MELATONIN ADMINISTRATION DURING THE FOLLICULAR PHASE AND EARLY PREGNANCY TO MINIMIZE SEASONAL INFERTILITY IN SWINE

BY

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THESIS

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ABSTRACT

It is well documented that fertility in pigs is reduced in summer and fall compared to winter and spring. Termed as seasonal infertility, problems with follicular development and corpora lutea formation and other unknown factors can combine to delay puberty in gilts, increase wean to estrus interval in sows, and increase rates of pregnancy failure and reduced litter size. The array of seasonal problems associate with the confounded effects of both heat stress and changing photoperiod. Although the modern domesticated pig is considered a "non-seasonal" breeder, its ancestor, the European wild boar, and other well characterized seasonal species, breed seasonally in response to changing photoperiod and hot temperature. Perception of photoperiod through the pineal gland alters patterns of melatonin which modulate the hypothalamic-pituitary axis and function of the ovary to control reproduction. Seasonal infertility problems are then at least partially mediated, through the seasonal changes in the duration of nighttime secretion of melatonin, which is shortest in summer and slowly increases into fall.

Because both photoperiod and heat stress are associated with seasonal infertility, two studies were designed to test whether short-term supplemental melatonin given to mimic the extended nighttime melatonin pattern observed in the peak fertility season of winter, could improve fertility in summer and fall under periods of heat stress. In both studies, exogenous melatonin was fed during proestrus and into early gestation, coinciding with the follicular and early luteal phases. Experiment 1 was performed in a single replicate using prepubertal gilts (n=36). Females were randomly assigned by age and weight and allocated in a 2 x 3 factorial treatment design to receive Melatonin (MEL, 5mg) or Control (CON, placebo) once a day while housed in one of three environmental rooms (32 °C) that provided: 1) 8 h of light and heat (8H); 2) 16 h light and heat (16H); or 3) 24 h light and heat (24H). All gilts received an i.m. injection

of P.G. 600, fence line boar exposure, and artificial insemination at estrus. Following P.G. 600, ovaries were assessed by ultrasound and reproductive tracts collected to assess pregnancy, litter size, fetal and placental measures at 33 days of gestation. Continuous and categorical data were analyzed using the GLM and GENMOD procedures of SAS for the main effects of treatment and room. Temperature, humidity, ammonia and light intensity for all the three rooms were assessed. There was no effect of MEL or room on estrus (91.6 ± 11.8%), or on follicle development (14.3 ± 1.5 large follicles/gilt in 91.6 ± 15.9% of the gilts). However, there was a tendency (P = 0.08) for MEL to improve pregnancy rate compared to CON (87.8% vs. 63.3%) but with no effect of room. The number of fetuses, healthy fetuses, abnormal fetuses and placental efficiency were not affected. The results of this study suggest that P.G. 600 alleviates the effects of heat stress on expression of estrus but only melatonin had beneficial effects on maintenance of pregnancy.

Experiment 2 was conducted at a 6,500 sow commercial farm in 12 sequential replicates starting from mid-summer and continuing into early-fall. In Expt. 2a, gilts (n = 420) that had expressed a second estrus were assigned by weight to receive once daily oral MEL (3 mg) or CON during the late follicular into the late luteal phase. In Expt. 2b, parity 1 (P1) sows (n = 470) were assigned by lactation length and backfat to MEL or CON during similar reproductive phases. Data were analyzed for the main effects of treatment, season (4 wk periods) and their interaction. In Expt. 2a there was no effect of MEL on age at 3rd estrus (203 ± 1.3 d), follicle size on 7th day of treatment (5.0 ± 0.3 mm), estrous cycle length (22.6 ± 0.4 d) or return to estrus (9.2 ± 4.0%). However, season affected number of follicles and gilts expressing estrus within 23 days (P = 0.03). There was no effect (P>0.10) of MEL or season on farrowing rate (80.0 ± 4.9%) or total born pigs (13.6 ± 0.4). In Expt. 2b, although there was no effect of MEL on follicle size (5.4 ± 0.3 mm), number (15.4 ± 1.2), or wean to estrus interval (8.9 ± 2.4 d), MEL reduced estrus

expression within 7 d of weaning by 8.5% compared to CON (73.5 vs. 82.0%, respectively). There was no effect of MEL on farrowing rate (83.0 ± 4.5%), but there was an effect of season (P = 0.001). Neither MEL nor season influenced total born (13.0 ± 1.3). In addition, environmental measures classified as lower or higher for lighting intensity, temperature, humidity in breeding and gestation, associated with fertility measures of estrus, pregnancy and litter size in both gilts and sows.

In Expt. 2, although there was clear evidence of seasonal fertility failures in gilts and sows, MEL treatment did not improve fertility effects in mature gilts, but the short-term MEL reduced and delayed estrus expression in parity 1 sows. It is possible that differences in the lighting and thermal environments for various periods of time prior to breeding might have affected the response to treatment in sows and gilts. The results of this experiment demonstrate season effects on commercial farms and that these change during summer and fall. Further, evidence of lighting and temperature effects on fertility during the different production phases is new, and has not been scientifically reported before, although much anecdotal evidence and opinion is accessible. Lastly, the studies show that short term MEL can affect seasonal fertility.

In order to help to alleviate seasonal infertility problems affecting the meat production globally, further studies in the follicle and luteal periods are needed to better understand how melatonin might be minimizing its effects on seasonality.

I dedicate this work to my family and my husband giving me support in every single decision that I have made to be here.

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LIST OF ABBREVIATIONS

AI: Artificial insemination

CL: C*orpus luteum*

CON: Control

Expt.: Experiment

FI: Fertility index

FSH: Follicle stimulating hormone

FR: Farrowing rate

GnRH: Gonadotropin-releasing hormone

HSP: Heat shock protein

IVF: In vitro fertilization

LH: Luteinizing hormone

LHRH: Luteinizing hormone-releasing hormone

Lx: Lux

MEL: Melatonin

PR: Pregnancy rate

P1 sow: Parity 1 sow / primiparous

RH: Relative humidity

RHT: Retinohypothalamic tract

ROS: Reactive oxygen species

SCG: Superior cervical ganglion

SCN: Suprachiasmatic nucleus

TNZ: Thermoneutral zone

UCT: Upper critical temperature

CHAPTER 1 - INTRODUCTION

Seasonal infertility has important implications on the production of meat, milk and eggs around the world in livestock species such as pigs, cattle, small ruminants and chickens (Chemineau et al., 2007). In the he dairy industry for instance, fertility is compromised during summer with increased number of days open, reduced conception rates and larger number of cows in anestrus (De Rensis et al., 2015). Small ruminants also show strong seasonality with many breeds of sheep being anestrual during spring, and milk goats showing negative correlations between production and fertility in summer (Nardone et al., 2010). As a consequence, financial losses occur due to reduced milk yield and the delays in production (Wakayo et al., 2015), while dairy producers can be affected by almost $1 billion when accounting for milk and animal losses (Nardone et al., 2010).

Pigs are also affected by seasonal infertility in many locations around the world. Within farms, seasonal infertility can be variable from one year to the next, but is most often reported during summer and autumn periods. Even among farms in the same region, the problem can be unpredictable in the months of occurrence, the duration of infertility, and the fertility measures affected. In the boar, normal sperm production is reduced, while in sows and gilts, follicular and luteal phase disturbances cause problems in follicle development, corpus luteum formation, pregnancy establishment and embryo survival. In the female, problems in expression and delays in estrus, as well as pregnancy failures, combine to increase nonproductive days, lower farrowing rates and litter sizes that reduce the number of pigs produced per sow each year. Collectively this becomes an important problem for the swine industry as it annually reduces the number of pigs that reach market in the following summer. In the USA, it is estimated that seasonal infertility is

responsible for approximately $420 million in losses (ISU, 2017). Also, the seasonal decline in production occurs on pig farms around the world to reduce production of pork, which is the most widely consumed animal protein source in the world (FAO, 2014; Waetke, 2014; Yeste, 2016).

In seasonal and non-seasonal livestock species, infertility and reduced productivity are evident and can be linked to both summer heat stress and changes in photoperiod during summer and fall. Several studies have shown that high temperatures (Bloemhof et al., 2013; Muns et al., 2016) can result in lower fertility and lead heat stress in pigs, cows, ewes, goats and chickens (Teague et al., 1968; Omtvedt et al., 1971; Wettemann and Bazer, 1985; Flowers and Day, 1990; Marai et al., 2007; Hansen, 2009; Bloemhof et al., 2013; Wakayo et al., 2015). In these cases, evidence of heat stress can be associated with behavioral, and physiological measures.

Yet photoperiod as a mediator of seasonality is also quite strong in several species, including the pig. In dairy cows, exposure to cooling systems during summer heat stress, while reducing temperature, fails to match the fertility achieved during winter (Rensis and Scaramuzzi, 2003). In pigs, fall infertility has been widely reported when temperatures are well below heat stress levels. Several studies suggest that the change in photoperiod during summer and fall is the main cause of seasonality and in modern swine breeds, is likely related to a genetic vestige of the European wild boar, a seasonal breeder (Mauget, 1985; Love et al., 1993; Peltoniemi and Virolainen, 2006). In the wild, the European pigs breed in late fall and winter and are anestrual during summer and early fall (Peltoniemi et al., 1999; Auvigne et al., 2010; Iida and Koketsu, 2013).

While the involvement of summer with heat stress and photoperiod with summer and fall, the problem is clearly confounded, and seasonal infertility will likely be attributed to a single factor but a complex interaction of multiple factors. For seasonal livestock species such as sheep

and horses as well as seasonal research animal models such as rodents, the endocrine response to photoperiod on fertility control has been well studied and partially defined (Rosa and Bryant, 2003; Williams et al., 2012). In these seasonal species, as well as other "non-seasonal" livestock species such as cattle and pigs, it seems clear that melatonin production and release in response to daily photoperiod is the key regulator of the hypothalamic pituitary axis that affects fertility throughout the seasons (Ortavant et al., 1988). Melatonin given exogenously or use of changing lighting intensity and schedule for some species can enhance fertility in the periods of lower productivity (Rosa and Bryant, 2003; Dardente et al., 2016). While lighting changes can take some time to influence the circadian cycle, exogenous melatonin may be able to induce more rapid responses.

Based on the fact that peak fertility in swine occurs during the winter season, and melatonin is released for longer periods during the longer nights, we hypothesized that longer melatonin exposure mimicking winter may be a method to improve summer and fall infertility. Previous evidence of melatonin effects in vitro and in vivo in pigs, suggested it might be possible to minimize seasonal infertility problems in summer and fall in sows and gilts through short term exogenous melatonin treatment during the follicular phases and during early pregnancy.

CHAPTER 2 - LITERATURE REVIEW

Seasonality

Seasonality is characterized by predictable changes that occur within animals and plants at certain times during the year. In animals, changes occur in metabolic control, immune function, behavior (Demas et al., 2009) and reproduction according to the environmental cues and the necessity for each specie to adapt for survival. Mostly observed in vertebrates, endogenous annual rhythms are evident in a wide range of species that include birds (Helm et al., 2009), fishes (Eriksson and Lundqvist, 1982) and mammals (Lincoln et al., 2006; Chemineau et al., 2007).

Avian species display some of the most pronounced seasonal changes and serve as an important model for studying the mechanisms underlying seasonality. Within seasons, birds may exhibit a range of changes including migration, and reproduction (Dawson et al., 2001). It appears that the main factor that regulates seasonality in birds is photoperiod, although this may depend upon bird species (Gwinner, 2003). In livestock species such as the chicken, changes in photoperiod has important effects on reproductive hormones such as prolactin and luteinizing hormone (LH) to influence ovulation, egg laying sequence, and egg incubation (Sharp et al., 1998).

In mammals, many wild species show strong seasonal fertility patterns, especially in global areas that show greater climate extremes. In certain important species that have been domesticated and selected for breeding, the strong seasonal fertility patterns may still predominate, have been reduced, or nearly diminished. Examples of these scenarios include the contrast between the wild sheep, horses, rodents, pigs, and poultry, and their domesticated

counterparts. In domesticated mammals where considerable data is available, seasonality affects fertility in cyclic activity, estrus expression, ovulation, spermatogenic activity and sexual behavior (Chemineau et al., 2008). Differences in seasonal classification can be made based on the frequency of breeding throughout the year (Vasantha, 2016). Animals defined as seasonal breeders successfully mate only in a certain time of the year as a natural intention to maximize the survival rates of their offspring based on optimal climate, favorable availability of nutrients, and conditions that favor lactation and progeny growth (Chemineau et al., 2007; Nelson et al., 2010). The breeding season also differs among different animal species as a result of the complex interaction of numerous factors including gestation length, body size, climate, and food availability. Within seasonal species, the number of estrous cycles that occur during the year can be classified as mono or polyestrus (Senger, 2012). Monoestrous animals typically have only one estrous cycle during the breeding season, and is observed in dogs, wolves, foxes and bears. Polyestrous females express several cycles during specific periods of the year, with the best studied of these including livestock species such as horses, sheep, goats, and deer. Some of the important seasonal breeders are fertile during short days, while others are classified as long-day breeders. Non-seasonal breeders do not limit their reproductive activity during the course of the year and thus are capable of having multiple estrous cycles throughout the year without regard of season (Vasantha, 2016). Included in this group are many species of rodents and domesticated livestock species such as cattle and pigs.

Seasonal fertility effects on livestock and food production

The classification of domesticated cattle, pigs, and poultry as non-seasonal and horses sheep and goats as seasonal livestock species is not as clear cut as that classification since each species shows fluctuations in their fertility throughout the year. This "seasonal infertility" had noted effects and raised awareness in response to increased costs of production and reduced profit for producers, resulting in instability in animal production, and the product availability at particular times of the year (Chemineau et al., 2007). Seasonal variation in the quantity and quality of milk produced from dairy cattle and goats, and produced meat from cattle, sheep and pigs, in addition to eggs from chickens, have all been documented to affect markets (Sharp, 1993; Rosa and Bryant, 2003; Chemineau et al., 2007; Chemineau et al., 2008). While the food production systems within the animal industry have been changing dramatically in the last decades, the domestication and selection of livestock species for traits that include high fertility has not been able to attenuate the effects of seasonality in many species or breeds (Dardente et al., 2016). Seasonal infertility persists under intensive livestock production systems, where it is possible to control natural variations in the environmental effects of temperature and light.

Cattle are one of the most widely used livestock species around the world due to their importance in producing large volumes of fluid milk and associated products. These animals serve as an interesting model for understanding the effects of season on fertility, in classically defined "non-seasonal" livestock species. When comparing different breeds and global locations, cattle can breed and ovulate during the course of the year, however variation in reproductive traits are noted with seasonal changes (Haugan et al., 2005). For example, during summer, variation in fertility increases during a time when cows are exposed to higher temperatures and longer photoperiods. It is also difficult to separate out the effects of temperature from

photoperiod. For example, breeding cows in summer to calve in spring is common in the Northern hemisphere for calf survival, while breeding in other seasons may have different fertility and calf survival effects depending upon global location and yearly climate. Important responses characterize seasonal infertility, such as abnormal expression of estrus (increase in length and decrease in signs of estrus), anestrus, and embryonic survival (Allen Tucker, 1982; Nardone et al., 2010). These effects may occur due to problems in follicular selection and prolonged dominance but also as a result of lower oocyte competence. Reduced oocyte quality may be due to disruption of normal maturation, followed by higher embryonic losses during periods of heat stress and longer photoperiod (Wakayo et al., 2015). The effects of season are also evident for heifers, as females born in autumn achieve puberty earlier than those born earlier during spring (Schillo et al., 1992). Interestingly, there are breeds better adapted to seasonal changes and locations with high environmental temperatures. While high temperatures decrease pregnancy rates in *Bos taurus* cattle, *Bos indicus* have evolved, adapted and been selected to achieve fertility while living in hotter climates (Randel, 2005). Furthermore, domesticated bulls also are affected by season with a higher percentage of sperm abnormalities, oxidative damaged sperm, reduced testicular size, and lower fertility during summer when compared to winter (Fields et al., 1979; Sekoni and Gustafsson, 1987; Mathevon et al., 1998; Nichi et al., 2006). Thus, semen collected during summer can also contribute to calving rates (Haugan et al., 2005). Similar to domesticated dairy cattle, domesticated breeds of buffalo are also classified as non-seasonal, but clearly display seasonal effects on ovarian activity. These species hold great importance for milk and meat production in certain areas around the world, such as India and Africa, and have been able to adapt to difficult environmental conditions (Perera, 2011). As a result, 60% of the economic losses in production result from heat stress and /or photoperiod

(Wolfenson et al., 2000), which, according to St-Pierre et al. (2003), averages $897 million to the dairy industry.

