

**Birth Litter Sex Ratio Affects Gilt Behaviour, Endocrine Status and
Reproductive Performance**

by

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Abstract

This thesis tested the hypothesis that females that developed in a male biased litter would be masculinised and therefore have impaired reproductive performance and be more aggressive than those born into female biased litters. One of the aims of this thesis was to investigate new tools for selection of gilts into the breeding herd. Current selection criteria are minimal, with genetics, weight, and body conformation as the main determinants. We investigated effects of the sex ratio of a gilt's birth litter, as in other litter bearing species it affects reproduction, behaviour, and physiology. One of the mechanisms that may be involved is the transfer of androgens from male to female fetuses during development which can occur either to neighbouring fetuses or to the whole litter. Therefore, females from male biased litters may have impaired reproduction and be more aggressive due to masculinisation during fetal development. A literature review found evidence that this phenomenon may affect pigs. We defined a male biased litter having $\geq 60\%$ males (including stillborns) and female biased as $\geq 60\%$ female. We examined sex ratio effects on induction of ovulation prior to puberty, and the endocrine milieu before and during natural oestrus. We also investigated behaviour by applying anxiety and aggression tests. Phenotypic differences were also assessed by measuring the anogenital distance (AGD; distance from anus to the vulva), which is commonly elongated in females from male biased litters. Interestingly, we found, in contrast to findings from other species, that the AGD of gilts from female biased litters was longer than those from male biased litters at 16 weeks of age. The anogenital distance at 21 weeks of age was then employed in a commercial study to examine associations with gilt fertility through two parities. Gilts with longer anogenital distances reached puberty younger, were more likely to be mated, and had a higher total born alive litter size. Based on our earlier data, we suggest these gilts were likely from female biased litters. In our behavioural studies we found that gilts from male

biased litters were bolder as they had a faster emergence time in an arena test, but they also showed trends towards increased aggression with increased scratch scores around weaning and greater likelihood to fight in a resident intruder test. We also found that when stimulated with exogenous gonadotrophins at 18 weeks of age, gilts from male biased litters has a higher ovulation rate. During second oestrus, gilts from male biased litters had impaired ovulatory luteinising hormone surges but no change in tonic luteinising hormone. These results highlight that gilts from male and female biased litters have different reproductive responses. Overall, our research shows that gilts from male biased litters are different to gilts from female biased litters in reproduction, behaviour, and physiology. Currently, it appears that gilts from female biased litters, or those with above average anogenital distances, are better suited to the breeding herd but further commercial work is required to assess the effect that this new selection tool has on overall performance.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of The University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Jemma Seyfang

Date: 09/02/2018

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Chapter 1.1: Introduction

General Background

Gilt wastage is an economic and animal welfare issue for the pig industry as many gilts that are selected for the breeding herd are subfertile, with 14% of the selected gilts not reaching first lactation in ideal production systems (Foxcroft *et al.* 2010). Many of the remaining gilts go on to be non-productive; they may come into puberty late, fail to conceive, miscarry, or produce small litters (Foxcroft *et al.* 2010). They may also be more aggressive which can be cause for removal from the herd, particularly as group housing increases, and aggression can lead to serious welfare concerns as well as a loss of production. In a typical pig production system, gilts will be selected for suitability to enter the breeding herd from about 18 weeks of age. The selection criteria are based on size, genetics, conformation and, occasionally, teat number. The process of gilt selection is poor at predicting lifetime reproductive performance. The overarching hypothesis of this thesis was that females that developed in a male biased litter will be masculinised and therefore have impaired reproductive performance and be more aggressive than those born into female biased litters. In this thesis, we explore if the sex ratio of a pig's birth litter can affect lifetime reproduction and behaviour, possibly through exposure to androgens during critical periods of fetal development *in utero*.

The lifetime reproductive potential of gilts, as well as their behaviour, may be influenced *in utero* by exposure to gonadal steroids with male pigs producing predominantly androgens and oestrogens, and females predominantly producing oestrogens. *In utero* exposure of female sheep to excessive levels of androgens had negative effects on the development of the reproductive axis and tract morphology as well as behaviour centres in the brain (Veiga-Lopez *et al.* 2009). This affected lifetime reproductive performance because of masculinisation of females (Veiga-Lopez *et al.* 2009). In some litter bearing species, masculinisation of females occurs *in utero* due to male synthesis of androgens and other male-specific compounds during gonadal differentiation, or

maternal testosterone, and not solely due to post-natal socialization with males (Raeside and Sigman 1975; Clemens *et al.* 1978; Hughes 2001; Arnold 2004; Navara and Nelson 2009; Tapp *et al.* 2011). Having a high proportion of male fetuses in a litter increases the chance of a female fetus being exposed to inappropriate levels of androgens. Sex biased litters can be produced by litter bearing species such as swine due to sex biased conception, implantation, and/or fetal mortality, that alters the expected 50:50 male:female sex ratio (Clark *et al.* 1993; James 2004; Grant *et al.* 2008; Rekiel *et al.* 2012). Throughout this thesis, a male biased litter is defined as one with $\geq 60\%$ males and a female biased litter with $\geq 60\%$ females as biases of 60% or more are common in commercial facilities and this percentage would affect both the proportion of males in the litter as well as the likelihood that a female develops between two males in male biased litters.

Although exposure to androgens *in utero* can permanently alter the lifetime reproductive performance of a gilt, these *in utero* effects are currently not considered in the gilt selection criteria. This is because there are currently no physical means to assess these traits until after the gilts have been selected and have entered the breeding herd. There is limited research into the development of gilts that are exposed to inappropriately high levels of androgens through fetal transfer of steroid hormones from males. It is currently unknown what effect being born into a male biased litter has on gilt reproductive development and behaviour. If the sex ratio of the birth litter of a gilt affects behaviour and lifetime reproductive performance, it could potentially be used as a tool for gilt selection to select for less aggressive pigs that have better lifetime reproductive performance under group-housed conditions. We hypothesised that gilts from a male biased litter would have impaired reproduction and an increase in aggressive behaviour compared to gilts from female biased litters.

This thesis tested the unifying hypothesis that females that developed in a male biased litter will be masculinised and therefore have impaired reproductive performance and be more aggressive than those born into female biased litters. This was explored through the effect that the sex ratio of the litter of a gilt can have on behaviour and reproduction, due to the effects that it can have on embryology, with females from male biased litters being more likely to be exposed to excessive androgen concentrations from their male littermates during fetal development. The possible effects of male biased litters on gilt development and behaviour and reproduction are covered in more detail in the literature review, chapter 1.2.

Thesis format

Chapter 1 covers the basic background and general introduction to the topic of gilt selection and sex biased litters as well as *in utero* effects on reproduction and behaviour and also outlines the thesis format and overall aims of the thesis. It also includes a published literature review entitled “The sex ratio of a gilt’s birth litter can affect her fitness as a breeding female”.

Chapter 2 entitled “The sex ratio of a litter affects the behaviour of its female pigs until at least 16 weeks of age” is the beginning of the experimental chapters and sets the scene for determining if there are differences in gilts from male biased and female biased litters. As we did find observable differences in gilts from each bias we move on to chapter 3 to determine if there are also physiological differences in reproduction for these gilts.

Chapter 3 entitled “Response to gonadotrophins differs for gilts from female- and male-biased litters” determined that there are physiological differences between gilts from male and female biased litters and their reproductive responses differ in response to

exogenous gonadotrophins. This gave evidence that the reproductive differences between these animals needed further investigation under normal endogenous condition and therefore we move on to chapter 4.

Chapter 4 entitled “Sex bias of the birth litter affects surge but not tonic LH secretion in gilts” looks at the differences in LH, which is a vital part of the reproductive axis responsible for ovulation, and found that again there were differences between gilts from male biased and female biased litters. This left the question of; could these endocrine differences result in reproductive performance differences? This was explored in chapter 5 on a commercial farm.

Chapter 5 looks at the effect sex bias can have on production and whether or not it can be identified with a physical marker, the anogenital distance. This chapter is entitled “Anogenital distance reflects the sex ratio of a gilt’s birth litter and predicts her reproductive success” and found that gilts from a female biased litter could be identified using the anogenital distance and were more suited to the breeding herd. This concluded the experimental chapters.

Chapter 6 is the discussion which connects the five experimental chapters and discusses the outcomes and implications of the research.

Project aims

The overall hypothesis of this thesis was that gilts from male biased litters would be masculinised and, therefore, have poorer reproductive performance, and would be more aggressive, than gilts from female biased litters. This information can then assist with the creation of a new selection criteria for gilts into the breeding herd and will also determine the effect that sex ratio has on behaviour and reproduction.

The individual experiments within this thesis aimed to:

1. Determine the effect sex ratio has on behaviour.
2. Determine the effect of sex ratio on ovulation in response to exogenous gonadotrophins.
3. Determine the effect of sex ratio on luteinising hormone concentrations around ovulation.
4. Determine the effect of sex ratio on the anogenital distance.
5. Assess the use of the anogenital distance as a selection tool on a commercial farm.

Chapter 1.2: Review of the Literature:

The sex ratio of a gilt's birth litter can affect her fitness as a breeding female

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Contribution to the Paper	Researched and wrote the paper.		
Overall percentage (%)	85		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- i. the candidate's stated contribution to the publication is accurate (as detailed above);
- ii. permission is granted for the candidate to include the publication in the thesis; and
- iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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The sex ratio of a gilt's birth litter can affect her fitness as a breeding female

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Abstract. Lifetime reproductive performance and behaviour are established *in utero* driven by genetics and the steroidal milieu of the uterine environment. Developing males synthesise androgens that can be absorbed by females in the litter. Consequently, the proportion of males in a litter has the potential to affect both lifetime reproductive performance and behaviour of female littermates. Although reproductive potential is understandably important for gilt selection, behaviour is becoming progressively more important as group housing of sows increases. Aggression can be a cause for removal from the breeding herd, and can also negatively affect reproductive performance and herd longevity. This review covers existing evidence of masculinisation in rodents and sheep, the mechanism causing masculinisation, and the knowledge gaps surrounding masculinisation in pigs. Premature culling of gilts is an economic and animal welfare issue in the pork industry resulting in low retention of gilts and reduced productivity. The selection of gilts needs to be improved to incorporate both lifetime reproductive potential and behaviour. We determined that there is cause to investigate the effect of the birth litter sex ratio of a gilt on her suitability for selection into the breeding herd.

Additional keywords: behaviour, fetal development, reproduction.

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Introduction

In this paper, we present the thesis that the steroidal milieu that a gilt is exposed to during critical periods of development *in utero* has the potential to influence her lifetime reproductive performance and behaviour. We hypothesise that gilts that develop in a male-biased litter will have impaired reproduction and will be more aggressive than gilts from female-biased litters due to increased exposure to androgens *in utero*, as this is seen across a wide range of rodent and livestock species. We review the literature relating to whether the sex ratio of the birth litter of a gilt can alter this steroidal milieu and, therefore, affect lifetime reproductive performance and behaviour.

There is evidence in a range of species, including rodents and sheep, that *in utero* exposure of females to excessive androgens, either exogenously or from male litter mates, creates masculinised females, and that these females have impaired reproduction and increased aggressive behaviour (Raeside and Sigman 1975; Clemens *et al.* 1978; Hughes 2001; Arnold 2004; Navara and Nelson 2009; Tapp *et al.* 2011). There is currently little evidence in pigs of masculinisation and its causes; however, it is known that the lifetime reproductive potential of gilts, as well as their behaviour, are influenced *in utero* by several

genetic and environmental factors. One critical environmental factor is exposure to gonadal steroids, with males predominantly producing androgens and females predominantly producing oestrogens.

Having a high proportion of males in a litter increases the chance of a female fetus being exposed to high concentrations of androgens. Often, litter-bearing species, such as the pig, produce litters with >60% males (male-biased) or >60% females (female-biased). Sex biased litters can be due to sex biased conception, implantation and/or fetal mortality. Each of these factors (among others) can alter the expected 50:50 male:female sex ratio (Clark *et al.* 1993; James 2004; Grant *et al.* 2008; Rekiel *et al.* 2012).

Although exposure to androgens *in utero* could permanently alter the lifetime reproductive performance of a gilt, these effects have not been widely researched in pigs. Premature culling of gilts is an economic and animal welfare issue for the pork industry. Approximately 14% of gilts selected to enter the breeding herd are culled before they produce a litter, with others coming into puberty late, initially failing to conceive, miscarrying or producing small litters (Foxcroft *et al.* 2010). In addition to poor reproductive performance, gilts (and sows)

can be removed from the breeding herd if they are highly aggressive. As the number of breeding animals that are group housed increases, high levels of aggression can lead to welfare concerns, as well as a loss of production. A method to select gilts that provides insight into the lifetime reproductive potential of a gilt and the likelihood that they will be aggressive would be of value to the pork industry to increase productivity and minimise welfare concerns. The currently used process of selection of gilts is poor at predicting lifetime reproductive performance, and the selection criteria do not account for potential behavioural development. If the sex ratio of the birth litter of a gilt affects behaviour and lifetime reproductive performance, it could potentially be used as a tool to select for less aggressive gilts that have better lifetime reproductive performance.

Effects of androgen exposure *in utero* on fetal development

Masculinisation of female mammals has been shown to occur *in utero* due to synthesis of androgens by male fetuses, maternal androgens or both (Raeside and Sigman 1975; Clemens *et al.* 1978; Hughes 2001; Arnold 2004; Navara and Nelson 2009; Tapp *et al.* 2011). Research in mammals, such as rats, mice, rabbits and sheep, concluded that androgens from developing male fetuses can pass through fetal membranes and masculinise females in the litter. This exposure to androgens could have been due to either the proximity of males to females *in utero* and/or the proportion of males compared with females (Raeside and Sigman 1975; Clemens *et al.* 1978; Hughes 2001; Arnold 2004; Navara and Nelson 2009; Tapp *et al.* 2011). This masculinisation has been shown to affect reproduction, behaviour and phenotype. Females are exposed to some androgens *in utero* and this is normal. However, when females are exposed to excessive androgen concentrations *in utero* due to either exogenous treatment or male littermates, many aspects of their physiology can be masculinised. Female rodents and sheep exposed to such conditions showed a hypothalamo–pituitary–gonadal (HPG) axis that was masculinised, as were aspects of behaviour that increase aggression. These changes have the potential to permanently affect the reproductive potential of an animal and its suitability for the breeding herd. Masculinisation affected neural pathways during development and altered responsiveness to steroid hormones, which resulted in a change in reproduction and behaviour (Ryan and Vandenberg 2002; Correa *et al.* 2013). Research has shown that masculinisation can create non-genetic variation, as well as diversity in reproductive behaviour and timing, and length of oestrous cycles in individuals within a litter (vom Saal 1989a; Uller *et al.* 2005). Masculinisation is an important trait for adaptation, but has negative implications in commercial settings where there is a desire for reproductive and behavioural uniformity.

