast, in 89 Alberta swine finishing farms, no significant difference in *Salmonella* shedding was observed among farms that did not clean or only scraped the pens or used pressure washing with or without disinfection between batches (Rajic et al., 2007b).

Although the findings are contradictory, it is well known that the cleaning and disinfection of the barn can reduce the *Salmonella* contamination level, but does not completely eliminate it. Several studies have shown residual *Salmonella* post−disinfection of barns and trucks (Funk et al., 2001b; Mannion et al., 2007; Dorr et al., 2009; Zewde et al., 2009b). In fact, residual environmental *Salmonella* contamination of the room was identified as a risk factor for *Salmonella* status of fattening pigs (Fablet et al., 2003; Beloeil et al., 2004; Beloeil et al., 2007). In addition, differential efficacy of cleaning and disinfection protocols have been reported (Mannion et al., 2007; Dorr et al., 2009); disinfectants (Mueller-Doblies et al., 2010) and increased antimicrobial *Salmonella* resistance has been described when certain biocides were applied in swine barns (Zewde et al., 2009a). Further information is needed to evaluate the efficacy of cleaning and disinfection protocols practices on *Salmonella* control in swine barns. Other personnel hygiene practices such as the presence of sanitary facilities at the entrance to facilities (e.g. changing room and toilet in farm) (Funk et al., 2001a; Lo Fo Wong et al., 2004a; Hautekiet et al., 2008), changing clothes or providing protective clothes and boots before entering and leaving the facilities (Beloeil et al., 2007; Rajic et al., 2007b; Hotes et al., 2010), and washing hands before handling pigs (Lo Fo Wong et al., 2004a) have been associated with a lower risk for *Salmonella* infection (seroprevalence and bacteriological culture). In addition, the use of disinfectant foot baths has been associated with a lower risk for *Salmonella* infection (Hautekiet et al., 2008; Twomey et al., 2010).

Humans, domestic and wild animals (e.g. dogs, cats, rodents and birds) are important vectors for the spread of *Salmonella* (Berends et al., 1996; Funk and Gebreyes, 2004; Fosse et al., 2009). Several studies have found an increased risk for *Salmonella* with increasing number of employees/visitors or personnel visits (Funk et al., 2001a; Cardinale et al., 2010). There was

an increased likelihood of detection of *Salmonella* with the presence of animals (e.g. birds, rodents, cats) other than pigs on the farm (Harris et al., 1997; Funk et al., 2001a; Cardinale et al., 2010). Protective features of the barn to avoid contact with animals (e.g. birds) such as fence enclosed pig facilities or closed barns were found to be protective in several studies (Beloeil et al., 2007; Poljak et al., 2008; Cardinale et al., 2010).

Housing has been recognized as a potential source of *Salmonella* (Letellier et al., 1999; Funk and Gebreyes, 2004; Gotter et al., 2011). Facilities allowing nose–to–nose contact between contiguous pens was associated with higher *Salmonella* prevalence in European (Lo Fo Wong et al., 2004a) and Canadian (Wilkins et al., 2010a) farms. Herds with fully slatted floors were less likely to be *Salmonella* positive when compared with other types of flooring (e.g. partially slatted or straw) (Davies et al., 1997b; Nollet et al., 2004; Hotes et al., 2010; Twomey et al., 2010). Conversely, van der Wolf et al. (2001) found no risk associated with different types of floors (van der Wolf et al., 2001b). In general, it is accepted that slatted floors decrease pig contact with fecal material, resulting in decreased fecal–oral transmission (Funk and Gebreyes, 2004).

Several epidemiological studies have found an association between high stocking density and *Salmonella* prevalence. Funk et al. (2001) reported that higher space allowance (more than 0.75$m^2$/pig) was associated with reduced *Salmonella* fecal prevalence (Funk et al., 2001a). However, the same research group found no difference between two levels of stocking density (0.60 $m^2$/pig versus 0.74 $m^2$/pig) on *Salmonella* fecal prevalence or antimicrobial resistance in a field trial study (Funk et al., 2007). More recently, Hautekiet et al. (2008) described higher risk of having high *Salmonella* seroprevalence when floor space per pig decreases in the fattening

period (Hautekiet et al., 2008). The association between the stocking density and *Salmonella* is not clear. The increased stocking density might increase pig to pig contact, and consequently, transmission. In addition, the stress caused by higher density might decrease the host immune defenses and make pigs prone to new infections (Funk and Gebreyes, 2004; Funk et al., 2007). The stocking density might also be related to batch size, as Beloeil et al. (2007) have reported a higher risk for *Salmonella* seroconversion prevalence when the number of pigs in a fattening room increased by 10–pig increments (Beloeil et al., 2007).

Herd size has been inconsistently associated with *Salmonella* prevalence*;* some studies show a higher risk in large herds (Carstensen and Christensen, 1998; Kranker et al., 2001; Hautekiet et al., 2008; Garcia-Feliz et al., 2009; Benschop et al., 2010) while others show inconsistent in small herds (van der Wolf et al., 2001b; Benschop et al., 2010). The difference among the studies might be related to biosecurity, hygiene measures and/or purchase of animals (Zheng et al., 2007) inherent to each size herd. Actually, "farm" contributed the largest component of model variability in a study of Ontario finishing pigs (Poljak et al., 2008), suggesting that farm level risk factors like herd size may significantly contribute to a farm's *Salmonella* risk. Moreover, mixing batches, continuous flow, and buying pigs from more than three suppliers increases the risk of *Salmonella* seroprevalence (Lo Fo Wong et al., 2004a; Farzan et al., 2006).

The effect of feed on *Salmonella* in swine can be divided into two categories: feed as source of salmonellae due to contamination and the impact of feed ingredients/structure on *Salmonella* proliferation in feed and the pig gastro–intestinal tract (Funk and Gebreyes, 2004). Feed is a potential *Salmonella* source to pig herds (Davies et al., 2004; Funk and Gebreyes, 2004; Molla et al., 2010; Kich et al., 2011). *Salmonella* has been isolated from feed trucks, feed

mill (Fedorka-Cray et al., 1997) or pig feed (Fedorka-Cray et al., 1997; Funk et al., 2001b; Molla et al., 2010). Feed-related serovars such as *S.* Cubana (Osterberg et al., 2006) and *S.* Yoruba (Osterberg et al., 2001) have been associated with feed–borne outbreaks of samonellosis in pig herds as result of contamination at feed plants. Many epidemiologic studies have demonstrated that feed composition and structure may be associated with *Salmonella* prevalence in pigs. Several factors have been investigated: wet or dry diets, feed particle size and form (pelleted diets, finely ground feed, meal), acidified diets (feed, water or both) and heat–treated feed (Funk and Gebreyes, 2004; Zheng et al., 2007). Numerous studies have showed higher bacteriological or serological prevalence in pig herds using dry feeding versus herds using wet or liquid feeding (van der Wolf et al., 1999; Kranker et al., 2001; van der Wolf et al., 2001b; Fablet et al., 2003; Beloeil et al., 2004; Bahnson et al., 2006b; Farzan et al., 2006; Benschop et al., 2008a; Hautekiet et al., 2008; Poljak et al., 2008; Hotes et al., 2010; Twomey et al., 2010). The liquid feed might include a fermention step, incorporation of organic acids or bio–products (e.g. whey) resulting in acidified feed (Funk and Gebreyes, 2004; Farzan et al., 2006). Herds fed whey, either to drink or as the liquid part of the diet, were at decreased risk of being seropositve (Lo Fo Wong et al., 2004a). Pigs fed non–pelleted feed have a lower risk of being serologically or bacteriologically *Salmonella* positive compared to pigs fed pelleted feed (Kranker et al., 2001; Leontides et al., 2003; Lo Fo Wong et al., 2004a; Rajic et al., 2007b; Poljak et al., 2008; Garcia-Feliz et al., 2009; Wilkins et al., 2010a). Although in those studies the category non–pelleted might include dry, wet feed, ground or floury feed, the authors are unanimous in noting that pelleted feed is a risk factor for *Salmonella*. In addition, in a recent systematic review by O'Connor et al. (2008) it was reported that the use of non-pelleted feed

shows the strongest evidence for reducing *Salmonella* prevalence in market finisher swine (O'Connor et al., 2008).

Water can be a potential source of *Salmonella*. Salmonellae have been isolated from water and drinkers (Barber et al., 2002; Gotter et al., 2011). In addition, water distribution systems might impact the risk of *Salmonella* transmission; as an example pigs from herds with water bowls were more likely to be *Salmonella* positive compared with herds with nipple drinkers (Bahnson et al., 2006b).

Several authors investigated the effect of health status on the risk of *Salmonella* in swine farms. Herds considered to have a high health status either by belonging to the Danish Specific Pathogen Free (SPF) program or by being a member of an Integrated Quality Control (IQC) program (Netherlands) were at lower risk of *Salmonella* infection (van der Wolf et al., 1999; Kranker et al., 2001; Benschop et al., 2008a). There was an increased risk of *Salmonella* shedding and seropositivity in herds with diarrhea and concurrent diseases such as Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Porcine Respiratory Coronavirus (PRCV), *Lawsonia intracellularis* and *Ascaris suum* (Møller et al., 1998; van der Wolf et al., 2001b; Fablet et al., 2003; Beloeil et al., 2004; Beloeil et al., 2007). On the other hand, LoFo Wong et al. (2004) found no association between health status and seroprevalence in European herds (Lo Fo Wong et al., 2004a). It is unclear if there is a direct relationship between the presence of other diseases on farms and *Salmonella* or if health status is a proxy of other unknown factors in herds that could be related to the *Salmonella* risk.

Antimicrobials have been widely used in swine production, both to prevent or treat diseases as well as to promote growth (Funk et al., 2006). There a lack of consistency among studies regarding the use of antimicrobials and the risk of *Salmonella* shedding. The use of

antimicrobials such as tylosin or a combination of chlortetracycline, procaine penicillin and sulphamethazine as growth promoters in finishing feed was associated with higher *Salmonella* seroprevalence (van der Wolf et al., 2001b; Leontides et al., 2003). In addition, pigs fed subtherapeutic chlortetracycline in the diet were at higher risk of shedding *Salmonella* (Funk et al., 2007). However, Rajic et al. (2007) reported the use of antibiotics through the water in all production phases was associated with lower farm seroprevalence (Rajic et al., 2007a). In contrast, no association was found between *Salmonella* shedding and the use of antibiotics (chlortetracycline and penicillin) in the finisher ration in an Ontario study (Poljak et al., 2008). The antibiotics in feed might be responsible for disrupting the normal microbial flora and consequently increasing the colonization of the gastrointestinal tract by gram–negative bacteria such as *Salmonella* (Funk et al., 2007; Rajic et al., 2007a). Nevertheless, differences in type of antibiotic, route of administration, purpose and characteristics of the farm must be considered when comparing studies. In addition, the spectrum of antibiotic resistance of the predominant *Salmonella* serovar might influence the magnitude of association regarding the prevalence and antibiotics use.

## SEASON, ENVIRONMENT FACTORS and FOODBORNE PATHOGENS

### Seasonal pattern of diseases

Many water−and foodborne diseases have a seasonal pattern. Seasonality is defined as a cyclic appearance of events over a period of time (course of a year) and in temperate latitudes is marked by three main factors: temperature, humidity and precipitation (Naumova, 2006). "The temporal variability in disease results of interactions between host susceptibility, periodicity in pathogen abundance and transmissibility, and the ever−changing environment that can support or repress a host or pathogen" (Naumova, 2006). The survival and transmissibility of pathogens are dependent on seasonal factors such as temperature, humidity and precipitation, when they combine to creat favorable conditions (e.g. high temperature, humidity, moisture) for pathogen proliferation and transmission (Naumova, 2006). Seasonal patterns of diseases often differs from location to location and can change over the years (Naumova, 2006). Lately, increased climatic variability may pose a higher risk of water and foodborne diseases at the various stages of the food chain, from primary production through to consumption. Climate−related factors such as changes in temperature and precipitation patterns, extreme weather events, ocean warming and acidification, can have an impact on the persistence and patterns of occurrence of bacteria and the patterns of their corresponding foodborne diseases, animal physiology and host susceptibility (Tirado et al., 2010). Seasonal host susceptibility is complex and not well defined or understood (Dowell, 2001; Naumova, 2006). Seasonal physiological changes occur in many mammalian species, which include reproductive and immunological alterations. The seasonal physiologic cycles are linked to light and dark cycles as mediated through the duration of daily melatonin release. Those physiologic responses might lead to higher susceptibility to

infections, because of an increased responsiveness of specific and nonspecific immunity, changes in the characteristics of mucosa surfaces and the expression of epithelial receptors (Dowell, 2001). The day−light length might even have an effect on *E. coli* O157:H7 seasonal shedding pattern in cattle. Cattle naturally infected with *E. coli* O157:H7 exhibit higher prevalences in lighted pens (Edrington et al., 2006). On the other hand, studies with melatonin were inconclusive regarding the physiological effect of day–light length on shedding in cattle (Edrington et al., 2008).

**Seasonality of foodborne pathogens in Humans**

Seasonal patterns of foodborne diseases in humans have been observed in temperate climates. For example human illness caused by *Salmonella spp* and *Campylobacter jejuni* tends to rise in summer and decrease in the winter (Naumova et al., 2007). Seasonal variation (or temporal variations) of those foodborne diseases has been correlated with oscillations of several environmental factors (Naumova et al., 2007). Among those environmental factors, ambient temperature has been consistently associated with human salmonellosis worldwide (Bentham and Langford, 2001; D'Souza et al., 2004; Fleury et al., 2006; Naumova et al., 2007). A linear association between ambient temperature and the number of human cases has been reported. In European countries, cases of salmonellosis increased about 5% to 12.5% for each one−degree increase in weekly temperature for ambient temperatures above 6 $^{\circ}$C (Kovats et al 2004). In Canada, the log relative risk of *Salmonella* weekly case counts increased by 1.2% for every degree increase in weekly mean temperature (Fleury et al., 2006). The association between the ambient temperature and incidence of human cases does not occur simultaneously. There is a

lag time between the change in ambient temperature and the respective onset of disease. Time series analysis studies of human salmonellosis in several in European countries, North America and Australia showed that, in general, human cases of salmonellosis increased 1–6 weeks after the peak in ambient temperature (Bentham and Langford, 2001; D'Souza et al., 2004; Fleury et al., 2006; Naumova et al., 2007; Lake et al., 2009). In United States, the peak of daily incidence of salmonellosis closely followed the peak in ambient temperature with a lag of 2–14 days in Massachusetts (Naumova et al., 2007). Short-term lag times between the peak of ambient temperature and illness could suggest that cross contamination and bacterial multiplication on food may occur from distribution systems to the point of consumption during food preparation (Bentham and Langford, 2001; Lake et al., 2009). Longer lag times were found in 5 Australian cities, a positive association between mean temperature of the previous month and number of salmonellosis notifications was found (D'Souza et al., 2004). In a Canadian study, the ambient temperature 0 to 6 weeks prior was found to be associated with the onset of human cases (Fleury et al., 2006). Long–term lag times might suggest the ambient temperature affects *Salmonella* at any point along the food chain, including at the farm, the slaughterhouse, distribution systems or the home (Bentham and Langford, 2001; D'Souza et al., 2004; Fleury et al., 2006; Lake et al., 2009). Lastly, we cannot ignore the fact that higher ambient temperature during the summer may influence consumer behavior; for example, via riskier cooking practices (e.g. barbecue) or consumption of raw foods (e.g. fruit or salads, uncooked meat) (Lake et al., 2009). Overall, the association between the mean and highest temperature several weeks prior the onset of the human cases suggest a hypotheses that the ambient temperature might affect the *Salmonella* dynamics at the farm level. Those effects might be mediated either by creating an environment favorable for the proliferation of bacteria in the environment and consequently

increasing the bacterial pressure and exposure to the livestock animals, or by increasing the animals's susceptibility to new infections or inducing recurrence of existing infections.

**Seasonality of salmonellosis in swine**

A number of studies have investigated the seasonality of *Salmonella* infections in swine with mixed results. Earlier studies based on the Danish *Salmonella* Surveillance and Control Program reported a seasonal pattern in *Salmonella* infection, with higher seroprevalence noted during the winter and fall (Carstensen and Christensen, 1998; Christensen and Rudemo, 1998; Hald and Andersen, 2001). However, more recent Danish studies have showed no seasonality of *Salmonella* seroprevalence. The distribution of *Salmonella* seroprevalence in meat juice (Danish–Mix ELISA) of slaughtered pigs did not follow a consistent seasonal pattern in a10–year time–series study. The authors explained that the differences observed with respected to the previous studies were likely due to the larger number of years included in this study. Consequently, they infered there was no need for targeted sampling during particular times of the year (Benschop et al., 2008b). In a second study, no seasonality was found in pooled carcass cultures (over six–year period) in Danish pig abattoirs (Baptista et al., 2010b). These findings are in contrast with those found by Hald et al. (2001) in a five–year study, which showed that the seroprevalence of *Salmonella*–positive slaughter pigs peaked in late winter and early fall, suggesting that the peak in swine seroprevalence was due to new infections that occur primarily in the late summer. In the same study, *S.* Typhimurium human incidence and pork prevalence started increasing in spring and peaked in late summer. Interestingly, late summer peak prevalence in pork appeared three to four weeks before the peak in human cases. Actually, the

weekly number of human *S.* Typhimurium cases was associated with the prevalence level in pork samples one to five weeks before case registration (Hald and Andersen, 2001). This suggests a potential impact of the thermal environment on *Salmonella* during distribution or in primary production. Nevertheless, the seroprevalence is representative of historical exposure to the bacteria that can occur at any point on the farm. In addition, pig *Salmonella* carcass contamination might result either from the intestinal carriage of *Salmonella* in the pig itself or cross−contamination in slaughter. Therefore, the disparity of the findings in those studies might be attributable to differences of unknown/unmeasured factors associated with farm and slaughter infections and/or contamination.

Seasonal peaks of *Salmonella* prevalence in pigs have been identified by other research groups in the US, UK and Belgium (Funk et al., 2001a; Hautekiet et al., 2008; Smith et al., 2010). Finishing pigs with higher *Salmonella* fecal prevalence were at greater odds of having been sampled in winter and spring in a North Carolina study (Funk et al., 2001a). Smith et al. (2010) reported higher seroprevalence during the fall in slaughter pigs in a UK monitoring Zoonosis Action Plan (ZAP) program (Smith et al., 2010). In contrast, Hautekiet et al. (2008) found that sampling in summer was associated with higher seroprevalence (Hautekiet et al., 2008).

It is evident that discrepancies in the seasonal patterns seen among various studies and countries exist. Season is a broad concept and seasonal patterns can vary from time to time and from region to region; consequently, patterns might easily change from country to country and even within. Seasonal cycles of infectious diseases involve complex interaction between three groups: pathogen appearance and disappearance, environmental changes, and host behavior changes (Dowell, 2001). In the case of livestock production systems, the host behavior changes

are dependent on management factors. Moreover, management practices might themselves exhibit seasonal variability and consequently affect the seasonality of *Salmonella* in swine (Hald and Andersen, 2001). Therefore, it is possible that some unobserved factors such as management may affect the seasonal pattern of *Salmonella* infection in swine. In addition, management practices specific to production system type vary from country to country and can contribute to different *Salmonella* seasonality patterns in swine. Another aspect that must be taken into account is the type of diagnostic test used to measure *Salmonella* infection (seroprevalence versus fecal culture). *Salmonella* infection and fecal shedding in swine might have seasonal peaks, which eventually were not found in corresponding seroprevalence peaks, because of antibodies persistency for long periods after the animal being infected.

**Thermal environment factors associated with salmonellosis in swine**

Season is characterized by environmental changes of temperature, humidity, precipitation and wind (Dowell, 2001; Naumova, 2006). Environmental factors such as temperature, rainfall and sunshine have been associated with *Salmonella* prevalence in swine. Finishing pigs exposed to wide variation in daily temperature high were at greater risk of *Salmonella* shedding (Funk et al., 2001a). In addition, large differences in long–term averages in the monthly mean temperature, as well as high actual rainfall and hours of sunshine were associated with higher *Salmonella* seroprevalence in UK pigs (Smith et al., 2010). In both studies the environmental parameters were retrieved from the closest weather station. Therefore, the environmental parameters might not reflect the actual environment in the barns.

Herds that had controlled and programmed barn temperatures above upper critical values (> 26°C, for pigs of 90 kg) had a higher seroprevalence (defined as average S/P) compared with herds with controlled programmed barn temperatures within the thermal neutral zone (Hautekiet et al., 2008). Herds with no temperature programming in the swine facilities (i.e. controlled temperatures) in fattening units had the highest *Salmonella* seroprevalence (Hautekiet et al., 2008). Controlled temperature and ventilation systems are important features of facilities to keep the animals in their thermo neutral zone in order to promote production and overall swine health. Nevertheless, controlling the environment of barns can be a challenge to keep them within optimal temperature ranges during certain seasons. Environment factors (e.g. temperature, rainfall and sunshine) might affect not only the host (pig) but also the sources and the survival of the pathogen in external environment. Certain environmental factors might support the survival and proliferation of *Salmonella* in the environment and consequently increase the bacterial exposure in the herd. Thus, increased prevalence associated with the thermal environment may be a combination of host susceptibility and increased exposure to the pathogens in environment.

**Thermal neutral zone and thermal stress**

Sub-optimal temperature and temperature variability appear to be an important factors associated with *Salmonella* infection in swine (Funk et al., 2001a; Funk and Gebreyes, 2004; Hautekiet et al., 2008; Smith et al., 2010). One of the biological explanations is that sub-optimal temperature might increase pig stress, which can lead to low immunity and increase susceptibility to new infections and recrudescence of *Salmonella* carriers (Funk et al., 2001a;

Hald and Andersen, 2001; Smith et al., 2010). The mechanisms behind of increased risk of infection when pigs are exposed to stress are complex and partially unknown (Mulder, 1995; Berends et al., 1996). Stress is generally considered to suppress the immune system and may lead to an increase of the occurrence of diseases (Salak-Johnson and McGlone, 2007). The term stress is broad and not well defined; everything that disrupts the normal state of the well being of the animal can be considered stress. Stress was initially defined as "exposure to nocuous environmental factors (stressors) elicits a nonspecific reaction, this reaction is characterized by enhanced pituitary-adrenal reactivity and facilitates the return to homeostasis" (Dantzer and Mormede, 1983). Lately, stress has been defined based on the type of insult, response to the aggression and effects on host. There are numerous environmental challenges, not only traditional environmental stressors (e.g. heat, cold, humidity, pollutants) but also the social environment that can cause disrupt the animal balance and consequently lead to a stress response in animals (Salak-Johnson and McGlone, 2007). Stress responses include physiologic, endocrinologic (hormonal), behavioral and production responses. Stress can be categorized in groups such as social stress, transport stress, environmental stress (e.g. temperature and humidity), and feed–related stress (e.g., feed withdrawal).

Animals have a range of comfortable temperatures within which they are able to maintain a relatively stable body temperature via behavioral and physiological means (Gaughan et al., 2008a) called the thermal neutral zone (TNZ) (Ames, 1980; Gaughan et al., 2008a). The TNZ is defined as a range of ambient temperatures (upper and lower critical temperatures; UCT and LCT, respectively) at which temperature regulation is achieved simply by control of sensible heat loss (i.e., without regulatory changes in metabolic heat production or evaporative heat loss) (Gaughan et al., 2008a). The term thermoneutrality can have different meanings in the

literature: 1) the range of environmental temperatures in which heat production remains basal, 2) range of environmental temperature over which the body temperature is normal and remains normal while sweating and panting do not occur, 3) the range of environmental temperature that provides a sensation of maximum comfort, and 4) the preferred thermal environment by the animal, 5) the optimal environment in which the animal have optimum health and performance (Ames, 1980). The different definitions are used depending on the outcome or the reason for describing thermoneutrality.

The thermo neutral range of air temperature is dependent on age and weight in swine (Jacobson et al.; Harmon and Hongwei, 1995) (Table 1.1). Younger pigs have a narrower TNZ range, and they are more susceptible to cold temperatures. Older pigs have a wider TNZ, and are less tolerant to higher temperatures. The UCT, LCT and TNZ are influenced by insulation (animal and external insulation), breed, nutrition, exercise, production, physiological status and health (Ames, 1980; Young et al., 1989; Gaughan et al., 2008a). Although the effective temperature depends mainly on the ambient air temperature, other factors have an important effect on effective temperature. Velocity of ambient air (wind and drafts), floor type, wet surfaces, bedding and building materials, and relative humidity all have an impact on the thermal environment of the animal (Young, 1981; Gaughan et al., 2008b). In addition, animal behavior (e.g., grouping, huddling) affects the temperature experienced by the animal (Young, 1981). Effective ambient temperatures below the LCT result in cold stress and those above UCT in heat stress (Ames, 1980; Gaughan et al., 2008a). Cold stress is due to the incapacity of the animal to increase its heat production and the losses to the surrounding environment are greater than the heat production rate (Young, 1981). Heat stress results from the animal's inability to dissipate sufficient heat or reduce the heat influx to maintain homeostasis of the

animal (homeothermy) (Gaughan et al., 2008a). From here forward, both (cold and heat stress) are addressed as the general term thermal stress, unless otherwise mentioned.

Temperature and humidity work in conjunction to effect overall environmental conditions on the animal. Thermal stress is caused by a combination of environmental factors including temperature, relative humidity, solar radiation, air movement, and precipitation (Bohmanova et al., 2007). Water vapor content of the air is an important factor because it has an impact on the rate of evaporative loss through the skin and lungs. The amount of moisture in the air is particularly important when the air temperature is outside of the comfort zone of the animal. Three measurement types are used by meteorologists to quantify water vapor content: 1) wet bulb temperature ($T_{wb}$), represents the equilibrium temperature of a thermometer covered with a cloth that has been wetted with pure water, relative humidity (RH), gives information about saturation of the air at a given temperature, dew point temperature ($T_{dp}$) is the temperature to which the air must be cooled for saturation to occur; and dry bulb temperature ($T_{db}$) refers to 'normal' air temperature (Bohmanova et al., 2007). Thus, thermal stress has been evaluated using both the TNZ temperature values and temperature–humidity–index (THI). THI combines within the same formula both air temperature and humidity (Lucas et al., 2000; St-Pierre et al., 2003; Bohmanova et al., 2007). There are numerous THI formulas with different weightings of dry bulb temperature, dew or wet point temperature. The most adequate formula to express the heat stress depends upon the species, production parameters to be measured, and climatic conditions (Lucas et al., 2000; St-Pierre et al., 2003; Bohmanova et al., 2007). Lucas et al., (2000) compared two THI formulas ($TH_1 = 0.72t_{wb} + 0.72t_{db} + 40.6$ and $THI_2 = 0.63t_{wb} +$

$1.17t_{db} + 32$, where $t_{db}$ and $t_{wb}$ were the dry and wet bulb temperatures of the ambient air in °C) to determine heat stress in swine during summer. Formula 2 predicted heat stress better especially under extreme conditions. A scale indicating the range of different combinations of temperature and humidity was published by NWSCR (National Weather Service Central Region, 1976) predicting the relative safety ranges of THI for livestock. The normal values for confined livestock were considered ≤ 74; alert values were those from 75 to 78, danger values those from 79 to 83, and emergency values were thoses ≥ 84 (Lucas et al., 2000). Temperature humidity index > 72 caused heat stress in growing–finishing pigs and consequently decreased dry matter intake with economic losses (St-Pierre et al., 2003).

Thermal challenges (thermal stressors) range from cold to hot and are life–cycle dependent (Nienaber and Hahn, 2007). The intensity and duration of the exposure to a given thermal stress factor will also determine animal responses (Gaughan et al., 2008a). Responses of animals vary according to the type of thermal challenge; that is acute events result in short–term adaptive changes in behavioral, physiological and immunological responses, while longer–term challenges will impact related performance responses (e.g., altered feed intake and heat loss which affect growth, reproduction and efficiency). When the thermal stress passes a certain threshold disrupted behavior is observed, with impaired immunity and physiology (Nienaber and Hahn, 2007). Acclimatization is observed through changes in physiological, immune and adapted performance when animals are challenged with moderate thermal stressors (Nienaber and Hahn, 2007; Gaughan et al., 2008a). The type of response and the lag time after the thermal challenge onset varies according to the insult (intensity and duration), and to the ability of the animal to recover (Nienaber and Hahn, 2007; Renaudeau et al., 2008). Genetics

might also play a role in thermal stress responses. New genetic lines of high–lean growth swine have become more susceptible to heat stress, because the total heat production is significantly higher compared with other lines (Brown-Brandl et al., 2001).

## Effects of thermal stress in swine

### Physiological changes

The effects of thermal stress on swine have been extensively studied and published in the literature. The majority of studies have been focused on heat stress. It is well established that heat stress causes physiological, behavioral and performance changes in pigs. There is no uniformity in terms of the threshold of the thermoneutrality, the duration of the exposure and lag time to observe changes in animals. Some authors defined heat stress based on a single value (such as 33°C) outside of the thermo neutral range (Collin et al., 2001). Other authors have investigated a range of temperatures with or without a period of adaptation (Brown-Brandl et al., 2001; Renaudeau et al., 2008), or have included the effect of humidity as well (Huynh et al., 2005b). Thus, summarizing the effects of thermal stress based on the literature is a challenge. The following review does not intend to be an exhaustive description of those effects but an overview of the range of effects of heat stress observed in swine.

Animal behavior and physiological changes occur when pigs are exposed to heat stress (Collin et al., 2001; Huynh et al., 2005b). The initial indicators of heat stress are increased respiration rate and water−to−feed consumption ratio, followed by decreased heat production and feed intake, and finally increased rectal temperature (Brown-Brandl et al., 1998; Brown-Brandl et al., 2001; Huynh et al., 2005b; Renaudeau et al., 2008). The time spent lying down

versus eating increases with a rise of ambient temperature (Collin et al., 2001; Aarnink et al., 2006). High temperature greatly affects the laying and excreting behavior; temperature is inversely related to huddling and positively related with wallowing, and the total number of bodily excretions increases with temperature (Huynh et al., 2005a; Aarnink et al., 2006). These behavior changes of the animal are an attempt of self–cooling when exposed to high temperatures.

These behavioral changes also might increase contact with fecal material and as a consequence, increase the risk of transmission of fecal–oral diseases as *Salmonella*. On the other hand, pigs exposed to cold stress show behavioral changes such as an increase in standing and feeding times, a decrease in laying and an overall increased activity (Hicks et al, 1998). How these differences in behavior might be related to susceptibility to infectious diseases or transmission rates is unknown.

**Production and reproductive changes**

Production and reproductive performance are affected when heat stress is present for extended periods. Growing pigs have reduced feed intake with a corresponding reduction in growth rate after exposure to heat stress (Brown-Brandl et al., 2000; Collin et al., 2001; Huynh et al., 2005b; Renaudeau et al., 2008). Average daily feed intake decreases about 100g/day each each 1 °C increase in ambient temperature between 24 °C and 36 °C, and from day 0 to 20 of heat stress exposure. In addition, the reduction of average daily gain during the same period is 55g/°C (Renaudeau et al., 2008). Moreover, a high relative humidity combined with high

temperature significantly affects the average daily gain, because it limits the animal's ability to dissipate heat via evaporation and accentuates the effect of heat stress (Huynh et al., 2005b). Higher fat deposition and lower protein deposition are observed in carcasses of pigs exposed to heat stress (Brown-Brandl et al., 2000). Heat stress affects the reproductive performance of sows and decreases milk yield and piglet growth rate (as a consequence of the reduction in milk yield) (Black et al., 1993; Bloemhof et al., 2008).

**Immune response changes**

Stress, including heat and cold stress, affects the immune response in swine. Exposure to stressors activates the hypothalamic–pituitary–adrenal axis, leading to a release adrenocorticotropic hormone (ACTH) and glucocorticosteroids into the blood of stressed animals (Dantzer and Mormede, 1983; Hicks et al., 1998). The levels of plasma corticosteroids (e.g., cortisol) increases when pigs are exposed to stress factors such as social stress, electrical stimulation, heat stress, and feed and water deprivation (Hicks et al., 1998). Cellular immune response is also affected by stress. Social and heat stress affect the cellular immune response by changing neutrophil and lymphocyte profiles, reducing cell proliferation, and reducing natural killer cell cytotoxicity (Morrowtesch et al., 1994; Hicks et al., 1998). In addition, the response to endotoxin is compromised in challenged piglets exposed to cold stress (Carroll et al., 2001). Those immune responses might compromise the immunologic defense and increase susceptibility to new infection with foodborne pathogens in farm animals (Rostagno, 2009).

In addition to the immune response, a complex network of interactions between the central nervous system, the enteric nervous system and the gastrointestinal tract is observed in

stressed animals. Those interactions include: the release of neuroendocrine and stress mediators (e.g. , glucocorticoid hormones and the catecholamines epinephrine and norepinephrine) that can have a significant effect either on the immune system and/or the gastrointestinal tract. The release of catecholamines during stress results in decreased gastric acid production, delayed gastric emptying, and accelerated internal motility and colonic transit and increased stomach pH. These changes can lead to a higher susceptibility to new infections due to greater survival and colonization of foodborne pathogens in gastrointestinal tract (Rostagno 2009). Recent research on microbial endocrinology has suggested a more complex interaction between the catecholomines released in gastrointestinal tract and bacteria growth and virulence factors (Freestone et al., 2008; Lyte et al., 2011).

In summary, stress results in a complex interaction between central nervous, immune and gastrointestinal systems that can predispose animals to a new infections or re–activate previous infections of several foodborne diseases in livestock species. The causal pathway between stress and foodborne pathogen infection is complex and involves central nervous system (CNS), gastrointestinal responses and interactions between bacteria and host (Rostagno, 2009). The interaction between the host, pathogens and environment are complex and imply host and pathogen adaptation mechanisms. In addition, other factors such as management can alter those mechanistic responses (Figure 1.1).