Certain livestock species such as sheep, goats and horses appear to show the strongest seasonal effects on reproductive activity in response to photoperiod alone. While the most primitive breeds of sheep exhibit a short breeding season from November to December in the northern hemisphere, domestication and selection has significantly attenuated the anestrual period and extended the breeding period from late summer until late winter (Malpaux et al., 1997). However, the longer breeding season did not increase the number of offspring throughout the year. The main manifestation of seasonality in the ewe involves changes in behavioral receptivity to the ram, silent ovulation during the anoestrus period, and measurable changes in steroid and LH production and release (Rosa and Bryant, 2003). Although the effects are not as pronounced, rams also show changes in sexual behavior, hormone production, spermatogenesis and testicular measures (Rosa and Bryant, 2003). The start of seasonal reproductive activity in sheep starts with their perception of shortening daylength in autumn, with the photoperiod effect working through melatonin to alter the release of gonadotropin releasing hormone (GnRH) and LH (Malpaux et al., 1997). Horses are also photosensitive but show their reproductive activity in the spring, when days are longer and photoperiod is increasing. Evolutionary determinants for the horse based on size, the 11 month gestation length combined with the effects of climate on food and survival, were likely involved in the long photoperiod pattern for the ovulatory and breeding seasons (Ginther et al., 2004). In mares, the breeding season induces active follicular waves, increases follicle size, and allows ovulation (Ginther et al., 2004). Stallions also show long day effects with higher circulating testosterone and increase in total scrotal width (Clay et al., 1988). The increasing duration of daily light results in decreased melatonin secretion

associated with the increases in GnRH and LH in mares, and also testosterone release in stallions (Johnson, 1986; Clay et al., 1988).

Avian livestock species such as chickens and turkeys also show reproductive activity in response to photoperiod and serve as an important model for understanding the mechanism of response. Depending on species of bird, the eye, pineal, or deep brain photoreceptors may act to control the response to photoperiod to regulate reproduction. However, the eye or pineal gland may not be required for seasonal rhythms (Sharp, 1993). Yet, there is an extra retinal photoreceptor (deep brain) close to the hypothalamus that does mediate the photoperiodic responses to synchronize the circannual rhythms (Sharp, 1993) and hypothalamic release of GnRH. Due to the high importance of poultry meat and egg production, seasonal effects on fertility hold great relevance in global food availability. Domestication of these animals for intensive production systems has focused on optimizing environmental conditions for lighting and temperature to improve meat production, laying performance and egg weight (Chemineau et al., 2007). In poultry production systems in temperate regions, increasing daylength in spring or provision of longer lighting regiments under intensive conditions, act directly through photoreceptors and GnRH neurons (not through melatonin) to stimulate GnRH release, secretion of LH and prolactin, gonad development, ovulation and oviposition (Sreekumar, 1997; Sharp et al., 1998; Dawson et al., 2001).

Seasonality in pigs

The modern domesticated pig used for commercial production of pork worldwide, is classified as a "non-seasonal" breeder. However, numerous reports of seasonal infertility and reduced production of pigs are available (Love, 1981; Claus and Weiler, 1985; Love et al., 1993;

Tast et al., 2002; Ramirez et al., 2009). The origin of the seasonality effects on fertility may be due to its ancestral relationship to the European wild pig. Many of the modern breeds of *Sus scrofa spp*. used in maternal and terminal lines include popular breeds such as Landrace, Large white, Duroc, Hampshire and Pietran. The wild pig is a true, short-day seasonal breeder, which breeds in fall and farrows in spring when temperature and food improve the chances of offspring survival. (Mauget, 1982). In the wild, sows produce one litter each year, and following farrowing, experience anestrus induced by lactation and season during summer and fall (Tast et al., 2001a). During the same summer season, boars show decreased testosterone and testicle size (Mauget, 1982).

Domestication of the pig initially focused on selection of prolific breeds of swine that could be efficient in production of pork in outdoor farms. However, with the move to indoor confinement systems, breeding and efficient production throughout the year became a priority (Tast et al., 2001a). Despite the move indoors, seasonal declines in fertility in summer and early fall occur globally in the domestic pig (Love, 1981; Peltoniemi et al., 2000; Tast et al., 2002; Auvigne et al., 2010; Bertoldo et al., 2011). Data from boars indicates problems in male fertility, with decreased libido, steroid synthesis and sperm counts during summer compared to winter (Claus and Weiler, 1985; Andersson et al., 1998; Knecht et al., 2013; Pinart and Puigmulé, 2013; Savi and Petrovi, 2015). In gilts and sows, exposure to long photoperiods in summer and fall, can have detrimental effects during the follicular and luteal phases (Love, 1981; Auvigne et al., 2010; Bertoldo et al., 2010). There is evidence to support photoperiod involvement in seasonal pregnancy failure as a result of lower progesterone during pregnancy (Love et al., 1993). Values were noted to be 10% lower in early fall and up to 50% lower in late fall compared to other seasons (Peltoniemi et al., 2000).

Seasonal effects on replacement gilt fertility

Seasonal effects on the fertility of replacement gilts have been documented over many years (Peltoniemi and Virolainen, 2006; Iida and Koketsu, 2013; De Rensis et al., 2017). Even when analyzing ordinary and high performing herds in Japan (Iida and Koketsu, 2013), Australia (Love et al., 1993; Bertoldo et al., 2010), Finland (Tast et al., 2002) and the US (Knox et al., 2013), the effects are quite clear. In young females, the problem is characterized by delayed puberty, higher return to estrus after insemination, and pregnancy losses in summer and early fall. Puberty is important, as age at first mating in gilts is associated with lifetime performance and its delay is related to reduced longevity of sows in the herd (Koketsu et al., 1999). The lower fertility of gilts during summer and fall has important implications as sow culling rates range between 45 to 63% annually (PigChamp, 2016a) and gilts are needed to replace these sows to maintain production (Houška, 2009) and improve genetics. Based on the research of Bortolozzo et al. (2009), optimization of gilt management to improve efficiency is critical and focus should be on the period from gilt arrival at the farm until entry into a breeding group. This 2 to 6 month timeframe would likely include periods of high temperature and changing photoperiods known to impact fertility (Love et al., 1993; Peltoniemi and Virolainen, 2006). Understanding and improving the critical environmental conditions related to lighting and temperature effects on gilts during summer and fall when they are developing or maturing, could help pubertal estrus expression and enable breeding at an optimal age and weight for reducing nonproductive days (Vargas et al., 2009) and improving lifetime productivity (Bortolozzo et al., 2009; Saito et al., 2011).

Infertility in weaned sows

Seasonal effects on weaned sow fertility has been consistently documented in reports over the last 50 years (Edwards et al., 1968; Love, 1981; Peltoniemi and Virolainen, 2006; Auvigne et al., 2010; Bertoldo et al., 2010; Bertoldo et al., 2011; Lopes et al., 2014). In these reports, wean to estrus interval (WEI) is longer, while ovulation rate, conception rate and litter size are all lower, starting in midsummer and extending into early autumn. Several studies indicate that estrual problems occur as a result of ovarian disturbances, more pronounced in primiparous (P1) than in multiparous sows (Prunier et al., 1996). This P1 problem can be explained by the metabolic imbalance experienced by these sows with their first litter, where susceptibility to heat stress, reduced feed consumption, and excessive body condition loss, combine to reduce fertility (Prunier et al., 1996). Delays in estrus and litter production increase nonproductive days. For consistent production flow, labor scheduling, and optimal farrowing rate and litter size, sows must be bred within 3-7 days after weaning (Koketsu et al., 1997a). Following breeding, the earliest indicator for failure is conception failure or return to estrus following service which is a prelude to lower farrowing rates, pig production, and economic return (Love et al., 1993). Ensuring that bred females produce a 3rd litter is required for return of investment. And, when analyzing rates, and reasons for culling, return to estrus after weaning, conceiving after insemination, and overall poor reproductive performance are the leading causes, and all are higher during summer and fall compared to other seasons (Kraeling and Webel, 2015). Despite the fact that genetic selection, nutrition, health, housing and breeding technologies have been advanced (Kraeling and Webel, 2015), some proportion of the sows are still susceptible to photoperiod and temperature, indicating that a better understanding of the problem may be required for finding solutions.

Introduction to heat stress and effects on fertility

The effect of elevated temperatures resulting in heat stress as a cause for seasonal infertility has been addressed for many years in numerous species such as dairy cattle, sheep, goats, mice and swine (Morrison, 1983; Flowers and Day, 1990; Kadzere et al., 2002; Rensis and Scaramuzzi, 2003; Hansen, 2009; De Rensis et al., 2015; Wakayo et al., 2015). Traditionally the lowest fertility occurs during the hottest months of the year (June to September in the northern hemisphere), although there is some indication that latent effects may also occur in the fall (Wolfenson et al., 2000). Animals perceive heat stress when their core body temperature exceeds the upper critical temperature (UCT) of the thermo-neutral zone (Bloemhof et al., 2008; Hansen, 2009). This happens when temperatures increase above the animal's capacity to loose heat to the environment (Hansen, 2009). Heat stress affects physiological and behavioral patterns involved in thermoregulation, which includes the balance of heat production and loss mechanisms (Renaudeau et al., 2012). Heat stress can have serious implications, as temperatures above the upper limit, can compromise health, feed intake, milk, meat and egg production, and fertility in different livestock species. Among species, variation in the TNZ is specie dependent and can be influenced by animal genetics, size (surface area), age, and metabolic state as well as many other factors. Measures of the the TNZ are most often generated in controlled chambers but in commercial settings it may differ considerably based on the environmental conditions for weather or control within a building. Besides high temperature alone, it is also important to consider the involvement of relative humidity (RH), solar radiation (when animals are not confined indoors) and wind speed within the climatic environment to better understand what can lead to heat stress conditions (Renaudeau et al., 2012).

Pathway to heat stress

The pathway to prevention of hyperthermia involves a series of steps whereby the animal uses simple physical and physiological modification within its environment to dissipate excess heat to the environment. Animals can lose heat via convection, conduction, evaporation, and radiation (Hansen, 2009). For convection, conduction and radiation to occur the animal's surface area, flooring and airflow are important, while for evaporation occur, the humidity in the air is the determining factor (Renaudeau et al., 2012). The change in blood flow from core towards the skin surface is one of the steps that an animal's body uses to lose heat in high temperatures. Many livestock species use this mechanism to increase evaporative heat loss through sweat glands. For instance cattle and sheep lose heat by sweating due to the high density of sweat glands in the skin (Renaudeau et al., 2012). However, other species do not have sweat glands (birds) or have only few with limited function (pigs), and therefore must rely on respiratory evaporation (Renaudeau et al., 2012). Changes in metabolism and endocrine systems, also work to increase heat loss once the temperature exceeds the point where convection, conduction, evaporation and radiant loss are not effective. Yet, the mechanism whereby hyperthermia causes negative effects on physiology and fertility at the systemic and cellular level in mammals and birds appears quite complex. At the cellular level, heat stress is known to activate heat-shock transcription factors and apoptosis cascades (Neuer et al., 2000). The heat shock proteins (HSP) are vital to cellular survival, since they are responsible to help other intracellular proteins as well as to act in cellular stress (Neuer et al., 2000). HSP are expressed in several tissues, including in the ovary cells, during the normal stages of folliculogenesis and oogenesis (Neuer et al., 2000). However, thermal stress can lead to over-expression and activation of these proteins, which can end up inducing cell damage and autophagy in the ovary, which can compromise the oocyte and

its development and perturb the hormonal environment (Hansen, 2009; Ross et al., 2017). The damage in the cells and tissues seems to generate reactive oxygen species (ROS), however, evidence in vitro and in vivo indicates that antioxidants such as melatonin can help protect cells from the damage (Matsuzuka et al., 2005; Hansen, 2009). The hypothalamic-pituitary-gonadal pathway appears sensitive to heat stress and when perceived, activated pathways stimulate cascades that will eventually suppress reproductive function through a negative feedback on GnRH production (Ross et al., 2017).

The thermo-neutral zone for dairy cows range between 5 to 25 °C, although breed and physiological state of the animals makes it vary (Kadzere et al., 2002). Exposure to heat stress (short or long) can affect follicle and luteal cell development, steroid production, oocyte quality, embryo development and endometrial function (Wolfenson et al., 2000). For cattle, the effects of high temperatures appear during summer and the consequence of the heat stress can follow into early and mid-autumn, decreasing female reproductive performance even when temperatures are lower (Wolfenson et al., 2000). In heifers and lactating cows exposed to heat stress, selection of the ovulatory follicle is changed as a result of reduced follicle dominance and fewer numbers of medium sized follicles which can be explained by a decrease in LH levels (Wolfenson et al., 1997; Wolfenson et al., 2000; De Rensis et al., 2005). Data also show a 60% reduction in the number of granulosa cells in follicles during summer compared to winter and a reduction of up to 50% in estradiol production (Wolfenson et al., 2000). It is estimated that approximately 15 to 30% of the in vitro maturing oocytes collected from heat stressed cows undergo apoptosis (Hansen, 2009). Available data in numerous species has been used to identify whether the origin of infertility arises at the level of the hypothalamus or at the level of the ovary. In well studied lab animal models such as the rat, heat stress effects can be associated with a reduction in

ovarian gonadotropin receptors, aromatase activity, and follicular fluid estradiol (Shimizu et al., 2005).

Data from classified "seasonal breeders" such as sheep, goats, and poultry, also helps show similarity in the pathways of heat stress on fertility. In ewes, heat stress reduces aromatase activity, LH receptors, estradiol, and delays ovulation (Hansen, 2009). In poultry, high temperatures have been shown to disrupt the normal levels of luteinizing hormone-releasing hormone (LHRH) in the hypothalamus, and pituitary LH (Novero et al., 1991; Lara and Rostagno, 2013). However, the mechanism for the effects may differ among species. For instance, hens exposed to thermal stress >35 °C for 30 minutes in the hours before ovulation had lower circulating levels of LH. The heat stress disruption of LHRH and LH would result in lower progesterone and abnormalities in egg laying (Novero et al., 1991).

Heat stress in pigs is a priority for the industry since high temperatures associate with economic losses from the low animal performance in breeding and grow-finish farms (Ross et al., 2015). Heat stress is known to cause reduced feed intake, alter metabolism and compromise oocyte and embryo development (Ross et al., 2015). Pigs are highly sensitive to heat stress as they have minimal sweat glands, and instead can only regulate heat loss when in confinement through increased respiratory rate, water consumption, and standing or lying (Wettemann and Bazer, 1985). Much of the attention of heat stress in pigs has focused on estrus related problems. Several reports have indicated that elevated temperatures during summer can delay age of gilts attaining puberty as a result of a reduction of gonadotropins and gonadotropin receptors on the ovaries and compromised follicle development (Flowers and Day, 1990; De Rensis et al., 2017). In mature gilts, D'Arce et al. (1970) reported that prolonged high temperatures in the environment during the estrous cycle, increased the incidence of abnormal corpora lutea

formation. In weaned sows, heat stress affects between 9 to 14% and result in delayed estrus expression (Peltoniemi and Virolainen, 2006; De Rensis et al., 2017) that is likely due to a reduction in follicle diameter. Further fertility problems may arise with delayed or even failed ovulation (Lopes et al., 2014; De Rensis et al., 2017).

Heat stress in pigs can also affect embryo survival and maintenance of pregnancy, although the origin of the problem is uncertain. It is suggested that perhaps reduced numbers of corpora lutea in females exposed to heat stress prior to breeding during summer may affect early pregnancy establishment and pregnancy maintenance (Edwards et al., 1968). However, according to Ross et al. (2015), during oocyte and embryo development sows exposed to short periods (five days) of hyperthermia following breeding have a significant reduction in embryo viability by day 27 of gestation. Previous studies showed that heat stress resulted in loss of almost 30% embryos by day 30 of gestation when compared to control gilts or sows (Edwards et al., 1968; Omtvedt et al., 1971). The early pregnancy failures and loss of embryos have been associated with heat stress >30 °C during early gestation (Edwards et al., 1968; Teague et al., 1968; Omtvedt et al., 1971). The high percentages of return to service after insemination due to high temperatures (Britt et al., 1983; Prunier et al., 1996) may approach 30% on individual farms (De Rensis et al., 2017) and associate with 10% of sow removals (Koketsu et al., 1997b). The effects of heat stress may also be latent, since regular returns around 21 and 42 days post insemination are an indicator of conception failure, and increase during summer. Irregular returns occur between 24 to 39 days after insemination, and associated with failure in pregnancy maintenance, and are known to increase in early fall (De Rensis et al., 2017).