Reproductive, behavioural and phenotypic traits of female offspring were affected by their proximity to males *in utero*, and this is likely due to transfer of androgens from male littermates (vom Saal 1989b; Parfet *et al.* 1990; Jubilan and Nyby 1992; Clark *et al.* 1993; Hernández-Tristán *et al.* 1999; Ryan and Vandenberg 2002; Nagao *et al.* 2004; Bánszegi *et al.* 2009). Intrauterine position refers to the location of the female in the uterus in relation to its male littermates, and the effect of

intrauterine position would be independent of litter size or litter sex ratio. In mice, it has been demonstrated that female pups with two male neighbours *in utero* were exposed to greater concentrations of testosterone than females that had no male neighbours (McDermott *et al.* 1978; Rines and vom Saal 1984; vom Saal 1989b; Ryan and Vandenberg 2002). Intrauterine position in relation to males permanently altered endocrine pathways, hormone levels and sensitivity to hormones, reproductive phenotypes, behaviour, and sex ratios of offspring. Although testosterone levels increase in masculinised females *in utero*, there was no difference in baseline testosterone concentrations in the plasma of females from various intrauterine positions after birth in rodents (Ryan and Vandenberg 2002; Correa *et al.* 2013). This suggests that the effects of exposure to high concentrations of androgens *in utero* have permanent organisational effects and therefore testosterone levels need not be maintained at an increased concentration after birth. The intrauterine position creates non-genetic variation in the future sex ratios and physiology of offspring. Female mice that had no male neighbours *in utero* were more likely to have 60% female litters, whereas females that developed next to one male produced a 50% sex ratio, and masculinised females were most likely to have 40% female offspring (Ryan and Vandenberg 2002; Rekiel *et al.* 2012). The sex ratio of the litter *in utero* can also affect fetal development through the proportion of males to females and the overall androgen concentration *in utero*. If androgens can affect the entire litter in some species, this could have negative implications for all females in that litter, particularly if the litter is male-biased. The mechanism of sex ratio deviations is still not well understood (Meikle *et al.* 1996) and requires further research.

Evidence of masculinisation includes changes in the anogenital distance, which is the distance between the anus and the genitals. Masculinised female mice with an increased anogenital distance had a shorter fertile lifespan, produced smaller and fewer litters, and these litters were also more likely to be male-biased (vom Saal *et al.* 1999; Bánszegi *et al.* 2012). In contrast, female mice with shorter anogenital distances were more likely to be mated and have more pregnancies than females with longer anogenital distance (vom Saal and Bronson 1978). This highlights that the anogenital distance can be reflective of reproductive potential in some species.

Reducing aggression can be difficult to achieve, as animals have individual temperaments, and varied levels of innate anxiety and aggression (Erhard *et al.* 1997). Although some correlations between weight and aggression have been found, the causes of individual aggressiveness in pigs are still unknown. Territory, limited resources and the size of the opponent are likely factors affecting aggression. However, the likelihood of an animal to be aggressive in these situations has been shown to be driven by their innate aggressiveness (D'Eath and Lawrence 2004). In rodents, there is evidence for genetic as well as developmental factors contributing to aggression (Mendel and Paul 1991; Erhard *et al.* 1997). For example, prenatal testosterone was positively correlated with aggression in adult male rats (Cruz and Pereira 2012), and increased exposure to sex steroids *in utero* caused aggressive female mice in male-biased litters (vom Saal 1989b; Parfet *et al.* 1990). It has been suggested that behavioural development is likely to occur during times

of rapid growth of the brain (Pond *et al.* 2000; D'Eath and Lawrence 2004). During development, androgens have been shown to modify the growth of the nervous system and hypothalamo–pituitary–adrenal axis (Broom 1981). Early life experiences affected behaviour; however, this is mediated by brain development that had already begun *in utero*. Masculinised female mice had heightened aggressive behaviour, particularly towards other females (vom Saal and Bronson 1978; vom Saal *et al.* 1999), and exhibited mounting behaviours throughout their lives (Rines and vom Saal 1984; vom Saal *et al.* 1999; Ryan and Vandenberg 2002). These masculinised females were less likely to show avoidance behaviours than non-masculinised females, potentially indicating lower anxiety levels (Ryan and Vandenberg 2002). The sex ratio of offspring may also affect behaviour through the rearing environment due to competition between siblings (Mendl and Paul 1991). Male mice reared with females were faster to attack than males reared with only males, indicating an effect of sex ratio during rearing on behaviour (Mendl and Paul 1991). In addition, litter size can affect behaviour, with more aggression seen in large litters, presumably due to competition for resources (Mendl and Paul 1991). Behavioural variation is important for formation of social hierarchies and survival in the wild, but can create problems for commercial livestock systems.

Impact of *in utero* androgen exposure on the hypothalamo–pituitary–ovarian axis

Testosterone negatively regulates the synthesis and secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus in males (Tilbrook and Clarke 2001). The steroid hormones are relayed back to the GnRH neurons via kisspeptin/neurokinin B/dynorphin (KNDy) neurons. The KNDy neurons co-express three neuropeptides: kisspeptin, neurokinin B and dynorphin, which were first identified in 2010 and are now accepted as the central regulator of GnRH production (Goodman *et al.* 2007; Navarro *et al.* 2009; Lehman *et al.* 2010; Wakabayashi *et al.* 2010; Merkley 2013). Male sheep have half the number of KNDy cells of females, and there are also fewer KNDy cells in female sheep treated with testosterone *in utero* (Cheng *et al.* 2010). This sexual dimorphism in the number of KNDy cells occurs *in utero* and is mediated by fetal androgen synthesis, with prenatal testosterone exposure resulting in fewer KNDy cells (Cheng *et al.* 2010; Lehman *et al.* 2010). Prenatal testosterone exposure of females leads to a decline in dynorphin and neurokinin B cells, but does not affect kisspeptin cells (Cheng *et al.* 2010). Although this still leads to a decline in the function of the HPG axis through a decreased ability of progesterone to inhibit GnRH, it also suggests that prenatal testosterone is not the only cause of sexual dimorphism, and that there may be different critical periods of development for the different peptides (Cheng *et al.* 2010). Prenatal testosterone exposure only partially mimicked sex differences in KNDy cell populations (Cheng *et al.* 2010). In sheep, prenatal testosterone disrupted female programming of the neuroendocrine system and reproductive axis due to its androgenic actions (Veiga-Lopez *et al.* 2009). Exposure to prenatal androgens also defeminised the oestradiol negative feedback mechanism that controls the tonic secretion of luteinising hormone in sheep (Jackson *et al.* 2008). Prenatal

testosterone disrupted oestrogen negative feedback, but not positive feedback, by reducing hypothalamic responsiveness to the negative feedback signal (Veiga-Lopez *et al.* 2009). In contrast, exposure of rats to testosterone *in utero* attenuated the activity of the HPG axis by suppressing the positive feedback mechanism, and over time it became progressively less responsive to circulating gonadotrophins (Gray and Wexler 1980). This difference in effect on positive compared with negative control may be due to the positive feedback centre not being programmed by testosterone directly, but rather, by the testosterone being aromatised to oestrogen (Veiga-Lopez *et al.* 2009). This induces changes in the dynamics of the luteinising hormone surge, causing a delay between the oestrogen increase and luteinising hormone surge, suggesting that the neural pathway that regulates the surge is compromised in androgenised female sheep (Veiga-Lopez *et al.* 2009).

There is no specific behavioural section of the brain, but rather, behaviours are regulated by many neural pathways throughout the brain and specific mechanisms within the brain (Broom 1981). The hypothalamus is thought to be important in regulating behaviour, as behaviour is associated with autonomic and endocrine responses (Roeling *et al.* 1994). A more comprehensive understanding of aggressive behaviour and its causes could provide the basis to reduce aggression and to identify animals that are likely to become aggressive. Androgens have organisational effects on the male neural circuit during fetal development, and later in life testosterone stimulates this circuit to elicit aggressive behaviour (Carlson 1998). The hypothalamo–pituitary–adrenal axis also regulates aggression, and individuals with low levels of glucocorticoids in response to stress have been shown to be more likely to be aggressive (Carlson 1998). The fetal development of the brain affects behaviour throughout life and therefore needs to be better understood.

Factors controlling the sex ratio of litters

Although Fisher's theory predicts that the sex ratio of a litter should be ~50 : 50, the Trivers–Willard hypothesis suggests that mothers can alter the sex ratio of their offspring, which can improve their chances of survival (Trivers and Willard 1973). As previously mentioned, litter-bearing species often produce litters that are >60% male. In general, it has been shown that females in good body condition are more likely to produce males, whereas females in poor condition are more likely to produce females (Trivers and Willard 1973; Sheldon and West 2004). Control of the sex ratio of offspring resulting from maternal body condition is thought to be possible through different mechanisms, such as selective fertilisation and selective mortality *in utero* (James 1996, 2004). All of these factors may contribute to the sex, phenotype and survival of the offspring through prenatal programming.

It is apparent that parental hormone levels at the time of mating can affect the sex ratio of the litter (James 2004). Hormone levels are influenced by the environment to create diversity in sex ratios. The sex of the offspring at conception is controlled by males, as the spermatozoa carry the X or Y chromosome (James 1996, 2004). Nonetheless, there is a level of maternal control of sex determination, with the maternal

hormone levels at time of conception influencing the probability that a particular X- or Y-bearing spermatozoon will fertilise a particular oocyte (James 1996, 2004; Grant and Irwin 2005). High testosterone or oestrogen in mothers at breeding increased the chances of conceiving a male, whereas elevated progesterone levels increased the chances of a female (James 2004). In humans, it has been shown that males are more likely to be conceived when fertilisation happens early or late in the fertile period as this is when testosterone and oestrogens are high in the mother (James 2004). In contrast, female conception is more likely in the middle of the fertile period when gonadotrophin concentrations are highest (James 2004). Oocytes originating from follicles having high levels of testosterone in the follicular fluid were more likely to be fertilised by Y chromosome-bearing spermatozoa, whereas progesterone in the follicular fluid was more likely to result in fertilisation by X-bearing spermatozoa (Grant and Irwin 2005; Grant *et al.* 2008). Therefore, maternal hormones can influence the sex bias of litters.

The social status of an animal can affect the sex of their offspring to benefit the survival of the parent and the offspring. In many species, subordinate females have been shown to be more likely to benefit from having female offspring, because they stay with the mother in the wild as opposed to males that disperse, and dominant females have been found to be more likely to conceive males than females (Grant 2007; Hohenbrink and Meinecke-Tillmann 2012). Dominant animals had better access to feed and improved nutrition, which enabled them to produce male-biased litters with high birthweights (Braastad 1998). In mammals, having *ad libitum* access to food in gestation or feeding a highly nutritious diet, a high-fat diet, or having ample body reserves leads to an increase in the number of males born (Navara and Nelson 2009). Dominant females produced more testosterone and had higher testosterone concentrations in maturing ovarian follicles, making the oocyte more susceptible to fertilisation by Y chromosome-bearing spermatozoa (Grant 2007; Navara and Nelson 2009). The social status of an animal can affect the sex of their offspring through nutrient availability, and benefits the survival of the parent and offspring for the current environmental conditions.

The loss of embryos or fetuses of one sex can also account for skewed sex ratios in litters that would otherwise have a 50 : 50 sex ratio. Compared with females, males are more susceptible to fetal mortality due to stress of the mother during gestation (Lane and Hyde 1973; Pratt and Lisk 1989; James 1996; Braastad 1998; Navara and Nelson 2009). Sex specific embryo mortality has also been observed in mice, hamsters and rabbits, with an increase in testosterone leading to an increase in female mortality, resulting in small male-biased litters (Pratt and Lisk 1989; Krackow 1992; Bánszegi *et al.* 2012). Subordinate female hamsters produced smaller litters than dominant females, and these litters had fewer males, but no difference in the number of females produced, adding evidence to the argument that males are more likely to be produced by dominant females (Pratt and Lisk 1989). As stress and maternal social status are linked with the sex of offspring, management strategies to utilise these effects would be an economically viable way of impacting the sex of offspring (Hohenbrink and Meinecke-Tillmann 2012). In addition, the Trivers–Willard hypothesis states that in an environment with limited resources, females will produce

more females. Therefore, it follows that the male fetuses are less robust and more sensitive to environmental disturbance. Consequently, sex specific embryo mortality can occur and affect the overall sex ratio of the litter.

Litter sex bias, and pig behaviour and reproduction

The causes and effects of masculinisation of females have not been rigorously investigated in pigs, despite the extensive research in other species. It is unknown if exposure of fetal pigs to high concentrations of androgens will have similar detrimental effects on reproductive and behavioural processes, as observed in females of other species. However, given the relative consistency across species of physiological and behavioural impacts on offspring exposed to high concentrations of androgens *in utero*, it is likely that the effects would be similar in pigs. If exposure of female pigs to high concentrations of androgens *in utero* results in impaired reproduction and increased aggression, there would be significant implications for management of these animals. Understanding the processes and effects would allow the development of means to improve production. For example, the gilt selection process could be improved by selecting against females that have been exposed to excessive androgens *in utero*, reducing premature culling of gilts.

Pigs often produce skewed sex ratio litters favouring one sex over the other. Meikle *et al.* (1996) reported that high ranking sows were more likely to give birth to a greater proportion of males than low ranking sows. However, Mendl *et al.* (1995) did not find a clear relationship in pigs between social status, nutritional status and sex ratio at birth. There are conflicting results around dominance and the sex ratio of offspring in pigs, as high ranked sows were found to wean more live females, but with no effect on sex ratio born (Mack *et al.* 2014). This could be due to different measures of stress and dominance across studies. When pregnant sows were stressed, male fetuses were more susceptible to mortality, resulting in a higher proportion of females born (Ryan and Vandenberg 2002). Mack *et al.* (2014) found that male piglets were also more likely to have disturbed growth and higher pre-weaning mortality after maternal stress in gestation. This is consistent with the findings in other species, and highlights that the sex ratio of offspring can be altered at conception or throughout gestation with sex specific fetal losses.

In contrast to the known effects of androgens on puberty in mice, the effect of excessive androgen exposure on the onset of puberty in pigs is unclear. Drickamer *et al.* (1997) reported failure to conceive and delayed puberty in gilts born into litters with $\geq 67\%$ males. Data are conflicting for age of puberty in gilts, with one other study demonstrating masculinised females exhibiting delayed puberty (Ford and Christenson 1987), and another finding no effect (Parfet *et al.* 1990). Nonetheless, the effect of sex ratio has not yet been adequately assessed. Reaching puberty at a relatively uniform age is important to commercial pig farming and, therefore, we need a better understanding of what controls time to puberty.