**Stress and food pathogens in swine**

Thermal stress may cause changes in gastro-intestinal bacterial species, genotypes and antimicrobial resistance phenotypes in swine. The relationship between thermal stress and the intestinal microflora of swine has been mainly reported with *E. coli* infections (Moro et al.,

1998; Moro et al., 2000; Jones et al., 2001; Mathew et al., 2003). Moro et al. (1998) investigated the effect of cold stress on the prevalence of antimicrobial resistance in *E. coli* from swine feces on a farm where no antimicrobial had been used in feed during the previous 10 years. Exposure to cold stress caused significant increases in ampicillin and tetracycline resistance of *E. coli* isolates in pigs exposed to a drop in temperature of at least 15°C within 24h before the sampling, compared to pigs within normal and stable temperatures. The cold stress samples were collected during the winter, below the lower critical temperature (10°C) of thermoneutrality for finishing pigs (Moro et al., 1998). In another study, pigs experimentally infected with enterotoxigenic *E. coli* (ETEC) have a significant increase in fecal shedding of ETEC when exposed to cold temperatures, compared with a non–stressed group (Jones et al., 2001). Heat stress has been associated with antibiotic resistance of *E. coli* as well. In an experimental study, a significantly higher proportion of *E. coli* collected after heat stress (34°C for 24h) were resistant to single or multiple antibiotics (amikacin, ampicillin, cephalothin, neomycin and tetracycline) as compared to samples from the same pigs pre–heat stress (Moro et al., 2000). In addition, only 25% of the pre–stress isolates showed multiple antimicrobial resistance patterns (equal or greater than two antimicrobial); in contrast to 85% of the isolates of post–stress having multiple resistances. Furthermore, a significant difference was observed for tetracycline resistance between isolates obtained from carcasses of a non–stressed group (40%) versus a stressed group (80%), suggesting that stressed animals were shedding higher numbers of resistant bacteria that subsequently contaminated the carcasses (Moro et al., 2000). Mathew et al. (2003) investigated the effect of cold stress on pigs fed with apramycin and the

antimicrobial resistance of fecal *E. coli*. Cold stress extended the duration of increased antimicrobial resistance to apramycin as compared to pigs kept at thermal neutral temperatures. Heat stress in combination with feeding apraymicin transiently increased the proportion of *E. coli* resistant to apramycin as compared to pigs fed apramycin and kept at a thermal neutral temperature (Mathew et al., 2003).

The mechanisms responsible for the increased antimicriobial resistance and bacterial growth when pigs were exposed to thermal stress are largely unknown. The thermal stress might result in physiological changes in the gastrointestinal tract such as motility, pH, and fermentation acid concentrations, which may then impact the gut flora. Those environmental changes may lead to an increased ability of bacteria to acquire resistance genes or allow the proliferation of resistance bacteria in gut microflora (Mathew et al., 2003). In addition, enteric bacteria respond directly to stress–related neuroendocrine hormones such as catecholamines that promote the enteric bacterial growth and alter the interactions between the intestinal mucosa and intraluminal microorganisms. The stress can influence the bacterial infectivity in non–immune manner, due to complex interactions designated by microbial endocrinology (Freestone et al., 2008; Rostagno, 2009; Lyte et al., 2011). The enteric growth and antimicrobial susceptibility of enteric flora alterations in response to thermal stress is the result of complex host–microbe interactions between the pig's physiology and the bacterial flora of the gastrointestinal tract.

Other stress factors have been associated with antimicrobial resistance and gastrointestinal pathogens shedding in swine. Social stress, transport, and feed and water withdrawal have each been associated with increased *Salmonella* and *E. coli* shedding in swine. Changes in antimicrobial resistance have been reported after transport and holding stress (Langlois et al., 1986; Molitoris et al., 1987) as well as after moving animals into and out of

their pens (Hedges and Linton, 1988). Weaning, mixing and handling all increased the ETEC and generic *E. coli* shedding in pigs (Jones et al., 2001; Dowd et al., 2007). Callaway et al. (2006) showed that social stress increased fecal shedding of *S.* Typhimurium in early weaned piglets (Callaway et al., 2006). This is particularly important because segregated early weaned piglets and re−grouping of piglets are practices that have been widely adopted by the swine industry; thus, the mixing of piglets might lead to social stress and consequently may increase the susceptibility to infections and shedding of *Salmonella*.

*Salmonella* shedding is significantly increased during transport and lairage (Berends et al., 1996; Hurd et al., 2002; Larsen et al., 2003). Transportation of pigs causes several levels of stress; each of crowding, social status, duration of trip and feed deprivation can affect the *Salmonella* shedding status during transportation. Factors such as high animal density, stress, and feed deprivation during transport can have a strong influence on the *Salmonella* status of pigs (Berends et al., 1996). Isaacson et al. (1999) demonstrated that pigs experimentally infected with *S.* Typhimurium had increased shedding after transportation. In contrast, Rostagno et al. (2005) did not find a difference in prevalence estimates when compared before and after transportation from farm to abattoir. Factors than transportation seem to contribute to the higher shedding observed after transport and lairage. Feed withdrawal is a common practice before pigs are transported to slaughter, in order to decrease the risk of carcass contamination during the evisceration (Martin-Pelaez et al., 2009). Increased pre-slaughter feed withdrawal and lairage times lead to cecal fermentation changes, increased pH and decreased concentrations of short chain fatty acids, and as a consequence there are increased numbers of Enterobacteriaceae including *Salmonella* in market pigs (Martin-Pelaez et al., 2008; Martin-Pelaez et al., 2009). On the other hand, a resting period on the transport vehicle decreased *Salmonella* shedding in pigs

(Rostagno et al., 2005b). In summary, several stress factors during transportation may contribute to higher *Salmonella* shedding after transportation.

Stress is a broad term used to identify a range of situations which alter the animal's homeostasis. Stressors such as transportation, social stress, and feed withdrawal are associated with increased *Salmonella* shedding in swine (Berends et al., 1996; Callaway et al., 2006; Martin-Pelaez et al., 2009). There is a lack of studies that evaluate the association between *Salmonella* shedding and sub–optimal thermal environment. One possible casual pathway for effects of the sub–optimal thermal environment on *Salmonella* shedding is in following diagram (Figure. 1.2). However, the causal pathway is not simple, due to the fact the stressors occur in combination and responses to stress involve multiple interactions between nervous, endocrine and immune systems. In order to address at least one component of this causal pathway, this study has as an objective to investigate the association between exposure to a sub–optimal thermal environment and *Salmonella* shedding in finishing swine.

# APPENDIX

**Table 1.1.** Upper and lower critical temperature criteria of thermal neutral zone of finishing pigs[a] used to assess the thermal (heat and cold) exposure.

| Pig age (weeks) | Pig weight (kg) | Lag time 12h, 24h, 48h, 72h, 1 week | | Lag time 1 month | |
|---|---|---|---|---|---|
| | | LCT[b] | UCT[c] | LCT[b] | UCT[c] |
| 10 | 25 | 21.1 | 27.8 | 22.8 | 27.8 |
| 12 | 36 | 18.9 | 26.7 | 21.1 | 27.8 |
| 14 | 47 | 16.7 | 26.7 | 18.9 | 26.7 |
| 16 | 58 | 14.4 | 26.7 | 16.7 | 26.7 |
| 18 | 70 | 13.3 | 26.7 | 14.4 | 26.7 |
| 20 | 85 | 12.2 | 26.7 | 13.3 | 26.7 |
| 22 | 98 | 12.2 | 26.7 | 12.2 | 26.7 |
| 24 | 109 | 11.1 | 26.7 | 12.2 | 26.7 |

[a] Adapted from Harmon and Hongwei, 1995.

[b] Lower critical temperature (°C)

[c] Upper critical temperature (°C)

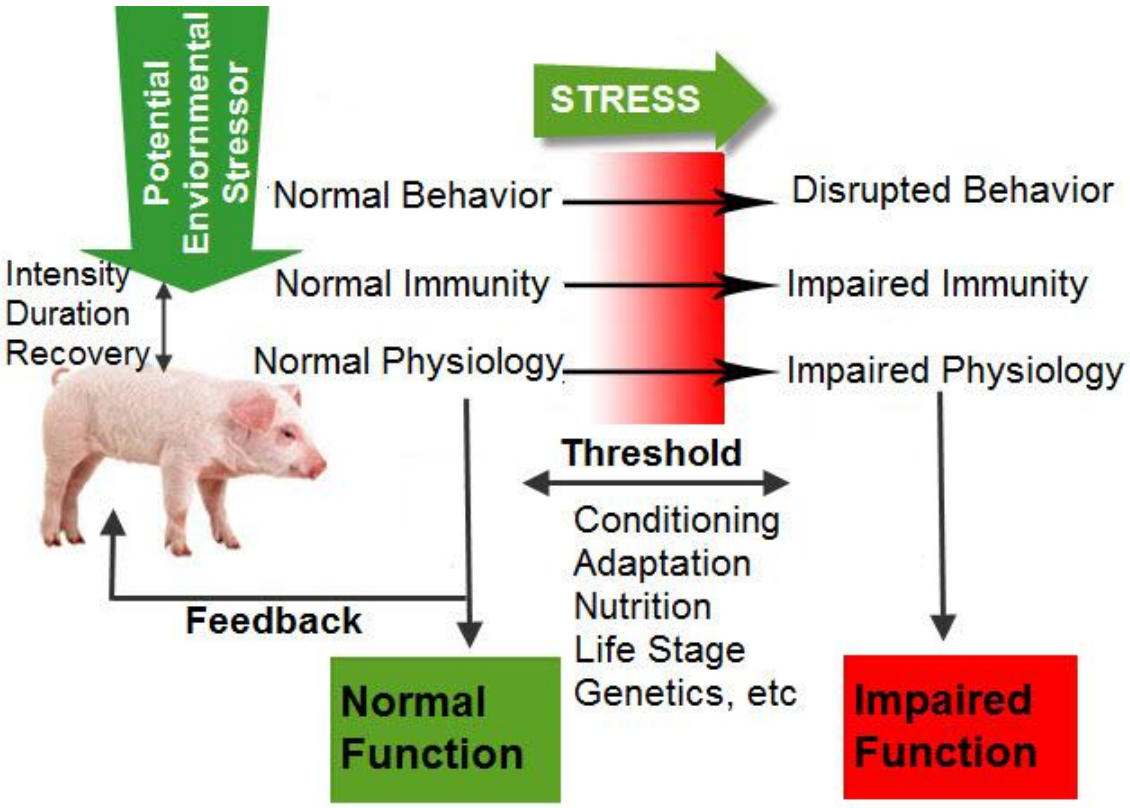


**Figure 1.1.** Responses of swine to potential environmental stressors that can have an effect on production, immunity and animal health (Adapted from Nienaber et al. (1999)). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

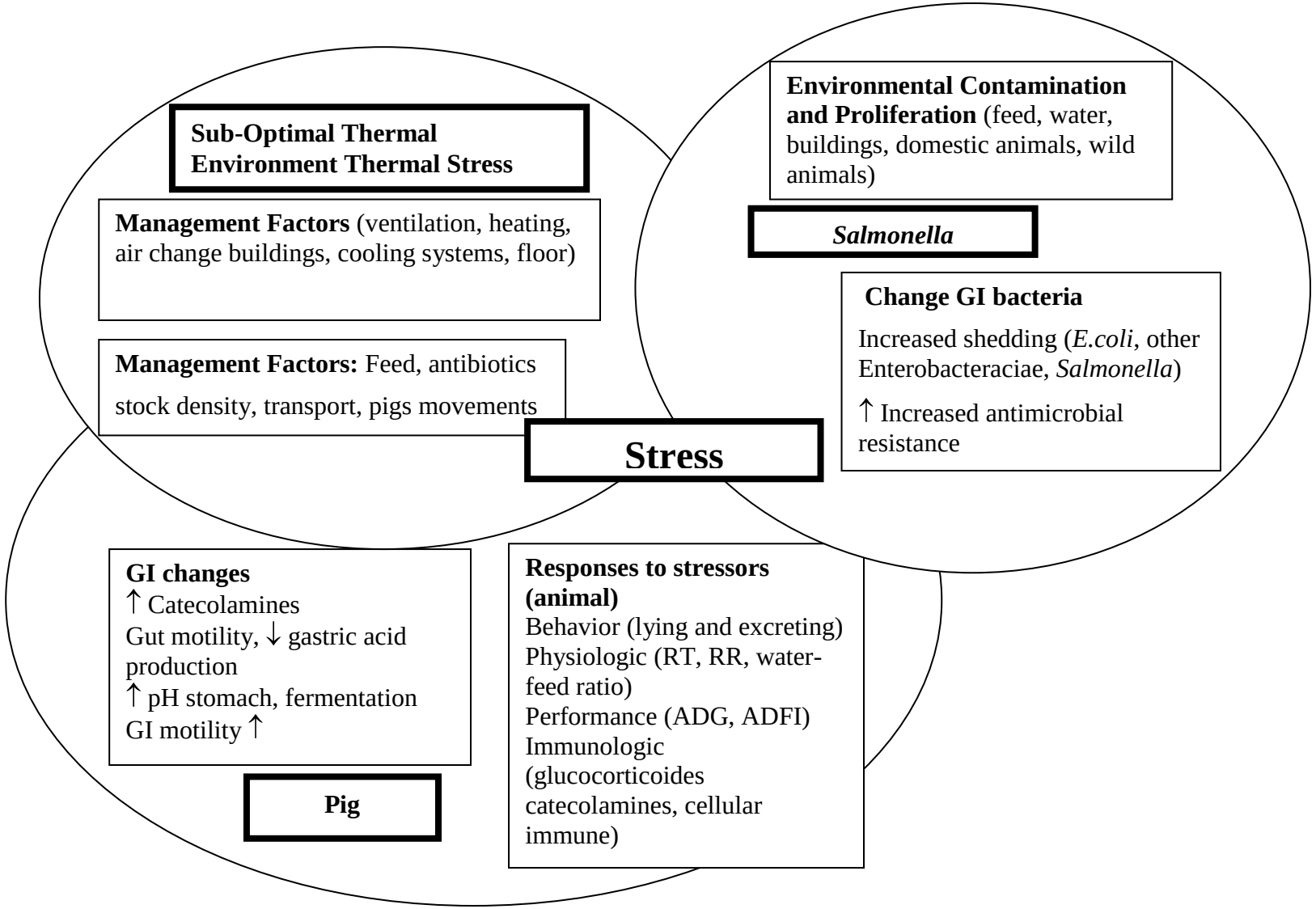


**Figure 1.2.** Potential casual pathway of the effect of suboptimal thermal environment on *Salmonella* shedding in swine.

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# CHAPTER 2

## Longitudinal study of *Salmonella* shedding in naturally infected finishing pigs

Manuscript based on these data was published on Epidemiology and Infection (Pires, A.F.A., Funk, J.A., Bolin, C. A. 2012. Longitudinal study of *Salmonella* shedding in naturally infected finishing pigs. Epidemiol.Infect. (Published online on November13$^{th}$ 2012))

## ABSTRACT

A 3 year longitudinal study was conducted on a multi-site farrow–to–finish production system. For each of 18 cohorts at three finishing sites, 50 pigs were randomly selected. Fecal samples were collected every 2 weeks for 16 weeks. *Salmonella* was cultured from 453 (6.6%) of 6836 fecal samples. The pig–level incidence of *Salmonella* was 20.8% (187/899 pigs). *Salmonella* prevalence varied between both sites and cohorts within sites. The proportion of positive samples decreased over the finishing period from 12.9% to 2.8%. Intermittent detection of *Salmonella* was found in more than 50% of pigs that were positive at more than one collection. The finding that the majority of pigs shed intermittently has implications for surveillance and research study design when determining *Salmonella* status. The variability in shedding over–time, as well as between and within sites, cohorts and pigs suggest that there may be time variant risk factors for *Salmonella* shedding in swine.

## INTRODUCTION

It has been well documented that *Salmonella species* are one of the major causes of foodborne diseases in the US and worldwide (Greig and Ravel, 2009; CDC, 2011; Scallan et al., 2011). In the US alone, it is estimated that 1,027 million nontyphoidal *Salmonella* human infections result in 19,336 hospitalizations and 378 deaths annually (Scallan et al., 2011), costing $ 365 billion in direct medical expenditures per year (CDC, 2011). Swine are a potential reservoir for human salmonellosis. The most common serotypes isolated in swine (*S.* Typhimurium, *S.* Heidelberg, *S.* Agona, and *S.* Infantis) are common to those found in human cases (Foley et al., 2008; CDC, 2010). It has been suggested that reduction of *Salmonella* contamination of pork requires interventions at three levels: pre−harvest (farm), harvest (slaughter) and post−harvest (distribution systems and consumer handling) (Lo Fo Wong et al., 2002; Boyen et al., 2008). In order to put in place on−farm control and intervention measures it is crucial to understand *Salmonella* infection dynamics in swine.

A large number of epidemiological studies have been conducted to determine prevalence and risk factors for *Salmonella* infection in swine. Most of these studies have used a cross−sectional study design. A limited number have assessed the fecal prevalence over time, with longitudinal studies showing high variability in *Salmonella* shedding at the farm, cohort and individual animal level (Funk et al., 2001; Beloeil et al., 2003; Kranker et al., 2003; Nollet et al., 2005; Rajic et al., 2005; Farzan et al., 2008; Dorr et al., 2009; Rostagno et al., 2012). Longitudinal studies at the pig level during the finishing phase have reported time variability of fecal shedding associated with cohort (or batch) of pigs (Funk et al., 2001; Beloeil et al., 2003; Kranker et al., 2003; Nollet et al., 2005). Intermittent fecal shedding is also commonly reported

in epidemiological studies of swine (Beloeil et al., 2003; Kranker et al., 2003; Nollet et al., 2005).

Therefore, longitudinal studies at the individual level based on bacteriological culture should be performed in order to investigate the dynamics of *Salmonella* infection in swine. The objective of this study was to describe the shedding pattern of *Salmonella* in feces of naturally infected finishing pigs.

## MATERIALS AND METHODS

A longitudinal study was conducted on a multi-site farrow–to–finish production system located in the Midwestern United States. The presence of *Salmonella* in the system had been confirmed by culture of pooled fecal samples prior to initiation of the study. Selection criteria for the production system were willingness to cooperate in a long–term research project and to share health management and production records. The production system had three–site management, meaning that overall production was separated into three stages of production, breeding and farrowing, nursery (weaning until approximately 10 weeks of age) and finishing (10 weeks to slaughter, 24 to 26 weeks), with each stage housed at separate sites. The production system had all–in all–out management in nursery and finishing sites. This system consisted of 2 farrowing sites (F1 and F2), 2 nursery sites (N1and N2), and 12 finishing sites. The farrowing sites had a total inventory of 3700 sows (F1=1300, F2=2400), the average one–time inventory of the finishing sites was 25000 (75000 finishing pigs/year marketed). During the study period the system transitioned from 2 farrowing sites to one farrowing site of 3000 sows. The number of nursery and finisher sites remained unchanged. Three finishing sites (A, B, and C) were conveniently selected, based on building design and willingness to participate in the study. At each finishing site one barn was selected for study inclusion. Site A had four barns in separate buildings. Pigs were allocated into 40 pens (20–25 pigs per pen). Sites B and C had identical building structures. Each site had four barns grouped in two buildings (two barns/rooms with one shared wall). Each barn housed approximately 1000 pigs. Pigs were housed in 12 pens; ten pens were initially stocked with pigs at placement (eight pens with a range of 100–125 pigs and two pens with a range of 40–50 pigs). The remaining two pens were

used for sick pens or pigs deemed to be at risk for illness. Sites A and B were finishing sites (10–26 weeks of age). Site C transitioned to a weaning to finishing site after the second cohort of pigs. For the wean to finish cohorts at site C, piglets were placed in the barn at 3 weeks of age and remained until marketing. Finishing site A received pigs from nursery N1 in all cohorts; site B received pigs from nursery N2 in 4 cohorts and from N1 and N2 in the last 2 cohorts of pigs. The first cohort for site C was supplied from nursery N2, for all other cohorts piglets were placed directly from the farrowing sites due to the transition to a weaning to finishing site.

## Sample collection

### Nursery sampling

In order to evaluate the *Salmonella* status of the cohort of pigs prior to sampling during the finishing phase, ten pools were collected from the nursery rooms approximately one week prior to movement to the finishing barn. A pool consisted of a minimum of five g of fresh fecal material collected from five different locations on the same pen floor (25g/pool). In the wean to finishing site (site C), ten pools were collected from ten random pens when pigs were approximately 9 weeks of age.

### Environmental sampling

Finisher barns were cleaned and disinfected between batches of pigs. The disinfectants (Synergize, Preserve International, Reno, NV, USA and VirkonS, Antec International, Suffolk, UK) were alternated following the standard operating procedure of the production system. In order to assess contamination, culture of environmental samples was performed after cleaning

and disinfection and before placement of pigs in the barn. Drag swabs and environmental swab samples were obtained from cleaned and disinfected floors, walls, gates and feeders/drinkers following previously described methods (Kingston, 1981). Briefly, swabs were moistened with ten ml of sterile buffered peptone water (BPW, Acumedia, Neogen Corporation, Lansing, MI,USA) before the collection. To sample floors, one drag swab was used for four pens in site A. In sites B and C, one drag swab was used per one to two pens depending on pen size. To sample other environmental surfaces, a single 4X4 gauze moistened with BPW was used to sample each surface. Ten, 5, 3 and 2 swabs were collected from floors, walls, gates and feeders/waterers, respectively in each barn prior to every cohort.

**Individual fecal sampling**

At the beginning of each cohort, 50 pigs (10 ± 2 weeks–old) were randomly selected and individually identified with ear tags. Random number generation was conducted in Microsoft Excel 2007 (Microsoft, Redmond, WA, USA). In site A, a simple random sample was generated to select one pig per every pen (n=40). Another ten pens were randomly selected to identify and select a second pig using a simple random sample (additional ten pigs for a total of n=50/barn; 30 pens with one study pig, ten pens with two study pigs). A random proportional sampling scheme based on the number of pigs in each pen was conducted in sites B and C within each cohort. A range of one to seven pigs per pen was selected. In sites B and C no pigs were selected for study inclusion from the pens identified for sick or at-risk pigs.

Individual fecal samples were collected from the rectum with gloved hand, and placed in sterile containers (Specimen cups, VWR International LLC, PA, USA). Gloves for collecting

the feces were changed between pigs. After collection, samples were stored at ambient temperature for transport to the laboratory. Individual pig fecal samples were collected every 2 weeks for 16 weeks (eight total sampling periods per pig). A total of 400 individual samples (50 pigs X 8 sample periods) per cohort and 7200 fecal samples overall (400 samples X 18 cohorts) were planned for collection.

## Bacteriological culture

### Fecal samples

Bacteriological culture for *Salmonella* was performed by the Diagnostic Center for Population and Animal Health, Michigan State University. Fecal samples were transported to the laboratory the day of collection, or stored for 48 hours at 2.8$^{o}$C.

Fecal samples were cultured using standard enrichment methods from Davies et al. (2000). Briefly: for pooled samples from the nursery, 25 g of the pooled fecal samples were diluted in 225 mL of Tetrathionate broth (TTB) (Becton Dickinson, Sparks, MD, USA) and incubated at 37$^{\circ}$C for 48 hours. For individual pig fecal samples 10 g of the individual fecal samples were inoculated into 90 mL of TTB and incubated at 37$^{\circ}$C for 48 hours. After incubation, an aliquot (100μL) of the fecal−TTB solution was inoculated into 9.9 mL of Rappaport-Vassiliadis broth (RV, Becton Dickinson, Sparks, MD, USA) and incubated at 42°C for 24 hours. The RV broth was plated onto Xylose Lysine Tergoitol 4 agar (XLT4, Remel, Thermo Fisher Scientific, Lenexa, KS, USA) selective agar plates and incubated at 37$^{\circ}$C,

overnight. Suspect *Salmonella* colonies from microbiological culture were screened using *Salmonella* poly O antisera antiglutination (Becton Dickinson, Sparks, MD, USA).

**Environmental samples**

Environmental samples were cultured following the same protocol (Davies et al., 2000) using a volume of TTB sufficient to submerge the swabs (~60ml).

**Data analysis**

Bacteriological culture data were entered into an Excel 2007 spreadsheet using appropriate coding and subsequently verified for accuracy by checking each entry with the original hard copy result. The spreadsheets were transferred to a relational database (Access 2007, Microsoft Corporation, Redmond, WA, USA). Data was retrieved from the database and imported into SAS 9.3 (SAS Institute, Cary, NC, USA) for data management and statistical analysis.

Descriptive statistics of demographic data (number of pigs sampled, gender), loss to follow-up and morbidity were presented in proportions.

Descriptive statistics of bacteriological culture were generated for the nursery, barn environment, site, cohort, pig, and fecal sample (observation). *Salmonella* apparent prevalence (proportion of positive samples/tested) and respective 95% confidence intervals were estimated at each unit of observation: cohort (*e.g.*, all collections combined within cohort), site (*e.g.*, all cohorts combined), pig age (by collection period) and individual sample. Pearson Chi-squared analysis with Bonferroni adjustment was used to compare apparent prevalence among sites. Chi-squared test for trend in proportions (Cochran-Armitage Test) was applied to test change of

apparent prevalence over time. Correlations between the proportion of positive fecal samples in a cohort (*e.g.*, all collections combined within cohort) and respective proportion of *Salmonella* positive samples in nursery and environment were determined using Spearman's rho. A significance level of 0.05 was used for all comparisons.

Patterns and duration of shedding were estimated for those pigs which met the following inclusion criteria: 1) survival until marketing (excluded n=3 dead and n=5 early shipment); 2) no more than one period from which a sample was not collected (excluded n=1); 3) had no more than two negative cultures between two positive culture results (excluded n=10).

In order to estimate the duration of shedding of individual pigs, we assumed that the shedding began 7 days prior the first detected positive culture and lasted until 7 days after the last isolation. The 7 days interval was selected taking into account data from experimental studies indicating that pigs start to shed *Salmonella* as early as 2 to 7 days post–exposure (Fedorka-Cray et al., 1994) and as late as 7 to 14 days (van Winsen et al., 2001; Osterberg et al., 2010) after exposure to a *Salmonella* contaminated environment or when commingled with pigs shedding *Salmonella*. This interval (7 days) was also the midpoint between two consecutive sampling periods.

# RESULTS

## Demographic results

A total of 900 pigs were selected for inclusion in the study. Forty six per cent were barrows or castrated males (410/900) and 54% were females (490/900). The total loss to follow-up for fecal sample collection was 5.1% (364/7200). Causes for loss to follow-up were: death, unable to collect a specimen (*e.g.*, empty rectum, sick animal), or shipped to market prior to final collection. A total of 17 pigs died during the study (17/900; 1.9%). The majority of the pigs were sampled 8 (71.4%; 643/900) or 7 times (23.1%; 208/900).

At the observation level (individual pig times number of sample periods pig was observed), diarrhea was described in 2.4% (164/6836) of the observations. At the pig level, 15.1% (136/900) were observed to have diarrhea at least once.

## Nursery and barn environment

The total proportion of positive samples and respective 95% C.I. of nursery, barn environment samples and sites, stratified by cohort are summarized in Table 2.1. Pooled fecal samples from the source nursery were collected and cultured in 17/18 cohorts. *Salmonella* was detected in at least one nursery pool in 76.5% (13/17) of the cohorts. A total of 36.5% (62/170) of the pooled nursery samples were *Salmonella* positive. The proportion of positive nursery samples ranged from 0% to 100% among cohorts (Table 2.1).

Environmental samples were collected for all cohorts. *Salmonella* was detected in at least one environmental swab in 61.1% (11/18) of the cohorts. The total number of positive

swabs was 40 (40/360; 11.1%). The proportion of positive barn environment swabs ranged from 0% to 85% among cohorts (Table 2.1).

**Site, cohort and age apparent prevalence**

*Salmonella* was isolated from at least one sample type (nursery, environmental or individual fecal) sample at all three sites. In 17/18 cohorts at least one individual fecal sample was positive. *Salmonella* was cultured from 6.6 % (453/6836; 95% C.I. 6.0–7.2%) of individual fecal samples. The proportion of positive fecal samples within a cohort (eight collection periods combined per cohort) ranged from 0% to 44.1%. Within site, the proportion of positive fecal samples per cohort (six cohorts/site) ranged from 1.5% (6/396; 95% C.I. 0.6-3.3%) to 12.0% (46/382; 95% C.I. 9.0–15.7%) in site A, 0.6% (2/362; 95% C.I. 0.1–2.0%) to 44.1% (156/354; 95% C.I. 38.8–49.4%) in site B and 0% to 6.1% (24/393; 95% C.I. 4.0–9.0%) in site C (Table 2.1).

For 17 cohorts with both nursery and environmental swab collections, there were 9 cohorts with at least one positive sample in both samples types that also had at least one individual fecal sample positive. Three cohorts were *Salmonella* positive in the nursery but *Salmonella* negative for environmental swabs. One cohort was negative in the nursery and had at least one environmental swab positive. Three cohorts were negative for both sample types. One cohort had at least one positive sample for both nursery and environmental samples but was negative for individual fecal samples. The proportion of *Salmonella* positive samples was significantly greater in those cohorts in which both the nursery and the barn environment were *Salmonella* positive (p−value<0.05) (Table 2.2). No significant difference was found among

cohorts negative for both types of samples and nursery positive and environment negative nor nursery negative and environment positive (p–value>0.05) (Table 2.2). There was a positive association between the proportion of positive samples in a cohort and the proportion of positive pooled nursery samples (rho=0.76, p–value=0.0002). There was also a positive association between the proportion of positive barn environmental swabs (rho=0.59, p–value=0.01) and the proportion of positive fecal samples in a cohort.

There was a significant difference between sites in the overall proportion of positive samples (p–value <0.0001). Site B (11.2%; 247/2203; 95% C.I. 9.9–12.6%) had a higher prevalence than site A (6.3%; 147/2338; 95% C.I. 5.3–7.4%) and site C (2.6%; 59/2295; 95% C.I. 2.0–3.3%). Site A also had a greater proportion of positive samples than Site C (p<0.0001).

For all 18 cohorts, the proportions of positive samples per cohort were plotted by age (Figure. 2.1). The overall median was 2.0%; 25%, 75% and 95% quartiles were 0%; 7.4%; 25.5%. The overall proportion of positive samples decreased significantly over the collection periods (p–value <0.0001). The *Salmonella* apparent prevalence decreased from 12.9% (115/890; 95% C.I. 10.8−15.3%) at the beginning of the finishing period (10 weeks−old) to 2.8% (20/706; 95% C.I. 1.7−4.3%) at the end of finishing phase (24–26 weeks old) (p−value <0.0001).

Variation in prevalence was seen between sites, between cohorts within site and within cohorts. For each site, the within-cohort *Salmonella* apparent prevalence was plotted by pig age (Figure. 2.2.a; Figure. 2.2.b; Figuere. 2.2.c). Within-site and across cohorts and age, the apparent prevalence ranged from 0 to 24.5% (95% C.I. 13.34–38.9%) in site A, from 0% to 71.4% (95% C.I. 56.7−83.4%) in site B, and from 0 to 20.4% (95% C.I. 10.2−34.4%) in site C.

**Pig apparent prevalence and duration of shedding**

Most pigs were detected as *Salmonella* positive for the first time at the first collection period (10 weeks of age, 61.5%; 115/187). This was followed by collections 2 and 3 (12 and 14 weeks of age; 14.4%, 27/187), collection 4 (16 weeks of age; 4.3%, 8/187), collection 6 (20 weeks of age 3.7%; 7/187) and collections 5, 7 and 8 (18, 22 and 24 weeks of age (0.5%; 1/187). Overall incidence of *Salmonella* was 20.8% (187 /899 pigs; 95% C.I. 18.2% –23.6%). Of the positive pigs, 87 were culture positive once (46.5%) and 27 (14.4%), 31 (16.6%), 17 (9.1%), 10 (5.4%), 6 (3.2%), and 7 pigs (3.7%) were positive, 2, 3, 4, 5, 6, and 7 times respectively. Only two pigs were *Salmonella*–positive in all eight collection periods. The duration of shedding was clustered within site and cohort. The majority of the pigs with two or more positive samples belonged to Site B (61/100 pigs), with sites A and C having 30 and 9 pigs detected as culture positive for *Salmonella* at two or more collection periods, respectively. In site B, two cohorts had the majority of pigs (53/61) with two or more positive samplings (40 and 13 pigs, in cohorts 2 and 4, respectively). In site A, two cohorts had the majority of pigs with two or more positive samples (19/30; 9 and 10 pigs in cohort 2 and 4, respectively).

There were 95 pigs detected positive in more than two sampling occasions that had consecutive sampling collections. Of these, 46.3% (44/95) had consecutive positive culture samplings, 23.2% (22/95) had one culture negative fecal sample between positive culture samples and 30.5% (29/95) were culture negative in two or more occasions between the first and last culture positive sample collection period for each pig.

A total of 168 pigs met the inclusion criteria for estimation of shedding period. The median time of shedding was 14 days (std = 32.5; range 14–112 days). Eighty five pigs (50.6%)

shed 14 days or less; 15 pigs (8.9%) shed for 28 days or less, 18 pigs (10.7%) shed for 42 days or less, 11 pigs (6.6%; 11/168) shed for 56 days or less and 39 pigs (23.2%; 39/168) shed between 70 to 112 days.

## DISCUSSION

Estimates of *Salmonella* prevalence in finishing pigs in the US range from 3.4% to 48% (Davies et al., 1997; Funk et al., 2001; Hurd et al., 2004; Bahnson et al., 2006; Gebreyes et al., 2006; Dorr et al., 2009; USDA-APHIS, 2009; Wang et al., 2010; Rostagno et al., 2012). The observed proportion of *Salmonella* positive samples and cohort prevalence were within the range of these reports. The overall incidence of positive pigs was 20.8%, which is, to the best of our knowledge, the first estimate of incidence in naturally infected swine in one large system in the US.