Heat stress in pigs during late gestation is linked to late pregnancy loss (Edwards et al., 1968; Omtvedt et al., 1971) and 12% lower farrowing rates during summer and fall compared to

the other seasons (Peltoniemi et al., 1999; Bloemhof et al., 2012; Bloemhof et al., 2013; Lopes et al., 2014; De Rensis et al., 2017). Further, genetic line differences for the upper critical temperature limit has been associated with farrowing rate and litter size (Bloemhof et al., 2008). The same group examined the herd records and noted a correlation between daytime temperature and reduced farrowing rate and litter size. They indicated that heat stress three weeks before breeding influenced farrowing rate while heat stress in the week before until two weeks after breeding had effects on number of total born pigs. The effects of heat stress may also extend into lactation, as lactating sows have an upper critical temperature limit that is lower (15 - 25 °C) compared to non-lactating (30 °C) sows (Kemp and Verstegen, 1987). Temperatures above the thermal limit for these sows could clearly compromise both sow and litter performance (Muns et al., 2016).

While the effects of heat stress occurring during summer are widespread, and the data from many studies convincing, there are other reports that suggest that the problem is more complex, and that other factors may be involved to cause these losses. For example, in controlled studies where sows or gilts were kept at 29°C (Renaudeau et al., 2001), 30°C (Auvigne et al., 2010) or 32°C (Williams et al., 2013) for extended periods, there were no major effects on reproductive physiology or fertility, despite significant changes in the animal's mechanisms for heat loss and production. There is the strong possibility for thermal adaption to a heat stress, which could occur through the brain and hypothalamic-pituitary-thyroid axis to alter thyroid hormones to regulate metabolic rate (Prunier et al., 1996) and alter neural mechanisms to influence behavioral changes to help animals lose heat. Both reproductive performance and heat tolerance are lowly heritable traits with variability between genetic lines and breeds quite high (Ross et al., 2015) making selection for either difficult (Ross et al., 2017).

Surprisingly, there is limited data on the beneficial cooling effects in swine. However, in cattle, application of cooling throughout the summer months is able to reduce mid-day temperatures by 2-7 °C resulting in reduced respiration rates and rectal temperatures (Khongdee et al., 2006). This approach is used as a strategy to maximize routes to exchange heat (Collier et al., 2006). Some studies show that these cooling systems are able to improve fertility although they still cannot match the winter fertility rates (Rensis and Scaramuzzi, 2003). Evidence shows that as internal body temperature increases, neural and endocrine changes occur to help maximize loss and minimize gain of heat. It would appear that many factors could alter the upper critical temperature among animals and the point at which certain changes take place for normal function. However, it is perhaps logical to assume that the response to heat stress is somewhat progressive, and upon reaching and exceeding a critical level, the damage at the cellular level in the tissues or organs increases to contribute to seasonal infertility symptoms. The heat stress scenario in pig fertility appears to involve its interaction with uncontrollable variables such as reproductive age and phase, metabolic state, and outdoor environmental conditions. When all are combined with other data, the information suggests that heat stress alone does not cause all the reproductive losses. As supported by previous investigators, hot temperatures interact with photoperiod to lower fertility in susceptible animals during summer and fall (Auvigne et al., 2010; Bertoldo et al., 2012; De Rensis et al., 2017).

Photoperiod and seasonal fertility

The effect of changing photoperiod in different seasons alters fertility in numerous livestock species, such as the classical seasonal (sheep, goats, horses, chicken) and also non-seasonal (cattle and pigs) species (Claus and Weiler, 1985; Bronson, 1988; Ortavant et al., 1988;

Sharp, 1993; Sreekumar, 1997; Dawson et al., 2001; Chemineau et al., 2007; Vasantha, 2016). Global position in relation to the equator has effects on climate, day length, and seasonal changes during the year. Evident annual variations in photoperiod and temperature are related to the high latitudes whereas those closest to the equator or in tropical areas may be slight and obvious mainly in birds (Dixit and Singh, 2011). Different species in each latitude are able to adapt and establish breeding patterns according to the climate. However, the domestication of livestock species brings complexity to the reproductive patterns, especially with selection for fertility in many or all seasons and their response to confinement housing with artificial photoperiod and different lighting intensity when compared to outdoor natural lighting. Each species has evolved its own physiological system to control breeding time, best suited for successful gestation and lactation length and offspring survival. However, they all seem to share a similar initial physiological response that perceives the changes in photoperiod to regulate the circadian pattern through hormonal regulation.

It is well stablished that vertebrates perceive the external environmental changes (lighting intensity and changes in photoperiod) through the eye (Simonneaux and Ribelayga, 2003). In mammals, the eye sensor is located in retinal ganglion cells which produce the photopigment melanopsin. The ganglion cells depolarize upon photo stimulation to convey the neural message via the retino-hypothalamic tract (RHT) of dark or light to the suprachiasmatic nuclei (SCN) (Vasantha, 2016). The polysynaptic signal is transmitted from the SCN to the superior cervical ganglia (SCG) which innervates the pineal gland through sympathetic nerves (Bearden and Fuquay, 1997).

Melatonin: synthesis, secretion and receptors

In vertebrates, the pineal gland is a small endocrine gland in the brain and located posterior to the hypothalamus and between the hemispheres. The main function of the pineal is to produce and release the hormone, melatonin (Simonneaux and Ribelayga, 2003). In amphibians and avian species, the pineal contains photoreceptors. However, in mammals, the pineal consists of neuroendocrine cells (pinealocytes) (Simonneaux and Ribelayga, 2003). The pineal responds to neural input on environmental lighting and changes in daylength to acutely activate or suppress melatonin synthesis (Melmed and Conn, 2007). Melatonin is released from the pineal gland into the surrounding dense network of blood vessels and into the cerebrospinal fluid (Simonneaux and Ribelayga, 2003).

The biosynthesis of melatonin starts with assimilation of tryptophan from circulation (Melmed and Conn, 2007) and then its conversion to 5-hydroxytryptophan by tryptophan-5-hydroxylase followed by conversion to seratonin by aromatic amino-acid decarboxylase. Seratonin is then converted to N-acetyl seratonin by N-acetyl transferase through an acetylation process (Vasantha, 2016). Finally, methylation of N-acetyl seratonin by hydroxyl-indole-methyltransferase forms melatonin (5-acetyl-N-methoxytryptamine) (Aleandri et al., 1996; Dubocovich and Markowska, 2005). The release of melatonin from the pineal allow binding to cells or tissues to encode the circadian rhythm (Reiter, 1991). Other tissues or organs such as the skin, gastrointestinal tract, immune cells, and the reproductive tract, produce small amounts of melatonin, but its function may be local and is still not clear (Acuna-Castroviejo et al., 2014). In addition, melatonin has been shown to be present in almost all biological fluids including saliva, breast milk and follicular fluid (Acuna-Castroviejo et al., 2014).

Melatonin is secreted into the blood during a 24 h period and is regulated by dusk and dawn (Zhdanova and Wurtman, 2005). Secretion into the blood begins at dusk or lights out, with the concentration in the plasma reaching its peak midway through the duration of darkness (Reiter, 1991; Aleandri et al., 1996). At dawn, subsequent exposure to light inhibits the production and secretion of melatonin. Among different species, the circadian pattern in peak concentration and duration of release of melatonin can change between summer and winter.

In the highly responsive Siberian hamster, melatonin levels are two times greater in winter than during the summer, while the European hamster shows ten times higher melatonin levels in winter compared to summer (de Almeida et al., 2011). In these animals, serum and pineal gland melatonin levels are two to four-fold higher in the middle of the dark phase compared the to middle of the daytime phase during active reproductive cycles (Tamura et al., 1998). Similar results have been reported in the domestic pig (Andersson, 2001) with melatonin 2 to 5 fold higher in the night (Paterson and Foldes, 1994). However, it appears that variation in the amplitude of the nocturnal melatonin rise occurs from one animal to another. Additionally, when comparing the domestic pig with other mammalian species, the pig's melatonin concentration appears to be lower, although it is believed that even the minor increase during the dark can be enough to alter the seasonal reproductive response (Andersson, 2001). Furthermore, gilts exposed to differing artificial lighting schedules show a significant increase in plasma concentration of melatonin 2 hours after dark and remains elevated until a decrease in the basal level of light is deleted (Paterson et al., 1992a). Melatonin disappears rapidly from circulation, with a 30 to 40 minute half-life, depending upon the species (Dubocovich et al., 2010). Other modifiers of when and how much melatonin is secreted may involve the availability of tryptophan in feedstuffs (Aleandri et al., 1996).

In mammals there are two main melatonin receptors (MT1 and MT2) with high affinity binding that are part of the G-protein-coupled receptor family (Dubocovich and Markowska, 2005; Melmed and Conn, 2007; Slominski et al., 2012). In some species only MT1 has been shown to be present (Malpaux et al., 2001). Recent studies reveal that these receptors are widely distributed in the body, such as retina, brain, skin, immune system, liver, blood vessels and gonads (Slominski et al., 2012; Acuna-Castroviejo et al., 2014). Other receptors such as MT3 and nuclear receptors activated by genes, have been identified in other species, but display a lower affinity compared to MT1 and 2 (Malpaux et al., 2001).

Melatonin and reproductive responses

In clearly seasonal breeders, fertility is regulated by the endocrine system as a result of photoperiod perception by the eye and neural signals to regulate pineal synthesis and release of melatonin into the bloodstream (Aleandri et al., 1996). Although the process seems to be quite complex and differ among species, melatonin is released from the pineal gland, enters the cerebrospinal fluid and binds the cells premammillary hypothalamic area (Misztal et al., 2002). It also activates discrete regions of the pituitary gland and together regulates the hypothalamus-hypophysial axis to controll reproductive function in mammals (Yasuo et al., 2009; Casey and Plaut, 2012). This includes the regulation of GnRH release (Aleandri et al., 1996; Malpaux et al., 2001), progesterone release and androgen production (Slominski et al., 2012). The effects of melatonin may depend upon species, and in rodents, melatonin has been shown to affect mainly GnRH and LH release (Slominski et al., 2012). In species such as the sheep, a short day breeder, when exposed to short daylength melatonin causes an increase in the frequency of GnRH (Malpaux et al., 1997) and LH (Aleandri et al., 1996) release. This ability of melatonin to

increase hormone release may involve some effects in the reduction in dopaminergic activity, which acts as an inhibitor to GnRH secretion (Malpaux et al., 2001). In the domestic pig, there are findings that suggest that photoperiod is directly related with melatonin and consequently with reproductive responses (Paterson and Pearce, 1990; Paterson et al., 1991) similar to the European wild pig (Mauget, 1982). While the data is clear that the modern domesticated pig shows clear melatonin responses to lights on and off, they appear to be more variable in their response to changing photoperiod and nocturnal melatonin patterns (Peltoniemi et al., 2005).

Melatonin as the main regulator of photoperiod and fertility in pigs

The regulation of seasonal reproduction and fertility in response to photoperiod and melatonin appears to act not only at the hypothalamus and pituitary, but also directly at the level of the ovary in mammals. In pigs, the effects of photoperiod and melatonin have been shown to alter follicle development, egg maturation, corpora lutea (CL) formation and progesterone production (Love et al., 1993; Xue et al., 1994; Chokoe and Siebrits, 2009). The presence of melatonin in the follicular fluid in pigs and other species suggests its importance in oocyte maturation, as the larger the diameter of a follicle, the less concentrated the hormone (Shi et al., 2009b). Evidence also supports the involvement of photoperiod in seasonal pregnancy failure in summer and fall due to low progesterone (Love et al., 1993). When looking for clues to seasonal pregnancy loss, a 10% reduction in progesterone has been observed in early fall which can progress to as much as 50% lower in late fall compared to other seasons (Peltoniemi et al., 2000). Bertoldo et al. (2012) proposed that in pigs, the mechanism of seasonal photoperiod response is similar to sheep, in that day length changes alter melatonin secretion to affect GnRH secretion. In pigs, during summer and fall, melatonin patterns may reduce GnRH and cause a decrease in

LH. This would result in lowered binding of LH to the CL to reduce production of progesterone. Lower levels of progesterone would have detrimental effects on embryo growth, development and survival, and impair maternal recognition of pregnancy. The final result of this can increase regular and irregular return rates, pregnancy loss, and decrease litter size.

Melatonin also has been shown to have potent effects as an antioxidant to protect against excess free radicals and reactive oxygen species (ROS) (Mayo et al., 2002; Rodriguez et al., 2004). Physiological stress in the cells, such as the ovulation process when the Graafian follicle goes through rupture and release the oocyte, is associated with local inflammation and release of ROS (Tamura et al., 2012). Excessive amount of ROS end up causing oxidative stress, which can accelerate oocyte deterioration; compromising its quality (Tamura et al., 2012; Cruz et al., 2014). Although the pineal is the primary source of circulating melatonin, the production of small amounts of this hormone by cells in the ovary such as the granulosa cells of the follicle and the oocyte may, play an important role in local protection of the cells from oxidative damage (Cruz et al., 2014).

Interestingly, Brzezinski et al. (1987) found that human follicular fluid contains higher concentrations of melatonin than in simultaneously collected serum, although there was a positive correlation between the two different sources. Different species, such as the pig, also have melatonin present in follicles (Shi et al., 2009a) as well as melatonin receptors on the granulosa and cumulus cells surrounding the eggs (Kang et al., 2009a). It is believed that this hormone may be synthesized in the granulosa cells and then released in the follicular fluid (Cruz et al., 2014). In addition, compared to immature follicles, those that are mature produce significantly greater amounts of melatonin, which is positively correlated with progesterone production (Nakamura et al., 2003).

Supplemental use of exogenous melatonin and lighting treatment to reduce seasonal infertility

The use of different lighting schedules, or provision of exogenous melatonin has been applied to overcome seasonal changes in reproductive responses in species such as sheep, horses and pigs. While in seasonal breeders such as sheep and horses, lighting changes help, at least partially, to improve fertility (Thimonier, 1981; Scraba and Ginther, 1985; Chemineau et al., 1992), in non-seasonal breeders, such as the pig, it is less effective (Diekman and Hoagland, 1983; Tast et al., 2001b; Canaday et al., 2013). However, Paterson and Pearce (1990) suggest that artificial lighting regimens can improve attainment of puberty in gilts, and helps provide compelling evidence that photoperiod is a major factor in onset of puberty. The use of lighting requires animals to be housed indoors under controlled lighting duration and intensity. Further, the response to light can require weeks to months to achieve an effect. This can be difficult for practical use on large commercial farms for some livestock species due to housing systems and phased animal relocation.

Data on the use of supplemental melatonin to overcome seasonal changes in the pig is limited. Of the available studies, the results have been either positive or show no effect on fertility. Exogenous melatonin orally administered to gilts once daily at concentrations of 1 to 5 mg resulted in increase from baseline within 10 to 20 minutes with a peak observed between 20 to 40 minutes (Diekman et al., 1991; Paterson et al., 1992b). The decline in melatonin began within 10 to 20 minutes after the peak and was still elevated above baseline at 2 to 8 hours depending on dose and daylength pattern (Paterson et al., 1992b). This administration regiment would likely result in two peaks of melatonin. The first immediately after oral treatment administration and the second peak occurring 6 to 8 hours after lights off (Tast et al., 2001a). Although it is not clear if the double peak has a physiological implication, the method of

administration that causes a double peak resulted in improvements in gilt fertility. Positive effects of melatonin on fertility have been reported by Paterson et al. (1992b) who fed melatonin once daily in the afternoon for one week and doubled the number of gilts reaching puberty. Diekman et al. (1991) also reported positive results of feeding melatonin daily to gilts during the four months from mid fall to early winter, for reducing age at puberty. However, the same authors also tested implants of melatonin which failed to alter the onset of puberty (Diekman et al., 1997), as result of the method of administration. The pattern of melatonin delivery seems to impact the duration of the nighttime melatonin rise, and continuous melatonin delivery from implants is consistently not effective for advancing puberty in gilts (Paterson et al., 1992b; Diekman et al., 1997) while daily melatonin feeding in the afternoon is used to simulate the longer dark phase in the fall. Further, melatonin is present in porcine follicular fluid and in vitro improves the development of oocytes (Shi et al., 2009b). When melatonin was added to in vitro culture media for only a few days, egg maturation, fertilization and embryo development were improved (Rodriguez-Osorio et al., 2007; Kang et al., 2009b; Do et al., 2015). Together, these data provide strong evidence for a local and rapid effect of melatonin acting directly at the level of the follicle and egg.

Collectively the data suggest exogenous melatonin during periods of seasonal infertility in pigs, can alter HPX and ovarian function to improve fertility. We designed two studies to test whether short term MEL administrational during the follicle and luteal phases, and early gestation could improve reproductive fertility in gilts and parity 1 sows.