Sows originating from female-biased litters have the potential for enhanced reproduction, as they have been shown to have larger litters than sows from male-biased litters (Edgerton and Cromwell 1987). Rekiel *et al.* (2012) also found that sows from female-biased litters tended to farrow and wean larger litters

than sows from male-biased litters, and these larger litters tended to have a greater proportion of females. Increased pigs weaned can also result from improved teat availability. The number of teats on gilts can be affected by both the number of teats on the mother and the proportion of males in the litter, with gilts born in female-biased litters having a higher number of teats than those in male-biased litters (Drickamer *et al.* 1999a), which is consistent with other litter-bearing species, such as rabbits and mice (Ryan and Vandenberg 2002; Hotchkiss *et al.* 2007; Bánszegi *et al.* 2010). This was due to testosterone *in utero* suppressing the development of mammary tissue (Kratochwil 1971). Therefore, males *in utero* can contribute to the physiology of their female littermates by altering the number of teats, thereby affecting her nursing capacity. If the sex ratio of the birth litter can affect future reproduction it could be used as a selection tool, but it is unknown if gilts from female-biased litters are better suited to the breeding herd than those from male-biased litters.

Aggression in pigs can adversely impact welfare and impair immunity, damage carcasses and can result in poor growth (Erhard *et al.* 1997; D'Eath 2002). As behavioural development occurs during the period of rapid brain growth, this would be the last 3 weeks of gestation and the first 2 weeks of postnatal life for pigs, highlighting the potential for both *in utero* effects on behaviour and early life experiences (Pond *et al.* 2000; D'Eath and Lawrence 2004). D'Eath (2002) highlighted that weight predicated success in fights, and that aggressive pigs persisted in showing aggressive behaviour. Drickamer *et al.* (1999b) found a positive relationship in pigs between the dominance status and level of aggression of a mother and her female progeny's social dominance. Aggressive pigs are also likely to have come from large litters in response to competition for nutrients in early life (D'Eath and Lawrence 2004). Piglet behaviour, such as aggression, can also be affected by early life experiences as the brain continues developing; for example, social isolation at an early age caused behavioural changes mediated by the neuroendocrine system (Kanitz *et al.* 2009). Nevertheless, the effect that the early life experience had on the young animal is guided by pathways established *in utero* (Stiles and Jernigan 2010). Exposure to testosterone prenatally may impair teat seeking ability, as masculinised females spent more time on agnostic behaviours rather than teat seeking, highlighting a sex difference in behaviour from birth (Bate *et al.* 1985). Based on limited data, social dominance in pigs does not appear to be predicted by anogenital distance or birth litter sex ratio (Drickamer *et al.* 1999b). Although it is known that masculinised females are more aggressive than non-masculinised females, it is unknown what effect this has in production systems that house pigs in groups. There is potential to decrease aggression in these group housing systems by selecting for less aggressive sows, which could be done by selecting against masculinised pigs for the breeding herd. Future research should aim to determine if selecting against masculinised females reduces aggression in group housing systems.

The effect of intrauterine position and/or sex ratio on anogenital distance in pigs is unclear, with conflicting results suggesting that sex ratio may or may not affect anogenital distance depending on whether fetal pigs are exposed to the steroidal milieu of every littermate rather than just the neighbouring fetus

(Parfet *et al.* 1990; Drickamer *et al.* 1999b; Ryan and Vandenberg 2002). Female pigs from male-biased litters had increased anogenital distances, but it is unknown if this was due to the intrauterine position effect or the sex ratio *per se*, as a high proportion of males increases the chances of females developing between two males (vom Saal *et al.* 1999). Gilts from male-biased litters were shown to have a masculinised phenotype with an increased anogenital distance, and were less likely to conceive to their first mating than gilts from female-biased litters (Drickamer *et al.* 1997). Feminisation of males, as indicated by a reduced anogenital distance, was linked to the number of females in a litter (Mack *et al.* 2014). The anogenital distance could be used as a marker of fertility if it is associated with masculinisation of female pigs that would then affect their reproduction. Again, there is a need for research to establish if this is the case.

The immunology of females can be altered through masculinisation. Masculinised female pigs show lower levels of thymosin β_4 than feminised females, suggesting varying thymic function and, therefore, potentially altered immunological function of masculinised females (Wise and Christenson 1992). The metabolism of selected toxins can also be altered by intrauterine position in rats (Ryan and Vandenberg 2002). Both factors could result in different susceptibilities to stress and disease between gilts with different intrauterine positions or sex ratios of their birth litter (Ryan and Vandenberg 2002).

There are limited data for pigs on the mechanisms responsible for the altered development of females in male-biased litters (Drickamer *et al.* 1997). It is currently unknown if the changes in fetal pigs occur in early development during the synthesis of testosterone by the fetal testes or if they occur later in development, from days 75 to 100 (Wise and Christenson 1992; Drickamer *et al.* 1997). Gilts exposed to exogenous testosterone in early fetal development were less likely to achieve puberty by 250 days of age, and had significantly lighter ovaries and uterus than did gilts not exposed to testosterone or that were treated later in gestation (Elsaesser and Parvizi 1979). Male pig fetuses reached a peak of testosterone production between 40–70 days post-conception (Colenbrander *et al.* 1978; Elsaesser and Parvizi 1979), and exposure of female pigs to testosterone in early fetal development (day 30–70 post-conception) significantly impaired the oestrogen feedback mechanism (Elsaesser and Parvizi 1979). Exposure to testosterone during late fetal development (90–106 days of fetal life) did not impair the oestrogen feedback mechanism, suggesting that the critical period of fetal development for the effects of testosterone on the female HPG axis is before day 90 (Elsaesser and Parvizi 1979). In pigs, there was transfer of fetal cells between siblings with male DNA being found in female fetuses and in the maternal circulation, highlighting the potential for transfer between siblings *in utero* (Karniyuchuk *et al.* 2012). Female DNA can also be found in male fetuses, which is most likely from both the female fetuses and the mother (Karniyuchuk *et al.* 2012). Fetal pigs have individual fetal membranes, and there are two extremities on these membranes that appear to connect to neighbouring fetuses from approximately day 39 of gestation providing a possible route for cellular transfer (Karniyuchuk *et al.* 2012). Androgens can cross fetal membranes and be absorbed by neighbouring fetuses, whereas placentae have

a role in regulating androgen metabolism to limit steroid transfer. Nevertheless, in male-biased litters or when testosterone levels are unusually high, the placenta may not be able to block all of the testosterone from crossing the fetal membrane and getting absorbed by the other fetuses in the litter (Hotchkiss *et al.* 2007). The sex ratio of the birth litter appears to be the most relevant cause of masculinisation of female pigs *in utero*. Sows derived from female-biased litters had better reproduction in terms of having more inseminations and less failure of pregnancies than sows derived from male-biased females (Drickamer *et al.* 1997). This is consistent with findings in rabbits (Bánszegi *et al.* 2010). Rekiel *et al.* (2012) suggest that the sex ratio of the litters should be considered when selecting gilts for the breeding herd as a way to improve herd fertility. In pigs, it is unknown whether androgens can be transferred throughout the litter or just to and from neighbouring fetuses. Although the intrauterine position via Caesarean section gives the most detailed information about the fetal development, it is only required if androgens are transferred solely via fetal membranes to neighbouring fetuses. Where androgens are able to circulate via the placentae or other means, then it is the total proportion of males that can affect prenatal androgen exposure, and hence the sex ratio would be more relevant than individual intrauterine position. Research into the mechanism of androgen transfer *in utero* will determine if it is the sex ratio of the litter or the individual intrauterine position that affects gilt masculinisation. Using the sex ratio of the birth litter to select gilts could improve fertility through female-biased selection. More conclusive research of the mechanisms responsible is needed to better understand fetal development and how masculinisation occurs *in utero* in pigs.

There is a need to select better breeders to increase gilt retention and productivity, as well as to improve uniformity in the onset of puberty, the length of the oestrous cycle and limit aggressive behaviour. If masculinised gilts are more aggressive and have poorer reproductive performance, it would be appropriate to select against these females. This may be achieved by selecting breeding stock from female-biased litters or from determining if anogenital distance is indicative of exposure to androgens *in utero*. If anogenital distance is a marker of masculinisation *in utero*, it could be a useful marker for lifetime reproductive performance and aggression. Anogenital distance and/or the sex ratio of the birth litter of a gilt may become a selection tool for gilts that can enhance the ability to predict lifetime reproductive performance and account for potential aggressive behavioural development. This would result in more efficient selection of breeding females, increased breeding efficiency and less aggressive pigs in the breeding herd, with the net benefit of improved production and increased gilt retention.

Conclusion

There is substantial evidence in other litter-bearing species to support the hypothesis that gilts that develop in a male-biased litter will have impaired reproduction and be more aggressive than gilts from female-biased litters due to increased exposure to androgen *in utero*. Masculinisation of females that results from exposure to high concentrations of androgens *in utero* can alter the function of the HPG axis and influence behaviour,

particularly aggression, while altering the morphology of the reproductive tract. The steroidal milieu of the prenatal environment, particularly the concentration of oestrogens and androgens that the developing fetus is exposed to, has potential to alter lifetime reproductive performance. Furthermore, it is likely that the sex ratio of the birth litter of a gilt will alter the concentration of oestrogens and androgens *in utero*. Future research in this area should aim to determine if the lifetime reproductive performance and behaviour of a gilt is altered by the sex ratio of its birth litter or its intrauterine position, and if anogenital distance is indicative of androgen exposure *in utero*.

Conflicts of interest

The authors declare no conflicts of interest.

References

- Arnold AP (2004) Sex chromosomes and brain gender. *Nature Reviews Neuroscience* **5**(9), 701–708. doi:10.1038/nrn1494
- Bánszegi O, Altbácker V, Bilkó Á (2009) Intrauterine position influences anatomy and behavior in domestic rabbits. *Physiology & Behavior* **98**(3), 258–262. doi:10.1016/j.physbeh.2009.05.016
- Bánszegi O, Altbácker V, Dúcs A, Bilkó Á (2010) Testosterone treatment of pregnant rabbits affects sexual development of their daughters. *Physiology & Behavior* **101**(4), 422–427. doi:10.1016/j.physbeh.2010.07.020
- Bánszegi O, Szenczi P, Dombay K, Bilkó Á, Altbácker V (2012) Anogenital distance as a predictor of attractiveness, litter size and sex ratio of rabbit does. *Physiology & Behavior* **105**(5), 1226–1230. doi:10.1016/j.physbeh.2012.01.002
- Bate LA, Hacker R, Kreukniet M (1985) The relationship between serum testosterone levels, sex and teat-seeking ability of newborn piglets. *Canadian Journal of Animal Science* **65**(3), 627–630. doi:10.4141/cjas85-074
- Braastad BO (1998) Effects of prenatal stress on behaviour of offspring of laboratory and farmed mammals. *Applied Animal Behaviour Science* **61**(2), 159–180. doi:10.1016/S0168-1591(98)00188-9
- Broom DM (1981) 'Biology of behaviour.' (Cambridge University Press: Cambridge)
- Carlson NR (1998) 'Physiology of behaviour.' (Pearson: Boston, MA)
- Cheng G, Coolen LM, Padmanabhan V, Goodman RL, Lehman MN (2010) The kisspeptin/neurokinin B/dynorphin (KNDy) cell population of the arcuate nucleus: sex differences and effects of prenatal testosterone in sheep. *Endocrinology* **151**(1), 301–311. doi:10.1210/en.2009-0541
- Clark MM, Karpiuk P, Galef BG (1993) Hormonally mediated inheritance of acquired characteristics in Mongolian gerbils. *Nature* **364**(6439), 712. doi:10.1038/364712a0
- Clemens LG, Gladue BA, Coniglio LP (1978) Prenatal endogenous androgenic influences on masculine sexual behavior and genital morphology in male and female rats. *Hormones and Behavior* **10**(1), 40–53. doi:10.1016/0018-506X(78)90023-5
- Colenbrander B, De Jong FH, Wensing CJ (1978) Changes in serum testosterone concentrations in the male pig during development. *Journal of Reproduction and Fertility* **53**(2), 377–380. doi:10.1530/jrf.0.0530377
- Correa LA, Frugone MJ, Soto-Gamboa M (2013) Social dominance and behavioral consequences of intrauterine position in female groups of the social rodent *Octodon degus*. *Physiology & Behavior* **119**, 161–167. doi:10.1016/j.physbeh.2013.06.002
- Cruz CD, Pereira OC (2012) Prenatal testosterone supplementation alters puberty onset, aggressive behavior, and partner preference in adult male rats. *The Journal of Physiological Sciences: JPS* **62**(2), 123–131. doi:10.1007/s12576-011-0190-7