Several longitudinal studies have been conducted at the farm (van der Wolf et al., 2001; Rajic et al., 2005; Rajic et al., 2007; Farzan et al., 2008; Rostagno et al., 2012) and cohort/pig group level (Merialdi et al., 2008; Dorr et al., 2009; Vigo et al., 2009). A limited number of studies have repeatedly sampled individual pigs (Funk et al., 2001; Beloeil et al., 2003; Kranker et al., 2003; Nollet et al., 2005). Similar to these previous studies, we report variability of prevalence by site, cohort and within pig. This may suggest there are risk factors at the site, cohort and pig level that might be associated with *Salmonella* prevalence. This variability reinforces that point estimates of prevalence might misclassify farm and pig status and that prospective studies are needed to assess time–dependent risk factors for *Salmonella* in swine with consideration for risk factors that may be distributed at different levels of organization (farm, cohort, pig) (Dohoo et al., 2010).

The majority of the pigs were detected as *Salmonella*–positive at the beginning of the finishing period (10 weeks–old). Although individual sampling during the nursery phase was not performed in this study, *Salmonella* was isolated in nursery pool samples from a majority of

the cohorts and there was a positive association between the nursery pool prevalence and the proportion of positive individual samples within a cohort. This suggests that pigs were exposed to *Salmonella* in the nursery and may have been shedding at arrival to the finishing barn. *Salmonella* shedding during the nursery phase has been reported (Nollet et al., 2005), in some cases representing the peak prevalence during the nursery period (Kranker et al., 2003). Several authors have reported increased prevalence when pigs were moved to finishing units (Nollet et al., 2005; Vigo et al., 2009), which may be a result of multiple potential factors: stress caused by transportation, comingling with new pigs, changes in feed type and exposure to residual contamination (Nollet et al., 2005; Vigo et al., 2009).

Contaminated facilities are a source of *Salmonella* (Funk et al., 2001; Mannion et al., 2007; Dorr et al., 2009; Zewde et al., 2009) and may in part explain the high prevalence of *Salmonella* at the first collection period. In agreement with other authors we observed that cleaning and disinfection did not eliminate *Salmonella* in the barn environment. The elimination of *Salmonella* from barn environments is difficult and residual contamination might be responsible for new infections (Funk et al., 2001; Beloeil et al., 2003; Mannion et al., 2007; Zewde et al., 2009). The positive association between the proportion of positive barn environment swabs and the proportion of positive individual samples within a cohort suggests that the contaminated environment may have contributed to *Salmonella* infections in the finishing phase.

Overall, prevalence decreased as pig age increased. Other authors have reported a decrease in prevalence during the finishing period (Kranker et al., 2003; Nollet et al., 2005; Vigo et al., 2009). It is unclear whether this association represents the natural history of *Salmonella* in swine, with young animals being more susceptible and ultimately clearing the infection over

time, or if other factors are involved. Further research to understand whether control of *Salmonella* in young pigs ultimately would decrease the risk of shedding at the time of harvest is warranted.

More than 50% of the *Salmonella* positive pigs were detected two times or more. Other studies that have followed pigs over time have reported a lower percentage of pigs that were detected more than two times. Beloeil et al. (2003) reported that a majority of pigs shed only once in weekly samplings. In other studies the comparison is not as direct, since in this study the sampling period was more frequent than other reports (Funk et al., 2001; Kranker et al., 2003). In this study, pigs identified as *Salmonella* positive more than two times were clustered within site and cohort. This is in agreement with Kranker et al. (2003), who reported characteristic patterns (shorter or longer periods of shedding) by cohort. This might suggest that there are cohort level effects that are related to duration of shedding or transmission dynamics.

The median and range of shedding duration in this study is similar to that described by Kranker et al. (2003), who reported a mean duration of shedding of 18 or 26 days, range of 7 to 101 days. Although our estimates are limited by an imperfect diagnostic test, the sampling frequency and the assumption of no new infections, these data present critical information regarding the duration of shedding in naturally infected swine. Further research to understand risk factors for duration of *Salmonella* shedding in swine are warranted.

There was intermittent detection of shedding in more than fifty percent of the pigs with multiple culture−positive collections. *Salmonella* carriers can shed intermittently and for long periods (Funk et al., 2001; Kranker et al., 2003; Scherer et al., 2008). It is difficult to separate intermittent shedding of *Salmonella* from intermittent detection or new infections. Despite being

an imperfect diagnostic test, fecal culture is considered the 'gold standard' for *Salmonella* isolation. Estimates of the relative sensitivity of fecal culture range from 6.5% to 95%, depending on culture method and parallel estimation of the sensitivity (Davies et al., 2000; Funk et al., 2000; Funk, 2003; Hurd et al., 2004; Rostagno et al., 2005; Love and Rostagno, 2008). Although a relative short sampling interval (two weeks) was conducted in this study, new infections could occur between sampling occasions. Therefore, the intermittent shedding could be either intermittent detection of an on–going infection or a new infection after clearance of a previous infection.

These data represent one production company in one region of the United States. Although this may limit external validity, we believe that this limitation is minimal. This farm is typical of many US swine production systems in size and production practices. Furthermore, there are many similarities between the results in this study compared to others both in the US and other countries. A further limitation for interpretation is in regards to the univariate analyses reported in this paper. Statistical inferences should be interpreted carefully, as the analyses did not take into account the clustered nature of the data (samples within pigs, pigs within pens, pens within barns, barns within sites), which may bias the results reported. Further analyses using multivariate analysis accounting for the clustered data structure is presented in chapter 4. The bias presented by the univariate analyses would tend to result in an increased risk of Type I error (Clarke, 2008). Despite this limitation, the findings presented in this paper are consistent with what has been previously reported in the literature (Funk et al., 2001; Beloeil et al., 2003; Kranker et al., 2003; Nollet et al., 2005; Mannion et al., 2007; Vigo et al., 2009; Zewde et al., 2009).

These descriptive data regarding the incidence, duration and pattern of shedding in swine provide critical data for understanding risk factors for *Salmonella* in finishing swine. The variability and clustering of *Salmonella* shedding by site, cohort and pig not only suggest a need to evaluate time variant risk factors, but also guide the design of future epidemiological studies for identification of potential risk factors at different levels of clustering (site, cohort and pig). Future research of the epidemiology of *Salmonella* in swine should focus on longitudinal study designs focused on multilevel and time variant risk factors. This study also reinforces that estimates of point−prevalence might misclassify herd or pig *Salmonella* status.

## ACKNOWLEDGMENTS

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# APPENDIX

**Table 2.1.** Proportion of samples positive for *Salmonella spp* by site and cohort (samples represent individual fecal samples, pooled fecal samples from the source nursery and barn environmental swabs) and respective 95% confidence intervals.

| | Environment[a] | | Nursery[b] | | Cohort | | |
|---|---|---|---|---|---|---|---|
| **Site /Cohort** | **%** | **95% C.I.** | **%** | **95% C.I.** | **Total positive fecal samples/ tested** | **%** | **95% C.I.** |
| Site A | | | | | | | |
| 1 | 5.0 | 0.1-24.9 | 60.0 | 26.2-87.9 | 30/388 | 7.7 | 5.3-10.9 |
| 2 | 25.0 | 8.7-49.1 | 80.0 | 44.4-97.5 | 42/396 | 10.6 | 7.8-14.1 |
| 3 | 0.0 | NA | NS | NS | 7/390 | 1.8 | 0.1-3.7 |
| 4 | 85.0 | 62.1-96.8 | 60.0 | 26.2-87.8 | 46/382 | 12.0 | 9.0-15.7 |
| 5 | 5.0 | 0.1-24.9 | 20.0 | 2.5-55.6 | 16/386 | 4.1 | 2.4-6.6 |
| 6 | 5.0 | 0.1-24.9 | 10.0 | 2.5-44.5 | 6/396 | 1.5 | 0.6-3.3 |
| Site B | | | | | | | |
| 1 | 10.0 | 1.2-31.7 | 10.0 | 0.25-44.5 | 12/383 | 3.1 | 1.6-5.4 |
| 2 | 10.0 | 1.2-31.7 | 100.0 | 69.2-100 | 156/354 | 44.1 | 38.8-49.4 |
| 3 | 0.0 | NA | 20.0 | 2.5-55.6 | 4/379 | 1.1 | 0.3-2.7 |
| 4 | 30.0 | 11.9-54.3 | 100.0 | 69.2-100 | 57/339 | 16.8 | 13.0-21.2 |
| 5 | 5.0 | 0.1-24.9 | 0.0 | NA | 2/362 | 0.6 | 0.1-2.0 |
| 6 | 0.0 | NA | 0.0 | NA | 16/386 | 4.1 | 2.4-6.6 |
| Site C | | | | | | | |
| 1 | 0.0 | NA | 0.0 | NA | 1/387 | 0.3 | 0.01-1.4 |
| 2 | 0.0 | NA | 80.0 | 44.4-97.5 | 24/393 | 6.1 | 4.0-9.0 |
| 3 | 0.0 | NA | 0.0 | NA | 6/390 | 1.5 | 0.6-3.3 |
| 4 | 15.0 | 3.2-37.9 | 60.0 | 26.2-87.8 | 18/371 | 4.9 | 2.9-7.6 |
| 5 | 5.0 | 0.1-24.9 | 10.0 | 2.5-55.6 | 0/376 | 0.0 | NA |
| 6 | 0.0 | NA | 10.0 | 2.5-44.5 | 10/378 | 2.6 | 1.3-4.8 |

NS: not sampled

NA: not applicable

[a]total of 20 environmental samples per cohort

[b]total of 10 pooled samples per cohort

**Table 2.2.** Distribution of cohorts and proportion of samples positive for *Salmonella spp* by the *Salmonella* status of nursery and environmental swabs[a].

| Nursery and environment status | Number of positive cohorts | Number of negative cohorts | Number of positive fecal samples/tested | Proportion of positive fecal samples (%)[b] | 95% C.I. |
|---|---|---|---|---|---|
| Nursery + environment + | 9 | 1 | 383/3771 | $10.2^{A}$ | 9.2-11.2 |
| Nursery + environment - | 3 | 0 | 38/1150 | $3.3^{BD}$ | 2.4-4.5 |
| Nursery - environment + | 1 | 0 | 2/362 | $0.6^{CE}$ | 0-2.0 |
| Nursery- environment - | 3 | 0 | 23/1163 | $2^{DE}$ | 1.3-3.0 |

[a] 17 cohorts are represented, 1 cohort was excluded as no nursery samples were collected

[b] different letters indicate a significant difference (p–value < 0.05) of proportion of positive fecal samples

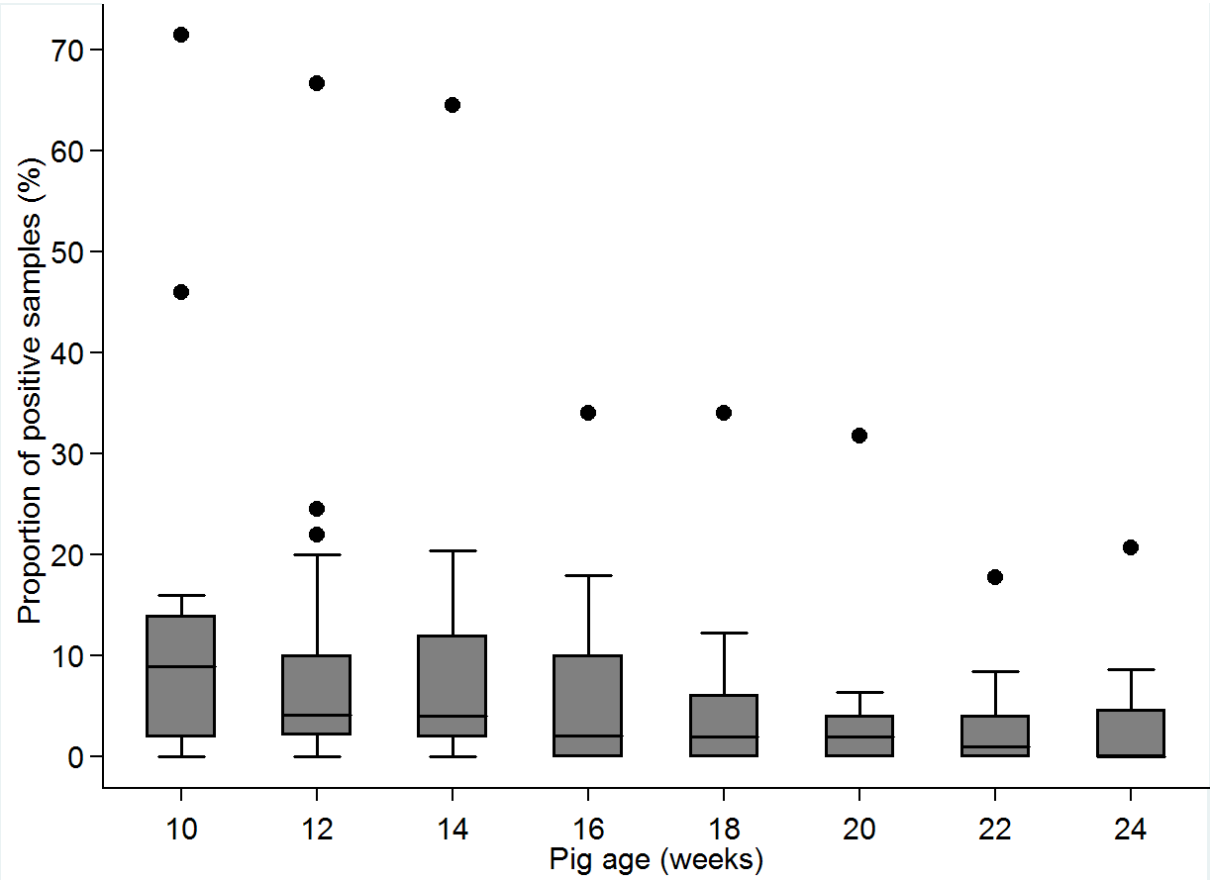


**Figure 2.1.** Box plot representing the distribution of *Salmonella* positive fecal samples within each cohort by pig age.

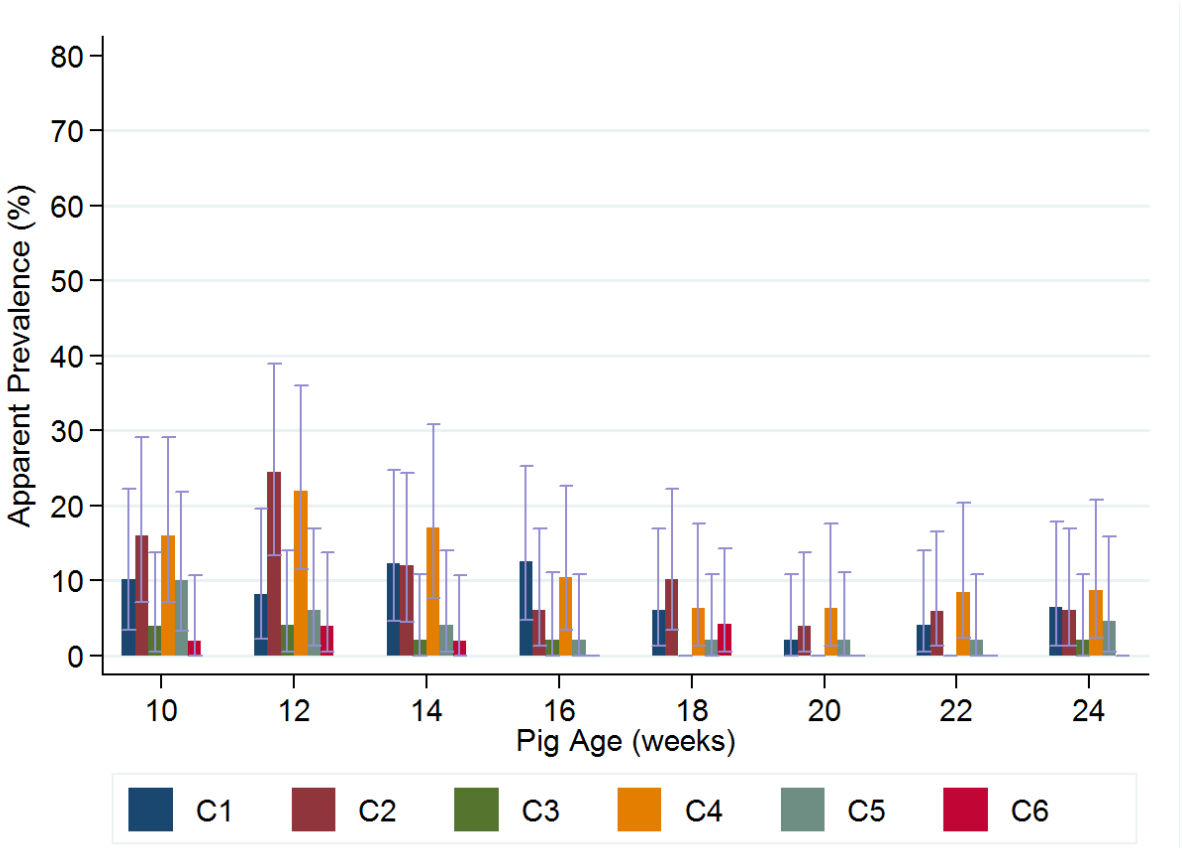


**Figure 2.2.a.** Apparent prevalence (individual fecal samples) for each cohort (C1–C6) by pig age in site A. Error bars represent 95% exact confidence intervals for proportions.

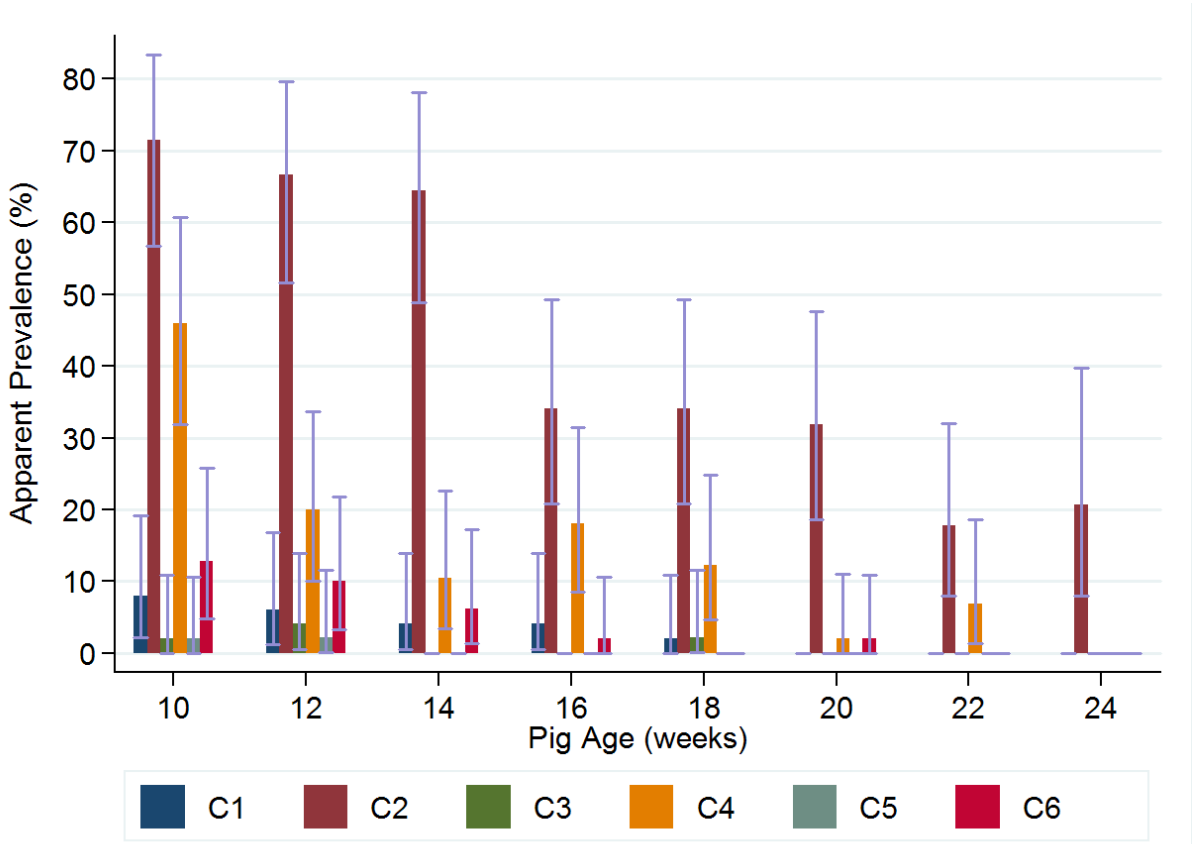


**Figure 2.2.b.** Apparent prevalence (individual fecal samples) for each cohort (C1–C6) by pig age in site B. Error bars represent 95% exact confidence intervals for proportions.

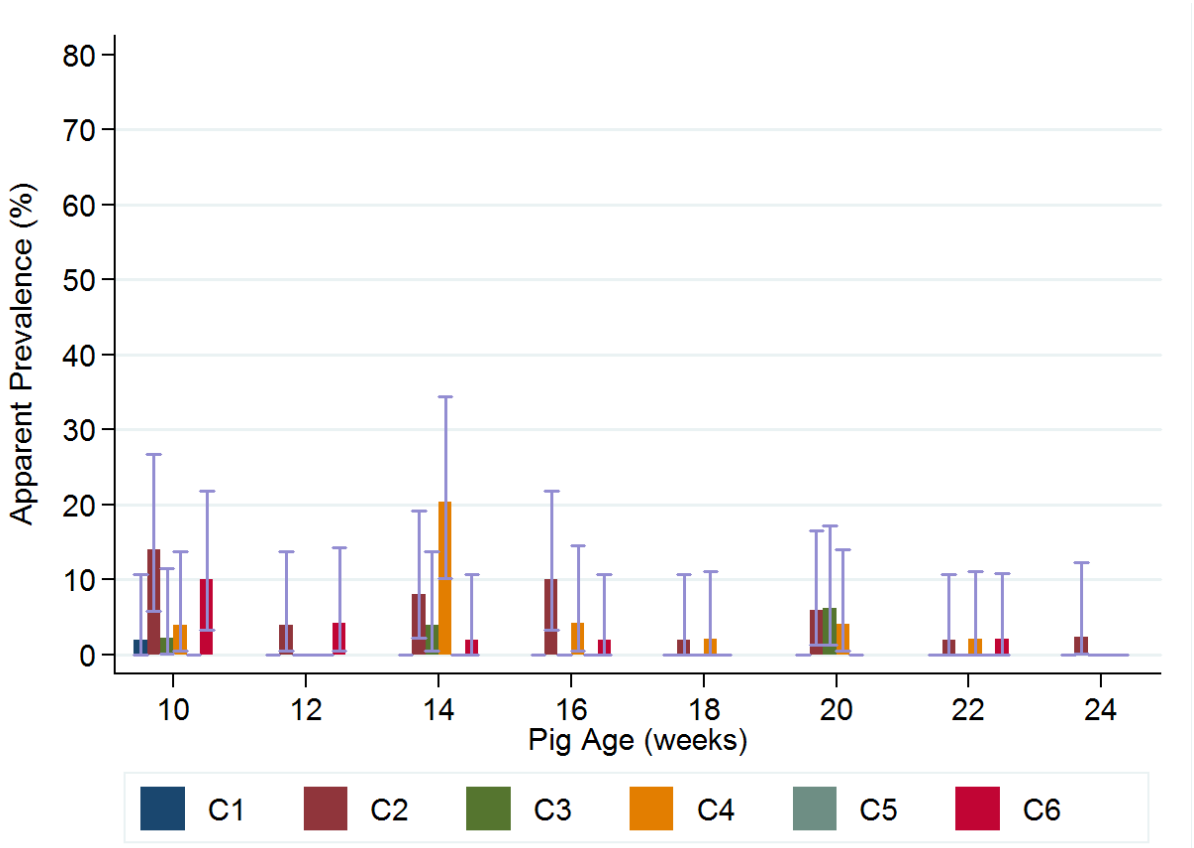


**Figure 2.2.c.** Apparent prevalence (individual fecal samples) for each cohort (C1–C6) by pig age in site C. Error bars represent 95% exact confidence intervals for proportions.

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# CHAPTER 3

## Direct quantitative real–time PCR for enumeration of *Salmonella* in feces of naturally infected pigs

## ABSTRACT

Quantification of *Salmonella* in asymptomatic pigs can be used to identify control measures and to assess the risk of carcass contamination during slaughter. The objectives of this study were: 1) to compare direct quantitative real-time PCR (q–PCR) detection of *Salmonella* to microbiological culture and 2) to quantify the fecal concentration of *Salmonella* in naturally infected pigs. Individual fecal samples (positive (n=443), negative (n=1225) determined by microbiological culture) were submitted to q–PCR. A receiver–operating characteristic curve was used to identify the quantification cycle (Cq) cut–off that optimized sensitivity and specificity. A Cq cut–off of 37.52 cycles optimized clinical sensitivity (15.4%) and specificity (99.6%). At this cut–off, direct q–PCR categorized 99.6% (1220/1225) of culture negative samples as negative. For culture positive samples, 15.4% (68/443) were detected by q–PCR, but only 3.4% (15/443) were within the q–PCR quantifiable range ($\geq 10^3$ CFU/g of feces). Of these latter samples, the concentration range was $1.06 \times 10^3$–$1.73 \times 10^6$ CFU/g feces. Of the samples with high *Salmonella* concentrations 7 were collected from one pig and 3 samples were collected from its pen–mates. Direct q–PCR may be an alternative to traditional culture–dependent methods for detection of pigs with high fecal concentrations of *Salmonella*, but not for detection of pigs shedding low concentrations of *Salmonella*, which represented the majority of pigs in this study. When high shedding was detected it was clustered within a single pig and its pen–mates. These data can contribute to quantitative risk assessments of the association between concentrations of *Salmonella* shed by pigs during the finishing phase and the risk of carcass contamination at slaughter.

## INTRODUCTION

*Salmonella* species are one of the major causes of foodborne diseases in the United States and worldwide (Greig and Ravel, 2009; Henao et al., 2011; Scallan et al., 2011). *Salmonella* is still one the most important bacteriological zoonotic hazards transmissible from pork to consumers (Fosse et al., 2009). A significant number of human cases of salmonellosis (1% to 25%) have been related to consumption of pork and pork products (Berends et al., 1998; Hald et al., 2006; Ravel et al., 2009; EFSA, 2010b; Guo et al., 2011). Swine are asymptomatic reservoirs for *Salmonella* and shed intermittently in their feces (Wood and Rose, 1992; Scherer et al., 2008), which can be a source of carcass contamination (Baptista et al., 2010; van Hoek et al., 2012) and subsequent transmission to humans (Bollaerts et al., 2009).

Since *Salmonella* is a ubiquitous organism, eradication as a control measure is not viable. Decreasing the concentration of *Salmonella* shed by swine may represent a more achievable disease control target. Enumeration of bacterial load can be used to identify contamination pressure and to identify effective control measures to reduce contamination in swine herds (Fravalo et al., 2003). In addition, data are needed for quantitative microbial risk assessments and for modeling transmission patterns of *Salmonella* (Bollaerts et al., 2009; Lanzas et al., 2011). Most of what is known about the concentration of *Salmonella* shed in pig feces is based on experimental studies (Wood and Rose, 1992; Gray et al., 1996; Osterberg and Wallgren, 2008; Scherer et al., 2008; Osterberg et al., 2009; Rostagno et al., 2011). A limited number of studies have quantified *Salmonella* concentration in feces of naturally infected swine. These were either

cross–sectional studies (Fravalo et al., 2003; Fablet et al., 2006; van Hoek et al., 2012) or estimates of pen–contamination in lairage (O'Connor et al., 2006; Boughton et al., 2007).

Traditionally, quantification of *Salmonella* in fecal samples has been based on culture–dependent methodologies. Those methodologies include use of enrichment and selective culture media, such as most probable number (MPN) technique; direct plating; use of modified semi–solid Rappaport–Vassiliadis (MRSV) medium; or use of the mini–MRSV–MPN technique (Fravalo et al., 2003; Fablet et al., 2006; O'Connor et al., 2006; Boughton et al., 2007; Osterberg and Wallgren, 2008; Osterberg et al., 2009). Quantitative methods based on culture are time consuming (3–7 days), labor intensive and costly; therefore, it can be impeditive to use in studies with a large number of samples.

Culture–independent methods such as quantitative real–time polymerase chain reaction (q–PCR) assays may be more efficient for high through–put diagnostic testing and may be more representative of the true bacterial concentration in the matrix tested. Q–PCR has been used to quantify *Salmonella* in food matrices, pig feces and on pig carcasses (Malorny et al., 2008; Park et al., 2008; Abley, 2011; Krämer et al., 2011; Löfström et al., 2011). Some of those methods included pre–enrichment of the sample before the DNA extraction (Malorny et al., 2008; Krämer et al., 2011), although the use of pre–enrichment media before the DNA extraction may misrepresent the "true" bacterial count. Direct quantification of *Salmonella* has been applied in food matrices (Fukushima et al., 2007; Cheng et al., 2009; Elizaquivel et al., 2011), pork carcass swabs (Guy et al., 2006; Löfström et al., 2011), chicken rinses (Wolffs et al., 2006) and fecal material (Harris et al., 2007; Pusterla et al., 2010; Abley, 2011). The quantification limit varies, depending on matrix, assay and processing. Few studies have enumerated *Salmonella* in swine

fecal samples using real–time PCR without enrichment (Harris et al., 2007; Abley, 2011). Quantitative real–time PCR is an alternative to the traditional quantitative culture-dependent method to directly quantify the bacterial concentration in swine feces. It allows enumeration of *Salmonella* in a large number and variety of samples in an efficient time-cost and automated way (Malorny et al., 2008; Elizaquivel et al., 2011; Löfström et al., 2011). The objectives of this study were: 1) to compare direct q–PCR detection of *Salmonella* in swine feces to the microbiological culture and 2) to quantify the fecal concentration of *Salmonella* in naturally infected pigs.

## MATERIALS AND METHODS

The samples were collected from a longitudinal study on a multi–site farrow–to–finish production system located in the Midwestern, United States. The primary aim of this study was to describe *Salmonella* shedding of naturally infected finishing pigs (Pires et al., 2012). Criteria used for the selection of production system, finishing sites, pig selection, sample collection and laboratory isolation of *Salmonella* have been described in detail elsewhere (Pires et al., 2012). Briefly, three sites from a multi–site farrow–to finish production system were selected for the study. At each 4–barn site, 1 barn was selected for the study inclusion. For each site selected, 6 consecutive cohorts of pigs were included in the study. Each 1000–head inventory barn housed pigs from 10 weeks of age until market (24–25 weeks of age). At the beginning of each cohort, 50 pigs (10 ± 2 weeks old) were randomly selected and individually identified. Individual fecal samples (10 g) were collected from pigs every 2 weeks for 16 weeks (8 total sample periods per cohort). Fecal samples were cultured using standard methods described elsewhere (Pires et al., 2012). Briefly, fecal samples (10 g) were inoculated into 90 mL of Tetrathionate broth (TTB) (Becton Dickinson, Sparks, MD) and incubated at 37°C for 48 hours. After incubation, an aliquot (100 μL) of the fecal–TTB solution was inoculated in into 9.9 mL of Rappaport–Vassiliadis broth (RV) (Becton Dickinson, Sparks, MD) and incubated at 42°C for 24 hours. The RV broth was plated onto Xylose Lysine Tergitol 4 agar (XLT4) (Thermo Fisher Scientific, Lenexa, KS) selective agar plates and incubated at 37°C, overnight. Suspect *Salmonella* colonies from the XLT4 were screened using triple sugar iron (Becton Dickinson, Sparks, MD) and urea agar slants (Becton Dickinson, Sparks, MD). *Salmonella*–suspect

colonies then were screened using *Salmonella* poly O antisera antiglutination (Becton Dickinson, Sparks, MD).

An aliquot of each fecal sample (200 mg) was stored at –80°C. A random selection of culture negative samples (n= 1225) and all culture positive samples (n=443) were submitted for q–PCR. A list of culture negative samples was generated by simple random sampling of negative samples from 17 out of 18 cohorts, using a commercial statistical software package (Proc surveyselect procedure; SAS 9.3; SAS Institute, Cary, NC). The sample size of culture negative samples was based on a Bayesian approach for sample size calculations for surveys to substantiate freedom from an infectious agent (Johnson et al., 2004) and using software available online (Bayesfreecalc2; http://www.epi.ucdavis.edu/diagnostictests/module02.html). Assumptions for calculation of sample size of culture negative samples for q–PCR evaluation were: less than 5% of the culture–negative samples would be false positive by direct q–PCR, based on expert opinion; microbiological culture sensitivity greater than 0.6 and mode of 0.7; microbiological culture specificity greater than 0.95 and a mode of 0.99. Based on these assumptions, a sample size of 1274 provided at least 93% confidence that the true prevalence was zero.

The DNA was extracted from feces using the Qiagen Qiamp Stool Mini Kit (Qiagen, Valencia, CA) according to manufacturer instructions. The PCR primers and probe targeting the *invA* gene were as described previously (Hoorfar et al., 2000). The q–PCR reaction conditions were modified as needed to accommodate use of reagents and equipment different from those described previously (Hoorfar et al., 2000). The cut–off for q–PCR assay was set at 45 cycles. The limit of detection of the q–PCR was determined using sterilized fecal samples spiked with

serial 10 fold dilutions of *Salmonella* isolated from a pig in the current study. The DNA extracted from the spiked fecal samples was used for generation of calibration curves. The lower quantification limit of the PCR was calculated to be 8.3 copies of target per reaction, which is equivalent to 917 CFU/g of feces. The linear dynamic range for quantification was determined to be $9.17 \times 10^2$ to $9.17 \times 10^6$ CFU/g of feces. The q–PCR was performed in triplicate for each and a calibration curve was generated for each plate of samples.