CHAPTER 3 – Fertility Responses of Gilts Orally Dosed with Melatonin During the Follicular and Early Luteal Phase When Housed in a Hot Environment Under Different Photoperiods

Abstract

It is well-documented that fertility in pigs is reduced in late summer and early fall compared to winter and spring. This seasonal infertility is characterized by delayed puberty, pregnancy failure, reduced litter size, and delayed return to estrus after weaning. Associated with abnormalities in follicular development and corpora lutea function in early gestation, these problems are thought to be mediated at least in part, through seasonal changes in the duration of nighttime secretion of melatonin. Coinciding with the periods of reduced fertility, nocturnal melatonin is minimal in late summer and early fall. In addition, numerous studies have also associated heat stress with reproductive failures in the same seasons. To determine whether seasonal infertility during heat stress might be mediated through changes in photoperiod, this study was designed to test if exogenous melatonin could mimic short days and minimize seasonal infertility problems in the follicular and early luteal phases. The experiment was performed in a single replicate with terminal line PIC gilts that were selected at 165 d of age and randomly assigned by age and weight (116.5 ± 1.1 kg) to treatment. On the first day of the experiment, (Day 1), gilts (n = 36) were allocated in a 2 x 3 factorial treatment design to receive Melatonin (MEL) or Control (CON, placebo) while housed in one of three environmental rooms that provided: 1) 8 h of light at 32 °C (8H); 2) 16 h light at 32 °C (16H); or 3) 24 h light at 32 °C (24H). Gilts were fed 1.8 kg in the AM and then MEL (5 mg) or CON treatment orally as a top

dress on the PM feed (0.9 kg). On Day 6, gilts received an i.m. injection of P.G. 600 and fence line boar exposure once daily until Day 13. Trans-rectal ultrasound was performed every other day from Day 6 to 10 to assess follicles. Gilts in estrus were inseminated twice on each day standing. On Day 14, all gilts were fed a control gestation diet once daily in the AM (2.7 kg), and all rooms were adjusted to similar lighting (16L : 8D) and temperature (22 °C). On Day 47 animals were slaughtered and reproductive tracts collected to assess pregnancy, litter size, fetal and placental measures (~Day 33 of gestation). Continuous and categorical data were analyzed using the GLM and GENMOD procedures of SAS for the main effects of treatment and room. Average 24 h temperature, humidity, and their index was assessed for 8H (26.9°C, 49.7%, and 74), 16H (28.3°C, 36.1%, and 75), and 24H (30.6 °C, 46.8%, and 79), respectively. Ammonia levels averaged 19, 8 and 11 ppm, respectively. Luminosity averaged 73, 157 and 266 lux during 24 h period and ranged between 0 and 218 lx, 0 and 240 lx, and 266 lx, respectively. There was no effect of treatment or room ($P>0.10$) on estrus (91.6 ± 11.8%), interval from P.G. 600 to estrus (4.1 ± 0.2 d), gilts with large follicles (91.6 ± 15.9%), or large follicles/gilt (14.3 ± 1.5). However, there was a tendency ($P = 0.08$) for MEL (87.8%) to improve pregnancy rate compared to CON (63.3%) but with no effect of room. Total fetuses (15.3 ± 3.1), healthy fetuses (14.7±2.9), abnormal fetuses (0.7 ± 0.5) and placental efficiency (0.09 ± 0.01, fetus wt./placenta wt.) were not affected by treatment or room ($P>0.10$). In this study, gilts were exposed to differing hours of heat stress throughout the day during the follicular and luteal phases, but responses were near optimal for large follicle development, estrus expression, and litter size. This suggests that under heat stress, a P.G. 600 induced follicular phase may negate melatonin effects on endogenous hormone release or even P.G. 600 is able to overcome heat stress and changes in photoperiod. However, the trend for improved pregnancy suggests luteal, embryo or

uterine response to melatonin supplementation during the first week of gestation and perhaps some antioxidant effects of this hormone during the first days of gestation. To better understand how melatonin might minimize effects of seasonal infertility, further studies in the follicle and luteal periods are needed.

Introduction

Heat stress and changing photoperiod during summer and early fall have been related to seasonal infertility in the swine industry worldwide. Declines in fertility in gilts and parity one (P1) sows is predominant during these periods of year. Delay in puberty, longer wean to estrus interval, higher return rates, pregnancy failures and lower farrowing rates characterize what leads to fewer pigs produced and available in the market in the following summer. While the cause of the problem is confounded by high temperatures and long photoperiod, the seasonal infertility pattern is clear. The problem seems to be particularly prevalent in the seasons that present environmental temperatures above the comfort zone leading to heat stress but also when hot days are followed by cooler nights. Moreover, during the same seasons, changes in photoperiod to longer days in summer followed by shorter days in early fall occur. Photoperiod in the pig appears to act through melatonin, with hours of release at night being minimum in summer and increasing in duration into early autumn. This hormone is produced and released by the pineal gland at night and may serve as a regulator of hormonal control as well as an antioxidant (Rodriguez et al., 2004; Tamura et al., 2012). Further, melatonin receptors are present on the granulosa cells of pig follicles (Shi et al. 2009) and the cumulus cells surrounding the egg (Kang et al. 2009). It is possible that seasonal photoperiod effects associated with melatonin could be partially impacting follicular development, corpus luteum formation, and embryo survival in

during early gestation. Although studies using supplemental melatonin in the pig are limited, positive effects of this hormone on fertility have been reported (Diekman et al., 1991; Paterson et al., 1992a; Love et al., 1993).

The present experiment was designed to simulate the periods of seasonal infertility for breeding gilts housed in confinement. The objective was to evaluate the fertility responses of gilts exposed to different durations of light and heat during the day and their response to exogenous melatonin during the follicle phase and early gestation.

Materials and Methods

The use of animals for this experiment was approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign (#14081).

Experimental design

The experiment was performed at the University of Illinois swine research farm in a single replicate in February 2015. Terminal line PIC® gilts (n = 36) that were 165 ± 2 d of age were selected and moved from a finishing barn into an environmentally controlled swine breeding and gestation facility. The females were housed in pens and weighed (116.5 ± 2.9 kg) and tagged for assignment to treatment. Gilts were randomly assigned by age and weight using a 2 x 3 factorial arrangement of treatments to receive either Melatonin (MEL) or Control (CON, placebo) while housed in one of three environmental rooms. The rooms were designed to provide either: 1) 8 h of light at 32°C (8H); 2) 16 h light at 32° C (16H); or 3) 24 h light at 32 °C (24H). The lighting for each room was set to 240 lux at pig level. Following assignment to treatment, on

Day 1, gilts were moved into a room and placed in a stall using an alternating treatment sequence. In their stalls, gilts had ad libitum access to water and received 2.7 kg of feed in the AM. In the PM (1500 h) gilts were fed MEL (5 mg) or CON (0 mg) as a top dress on a small amount of fed (0.9 kg) starting on Day 1 and continuing for the next 13 d. On Day 6, all gilts received an i.m. injection of P.G. 600® (400 IU of PMSG and 200 IU of hCG, Merck Animal Health, NJ) to induce a follicle phase and estrus. Fence line boar exposure was provided once daily for 5 min for each gilt and estrus was determined using the back-pressure test until Day 13. Trans-rectal ultrasound was performed every other day at 1530 to 1730 h following treatment on Days 6 to 10 to assess number and size of follicles. Ultrasound was performed using an Aloka 500 V ultrasound (Hitachi Aloka Medical, Ltd., Wallingford, CT) with a 7.5 MHz linear array transducer attached to a PVC stabilizing rod. Scans of both ovaries were digitally recorded to determine the presence, number and size of ovarian follicles for later assessment. Gilts in estrus were artificially inseminated twice, once on each day standing. Semen was obtained from a commercial boar stud (PIC®) with each dose containing 3.0×10^9 sperm from a pool with 5 boars. Any gilt not detected in estrus by Day 12 of the experiment was considered anestrous. On Day 14, all gilts were fed a standard gestation diet once daily in the AM (1.8 kg), and all rooms were adjusted to similar lighting (16L : 8D) and temperature (22.0 °C). All animals were maintained in their crate in the rooms until Day 47, when gilts were sent to a local slaughterhouse and reproductive tracts collected to assess ovaries, and Day 33 pregnancy status. Reproductive tracts were assessed for number of corpora lutea (CL). Healthy fetuses were counted and weighed (± 0.01 g) and crown-rump length measured using a caliper (± 1.0 mm). Degenerating fetuses were also counted and identified based on appearance and size, along with placental size and weight.

Preparation of Melatonin (MEL) and Control (CON) treatments

Melatonin (N-acetyl-5-methoxytryptamine) was purchased from Sigma Chemical (>98% pure) for oral delivery based on the approach and dose previously reported for use in swine (Diekman et al., 1991; Paterson et al., 1992b). The MEL stock solution (5 mg/mL) was prepared every 3 d by diluting MEL (270 mg : 54 mL) in absolute ethanol with storage at RT in a glass bottle, protected from light. The CON solution was 100% ethanol. To prepare the oral dosing solutions for each animal, 1 mL of the MEL or CON stock was pipetted into a 50 mL conical tube filled with feed. The tube was not capped in order to allow the ethanol to evaporate overnight. For treatment, the 50 mL treatment tube with feed was top dressed on the 0.9 kg of feed given in the PM.

Environmental Rooms

The three rooms were identical in dimension, and designed for air control, flooring, insulation and lighting with each sharing a common pit. In each room, 6 pairs of fluorescent tube lights mounted on the ceiling provided illumination based on automatic timers. Each room had its own thermostatic control system, with air inlets and exhaust, electric heat, and fans. Each room contained 12 gestation stalls that were 0.61 x 2.13 m in dimension on solid and concrete slatted flooring (Canaday et al., 2013). **Figure 1** illustrates the layout of the experimental facility. In order to modify the rooms to gradually gain or lose temperature similar to a normal day and night, solid blackout curtains were installed in each doorway for the 8H and 16H rooms to block the gestation room lighting from the experimental rooms, while allowing free flow of air through the curtain. Outside the rooms, the gestation barn was maintained at 22.0 °C and low lux

lighting. In the daytime, the doors would be closed and the rooms allowed to heat for the appropriate time and at the set time, and the doors would be opened (8H and 16H) and the curtains unrolled to prevent light entry, but allow exchange of the cooler gestation barn air with the hot air inside the rooms.

Environmental Measures

Daily room temperature, humidity, ammonia levels and light intensity measurements were obtained for each week of the study. Measurements were obtained at pig level (0.5 m from the floor) at the middle location of the room. Relative humidity (RH) and temperature readings within each room were obtained using a MicroLite Temperature Logger with an accuracy of ± 0.3 °C and ± 2% RH. Data was obtained on the sampling days every hour. Light intensity was measured using a Digital Lux Meter with an operational detection limit of 0 to 400,000 lx and accuracy: ± 3%. Ammonia levels were measured using Gastec standard detector tube system (GASTEC GV-100/GV-110) with 0.5 – 78 ppm.

Statistical Analysis

Data were analyzed using ANOVA procedures in SAS (SAS Institute Inc., Cary, NC USA). Continuous response measures were analyzed using the PROC MIXED procedure for significance of the main effects using the *F*-test and differences between least squares means identified using the *t*-test. Binary response measures were analyzed using PROC GENMOD using a binary distribution and a logit-link function. Significant main effects and differences between least squares means were identified using the x^2 test**.** The models contained the main

effects of treatment (MEL and CON) and room (8H, 16H and 24H) and their interaction with age and weight included as covariates and removed when not significant. The assumptions of the ANOVA were tested for normal distribution using PROC UNIVARIATE and Levene's test for homogeneity of variance. When data could not meet the assumptions, they were transformed for analysis. Significance was identified when $P \leq 0.05$ and trends when $P>0.05$ but ≤ 0.10.

Results

Environmental measures for each room

The average 24h room temperature (mean ± SE) was 26.9 ± 0.4 °C (24.1 - 31.0 °C) for 8H, 28.3 ± 0.4 °C (24.3 - 30.8 °C) for 16H, and 30.6 ± 0.4 °C (29.5 - 31.1 °C) for 24H (**Figure 2**). The average 24h room relative humidity was 49.7 ± 1.1% (42.2 - 63.6%) for room 8H, 36.1 ± 1.1% (24.6 - 50.0 ± 1.1%) for 16H and 46.8 ± 1.1% (33.4 - 63.4%) for 24H (**Figure 3**). Ammonia levels were measured for 5 consecutive days at the moments of doors closed for 5 to 8 hours and averaged in 19.0 ± 2.4 ppm (12 - 28 ppm) for 8H, 8.4 ± 0.7 ppm (6 - 10 ppm) for 16H, and 10.8 ± 1.0 ppm (9 - 15 ppm) for 24H. Light intensity was 0Lx with lights off and 218 lux for 8H, 243 lux for 16H, and 266 lux for 24H.

Reproductive responses to treatment and environmental rooms

Results for reproductive measures in response to treatment or room are shown in **Table 1.** Overall there was no interaction between rooms and treatment ($P>0.10$). There was no effect of treatment or room ($P>0.10$) on average vulva score on Days 3 and 4 after P.G. 600, estrus expression within 5 days (91.6%), interval from P.G. 600 to estrus (4.1 d), duration of estrus (1.6

d), gilts with large follicles (88.3%) or numbers of large follicles (14.3) at estrus. On Day 33 following estrus and AI, there was a statistical tendency (P = 0.08) for MEL to improve pregnancy rate (87.8%) compared to CON (63.3%), but with no effect of room. Neither treatment nor room had any effect on number of CL (15.2), normal fetuses (14.7) or degenerating fetuses (0.7). A fertility index of pregnancy rate and healthy fetuses was not tested but suggested a treatment effect, with 24 h (1,242) 16H (1,038) and 8H (980) and MEL 1229 with CON (943). No effect of treatment or room was identified for any measure for fetal or placental weights.

Discussion

The present study was designed to assess whether melatonin could enhance the fertility of gilts housed under environmental conditions associated with seasonal infertility. Seasonal infertility in summer and early fall is confounded by high temperature and long photoperiods that shorten. Higher occurrence of anestrus, longer periods to estrus, and pregnancy loss (Love, 1981; Britt et al., 1983; Tast et al., 2002; Auvigne et al., 2010) are the causes for reduced pig production in the following year likely resulting from abnormalities during the follicle and early luteal phases of gestation. In this study, during the first week of treatment, temperature (26.9 to 30.6 °C) and humidity (36.1 to 49.7%) were above thermoneutral, and similar to patterns recorded in summer and early fall even for indoor housed pigs (Edwards et al., 1968; Wegner et al., 2014). At the same time, the duration of lighting provided a range of exposure that a pig would receive during summer and early fall whether indoors or outside. There were no effects of melatonin, high temperature or photoperiod on any estrus measure or litter trait for prepubertal gilts treated with P.G. 600 that were inseminated. However, there was a trend for pregnancy rate to be reduced in CON when compared to MEL treated gilts with, the reduction most associated

with the CON 8H and 16H. Although conception failure is a critical response to seasonal infertility, the data in the present study should be interpreted with caution due to the limited numbers of animals. Yet if the effect would persist with increased numbers of observations, it might be logical associate this with melatonin ability to improve in vitro development and maturation of the pig oocyte and embryo (Kang et al., 2009b). This may occur since melatonin has antioxidative ability that can help to protect the oocyte and embryo from ROS. Furthermore, another in vitro study with pig oocytes showed that embryos treated with melatonin improved its performance (Rodriguez-Osorio et al., 2007).

Our model to observe the effects of heat stress, lighting, and melatonin, was based on induction of a gonadotropin synchronized follicle phase that shows variable (50 – 80%) estrual responses (Britt et al., 1989; Garcia et al., 2004; Estienne and Crawford, 2015) within 6 d following injection. However, this study, P.G. 600 induced most gilts into estrus, and appeared to overcome most effects of season for failed follicle development and estrus expression (Knox and Zas, 2001; Bertoldo et al., 2010; De Rensis et al., 2017). As a result, at least for estrus, P.G. 600 would negate any beneficial effect of melatonin, if gonadotropin deficiency was the cause of infertility. However, with respect to pregnancy, there are no reports to suggest P.G. 600 would have any beneficial effect. Further, the prepubertal gilt induced with P.G. 600 and inseminated, is considered a low fertility model as has been noted previously (Estienne and Crawford, 2015). From a practical standpoint, short-term melatonin treatment during the sensitive fertility weeks within season, could be accomplished, especially if it had an important economic outcome. Long-term daily feeding (4 to 5 months) of melatonin (Diekman et al., 1991) and even short-term feeding for only 7 d (Paterson et al., 1992b) were able to advance puberty in gilts during periods not associated with heat stress. The data appear clear however, that the diurnal pattern

for melatonin exposure is important, as continuous implant exposure does not advance puberty in gilts (Diekman et al., 1997; Kennaway et al., 2015). The implant approach possibly does not work properly in pigs, continuous exposure simulates continuous dark. The acute rise of the hormone from oral dosing allows a diurnal increase and decrease to send a message for perception of photoperiod.