- D'Eath RB (2002) Individual aggressiveness measured in a resident-intruder test predicts the persistence of aggressive behaviour and weight gain of young pigs after mixing. *Applied Animal Behaviour Science* **77**(4), 267–283. doi:10.1016/S0168-1591(02)00077-1
- D'Eath RB, Lawrence AB (2004) Early life predictors of the development of aggressive behaviour in the domestic pig. *Animal Behaviour* **67**(3), 501–509. doi:10.1016/j.anbehav.2003.06.010
- Drickamer LC, Arthur RD, Rosenthal TL (1997) Conception failure in swine: importance of the sex ratio of a female's birth litter and tests of other factors. *Journal of Animal Science* **75**(8), 2192–2196. doi:10.2527/1997.7582192x
- Drickamer LC, Rosenthal TL, Arthur RD (1999a) Factors affecting the number of teats in pigs. *Journal of Reproduction and Fertility* **115**(1), 97–100. doi:10.1530/jrf.0.1150097
- Drickamer LC, Arthur RD, Rosenthal TL (1999b) Predictors of social dominance and aggression in gilts. *Applied Animal Behaviour Science* **63**(2), 121–129. doi:10.1016/S0168-1591(99)00005-2
- Edgerton LA, Cromwell GL (1987) Sex of siblings may influence reproductive performance in sows. *Journal of Animal Science* **63**(1), 365.
- Elsaesser F, Parvizi N (1979) Estrogen feedback in the pig: sexual differentiation and the effect of prenatal testosterone treatment. *Biology of Reproduction* **20**(5), 1187–1193. doi:10.1095/biolreprod20.5.1187
- Erhard HW, Mendl M, Ashley DD (1997) Individual aggressiveness of pigs can be measured and used to reduce aggression after mixing. *Applied Animal Behaviour Science* **54**(2–3), 137–151. doi:10.1016/S0168-1591(97)00068-3
- Ford JJ, Christenson RK (1987) Influences of pre- and postnatal testosterone treatment on defeminization of sexual receptivity in pigs. *Biology of Reproduction* **36**(3), 581–587. doi:10.1095/biolreprod36.3.581
- Foxcroft G, Patterson J, Dyck M (2010) Improving production efficiency in a competitive industry. In '24th Manitoba swine seminar. Sharing ideas and information for efficient pork production', 3–4 February 2010, Manitoba, Canada. pp. 81–98. (Manitoba Pork Council: Winnipeg, Manitoba, Canada)
- Goodman RL, Lehman MN, Smith JT, Coolen LM, De Oliveira CV, Jafarzadehshirazi MR, Pereira A, Iqbal J, Caraty A, Ciofi P, Clarke IJ (2007) Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. *Endocrinology* **148**(12), 5752–5760. doi:10.1210/en.2007-0961
- Grant VJ (2007) Could maternal testosterone levels govern mammalian sex ratio deviations? *Journal of Theoretical Biology* **246**(4), 708–719. doi:10.1016/j.jtbi.2007.02.005
- Grant VJ, Irwin RJ (2005) Follicular fluid steroid levels and subsequent sex of bovine embryos. *Journal of Experimental Zoology. Part A, Comparative Experimental Biology* **303A**(12), 1120–1125. doi:10.1002/jez.a.233
- Grant VJ, Irwin RJ, Standley NT, Shelling AN, Chamley LW (2008) Sex of bovine embryos may be related to mothers' preovulatory follicular testosterone. *Biology of Reproduction* **78**(5), 812–815. doi:10.1095/biolreprod.107.066050
- Gray GD, Wexler BC (1980) Estrogen and testosterone sensitivity of middle-aged female rats in the regulation of LH. *Experimental Gerontology* **15**(4), 201–207. doi:10.1016/0531-5565(80)90025-X
- Hernández-Tristán R, Arevalo C, Canals S (1999) Effect of prenatal uterine position on male and female rats sexual behavior. *Physiology & Behavior* **67**(3), 401–408. doi:10.1016/S0031-9384(99)00077-3
- Hohenbrink S, Meinecke-Tillmann S (2012) Influence of social dominance on the secondary sex ratio and factors affecting hierarchy in Holstein dairy cows. *Journal of Dairy Science* **95**(10), 5694–5701. doi:10.3168/jds.2011-5281
- Hotchkiss AK, Lambright CS, Ostby JS, Parks-Saldutti L, Vandenberg JG, Gray LE (2007) Prenatal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague-Dawley rats. *Toxicological Sciences* **96**(2), 335–345. doi:10.1093/toxsci/kfm002
- Hughes IA (2001) Minireview: sex differentiation. *Endocrinology* **142**(8), 3281–3287. doi:10.1210/endo.142.8.8406
- Jackson LM, Timmer KM, Foster DL (2008) Sexual differentiation of the external genitalia and the timing of puberty in the presence of an antiandrogen in sheep. *Endocrinology* **149**(8), 4200–4208. doi:10.1210/en.2007-1382
- James WH (1996) Evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels at the time of conception. *Journal of Theoretical Biology* **180**(4), 271–286. doi:10.1006/jtbi.1996.0102
- James WH (2004) Further evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels around the time of conception. *Human Reproduction (Oxford, England)* **19**(6), 1250–1256. doi:10.1093/humrep/deh245
- Jubilan BM, Nyby JG (1992) The intrauterine position phenomenon and precopulatory behaviors of house mice. *Physiology & Behavior* **51**(4), 857–872. doi:10.1016/0031-9384(92)90127-N
- Kanitz E, Puppe B, Tuchscherer M, Heberer M, Viergutz T, Tuchscherer A (2009) A single exposure to social isolation in domestic piglets activates behavioural arousal, neuroendocrine stress hormones, and stress-related gene expression in the brain. *Physiology & Behavior* **98**(1–2), 176–185. doi:10.1016/j.physbeh.2009.05.007
- Karnychuk UU, Van Breedam W, Van Roy N, Rogel-Gaillard C, Nauwynck HJ (2012) Demonstration of microchimerism in pregnant sows and effects of congenital PRRSV infection. *Veterinary Research* **43**(1), 19. doi:10.1186/1297-9716-43-19
- Krackow SV (1992) Sex ratio manipulation in wild house mice: the effect of fetal resorption in relation to the mode of reproduction. *Biology of Reproduction* **47**(4), 541–548. doi:10.1095/biolreprod47.4.541
- Kratochwil K (1971) *In vitro* analysis of the hormonal basis for the sexual dimorphism in the embryonic development of the mouse mammary gland. *Development* **25**(1), 141–153.
- Lane EA, Hyde TS (1973) Effect of maternal stress on fertility and sex ratio: a pilot study with rats. *Journal of Abnormal Psychology* **82**(1), 78–80. doi:10.1037/h0034851
- Lehman MN, Coolen LM, Goodman RL (2010) Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology* **151**(8), 3479–3489. doi:10.1210/en.2010-0022
- Mack LA, Lay DC, Eicher SD, Johnson AK, Richert BT, Pajor EA (2014) Growth and reproductive development of male piglets are more vulnerable to midgestation maternal stress than that of female piglets. *Journal of Animal Science* **92**(2), 530–548. doi:10.2527/jas.2013-6773
- McDermott NJ, Gandelman R, Reinisch JM (1978) Contiguity to male fetuses influences ano-genital distance and time of vaginal opening in mice. *Physiology & Behavior* **20**(5), 661–663. doi:10.1016/0031-9384(78)90261-5
- Meikle DB, Drickamer LC, Vessey SH, Arthur RD, Rosenthal TL (1996) Dominance rank and parental investment in swine (*Sus scrofa domestica*). *Ethology* **102**(8), 969–978. doi:10.1111/j.1439-0310.1996.tb01174.x
- Mendl M, Paul ES (1991) Litter composition affects parental care, offspring growth and the development of aggressive behaviour in wild house mice. *Behaviour* **116**(1), 90–108. doi:10.1163/156853990X00374
- Mendl M, Zanella AJ, Broom DM, Whitemore CT (1995) Maternal social status and birth sex ratio in domestic pigs: an analysis of mechanisms. *Animal Behaviour* **50**(5), 1361–1370. doi:10.1016/0003-3472(95)80051-4
- Merckley CM (2013) The role of kisspeptin and KNDy cells in the reproductive neuroendocrine system. PhD thesis, The University of Western Ontario, London, Ontario, Canada.
- Nagao T, Wada K, Kuwagata M, Nakagomi M, Watanabe C, Yoshimura S, Saito Y, Usumi K, Kanno J (2004) Intrauterine position and postnatal growth in Sprague-Dawley rats and ICR mice. *Reproductive Toxicology (Elmsford, N.Y.)* **18**(1), 109–120. doi:10.1016/j.reprotox.2003.10.009

- Navara KJ, Nelson RJ (2009) Prenatal environmental influences on the production of sex-specific traits in mammals. *Seminars in Cell & Developmental Biology* **20**(3), 313–319. doi:10.1016/j.semcdb.2008.12.004
- Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, Steiner RA (2009) Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *The Journal of Neuroscience* **29**(38), 11859–11866. doi:10.1523/JNEUROSCI.1569-09.2009
- Parfet KR, Ganjam VK, Lamberson WR, Rieke AR, vom Saal FS, Day BN (1990) Intrauterine position effects in female swine: subsequent reproductive performance, and social and sexual behavior. *Applied Animal Behaviour Science* **26**(4), 349–362. doi:10.1016/0168-1591(90)90034-B
- Pond WG, Boleman SL, Fiorotto ML, Ho H, Knabe DA, Mersmann HJ, Savell JW, Su DR (2000) Perinatal ontogeny of brain growth in the domestic pig. *Experimental Biology and Medicine* **223**(1), 102–108. doi:10.1046/j.1525-1373.2000.22314.x
- Pratt NC, Lisk RD (1989) Effects of social stress during early pregnancy on litter size and sex ratio in the golden hamster (*Mesocricetus auratus*). *Journal of Reproduction and Fertility* **87**(2), 763–769. doi:10.1530/jrf.0.0870763
- Raeside JJ, Sigman DM (1975) Testosterone levels in early fetal testes of domestic pigs. *Biology of Reproduction* **13**(3), 318–321. doi:10.1095/biolreprod13.3.318
- Rekiel A, Więcek J, Wojtasik M, Ptak J, Blicharski T, Mroczko L (2012) Effect of sex ratio in the litter in which Polish Large White and Polish Landrace sows were born on the number of piglets born and reared. *Annals of Animal Science* **12**(2), 179–185. doi:10.2478/v10220-012-0015-5
- Rines JP, vom Saal FS (1984) Fetal effects on sexual behavior and aggression in young and old female mice treated with estrogen and testosterone. *Hormones and Behavior* **18**(2), 117–129. doi:10.1016/0018-506X(84)90037-0
- Roeling TA, Veening JG, Kruk MR, Peters JP, Vermelis MEJ, Nieuwenhuis R (1994) Efferent connections of the hypothalamic 'aggression area' in the rat. *Neuroscience* **59**(4), 1001–1024. doi:10.1016/0306-4522(94)90302-6
- Ryan BC, Vandenbergh JG (2002) Intrauterine position effects. *Neuroscience and Biobehavioral Reviews* **26**(6), 665–678. doi:10.1016/S0149-7634(02)00038-6
- Sheldon BC, West SA (2004) Maternal dominance, maternal condition, and offspring sex ratio in ungulate mammals. *American Naturalist* **163**(1), 40–54. doi:10.1086/381003
- Stiles J, Jernigan TL (2010) The basics of brain development. *Neuropsychology Review* **20**(4), 327–348. doi:10.1007/s11065-010-9148-4
- Tapp AL, Maybery MT, Whitehouse AJ (2011) Evaluating the twin testosterone transfer hypothesis: a review of the empirical evidence. *Hormones and Behavior* **60**(5), 713–722. doi:10.1016/j.yhbeh.2011.08.011
- Tilbrook AJ, Clarke IJ (2001) Negative feedback regulation of the secretion and actions of GnRH in Males. *Biology of Reproduction* **64**, 735–742. doi:10.1095/biolreprod64.3.735
- Trivers RL, Willard DE (1973) Natural selection of parental ability to vary the sex ratio of offspring. *Science* **179**(4068), 90–92. doi:10.1126/science.179.4068.90
- Uller T, Meylan S, De Fraipont M, Clobert J (2005) Is sexual dimorphism affected by the combined action of prenatal stress and sex ratio? *Journal of Experimental Zoology. Part A, Comparative Experimental Biology* **303A**(12), 1110–1114. doi:10.1002/jez.a.231
- Veiga-Lopez A, Astapova OI, Aizenberg EF, Lee JS, Padmanabhan V (2009) Developmental programming: contribution of prenatal androgen and estrogen to estradiol feedback systems and periovulatory hormonal dynamics in sheep. *Biology of Reproduction* **80**(4), 718–725. doi:10.1095/biolreprod.108.074781
- vom Saal FS (1989a) The production of and sensitivity to cues that delay puberty and prolong subsequent oestrous cycles in female mice are influenced by prior intrauterine position. *Journal of Reproduction and Fertility* **86**(2), 457–471. doi:10.1530/jrf.0.0860457
- vom Saal FS (1989b) Sexual differentiation in litter-bearing mammals: influence of sex of adjacent fetuses in utero. *Journal of Animal Science* **67**(7), 1824–1840. doi:10.2527/jas1989.6771824x
- vom Saal FS, Bronson FH (1978) *In utero* proximity of female mouse fetuses to males: effect on reproductive performance during later life. *Biology of Reproduction* **19**(4), 842–853. doi:10.1095/biolreprod19.4.842
- vom Saal FS, Clark MM, Galef BG, Drickamer LC, Vandenbergh JG (1999) Intrauterine position phenomenon. *Encyclopedia of Reproduction* **2**, 893–900.
- Wakabayashi Y, Nakada T, Murata K, Ohkura S, Mogi K, Navarro VM, Clifton DK, Mori Y, Tsukamura H, Maeda KI, Steiner RA (2010) Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. *The Journal of Neuroscience* **30**(8), 3124–3132. doi:10.1523/JNEUROSCI.5848-09.2010
- Wise TH, Christenson RK (1992) Relationship of fetal position within the uterus to fetal weight, placental weight, testosterone, estrogens, and thymosin beta 4 concentrations at 70 and 104 days of gestation in swine. *Journal of Animal Science* **70**(9), 2787–2793. doi:10.2527/1992.7092787x

Chapter 2:

The sex ratio of a litter affects the behaviour of its female pigs until at least 16 weeks of age

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Name of Principal Author (Candidate)	Jemma Seyfang
Contribution to the Paper	Assisted with the design of the experiment, managed and carried out the experimental trial, analysed statistics, drafted and edited manuscript.
Overall percentage (%)	85
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date <u>29/1/18</u>

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

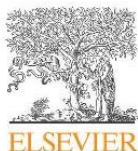
- i. the candidate's stated contribution to the publication is accurate (as detailed above);
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The sex ratio of a litter affects the behaviour of its female pigs until at least 16 weeks of age



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ABSTRACT

There are sex differences in behaviour with males tending to be more aggressive and less fearful than females. When females are treated with androgens they display more aggressive, male like, behaviours and this suggests that androgen exposure has a role in the development of aggression. Female pigs born into litters that are more than 60% male (male biased) are likely exposed to greater androgen concentrations *in utero* than female pigs born into litters that are greater than 60% female (female biased). We hypothesise that the proportion of males in the litter *in utero* affects female pig behaviour. We predict that gilts from male biased litters will be more aggressive and less anxious than gilts from female biased litters. The behaviour of females from male biased ($n = 24$) and female biased ($n = 30$) litters was assessed at day 11 and day 21 of life with an arena test, at weaning (28 days of life) by assessing fight number and duration as well as injury scores and at 16 weeks of age with a resident intruder test. In the day 21 arena test, the pigs from female biased litters took longer to emerge into the arena than pigs from male biased litters (1.13 ± 0.05 (42.2 s) vs. 1.32 ± 0.09 (15.5 s) respectively, $P = 0.016$). At weaning there was a higher scratch score for piglets from male biased litter (0.84 ± 0.2 vs 0.69 ± 0.15 , $P = 0.015$) and this continued as a trend on the day following weaning (1.76 ± 0.10 vs 1.57 ± 0.13 , $P = 0.080$). At 16 weeks of age in the resident intruder test 48% of gilts from male biased litters initiated a fight compared to 24% of gilts from female biased litters ($P = 0.08$). Our data suggest that the sex ratio of the birth litter can affect behaviour of gilts, potentially having long term implications for sow aggression.

1. Introduction

Males behave differently to females, with males often being more aggressive and less fearful and this is likely due to the effect of androgens. There is evidence in a wide range of species, including rodents and sheep, that exposure of females to excessively high concentrations of androgen either exogenously (*in utero* or as adults) or from male litter mates (*in utero*) creates masculinised females and that these females display masculine behaviours (Raeside and Sigman, 1975; Clemens et al., 1978; Hughes, 2001; Arnold, 2004; Navara and Nelson, 2009; Tapp et al., 2011). Although litter bearing species such as pigs may be expected to produce litters of equal numbers of males and females, sows often produce sex-biased litters (male-biased litters $\geq 60\%$ males or female-biased litters $\geq 60\%$ females). There are a variety of reasons for this including sex-biased conception, and implantation and fetal mortality favouring one sex over the other (Clark et al., 1993; James, 2004; Grant et al., 2008; Rekiel et al., 2012; Raeside and Sigman, 1975).