The Cq value from the real time q–PCR was used as a proxy measure of fecal bacterial load. The Cq value is inversely proportional to the amount of bacteria load in fecal sample, the lower the Cq value the higher the fecal concentration. The Cq values were recorded for all tested samples, a Cq average was obtained for those samples detected by the q–PCR assay (Cq average was calculated when 2 or 3 wells were detected positive). All other samples that were tested and not detected by q-PCR were assigned a single value (Cq = 45) for the purpose of the comparative study.

Descriptive statistics (median, mean standard error, 95% confidence intervals) of Cq values were described for those samples detected by q–PCR based on the analytical cut–off. In order to optimize the best compromise between the diagnostic sensitivity and specificity, the Cq was compared to the 'gold standard' (fecal culture) by means of receiver–operating characteristic curve (ROC) (Greiner et al., 2000). A pig was *Salmonella*–positive if the fecal sample tested culture positive at each sample period. Statistical analyses were performed using a commercial statistical software package (MedCalc for Windows, version 12.1.3.0, MedCalc Software, Mariakerke, Belgium). For each q–PCR result (Cq value), sensitivity, specificity, positive and negative likelihood ratios and respective 95% confidence intervals (exact binomial estimation)

were estimated, relative to the fecal culture, and the ROC curve was constructed by plotting the sensitivity versus 1–specificity. The area under the curve (AUC), standard error and 95% confidence intervals (binomial exact estimation) were estimated based on non–parametric methodology (DeLong et al., 1988). TheYounden index of diagnostic accuracy (sensitivity + specificity–1) (Greiner et al., 2000) was calculated for the Cq cut–off which optimizes the sensitivity and specificity. This cut-off was defined as the diagnostic Cq cut–off. Individual fecal samples were re–categorized in negative versus positive based on diagnostic Cq cut–off in order to estimate the *Salmonella* fecal concentration.

Descriptive statistics of concentrations were presented as copy numbers of *invA* gene/g of feces as well as with a scoring system. Individual fecal samples were classified into 4 scores based on q–PCR and culture results. The four scores were: 0) culture-negative and q–PCR–negative; 1) culture–positive and q–PCR negative; 2) culture–positive and q–PCR positive and culture-negative and q–PCR positive, not within quantifiable range; 3) culture–positive and q–PCR positive within the quantifiable range. The concentration gradient was assumed to increase from score 0 to score 3.

## RESULTS

Cq values were generated for 69 culture positive samples (15.6%, 69/443), with a median Cq of 35.5 (95% C.I. 35.4−35.7, range = 24.6 to 38.6) and 25 culture negative samples (2.0%; 25/1225) with a median Cq of 38.4 (95% C.I. 38.1–39.4, range = 34.7 to 42.8). For the purpose of ROC analysis, a Cq value of 45 was attributed to 374 culture positive and 1200 culture negative samples.

The receiver−operating characteristic curve (ROC) curve for the q−PCR test is shown in Figure 3.1. The area under the curve (AUC) was 0.569 (95% C.I. 0.545–0.593), which can be interpreted that a randomly selected *Salmonella* culture positive sample has a lower Cq value than a randomly selected *Salmonella* culture negative sample 56.9% of the time. The AUC was significantly different from a non−informative curve (p−value<0.0001). The direct fecal q−PCR test was of low accuracy (0.5<AUC<0.7) as compared to the gold standard of fecal culture (Greiner et al., 2000; Gardner and Greiner, 2006). The optimal diagnostic cut−off of Cq value was 37.52, which maximized clinical sensitivity (15.4%; 95% C.I. 12.1–19.0%), specificity (99.6%; 95% C.I. 99.1–99.9%) and corresponds to the maximum Youden index (0.149). The positive likelihood ratio was 37.6 (95% C.I. 30.2–46.8%) and the negative likelihood ratio was 0.85 (95% C.I. 0.4–2.0%). The likelihood ratio of a positive test represents the link between the odds of the pretest and post−test probability of disease, given a positive test result (Greiner and Gardner, 2000; Gardner and Greiner, 2006). In this case, a q−PCR positive is 37.6 times more likely in a culture positive fecal sample than in a culture negative fecal sample.

Based on the optimal diagnostic cut–off (Cq = 37.52) and the maximum Younden index, 99.6% (1220/1225) of the *Salmonella* culture negative samples were q–PCR negative. Of the culture positive samples, 15.4% (68/443) were detected by q–PCR, but only 3.4% (15/443) of the samples were detected in triplicate and within the quantifiable range ($\geq 10^3$ copies of the *invA* gene).

The distribution of samples in *Salmonella* concentration score categories, were classified as follows: Score 0) (negative on both tests) 1220; Score 1) (positive culture /negative q–PCR) 375; Score 2 (positive culture /positive q–PCR and negative culture/positive q–PCR) 58; Score 3) (positive culture/positive q–PCR, within quantifiable range) 15 (Figure 3.2).

For the 15 samples in score 3 the concentration ranged from $1.06 \times 10^3$ to $1.73 \times 10^6$ copies of *invA* gene/g feces (median = $2.97 \times 10^5$ and std = $6.16 \times 10^6$). These 15 samples were collected from a total of 9 pigs (Figure 3.3). Forty seven percent (7/15) of the fecal samples in the score 3 group were collected from the same pig (Pig ID 4, Figure 3.3). This pig also had the highest concentration of *Salmonella* shed (range $4.08 \times 10^5$ to $1.73 \times 10^6$ copies of the *invA* gene/g feces), which occurred at the collection period immediately prior to marketing. Forty-four percent (4/9) of the pigs in the score 3 group were not only from the same cohort, but also the same pen.

## DISCUSSION

There are significant knowledge gaps regarding quantitative risk for *Salmonella* shedding on farm and risk of carcass contamination. It is intuitive that the concentration of bacteria shed in feces is related to both transmission dynamics on farm, as well as to the risk for carcass contamination, however, to the date data are limited. This is likely due to many factors, not the least of which is the challenge of quantifying *Salmonella* concentrations in complex matrices such as feces. This study evaluates the potential application of direct q–PCR to identify pigs shedding high concentrations (>$10^3$ CFU/g) of *Salmonella* in their feces. This approach removes the impediments of both logistics for labor and the challenges of interpretation of concentration after enrichment. The detection limit of this PCR assay without enrichment is in agreement with other reports of $10^3$ to $10^4$ gene copies per gram of feces (Malorny and Hoorfar, 2005; Harris et al., 2007; Malorny et al., 2008; Abley, 2011). One of the studies evaluated the performance of the real−time PCR assay in fecal samples inoculated with *Salmonella enteritidis* (ATCC 13076) with final concentrations ranging from $10^1$ to $10^8$ CFU/mL. There was a strong positive correlation between the sample concentrations and q–PCR results, but the q–PCR concentration estimates were 10 fold lower than the inoculated concentration (Abley, 2011). Despite this limitation, a practical application of this methodology may be to detect and determine fecal load in swine shedding high concentrations of *Salmonella*.

In a recent study, a method was developed to quantify *Salmonella*, combining a short non-selective enrichment (8h) followed by q–PCR (Krämer et al., 2011). This allowed enumeration of low numbers (1.4 CFU/10g) in cork borer samples (skin) from pig carcasses by

harvesting the cells in log phase of bacterial growth (Krämer et al., 2011). However, this methodology was applied to a specific type of sample and sample processing method (DNA extraction, pre−enrichment, etc); therefore, its applicability to swine fecal samples must be further tested (Krämer et al., 2011). A further consideration is the lack of knowledge regarding whether it is critical to food safety outcomes to be able to quantify *Salmonella* concentrations in animal feces at less than $10^3$ CFU/g. Further research to understand the association between fecal concentration and carcass contamination can elucidate what analytical sensitivity is required.

One potential explanation for the low sensitivity of the q−PCR is that the targeted gene (inv*A* gene) might not be present in all the *Salmonella* strains present on the farm and as a result, positive culture samples might not be detected by q−PCR. The assay used in the current study has been shown to detect 110 *Salmonella* strains (Hoorfar et al., 2000), among those are the most common serovars found in swine (e.g., *S.* Typhimuirium, *S.* Heidelberg, *S.* Agona , *S.* Derby). It is known that some *Salmonella* strains (*S.* Senftenberg and *S.* Litchfield) have natural deletions within the *Salmonella* pathogenecity island 1 involving the *inv*, *spa*, and *hil* loci (Ginocchio et al., 1997). We did not determine the serovars of the *Salmonella* isolates in this study. Future research to classify the serovars isolated in this study is planned.

The quantitative limit of real−time PCR without enrichment should be taken into account in studies that use this methodology for enumeration of *Salmonella* in swine fecal material. Quantitative real−time PCR might be a good alternative to the traditional quantitative culture−dependent methods, because it allows enumeration of *Salmonella* in a large number and variety of samples in an efficient time−cost and automated way (Malorny et al., 2008; Löfström

et al., 2011). One of the potential applications of this methodology is for identification of high shedders (>$10^3$ CFU/gram), either at the farm or in lairage. This might be of particular interest to identify high shedders in order to apply control measures, such as segregation of pigs during transportation and during harvest.

The majority of the pigs in this study shed low concentrations, below the quantitative limit of q–PCR. These results are in agreement with those using other diagnostic tests for quantification. A study using the mini-MRSV–MPN technique, reported that 86% of fecal samples from naturally infected pigs sampled at the abattoir had less than 200 organisms /g (Fravalo et al., 2003). Using the same technique, estimated concentrations of 2.4 to 350 organisms per gram of feces were reported in pooled fecal samples of finishing pigs on French farms (Fablet et al., 2006). Quantitative studies in lairage environments, using enrichment media and MPN technique have reported variable and relatively low bacterial concentrations; median pen surface concentrations ranged between 1.8–11.5 organisms/100 $cm^2$ (Boughton et al., 2007) and 457–1071 organisms/ml of slurry collected from lairage pens (O'Connor et al., 2006). More recently, a study in a Dutch slaughterhouse reported an estimated mean concentration of 1.88 ±1.42 $\log_{10}$ MPN/g on rectal swabs of carcasses sampled after exsanguination (van Hoek et al., 2012). In experimental studies, carrier pigs shed intermittently in concentrations below the detection limit (< 10CFU/gram) for two months after being infected (Scherer et al., 2008).

To the best of our knowledge this is first study to quantify the fecal concentration of *Salmonella* in repeated sampling of individual, naturally infected finishing pigs. The few fecal samples with high concentrations of *Salmonella* in the feces were clustered within pig and pen. Further investigation of whether there are potential risk factors for shedding high concentrations

of *Salmonella*, and the transmission dynamics of *Salmonella* in groups with and without "high shedders" is an area worth investigating. Quantitative risk assessment studies have suggested that most exposures of swine to *Salmonella* are at doses below the infectious dose (EFSA, 2010a). Doses greater than $10^3$ CFU increase the probability of infection in swine (Osterberg and Wallgren, 2008; EFSA, 2010a). The infectious dose of *Salmonella* is dependent upon serovar, exposure to contaminated fecal material (mass fecal material/fecal mass ingested) and duration of exposure (van Winsen et al., 2001; Jensen et al., 2006; Osterberg et al., 2010). There are likely interactions between risk of infection with both concentration shed as well as the number of animals shedding. These data may provide insight into comparison of intervention strategies targeted at control of pigs that shed high concentrations, for perhaps long periods of time, as compared to interventions more generally targeted at control of prevalence at the group level.

The importance of high shedders for risk of contamination at the slaughterhouse is unknown. For example, is the greater public health risk associated with having a large population of pigs shedding very low concentrations of *Salmonella*, or a small proportion of pigs shedding high concentrations? Current efforts for surveillance at slaughter focus on prevalence outcomes (Baptista et al., 2010; USDA-FSIS, 2010). Quantitative risk assessment studies have reported that interventions to reduce *Salmonella* cases in humans due to pork–related products includes reducing slaughter pig prevalence by reducing the number of infected pigs with high infection/contamination loads entering the slaughterhouse (EFSA, 2010a). Identification of high shedders may be more effective for preventing carcass contamination. The main factor determining risk of human illness reported in these studies was gross contamination (i.e. large

numbers of CFUs per carcass), where such contamination is usually via fecal leakage from a heavily–infected pig, then cross–contamination to a substantial number of carcasses further down the processing line (EFSA, 2010a). Understanding the relationship between the concentration of *Salmonella* shed and public health risk is an area of critical concern for food safety. A combination of further risk analyses as well as economic analyses of the cost of identifying high shedders relative to overall prevalence is needed to develop appropriate surveillance and intervention strategies.

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# APPENDIX

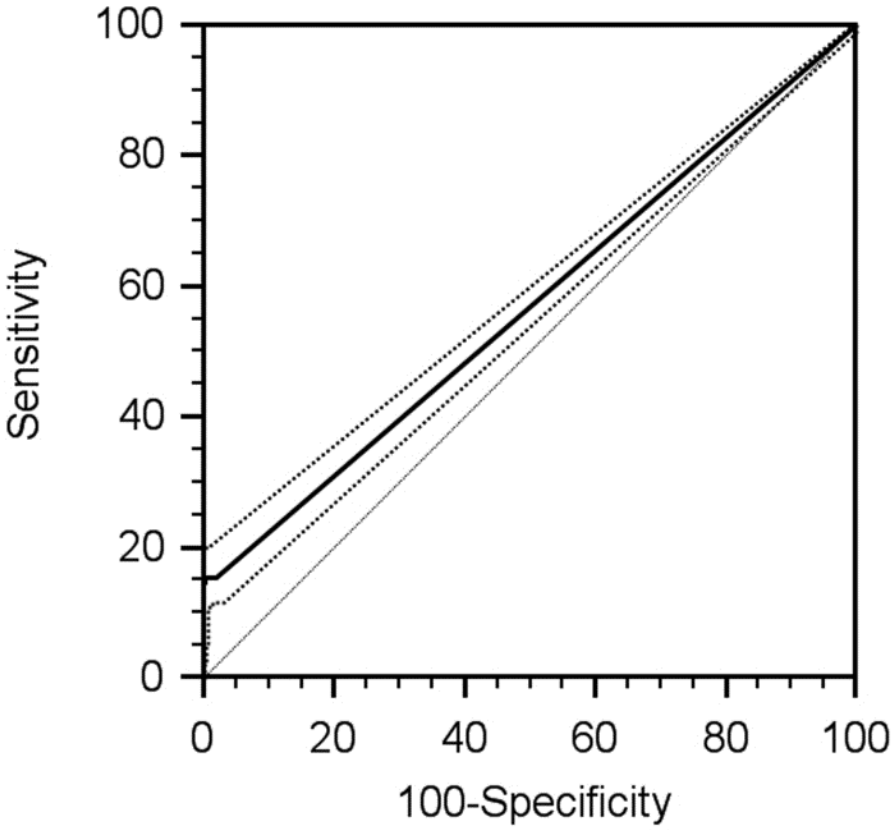


**Figure 3.1.** Receiver–operating characteristic curve (ROC) for the real time PCR to detect *Salmonella* in 1668 pig fecal samples (AUC = 0.569, Sensitivity = 15.4% Specificity = 99.6%). Solid line AUC, dashed lines 95% CI, light line AUC = 0.50).

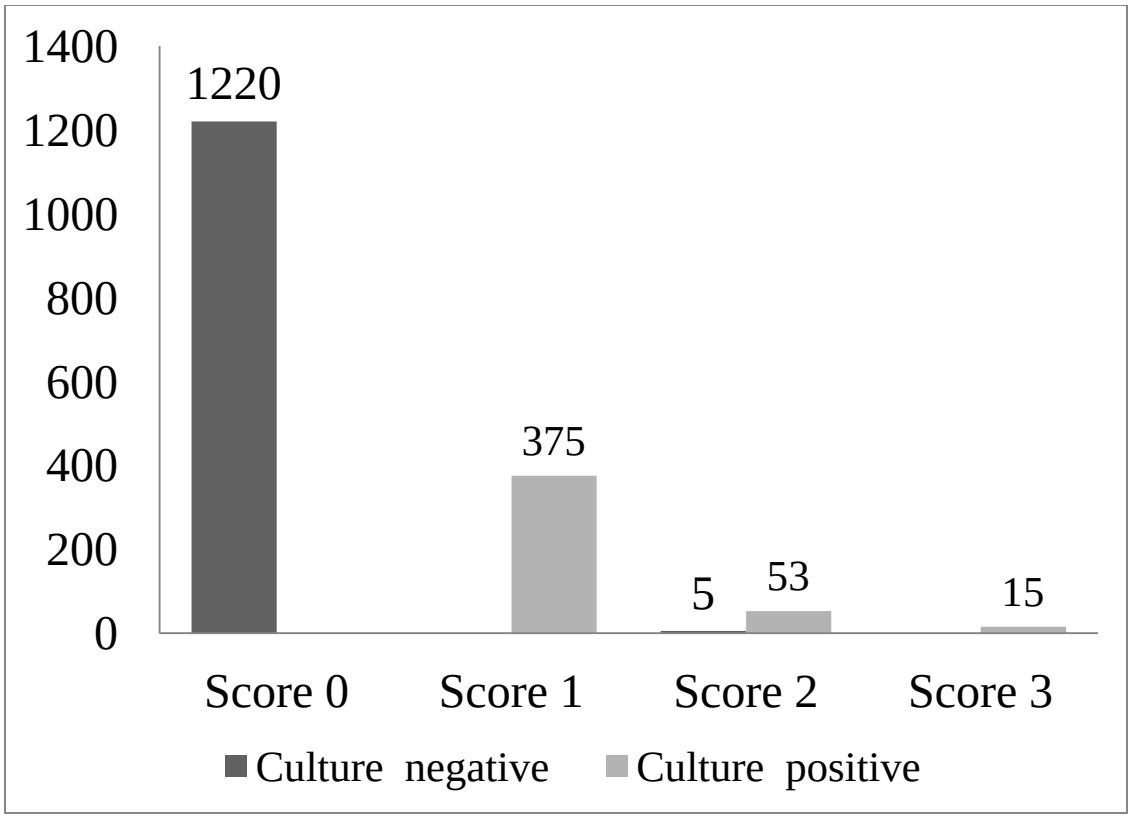


**Figure 3.2.** Distribution of 1668 pig fecal samples classified in scores, based on culture and direct quantitative real–time PCR (score 0: culture–negative and q–PCR negative; score1: culture-positive and q–PCR negative; score 2: culture-positive and q–PCR positive and culture–negative and q–PCR negative, not within quantifiable range; score 3: culture–positiveand q–PCR positive within quantifiable range, $>10^3$CFU/g).

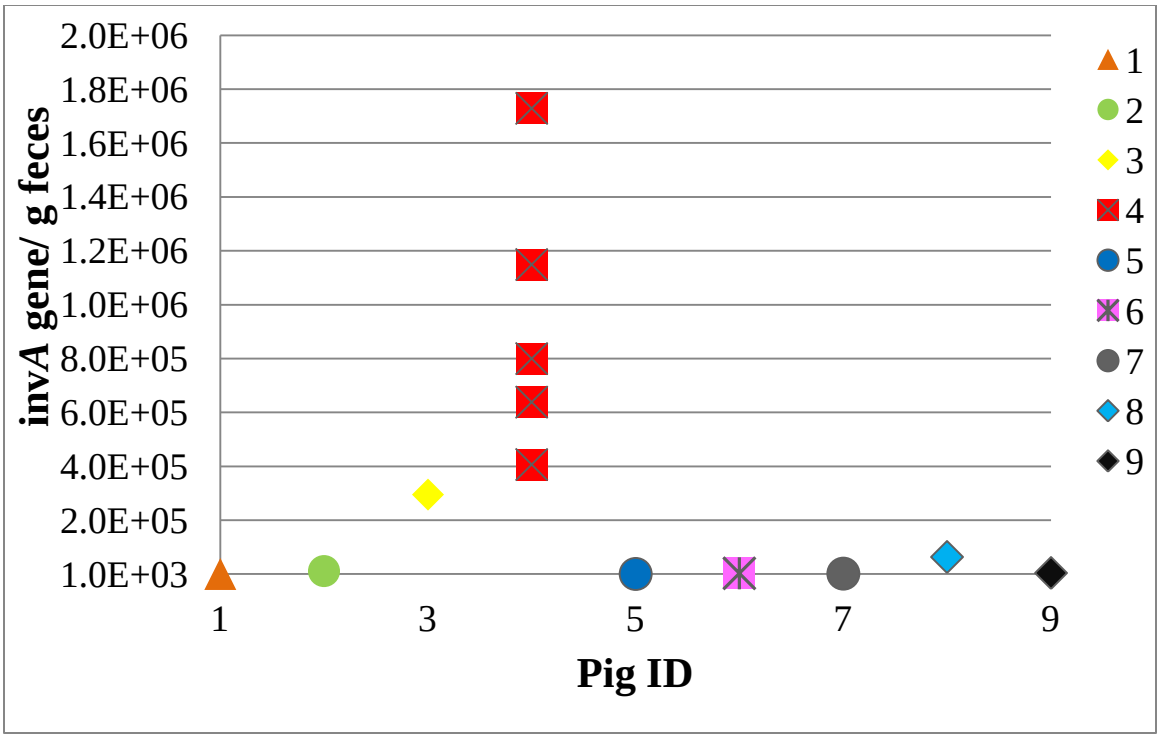


**Figure 3.3.** Concentration of *Salmonella invA* genes in fecal samples of 9 pigs, belonging to score 3 (culture–positive and quantitative real–time PCR positive in the quantifiable range, $>10^3$CFU/g).

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# CHAPTER 4

## Multilevel analysis to evaluate the association between environmental thermal parameters and *Salmonella* shedding in finishing pigs

## ABSTRACT

The objectives of this study were to evaluate the association between the thermal environment in the barn and *Salmonella* status in finishing pigs and to estimate the proportion of the model variance attributable to cohort, pig and individual sample level effects. For these purposes, individual fecal samples from 900 finishing pigs (8 collections per pig) were repeatedly collected from 18 cohorts (50 pigs per cohort) on 3 sites of a multi–site farrow–to–finish production system in a longitudinal study. Pen temperature and humidity were measured every 2 minutes during the study period. The thermal parameters of interest were: hourly average, minimum and maximum lagged temperatures, hourly temperature variation, temperature humidity index (THI) and cumulative number of hours/degree above and below the thermal of neutral zone at the pen level prior to fecal sampling for 6 time periods (12h, 24h, 48h, 72h, 1 week and 1 month). Additional potential risk factors at the individual (e.g., gender, health events), cohort (e.g., mortality, morbidity, *Salmonella* nursery status) and pen level were also evaluated. Multilevel logistic models using generalized linear models, with random intercepts at pig, pen and cohort levels to account for clustering (individual samples nested within pigs, pigs nested within pens, pens within cohorts) were constructed. The outcome variable was *Salmonella* fecal status of the individual sample. Cold exposure (temperatures below the thermal neutral zone) and exposure to a THI >72 were both associated with risk *Salmonella* shedding. Nursery *Salmonella* status, site, pig age and cohort mortality rate were also associated with *Salmonella* shedding.

The largest proportion of model variance was associated with the individual fecal sample (44.7%) followed by cohort (24.1%) and pen (20.7%). The present study allowed investigating

the association of time–variant thermal factors and *Salmonella* shedding. Interventions that target the thermal environment may have an effect on reducing *Salmonella* shedding in swine and also improve pig well–being and production efficiency. Alternatively, thermal parameters may be used to identify groups of pigs at high risk for *Salmonella* shedding. Future studies should be performed to investigate the cost–efficacy of interventions to improve the thermal environment to decrease *Salmonella* in swine.

## INTRODUCTION

Salmonellosis remains a major foodborne disease threat to public health worldwide (Greig and Ravel, 2009; CDC, 2011; Scallan et al., 2011). A seasonal pattern of human salmonellosis is well–described, with the highest incidence in summer (Naumova et al., 2007). Seasonal variation of foodborne diseases has been related with oscillations of several environmental factors (*e.g.*, temperature, humidity and precipitation) (Naumova et al., 2007). Among those environmental factors, ambient temperature has been consistently associated with human salmonellosis worldwide (Bentham and Langford, 2001; D'Souza et al., 2004; Kovats et al., 2004; Fleury et al., 2006; Naumova et al., 2007; Castronovo et al., 2009). In general human cases increased 1 to 6 weeks after peak ambient temperature (Bentham and Langford, 2001; D'Souza et al., 2004; Fleury et al., 2006; Naumova et al., 2007; Lake et al., 2009). While short–term lag times between high ambient temperature and illness may suggest that cross contamination and bacterial multiplication on food occur close to the point of consumption during food preparation (Bentham and Langford, 2001; Lake et al., 2009), long–term lag times suggest that ambient temperature affects *Salmonella* risk earlier in the food chain, including at the farm, the slaughterhouse, distribution systems or in the home (Bentham and Langford, 2001; D'Souza et al., 2004; Fleury et al., 2006; Lake et al., 2009). Overall, the association between the ambient temperature several weeks prior to the onset of the human cases suggests that temperature might affect *Salmonella* dynamics at the farm level. Those effects might be caused either by creating an environment favorable for the proliferation of bacteria in the environment and consequently increasing bacterial pressure and exposure to livestock, or by increasing the animal susceptibility to new or recurring infections.

A number of studies have investigated the seasonality of *Salmonella* infection in swine with mixed results. On one hand, some studies reported no seasonality (Benschop et al., 2008; Baptista et al., 2010) while others reported higher prevalence during different seasons; either with higher seroprevalence in winter and fall (Carstensen and Christensen, 1998; Christensen and Rudemo, 1998; Hald and Andersen, 2001; Smith et al., 2010) or summer (Hautekiet et al., 2008) and higher fecal prevalence in winter and spring (Funk et al., 2001a).

Season may serve as a proxy for many potential risk factors. Seasonality may represent other changes in management practices during those periods which could increase *Salmonella* risk or potentially a relationship between changes in environmental factors (e.g., temperature, humidity) and *Salmonella* shedding. Environmental factors such as temperature, rainfall and sunshine have been associated with *Salmonella* prevalence in swine. Finishing pigs exposed to wide variations in daily high temperature were at greater risk of high *Salmonella* prevalence (Funk et al., 2001a). In addition, large differences in long–term averages of the monthly mean temperature, as well as high rainfall and hours of sunshine were associated with higher *Salmonella* seroprevalence in UK pigs (Smith et al., 2010). In both studies the environmental parameters were retrieved from the closest weather station. Therefore, the recorded environmental parameters might not reflect the environment in closed barns. Moreover, herds that had ventilation control settings above the upper critical values (> 26 °C, for pigs of 90 kg) had a higher seroprevalence compared with herds with controlled programmed temperature within the thermal neutral zone (TNZ) (Hautekiet et al., 2008). A limitation of all of these studies is that they focused on investigation of risk factors at the herd level and were cross–sectional study designs. There is a lack of knowledge of risk factors at the pig–level and

of time–dependent factors; esprcially namely, environmental thermal parameters within the barn and the association with *Salmonella* dynamics.

The objectives of this study were to evaluate the association between environmental thermal parameters in the barn and *Salmonella* shedding in finishing pigs, and to estimate the proportion of total model variance attributable to cohort, pig and individual sample level effects. We hypothesize that there is an association between sub–optimal thermal parameters in the barn and *Salmonella* shedding in finishing pigs.

## MATERIALS AND METHODS

### Study design

A longitudinal study was conducted on a multi−site farrow−to−finish production system located in the Midwestern United States. The presence of *Salmonella* had been confirmed by culture of pooled fecal samples prior to initiation of the study. Selection criteria for the production system were willingness to cooperate in a long−term research project and to share health management and production records. The production system had three−site management, meaning that overall production was separated into three stages of production: breeding and farrowing, nursery (weaning until approximately 10 weeks of age) and finishing (10 weeks of age until slaughter, 24 to 26 weeks), with each stage housed at separate sites. The production system had all−in all−out (AIAO) management in nursery and finishing sites. Three finishing sites (A, B, and C) were conveniently selected, based on building design and willingness to participate in the study. At each finishing site one barn was selected for study inclusion. Site A had four barns in separate buildings, with cold weather mechanical ventilation and natural ventilation for warm weather ventilation. Pigs were allocated into 40 pens (20−25 pigs per pen). Dry feeders were shared in every two pens (20 feeders per barn) and each pen had two nipple waterers. Sites B and C had identical building structures: each site had four barns grouped in two buildings (two barns / building with one shared wall) and total mechanical ventilation (tunnel ventilation capable for warm weather). Each barn housed approximately 1000 pigs. Pigs were housed in 12 pens; ten pens were initially stocked with pigs at placement (eight pens with a range of 100−125 pigs and two pens with a range of 40−50 pigs). The remaining two pens were used for sick pens or pigs deemed to be at risk for illness. Double tube feeders (wet/dry) were

located in 8 larger pens and single tube feeders (wet/dry) in 4 smaller pens. Pens were separated by open, metallic gates and a central alley divided the barn. All buildings (A, B and C) had total slatted concrete floors and a deep pit that was emptied at least once a year. Propane heaters were used for heating at all sites. Sites A and B were finishing sites (10–26 weeks of age). Site C transitioned to a weaning to finishing site after the second cohort of pigs. For the wean to finish cohorts at Site C, piglets were placed in the barn at 3 weeks of age and remained until marketing. Site A cohorts were sampled from June 2008 to August 2010; site B cohorts from July 2008 to September 2010; site C cohorts from June 2009 to August 2011.

**Sample size**

The sample size employed in this study was selected to detect an expected difference of 6% in the prevalence between the exposed group (12%) and non-exposed group (6%) to ambient temperature greater than 23.9 °C (based on preliminary data of pigs in the 18 to 22 week old range). Assumptions were a fixed type 1 error of 5% (two-tailed test), 80% confidence and adding 20% due to loss of power associated with the inclusion of confounders and loss to follow-up. Considering intra-class correlations of 0.75 and 0.90 due to repeated sampling within individual pigs (8 sampling periods), a total of 853 pigs and 996 pigs were estimated, respectively (Twisk, 2007). As a compromise between the two estimates a total of 900 pigs were sampled in 18 cohorts (50 pigs per cohort, 8 samples per pig, 7200 total individual pig fecal samples).

**Sampling of individual fecal samples**

At the beginning of each cohort, 50 pigs (10 ± 2 weeks–old) were randomly selected and individually identified with ear tags. Random number generation was conducted in Microsoft Excel 2007 (Microsoft, Redmond, WA, USA). In site A, a simple random sample was generated to select 1 pig per every pen (n = 40). Another 10 pens were randomly selected to identify and select a second pig using a simple random sample (additional 10 pigs for a total of n = 50 / barn; 30 pens with 1 study pig, 10 pens with 2 study pigs). A random proportional sampling scheme based on the number of pigs in each pen was conducted in sites B and C within each cohort. A range of 1 to 7 pigs per pen was selected. In sites B and C no pigs were selected for study inclusion from the pens identified for sick or at–risk pigs.

Individual fecal samples were collected from the rectum with gloved hand, and placed in sterile containers (Specimen cups, VWR International LLC, PA, USA). Gloves for collecting the feces were changed between pigs. After collection, samples were stored at ambient temperature for transport to the laboratory. Individual pig fecal samples were collected every 2 weeks for 16 weeks (eight sampling periods per pig).

**Laboratory protocol for isolation of *Salmonella***

Bacteriological culture for *Salmonella* was performed by the Diagnostic Center for Population and Animal Health (DCPAH), Michigan State University. Fecal samples were transported to the laboratory the day of collection or stored for 48 hours at 2.8 °C. Fecal samples were cultured using standard methods described previously (Pires et al., 2012).

In order to evaluate the *Salmonella* status of the cohort of pigs prior to sampling during the finishing phase, 10 pooled fecal samples were collected from the nursery rooms approximately one week prior to movement to the finishing barn in sites B and C. In the wean to finishing site (Site C), 10 pools were collected from 10 random pens (1 pool/pen) when pigs were approximately 9 weeks of age. A pool consisted of a minimum of 5 g of fresh fecal material collected from 5 different locations on the same pen floor (25 g/pool). Contamination of the study barns was assessed by culture of barn environmental samples after cleaning and disinfection and before placement of each cohort of pigs in the barn. Drag swabs and environmental swab samples were obtained from cleaned and disinfected floors, walls, gates and feeders/drinkers (total of 20 samples per cohort) following previously described methods (Pires et al., 2012). Nursery and environmental samples were cultured using methods described previously (Pires et al., 2012).

**Environmental data collection and description of barn ventilation systems**

The thermal environment of the barns was monitored using a real−time system for the continuous measurement of temperature and humidity. Wireless network temperature sensors were used to monitor the temperature (Darr and Zhao, 2008) and commercially available weather resistant temperature and humidity data loggers (Hobo U23 Temperature / Relative Humidity, Onset HOBO Data Loggers, Bourne, MA, USA) were distributed to obtain humidity data and served as an alternative back−up system for temperature data.

The Site A barn used natural ventilation during summer, with temperature controlled by means of automatic curtains located on the north and south side walls of the building. In colder weather, the curtain was fully closed and temperature was controlled by exhausting air via three 12–inch ventilation fans. Two fans were located on the west end of the building while one was located near the east end wall. The barn used 4 ceiling–mounted mixing fans to promote equal temperature distribution. The barn had 10 box inlets equally distributed in the roof of the building for negative pressure drawing of air from the barn attic during cold weather. An automatic controller operated the curtains and fans with the set points manually adjusted at least monthly or as deemed necessary by the farm personnel. Twenty wireless temperature sensors were installed in the barns with one sensor placed every 2 pens (10 sensors per side). Ten weather resistant temperature and humidity loggers were distributed every four pens.