Despite the evidence that melatonin can effect reproductive fertility in pigs, and that patterns of melatonin are associated with seasonal fertility in the wild pig, few studies have been able to show any effect of lighting on pig reproduction. Insight as to how or why this may be occurring could be complicated by the time required for an animal to become set to a photoperiod pattern whether indoors or out. Data in pigs indicate that lighting changes may require days, weeks or months to have an effect on return to estrus after weaning (Stevenson et al., 1983; Paterson and Pearce, 1990). However, other studies find that lighting changes do not have any effect on fertility (Diekman and Hoagland, 1983; Canaday et al., 2013). It may also be important to observe what type of fertility response was being assessed. In the pig, the underlying mechanisms of estrus and conception failures are of industry priority. Melatonin is a hormone that is released at night in response to patterns of light and darkness, and appears to simply respond to decreasing or increasing intensity or lights on and off. The time required for the natural melatonin pattern to have some impact on reproduction is far less certain, although as previously noted, it may require days to weeks for exogenous MEL to effect pig fertility in vitro and in vivo (Diekman et al., 1991; Paterson et al., 1992b; Paterson and Foldes, 1994; Shi et al., 2009b; Do et al., 2015). It is not clear how the existing melatonin pattern interacts with exogenous melatonin. In this case, too little would have no impact, while too much may have an opposite outcome. The data suggest that the approach used with feeding melatonin in the PM

would extend the hours of nighttime MEL. Exogenous melatonin orally administered to gilts once daily at concentrations of 1 to 5 mg resulted in increase from baseline within 10 to 20 minutes with a peak observed between 20 to 40 minutes (Diekman et al., 1991; Paterson et al., 1992b). The decline in melatonin began within 10 to 20 minutes after the peak and was still elevated above baseline at 2 to 8 hours depending on dose and daylength pattern (Paterson et al., 1992b). This administration regiment would likely result in two peaks of melatonin. The first immediately after oral treatment administration and the second peak occurring 6 to 8 hours after lights off (Tast et al., 2001a). Although it is not clear if the double peak has a physiological implication, the method of administration that causes a double peak resulted in improvements in gilt fertility (Paterson et al., 1992b).

With respect to the effect of high temperature, it is possible, that prepubertal gilts treated with P.G. 600 are less sensitive to heat stress than sows. There is data to indicate certain levels of heat stress may or may not affect fertility. It is also possible, that the level or duration of heat stress we imposed was not a true stress for growing gilts that had ad libitum access to water, concrete, greater crate space, and air movement. High and varying ambient temperatures and relative humidity did not affect follicles, estrus responses, conception rates and embryo survival in this study. According to Nienaber et al. (2004) categories of weather safety associated with the temperature humidity index (THI) suggest that the 16H and 24H treatments, exposed gilts to 'alert' and 'danger' categories, respectively, but not the 8H, which was considered normal. However, based on the reproductive responses obtained, there was no evidence that females were affected by heat stress. Even though the THI was on the pathway for dangerous consequences for animals, perhaps this was not enough to result in delayed estrus, reduced in ovulation rate or

reduced number of embryos, as previously reported for gilts exposed to 30.0 °C (Edwards et al., 1968).

It has hypothesized that under heat stress, lighting and melatonin may interact to alter fertility. A previous study (Canaday et al., 2013) showed that reproductive responses were not affected for mature gilts housed under heat stress and low lighting during breeding and gestation. Due to limited numbers of gilts and design of the present study, it was not possible to identify effects on fertility, although there was an indication of a trend on pregnancy. In this record, melatonin may not be acting through a gonadotropin pathway as much as through an antioxidant pathway.

Conclusion

In the present study gilts exposed to differing duration of light and heat during the follicular and luteal phases, had no effect on follicle development, estrus expression, and litter size which were near optimal. Under these conditions a follicular phase induced by P.G. 600 seemed to negate any effects of exogenous melatonin in photoperiod, and high temperature. The tendency for improved pregnancy rate for the melatonin supplemented females suggests that the hormone might have acted on the oocyte or embryo, CL or uterus in the follicle or early gestation weeks. Although it is believed that the antioxidative effect of melatonin may be the mechanism for improved fertility, further studies using this hormone during follicular and luteal periods with and without P.G. 600 may be required to better understand the pathways and actions for melatonin, photoperiod and heat stress.

Acknowledgements

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Tables and Figures - Experiment 1

Figure 1. Experimental facility layout of the three individual rooms. A. Measures of room and crate size and placement of 2 doors per room with blackout curtains in the first two rooms. B. Placement of regulation devices: fluorescent lighting, pit curtain to prevent air flow between rooms through the pit, heating unit, and exhaust vents. C. Arrows indicate animal orientation within the facility and crate location over partially slatted floors. Adapted from (Canaday et al., 2013).

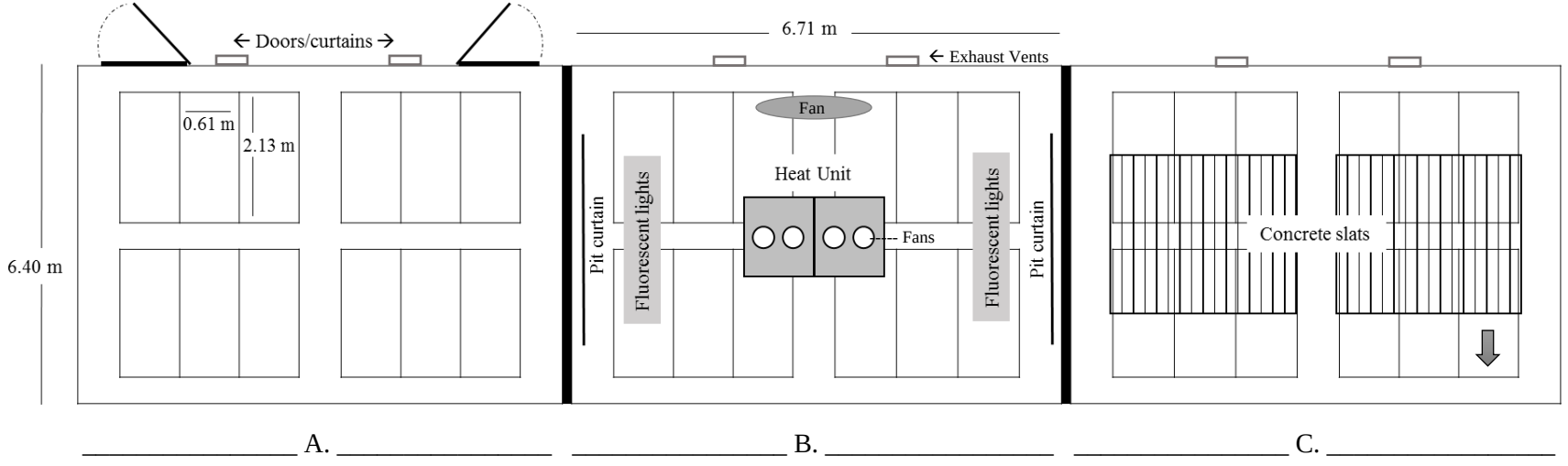

Figure 2. Temperature (°C) and lighting schedule during the 24 hour period in each room providing: 8 h of light (240 lux) and heat (32°C, 8H), 16 h of light and heat (16H), or 24 h of light and heat (24H).

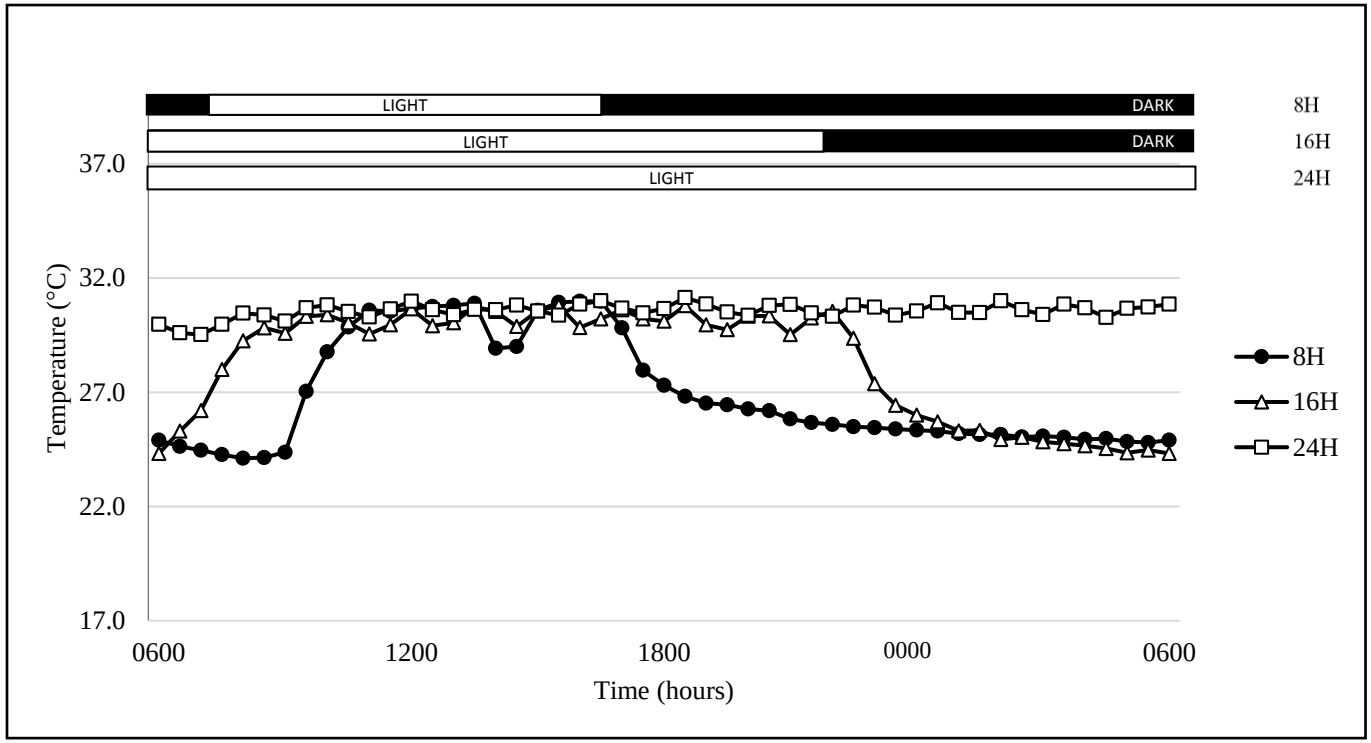

Figure 3. Relative humidity (RH, %) and lighting schedule during a 24 hour period in the three treatment rooms providing: 8 h of light (240 lux) and heat (32°C, 8H), 16 h of light and heat (16H), or 24 h of light and heat (24H).

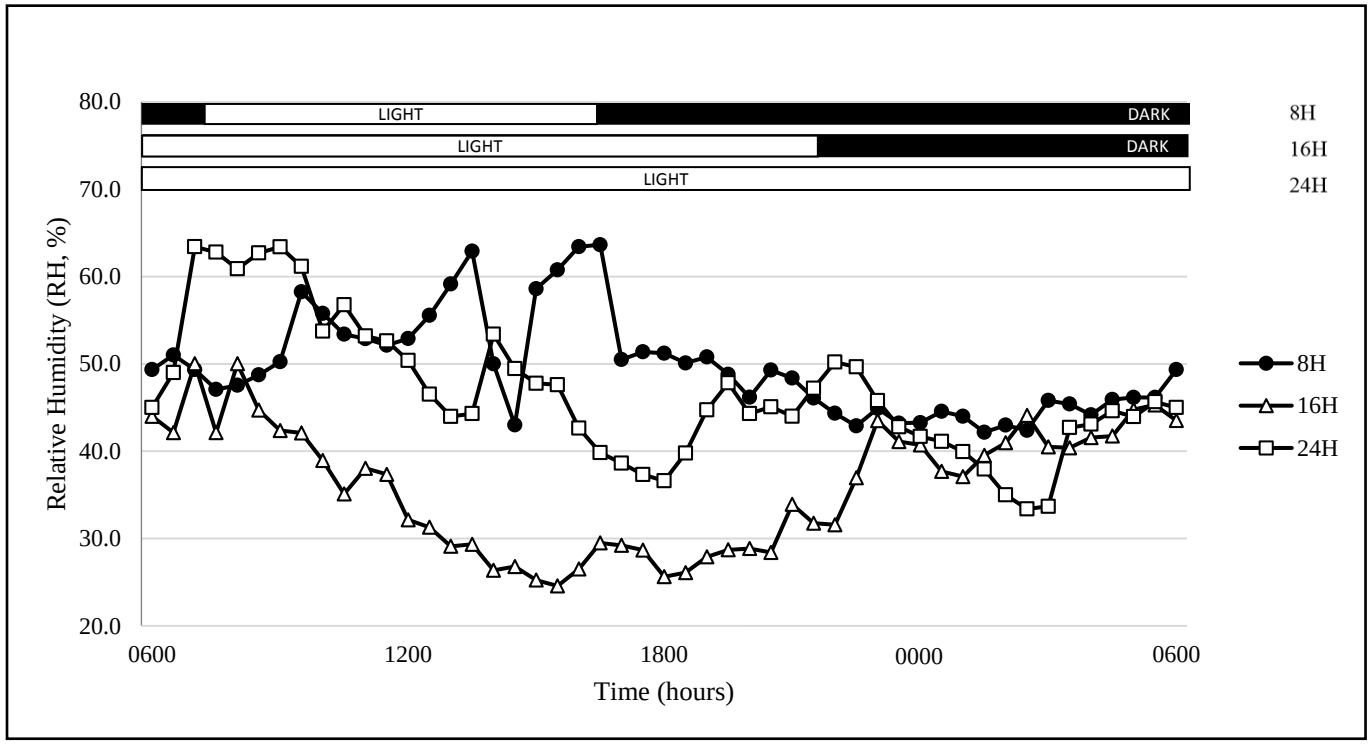

Table 1. Effect of Melatonin (MEL) or Control (CON) treatment during a P.G. 600 induced follicle phase and early gestation for gilts housed under 8 h (8H), 16 h (16H) and 24 h (24H) of heat (32 °C) and light (240 Lx).