Having a high proportion of males in the litter increases the likelihood that a female will develop between two males and the likelihood that she will be exposed to excessively high androgen concentrations due to androgen production by male littermates. In female rodents, the effects of exposure to androgens from male litter mates can be a result of their intrauterine position, with a female developing between two males exhibiting a masculine phenotype, or it can be related to the total proportion of males in the litter (Clark et al., 1993; vom Saal et al., 1999). Having more males in a litter or being born into a male biased litter increases the likelihood that a female will be exposed to excessive androgen concentrations *in utero*. Therefore, a male biased litter is more likely to produce masculinised females.

Although masculinisation of females can have negative effects on the development of behavioural centres in the brain, and may cause females to be more aggressive (Tapp et al., 2011; Veiga-Lopez et al., 2009), masculinised females can be less anxious and less fearful, or bolder (Hauser and Gandelman, 1983). Rams are less fearful than ewes,

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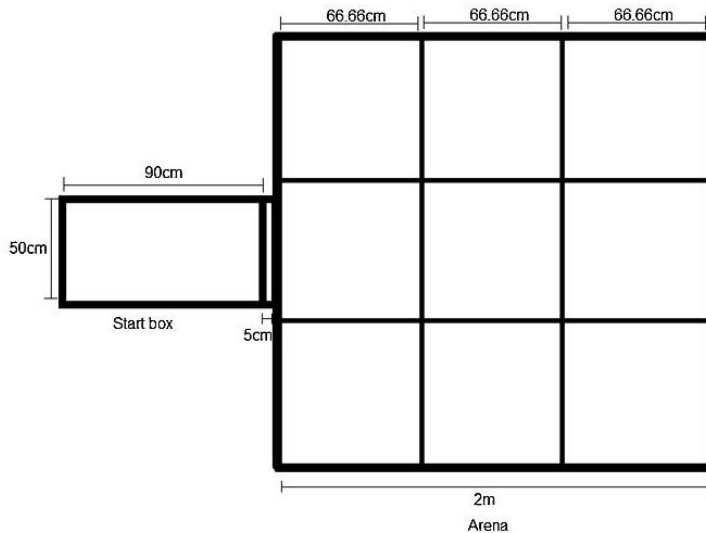


Fig. 1. Diagram of the arena test with start box and grids to scale.

and when ewes are given a daily injection of testosterone propionate they displayed reduced fear reactions to previously fearful situations (Vandenheede and Bouissou, 1993). Injection of testosterone had an immediate effect on the behaviour of ewes, but the reduction in fearfulness persisted for at least 8 months after the testosterone injections ceased (Bouissou and Vandenheede, 1996). Likewise, heifers treated with testosterone showed reduced fear reactions (Boissy and Bouissou, 1994). Combined, these experiments suggest that testosterone exposure can reduce fearfulness in females and that this reduced fearfulness can persist for a long period.

The steroidal milieu under which a gilt develops *in utero* has the potential to influence her lifetime behaviour and the concentration of androgens *in utero* likely affects the development of aggression and fear in females. In addition, behavioural changes that result from exposure to excessively high concentrations of androgens *in utero* are likely to persist long term. We tested the hypothesis that female pig behaviour would be affected by the proportion of males in the birth litter, with gilts from male biased litters being more aggressive and less anxious than gilts from female biased litters.

2. Materials and methods

This experiment was conducted at the University of Adelaide Roseworthy Piggery (Roseworthy, South Australia) in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC 2013) and the *Animal Welfare Act 1985* and approved by the University of Adelaide Animal Ethics Committee (approval number S-2015-060B). Large White x Landrace gilts were used with an average 28-day weaning age and raised under standard commercial conditions. Up to three females per litter were selected at birth from litters consisting of > 60% male (male biased) or > 60% female (female biased) piglets from a total of 36 litters. We considered over 60% a sufficiently large skew in the sex ratio to allow us to see differences between the two biases. This ratio also took in to account piglets that were born dead. The total born litter size, including stillborns, ranged from 8 to 15, with male biased litters having a range of 5–14 males and female biased litters having a range of 1–6 males. The litter sizes reared ranged from 8 to 13 piglets. The sex ratio reared was not always the same as the sex ratio born due to stillborns, fostering, and early deaths. The three females selected were tagged for identification and used as focus piglets for behavioural testing. There was a total of 45 gilts from male biased litters and 44 gilts from female biased

litters.

2.1. Husbandry

Piglets were born into traditional farrowing crates with an overall pen size of 2.2 m × 1.7 m and a stall area of 2.2 m × 0.5 m with plastic flooring. Piglets had a creep area with a heat mat throughout lactation, as well as a heat lamp for the first 24 h after farrowing. At approximately 28 days of age piglets were weaned into pens for weaning behaviour analysis over two days. These pens were 1.3 m × 3.0 m in size with 0.9 m walls and housed 20 piglets per pen. These pens were concrete with partial slats in design and contained one nipple drinker and one *ad libitum* feeder with three access points. After two days they were then moved into straw-based ecoshelters with the commercial herd until 16 weeks of age and were not used for any experiments during this period. At 16 weeks of age focus animals were then moved for the resident intruder test into pens that were 1.3 m × 3.0 m in size with 0.9 m walls with partially slatted concrete flooring. These pens housed two pigs per pen and contained one nipple drinker and the animals were floor fed. They remained here for two weeks before rejoining the commercial herd permanently.

2.2. Arena and novel object test

An arena test, modified from a novel arena and novel object test designed for sheep by Roussel et al. (2004), was used on young piglets. The arena test was performed twice, once on day 11 of life and again on day 21 of life and video recorded using a Kaiser Baas camera (Kasier Baas X80 edition, Kaiser Baas, Rowville, VIC, AUS). The test was performed on individual male biased ($n = 24$) and female biased piglets ($n = 30$) from a subset of the total selected focus piglets. The piglet was placed into a start box for 1 min acclimatisation, and then allowed to emerge into a 2 m × 2 m test arena (as shown in Fig. 1), with the time taken to emerge recorded. For the first 3 min in the arena the piglet's movement (number of lines crossed), vocalisations (grunts and squeals), defecation, and number of jumps (at least the front two feet off the ground) were recorded. The floor of the arena was marked with tape into nine equal squares to determine the piglets movement through number of lines crossed. After 3 min, a red bucket suspended above the arena with rope was introduced to the middle square of the arena, which marked the beginning of the novel object test. For a further 1 min, movement, vocalisation, defecation, and jumps as well as

Table 1

Ethogram describing the behaviours recorded and analysed for the arena and novel object test on days 11 and 21 of age for female piglets that had originated in female biased (< 60% female) or male biased (< 60% male) litters.

Behaviour	Description
Movement	Number of lines crossed within the arena. Lines were counted as crossed when the snout and front two feet had crossed the line.
Vocalisation	
<i>i.</i> Grunt	A low frequency sound
<i>ii.</i> Squeal	A high frequency sound
Defecation/urination	Discharge of faeces or urine from the body
Jumps	The number of times the piglet jumped with at least the front two feet off the ground.
Latency to touch bucket	Time taken between the bucket entering the centre of the arena and the piglet touching it with snout contact
Duration of contact with bucket	Total time the piglet had its snout against the bucket
Frequency of contact	Number of times the piglet touched the bucket with its snout

latency to touch the bucket with snout, duration of contact with the bucket, and frequency of contact were recorded. These behaviours are defined in Table 1. Piglets that did not touch the bucket were given the maximum value of 60 s for latency. The test then concluded and the piglets were returned to their litter.

2.3. Post weaning aggression

At 28 days of age piglets were weaned into pens in groups of 20. This was run over four batches with two pens per batch; one for piglets from male biased litters and one for piglets from female biased litters. Twenty piglets were used each batch per pen to ensure the pens were filled to the same capacity each block, however, there were not always enough focus piglets to fill each pen. Therefore, non-focus females of the same age and from the same litter bias were also added to the pen. Over four blocks 80 females from male biased litters were weaned into pens including 45 focus animals, and 80 females from female biased litters with 44 focus animals. Redness/irritation scores as well as a scratch/abrasion scores were recorded and are defined in Table 2 (Widowski et al., 2003). These were recorded immediately prior to weaning and the following day. A change in injury scores was also calculated. Pens were video recorded for 1 h at 3 h post weaning and again 24 h later. Video footage was analysed for the number of fights in each pen, as well as the duration of fight events in each pen. A fight was recorded when there was reciprocal aggression as defined by Verdon et al. (2016) with both piglets involved in the fight delivering aggression for a period of 5 s or longer.

2.4. Resident intruder test

A subset of gilts were used for a resident intruder test (male biased $n = 25$ and female biased $n = 26$). At 16 weeks of age the focus individuals became residents and were moved into a home pen that was randomly assigned. The residents were acclimatized for 1 week in their home pen with a companion. On the day of the test, the companion was removed and replaced with a female intruder and a 5 min interaction

was observed where latency to attack and initiator of attack were recorded. The intruder pig was removed as soon as an aggressive encounter occurred or at 5 min if no aggressive encounter occurred. The intruders were allocated randomly to a resident and were within a 10 kg weight range of the resident. An attack was defined as consecutive biting events, head knocking, or mounting that lasted longer than 5 s and resulted in the receiving pig reciprocating or fleeing.

2.5. Statistical analysis

IBM SPSS Statistics v22 was used for statistical analysis. For all behavioural tests, data that were not normally distributed were log transformed for analysis. Arena test results were analysed using a linear mixed model analysis of variance. Day 11 and day 21 were analysed separately with fixed factors being litter size reared (8–13), day of farrowing, batch (one to three), piglet test order, birth weight category, and the sex bias of litter (male biased or female biased), with birth sow as a random factor. The data from weaning were analysed separately for each day (weaning and 1 day post weaning) in a general linear mixed model with replicate and pen as random terms, and sex bias of the litter as the fixed effect. For the resident intruder test, Pearson chi-squared analysis was used to analyse the proportion of fights instigated by gilts from male biased litters compared to female biased litters. For gilts that did fight, the latency to fight was analysed in a linear mixed model with fixed factors of batch, age at test, and sex bias of litter, with sow as a random factor. Fights that were instigated by the intruder were rare, and not included in the analysis as it did not express the resident's aggressive behaviour. All video analyses were completed by one person to remove inter-observer variation. P-values of less than 0.05 were considered significant and values between 0.05 and 0.10 were considered as a trend. Data are shown as the mean \pm the standard error of the mean (SEM) with the non-transformed means in parenthesis.

3. Results

3.1. Arena test

The arena test demonstrated that the time taken to emerge from the start box was greater for piglets from female biased litters than for piglets from male biased litters, although the difference was only significant at 21d of age ($F(1,35) = 6.49, P = 0.016$). There were no other behavioural differences between piglets from male biased litters or piglets from female biased litters at day 21. The arena test at 11 days of age identified no difference in the behaviours recorded between piglets from male biased litters and piglets from female biased piglets (Table 3).

3.2. Post weaning aggression

The scratch score for piglets from male biased litters was higher at weaning than for piglets from female biased litters ($F(1) = 6.471, P = 0.013$, Table 4). The day following weaning this continued as a trend toward a greater number of scratches on piglets from male biased litters than on piglets from female biased litters ($F(1) = 3.114, P = 0.080$, Table 4). There were no differences in the number of fights

Table 2

Redness and irritation score and scratches and abrasions score adapted from Widowski et al. (2003). The score was used to calculate an injury score for female piglets that were approximately 28 days of age. The head and body were assessed for redness and scratches.

Score	Classification	Redness	Scratches
0		No redness, swelling, or hair loss.	No scratches or skin loss evident.
1	Mild	Reddening, swelling, or hair loss barely detectable.	One to three small (≤ 2 cm) scratches or areas of abraded skin evident.
2	Moderate	Reddening, swelling, or hair loss obvious.	One to three larger (> 2 cm) scratches or areas of abraded skin observed.
3	Severe	Easily observed, darker reddening, swelling, or patches of hair loss.	More than 3 scratched (usually > 2 cm) or larger areas of skin loss.

Table 3

Effect of sex ratio of the birth litter on a range of behaviours at 11 and 21 days of age using an arena and novel object test (mean \pm SEM). The values in parenthesis are the transformed means. Asterisks indicates significance as $P < 0.05$.

	Day 11			Day 21		
	Male biased n = 24	Female biased n = 30	P value	Male biased n = 24	Female biased n = 30	P value
Arena test Emergence (s)	1.76 \pm 0.22 (28.81)	1.44 \pm 0.11 (49.30)	0.280	1.13 \pm 0.05 (15.54)	1.32 \pm 0.09 (42.20)	0.016*
Grunts	13.82 \pm 2.46 (190.38)	13.30 \pm 1.35 (199.19)	0.858	8.79 \pm 0.72 (89.29)	9.24 \pm 0.60 (95.80)	0.294
Squeals	0.804 \pm 0.51 (57.08)	1.58 \pm 0.27 (65.11)	0.205	1.38 \pm 0.40 (5.50)	1.40 \pm 0.38 (6.10)	0.968
Lines crossed	6.48 \pm 0.72 (43.62)	6.28 \pm 0.38 (40.70)	0.815	4.28 \pm 0.30 (20.45)	4.44 \pm 0.26 (21.73)	0.859
Jumps	0.40 \pm 0.13 (1.23)	0.44 \pm 0.13 (1.7)	0.695	0.17 \pm 0.10	0.47 \pm 0.23	0.455
Defecation/urination	0.08 \pm 0.05	0.26 \pm 0.10	0.434	0.42 \pm 0.12	0.20 \pm 0.09	0.853
Novel object test Grunts	7.67 \pm 1.75 (43.58)	6.65 \pm 0.95 (54.44)	0.556	1.29 \pm 0.08 (24.50)	1.31 \pm 0.07 (26.80)	0.539
Squeals	0.95 \pm 0.18 (7.88)	1.13 \pm 0.34 (14.15)	0.438	2.29 \pm 1.02	1.60 \pm 0.80	0.771
Lines crossed	3.77 \pm 0.64 (9.38)	2.87 \pm 0.33 (10.44)	0.264	2.10 \pm 0.20 (5.33)	2.27 \pm 0.12 (5.57)	0.516
Jumps	0.15 \pm 0.12	0.41 \pm 0.23	0.662	0.13 \pm 0.09	0.17 \pm 0.12	0.918
Defecation	0.12 \pm 0.06	0.04 \pm 0.04	0.232	0.08 \pm 0.06	0.07 \pm 0.05	0.986
Object contact latency (s)	4.62 \pm 1.85 (41.42)	5.83 \pm 0.99 (45.48)	0.578	1.29 \pm 0.12 (39.50)	1.49 \pm 0.10 (56.00)	0.383
Duration of contact (s)	0.613 \pm 0.24 (9.27)	0.816 \pm 0.11 (5.00)	0.534	0.95 \pm 0.09 (10.21)	1.00 \pm 0.08 (8.00)	0.957
Frequency of contact	2.58 \pm 0.49	1.78 \pm 0.32	0.463	1.79 \pm 0.29	1.33 \pm 0.24	0.176
Proportion of piglet to touch novel object	0.81 \pm 0.08	0.78 \pm 0.08	0.788	0.79 \pm 0.08	0.63 \pm 0.09	0.205

Table 4

Effect of sex ratio of a gilts birth litter on aggression at approximately 28 days of age at weaning and the day following weaning using video footage for 1 h each day to assess fight number and time as well as injury scores at these time points and the change in injury score across days (mean \pm SEM).