The barns in site B and C barn were fully mechanical tunnel–ventilated buildings. The barns operated with 5 stages of ventilation. The minimum stage utilized a 36–inch variable fan while the remaining four fans were 48–inch variable fans. The barn had one curtain walls, one curtain opened to the outside environment, while the second curtained wall was shared with the adjoining barn. While the curtain associated with the outside lowered based on ventilation needs, the shared curtain wall remained closed at all times. The barns had six box inlets equally distributed along in the ceiling for negative pressure ventilation during winter. Twenty two wireless temperature sensors were installed inside the barn, for each pen three were 3 sensors, 2 on gates of adjacent pens and 1 placed in the center near the feeders. Peripheral sensors were placed between pens, contributing data for the closest two pens. Ten temperature and humidity

data loggers (Hobo U23 Temperature/Relative Humidity, Onset HOBO Data Loggers, Bourne, MA, USA) were installed near the feeders.

**Environmental thermal parameters**

Temperature and humidity were recorded every 2 minutes twenty–four hours/day for the entire placement period of each cohort. Data was manually downloaded from the barns every two weeks. An Excel macro (Excel 2007, Microsoft, Redmond, WA, USA) was used to sort temperature and humidity data and calculate the environmental parameters at each sensor and pen for the measurement period. The pen averages of sites B and C were estimated using 3 sensors, and 1 sensor monitored every two pens in site A. The environmental parameters defined as exposures of the interest were: 1) absolute temperature at the sampling time; 2) hourly average temperature; 3) hourly variation (variance of average temperature); 4) maximum lagged temperature; 5) minimum lagged temperature; 6) the cumulative degrees and hours below the lower critical temperature of the TNZ; 7) the cumulative degrees and hours above the upper critical temperature of TNZ ; 8) temperature humidity index (THI); 9) the cumulative degree and hours above the THI threshold (72) for finishing pigs (St-Pierre et al., 2003). The upper and lower critical temperature of TNZ ($^{\circ}$C) criteria for lag times 12h to 1 week and 1 month are presented in Table 4.1 and were based on pig age (Harmon and Hongwei, 1995). The temperature humidity index used in this study was $THI = 0.63t_{wb} + 1.17t_{db} + 32$, where $t_{db}$ and $t_{wb}$ are the dry and wet bulb temperatures of the ambient air in °C (Lucas et al., 2000). For all parameters except absolute temperature, hourly and cumulative calculations for each parameter

were calculated for every pen for 6 time periods prior to the time of fecal sampling (12h, 24h, 48h, 72h, 1week and 1 month).

**Description of other variables**

The following data were recorded at each respective unit of observation (e.g., pig, pen and cohort). At the pig–level, gender, age (in weeks), diarrhea or any other symptom of illness, movement to a sick or subject pen, were recorded by the project personnel at each collection time. A pig was considered to have an abnormal health status if one of the following events occurred at the sampling time: 1) diarrhea; 2) sick or being moved to the sick pen; 3) undersized pig; 4) subject pig (defined by farm personnel: a pig that appears abnormal for any reason and is tagged with a unique tag and housed separately); 5) any sign of disease observed by research team personnel (e.g. lameness, diarrhea, respiratory signs). Pens were categorized as 'sick',' subject' and 'normal pen' in addition to the environmental parameters which were measured at the pen–level. At the cohort level, the mortality, morbidity (total number of treatments as a proxy for illness), total number of subject pigs, type of treatment (e.g., antimicrobial, anti–inflammatory therapy), *Salmonella* status of the nursery and barn environment, and season of each collection were recorded. Season was defined as follows: spring (March to May), summer (June to August), fall (September to November) and winter (December to February).

## Software used for data base management and statistical analyses

### Data management: exclusions and validation of data

An electronic database was created using Microsoft Access (Access 2007, Microsoft Corporation, Redmond, WA, USA) to record all the laboratory, environmental and field data (pig, cohort, farm). Data were imported into SAS 9.3 (SAS Institute, Cary, NC, USA) for data management and statistical analysis (descriptive statistics and model building). All the statistical analyses were performed in SAS 9.3 unless stated otherwise.

Bacteriological culture data were entered into a spreadsheet (Excel 2007) using appropriate coding and subsequently verified for accuracy by checking each entry with the original hard copy results. The spreadsheets were transferred to the relational database. A subset of individual pig observations data (n = 860) was verified using a random selection of the records for each pig variable. The sample size for data verification was based on an estimated 20% record entry error + 5% error with 95% of confidence interval, using an internet based calculator (available online at http://epitools.ausvet.com.au/content.php?page=1Proportion). The environmental parameter data were transferred to the database. Prior to statistical analysis of environmental parameters, data were explored by means of descriptive statistics and graphical visualization and evaluated for unlikely values. Outliers and extreme values outside of biologically plausible ranges were replaced as missing values before performing the statistical analysis.

**Model building**

Associations between the *Salmonella* status of finishing pigs and the risk factors at the cohort–, pig– and pen– level were evaluated. In order to account for the clustering of the data in a four–level hierarchical structure, multilevel models were applied, since individual fecal samples were nested within pigs, pigs within pens, and pens within cohorts. A multilevel logistic model with random intercepts at the pig–, pen– and cohort– levels was fitted using PROC GLIMMIX using a residual pseudo–likelihood subject–specific expansion method (RSPL) with optimization technique of Newton –Raphson with ridging. The dependent variable was at the individual fecal sample *Salmonella* status (yes/no). Site (A, B, C) was considered as a fixed effect. The final models were fitted with random intercepts and a random slope on age (time) at the pig–level in order to account for auto–correlation of sampling within pig (trend model) (Masaoud and Stryhn, 2010; Snijders and Bosker, 2012b).

Correlations between the independent variables were assessed based on Pearson's and Spearman's coefficients depending on whether the normality condition was met or not. If the value of the correlation statistic between two independent variables was equal to or greater that 0.8 at a $p \leq 0.05$, different approaches were conducted as follows. Independent models were built for each environmental variable (i.e., hourly average and maximum lagged temperature) for each time period (12 h, 24 h, 48 h, 72 h, 1 week and 1 month). Environmental variables measured in the same lag time were tested in same model when pair–wise correlation was less than 0.8% (maximum lagged and minimum temperatures; average hourly temperature and variation).

Before model building, the linearity assumption between the log odds of outcome and continuous predictors was tested using the following approaches: testing the quadratic term in the model, categorizing the predictor to see if the coefficients increased uniformly and/or plotting the continuous predictor against the logit of the outcome using the lowess curve (STATA version 11 StataCorp, College Station, TX, USA). If the linearity assumption was not met, a quadratic term was added based on visualization of a curvilinear shape of the lowess curve, or the variable was transformed using adequate transformation (natural log) or categorized, depending on the variable (Dohoo et al., 2010d).

Initially, a total of 61 environmental variables were examined and analyzed using descriptive statistics and graphics. The independent variables tested included thermal parameters measured at the pen–level (environmental parameters, type of pen), pig – level factors (gender, health status and age), cohort – level (mortality, morbidity, nursery and barn *Salmonella* status, season) and site. Independent variables were screened in univariable analysis using a 25% significance level (p–value < 0.25). All environmental variables of interest were included in multivariable models even if not significant in univariable screening. Manual building was conducted in multivariable models by backward elimination and independent models were constructed for the environmental parameters at different lag times and for highly correlated thermal variables (r >= 0.8). First-order interactions with biological plausibility and main effects were tested using Wald's test (p–value < 0.05). The variable site (A, B and C) was forced into the models. Interactions and main effects were removed one at a time. In the final model potential confounders based on causal diagrams (pig health status, morbidity, and season) were evaluated. A variable was considered a confounder if it caused a change greater than 20% to the coefficient of a statistically significant variable when the potential confounder was removed from

the model (Dohoo et al., 2010a). Hosmer-Lemeshow goodness-of-fit test was assessed on final models (Dohoo et al., 2010b).

**Variance components**

The proportion of model variance at each hierarchical level was estimated using a method based on latent response variables (or logistic threshold method) (Dohoo et al., 2010c; Snijders and Bosker, 2012a). The latent variable technique allows estimation of variance components and intra-class correlation by fixing error variance at $\pi^2/3$ and a mean of zero at the individual fecal sample level (Dohoo et al., 2010c). A random effect model with intercept as the only fixed term (null model), and final models (with one of the main exposures), and with the random effects of cohort, pen nested within cohort, pig nested within pen, and sampling nested within pig were fitted using residual pseudo–likelihood in PROC GLIMMIX, SAS 9.3. The total variance was estimated as follows: Var (Zijkl= var ($\mu_{Cohort\ (i)}$) + var ($\mu_{pen\ (j)}$ + var ($\mu_{pig\ (k)}$) + var ($\varepsilon_{(ijkl)}$) = $\sigma^2_{Cohort} + \sigma^2_{pen} + \sigma^2_{pig} + \pi^2/3$. Where $\pi^2/3$ is the variance occurring at the individual fecal sample level, $\sigma^2_{pig}$ at the pig level, $\sigma^2_{pen}$ at the pen level, and $\sigma^2_{Cohort}$ at the cohort level.

# RESULTS

## Assessing linearity of continuous variables and transformation of variables

Age was the only statistically significant continuous variable that had a linear relationship with the log odds of *Salmonella*. For the remaining variables, the following approaches were taken: as the lowess curves demonstrated a curvilinear shape, a quadratic term was tested for significance for the following variables: absolute temperature, hourly average temperature, maximum lagged temperature and temperature humidity index. The hourly variance and minimum lagged temperature were categorized in quartiles. The following environmental parameters had a correlation less than 0.8; therefore these variables were included in same model: 1) average hourly temperature and variance (quartiles); 2) maximum lag temperature and minimum lag temperature. The cumulative degree and hours above and below the TNZ and the degree and hours above THI threshold were transformed as described below.

A categorical transformation of cold and heat exposures was conducted. Cold exposure was defined as any time in each respective lag time that the pen had a temperature below the lower critical value of TNZ adjusted for pig age (Table 4.1); the reference group was pigs exposed to temperatures within the TNZ or above the UCT. An identical approach was conducted for the heat exposure, such that any time the pen had a temperature above the upper critical value of the TNZ for the respective pig age, it was categorized as heat exposed; for this variable the reference group was those pigs exposed to temperatures within the TNZ or below the LCT. Because of the distribution of the data of number of hours and units above THI of 72, the heat index exposure was transformed into a categorical variable, with heat exposure being

exposure to a THI greater than 72 (reference group was pigs exposed to THI less than or equal 72).

Significant risk factors at the cohort−level that did not meet the assumption of linearity were categorized into a binary variable: 1) mortality, morbidity (i.e., total of treatments) and the proportion of subject pigs that was estimated as a proportion based on total number of pigs placed at the beginning of each cohort and then categorized in 2 levels depending on central tendency; less than or equal to the mean (mortality = 1.78%; subject pigs = 1.87%) or median (morbidity = 0.48%) (reference level) and greater than the mean/median for all cohorts; 2) nursery *Salmonella* status was categorized into 2 levels based on the overall mean of the total positive pools (less than or equal to the mean, 3.64% (reference level) and greater than the mean); 3) barn environmental status was categorized as positive when at least one sample was *Salmonella* positive (reference level, all swabs were negative).

## Descriptive Results

### *Salmonella* prevalence results/ isolation of *Salmonella*

A total of 900 pigs was selected for inclusion in the study, 899 pigs were sampled at least once for fecal culture. The total loss due to missing sample for fecal collection was 5.1% (364/7200). Causes of missing sample and detailed description of *Salmonella* prevalence are described elsewhere (Pires et al., 2012). Nursery sampling could not be conducted in one of the cohorts (site A, cohort 3); therefore, the average of the positive pools among the nurseries of the site was attributed to that cohort in order to be able include the cohort in multilevel analysis. *Salmonella* was cultured from 6.6% (453/6836) of individual fecal samples. The distribution of

*Salmonella* prevalence by categorical variables at the pig–, pen– and cohort–levels is presented on Table 4.2.

## Descriptive statistics of environmental thermal parameters

### Missing observations for the environment pens

Due to a mechanical failure, temperature and humidity were not recorded during the first visit for cohort 1 at site A. Accordingly, this visit was excluded in all risk factor analyses. Therefore, the dataset used in model building for risk factor analyses was reduced to 6787 samples with a prevalence of 6.6% (448/6787) (Table 4.2). The risk factor analyses were conducted for the lag times 12 and 24 hours for all remaining cohorts (17/18) and for 48 and 72 hours in almost all cohorts (16/18) except visit 1 cohort 5, site A (the recorded period time was less than 72 hours). Since environmental monitoring was not possible prior to pig placement, risk analyses for the lag times of 1 week and 1 month were conducted for visit 2 and greater and visit 4 and greater, respectively. Hourly average, maximum lagged and minimum temperature , and temperature humidity index within 24h are graphically represented in in Figures 4.1 to 4.12, and stratified by site, cohort and pig age. Descriptive statistics of the environmental parameter variables used in the univariable and multivariable models are summarized in Tables 4.3 and 4.4.

**Risk analyses**

The data represent a four–level hierarchical structure with cohort at the highest level (N=18), followed by pen (N = 361), pig (range = 898–899) and individual fecal samples (range = 6412–6751) (Table 4.5.). Note that site (A, B and C) were treated as fixed effect.

The significant explanatory variables in the multivariable models are presented in Table 4.5; a separate model is presented for each significant main thermal exposure (n=5): 1) cold exposure at 12 hours, 2) 24 hours and 3) 72 hours; 4) heat index exposure at 24 hours and 5) 48 hours. There was a significant association between cold exposure and the odds of *Salmonella* shedding; pigs exposed to temperatures below their TNZ were more likely to be *Salmonella* positive (OR 1.51, 1.58 and 1.43 for temperatures measuered 12, 24 and 72 hours prior to sampling, respectively). Likewise, pigs exposed to an excessive heat index (THI > 72) 24 and 48 hours prior to sampling were at higher risk for shedding *Salmonella* (OR (24h) = 1.46; (95% C.I. 1.03–2.07); OR (48h) =1.45; (95% C.I. 1.01–2.09)). Nursery *Salmonella* status and cohort finisher mortality were significant in the final models. Pigs from nurseries with the proportion of positive pools greater than the mean were more likely to shed *Salmonella*. Pigs from cohorts with mortality greater than the mean were more likely to be *Salmonella* positive. There was also a significant effect of site; pigs from site A were at greater odds of being *Salmonella* positive compared to site C. Age was significantly associated with *Salmonella* status. As pig age increased, the risk of *Salmonella* shedding decreased linearly. For instance, in model 1 the relative odds of *Salmonella* shedding decreased 30% (OR (12h) = 0.7; (95% C.I. 0.65–0.74)) for each 2 week increase in age. No evidence of confounding was found regarding pig health status, morbidity or season. No significant interaction effects were identified (p –value > 0.05).

A random slope for pig age was introduced in the final models in order to account for auto-correlation; however, the models failed to converge with the random slope included in the models. Different estimation methods (e.g., Laplace approximation and Quad, Gauss-Hermit quadrature) were tried without success. The Hosmer-Lemeshow goodness–of–fit test was assessed on fixed effect models. The test was significant for the 5 models (p–value < 0.001).

**Variance components**

Estimates of variance, standard error of variance and proportion of variance for *Salmonella* shedding at each level are presented in Table 4.6. In the multi–level intercept only model, the proportion of variation explained at the sample, pig, pen and cohort levels was 44.8%, 10.2%, 20.5% and 24.5%, respectively. Only one model (model 1) was presented to explain the proportion of variation as the relative proportion at different levels were identical in all 8 final models. The proportion of variance explained at sample, pig, pen and cohort levels was 50.8%, 14.8%, 26.5%, 7.9%, respectively (Table 4.6).

## DISCUSSION

To our knowledge, this is the first study to evaluate time–dependent environmental risk factors influencing *Salmonella* shedding in finishing pigs. Multilevel models with 4 levels (cohort, pen, pig and individual fecal sample) were used to take into account the hierarchical data structure and to estimate the contribution of different levels to total variation of *Salmonella* shedding.

Sub–optimal thermal conditions in the barns were associated with *Salmonella* shedding and this association was significant at time periods relatively proximal to fecal sampling (72 h or less). Those conditions represented extremes of thermal conditions, either exposure to cold or heat index in a short–term time period, which might imply that only extremes have a significant effect on *Salmonella* shedding. The short time period prior to sampling being associated with *Salmonella* shedding may be a function of a true, short-term effect, and/or may suggest that pigs could have adapated to these temperatures with more time exposure. A biological explanation for the association between the thermal environment and *Salmonella* shedding is that sub–optimal temperature might increase pig stress, which can lead to lowered immunity and increased susceptibility to new infections and/or recrudescence of shedding in *Salmonella* carriers (Funk et al., 2001a; Hald and Andersen, 2001; Smith et al., 2010). The mechanisms behind the increased risk of infection when pigs are exposed to stress are complex and partially unknown (Mulder, 1995; Berends et al., 1996; Rostagno, 2009). But stress is generally considered to suppress the immune system and may lead to an increase in the occurrence of diseases (Salak-Johnson and McGlone, 2007). These data suggest that thermal environment may

at least be a component of this causal pathway and that decreasing the exposure to sub–optimal thermal parameters might decrease *Salmonella* in swine.

These data do represent the challenges producers face to keep the thermal environment within the TNZ, in particular during periods of extreme outdoor temperature even though the buildings have a controlled programmed temperature and ventilation system. The range of the TNZ thresholds are narrower in young pigs than older pigs (Jacobson et al.; Harmon and Hongwei, 1995; Fangman and Zulovich, 2000), and as consequence the likelihood for a pig being outside the TNZ at younger ages is greater than the older ages (end of the finishing period). The implications are that young pigs are at higher risk of being exposed to temperatures outside of the TZN ia also associated with the fact that young pigs are more susceptible to cold temperatures (Young, 1981; Moro et al., 1998; Carroll et al., 2001; Jones et al., 2001) and they are more prone to infection (Carroll et al., 2001; Jones et al., 2001) it might have a maginification effect of the both factors combined in the earlier stages of the finishing phase.

The available studies to evaluate heat and cold stress in swine are based mainly on outcomes such as production, animal behavior, metabolic/physiologic parameters and reproduction (Bloemhof et al., 2008). Few studies have investigated the relationship between foodborne pathogens and exposure to temperatures outside of the TNZ. The relationship between thermal stress and the intestinal microflora of swine has been mainly reported related to *E. coli* infections (Moro et al., 1998; Moro et al., 2000; Jones et al., 2001; Mathew et al., 2003). In these studies, exposure to thermal stress was associated with shifts in the antimicrobial resistance profile of *E. coli* isolated from the feces, potentially suggesting a shift in microbial populations or increases in antimicrobial resistance transfer under sub–optimal thermal conditions. In the present study it is unclear if the association between the sub-optimal thermal

exposures and *Salmonella* is due to a change of gastro–intestinal microflora and/or an increased risk of *Salmonella* infection or recrudescence of a previous infection; nevertheless, it resulted in increased shedding in *Salmonella* during those periods when pigs were outside of the TNZ.

Perharps unexpectedly, an association between *Salmonella* shedding and the high lagged temperature or heat load was not found; this is in contrast with reports of human salmonellosis, which have been associated with high ambient temperatures worldwide (Bentham and Langford, 2001; Kovats et al., 2004; Fleury et al., 2006; Naumova et al., 2007; Lake et al., 2009). On one hand, the lack of association might instead reflect the involvement of food contamination at other levels of the food chain; such as, from contamination or during food processing, transport, during commercial or home preparation of food. On the other hand, the association between heat index and *Salmonella* shedding may re–enforce the importance of exposure to high temperature and high relative humidity simultaneously for *Salmonella* risk in swine. The negative impact of heat stress on swine performance and health are well described in literature. The impact of heat abatement on loss of daily gain and death of grow–finishing swine when exposed heat stress has been associated with economic losses in multi–state study (St-Pierre et al., 2003). Cost–effectiveness of heat abatement interventions needs to be further investigated regarding *Salmonella* risk and production improvement in commercial swine systems. It may be that heat abatement costs that benefit public health may be economically viable for the producer through gains in production performance.

Others authors have reported an association between sub–optimal temperature and temperature variability and *Salmonella* infection in swine (Funk et al., 2001a; Hautekiet et al., 2008; Smith et al., 2010); however, these were limited by study design and source of thermal

data, which often did not represent the exposure inside the barn. The present study evaluated the effect of environmental parameters on *Salmonella* shedding by taking into account the time−variant nature of thermal risk factors by monitoring the thermal environment in the barn in real−time, as opposed to those that either considered the temperature retrieved from the closest weather station (Funk et al 2001; Smith 2010) or based on ventilation control settings above the TNZ in the barn (Hautekiet et al 2008).

The reference values of TNZ considered in this paper were developed based on performance and physiological responses to thermal stress and not on susceptibility to infections. One of the challenges to evaluate the effect of sub−optimal thermal parameters is to define the ideal TNZ range for finishing pigs. There is some divergence among the recommendations for TNZ (Jacobson et al.; Harmon and Hongwei, 1995; Fangman and Zulovich, 2000). Moreover, the effective temperature experienced by the pig will be different from air temperature measured in this study due to several factors such as drafts at the animal level, building insulation, and floor type (dry versus wet concrete, use of mats for draft protection) (Young, 1981; Gaughan et al., 2008). Adjustments to air temperature, accounting for drafts, building insulation and floor type, have been suggested to estimate the effective temperature (Fangman and Zulovich, 2000). Other factors might have an effect on stress response such as presence of environmental gases in barn (e.g., ammonia, carbon monoxide or dioxide, hydrogen sulfide) (Jacobson et al.; Fangman and Zulovich, 2000). Those factors were not taken into account in this study. Nor were adaptive behavioral changes such as grouping or huddling, which can affect the experienced temperature (Young, 1981). Pig acclimation to the thermal environment was not evaluated. Pigs can adapt to thermal changes, and the time interval of adaptation depends on many factors (e.g., time and magnitude of exposures) (Renaudeau et al., 2008). Pig acclimation to the environmental

temperatures may in part explain why only short–term exposures were found to be significant in this study.

Season was not significant in the multivariable models. As previously discussed, there is significant variability in the literature regarding season and *Salmonella* shedding on swine (Funk et al., 2001a; Hald and Andersen, 2001; Hautekiet et al., 2008; Smith et al., 2010). The lack of consistency in the literature might reflect not only variations of temperature, humidity and precipitation (Naumova, 2006) but also management factors (Hald and Andersen, 2001). Therefore, season might be a proxy of the thermal environmental oscillations in the barn, which could explain the lack of association in this study. This could be due to the fact that the effect of season can be off–set by management of the barn environment, so that actual the variability reported reflects varying capabilities for producers to keep pigs within the TNZ. Ventilation and heating of the barns are adjusted in response to the seasonal climatic changes, which might be a challenge in certain seasons (Funk and Gebreyes, 2004). Therefore, it is possible that some unobserved factors such as management may affect the seasonal pattern of *Salmonella* infection in swine. Moreover, management practices specific to production system type from country to country can contribute to different of *Salmonella* seasonality patterns in swine described in literature. Another justification to the fact season is not significant in this study is that the variability associated with cohort (either due to seasonal or management differences among cohorts) is that season may have been accounted for by controlling for cohort as a random effect, since the season and cohort are related because each cohort occurred over two (17 cohorts) or three (1 cohort) consecutive seasons.

There was a strong association between a fecal sample being *Salmonella* positive and cohorts with positive nursery pools greater than the mean. Pigs entering the finisher from these

cohorts were exposed to *Salmonella* in the nursery and may have been shedding at arrival to the finishing barn. Because of the exposure during the nursery phase, it is unclear if the effect of sub−optimal thermal environment, in particular cold exposure in young pigs, increases susceptibility to new infections, and/or the shedding duration during the finishing phase in pigs that were *Salmonella* positive at arrival. Nevertheless, the negative impact of cold exposure for production and health outcomes in young pigs is well−described (Young, 1981; Moro et al., 1998; Carroll et al., 2001; Jones et al., 2001), so improvement of environmental temperature management in the barn by keeping pigs within the TNZ range may not only decrease *Salmonella* shedding, but also will contribute to improve growing pig performance.

Despite the fact that environmental contamination can be a source of *Salmonella* infection, exposure to a *Salmonella* contaminated barn was not significant in the final models. Pigs exposed to a contaminated environment have been found to be at higher risk for *Salmonella* in previous studies (Beloeil et al., 2003; Beloeil et al., 2004; Beloeil et al., 2007). The presence of residual contamination is related to cleaning and disinfection of the facilities and equipment; those practices have been inconsistently associated with decrease of *Salmonella* prevalence on swine farm (Funk and Gebreyes, 2004; Fosse et al., 2009), either by decreasing the risk (Hautekiet et al., 2008; Cardinale et al., 2010) or by increasing the risk (van der Wolf et al., 2001; Poljak et al., 2008), or no difference among different practices (Rajic et al., 2007). One of the explanations for lack of significance could be due to a non differential exposure among cohorts by losing information when this risk factor was categorized into a binary variable. In fact, the majority of the positive fecal samples (85%; 383/453) were from cohorts with at least one positive environmental swab sample. Since *Salmonella* is difficult to eliminate from the barn (Funk and Gebreyes, 2004), and cleaning and disinfection only reduces the contamination

pressure (Mannion et al., 2007; Zewde et al., 2009), combined with the use of an imperfect diagnostic test to isolate *Salmonella* (Love and Rostagno, 2008), all the cohorts might have identical exposure regarding *Salmonella* contamination of the barn.

A decrease in *Salmonella* risk occurred with increasing pig age. Other authors have also reported a decrease in prevalence during the finishing period (Kranker et al., 2003; Nollet et al., 2005; Vigo et al., 2009; Molla et al., 2010).

There was a significant association between cohort mortality and *Salmonella* shedding. *Salmonella* infection in swine is mainly subclinical; mortality associated with clinical cases in swine are tipically associated with two main serovars (*S.* Cholerasuis var. Kunzendorf and *S.* Typhimurium) (Fedorka-Cray et al., 2000; Barrow et al., 2010). The servovars of the *Salmonella* isolates in this study were not identified. No clinical cases of salmonellosis were reported during the study period. The significant association with mortality for *Salmonell*a shedding might a result of mortality being a proxy of overall cohort health and/or management practices. An association between *Salmonella status* and several swine diseases has been reported (Møller et al., 1998; van der Wolf et al., 2001; Fablet et al., 2003; Beloeil et al., 2004; Beloeil et al., 2007). On the other hand, Lo Fo Wong et al., (2004) reported no association between health status and seroprevalence in European herds (Lo Fo Wong et al., 2004). Despite the limitation of not knowing the causes of mortality, there was no association with individual health status at the pig level or cohort morbidity and *Salmonella* shedding.

A significant difference was observed among sites despite belonging to the same production system and having an identical pig source, feed and overall management procedures. Other authors have reported variability in *Salmonella* prevalence among herds and within the

same herd over time (Funk et al., 2001b; Rajic et al., 2005). The observed difference might be due to unmeasured factors associated with site, such as producer behaviors and biosecurity.

In this study, the variance estimates for *Salmonella* shedding calculated in both models (null and full model with significant fixed effects) differed numerically and in relative variance regarding the cohort and pen levels. The individual fecal sample was the level with highest variance in both models. Taking into consideration the type of approach used to estimate the proportion of variation, the use of the latent variable usually attributes the highest variability to the lowest level (Dohoo et al., 2001; Funk et al., 2007). In the null model, after the sample level, the next highest relative proportions of variance were at the cohort and pen levels (24.5% and 20.5%, respectively). Organizational levels that explain the greatest amount of variation are considered the best for targeting interventions (Dohoo et al., 2001; Funk et al., 2007). Based on these data the cohort appears to be appropriate level to target interventions to reduce *Salmonella* shedding. Moreover, the highest variability levels (cohort and pen) should be taken into account when a sampling scheme is put in practice in epidemiological studies. Comparison of distribution of sources of variation between the null model and model with significant fixed effects (model 1) showed a reduction of the overall variance and change in the variance distribution. The proportion of variance attributable to cohort decreased, which can be explained by the inclusion of significant fixed effects at the cohort level (mortality and *Salmonella* positive pools in nursery). Nevertheless, pen and cohort remained significant sources of variation. Other authors have reported pen as a significant source of variation as well (Funk et al., 2007), as opposed to others that identified the highest source of variation as being the farm level (Poljak et al., 2008). This study differs from those by being a longitudinal study which investigated the association of time dependent variables at the pen level with repeated sampling within pig. We

did not measure previously described pen–level risk factors such as pen density (Funk et al., 2001a; Hautekiet et al., 2008) or pen weight (Poljak et al., 2008). However, those two factors were not taken into account because the pen-density was kept relatively constant over the study period and pig weight is highly correlated with pig age. Clustering of *Salmonella* shedding within the pen has been described in several studies (Davies et al., 1997; Beloeil et al., 2003; Funk et al., 2007; Poljak et al., 2008; Rao et al., 2010) and is primarily hypothesized to be a result of increased risk of transmission among pen–mates (Beloeil et al., 2003; Funk et al., 2007; Rao et al., 2010).

Despite the fact that the majority of variance was associated with individual fecal samples, the interpretation of the sources of variation and comparison with other studies should be done carefully because of the latent variable approach (Dohoo et al., 2010c). The estimates of the variance and respective standard errors calculated using the restricted pseudo–likelihood can lead to bias due to underestimation of the variance and standard errors (Dohoo et al., 2001; Masaoud and Stryhn, 2010). However, studies comparing methods to estimate model variance structure (using different estimating algorithms) of random effects have shown different numeric values, but with same trend in proportional distribution of the variation (Dohoo et al., 2001; Poljak et al., 2008).

One of limitations of this study is the using only one swine production company. One unique production company was selected in order to improve internal validity of the study, to control for potential confounders such as genetics, feed, treatment and vaccination protocols, biosecurity, and management practices. Moreover, due to the type of study, with monitoring of environmental thermal parameters in real–time, it would be difficult to implement in several production companies simultaneously. Nevertheless, the selected production system is

representative of the swine industry in the US and two types of buildings were included in order to account for different ventilation systems used in swine barns.

The other limitation is related to study design; because the study began at the time of placement in the finisher barn (10 weeks of age) there were differences in the duration of lag times available to be recorded among cohorts, particularly in first visit. Therefore analysis of thermal environmental effects was restricted to shorter lag times (12 to 72 hours) for the first collection (10 weeks of age) as well as exclusion of longer lag times from the second visit (longest recorded lag period was 1 week) and the monthly lag period could not be evaluated until the $4^{th}$ collection (16 weeks of age). The reduction of sample size due to no recording of environmental parameters might be to compromise the ability to find an effect in long term exposures, not only as a result of reduced power of detection in the older age groups, but also as a consequence of not being measured in younger pigs. Although a lack of association due to study limitations cannot be ruled out, previous studies based on effect of thermal stress on gastro-intestinal pathogen changes have been focused on the effects of short-term exposures (Moro et al., 1998; Moro et al., 2000; Jones et al., 2001).

Mechanical failure of the sensors resulted in loss of data regarding thermal parameters in some of the cohorts leading to reduction of the sample size, and consequently power to discern a difference in the dataset for the temperature parameters. This mechanical failure contributed to a decrease in sample size of 0.03% – 4.8%.

Statistical analysis of binary data, repeated measures and with hierarchical structure is a challenge (Dohoo et al., 2001; Masaoud and Stryhn, 2010) and some procedures can be computationally intensive. In order to reduce the unexplained auto–correlation of repeated sampling within–pig a time–varying variable (age) was included as fixed effect in models and a

random slope for age was offered in final models (Masaoud and Stryhn, 2010). Convergence problems were found when the random slope for age was tested; therefore a simpler model, without a random slope, was selected. The algorithm used in this study, restricted pseudo–likelihood, under certain conditions might be prone to bias towards the null (Masaoud and Stryhn, 2010). In simulation models of repeated measures studies, this approximation method has been shown to perform worse as compared to algorithms, leading to a downward bias of the estimates (Masaoud and Stryhn, 2010). Despite the possible bias towards to the null, the findings here reported support that there is a significant association among sub–optimal thermal environment and *Salmonella* shedding in swine. Discrepancy among the results from different estimation procedures for binary responses and multilevel data suggests that multiple procedures should be considered when fitting those models (Dohoo et al., 2001; Masaoud and Stryhn, 2010). Simultation studies accommodating both the data structure of repeated measures with binary outcoms and multilevel structures of the data are lacking in order to determine the best approach to analyze this type of data. A comparative study using this dataset might be useful to compare the estimates using different statistical approaches and is recommended for future research.

# CONCLUSION

Sub-optimal thermal conditions in the barns were associated with *Salmonella* shedding and this association was significant at time periods relatively proximal to fecal sampling (72 h or less). Those sub–otimal conditions were extremes, either exposure to cold (temperatures below the thermal neutral zone) or to heat index value of > 72, which reflects the challenge to keep the thermal environment within pigs'comfort zone even in mechanically controlled environments such as swine buildings. Interventions that target the thermal environment may reduce *Salmonella* shedding in swine and improve pig well–being and production efficiency. These types of interventions are encouraging, as the production benefits may provide incentive for producers to use environmental management as an intervention for *Salmonella* control. Alternatively, thermal parameters may be used to identify groups of pigs at high risk for *Salmonella* shedding. Future studies to identify efficacious and cost effective thermal environmental interventions are needed.