	Treatment									
	CON			MEL						
	Room			Room				Treatment	Room	Interaction
	8H	16H	24H	8H	16H	24H	SE	P-value	P-value	P-value
n	6	6	6	6	6	6				
Weight, Kg	116.7	117.3	115.2	115.8	119.8	114.3	2.9	0.91	0.42	0.80
Vulva score (1,2,3)	2.0	2.3	2.2	2.2	2.0	2.3	2.2	1.00	0.84	0.60
Estrus, %	83.3	100	100	83.3	100	83.3	11.8	0.57	0.38	0.72
Duration of estrus (d)	1.2	1.8	1.6	1.5	2	1.6	0.3	0.43	0.09	0.81
P.G.600 to estrus interval (d)	4.6	4.2	3.8	4.0	4.0	4.0	0.2	0.19	0.14	0.14
Large follicles, %	100.0	83.0	94.0	78.0	79.0	96.0	15.9	0.34	0.50	0.49
Large follicles number	16.3	13.0	12.4	15.3	14.0	15.0	1.5	0.51	0.15	0.31
Pregnancy rate on d 33, %	40.0	66.7	83.3	80.0	83.3	100.0	18.4	0.08	0.19	0.74
CL number on d 33 of gestation	19.3	14.8	19.5	17.8	17.8	15.0	3.7	0.68	0.74	0.38
n[1]	34	58	71	64	53	65				
No. of healthy embryos	17.0	14.5	14.2	16.0	13.3	13.0	2.9	0.64	0.62	1.00
No. of abnormal embryos	1.5	0.5	0.8	0.5	0.3	0.4	0.5	0.21	0.53	0.78
Fertility Index	680	967	1183	1280	1108	1300				
Embryo survival, %	81.0	90.5	70.3	90.4	74.5	81.0	7.0	0.75	0.41	0.09
Avg. fetus weight (g)	3.1	3.4	3.2	2.9	3.0	3.5	0.2	0.51	0.45	0.21
Avg. fetus length (cm)	3.1	3.5	3.2	3.3	3.4	3.4	0.1	0.35	0.23	0.58
Avg. placenta weight (g)	33.1	44.8	34.9	29.0	34.0	39.2	5.1	0.41	0.32	0.29
Avg. placenta length (cm)	38.4	46.8	43.0	39.3	40.1	48.5	4.2	0.97	0.31	0.31
Placenta efficiency (fw:pw)	0.10	0.08	0.10	0.11	0.10	0.09	0.0	0.63	0.56	0.52

[1] n values representing total number of fetuses present on females tract

CHAPTER 4 – Effects of Feeding Melatonin During Proestrus and Early Gestation to Gilts and Parity 1 Sows to Minimize Effects of Seasonal Infertility

Abstract

Seasonal infertility associated with the effects of heat stress and changing photoperiod in summer and fall is thought to be the cause of delayed puberty, increased wean to estrus interval, pregnancy failures, and reduced litter size. In seasonal species, changing photoperiod alters melatonin to modulate the hypothalamic-pituitary axis and function of the ovary to control reproduction. This study was designed to test whether supplemental melatonin given to mimic the extended nighttime melatonin pattern observed in the winter season could improve fertility in summer and fall. Exogenous melatonin was fed during proestrus and into early gestation, coinciding with follicle selection, corpus luteum formation, pregnancy recognition and embryo survival. Two experiments (Expt.) were conducted at a 6,500 sow, breed to wean farm in 12 sequential replicates from Jun to Sep. In Expt. 2a, gilts (n = 420) that had expressed a second estrus were assigned by weight to receive once daily oral Melatonin (MEL, 3 mg) or Control (CON, placebo) in a syrup solution at 1400 h each day for 3 wk starting 1 wk before insemination. In Expt. 2b, parity 1 sows (n = 470) were randomly assigned by lactation length and back fat to receive MEL or CON for 3 wk, starting 2 d prior to weaning. Data were analyzed for the main effects of treatment and season (4 wk periods) and their interaction. Environmental measures were also analyzed for reproductive responses. In Expt. 2a there was no effect (P>0.10) of MEL on age at 3rd estrus (203 ± 1.3 d), follicle size on Day 7 of treatment (5.0 ± 0.3 mm),

estrous cycle length (22.6 ± 0.4 d) or return from service (RE, 9.2 ± 4.0%). However, there was an effect of season (P = 0.03) on number of follicles and on gilts expressing estrus within 23 d of the previous estrus (P < 0.005). There was also no effect of MEL or season on farrowing rate (FR, 80.0 ± 4.9%) or total born pigs (TB, 13.6 ± 0.4). In Expt. 2b, there was no effect of MEL on follicle size or number, or on wean to estrus interval, but MEL (P = 0.03) reduced estrus expression within 7 d of weaning compared to CON (73.5 vs 82.0%, respectively). There was no effect of MEL on FR, but there was an effect of season (P = 0.001). Neither MEL nor season influenced TB (13 ± 1.3, P>0.10). Further, gilts and parity 1 sows exposed to low light intensity during breeding had lower conception and farrowing rates, respectively. As well, high temperatures during breeding also reduced gilt conception rates. Although there was clear evidence of seasonal fertility failures in gilts and sows in breeding and conception measures, MEL treatment did not improve fertility in mature gilts, but short-term MEL reduced and delayed estrus expression in parity 1 sows. It is possible that differences in the lighting and thermal environments for various periods of time prior to breeding, might help explain the differential response to MEL in sows and gilts.

Introduction

Seasonal infertility in swine breeding herds during summer and fall is the primary contributor for fewer pigs available for market the following summer (USDA, 2017). Delayed puberty, extended wean to estrus interval, and higher rates of anestrus reduce the numbers inseminated, and when added to increased conception failure and pregnancy loss, combine to dramatically reduce the numbers of pigs born in winter. Some of these failures have been reported in summer when daylength is longer and temperatures hotter as well as in fall when

daylength is shorter and temperatures cooler (Britt et al., 1983; Tast et al., 2002; Wegner et al., 2014). During these seasons prolonged wean to estrus interval and conception failures have been reported (Xue et al., 1994; Prunier et al., 1996; Bertoldo et al., 2011). The underlying mechanisms causing seasonal infertility in the pig have not been clearly elucidated, but high daytime temperatures, large temperature fluctuations between night and day, and shifting patterns of light and dark phases in summer and fall are linked to this syndrome (Iida and Koketsu, 2013). Seasonal decline in fertility for modern breeds of swine may be partly explained by remnants of ancestral genes from the wild pig (Tast et al., 2001a). Since the European wild boar is a seasonal breeder and fertile during short days in fall but infertile during the long days of summer, the presence of seasonally responsive genes could explain why certain breeds or genotypes show seasonal patterns more than others (Bergsma and Hermesch, 2012).

Despite advanced animal housing systems where the breeding herd is maintained in confinement buildings that control much of the duration and intensity of light while maintaining temperatures within acceptable comfort limits, seasonal effects are still reported. Data on the effects of photoperiod suggest that seasonal fertility may be controlled by the pineal gland in response to the number of days and patterns of light exposure leading to different concentrations and duration of nighttime melatonin in circulation (Vasantha, 2016). In the seasons of low fertility such as summer, long days are associated with shorter duration of nighttime melatonin release while in the seasons of highest fertility, the longest periods of nighttime melatonin release are observed. Data to support the theory of seasonality in pigs as a result of changes in lighting duration, comes from studies where gilts received oral melatonin late in the day to extend the duration of circadian melatonin exposure and which resulted in advanced onset of puberty (Diekman et al., 1991; Paterson et al., 1992a; Love et al., 1993). In-vitro data also

support the concept as melatonin added into the culture media with porcine oocytes advanced their maturation and resulted in improved numbers and quality of embryos (Shi et al., 2009b; Do et al., 2015).

Based on the available information, the objectives of these studies were to determine whether seasonal infertility in summer and fall could be alleviated in mature gilts and P1 sows by feeding melatonin once daily for 1 wk during proestrus and continuing into the 2 wk following breeding. Measures were obtained to assess effects during the follicle and luteal phases and included measures of estrus, ovarian follicles, conception, farrowing, and litter size.

Materials and Methods

The use of animals for these experiments were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign (#14081).

Animal housing

Experiments (Expt.) 2a (gilts) and 2b (parity 1 sows, P1) were performed at a 6,500 sow breed-to-wean commercial research farm in western Illinois. The farm was designed to breed and house gilts and P1 sows in individual stalls (1.24 m^2) for 30 ± 2 d after breeding. Thereafter, they were moved into static group gestation pens (56 gilts and P1 sows/ 93.9 m^2 per pen) before movement into farrowing on d 110 of gestation. The feed composition used in breeding and gestation was the same, and was a corn-soybean meal, DDGS diet, that met or exceeded the recommendations of the 2012 NRC requirements.

The replacement gilts used in this study were received from a genetic supplier once every 5 mo. and developed on farm. Gilts were held in isolation for 8 wk as newly weaned pigs (~20 d of age). Each month a group of gilts (n = 280) was moved into the 1st six pens (n = 46/ pen) in the grower. The next month, they were moved to the next set of six pens and then the next set of pens the following month. At selection at 150 d of age, gilts in different pens were mixed and moved into six pens with 46 gilts/ pen. Daily physical boar exposure was performed starting at 165 d of age. Gilts in each pen were checked for estrus once daily for 20 min using physical boar exposure with three mature boars that were 10 to 14 mo. of age. Boars were selected from a pool (n = 15) and use was rotated daily. Gilts were identified in estrus when they showed immobility to either boar mounting or the back-pressure test applied by the technician. Once they were found in estrus they were moved to another set of four pens with 56 gilts/ pen. For Expt. 2a, gilts were maintained in pens until detection of 2nd estrus and treatment assignment by week (replicate) of relocation into a stall. While in pens, gilts had ad libitum access to a grow-finish diet and water. Gilts that weighed ≥100 kg on a digital scale (TRU-TEST, Aukland, NZ) at second estrus were assigned to treatment while gilts that were less than the target weight or were lame, were not assigned. Gilt development occurred in a grow-finish facility connected to the sow farm. The animal rooms were tunnel ventilated with fully slatted concrete flooring. Following the start of the estrus detection process, and prior to detection of first estrus and their movement into stalls, gilts received daily training for the use of electronic sow feeding stations.

Experiment 2a. Mature gilts

Experimental design

This experiment was performed in 12 replicates starting in the 3rd week of Jun and continuing through the end of Sep 2015. Mature PIC Camborough® 1050 gilts (n = 420) housed in pens in a development unit on the farm were initially selected for allocation to treatment upon detection of 2nd estrus within the same wk. Assignment to treatment required a minimum body weight of 100 kg on an electronic scale when assessed on Day 14 (± 2) of the 2nd cycle when gilts were 176 to 282 d of age. Upon meeting the estrus and weight requirements (range: 100 to 160 kg) , gilts were randomly assigned by weight to Melatonin (MEL) or Control (CON, placebo) treatment and moved from their pen into individual stalls using an alternating treatment sequence. Treated gilts received 3 mg of MEL or 0 mg (CON) at 1400 to 1500 h for 21 d. The dose was administered orally in 5 mL of diluted corn syrup using a calibrated dosing gun. The treatment was targeted to begin at the start of the 7 d follicle phase, coinciding with Day 14 of the 2nd estrous cycle. The estimate for the days of the follicle and luteal phases were based on previous endocrine data (Knox et al., 2003) and on the 21 d cycle length previously reported for the gilts on this farm (Knox et al., 2016). Treatment continued for the next 14 d and was designed to cover the periods for formation of the corpora lutea, embryo development and establishment of pregnancy. Detection of estrus began on Day 14 of the 2nd cycle and continued for the next 28 d using twice daily fence line exposure to a mature boar and application of the back pressure test. On the day of estrus, gilts were inseminated once, and then at 24 h intervals each day standing. Cervical artificial insemination (AI) was performed using 3.0×10^9 sperm/dose. Two days after the last insemination, gilts were relocated to stalls in the gestation barn. Gilts were checked for return to estrus using once daily boar exposure. At 30 ± 2 d after

first insemination, trans-abdominal ultrasound was performed to detect pregnancy. Gilts confirmed pregnant were moved into static group pens with 56 gilts/pen and a single electronic sow feeding station.

Experiment 2b. Parity 1 sows

Experimental design

This experiment was performed in the same timeline for the 12 replicates as in experiment 2a. PIC Camborough® 1050 P1 sows (n = 470) that had farrowed their first litter were selected during lactation. Backfat was measured using a Lean-Meater (Renco, Golden Valley, MN) 5 ± 2 d before weaning. Sows were assigned to receive 3 mg (MEL) or 0 mg (CON, placebo) of melatonin for 21 d based on backfat (range: 8 to 13 mm) and length of lactation (range: 23 to 38 d). Treatment dosing began in farrowing 2 d before weaning and continued after relocation to stalls in breeding and gestation. Sows assigned to MEL and CON were housed in alternating farrowing crates and upon relocation, alternating breeding and gestation stalls. Sows were treated between 1400 to 1500 h using the 5 mL oral dosing method that coincided with the follicle, luteal and early gestation periods similar to that described in Expt. 2a. Estrus detection was performed twice daily and sows inseminated using post-cervical artificial insemination with 3.0×10^9 sperm at onset estrus and at 24 h intervals until no longer standing. One day after the last insemination, sows were moved into stalls in the gestation barn where they were checked once daily for return from service. Following confirmation of pregnancy on Day 30 after breeding, P1 sows were moved into the parity segregated group with 56 females/pen (P1 sows and gilts) and a single electronic feed station.

Preparation of melatonin (MEL) and control (CON) treatments

For Expt. 2a and b, melatonin was purchased from Sigma-Aldrich (≥98% pure N-Acetyl-5-methoxytryptamine) as previously reported for use in swine (Diekman et al., 1991; Paterson et al., 1992a). The stock treatment solutions were prepared each wk at 3 mg/mL MEL and 0 mg/mL CON in absolute ethanol. The oral dosing solution was prepared by diluting the stock solution 1:5 (100:400 mL) in a syrup-water solution containing 100 mL of corn syrup and 300 mL of H_20. Throughout the replicates, the oral dosing solutions were prepared once each week in volumes that enabled all animals to be treated. The stock and oral dosing solutions were stored in 500 mL bottles at room temperature until treatment dosing.

Ultrasound evaluation of the ovaries

Transrectal ultrasound was performed using an Aloka 500 V with a 7.5 MHz linear array transducer (Hitachi Aloka Medical, Ltd., Wallingford, CT) as previously described (Knox and Althouse, 1999) with continuous digital recording. Ultrasound scanning of the ovaries was performed in every other replicate for a sub-population of gilts (n = 104) and sows (n = 65) that included animals from each treatment. Transrectal ultrasound scanning of the ovaries was performed on Day 7 of treatment between 0800 and 1200 h while females were in stalls in the breeding barn. The scanning was timed to assess the ovaries near or on the expected day of estrus. After the end of the experiments, digital video playback of the recordings was used to count and measure the number and size of the three largest follicles on both ovaries. For analyses, the average size of the three largest follicles was used for classification as small (< 3 mm), medium (3 to 6.49 mm) or large (≥ 6.5 mm). Follicle number was included the total number of medium and large follicles. The follicle average size was used to determine the

percentage of females with follicles ≥5 mm, previously defined as potentially ovulatable (Soede et al., 1998; Knox et al., 2009).

Reproductive measures

For Expt. 2a and 2b, estrus measures were collected for all gilts and sows in each replicate. All gilts were included in estrus expression within 42 d of the 2nd estrus for determining estrus duration and inter-estrus interval. For sows, estrus data within 21 d following weaning was used to calculate the wean to estrus interval. For both experiments, number of services, regular and irregular returns, conception and farrowing, and litter measures for total born, born alive, stillborn and mummies were obtained.

Ambient light intensity, temperature and humidity

Weekly measures for ambient light intensity, temperature, and relative humidity (RH) were obtained at 1100 h for all replicates of experiment 2a and 10 replicates of 2b. Light intensity originated from natural and/or indoor lighting depending upon the location and phase of production. The lux (lx) level in the gilt development unit was assessed in the middle of the pens. Each pen was located close to an open curtain where natural light entered. In breeding, light intensity was measured in rooms with curtains partially open and with indoor lights on. Light intensity in the gestation barns were assessed in areas that may have had illumination from natural light originating from open curtains and indoor lighting or indoor lighting alone, depending upon the distance of the row and stall from the curtains. Measurements were obtained at gilt and sow level (~0.5 m from the floor) at three representative locations in each barn and

row in each replicate to obtain an average. Light intensity was measured using a Mini Environmental Quality Meter (Sper Scientific, Scottsdale, AZ) with an operational detection limit of 0 to 20,000 lx and accuracy of ± 5%. Temperature and RH readings were obtained using the same device with a detection range of 0 to 50 ºC (accuracy ± 0.1 ºC) and 10 to 95% RH (accuracy ± 6%). In addition, the corresponding data for average daily outdoor temperature and RH, with daily High and Low, was obtained from Weather Underground (wunderground.com, San Francisco, CA) using a location < 32 km from the farm.

Statistical Analysis

Data were analyzed using ANOVA procedures in SAS (SAS Institute Inc., Cary, NC USA). Continuous response measures were analyzed using the PROC MIXED procedure for significance of the main effects using the *F*-test and differences between least squares means identified using the *t*-test. Binary response measures were analyzed using PROC GENMOD using a binary distribution and a logit-link function. Significant main effects and differences between least squares means were identified using the x^2 test**.** The models included the main effects of treatment (MEL and CON) and season and their interaction. Season was created by combining the data from 4 consecutive weeks starting the 3rd week of June. The seasons were classified as Mid-summer (Jun-Jul), Late-summer (Jul-Aug) and Early-fall (Aug-Sept). Other variables such as lactation length, body condition score and body weight were included as covariates in the model and removed if not significant. For assessing differences in environmental measures, the models included the main effects of location (breeding or gestation) and season. Fertility data were also submitted to analysis using the environmental measures as explanatory variables. Although measures were limited in variation, higher and lower categories

with sufficient numbers of observations were created for RH (≥ or < 70%), temperature (≥ or < 25 °C) and light intensity (≥ or < 45 lx). Because there were not adequate numbers of observations when evaluating more complex models that also contained the RH, °C, and lx categories by location and season, we employed GLMSELECT to identify significant explanatory variables in a model for the fertility measures of estrus, conception, farrowing and litter size. The assumptions of the ANOVA were tested for normal distribution using PROC UNIVARIATE and Levene's test was used to test homogeneity of variance. When data could not meet the assumptions, they were transformed for analysis. Significance was identified when $P \leq 0.05$ and trends when $P>0.05$ but ≤ 0.10.