	Male biased n = 45	Female biased n = 44	P value
Weaning Redness	0.43 \pm 0.08	0.55 \pm 0.11	0.183
Scratches	0.84 \pm 0.12	0.69 \pm 0.15	0.013
Fight number	4.15 \pm 0.66	2.88 \pm 0.82	0.488
Fight duration (s)	1.27 \pm 0.13 (69.84)	1.36 \pm 0.17 (53.18)	0.850
24 h later Redness	1.21 \pm 0.10	1.09 \pm 0.13	0.157
Scratches	1.75 \pm 0.10	1.57 \pm 0.13	0.080
Change in redness	0.83 \pm 0.10	0.64 \pm 0.12	0.480
Change in scratches	1.03 \pm 0.11	0.86 \pm 0.15	0.439
Fight number	1.18 \pm 0.27	1.01 \pm 0.34	0.146
Fight duration (s)	1.18 \pm 0.19 (10.51)	0.98 \pm 1.76 (48.58)	0.718

or their duration on the day of weaning or the following day, or in redness scores at these times. There was also no difference in change of redness and scratch scores the day following weaning.

3.3. Resident intruder test

Gilts from male biased litters tended to be more likely to fight compared to gilts from female biased litters (48% vs. 24% respectively; $P = 0.08$). For the gilts that did fight, the latency for gilts from male biased litters to fight was 97.9 ± 0.6 s compared to 90.5 ± 0.8 s for gilts from female biased litters ($F(1,15) = 0.54$, $P = 0.820$). This is presented as a point of interest but the sample size does not allow for meaningful statistical interpretation as only 11 gilts from male biased litters and 6 gilts from female biased litters fought.

4. Discussion

Our data suggest that there are differences in behaviour between gilts from male biased litters and gilts from female biased litters. In particular, we present evidence that piglets from male biased litters emerged more quickly from a start box in an arena test, had a greater number of scratches at weaning, and had an increased tendency to fight than piglets from female biased litters. This suggests that the sex ratio of the birth litter of gilts can affect behaviour and these effects last until at least 16 weeks of age. Our research suggests that gilts from male biased litters are less likely to be fearful and more likely to be aggressive than gilts from female biased litters. Further research should be conducted to determine if aggression in group housing systems can be alleviated by

selecting against gilts from male biased litters.

The decreased time to emerge from a start box into an arena test for piglets from male biased litters suggests that these individuals were bolder than piglets from female biased litters. Behavioural inhibition such as increased emergence time is a fear-related behaviour and can be interpreted as an expression of fear, which has been demonstrated in numerous studies in poultry, rodents, sheep, and cattle (Boissy and Bouissou, 1995; Hogan, 1965; Jones, 1996; Jones et al., 1999; McClean and Meredith, 1964; Romeyer and Bouissou, 1992; Thiessen et al., 1962). Previous research from our group has shown that piglets born after a difficult parturition are less bold, identifying that the environment experienced prenatally has the ability to influence post-natal behaviour (Ralph et al., 2015). Masculinised animals behave differently to non-masculinised animals and females exposed to greater concentrations of androgens *in utero* display male like behaviours (Hauser and Gandelman, 1983; Ryan and Vandenberg, 2002). As female pigs from male biased litters are likely masculinised due to greater exposure to androgens *in utero* it follows that the female pigs from male biased litters in the current experiment were faster to emerge from the start box than female pigs from female biased litters. It remains unknown whether androgens are directly responsible for the behavioural differences or whether there is an indirect effect of androgens through aromatisation to oestrogens (Vandenheede and Bouissou, 1993). Nonetheless, we present evidence that piglets from males biased litters may be less fearful than piglets from female biased litters.

The greater number of scratches on piglets from male biased litters at weaning indicates that there was more aggression at weaning between piglets from male biased litters than piglets from female biased litters. There was, however, no recorded differences in the incidence of aggression within the pens. This may suggest that the intensity of the fights was greater between piglets from male biased litters or that our sampling regime was insufficient to record all aggressive interactions. It also indicates that gilts from male biased litters may have had more scratches in the first four weeks of life and this weaning score is inclusive of prior injuries during lactation as well as at weaning. The 1 h of video may not have been recorded at the most suitable time to record displays of aggression. Aggression is usually higher leading up to and immediately after feeding but as the piglets in the current experiment were fed *ad libitum* we were unable to record behaviours around discrete feeding events. As such we may have recorded more aggressive behaviours if the piglets were fed at discrete times throughout the day rather than *ad libitum*. It is also possible that 1 h of video footage was insufficient to accurately represent what was occurring throughout the day. There was a trend towards gilts from male biased litters showing an increased likelihood to attack during the resident intruder test and

this supports the notion that gilts from male biased litters displayed greater levels of aggression. Time spent fighting during a resident intruder test is a good indicator of individual aggressiveness, however, during the resident intruder test we were unable to record the time spent fighting and instead ended the test after the first attack as done by D'Eath and Pickup (2002) and Erhard et al. (1997). The intruder was removed as soon as a fight began to minimise injuries and stress to the test animals. Residents are more likely to instigate fights, therefore, making the focus pig the resident affords them greater opportunity to display aggressive behaviours. Combined, the differences and trends seen in the number of scratches at weaning and in likelihood to attack during the resident intruder test highlight that there are differences in behaviour between gilts from male biased litters and gilts from female biased litters. These differences may have been more pronounced if we had more animals that had fought to assess the latency to fight, as well as aggression around discrete feeding events. The birth sex ratio is also likely to affect the reared sex ratio, with gilts from male biased litters being more likely to grow up in a male dominated pen. In the current experiment the birth sex ratio included stillborns and so the reared sex ratio was not always the same as the sex ratio born, this was also altered by early deaths and fostering. However, it does still increase the likelihood of male biased gilts being reared in a male biased farrowing crate and this may also influence their behaviour, further contributing to increased aggression in gilts from male biased litters.

In the current experiment we have recorded differences in behaviour between gilts from male biased litters and gilts from female biased litters up to 16 weeks of age. It is yet to be determined whether these differences will persist for the lifetime of the pig. Fraser (1974) found that individual differences in fear behaviours in pigs were consistent over time and von Borell and Ladewig (1992) found that individual differences in pig behavior were consistent over 14 weeks. von Borell and Ladewig (1992) also found that there was only a small amount of variation within litters for all behaviours, highlighting that behaviour is influenced by prenatal development through either genetics or environment or both. Van Reenen et al. (2005) found that in cattle, individual differences in behaviour were consistent at 3 weeks, 16 weeks, and 26 weeks of age, with the relationship between 16 weeks to 26 weeks being the strongest. They concluded that individual behaviours must be mediated by a stable underlying characteristic or biological process. The behaviour changes in gilts from male biased litters tended to persist until at least 16 weeks of age. Further study is needed to determine if the differences in aggressive behaviours seen in early life persist into adulthood and this is particularly important for gilts as they enter the breeding herd. If the trends towards increased aggression in gilts from male biased litters remained throughout life this could impact welfare and production in group housing systems. As behaviour in pigs and cattle remains consistent over time in previous studies (Borell and Ladewig 1992; Fraser 1974; Van Reenen et al., 2005), we suggest that the trend towards increased aggressive behaviors for pigs from male biased litters in the current study would continue throughout life.

5. Conclusion

The behaviour of a gilt is likely affected by the sex ratio of the litter from which it was born. Gilts from male biased litters are more likely to be aggressive and less fearful than gilts from female biased litters. This is in keeping with the effects of exposure to excessive concentrations of androgens *in utero* on female behaviour. Piglets from male biased litters were faster to emerge from a start box and enter an arena and there were trends towards fights of greater intensity based on the number of scratches and an increase in number of fights initiated by gilts from male biased litters. Further work is required to determine if the difference in behaviour detected in young pigs persists into adulthood particularly around puberty, during oestrus and gestation. If the sex ratio of a gilt's birth litter can predict the likelihood that a gilt will

display greater levels of aggression it could be included in the criteria for selecting gilts to enter the breeding herd. Reducing the number of aggressive pigs selected into the breeding herd may result in an increase in the retention of gilts in the breeding herd. This would have benefits for the welfare of sows and for the efficiency of the breeding herd.

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References

- Arnold, A.P., 2004. Sex chromosomes and brain gender. *Nat. Rev. Neurosci.* 5 (9), 701–708.
- Boissy, A., Bouissou, M.F., 1994. Effects of androgen treatment on behavioral and physiological responses of heifers to fear-eliciting situations. *Horm. Behav.* 28 (1), 66–83.
- Boissy, A., Bouissou, M.F., 1995. Assessment of individual differences in behavioural reactions of heifers exposed to various fear-eliciting situations. *Appl. Anim. Behav. Sci.* 46 (1–2), 17–31.
- Bouissou, M.F., Vandenheede, M., 1996. Long-term effects of androgen treatment on fear reactions in ewes. *Horm. Behav.* 30 (1), 93–99.
- Clark, M.M., Karpiuk, P., Galef, B.G., 1993. Hormonally mediated inheritance of acquired characteristics in Mongolian gerbils. *Nature* 364 (6439), 712.
- Clemens, L.G., Gladue, B.A., Coniglio, L.P., 1978. Prenatal endogenous androgenic influences on masculine sexual behavior and genital morphology in male and female rats. *Horm. Behav.* 10 (1), 40–53.
- D'Eath, R.B., Pickup, H.E., 2002. Behaviour of young growing pigs in a resident-intruder test designed to measure aggressiveness. *Aggress. Behav.* 28 (5), 401–415.
- Erhard, H.W., Mendl, M., Ashley, D.D., 1997. Individual aggressiveness of pigs can be measured and used to reduce aggression after mixing. *Appl. Anim. Behav. Sci.* 54 (2), 137–151.
- Fraser, D., 1974. The vocalizations and other behaviour of growing pigs in an open field test. *Appl. Anim. Ethol.* 1 (1), 3–16.
- Grant, V.J., Irwin, R.J., Standley, N.T., Shelling, A.N., Chamley, L.W., 2008. Sex of bovine embryos may be related to mothers' preovulatory follicular testosterone. *Biol. Reprod.* 78 (5), 812–815.
- Hauser, H., Gandelman, R., 1983. Contiguity to males *in utero* affects avoidance responding in adult female mice. *Science* 220, 437–438.
- Hogan, J.A., 1965. An experimental study of conflict and fear: an analysis of behavior of young chicks toward a mealworm. Part I. The behavior of chicks which do not eat the mealworm. *Behaviour* 25 (1), 45–96.
- Hughes, I.A., 2001. Minireview: sex differentiation. *Endocrinology* 142 (8), 3281–3287.
- James, W.H., 2004. Further evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels around the time of conception. *Hum. Reprod.* 19 (6), 1250–1256.
- Jones, R.B., Satterlee, D.G., Cadd, G.G., 1999. Timidity in Japanese quail: effects of vitamin C and divergent selection for adrenocortical response. *Physiol. Behav.* 67 (1), 117–120.
- Jones, R.B., 1996. Fear and adaptability in poultry: insights, implications and imperatives. *World's Poultry Sci. J.* 52 (02), 131–174.
- McClearn, G.E., Meredith, W., 1964. Dimensional analysis of activity and elimination in a genetically heterogeneous group of mice (*Mus musculus*). *Anim. Behav.* 12 (1), 1–10.
- Navara, K.J., Nelson, R.J., 2009. Prenatal environmental influences on the production of sex-specific traits in mammals. *Semin. Cell. Dev. Biol.* (Elsevier).
- Ralph, C.R., Staveley, L.M., Burnard, C.L., Plush, K.J., 2015. Piglets born with a high degree of meconium staining display altered behaviour throughout lactation. *Anim. Prod. Sci.* 55 (12), 1475.
- Rekiel, A., Więcek, J., Wojtasik, M., Ptak, J., Blicharski, T., Mroczko, L., 2012. Effect of sex ratio in the litter in which Polish Large White and Polish Landrace sows were born on the number of piglets born and reared. *Ann. Anim. Sci.* 12 (2), 179–185.
- Romeyer, A., Bouissou, M.F., 1992. Assessment of fear reactions in domestic sheep, and influence of breed and rearing conditions. *Appl. Anim. Behav. Sci.* 34 (1), 93–119.
- Roussel, S., Hemsworth, P.H., Boissy, A., Duvaux-Ponter, C., 2004. Effects of repeated stress during pregnancy in ewes on the behavioural and physiological responses to stressful events and birth weight of their offspring. *Appl. Anim. Behav. Sci.* 85 (3), 259–276.
- Ryan, B.C., Vandenberg, J.G., 2002. Intrauterine position effects. *Neurosci. Biobehav. Rev.* 26 (6), 665–678.
- Tapp, A.L., Maybery, M.T., Whitehouse, A.J., 2011. Evaluating the twin testosterone transfer hypothesis: a review of the empirical evidence. *Horm. Behav.* 60 (5), 713–722.
- Thiessen, D.D., Zolman, J.F., Rodgers, D.A., 1962. Relation between adrenal weight, brain cholinesterase activity, and hole-in-wall behavior of mice under different living conditions. *J. Comp. Physiol. Psychol.* 55 (2), 186.
- Van Reenen, C.G., O'Connell, N.E., Van der Werf, J.T., Korte, S.M., Hopster, H., Jones, R.B., Blokhuis, H.J., 2005. Responses of calves to acute stress: individual consistency and relations between behavioral and physiological measures. *Physiol. Behav.* 85 (5), 557–570.
- Vandenheede, M., Bouissou, M.F., 1993. Effect of androgen treatment on fear reactions in ewes. *Horm. Behav.* 27 (4), 435–448.
- Veiga-Lopez, A., Astapova, O.I., Aizenberg, E.F., Lee, J.S., Padmanabhan, V., 2009.