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# APPENDIX

**Table 4.1.** Upper and lower critical temperature criteria of thermal neutral zone of finishing pigs[a] used to assess the thermal (heat and cold) exposure.

| Pig age (weeks) | Pig weight (kg) | Thermal Neutral Zone (Temperature°C) | | | |
|---|---|---|---|---|---|
| | | Lag time 12h, 24h, 48h, 72h, 1 week | | Lag time 1 month | |
| | | LCT[a] | UCT[b] | LCT[a] | UCT[b] |
| 10 | 25 | 21.1[d] | 27.8[d] | NA[e] | NA[e] |
| 12 | 36 | 18.9 | 26.7 | NA[e] | NA[e] |
| 14 | 47 | 16.7 | 26.7 | NA[e] | NA[e] |
| 16 | 58 | 14.4 | 26.7 | 16.7 | 26.7 |
| 18 | 70 | 13.3 | 26.7 | 14.4 | 26.7 |
| 20 | 85 | 12.2 | 26.7 | 13.3 | 26.7 |
| 22 | 98 | 12.2 | 26.7 | 12.2 | 26.7 |
| 24 | 109 | 11.1 | 26.7 | 12.2 | 26.7 |

[a] Adapted from Harmon and Hongwei, 1995

[b] Lower critical temperature (°C)

[c] Upper critical temperature (°C)

[d] Thermal environment variables lag time 1 week not estimated at 10 week–old

[e] Not applicable, thermal environment variables lag time 1 month not estimated at 10–14 week old

**Table 4.2.** Proportion of *Salmonella* positive samples stratified by pig, pen and cohort variables (risk factors) among 6787 individual pig fecal samples. Multilevel univariable analysis.

| Measured at level | Variable (level) | Total samples % | *Salmonella* positive individual samples (%) | *P*–value[a] |
|---|---|---|---|---|
| Pig | Gender | | | 0.36 |
| | Male | 45.68 | 2.87 | |
| | Female | 54.32 | 3.73 | |
| | Abnormal health status[b] | | | 0.25 |
| | No | 93.37 | 6.19 | |
| | Yes | 6.63 | 0.41 | |
| Cohort | | | | |
| | Nursery[c] | | | <0.001 |
| | Greater than mean | 43.67 | 5.53 | |
| | Less than or equal to mean | 56.33 | 1.08 | |
| | Environment[d] | | | 0.18 |
| | Positive | 60.17 | 5.6 | |
| | Negative | 39.93 | 1 | |
| | Mortality[e] | | | 0.11 |
| | Greater than mean | 50.27 | 4.71 | |
| | Less than or equal to mean | 49.73 | 1.89 | |
| | Morbidity[f] | | | 0.3 |
| | Greater than median | 44.45 | 1.69 | |
| | Less than or equal to median | 55.55 | 4.91 | |
| | Subject[d] | | | 0.12 |
| | Greater than mean | 33.65 | 0.74 | |
| | Less than or equal to mean | 66.35 | 5.86 | |
| | Season | | | 0.01 |

**Table 4.2. (cont'd)**

| Measured at level | Variable (level) | Total samples % | *Salmonella* positive individual samples (%) | *P*–value[a] |
|---|---|---|---|---|
| | Spring | 16.55 | 0.47 | |
| | Summer | 36.98 | 1.12 | |
| | Fall | 17.17 | 2.21 | |
| | Winter | 28.7 | 2.8 | |
| Pen | | | | 0.28 |
| | Subject Pen | 1.84 | 0.13 | |
| | Sick Pen | 1.27 | 0.01 | |
| | Other Pens | 96.89 | 6.45 | |
| Farm | | | | 0.35 |
| | Site A | 33.73 | 2.09 | |
| | Site B | 32.46 | 3.64 | |
| Farm | | | | |
| | Site C | 33.81 | 0.87 | |

[a] Univariable analysis, multilevel logistic models with random intercepts at pig–, pen– and cohort–levels

[b] Abnormal health status (Yes) when one of the events occurred at the sampling time:1) diarrhea; 2) sick or being moved to the sick pen; 3) undersized pig; 4) 'subject' pig; 5) any sign of disease observed by research personnel

[c] nursery *Salmonella* status: overall mean of the total *Salmonella* positive pools (reference: less than or equal to the mean 3.64 %)

[d] Barn environmental status positive when at least one sample was *Salmonella* positive

[e] Mortality: overall mean of proportion of dead pigs(reference: less than or equal to the median, 1.78%), based on total of pigs placed at the beginning of each cohort

[f] Morbidity: median of proportion of total treatment (reference: less than or equal to the median, 0.48%), based on total of pigs placed at the beginning of each cohort

[g] Subject status: median (reference: less than or equal to the median, 1.87%) of proportion of total pigs that were deined by farm personnel as abnormal and housed separately, based on total of pigs placed at the beginning of each cohort

[h] Type of pen

**Table 4.3.** Descriptive statistics of the continuous thermal environment risk factors, univariable analysis.

| Variable | *Salmonella* positive fecal samples (%) | | *Salmonella* negative fecal samples | | N | *P*–value[c] |
|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | | |
| Absolute temperature[d] | 21.35 | 2.54 | 21.56 | 2.83 | 6644 | 0.004 |
| Hourly average temperature[d] | | | | | | |
| 12 hours | 21.22 | 2.33 | 21.37 | 2.62 | 6743 | <0.001 |
| 24 hours | 21.87 | 2.52 | 22.4 | 3 | 6746 | <0.001 |
| 48 hours | 21.9 | 2.48 | 22.4 | 2.91 | 6698 | <0.001 |
| 72 hours | 21.86 | 2.44 | 22.38 | 2.84 | 6698 | <0.001 |
| 1 week[a] | 21.77 | 2.32 | 22.32 | 2.87 | 5919 | <0.001 |
| 1 month[b] | 21.45 | 2.18 | 22.26 | 2.72 | 4187 | 0.63 |
| Hourly variation temperature[d] | | | | | | |
| 12 hours | 0.59 | 0.99 | 0.7 | 0.95 | 6787 | 0.73 |
| 24 hours | 2.24 | 4.53 | 3.45 | 5.18 | 6787 | 0.44 |
| 48 hours | 2.58 | 4.52 | 4.03 | 5.2 | 6732 | 0.33 |
| 72 hours | 2.82 | 5.08 | 4.51 | 5.78 | 6732 | 0.94 |
| 1 week[a] | 3.42 | 6.13 | 5.56 | 5.78 | 5941 | 0.44 |
| 1 month[b] | 4.8 | 8.06 | 7.62 | 15 | 4182 | 0.38 |
| Maximum lagged temperature[d] | | | | | | |
| 12 hours | 22.77 | 2.69 | 23.5 | 3.23 | 6719 | 0.02 |
| 24 hours | 24.2 | 3.25 | 25.37 | 3.96 | 6745 | 0.02 |
| 48 hours | 24.71 | 3.39 | 26.06 | 4.04 | 6690 | 0.06 |
| 72 hours | 25.02 | 3.44 | 26.6 | 4.18 | 6690 | 0.7 |
| 1 week[a] | 25.7 | 3.55 | 27.57 | 4.51 | 5887 | 0.24 |
| 1 month[b] | 26.56 | 3.39 | 29.34 | 4.4 | 4024 | 0.11 |

**Table 4.3. (cont'd)**

| Variable | *Salmonella* positive fecal samples (%) | | *Salmonella* negative fecal samples | | N | *P*–value[c] |
|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | | |
| Minimum lagged temperature[d] | | | | | | |
| 12 hours | 19.96 | 2.35 | 19.94 | 2.75 | 6717 | <0.001 |
| 24 hours | 19.64 | 2.48 | 19.61 | 2.93 | 6719 | <0.001 |
| 48 hours | 19.07 | 2.71 | 18.99 | 2.95 | 6673 | <0.001 |
| 72 hours | 18.73 | 2.68 | 18.66 | 2.94 | 6673 | <0.001 |
| 1 week[a] | 17.81 | 2.4 | 17.81 | 2.92 | 5891 | 0.002 |
| 1 month[b] | 15.82 | 2.69 | 16.09 | 3.16 | 4085 | 0.002 |
| Temperature Humidity Index | | | | | | |
| 12 hours | 68.31 | 6.09 | 69.82 | 6.38 | 6617 | 0.001 |
| 24 hours | 67.67 | 5.89 | 68.71 | 5.77 | 6622 | 0.003 |
| 48 hours | 67.68 | 5.9 | 68.7 | 5.71 | 6572 | <0.001 |
| 72 hours | 67.64 | 5.71 | 68.71 | 5.52 | 6577 | <0.001 |
| 1 week[a] | 67.18 | 5.84 | 68.61 | 5.6 | 5931 | 0.001 |
| 1 month[b] | 66.33 | 4.75 | 68.51 | 4.93 | 4187 | 0.9 |

[a]Estimates of collections 2 and greater

[b]Estimated of collections 4 and greater

[c]Univariable analysis, multilevel logistic models with random intercepts at pig–, pen– and cohort–levels

[d]Temperature units ° C

**Table 4.4.** Descriptive statistics of the categorical thermal environment risk factors, univariable analysis.

| Variable (level) | Total samples (%) | *Salmonella* positive individual samples (%) | N | *P*–value[a] |
|---|---|---|---|---|
| Hourly variation 12 hours | | | | 0.03 |
| Q1 | 22.32 | 1.5 | 6787 | |
| Q2 | 24.72 | 1.92 | | |
| Q3 | 27.89 | 2.09 | | |
| Q4 | 25.06 | 1.09 | | 0.7 |
| Hourly variation 24 hours | | | 6787 | |
| Q1 | 24.85 | 2.31 | | |
| Q2 | 25.12 | 2.03 | | |
| Q3 | 24.87 | 1.49 | | |
| Q4 | 25.05 | 0.77 | | |
| Hourly variation 48 hours | | | 6732 | 0.46 |
| Q1 | 24.85 | 2.3 | | |
| Q2 | 25.03 | 2.24 | | |
| Q3 | 24.99 | 1.17 | | |
| Q4 | 25.13 | 0.86 | | |
| Hourly variation 72 hours | | | | |
| Q1 | 23.14 | 2.63 | 6732 | 0.71 |
| Q2 | 26.62 | 1.93 | | |
| Q3 | 25.16 | 1.14 | | |
| Q4 | 25.07 | 0.88 | | |
| Hourly variation 1 week | | | 5941 | 0.53 |
| Q1 | 24.34 | 2.49 | | |
| Q2 | 24.91 | 1.6 | | |
| Q3 | 25.58 | 0.98 | | |
| Q4 | 25.16 | 0.62 | | |

**Table 4.4. (cont'd)**

| Variable (level) | Total samples (%) | *Salmonella* positive individual samples (%) | N | *P*–value[a] |
|---|---|---|---|---|
| Hourly variation 1 month | | | 4182 | 0.25 |
| Q1 | 31.35 | 2.13 | | |
| Q2 | 25.32 | 1.03 | | |
| Q3 | 17.74 | 0.45 | | |
| Q4 | 25.59 | 0.38 | | |
| Lowest lagged temperature 12 hours | | | 6719 | 0.005 |
| Q1 | 20.93 | 1.52 | | |
| Q2 | 24.1 | 1.85 | | |
| Q3 | 25.35 | 1.83 | | |
| Q4 | 25.63 | 1.38 | | |
| Lowest lagged temperature 24 hours | | | 6719 | <0.001 |
| Q1 | 24.93 | 1.52 | | |
| Q2 | 24.1 | 1.85 | | |
| Q3 | 25.35 | 1.83 | | |
| Q4 | 25.63 | 1.38 | | |
| Lowest lagged temperature 48 hours | | | | |
| Q1 | 24.55 | 1.38 | 6673 | <0.001 |
| Q2 | 25.27 | 2.05 | | |
| Q3 | 24.79 | 1.74 | | |
| Q4 | 25.4 | 1.38 | | |
| Lowest lagged temperature 72 h | | | 6673 | <0.001 |
| Q1 | 24.94 | 1.41 | | |
| Q2 | 24.64 | 1.84 | | |
| Q3 | 25.36 | 1.84 | | |
| Q4 | 25.07 | 1.45 | | |

**Table 4.4. (cont'd)**

| Variable (level) | Total samples (%) | *Salmonella* positive individual samples (%) | N | *P*–value[a] |
|---|---|---|---|---|
| Lowest lagged temperature 1 week | | | 5891 | 0.08 |
| Q1 | 24.49 | 1.37 | | |
| Q2 | 25.39 | 1.78 | | |
| Q3 | 24.7 | 1.41 | | |
| Q4 | 25.41 | 1.1 | | |
| Lowest lagged temperature 1 m | | | 4085 | 0.96 |
| Q1 | 24.77 | 0.91 | | |
| Q2 | 23.53 | 1.49 | | |
| Q3 | 26.02 | 1.03 | | |
| Q4 | 25.68 | 0.54 | | |
| Cold exposure 12 hours | | | 6751 | 0.001 |
| Yes | 11.6 | 1.39 | | |
| No | 88.4 | 5.18 | | |
| Cold exposure 24 hours | | | 6751 | 0.001 |
| Yes | 13.15 | 1.48 | | |
| No | 88.65 | 5.1 | | |
| Cold exposure 48 hours | | | 6701 | 0.001 |
| Yes | 17.04 | 1.9 | | |
| No | 82.96 | 5.61 | | |
| Cold exposure 72 hours | | | | 0.001 |
| Yes | 18.13 | 1.97 | | |
| No | 81.87 | 4.58 | | |
| Cold exposure 1 week | | | 5920 | 0.001 |
| Yes | 14.9 | 1.33 | | |
| No | 85.1 | 4.32 | | |

**Table 4.4. (cont'd)**

| Variable (level) | Total samples (%) | *Salmonella* positive individual samples (%) | N | *P*–value[a] |
|---|---|---|---|---|
| Cold exposure 1 month | | | 4160 | 0.04 |
| Yes | 21.63 | 1.08 | | |
| No | 78.37 | 2.88 | | |
| Heat exposure 12 hours | | | 6782 | 0.1 |
| Yes | 13.96 | 0.27 | | |
| No | 86.04 | 6.34 | | |
| Heat exposure 24 hours | | | 6782 | 0.08 |
| Yes | 32.88 | 1.28 | | |
| No | 67.18 | 5.32 | | |
| Heat exposure 48 hours | | | 6732 | 0.13 |
| Yes | 39.81 | 1.56 | | |
| No | 60.19 | 5.02 | | |
| Heat exposure 72 hours | | | 6732 | 0.46 |
| Yes | 43.52 | 1.62 | | |
| No | 56.48 | 4.96 | | |
| Heat exposure 1 week | | | 5941 | 0.99 |
| Yes | 51 | 1.48 | | |
| No | 49 | 4.21 | | |
| Heat exposure 1 month | | | 4182 | 0.23 |
| Yes | 60.9 | 1.12 | | |
| No | 39.1 | 2.87 | | |
| Heat Index exposure 12 hours | | | 6462 | <0.001 |
| Yes | 38.19 | 1.92 | | |
| No | 61.81 | 4.49 | | |

**Table 4.4. (cont'd)**

| Variable (level) | Total samples (%) | *Salmonella* positive individual samples (%) | N | *P*–value[a] |
|---|---|---|---|---|
| Heat Index exposure 24 hours | | | 6462 | <0.001 |
| Yes | 51.32 | 2.29 | | |
| No | 48.68 | 3.92 | | |
| Heat Index exposure 48 hours | | | | <0.001 |
| Yes | 57.72 | 2.81 | 6412 | |
| No | 42.28 | 3.57 | | |
| Heat Index exposure 72h | | | 6412 | 0.002 |
| Yes | 64.19 | 3.01 | | |
| No | 35.81 | 3.37 | | |
| Heat Index exposure 1 week | | | 5668 | 0.05 |
| Yes | 70.17 | 2.86 | | |
| No | 29.83 | 2.61 | | |
| Heat Index exposure 1 month | | | 3908 | 0.39 |
| Yes | 85.44 | 2.87 | | |
| No | 14.56 | 0.69 | | |

[a] Univariable analysis, multilevel logistic models with random intercepts at pig–, pen– and cohort–levels

**Table 4.5.** Final multivariable random effects logistic regression models of associations between thermal environment parameters, pig–level and cohort–level risk factors and *Salmonella* shedding in finishing pigs in three sites.

| Models | Independent variable | Beta[a] | SE[b] | OR[c] | 95% CI[d] | *P*-value[e] |
|---|---|---|---|---|---|---|
| Model 1 | | | | | | |
| | Intercept | -3.39 | 0.64 | … | … | … |
| | Cold exposure 12 hours | 0.41 | 0.2 | 1.51 | 1.02-2.25 | 0.04 |
| | Nursery status[f] | 1.93 | 0.46 | 6.91 | 2.79-17.15 | <0.001 |
| | Mortality[g] | 1.08 | 0.48 | 2.95 | 1.15-7.55 | 0.02 |
| | Age[h] | -1.181 | 0.017 | 0.7 | 0.65-0.74 | <0.001 |
| | Site[i] | | | | | 0.01 |
| | A vs C | 0.7 | 0.58 | 2.01 | 0.65-6.21 | |
| | B vs C | 1.8 | 0.61 | 6.06 | 1.84-19.98 | |
| | cohorts (n=18); pens (n=361); pigs (n=899); individual fecal samples (n=6751); *Salmonella* prevalence (6.58%) | | | | | |
| Model 2 | | | | | | |
| | Intercept | -3.42 | 0.64 | … | … | … |
| | Cold exposure 24 hours | 0.45 | 0.19 | 1.58 | (1.07-2.30) | 0.02 |
| | Nursery status[f] | 1.93 | 0.47 | 6.92 | (2.77-17.31) | <0.001 |
| | Mortality[g] | 1.08 | 0.48 | 2.94 | (1.14-7.59) | 0.02 |
| | Age[h] | -0.18 | 0.017 | 0.7 | (0.65-0.74) | <0.001 |
| | Site[i] | | | | | 0.01 |
| | A vs C | 0.7 | 0.58 | 2.02 | (0.65-6.29) | |
| | B vs C | 1.81 | 0.61 | 6.12 | (1.84-20.37) | |
| | cohorts (n=18); pens (n=361); pigs (n=899); individual fecal samples (n=6751); *Salmonella* prevalence (6.54%) | | | | | |

**Table 4.5. (cont'd)**

| Models | Independent variable | Beta[a] | SE[b] | OR[c] | 95% CI[d] | *P*-value[e] |
|---|---|---|---|---|---|---|
| Model 3 | | | | | | |
| | Intercept | -3.44 | 0.65 | … | … | … |
| | Cold exposure 72hours | 0.36 | 0.18 | 1.43 | (1-2.04) | 0.05 |
| | Nursery status[f] | 2 | 0.47 | 7 | | <0.001 |
| | Mortality[g] | 1.04 | 0.48 | 7.35 | (2.93-18.42) | 0.03 |
| | Age[h] | -0.18 | 0.018 | 0.7 | (0.65-0.75) | <0.001 |
| | Site[i] | | | | | 0.01 |
| | A vs C | 0.58 | 0.58 | 1.78 | (0.57-5.57) | |
| | B vs C | 1.76 | 0.61 | 5.81 | (1.77-19.13) | |
| | cohorts (n=18); pens (n=361); pigs (n=898); individual fecal samples (n=6701); *Salmonella* prevalence (6.55%) | | | | | |
| Model 4 | | | | | | |
| | Intercept | -3.68 | 0.65 | … | … | … |
| | Heat Index exposure 24 hours | 0.38 | 0.18 | 1.46 | (1.03-2.07) | 0.032 |
| | Nursery status[f] | 1.96 | 0.44 | 7.14 | (2.99-16.95) | <0.001 |
| | Mortality[g] | 1.24 | 0.46 | 3.47 | (1.41-8.54) | 0.007 |
| | Age[h] | -0.18 | 0.017 | 0.7 | (0.65-0.74) | <0.001 |
| | Site[i] | | | | | 0.006 |
| | A vs C | 0.65 | 0.55 | 1.92 | (0.65-5.65) | |
| | B vs C | 1.81 | 0.59 | 6.09 | (1.92-19.30) | |
| | cohorts (n=18); pens (n=361); pigs (n=899); individual fecal samples (n=6462); *Salmonella* prevalence (6.41%) | | | | | |

**Table 4.5. (cont'd)**

| Models | Independent variable | Beta[a] | SE[b] | OR[c] | 95% CI[d] | *P*-value[e] |
|---|---|---|---|---|---|---|
| Model 5 | | | | | | |
| | Intercept | -3.61 | 0.64 | | | |
| | Heat Index exposure 48 hours | 0.37 | 0.19 | 1.45 | (1.01-2.09) | 0.046 |
| | Nursery status[f] | 2.02 | 0.44 | 7.54 | (3.18-17.91) | <0.001 |
| | Mortality[g] | 1.22 | 0.46 | 3.4 | (1.39-8.33) | 0.007 |
| | Age[h] | -0.18 | 0.017 | 0.7 | (0.65-0.74) | <0.001 |
| | Site[i] | | | | | 0.005 |
| | A vs C | 0.54 | 0.55 | 1.75 | (0.59-5.05) | |
| | B vs C | 1.8 | 0.58 | 7.54 | (3.18-17.91) | |
| | cohorts (n=18); pens (n=361); pigs (n=898); individual fecal samples (n=6412); *Salmonella* prevalence (6.38%) | | | | | |

[a] Regression coefficient

[b] Standard error of the mean

[c] Odds ratio

[d] 95% confidence interval

[e] Wald test

[f] Reference less than or equal to mean ( %)

[g] Reference less than or equal to mean ( %)

[h] Age 2 weeks unit

[i] Reference: site C

[j] Thermal neutral zone

**Table 4.6.** Variance components and proportion of variance at the cohort–, pen–, pig and individual fecal–level of the null model and final model (model 1, cold exposure at 12 hours).

| Data hierarchy | Null–Model | | | Final model–Model 1 | | |
|---|---|---|---|---|---|---|
| | Variance estimate | Se[a] | Proportion (%) | Variance estimate | Se[a] | Proportion (%) |
| Cohort | 1.8 | 0.75 | 24.5 | 0.51 | 0.35 | 7.9 |
| Pen | 1.51 | 0.3 | 20.5 | 1.72 | 0.35 | 26.5 |
| Pig | 0.75 | 0.18 | 10.2 | 0.96 | 0.21 | 14.8 |
| Individual fecal sample[b] | 3.29 | … | 44.8 | 3.29 | … | 50.8 |
| Total variance | 7.35 | … | 100 | 6.48 | … | 100 |

[a] Standard error

[b] Individual fecal sample variance: $\pi^2/3=3.29$ (latent–variable technique)

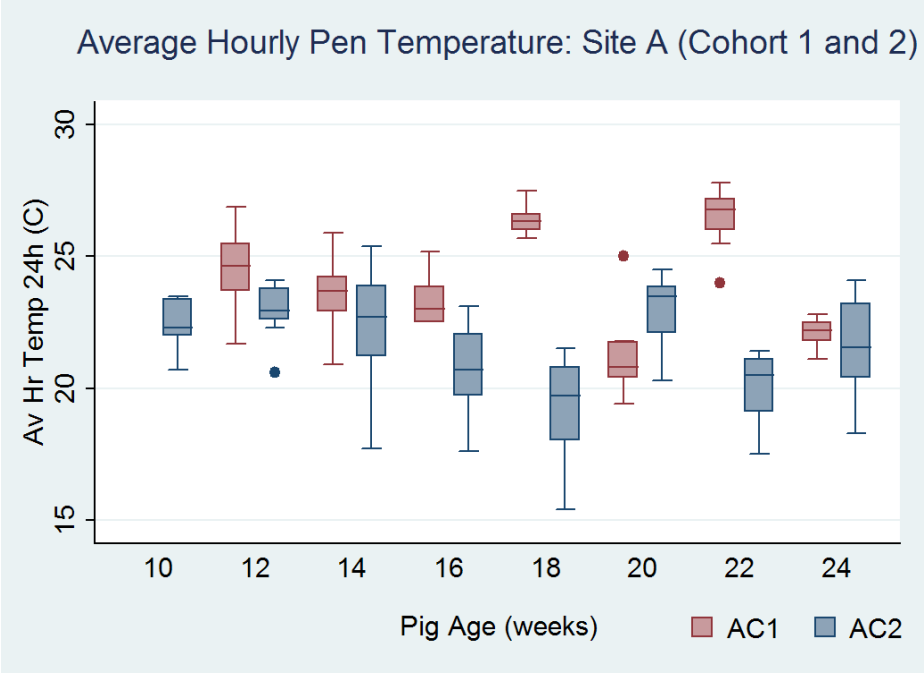


**Figure 4.1.a.** Box plot of the average hourly pen temperature (°C) within 24 hours for cohort 1 (AC1, 06/3/2008 – 09/06/2008) and 2 (AC210/08/2008 – 01/24/2009) by pig age in site A. Note no data on first visit of the cohort 1 (10 week–old).

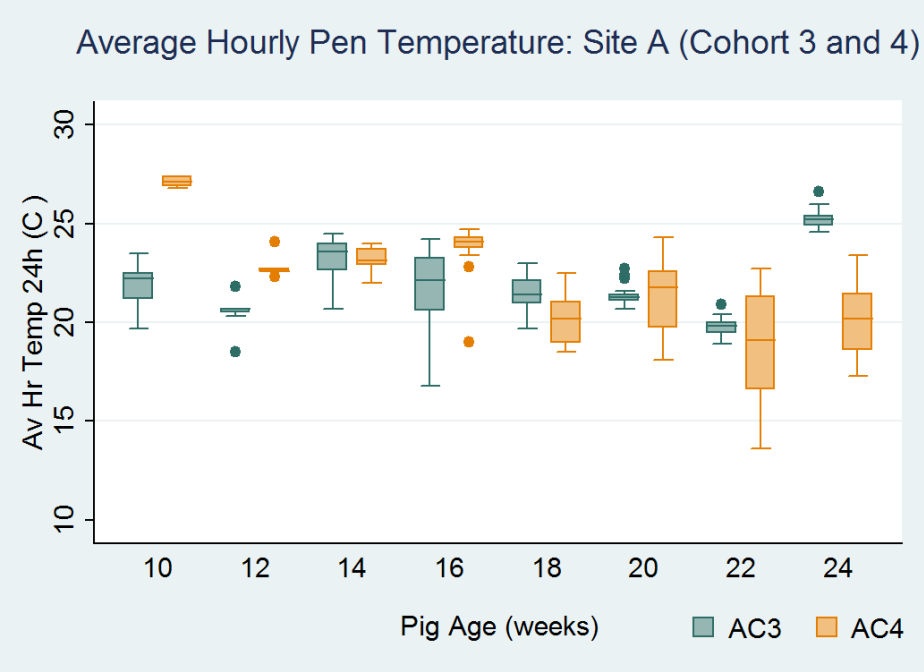


**Figure 4.1.b.** Box plot of the average hourly pen temperature (°C) within 24 hours for cohort 3 (AC3, 03/21/2009 – 06/29/2009) and 4 (AC4, 08/10/2009 – 11/17/2009) by pig age in site A

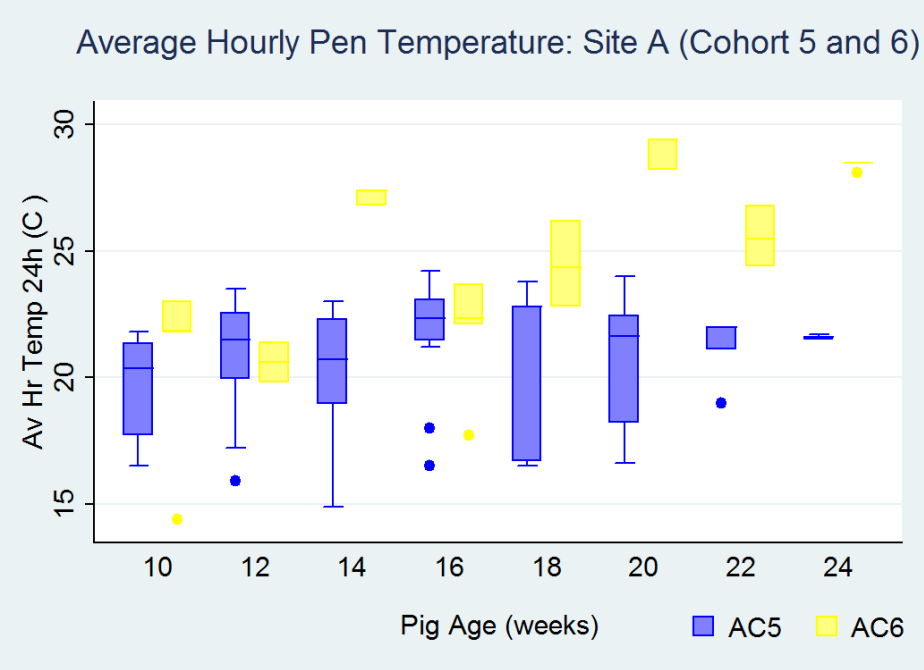


**Figure 4.1.c.** Box plot of the average hourly pen temperature (°C) within 24 hours for cohort 5 (AC5, 12/16/2009 – 03/22/2010) and 6 (AC6, 04/24/2010 – 08/02/2010) by pig age in site A.

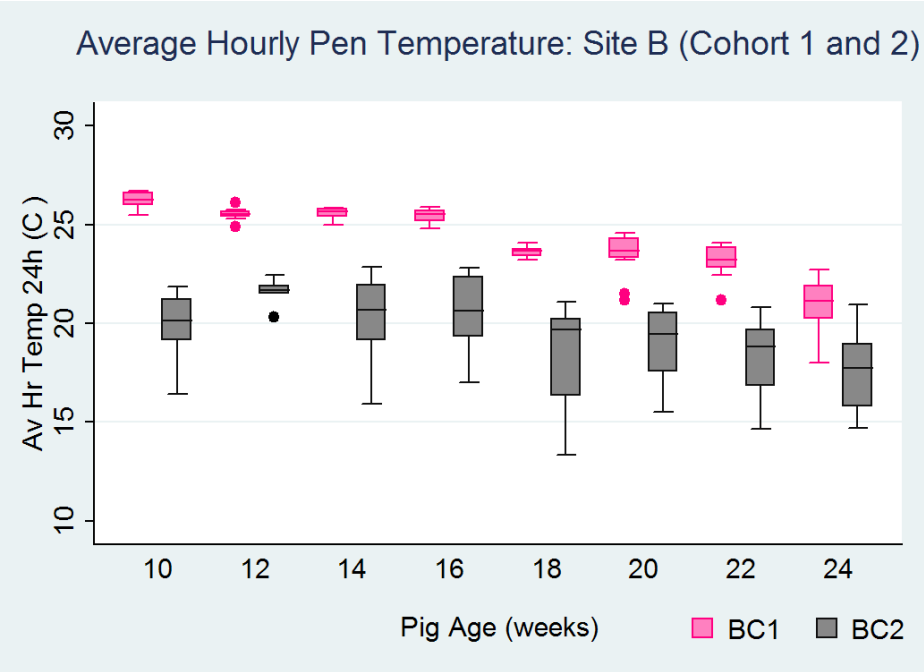


**Figure 4.2.a.** Box plot of the average hourly pen temperature (°C) within 24 hours for cohort 1 (BC1, 07/07/2008 – 10/11/2008) and 2 (BC2, 11/22/2008 – 02/28/2009) by pig age in site B.

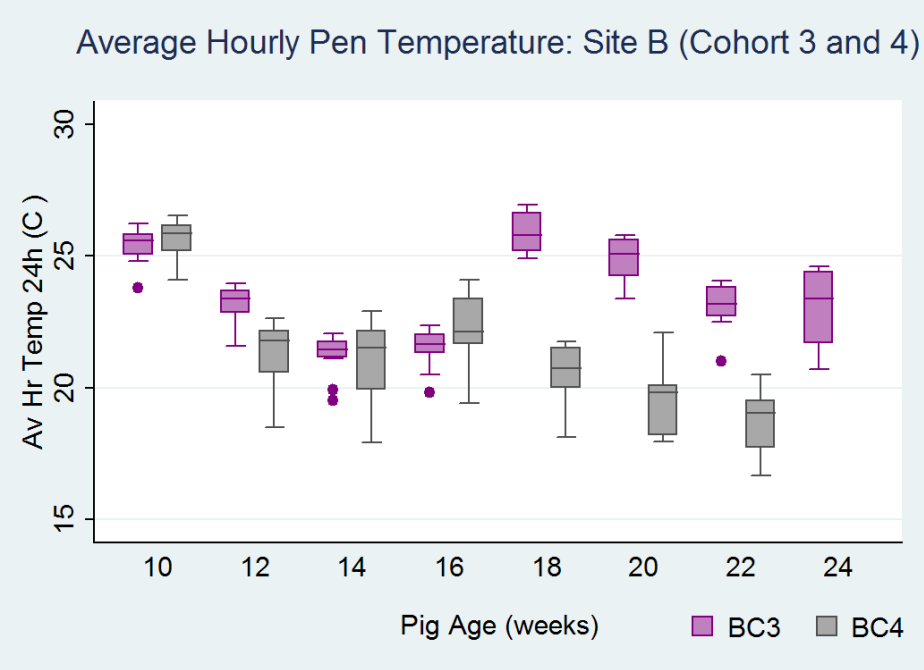


**Figure 4.2.b.** Box plot of the average hourly pen temperature (°C) within 24 hours for cohort 3 (BC3, 04/25/2009 –08/03/2009) and 4 (BC4, 09/15/2009 – 12/08/2009) by pig age in site B. Note no data on last visit of the cohort 4 (24week–old).