Results

Outdoor environmental measures corresponding to days of barn assessment

During both experiments, outdoor environmental measures were obtained for the same time and day as the inside measures for breeding and gestation and averaged for seasonal means. Also, average, maximum and the lowest temperature of the corresponding day were obtained for the 12 replicates (**Table 2**). The seasonal outdoor temperatures were higher in Late-summer and Early-fall and matched the indoor temperature in the gilt breeding location, but was opposite in gestation. Relative humidity in either location, did not reflect outdoor RH. For the P1 sows in breeding and gestation neither temperature nor RH reflected outdoor measures at 1100 across seasons.

Experiment 2a. Environmental assessment of the gilt housing areas

Environmental measures obtained during the study in different locations (breeding and gestation) are shown in **Table 3**. Lighting intensity in breeding did not differ across seasons and averaged 192.7 ± 77.0 lx, but in gestation, tended to be nearly 3-fold greater in Late-summer than the other seasons ($P = 0.06$). Lighting intensity was greater ($P \leq 0.05$) in breeding than gestation only in Mid-summer but did not differ in the later seasons. Temperature and RH effects were evident across seasons for breeding with higher temperature (+2.7 °C) and RH (+12.6%) in Late-summer compared to Mid-summer. However, in gestation, temperature (+2.6 °C) and RH (+7.2%) were greater in Mid-summer than Late-summer and Early fall. Temperature and RH comparisons between breeding and gestation occurred within season, and were both higher in the gestation barn compared to the breeding barn in Mid-summer, but reversed in Late-summer, when temperature and RH were greater in the breeding barns compared to gestation, but not different in Early-fall.

Experiment 2a. Gilt measures and response to treatment

Reproductive responses for gilts are shown in **Table 4**. There was no significant interaction ($P>0.10$) of treatment and season for any of the reproductive measures.

Follicle phase and estrus responses

In the sub-population of gilts assessed for ovarian responses after 7 d of treatment during the follicle phase, MEL increased ($P = 0.03$) the total number of follicles (14.6 ± 0.8) compared to CON (13.1 ± 0.8) but had no effect on average follicle size (5.0 ± 0.3 mm). The number of

follicles (P = 0.03) were different among seasons and greatest in Mid-Summer (14.9 ± 0.8), followed by Late-Summer (13.8 ± 0.8) and Early-Fall (12.8 ± 0.8). Similarly, season (P = 0.04) but not treatment (P>0.10), affected the percentage of females that had follicles ≥5 mm, indicating a lower percentage of gilts in Mid-summer had follicles of a size potentially capable of ovulation (35.6 ± 13.7%) compared to Late-summer (69.9 ± 13.7%), while gilts from Early-fall were intermediate (54.2 ± 13.7%) and not different from the other seasons. Treatment had no effect (P>0.10) on the proportion of gilts expressing estrus within 23 d of the previous estrus (72.2 ± 5.8%) and no effect on the inter-estrus interval (23.0 ± 0.7 d). However, there was an effect of season (P = 0.005) on the percentage of gilts expressing estrus within 23 d and was lowest in Mid-summer (61.5 ± 4%) compared to Late-summer (74.8 ± 4.0%) and Early-fall (80.3 ± 5%).

Conception and litter performance

There was no effect of treatment on return from service (9.2 ± 4.0%), conception rate (84.5 ± 4.3%), farrowing rate (80.0 ± 4.9%), total born pigs (13.6 ± 0.4), number born alive (12.8 ± 0.4), stillborn (0.6 ± 0.1) or mummified fetuses (0.2 ± 0.1). Season did not affect conception or farrowing rates, but there was tendency (P < 0.10) for total born pigs to be lower in Late-summer (-1 pig) when compared to Mid-summer, and number born alive to be lower in Late-summer than Mid-summer and Early-fall. For gilts detected in estrus and receiving artificial insemination, conception rate (P = 0.002), farrowing rate (P = 0.04) and total born pigs (P = 0.02) were all reduced. Inter-estrus intervals classified as short (≤ 18 d) reduced (P < 0.05) conception (-15%) and farrowing rates (- 19%) when compared to normal intervals of 19 to 23 d. In addition, when cycles were long (≥ 24 d) conception (-5%) and farrowing (-4%) were reduced

compared to gilts expressing a normal interval. In addition, conception rates were reduced in response to lower lighting intensity (-8%, P = 0.007) and higher temperature (-7%, P = 0.02) in breeding. Also, higher RH in breeding (>78%) was associated with a decrease (P = 0.009) in total born pigs (-1) compared to lower RH. In gestation, higher RH tended (P = 0.06) to be associated with reduced farrowing rate (-15%).

Experiment 2b. Environmental assessment of the Parity 1 sow housing areas

Environmental measures obtained for housing locations for P1 sows are shown in **Table 5.** Lighting intensity in breeding did not differ among seasons and averaged 39.5 ± 62.4 lx, but was different in gestation with lighting intensity ~ 100 lx lower in Mid-summer and Late-summer compared to Early-fall. Light intensity did not differ between breeding and gestation within season. Temperature (27.0 ± 1.8 °C) and RH (82.6 ± 4.6%) in breeding did not differ among seasons. However, in gestation, temperatures in Mid- and Late- summer were 4.5 °C higher than Early-fall. Gestation RH was also 6.7% higher in Late-summer compared to the other seasons ($P \leq 0.05$). Temperature differences between breeding and gestation within seasons were only evident in Early-fall, when the breeding barn was 6.4 °C higher than gestation. For RH, the breeding barn was 20.8% higher in Mid-summer and 9.6% higher in Early fall compared to gestation locations.

Experiment 2b. Parity 1 sow measures and response to treatment

Reproductive responses for gilts are shown in **Table 6**. There was no significant interaction of treatment and season for any of the reproductive measures.

Follicle phase and estrus responses

In the sub-population of P1 sows assessed for ovarian responses following 7 d of treatment during the follicle phase, there was no effect of treatment on total number of follicles (15.4 ± 1.2) nor follicle size (5.4 ± 0.3 mm). However, season did affect follicle numbers (P = 0.0002) with lower numbers (- 5) observed in Mid-summer and Early-fall compared to Late-summer. The proportion of sows with potentially ovulatable follicles ≥5 mm was not influenced by treatment or season (71.4 ± 14.7%). There was a tendency for MEL (P = 0.09) to extend wean to estrus interval (8.9 ± 2.4 d) compared to CON (7.5 ± 2.4 d), while season had no effect. The proportion of P1 sows that expressed estrus within 3 to 7 d after weaning was reduced (P = 0.03) by MEL (73.5 ± 5%) compared to CON (81.9 ± 5%). Other factors also influenced estrus responses, and sows with an average follicle size ≥5 mm on Day 7, were 11.4% more likely to display estrus within 7 d, and also presented a 2.6 d shorter wean to estrus interval. Only higher RH in the breeding barn (>75%) was associated with a 20% reduction of estrus expression within 7 d of weaning (P = 0.02).

Conception and litter performance

There was no effect of treatment on return from service (14.5 ± 4.5%), conception rate (88.3 ± 3.8%), farrowing rate (83.0 ± 4.5%), total born pigs (13.1 ± 1.3), number born alive (12.3 ± 1.2), stillborn (0.5 ± 0.3) or mummified fetuses (0.3 ± 0.3). However, season did affect fertility, and Mid-summer had the greatest impact on increasing (P = 0.0003) return from service (+13 to 17%), decreasing (P = 0.001) conception (- 8.5 to 13%) and farrowing (- 12 to 16%) rates, and tending (P = 0.07) to increase the number of stillborn pigs (+ 0.2) and mummified (+ 0.3) fetuses (P = 0.09). Late-summer also showed a lower conception (- 4.6%) and farrowing rate

(- 3.7%) when compared to Early fall. Of the environmental measures, lower light intensity (-14.9%) and higher temperature (-24.7%) in breeding were associated with a reduction (<0.01) farrowing rate.

Discussion

During periods of seasonal infertility starting in Mid-summer and following into Early-fall, oral dosing of mature gilts during the follicle, luteal, and early gestation periods indicated melatonin had only a slight effect on increasing total number of follicles, but without any measurable effect on subsequent fertility. In contrast, P1 sows treated with melatonin during the same phases, showed no effect on follicles, but did reduce and delay expression of estrus after weaning, but without other effects on fertility. It is important to note that the effects on estrus will have important downstream effects, and when evaluating treatment impact using an index of farrowing rate x live born pigs (x 100), there is a 16% reduction in live pigs produced in the melatonin treated P1 sows compared to controls. Although we could not identify melatonin interacting with any other measure, other variables such as season, follicle size, and RH, had significant effects on estrus, depending upon parity of the female. And while melatonin had no effect on subsequent fertility, conception, farrowing, and litter performance were negatively affected by season, interval to estrus, lux, temperature, and relative humidity, depending upon whether the female was a sow or gilt. Even though melatonin treatment had an unexpected negative effect on estrus expression in 8% of the weaned sows, it was intriguing that only 7 d of treatment affected this key fertility measure. These results lead us to hypothesize that illumination for gilts and sows, perhaps acting through melatonin, alone or in combination with temperature and humidity, influences fertility in summer and fall. It is also evident that

considerable variation in lighting, temperature, and RH can be observed between breeding and gestation housing areas within and across seasons. These measures can also differ for females housed by parity segregated production. It is not clear why a small percentage of P1 sows were affected by melatonin and not the majority of P1 sows and gilts. However individual metabolic state or change in body measures in lactation were not assessed, nor the ability to assess every single animal location in each replicate. Since the circadian pattern of response to a set lighting regime or melatonin treatment, may involve complex relationships with previous exposure to different lighting and intensity patterns over some unknown period of time, it would seem necessary to assess individual female measures over phases of production in order to establish lighting or the sequence as a clear factor involved with fertility in summer and fall.

Our approach for addressing seasonal infertility in the present experiments was to alter the pig's perception of seasonal photoperiod, by providing supplemental melatonin to simulate the longer nights associated with peak winter fertility (Paterson et al., 1992a; Love et al., 1993). Melatonin is a hormone derivative of the amino acid tryptophan and the neurotransmitter serotonin, and has been reported to be the key regulator of seasonal reproductive patterns in wild and domestic pigs (Tast et al., 2001a). Much of what is known about melatonin effects on seasonal fertility originates from studies in seasonal species such as the sheep, horses, and rodents (Peltier et al., 1998; Ishizuka et al., 2000; Abecia et al., 2011). Data from these species shows that light entering through the eye and stimulating the photo receptors generate a rhythm in the suprachiasmatic nucleus (SCN), transmitting it to the pineal gland to synthesize and release melatonin (Arendt, 1998; Malpaux et al., 2002). The increased amount of melatonin secretion during nocturnal periods send signals to the hypothalamus-pituitary-gonadal axis depending upon season, alter the secretion during changing photoperiod (Abecia et al., 2011;

Vasantha, 2016). In some species, increasing or decreasing melatonin synthesis works as a positive (sheep, goats and rodents) or negative (horses) feedback respectively, to stimulate GnRH and LH release from the pituitary (Vasantha, 2016). Interestingly, melatonin receptors are present in different cells including granulosa cells, oocytes, and luteal cells of the ovaries. In many cases, melatonin appears to be acting as an antioxidant for radical damage by ROS (Cruz et al., 2014). A possible reason for the negative estrus response to exogenous melatonin for P1 sows, could be related to removal of too much of the needed ROS by melatonin. Furthermore, melatonin is present in porcine follicular fluid and its concentration is directly proportional to follicle size (Shi et al., 2009b). Additionally, melatonin receptors in the pig (MTNR1A gene) is associated with litter depending upon season (Ramirez et al., 2009).

Use of exogenous melatonin to control seasonal fertility during summer and fall was shown in studies with sheep and goats which breed under short photoperiod. Thus gonadotropins secretion eventually initiates reproduction function, encouraging estrus and lambing synchronization (Paterson and Foldes, 1994; Abecia et al., 2011). In ewes, positive results from exogenous long term MEL (65 days) using implants during the anestrous period until day 5 after estrus, improved the number and viability of embryos and pregnancy rates (Vazquez et al., 2010). Also, increased number of follicular waves and rate of cleaved oocytes have been reported when treating goats with MEL implants for 40 to 45 days (Berlinguer et al., 2009). However, in other non-seasonal breeders such as rats, a negative reproductive response in estrus expression occurs when receiving daily injection of melatonin, (Wurtman et al., 1963). When given daily treatment with melatonin during periods of proestrus, ovulatory surge of gonadotropin is observed (Ying and Greep, 1973). However, melatonin given to prepubertal rats

advances puberty (Agrasal and Esquifino, 1989). These findings are interesting and mimic the results in P1 sows in the present study and for advancing puberty in gilts.

Despite the classification of the pig as a non-seasonal breeder, estrus and conception failures in gilts and P1 sows in summer (Xue et al., 1994; Peltoniemi et al., 1999; Iida and Koketsu, 2013) and early fall (Wegner et al., 2014) are reported. Additionally, changing photoperiods have been shown to influence boar and sow fertility in different seasons (Sancho et al., 2004; Chokoe and Siebrits, 2009). Problems in delayed age at first breeding in gilts has been associated with decreasing photoperiod (Iida and Koketsu, 2013), while the overall proportion of gilts reaching puberty is lower during long days compared to short days (Paterson et al., 1991). In vitro data support the concept for melatonin effects, and when added into the culture media, porcine oocytes advance maturation, and improves numbers and quality of embryos (Shi et al., 2009b; Do et al., 2015). Much of the in vivo photoperiod research of season has been performed using age of puberty as the response for the gilt. Research indicates that exogenous long term melatonin advances and improves gilt puberty during decreasing and increasing photoperiods (Diekman et al., 1991; Paterson et al., 1992b). This provides some evidence that photoperiod, is at least part of the seasonal fertility problem. Although opinions are controversial about pigs being responsive to long (Diekman and Hoagland, 1983; Diekman et al., 1992) rather than short days (Paterson and Pearce, 1990; Paterson and Foldes, 1994), it appears that the short day model would better fit the approach for providing exogenous melatonin during long days, similar to the present study. Exogenous melatonin orally administered to gilts once daily at concentrations of 1 to 5 mg resulted in increase from baseline within 10 to 20 minutes with a peak observed between 20 to 40 minutes (Diekman et al., 1991; Paterson et al., 1992b). The decline in melatonin began within 10 to 20 minutes after the peak and was still elevated above baseline at 2 to 8 hours

depending on dose and daylength pattern (Paterson et al., 1992b). This administration regiment would likely result in two peaks of melatonin. The first immediately after oral treatment administration and the second peak occurring 6 to 8 hours after lights off (Tast et al., 2001a). Although it is not clear if the double peak has a physiological implication, the method of administration that causes a double peak resulted in improvements in gilt fertility. In contrast, in untreated gilts natural melatonin peaks at 2 to 4 hours after lights off and remains elevated for the duration of darkness. Melatonin rapidly declines and reaches baseline within 2 hours after lights are turned on for gilts housed in confinement (Paterson et al., 1992a).

The results of the present study indicated that melatonin given to P1 sows starting 2 d before weaning, reduced expression of estrus in ~8.0% and tended to extend the wean to estrus interval by ~1 d. In attempting to understand why this occurred, the data indicated that while melatonin had no measurable effect on follicles in sows, it did in gilts. This was somewhat unexpected, since there was no effect of melatonin on estrus in gilts. However, the differences in follicle numbers and sizes were slight, and would not be expected to affect fertility. Therefore it is perhaps more likely, that melatonin may have had its effect as an antioxidant affecting follicle health or function, rather than gonadotropin control. This may be supported by data showing that FSH and LH measured just after MEL treatment was administered for 90 days, gilts were not affected. It is also very possible that the negative effects on estrus in the sub-population of P1 sows, was affected by the response as a result of their prior circadian programming, due prior lighting exposure in farrowing or even earlier. It may also be possible that the low lux level in breeding for some sows, housed in certain areas, was already perceived as long days, and with exogenous melatonin, we extended the nighttime pattern to simulate housing sows in the dark or with only a few hours of daylight.