- Developmental programming: contribution of prenatal androgen and estrogen to estradiol feedback systems and periovulatory hormonal dynamics in sheep. *Biol. Reprod.* 80 (4), 718–725.
- Verdon, M., Morrison, R.S., Hemsworth, P.H., 2016. Rearing piglets in multi-litter group lactation systems: effects on piglet aggression and injuries post-weaning. *Appl. Anim. Behav. Sci.* 183, 35–41.
- vom Saal, F.S., Clark, M.M., Galef, B.G., Drickamer, L.C., Vandenbergh, J.G., 1999. Intrauterine position phenomenon. *Encyclopedia of Reproduction* 2. pp. 893–900.
- von Borell, E., Ladewig, J., 1992. Relationship between behaviour and adrenocortical response pattern in domestic pigs. *Appl. Anim. Behav. Sci.* 34 (3), 195–206.
- Widowski, T.M., Cottrell, T., Dewey, C.E., Friendship, R.M., 2003. Observations of piglet-directed behavior patterns and skin lesions in eleven commercial swine herds. *J. Swine Health Prod.* 11 (4), 181–185.

Chapter 3:

Response to gonadotrophins differs for gilts from female- and male-biased litters

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Principal Author

Name of Principal Author (Candidate)	Jemma Seyfang
Contribution to the Paper	Assisted with the design of the experiment, managed and carried out the experimental trial, analysed statistics, drafted and edited manuscript.
Overall percentage (%)	85
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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By signing the Statement of Authorship, each author certifies that:

- i. the candidate's stated contribution to the publication is accurate (as detailed above);
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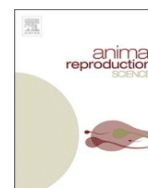
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Response to gonadotrophins differs for gilts from female- and male-biased litters

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ABSTRACT

In several species, females masculinised by abnormal androgen exposure in utero have poor reproductive performance and gilts born into litters with a male bias are likely exposed to greater androgen concentrations prenatally than gilts born into female-biased litters. At 24 h of age, piglet plasma testosterone concentrations in gilts from male-biased litters (> 60% male; n = 22) or female-biased litters (> 60% female; n = 27) were not different. At 18 wks of age, all gilts received an injection of 400 IU equine chorionic gonadotrophin plus 200 IU human chorionic gonadotrophin to stimulate oestrus. Two weeks after the injection gilts were slaughtered and ovaries collected for determination of numbers of corpora lutea (CL). Compared to gilts from female-biased litters, gilts from male-biased litters were more likely to ovulate (86.0% vs 59.5%, $P = 0.047$) and had more CL (13.1 ± 1.5 vs 7.2 ± 1.7 , $P = 0.015$). The present data indicate an effect of birth litter sex-bias on pre-pubertal physiological development, possibly involving organisational effects at the ovarian cellular level impacting on future ovarian function. Potential impacts on subsequent fertility remain to be determined.

1. Introduction

Female lifetime reproductive performance can be affected by exposure to abnormal androgen concentrations in utero (Veiga-Lopez et al., 2009), with abnormally high exposure to androgens in utero causing masculinisation of females. In litter bearing species, female fetuses can be exposed to androgens from male fetuses secreting testicular androgens during gonad differentiation (Raeside and Sigman, 1975). Although litter bearing species such as pigs may be expected to produce litters of 50:50 male:female, sows often produce sex-biased litters (male-biased litters $\geq 60\%$ males or female-biased litters $\geq 60\%$ females) and there are a variety of reasons for this including sex-biased conception, and implantation and fetal mortality favouring one sex over the other (Clark et al., 1993; James, 2004; Grant et al., 2008; Rekiel et al., 2012). In rodents, the effects of androgen exposure from male litter mates can be a result of their intrauterine position, with a female developing between two males exhibiting a masculine phenotype, or it can be related to the total proportion of males in the litter (Clark et al., 1993; vom Saal et al., 1999). Female sheep exposed to abnormally high exogenous testosterone during their fetal development variably exhibit an advanced onset of puberty, irregular ovulatory cycles, anovulation, or polycystic ovaries (Padmanabhan and Veiga-Lopez, 2013). These effects could also be seen in viviparous lizard females exposed to endogenous testosterone in litters with a high proportion of males (Uller et al., 2005). Having a high proportion of males in the litter increases the likelihood that a female will develop between two males. For this reason, a male-biased litter is more likely to produce masculinized females. We hypothesize that due to increased androgen exposure in utero, neonatal gilts from male-

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biased litters will have increased basal testosterone and subsequent impaired ovarian function compared to gilts from female-biased litters.

2. Materials and methods

This experiment was conducted at the University of Adelaide Roseworthy Piggery (South Australia) in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC 2013) and approved by the University of Adelaide Animal Ethics Committee (approval number S-2015-060B). A total of 49 terminal line gilts from Large White x Landrace sows were selected from 20 litters consisting of > 60% male (male-biased; $n = 22$) or female (female-biased; $n = 27$) piglets, including stillborn piglets. Up to three gilts per litter were selected and tagged as focus pigs and were monitored from birth until slaughter at 20 wks of age. They were raised under standard Australian commercial conditions with pigs being weaned at 28 d into a straw-based ecoshelter. The study began in May 2015 and was completed in November 2015 over two farrowing batches. All pigs were weighed at 24 h and 21 d, and focus pigs also at 16 wks. Pigs were allowed free access to feed and water at all times.

2.1. Testosterone

At 24 h of age a 3 mL blood sample was taken from all focus pigs via jugular venepuncture into heparinised tubes and the plasma harvested after centrifugation at 1500 x g for 10 min at 4 °C and then stored at –20 °C until required for assay. Plasma was assayed for testosterone using a testosterone enzyme immunoassay kit (Arbor Assays, catalogue number K032-H5) as described by [Graham et al. \(2015\)](#), following the manufactures instructions and using a double extraction with diethyl ether. All samples were assayed in triplicate and the intra- and inter-assay coefficients variation were 12% and 0.77%, respectively, and assay sensitivity was 9.92 pg/mL.

2.2. Ovarian response to stimulation

At 18 wks of age, all gilts were injected with 400 IU equine chorionic gonadotrophin plus 200 IU human chorionic gonadotrophin (PG600[®], Intervet Australia, Bendigo, Victoria). Gilts were then exposed to fenceline contact with boars in a detection-mating area for 20 min/d for 6 d for oestrus detection. Gilts were deemed to be oestrous if they exhibited a standing response to back pressure in the presence of a boar. At 20 wks of age the gilts were slaughtered and their ovaries collected for determination of numbers of corpora lutea (CL). Gilts without CL were classified as anovular.

2.3. Statistical analysis

Testosterone concentrations were analysed using a one way ANOVA (IBM SPSS Statistics 22) with testosterone concentration as the dependent variable and the sex-bias (male- vs female-bias) as the independent variable. Oestrus and ovulation data were analysed using a general linear model for the proportion of gilts that exhibited oestrus and ovulated, and numbers of CL from each sex-bias with birth weight and weaning weight as covariates. Regression analysis was conducted to examine the relationship between the sex ratio of the birth litter and the number of CL. A one-way ANOVA was used to analyse differences in farrowing data, rearing data, and weights for each bias.

3. Results

3.1. Testosterone

There was no detectible effect of birth litter sex ratio on plasma testosterone concentrations in female piglets 24 h after birth. The mean (\pm SEM) testosterone concentrations for female piglets from female-biased litters was 0.52 ± 0.06 ng/mL compared to 0.66 ± 0.08 ng/mL for female piglets from male-biased litters ($P = 0.228$).

3.2. Ovarian response

Although there were differences in total born litter size ($P = 0.003$) and pigs weaned ($P = 0.003$) in the birth litters, there no treatment differences in piglet weights at any time ([Table 1](#)). By 20 wks of age fewer gilts from female-biased litters had exhibited oestrus than did gilts from male-biased litters (59.5% vs 86.0%, $P = 0.047$). All gilts that exhibited a standing response during oestrus detection had ovulated and all gilts that did not stand were anovular, as determined by presence or absence of CL on the ovaries. There was no difference in the PG600 injection to oestrus detection interval. For gilts that did ovulate, those from female-biased litters had fewer CL than did those from the male-biased litters (7.2 ± 1.7 vs 13.1 ± 1.5 , $P = 0.015$).

The gilts from male-biased litters had a greater range of CL counts (3–31) compared to gilts from female-biased litters (3–14). There was a negative linear relationship between the sex ratio of the litter and CL number, with CL number decreasing with an increase in proportion of females ($R^2 = 0.242$; $P = 0.003$; [Fig. 1](#)).

Table 1The mean \pm SEM farrowing and rearing data, as well as weights for focus pigs from male biased (n = 22) and female biased litters (n = 27).

	Male biased	Female biased	P value
Total born	13.77 \pm 0.48	11.74 \pm 0.44	0.003
Total born alive	12.82 \pm 0.60	11.67 \pm 0.43	0.118
Stillborns	0.96 \pm 0.28	0.07 \pm 0.05	0.001
Mummified	0.73 \pm 0.15	0.11 \pm 0.06	0.000
Percent female	29.69 \pm 1.68	70.87 \pm 1.74	0.000
Litter size reared	11.27 \pm 0.37	10.67 \pm 0.43	0.302
Number weaned	11.00 \pm 0.38	9.48 \pm 0.29	0.003
Weight at 24 h	1.58 \pm 0.07	1.68 \pm 0.07	0.316
Weight at 21 d	6.97 \pm 0.32	7.37 \pm 0.27	0.339
Weight at 16 wk	69.96 \pm 1.80	69.98 \pm 1.34	0.990

4. Discussion

Our data indicate that at 18 wks of age gilts from male-biased litters were more likely to exhibit oestrus, ovulate, and have more CL in response to exogenous gonadotrophins than gilts from female-biased litters. Our hypothesis is, therefore, not supported. The mechanism controlling the observed sex-bias effect on the ovarian response in pigs is not known but clearly involves an effect on the sensitivity of ovarian follicles to hormonal stimulation. Interestingly, higher ovulation rates have been shown in gilts treated with dihydrotestosterone (DHT), and CL development was impaired in gilts when androgenic actions were blocked with flutamide (Cardenas et al., 2002; Grzesiak et al., 2014). In addition, gilts that were injected with either 0, 6, 60 or 600 μ g/kg DHT daily from day 13 of the oestrous cycle had a dose-dependent increased ovulation rate (Cardenas et al., 2002). Since DHT is not aromatised, this suggests that androgens per se are important for follicular and CL development in pigs. Gilts from male-biased litters are more likely to be exposed to higher androgen concentrations in utero and this may have organisational effects at the ovarian cellular level that potentiates future follicle growth and CL development. As discussed by Pope and Cardenas (2006), the mechanism involved remains unknown although an effect mediated through changes in FSH receptor induction seem possible. Although we provide no direct evidence of androgenic action and could demonstrate no effect on neonatal plasma testosterone concentrations, our results are consistent with those evoked by androgen treatment and suggest that gilts from male-biased litters may be exposed to endogenous androgen therapy due to androgen production by their male litter mates. This prenatal androgen exposure may have benefits for ovarian function in response to exogenous stimulation.

The literature suggests that females from male-biased litters of various species may be relatively poor reproductive performers as they may be less likely to conceive, may reach puberty later, and may produce less offspring than gilts from female-biased litters (Edgerton and Cromwell, 1987; Drickamer et al., 1997; vom Saal et al., 1999; Bánszegi et al., 2010). Masculinised female mice exhibit delayed onset of puberty, extended oestrous cycles, and stop producing pups at a younger age than non-masculinised female mice (vom Saal et al., 1999). However, masculinised ewe lambs (vom Saal et al., 1999; Veiga-Lopez et al., 2009) and gilts (Lamberson et al., 1988; Rohde Parfet et al., 1990) had an advanced onset of puberty indicating species variation. Interestingly, we have also performed a small pilot study using post-pubertal gilts slaughtered at 34 weeks of age that indicated no effect of birth litter sex-bias on numbers of CL (unpublished data). This may indicate that there were differences in gilt physiological age at 18 weeks but this difference was no longer evident at maturity. Rohde Parfet et al. (1990) also found no difference in numbers of CL for the first three

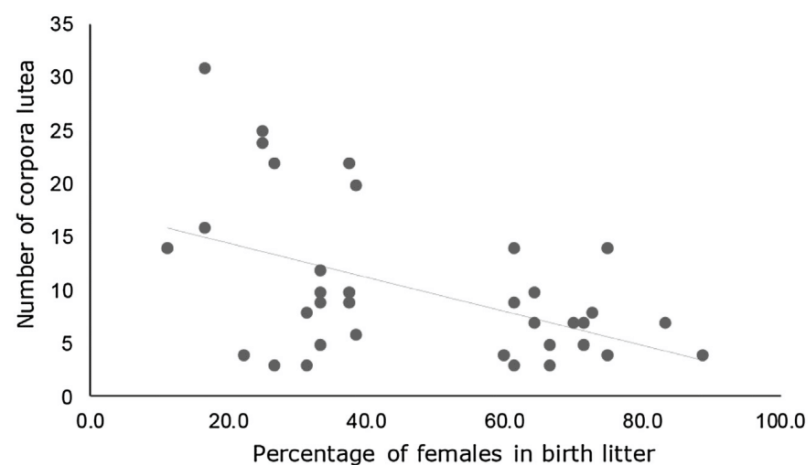


Fig. 1. The relationship between number of corpora lutea and percentage of females in the birth litter. There was a significant negative linear relationship, with the number of corpora lutea increasing with a decrease in the percentage of females in the birth litter, $R^2 = 0.242$; $P < 0.01$.

oestrous cycles of gilts that had developed between one male, two males, or no males.

In ewe lambs, prenatal testosterone exposure resulted in advanced onset of puberty but the LH surge was delayed and the surge amplitude was decreased, suggesting that the hypothalamo-pituitary-gonadal (HPG) axis was compromised by androgen exposure in utero (Veiga-Lopez et al., 2009). If similar endocrine consequences occur in gilts, a delayed LH surge with a decreased surge amplitude could have negative reproductive implications for gilts including poor timing of insemination relative to ovulation and impaired follicular luteinisation with downstream effects on fetal survival, and possibly pregnancy maintenance (Kirkwood and Aherne, 1985). It has been noted that conditions adversely affecting sow fertility, including poor nutrient intake during lactation and short lactation lengths, are also associated with smaller LH surges (Kirkwood et al., 1984; Baidoo et al., 1992). Further research is needed to assess if litter sex-bias has an impact on lifetime reproductive performance of gilts.

The sex ratio of a gilt's birth litter affects ovarian development and ovulation, with male-biased gilts having greater ovulatory responses to exogenous hormone stimulation. The negative linear relationship between ovulation rate and proportion of females in the litter suggests that the extremes in birth litter sex-bias should be considered when selecting gilts, especially sex-biases greater than 60%. Nonetheless, ovulation rate is only one aspect of reproduction and it is important to fully characterise other aspects of the pig's reproductive physiology that may be impacted by the sex ratio of its birth litter. This will determine if being born into a male-biased litter results in greater lifetime reproductive performance and if the sex ratio of the birth litter could be used in selection of gilts for the breeding herd. Further research is required on ovulation rate and LH secretion without exogenous gonadotrophin intervention.