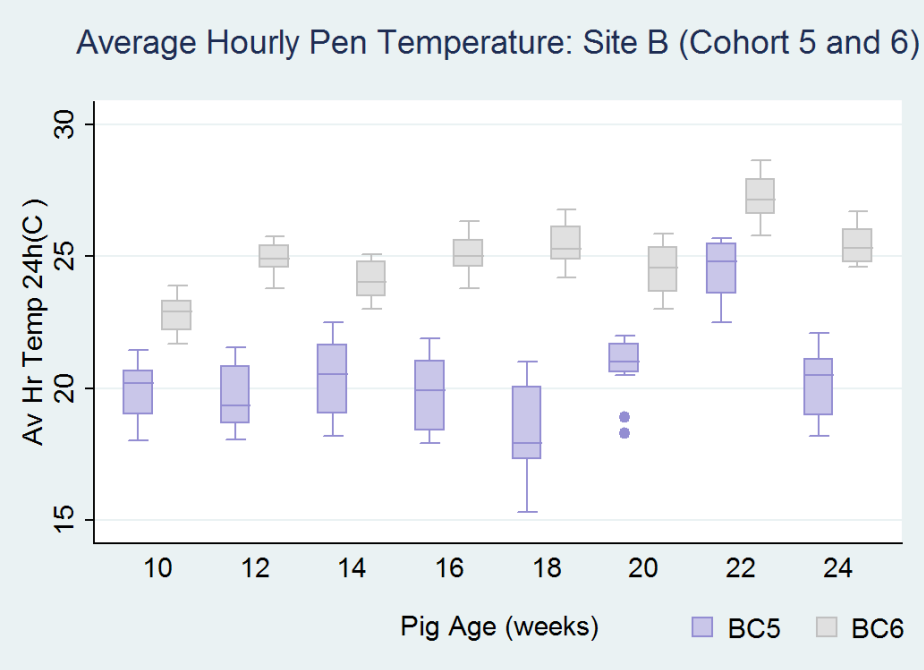


**Figure 4.2.c.** Box plot of the average hourly pen temperature (°C) within 24 hours for cohort 5 (BC5, 01/11/2010 – 04/17/2010) and 6 (BC6, 05/19/2010 – 08/23/2010) by pig age in site B.

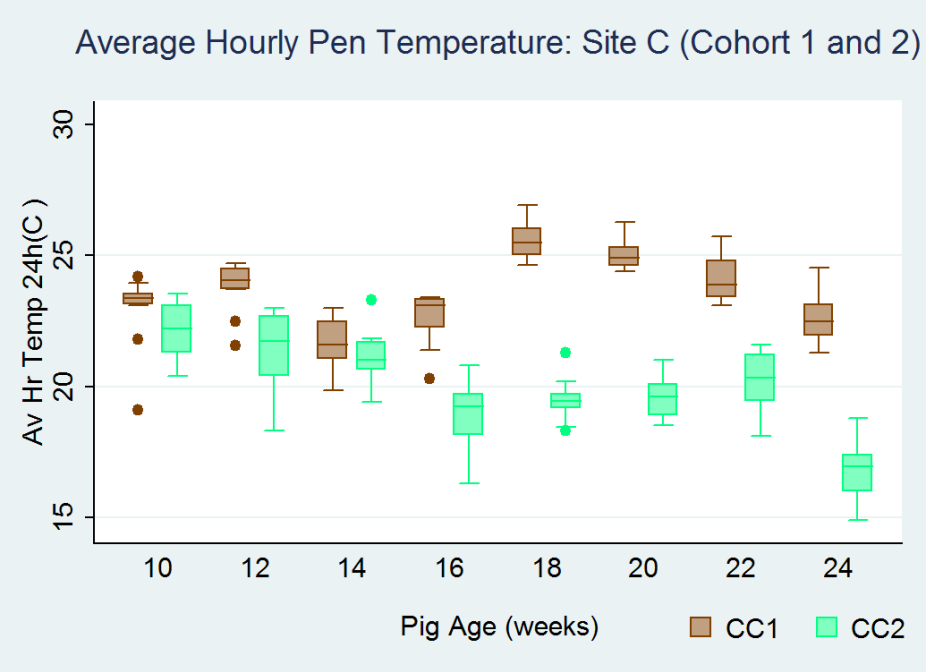


**Figure 4.3.a.** Box plot of the average hourly pen temperature (°C) within 24 hours for cohort 1 (CC1, 06/02/2009 – 09/08/2009) and 2 (CC2, 12/21/2009 – 03/27/2010) by pig age in site C.

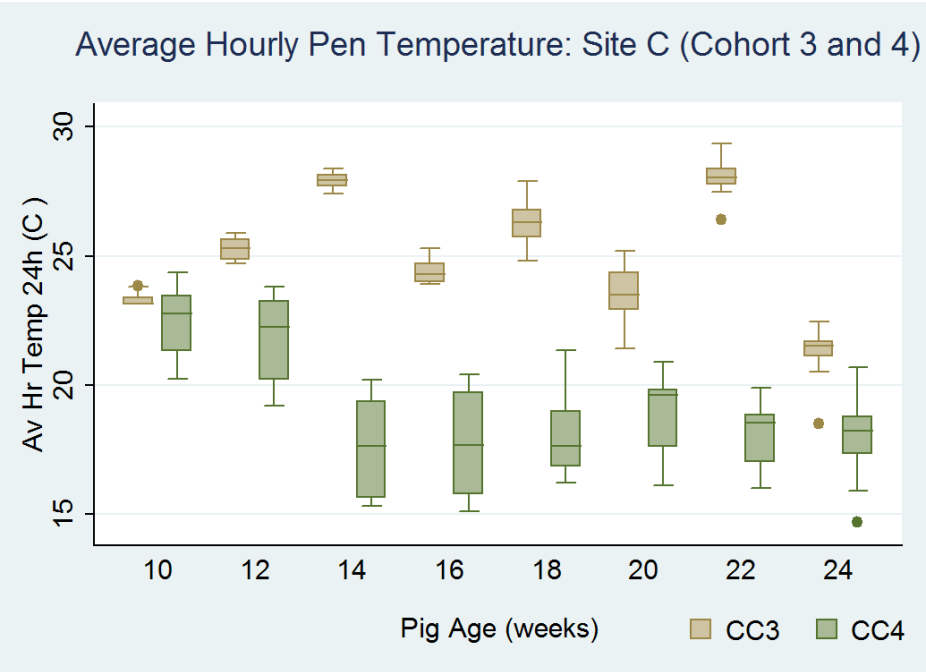


**Figure 4.3.b.** Box plot of the average hourly pen temperature (°C) within 24 hours for cohort 3(CC3, 06/07/2010 – 09/11/2010) and 4 (CC4, 11/20/2010 – 02/26/2010) by pig age in site C.

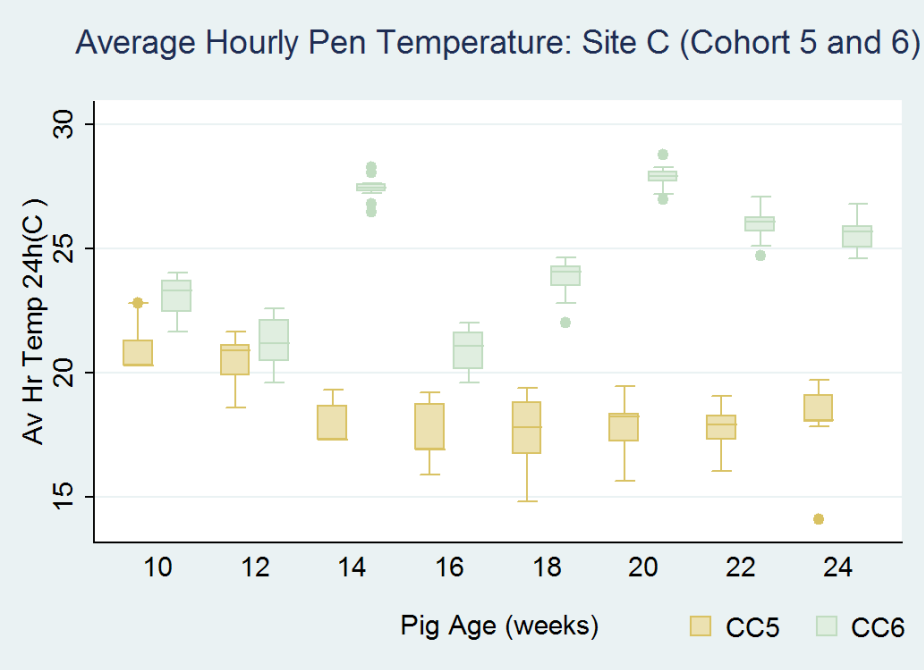


**Figure 4.3.c.:** Box plot of the average hourly pen temperature (°C) within 24 hours for cohort 5 (CC5, 11/27/2010 – 03/05/2011) and 6 (CC6, 04/30/2011 – 08/08/2011) by pig age in site C.

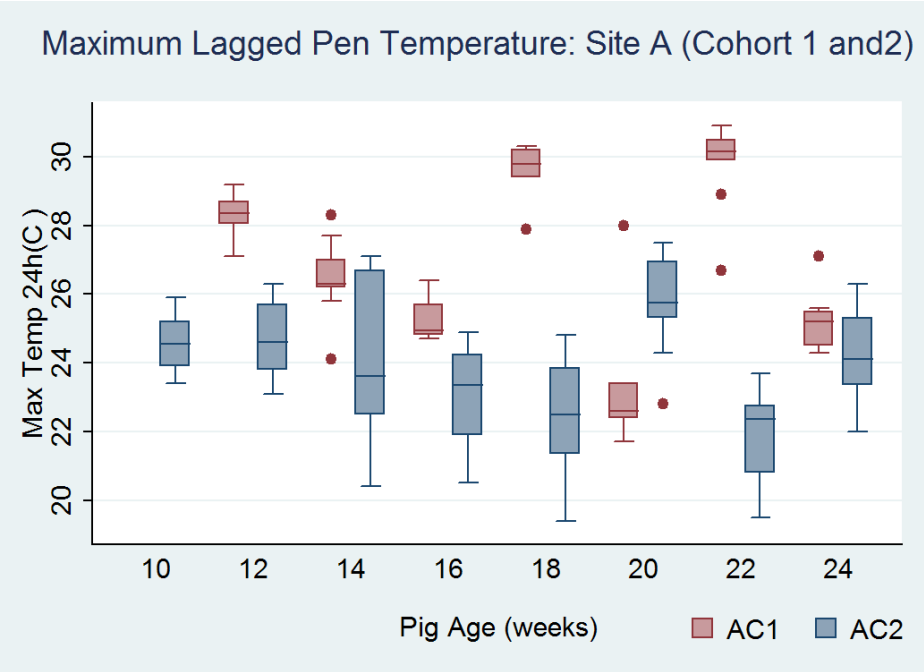


**Figure 4.4.a.** Box plot of the maximum lagged pen temperature (°C) within 24 hours for cohort 1 (AC1, 06/3/2008 – 09/06/2008) and 2 (AC2,10/08/2008 – 01/24/2009) by pig age in site A. Note no data on first visit of the cohort 1 (10 week–old).

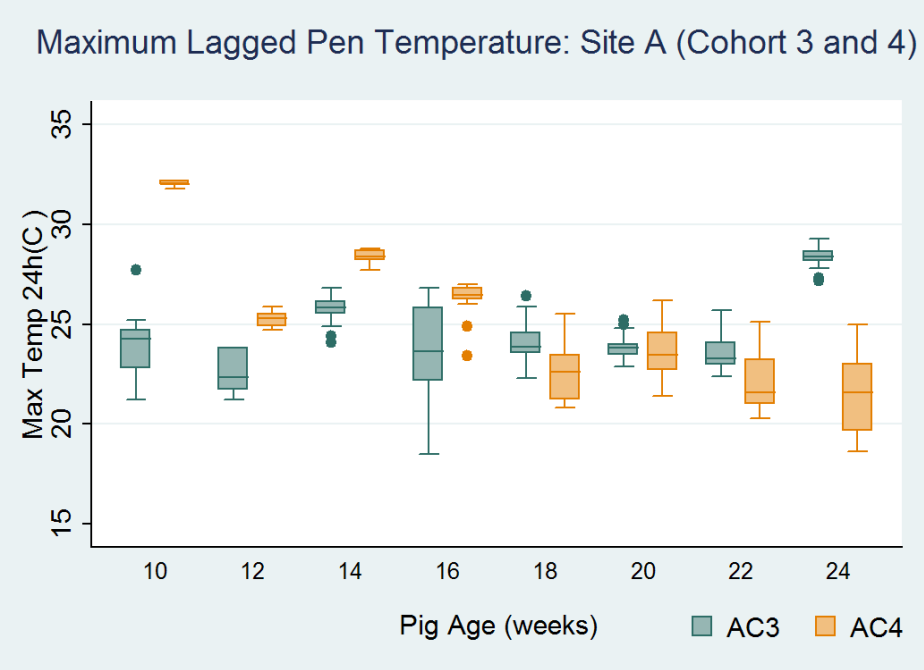


**Figure 4.4.b.** Box plot of the maximum lagged pen temperature (°C) within 24 hours for cohort 3 (AC3, 03/21/2009 – 06/29/2009) and 4 (AC4, 08/10/2009 – 11/17/2009) by pig age in site A.

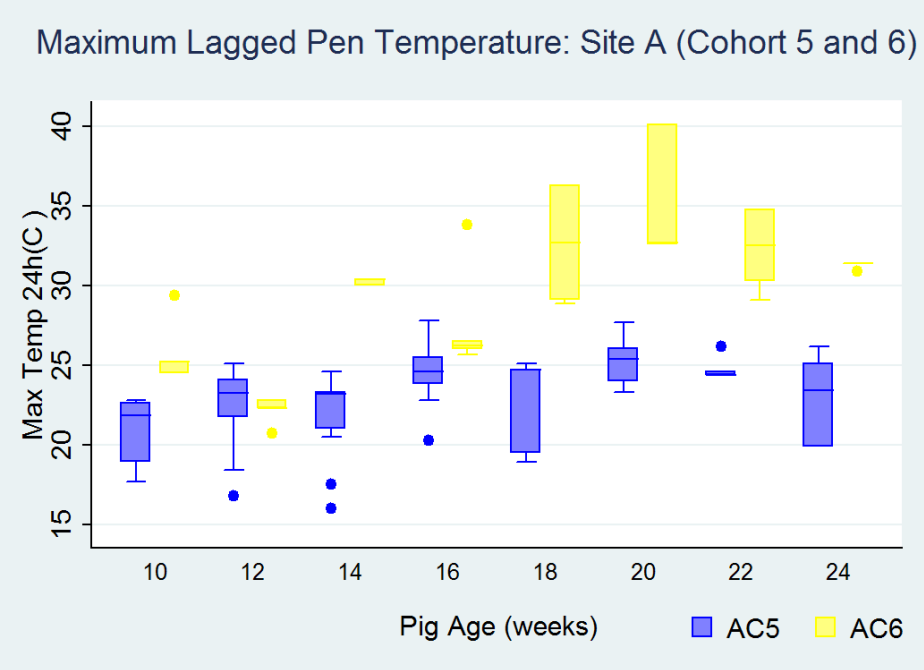


**Figure 4.4.c.:** Box plot of the maximum lagged pen temperature (°C) within 24 hours for cohort 5 (AC5, 12/16/2009 – 03/22/2010) and 6 (AC6, 04/24/2010 – 08/02/2010) by pig age in site A.

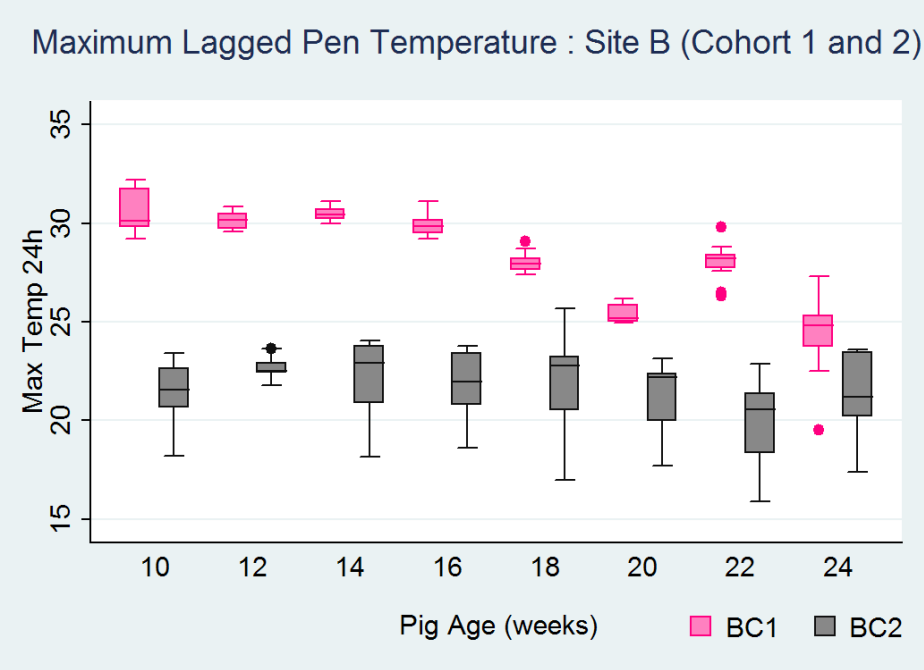


**Figure 4.5.a.** Box plot of the maximum lagged pen temperature (°C) within 24 hours for cohort 1 (BC1, 07/07/2008 – 10/11/2008) and 2 (BC2, 11/22/2008 – 02/28/2009) by pig age in site B.

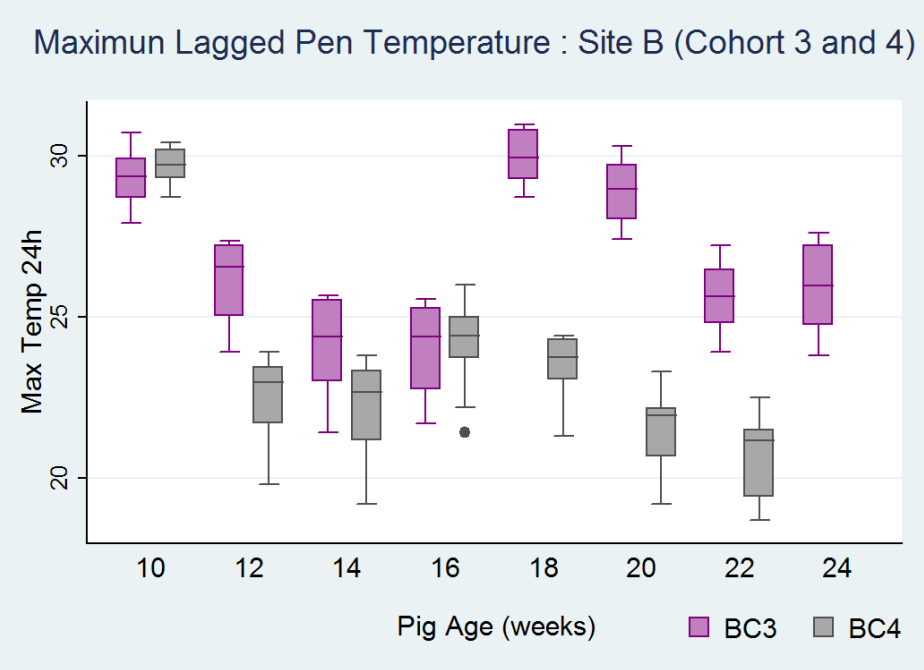


**Figure 4.5.b.:** Box plot of the average hourly pen temperature (°C) within 24 hours for cohort 3 (BC3, 04/25/2009 –08/03/2009) and 4 (BC4, 09/15/2009 – 12/08/2009) by pig age in site B. Note no data on last visit of the cohort 4 (24week–old).

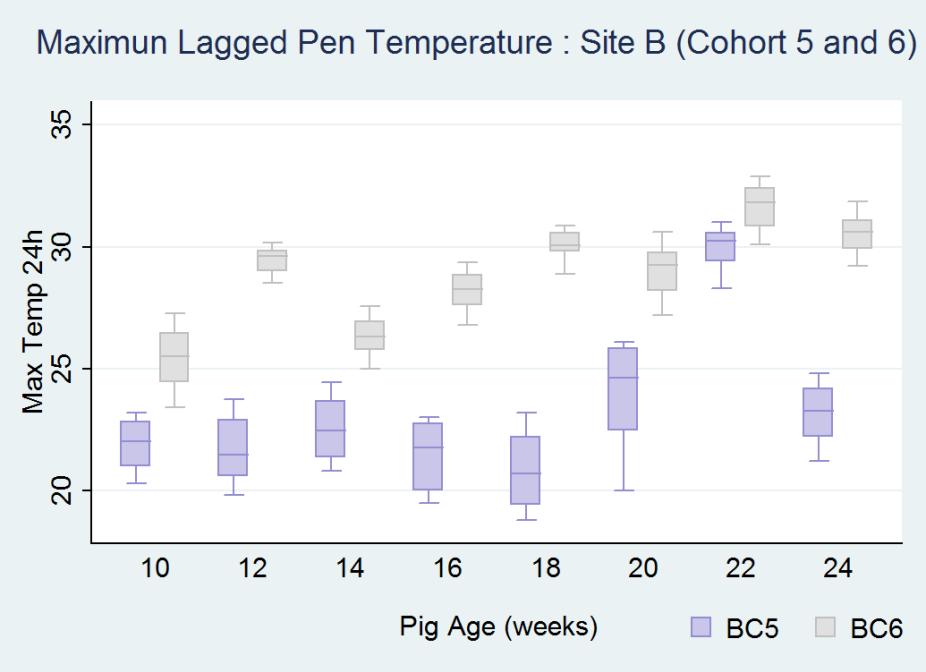


**Figure 4.5.c.** Box plot of the maximum lagged pen temperature (°C) within 24 hours for cohort 5 (BC5, 01/11/2010 – 04/17/2010) and 6 (BC6, 05/19/2010 – 08/23/2010) by pig age in site B.

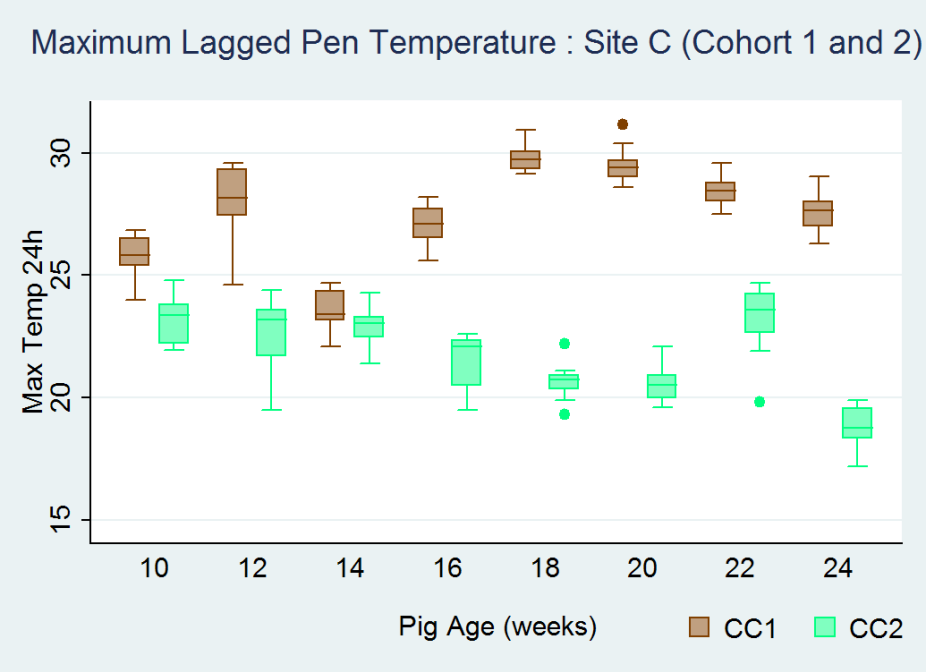


**Figure 4.6.a.:** Box plot of the maximum lagged pen temperature (°C) within 24 hours for cohort 1 (CC1, 06/02/2009 – 09/08/2009) and 2 (CC2, 12/21/2009 – 03/27/2010) by pig age in site C.

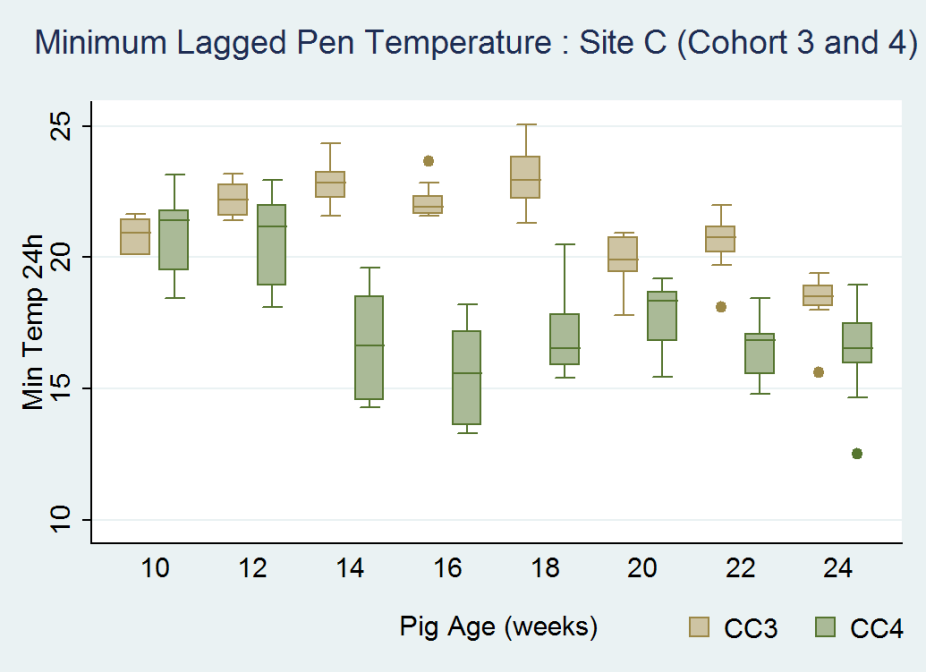


**Figure 4.6.b.** Box plot of the maximum lagged pen temperature (°C) within 24 hours for cohort 3(CC3, 06/07/2010 – 09/11/2010) and 4 (CC4, 11/20/2010 – 02/26/2010) by pig age in site C.

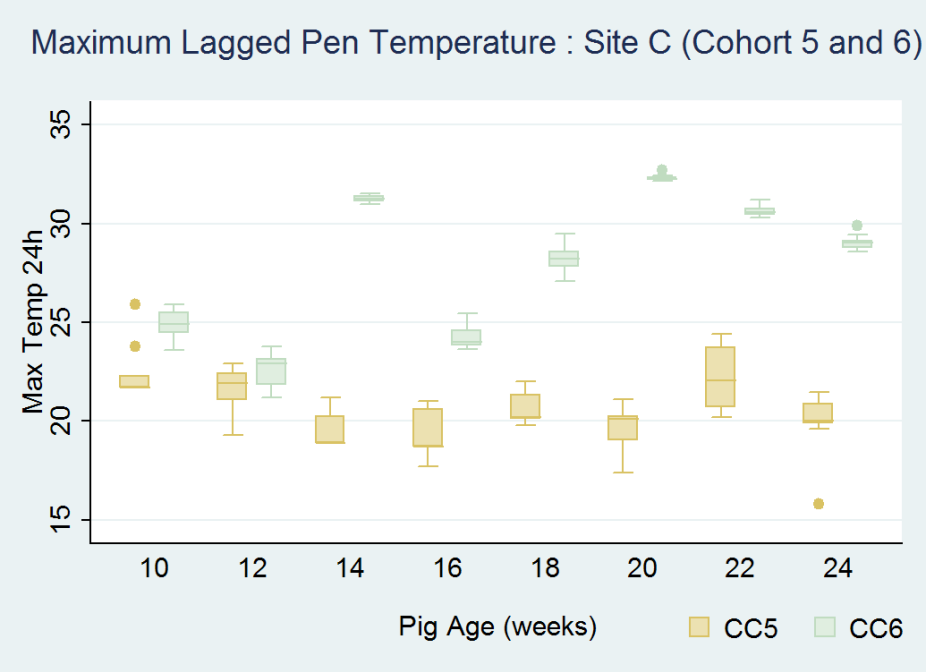


**Figure 4.6.c.** Box plot of the maximum lagged pen temperature (°C) within 24 hours for cohort 5 (CC5, 11/27/2010 – 03/05/2011) and 6 (CC6, 04/30/2011 – 08/08/2011) by pig age in site C.

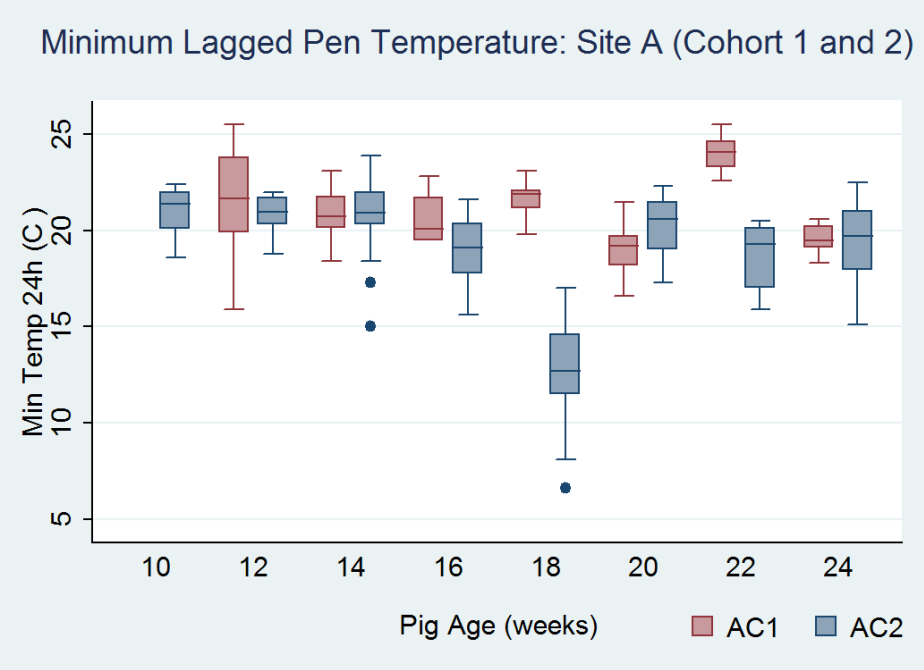


**Figure 4.7.a.** Box plot of the minimum lagged pen temperature (°C) within 24 hours for cohort 1 (AC1, 06/3/2008 – 09/06/2008) and 2 (AC2, 10/08/2008 – 01/24/2009) by pig age in site A. Note no data on first visit of the cohort 1 (10 week–old).

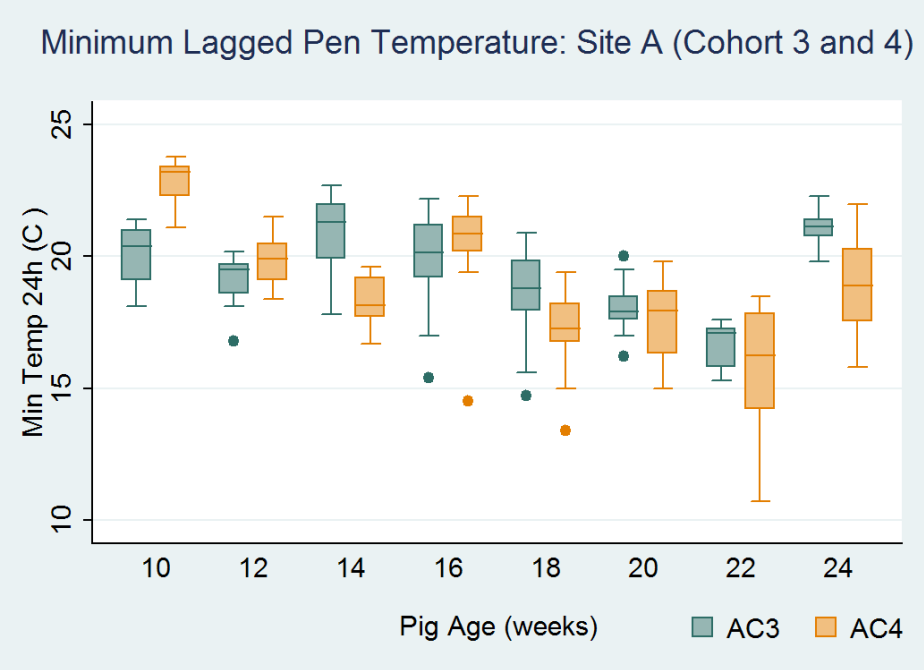


**Figure 4.7.b.** Box plot of the minimum lagged pen temperature (°C) within 24 hours for cohort 3 (AC3, 03/21/2009 – 06/29/2009) and 4 (AC4, 08/10/2009 – 11/17/2009) by pig age in site A.

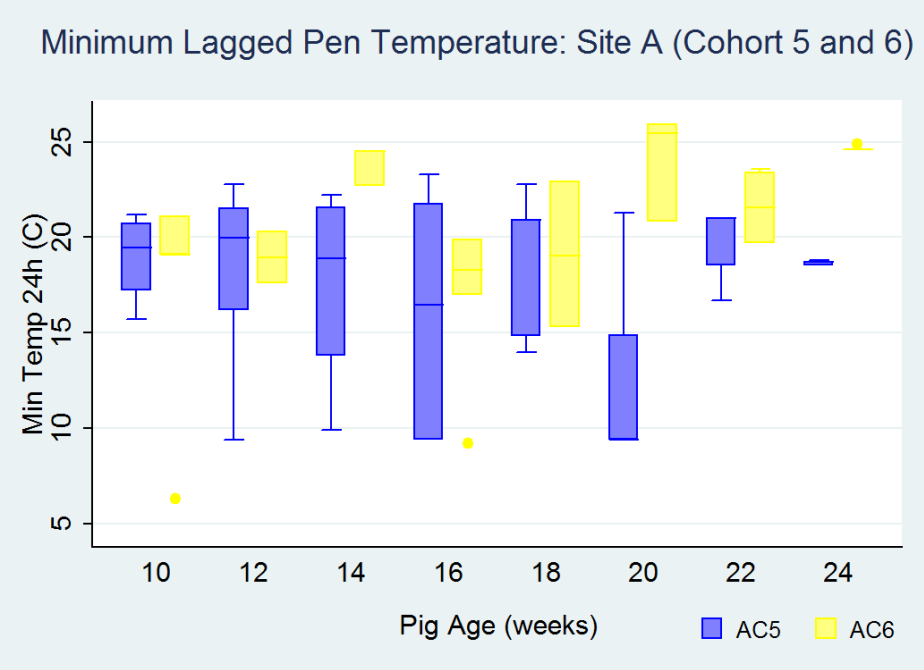


**Figure 4.7.c.** Box plot of the minimum lagged pen temperature (°C) within 24 hours for cohort 5 (AC5, 12/16/2009 – 03/22/2010) and 6 (AC6, 04/24/2010 – 08/02/2010) by pig age in site A.