Season had major effects on estrus expression in mature gilts in Expt. 2a, most notably in Mid-summer. Although there was no effect of season on estrus in P1 sows, compared to industry averages for multiparous breeding herds throughout much of the year, a 10% reduction is evident (PigCHAMP, 2016b). Gilts and P1 sows are known to have lower fertility when compared to higher parity sows, with higher rates of reproductive failure in summer (Peltoniemi et al., 1999). Reports also indicate that in higher parity sows, prolonged wean to estrus intervals are observed in summer and autumn (Britt et al., 1983; Xue et al., 1994; Knox and Zas, 2001; Dojana et al., 2017). Since mature gilts and P1 sows are still growing, perception of seasonal change or stress in some of the females could affect feed intake and metabolism, resulting in a negative energy balance in lactating sows, and only neutral energy balance for growing gilts. This could lead to decreased gonadotropin secretion and restricted follicle growth, resulting in altered estrous cycles or prolonged wean to estrus intervals (Koketsu et al., 2017). In our study, season was associated with differences in follicle number and size depending upon parity, with follicle size associated with estrus. Estrus failure or delay could be associated with smaller sized follicles, typical of sows further from estrus (Lucy et al., 2001). Although season is confounded with light intensity and photoperiod, elevated temperature alone or in combination with high humidity, have been shown to affect fertility (Bloemhof et al., 2013; Muns et al., 2016) and estrous cycles in mature gilts (Iida and Koketsu, 2013; Ross et al., 2015). However, in the present experiments, only increased humidity for P1 sows in breeding could be associated with changes in estrus.

If melatonin is hypothesized to affect conception, farrowing or litter traits, we proposed it would need to be given before breeding and into the major periods of embryo loss. But the 3 wk treatment used during these experiments had no such effects in gilts or sows. Yet season did have

negative effects on conception and farrowing rates in P1 sows. This effect is interesting, as it does confound with other factors such as lighting, temperature and RH during breeding and gestation, which in some cases, can extend into the next season. Previous studies report conception and farrowing rates are reduced in summer and early fall (Love et al., 1993; Peltoniemi et al., 1999; Tast et al., 2002; Bertoldo et al., 2011). Most studies group season into blocks of months. However, it is clear, that the months of summer and fall do not affect fertility similarly (Peltoniemi et al., 1999). Production records that examine performance by week show extensive variation, and logical groupings of weeks. In the present study, season groupings were designed to provide 4 wk data sets. However, the time period from weaning to breeding and day 35 of gestation requires 7 weeks when embryo loss is complete. Females bred in the 2nd to 4th weeks of any season would extend into the next season. In mature gilts, season affected inter-estrous interval with both short and long intervals having negative effects on farrowing and litter measures. Although the effects of abnormal interval have been previously identified, this is the first report in response to season. Another new association identified that low lux in breeding, for gilts and sows, had negative effects on either conception or farrowing rate. The negative association of low levels of light (~30 to 40 lx) in breeding is interesting, and we are not aware of other data that has reported this response (Tast et al., 2001b). Although the mechanism is unclear, it is logical to speculate that the lux effect could be on oocyte or embryo quality, since supplemental melatonin for in vitro pig oocytes and embryos improves development (Shi et al., 2009b; Do et al., 2015). Since the levels of light were quite different within and between locations for gilts and sows, the classes were not identical between experiments. Nevertheless, despite a limited range of values, low lighting in breeding reduced fertility in gilts and sows. Elevated temperature in breeding also had negative effects on conception, or farrowing rate,

depending on parity. And, there was tendency for higher RH in breeding and gestation to reduce farrowing rate and litter size (Love et al., 1993; Bertoldo et al., 2011). Previous studies have examined the effect of heat and humidity stress in breeding and gestation and it is clear that classification for high and low varies among experiments (Warnick et al., 1965; van Rensburg and Spencer, 2014; Muns et al., 2016).

Our experimental model for sows and gilts bred during summer and early fall, was designed to mimic the long nighttime pattern of release for melatonin observed in winter. The months in winter, are consistently associated with the highest levels of breeding herd fertility (Kraeling and Webel, 2015; Koketsu et al., 2017). Since melatonin is the mediator of seasonal fertility, and is synthesized and released for longer hours in winter, our approach was to dose females during the follicular and early luteal phase after breeding as a method to alleviate estrus and conception problems. The 3 mg dose has previously proved effective (Diekman et al., 1991) for increasing circulating duration of MEL above baseline for animals reaching early puberty (Diekman et al., 1991; Paterson et al., 1992b). The method of administration may not have been long enough to have the desired effect, however, daily MEL has had positive effects in pigs but continuous treatment with implants has not been successful (Paterson et al., 1992a; Paterson et al., 1992b; Paterson and Foldes, 1994; Kennaway et al., 2015).

The evidence from the current studies as well as that from several other published works, indicates that lower lux (Prunier et al., 1996), and higher temperature and humidity (Omtvedt et al., 1971; Ross et al., 2015) can reduce fertility. Further, light intensity and exogenous melatonin affect fertility responses differently in gilts and sows. We hypothesize that the different responses are likely due to previous lighting setting the neuroendocrine response to illumination and melatonin. In gilts, this could occur within the development unit while in sows, might occur

while in the farrowing room. Because of the endless number of combinations for female metabolic state, parity, stage of reproduction, light duration and intensity, temperature and humidity, and season, the probability of identifying any single or combination of factors that influence fertility become low. Perhaps an approach that could work, would be to identify the specific environmental measures for individual female starting in development and following them into the breeding and gestation up until the 2nd parity. With adequate numbers of observations and collection of body measures as well, the probability of identifying effects and interactions would improve significantly.

Conclusion

In conclusion, melatonin treatment increased number of follicles at estrus in mature gilts, but reduced expression of estrus in P1 sows. While having no effects on conception, farrowing or litter traits. Depending upon parity and season, fertility could be affected by lighting, temperature or humidity. These results identify the complex effects of season on reproductive function and fertility in gilts and P1 sows. These two parity groups comprise ~33% of the breeding herd and directly associated with herd productivity, culling, and longevity. The results of the present study extend the existing literature indicating that exogenous melatonin affects fertility, perhaps through follicle health and development to affect estrus. However, responses to melatonin treatment appear to depend upon parity and either metabolic state or prior or current exposure to lighting. Further research is needed to understand how illumination affects melatonin release, and how and where melatonin acts to impact reproductive fertility.

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Tables and figures - Experiment 2a and b

Table 2. Experiment 2a and b. Outside temperature and humidity during seasons[1]

	Season			SEM
	Mid-summer	Late-summer	Early-fall	
n[3]	4	4	4	
Temperature at 1100 h (°C)	20.8	27.1	24.2	1.9
Average temperature (°C)	22.2	23.3	22.1	1.5
Maximum temperature (°C)	26.2	28.3	27.4	1.6
Lowest temperature (°C)	17.9	17.9	16.4	1.5
Relative humidity at 1100 h (RH, %)	80.8	56.0	60.5	5.0
Average relative humidity (RH, %)	82.0	73.8	70.8	3.6
Maximum relative humidity (RH, %)	96.8	96.0	93.0	1.8
Lowest relative humidity (RH, %)	66.3	51.0	48.0	6.1

[1]Season was classified in 4 wk period starting from the 3rd week of Jun (Mid-summer), 3rd wk of Jul (Late-summer) and 3rd wk of Aug (Early-fall)
[3]N values representing number of weeks that were used to obtain the values

Table 3. Experiment 2a. Location measures of lighting intensity, temperature and humidity at 1100 h for gilts housed in breeding and gestation during seasons[1,2]

	Season			SEM	P-value Season
	Mid-summer	Late-summer	Early-fall		
n[3]	4	4	4		
Breeding light (Lx)	235.3[a]	124.4	218.3	77.0	0.49
Gestation light (Lx)	65.3[b]	206.8	55.3	82.7	0.06
Breeding temperature (°C)	22.7[ax]	25.4[ya]	24.6[xy]	0.7	0.0008
Gestation temperature (°C)	26.0[bx]	23.8[by]	23.1[y]	0.7	0.0045
Breeding relative humidity (RH, %)	68.8[ax]	81.4[ay]	74.6[xy]	2.3	<0.0001
Gestation relative humidity (RH, %)	77.1[bx]	71.1[by]	69.0[y]	2.5	0.04

[1]Season was classified in 4 wk period starting from the 3rd week of Jun (Mid-summer), 3rd wk of Jul (Late-summer) and 3rd wk of Aug (Early-fall)
[2]Location refers to measures obtained during the week of breeding and the 1st wk of gestation
[3]N values representing number of weeks that were used to obtain the values
[a-b]Within a column location means with different superscripts are different ($P \leq 0.05$)
[x-y] Within a row seasonal means with different superscripts different ($P \leq 0.05$)

Table 4. Experiment 2a. Seasonal reproductive performance of gilts orally dosed with Melatonin (MEL) or Control (CON, placebo) during the presumptive follicle phase before insemination, and then for the next 14 d of the luteal phase[1,2]

	Treatment								
	MEL			CON					
	Season								
	Mid-summer	Late-summer	Early-fall	Mid-summer	Late-summer	Early-fall	SEM	P-value Treatment[3]	P-value Season
n[4]	19	16	16	21	18	14			
Follicle size, mm	4.6	5.4	5.1	4.8	5.1	5.1	0.3	0.87	0.11
Follicle number	16.3[a]	13.9[b]	13.5[c]	13.6[a]	13.7[b]	12.1[c]	0.8	0.03	0.05
Females with follicles ≥ 5 mm, %	27.3[a]	70.6[b]	53.8[ab]	43.8[a]	69.2[b]	54.5[ab]	13.7	0.64	0.04
n	86	77	52	81	74	50			
Inter estrus interval, d	24.3[a]	22.8[b]	22.9[b]	24.9[a]	24.0[b]	22.4[b]	0.8	0.24	0.02
Estrus within 23 d, %	58.6[a]	77.2[b]	75.5[c]	64.4[a]	72.5[b]	85.0[c]	5.8	0.53	0.005
Return to estrus following A.I., %	10.1	7.6	3.6	14.6	11.7	7.7	4.0	0.16	0.26
Conception, %	83.5	84.7	89.2	82.1	81.5	86.0	4.3	0.42	0.57
Farrowing, %	81.4	73.0	86.0	75.3	80.5	83.7	4.9	0.93	0.26
Total born pigs	13.9	12.8	13.5	14.0	13.4	13.8	0.4	0.41	0.09
Number born alive	13.1	11.9	12.9	13.2	12.6	12.9	0.4	0.41	0.08
Stillborn pigs	0.6	0.7	0.4	0.6	0.6	0.6	0.1	0.72	0.76
Mummified fetuses	0.2	0.2	0.2	0.2	0.2	0.3	0.1	0.51	0.98

[1]Season was classified in 4 wk period starting the 3rd week of Jun (Mid-summer), 3rd wk of Jul (Late-summer) and 3rd wk of Aug (Early-fall)

[2]Treatment was administered at 1400 h for 21 d starting 14 d after 2nd estrus

[3]Treatment x Season not significant (P >0.10)

[4]Sub-population of gilts assessed by ultrasound on Day 7 of treatment

[a-c]Within a row seasonal means with different superscripts are different (P ≤ 0.05)

Table 5. Experiment 2b. Location measures of lighting intensity, temperature and humidity at 1100 h for Parity 1 sows housed in breeding and gestation during seasons[1,2]

	Season			SEM	P-value Season
	Mid-summer	Late-summer	Early-fall		
n[3]	4	4	2		
Breeding light (Lx)	57	31	30.5	62.4	0.77
Gestation light (Lx)	40.3^{x}	61.5^{x}	152.6^{y}	25.1	0.028
Breeding temperature (°C)	27.1	26.8	27.4^{a}	1.8	0.58
Gestation temperature (°C)	25.5^{x}	25.4^{x}	21.0^{by}	0.6	0.0002
Breeding relative humidity (RH, %)	88.1^{a}	82.5	77.1^{a}	4.6	0.51
Gestation relative humidity (RH, %)	67.3^{bx}	74.2^{y}	67.5^{bx}	1.7	0.0006

[1]Season was classified in 4 wk period starting from the 3rd week of Jun (Mid-summer), 3rd wk of Jul (Late-summer) and 3rd wk of Aug (Early-fall)

[2]Location refers to measures obtained during the week of breeding and the 1st wk of gestation

[3]N values representing number of weeks that were used to obtain the values

[a-b]Within a column location means with different superscripts are different ($P \leq 0.05$)

[x-y] Within a row seasonal means with different superscripts different ($P \leq 0.05$)

Table 6. Experiment 2b. Seasonal reproductive performance of Parity 1 sows orally dosed with Melatonin (MEL) or Control (CON, placebo) during the presumptive follicle phase before insemination, and then for the next 14 d of the luteal phase[1,2]

	Treatment								
	MEL			CON					
	Season								
	Mid-summer	Late-summer	Early-fall	Mid-summer	Late-summer	Early-fall	SEM	P-value Treatment[3]	P-value Season
n[4]	16	9	13	9	9	9			
Follicles size, mm	5.9	5.3	5.2	5.7	4.8	5.5	0.3	0.67	0.13
Follicle number	13.1	19.1	16.4	13.0	18.2	12.6	1.2	0.15	0.002
Females with follicles ≥ 5 mm, %	80	83.3	63.6	86.7	40.0	75.0	14.7	0.50	0.30
n	56	90	93	53	82	96			
Wean to estrus interval, d	9.9	8.5	8.5	7.2	7.9	7.5	2.4	0.09	0.82
Estrus within 7 days, %	72.2	71.9	76.5	76.9	82.0	86.8	4.8	0.03	0.29
Return to estrus following A.I., %	27.8[a]	11.8[b]	6.5[b]	21.5[a]	11.5[b]	8.4[b]	4.5	0.79	0.0003
Conception, %	75.9[a]	90.6[b]	95.6[c]	86.3[a]	88.5[b]	92.6[c]	3.8	0.89	0.003
Farrowing, %	72.2[a]	85.7[b]	90.2[b]	75.0[a]	86.0[b]	88.8[b]	4.5	0.95	0.001
Total born pigs	13.5	12.7	13.3	12.6	13.6	12.6	1.3	0.73	0.81
Number born alive	12.3	12.2	12.6	11.7	13.0	11.8	1.2	0.66	0.48
Stillborn pigs	0.6	0.3	0.4	0.6	0.4	0.5	0.3	0.64	0.07
Mummified fetuses	0.6	0.2	0.3	0.3	0.2	0.3	0.3	0.72	0.09

[1]Season was classified in 4 wk period starting the 3rd week of Jun (Mid-summer), 3rd wk of Jul (Late-summer) and 3rd wk of Aug (Early-fall)

[2]Treatment was administered at 1400 h for 21 d starting 2 d before weaning

[3]Treatment x Season not significant (P >0.10)

[4]Sub-population of Parity 1 sows assessed by ultrasound on Day 7 of treatment

[a-c]Within a row seasonal means with different superscripts are different (P ≤ 0.05)

CHAPTER 5 – Overall Conclusions and Future Directions

Currently the swine industry in the United Stated and worldwide faces significant losses due to seasonal infertility. Although not consistent, the impact in reproductive losses is visible in many farms during summer and fall seasons and the consequences on markets in the following year. The objectives of the literature review were to help define what is known about seasonal infertility, the pathway of control and the effects and involvement of melatonin. Both experiments 1 and 2a and b were designed to determine whether estrus, pregnancy and litter size could be improved during periods of seasonal infertility for prepubertal, mature gilts, and parity 1 sows. Our approach to use melatonin was to feed it during proestrus, estrus and during early gestation. The results of the experiments performed in pigs corroborated some of the literature that melatonin does affect reproductive responses, but in different ways. In study 1, performed in controlled rooms on a research farm, high temperatures and differing lighting schedules had no effect on reproductive responses, while exogenous melatonin tended to improve pregnancy rates. In study 2, performed on a large commercial farm, melatonin had only a negative effect on estrus in parity 1 sows, but not mature gilts. However, season and environmental lighting, temperature and humidity inside the barn were associated with important fertility measures such as follicle development, estrus, conception, farrowing, and litter size. The somewhat conflicting data seems to indicate how complex this issue is really commercial swine farms. The recognition of different fertility responses between summer and early-fall confirm that temperature and photoperiod are contributing to seasonal infertility. Yet, although undesirable results occurred it is remarkable that for only feeding melatonin a short period (1 week) during summer and fall could impact estrus expression. Melatonin treatment did not improve fertility measures in mature gilts or parity one sows. And in fact, the detrimental effects in reduction of estrus expression in parity 1

sows and a lack of an effect in gilts suggests that the melatonin response could have been determined based on prior photoperiod priming. Further investigation is still necessary to better understand how and where melatonin may act when given orally, the melatonin response based on prior lighting, and its role perhaps as an antioxidant. On commercial farms it is difficult sometimes to control temperatures and humidity, while lighting might be more simply controlled. However, it will be important to know what lighting to use in the different phases of production, and perhaps when a change in lighting should occur before a fertility event. As an alternative, melatonin treatment for as short as 1 week and as long as 3 weeks, could be associated with changes in fertility, regardless of prior lighting or temperature. The exogenous feeding of melatonin would seem more practical if the scenarios for improvement in fertility can be identified.

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