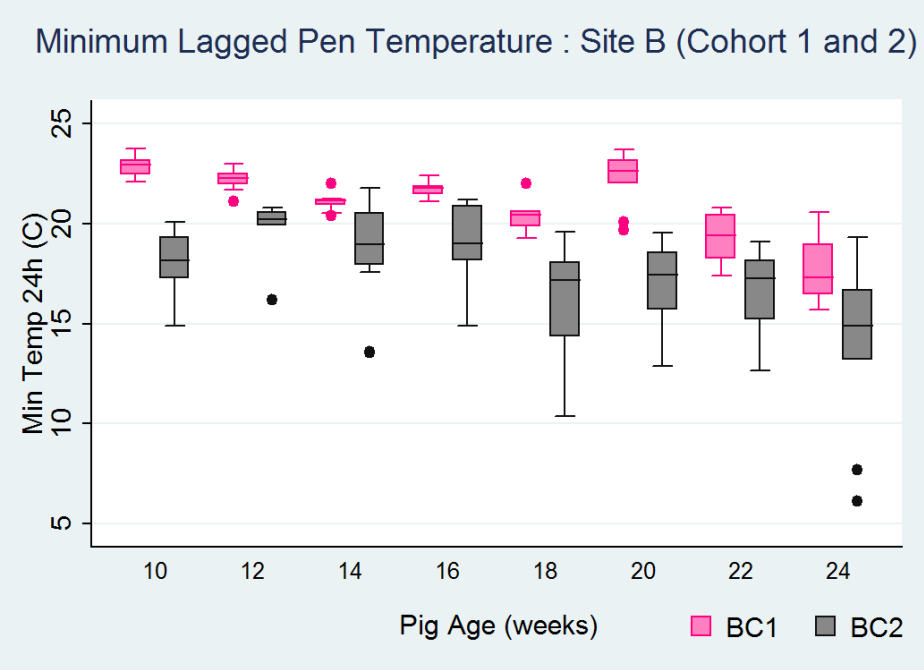


**Figure 4.8.a.** Box plot of the minimum lagged pen temperature (°C) within 24 hours for cohort 1 (BC1, 07/07/2008 – 10/11/2008) and 2 (BC2, 11/22/2008 – 02/28/2009) by pig age in site B.

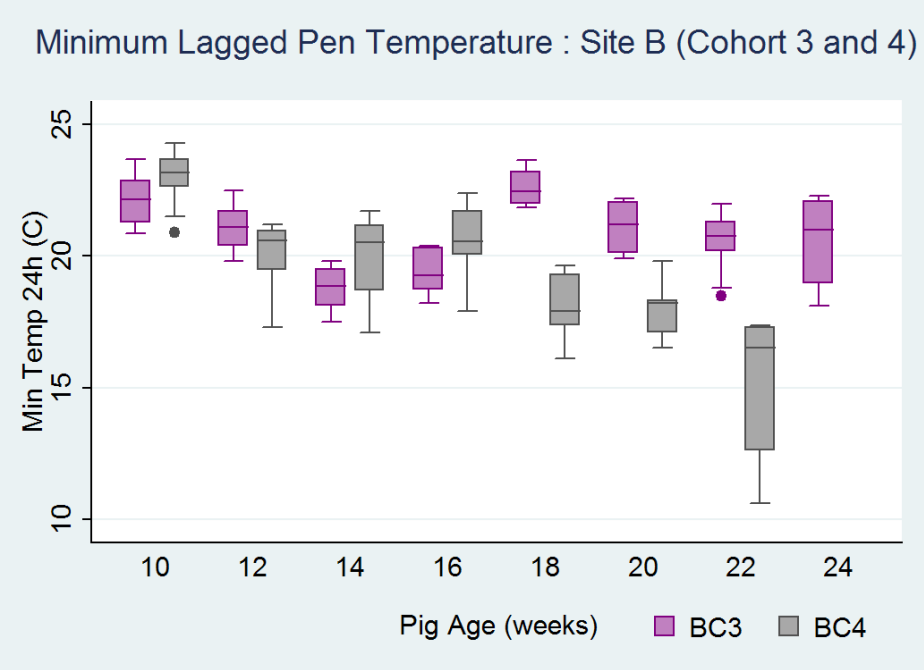


**Figure 4.8.b.** Box plot of the minimum lagged pen temperature (°C) within 24 hours for cohort 3 (BC3, 04/25/2009 –08/03/2009) and 4 (BC4, 09/15/2009 – 12/08/2009) by pig age in site B. Note no data on last visit of the cohort 4 (24week–old).

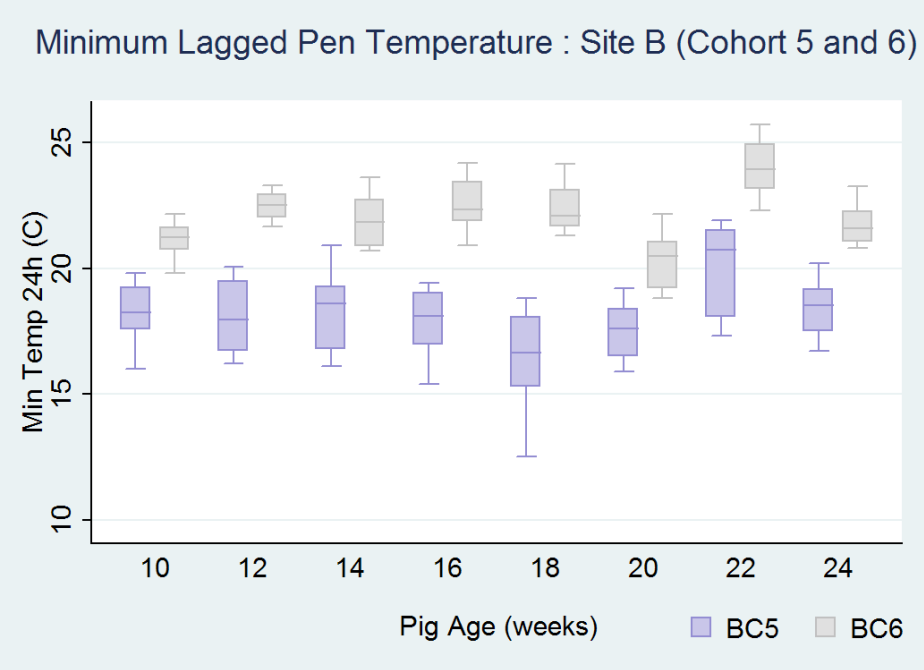


**Figure 4.8.c.** Box plot of the minimum lagged pen temperature (°C) within 24 hours for cohort 5 (BC5, 01/11/2010 – 04/17/2010) and 6 (BC6, 05/19/2010 – 08/23/2010) by pig age in site B.

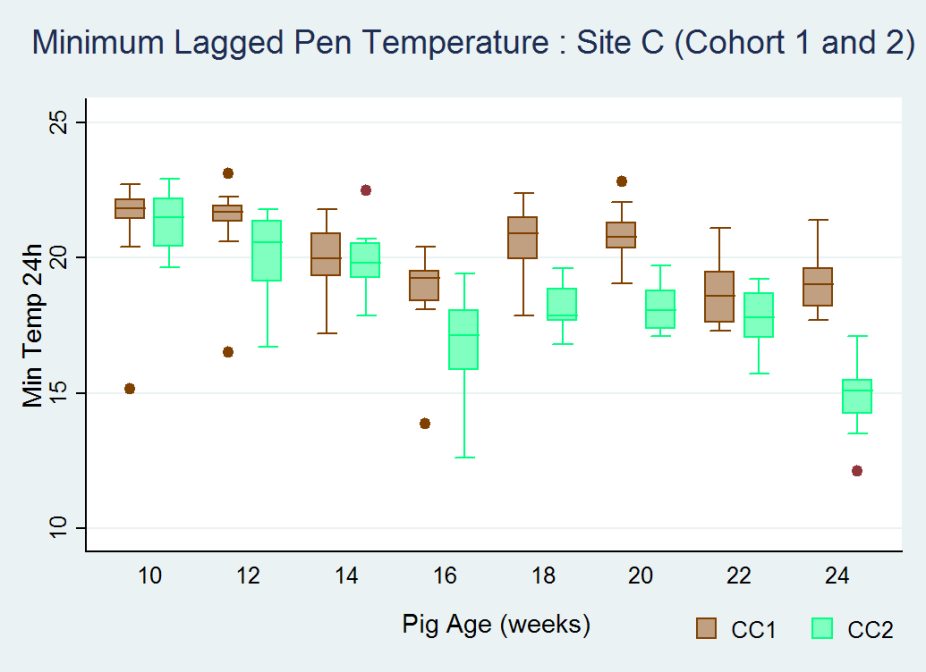


**Figure 4.9.a.** Box plot of the minimum lagged pen temperature (°C) within 24 hours for cohort 1 (CC1, 06/02/2009 – 09/08/2009) and 2 (CC2, 12/21/2009 – 03/27/2010) by pig age in site C.

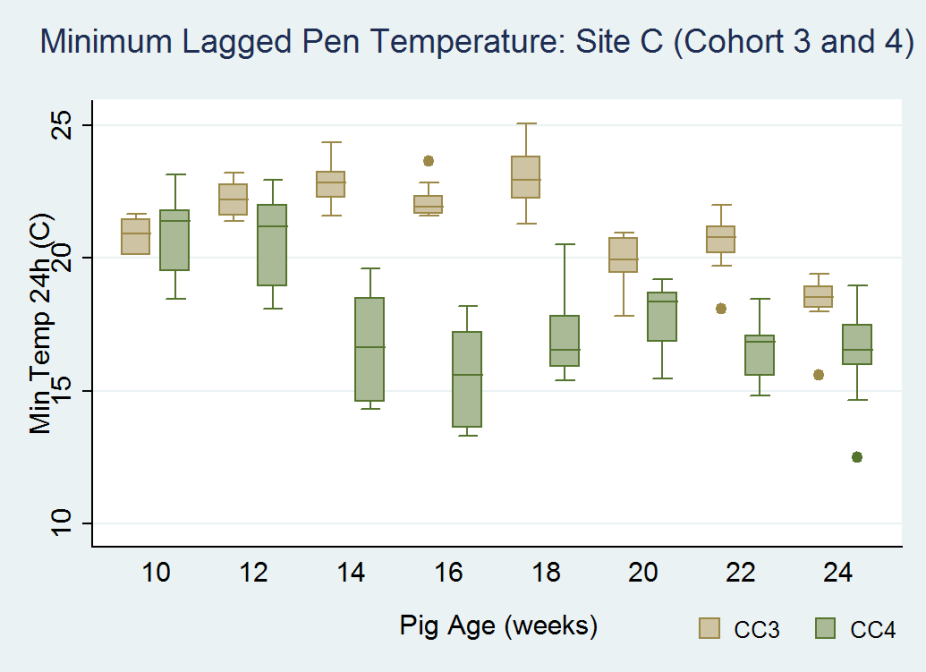


**Figure 4.9.b.** Box plot of the minimum lagged pen temperature (°C) within 24 hours for cohort 3(CC3, 06/07/2010 – 09/11/2010) and 4 (CC4, 11/20/2010 – 02/26/2010) by pig age in site C.

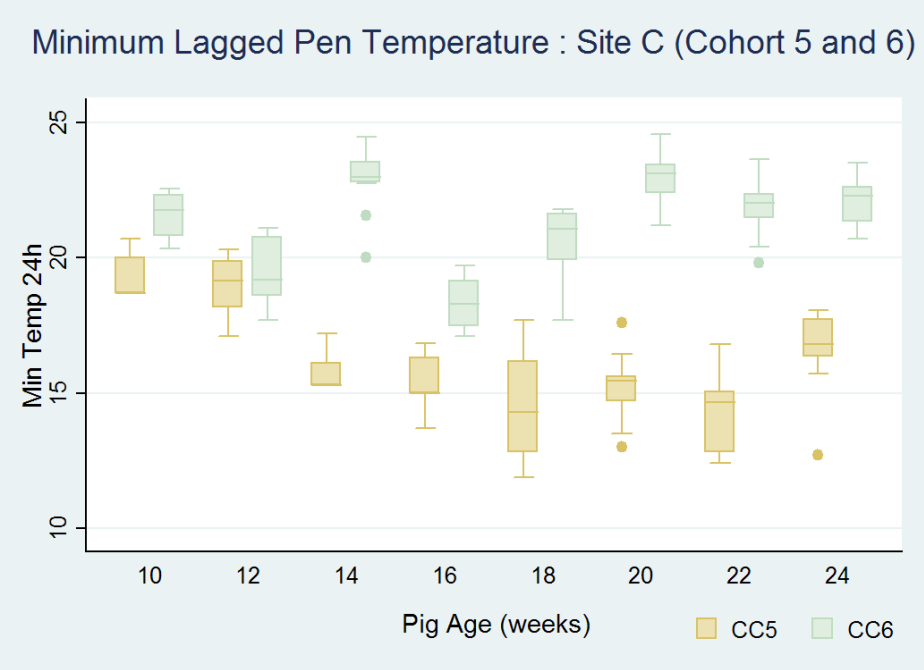


**Figure 4.9.c.** Box plot of the minimum lagged pen temperature (°C) within 24 hours for cohort 5 (CC5, 11/27/2010 – 03/05/2011) and 6 (CC6, 04/30/2011 – 08/08/2011) by pig age in site C.

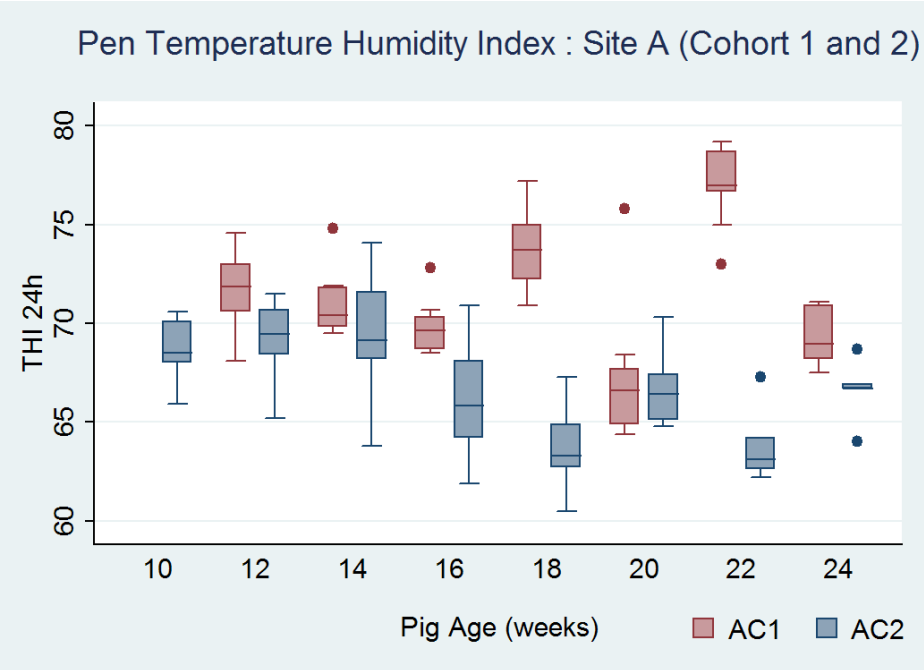


**Figure 4.10.a.** Box plot of the pen temperature temperature humidity (THI) index within 24 hours for cohort 1 (AC1, 06/3/2008 – 09/06/2008) and 2 (AC2,10/08/2008 – 01/24/2009) by pig age in site A. Note no data on first visit of the cohort 1 (10 week–old).

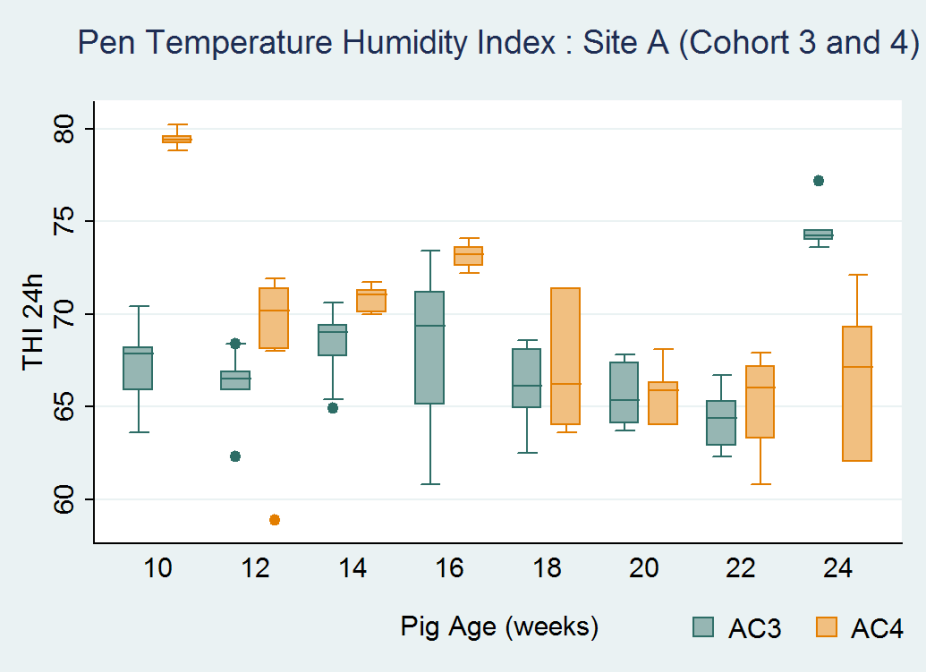


**Figure 4.10.b.** Box plot of the pen temperature humidity index (THI) within 24 hours for cohort 3 (AC3, 03/21/2009 – 06/29/2009) and 4 (AC4, 08/10/2009 – 11/17/2009) by pig age in site A.

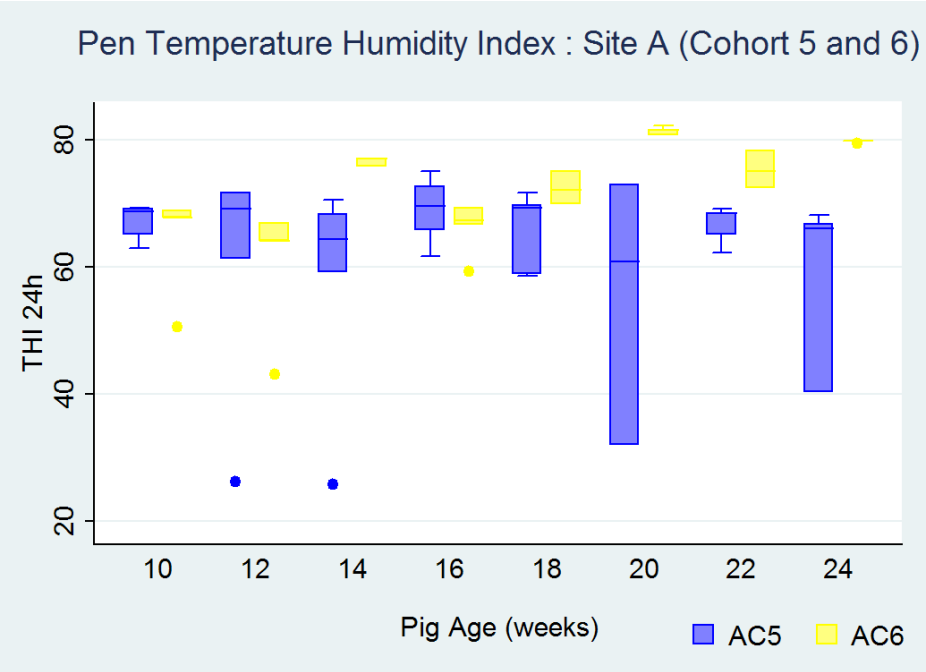


**Figure 4.10.c.** Box plot of the pen temperature humidity index (THI) within 24 hours for cohort 5 (AC5, 12/16/2009 – 03/22/2010) and 6 (AC6, 04/24/2010 – 08/02/2010) by pig age in site A.

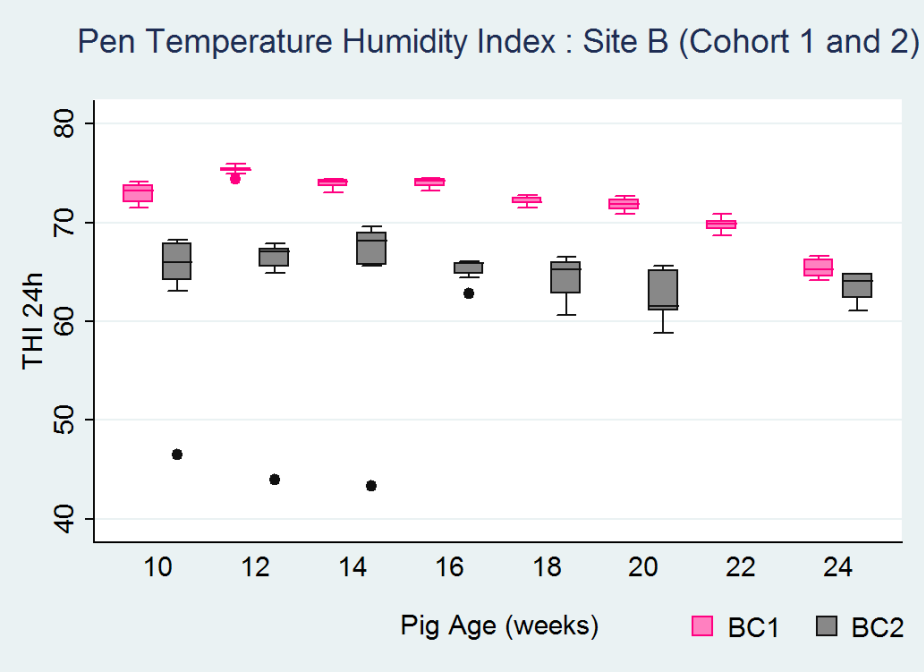


**Figure 4.11.a.** Box plot of the pen temperature humidity index (THI) 24 hours for cohort 1 (BC1, 07/07/2008 – 10/11/2008) and 2 (BC2, 11/22/2008 – 02/28/2009) by pig age in site B.

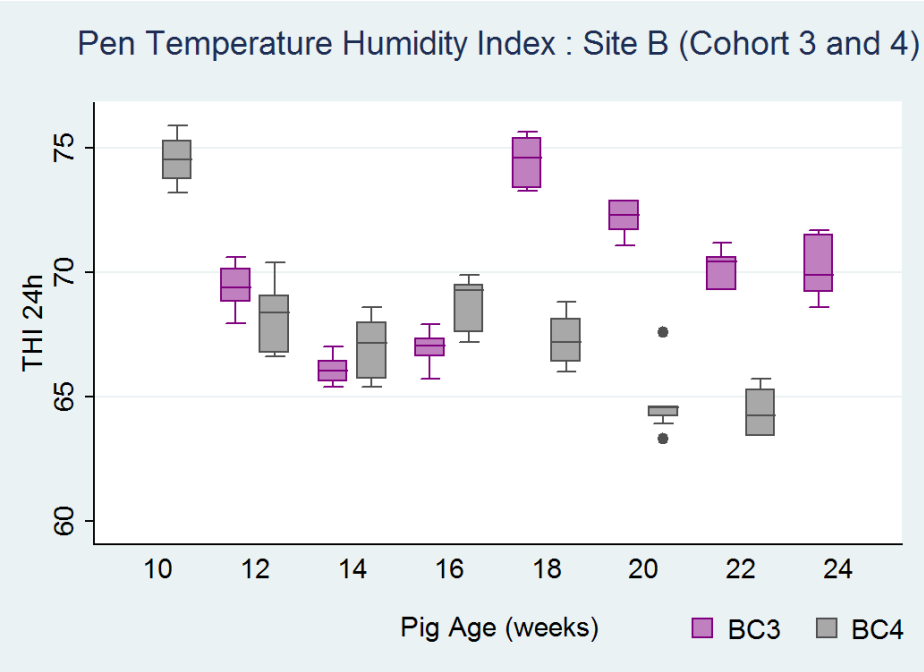


**Figure 4.11.b.** Box plot of the pen temperature humidity index (THI) within 24 hours for cohort 3 (BC3, 04/25/2009 –08/03/2009) and 4 (BC4, 09/15/2009 – 12/08/2009) by pig age in site B. Note no data on last visit of the cohort 4 (24week-old).

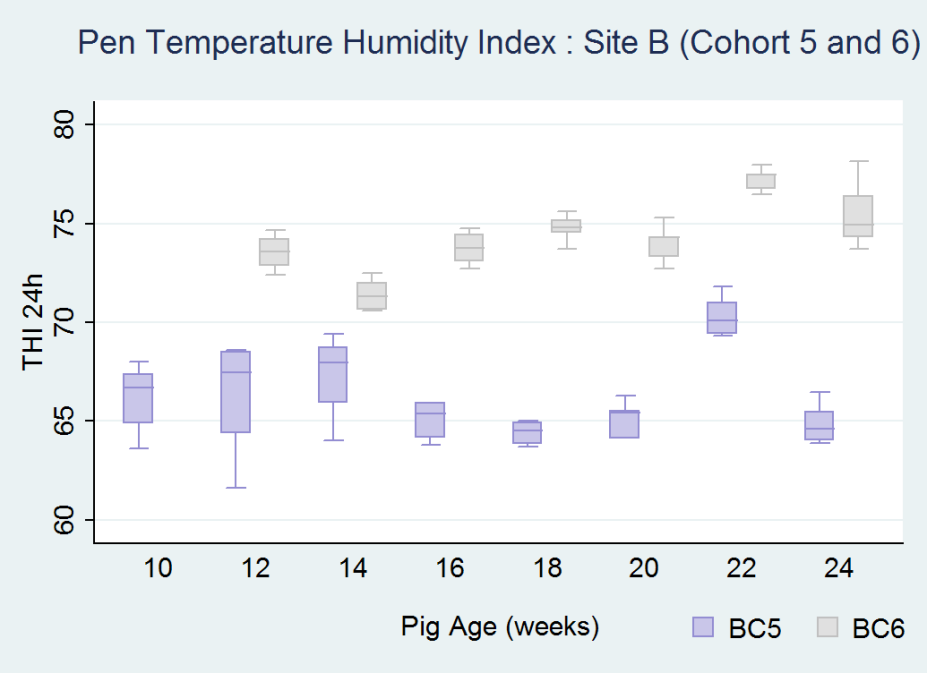


**Figure 4.11.c.** Box plot of the pen temperature humidity index (THI) within 24 hours for cohort 5 (BC5, 01/11/2010 – 04/17/2010) and 6 (BC6, 05/19/2010 – 08/23/2010) by pig age in site B.

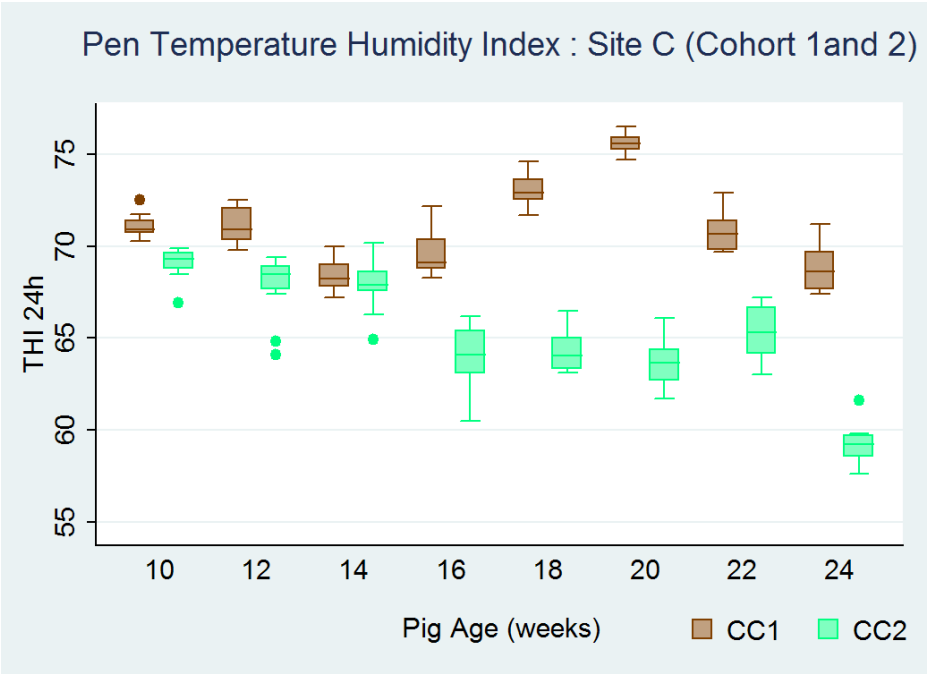


**Figure 4.12.a.** Box plot of the pen temperature humidity index (THI) within 24 hours for cohort 1 (CC1, 06/02/2009 – 09/08/2009) and 2 (CC2, 12/21/2009 – 03/27/2010) by pig age in site C.

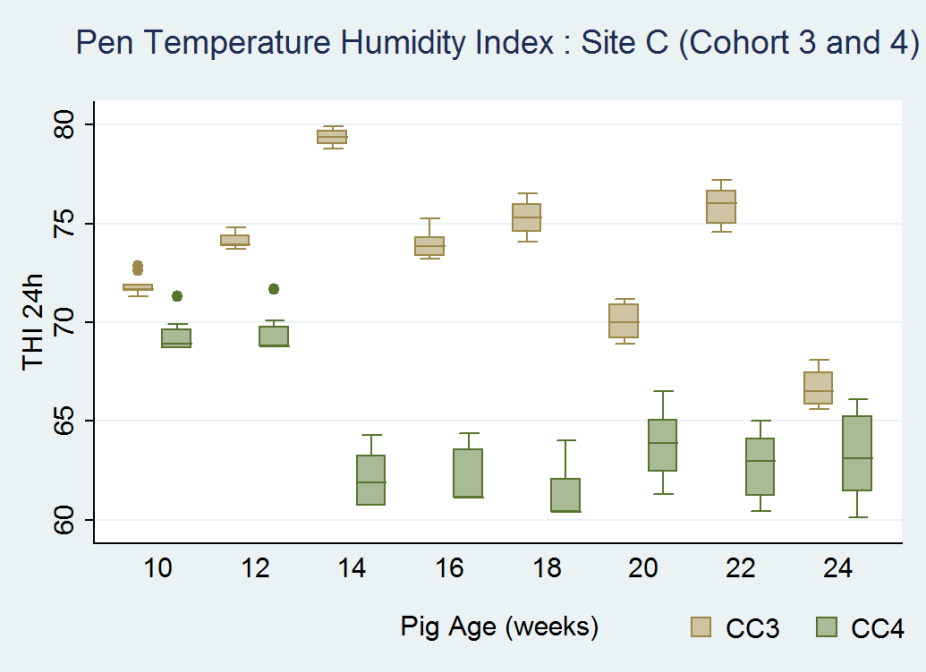


**Figure 4.12.b.** Box plot of pen temperature humidity index (THI) within 24 hours for cohort 3(CC3, 06/07/2010 – 09/11/2010) and 4 (CC4, 11/20/2010 – 02/26/2010) by pig age in site C.

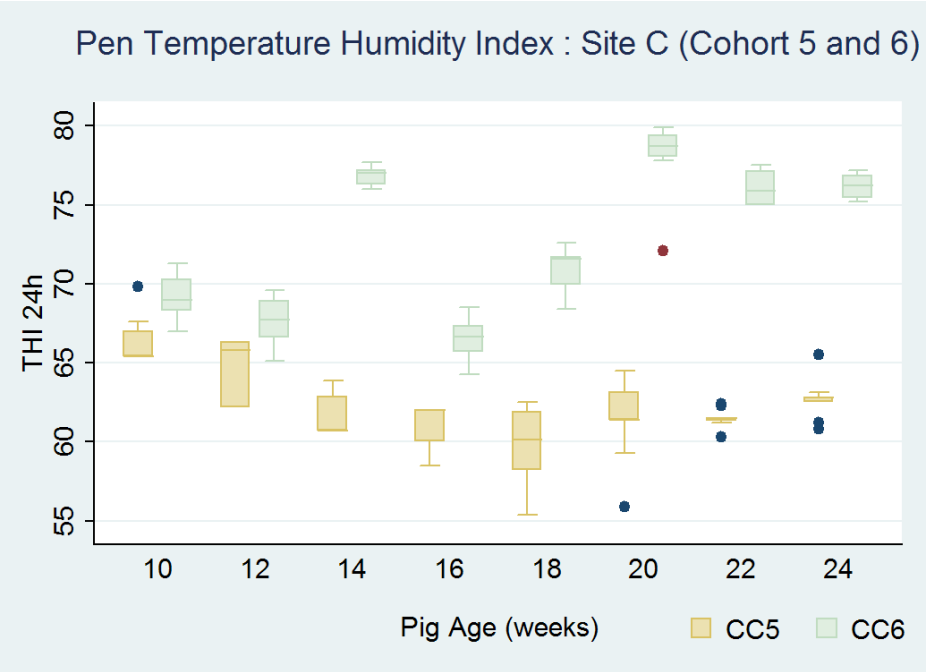


**Figure 4.12.c.** Box plot of the pen temperature humidity index (THI) within 24 hours for cohort 5 (CC5, 11/27/2010 – 03/05/2011) and 6 (CC6, 04/30/2011 – 08/08/2011) by pig age in site C.

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