In order to better understand the impact of the sex ratio of a gilts birth litter further research should focus on the secretory pattern of luteinizing hormone and the neuroendocrine control of the HPG axis and long term reproductive outcomes. The current experiment provides evidence that ovarian function in pre-pubertal gilts from male-biased litters is different to that of gilts from female-biased litters in response to exogenous hormones and this provides an excellent platform to further explore the capacity for the reproductive performance of gilts to be influenced by their littermate's gender in utero.

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Conflicts of interest

None.

References

- Baidoo, S.K., Aherne, F.X., Kirkwood, R.N., Foxcroft, G.R., 1992. Effect of feed intake during lactation and after weaning on sow reproductive performance. *Can. J. Anim. Sci.* 72, 911–917.
- Bánszegi, O., Altbácker, V., Dúcs, A., Bilkó, Á., 2010. Testosterone treatment of pregnant rabbits affects sexual development of their daughters. *Physiol. Behav.* 101, 422–427.
- Cardenas, H., Herrick, J.R., Pope, W.F., 2002. Increased ovulation rate in gilts treated with dihydrotestosterone. *Reproduction* 123, 527–533.
- Clark, M.M., Karpiuk, P., Galef, B.G., 1993. Hormonally mediated inheritance of acquired characteristics in Mongolian gerbils. *Nature* 364 (6439) 712–712.
- Drickamer, L.C., Arthur, R.D., Rosenthal, T.L., 1997. Conception failure in swine: importance of the sex ratio of a female's birth litter and tests of other factors. *J. Anim. Sci.* 75, 2192–2196.
- Edgerton, L.A., Cromwell, G.L., 1987. Sex of Siblings May Influence Reproductive Performance in Sows. Progress Report-Kentucky Agricultural Experiment Station, USA.
- Graham, K.M., Mylniczko, N.D., Burns, C.M., Bettinger, T.L., Wheaton, C.J., 2015. Examining factors that may influence accurate measurement of testosterone in sea turtles. *J. Vet. Diagn. Invest.* 28, 12–19.
- Grant, V.J., Irwin, R.J., Standley, N.T., Shelling, A.N., Chamley, L.W., 2008. Sex of bovine embryos may be related to mothers' preovulatory follicular testosterone. *Biol. Reprod.* 78, 812–815.
- Grzesiak, M., Knapczyk-Stwora, K., Ciereszko, R.E., Golas, A., Wiecech, I., Slomczynska, M., 2014. Androgen deficiency during mid-and late pregnancy alters progesterone production and metabolism in the porcine corpus luteum. *Reprod. Sci.* 21, 778–790.
- James, W.H., 2004. Further evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels around the time of conception. *Hum. Reprod.* 19, 1250–1256.
- Kirkwood, R.N., Aherne, F.X., 1985. Energy intake, body composition and reproductive performance of the gilt. *J. Anim. Sci.* 60, 1518–1529.
- Kirkwood, R.N., Lapwood, K.R., Smith, W.C., Anderson, I.L., 1984. Post weaning plasma levels of LH, prolactin, oestradiol 17 β and progesterone in sows following lactations of 10 or 35 days. *J. Reprod. Fertil.* 70, 95–102.
- Lamberson, W.R., Blair, R.M., Rohde Parfet, K.A., Day, B.N., Johnson, R.K., 1988. Effect of sex ratio of the birth litter on subsequent reproductive performance of gilts. *J. Anim. Sci.* 66, 595–598.
- Padmanabhan, V., Veiga-Lopez, A., 2013. Sheep models of polycystic ovary syndrome phenotype. *Mol. Cell. Endocrinol.* 373, 8–20.
- Pope, W.F., Cardenas, H., 2006. Androgens in female pig reproduction: actions mediated by the androgen receptor. *Soc. Reprod. Fertil. Suppl.* 62, 55–67.
- Raeseide, J., Sigman, D., 1975. Testosterone levels in early fetal testes of domestic pigs. *Biol. Reprod.* 13, 318–321.
- Rekiel, A., Więcek, J., Wojtasik, M., Ptak, J., Blicharski, T., Mroczko, L., 2012. Effect of sex ratio in the litter in which polish large white and polish landrace sows were born on the number of piglets born and reared. *Ann. Anim. Sci.* 12, 179–185.
- Rohde Parfet, K.A., Ganjam, V.K., Lamberson, W.R., Rieke, A.R., Vom Saal, F.S., Day, B.N., 1990. Intrauterine position effects in female swine: subsequent reproductive performance, and social and sexual behavior. *Appl. Anim. Behav. Sci.* 26, 349–362.
- Uller, T., Meylan, S., De Fraipont, M., Clobert, J., 2005. Is sexual dimorphism affected by the combined action of prenatal stress and sex ratio? *J. Exp. Zool.* 303, 1110–1114.
- Veiga-Lopez, A., Astapova, O.I., Aizenberg, E.F., Lee, J.S., Padmanabhan, V., 2009. Developmental programming: contribution of prenatal androgen and estrogen to estradiol feedback systems and periovulatory hormonal dynamics in sheep. *Biol. Reprod.* 80, 718–725.
- vom Saal, F.S., Clark, M.M., Galef, B.G., Drickamer, L.C., Vandenberg, J.G., 1999. Intrauterine position phenomenon. In: Knobil, E., Neill, J. (Eds.), *Encyclopaedia of Reproduction*. Academic Press, New York, pp. 893–900.

Chapter 4:

Sex bias of the birth litter affects surge but not tonic LH secretion in gilts

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Overall percentage (%)	85
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- i. the candidate's stated contribution to the publication is accurate (as detailed above);
- ii. permission is granted for the candidate to include the publication in the thesis; and
- iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Sex bias of the birth litter affects surge but not tonic LH secretion in gilts¹

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ABSTRACT: The physiology and behavior of gilts that develop in a male-biased litter can differ from gilts that develop in a female-biased litter. We hypothesized that gilts from male-biased litters will have a delayed and attenuated luteinizing hormone (LH) surge, and reduced LH pulse frequency and amplitude compared to gilts from female-biased litters. Gilts were selected at birth from male-biased (>60% males $n = 10$) or female-biased (>60% females $n = 9$) litters. From 18 wk of age, detection of puberty using daily boar contact began and their subsequent estrous periods were synchronized with oral progestogen (altrenogest). On day 3 after altrenogest withdrawal, blood samples were obtained from 6 gilts per sex bias group at 10 min intervals from 0900 to 2100 h to determine LH pulse amplitude and

frequency. From 0900 on day 4, all 19 gilts were sampled every 4 h until the end of estrus to characterize LH surge dynamics. There were no differences between groups in LH pulse characteristics. Compared to gilts from female-biased litters, the LH surge in gilts from male-biased litters was delayed [56.00 ± 3.32 h vs. 43.11 ± 3.76 h (mean \pm standard error of the mean (SEM)), $P < 0.05$], the duration was decreased [29.78 ± 2.12 h vs. 37.71 ± 1.19 h (mean \pm SEM), $P < 0.05$] and the total secretion as measured by area under the curve was decreased (91.42 ± 9.52 ng/mL vs. 120.28 ± 9.48 ng/mL, $P < 0.05$). Our results indicate that a male-biased uterine environment has different effects on the tonic secretion of LH than the LH surge, with only some elements of the LH surge being affected.

Key words: gilts, litter sex bias, luteinizing hormone

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INTRODUCTION

The physiology and behavior of gilts that develop in a male-biased litter can differ from gilts that develop in a female-biased litter. For example, as the number of males in a litter increased the age of first estrus for gilts in the litter declined (Lamberson et al., 1988).

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Gilts that failed to conceive on their first mating were more likely to be from a male-biased litter and gilts from a male-biased litter were more sensitive to gonadotropins, more aggressive and more males in a litter resulted in less teats (Drickamer et al., 1997; Drickamer et al., 1999; Seyfang et al., 2017, 2018). The physiology and behavior of a gilt can also be affected by its intrauterine position. Gilts that developed in utero between 2 males reached puberty earlier than gilts that developed between 2 females while gilts that developed between 2 females showed enhanced receptivity (Parfet et al., 1990a, 1990b). These differences resemble the effects of exposure to excessive concentrations of testosterone in utero. Exogenous treatment of sows with testosterone increased

testosterone in amniotic fluid and in maternal and fetal circulation. Responsiveness to the positive feedback effects of estrogen on luteinizing hormone (LH) secretion was reduced when gilts born to sows treated with testosterone were 160 d of age (Elsaesser and Parvizi, 1979; Petric et al., 2004). This indicates that gilts exposed to excessive concentrations of testosterone in utero had deficits in the secretion of LH. Gilts that developed in utero between 2 males, however, did not have greater testosterone concentrations than females that developed between 2 females (Framstad et al., 1990; Wise and Christenson, 1992). We aimed to determine if there were differences in the secretion of LH between gilts that developed in a male-biased litter and gilts that developed in a female-biased litter and tested the hypothesis that gilts from a male-biased litter would have a delayed LH surge onset, an attenuated LH surge amplitude, reduced LH pulse frequency, and reduced LH pulse amplitude.

MATERIALS AND METHODS

This experiment was approved by the University of Adelaide Animal Ethics Committee (approval number S-2015-060C). It was conducted at the Roseworthy Piggery, The University of Adelaide (South Australia) in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013) and the Animal Welfare Act, 1985.

An indicative timeline of the methods is illustrated in Fig. 1. Nineteen terminal line gilts from Large White \times Landrace sows were selected from 14 litters consisting of > 60% male (male biased; $n = 10$) or > 60% female (female biased; $n = 9$) piglets, including stillborn piglets. Gilts were selected at birth and only 1 or 2 females from each litter were selected to minimize the effect of litter. Pigs were weaned at 28 d into a straw-based open sided group shed and raised under standard Australian commercial conditions. At 16 wk of age, the gilts

were moved from the ecoshelter to a controlled environment barn where they were kept in pens (1.3 m \times 3.0 m) in groups of 4. They had access to water ad libitum and access to 4 kg/d each of a gilt developer diet formulated to provide 13 MJ digestible energy (DE)/kg and 0.87% total lysine.

Detection of puberty began at 18 wk of age, with the gilts having fenceline contact for 60 min/d with mature boars in a detection-mating area. Detection of puberty was confirmed by the standing response to back pressure in the presence of a boar.

From 12 d after detection of puberty, gilts were orally administered 20 mg/d of a progestogen, altrenogest, to synchronize estrus. Gilts remained on altrenogest daily until all gilts had exhibited puberty and had been on altrenogest for a minimum of 16 d. Altrenogest was withdrawn from all gilts on the same day.

All gilts were moved into individual stalls (225 cm \times 62 cm) 1 mo prior to altrenogest removal. During this time all gilts were acclimatized to the stalls, feeding schedule and human interactions. This minimized the effects of housing on any measurements. On day 2 after altrenogest withdrawal gilts were fitted with indwelling jugular catheters accessed via their ear veins. On day 3 after altrenogest withdrawal, a subset of 12 gilts (6 gilts from male-biased litters and 6 gilts from female-biased litters) were sampled to assess tonic LH secretion. Blood samples were collected into heparinized tubes at 10 min intervals from 0900 to 2100 h from 6 gilts from male-biased litters and 6 gilts from female-biased litters to characterize the dynamics of tonic LH secretion indicated by the frequency and amplitude of LH pulses.

To characterize the dynamics of the LH surge, starting at 0900 on day 4 after altrenogest withdrawal, all 19 gilts were sampled every 4 h until the end of estrus as determined by a failure to stand to back pressure in the presence of a boar. All samples were centrifuged at 1,360 g for 10 min and the plasma harvested and stored at -20°C until

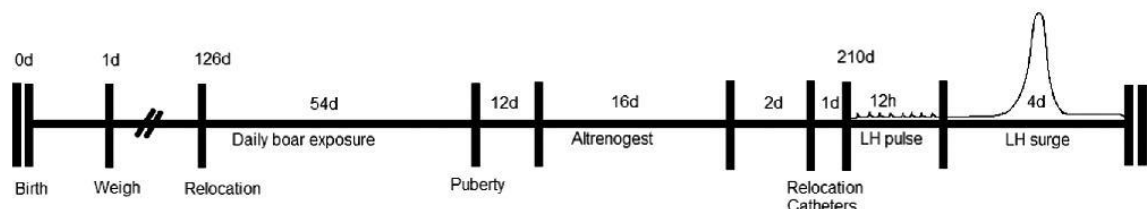


Figure 1. Timeline of methods from when the gilts were selected at birth, then weighed at 24 h, boar exposed from 18 w of age until puberty, administered altrenogest, altrenogest withdrawn, and then blood sampled for luteinizing hormone (LH) concentrations before and during ovulation, as well as progesterone (P4) concentrations after ovulation. The LH pulse samples were collected every 10 min and the LH surge samples collected every 4 h. Each gilt had a minimum of 16 d on altrenogest.

required for LH assay. A double antibody radioimmunoassay was used to measure LH, using an AFP-15103194 antibody with porcine LH preparation AFP-11043B as standard, as described previously (Niswender et al., 1969). The lowest detectable concentration was 0.4 ng/mL. The intra- and interassay coefficients of variation were 11.9% and 20.3%, respectively.

Statistical Methods

Pulses of LH were defined as increases in concentration of LH that exceeded the preceding value by at least 3 times the mean standard deviation of the LH concentrations and was followed by a decline at a rate consistent with the half-life of LH in pigs of 28 min (Turner et al., 1999). The number of pulses (frequency) per hour, the pulse amplitude, and overall mean LH concentrations were analyzed using a 1-way analysis of variance (ANOVA). The LH surge was defined as described by previous literature (Barb et al., 1982), with the onset beginning when the concentration was 50% greater than the baseline and ended when the concentration decreased to 50% below the baseline. The baseline was calculated by averaging the data prior to an increase in concentration and removing outliers until the values were all within 2 standard deviations of the mean. The data were first analyzed with a repeated measures ANOVA to determine that there was a time \times treatment interaction in the LH surge between gilts from male-biased litters and gilts from female-biased litters. The time of onset of the LH surge, the duration of the LH surge, the total secretion as determined by the area under curve, and the maximum concentration of the LH surge (peak amplitude) were analyzed using a 1-way ANOVA. The normality of the data was tested using the homogeneity of variance test, and Kolmogorov-Smirnov and Shapiro-Wilk tests of normality. The duration of the LH surge and the time to the peak of the LH surge were not normally distributed, however, this was not improved by log 10 or square root transformation so the raw data were used.

RESULTS

Descriptive Data

Gilts from male-biased litters came from litters of 11.80 ± 0.74 total born piglets with 3.60 ± 0.37 females and 8.20 ± 0.49 males. Gilts from female-biased litters came from litters of 12.89 ± 0.66

total born piglets with 8.56 ± 0.53 females and 4.33 ± 0.23 males. There was no difference in litter size between gilts from male-biased litters and gilts from female-biased litters ($P = 0.291$). The percentage of females in male-biased litters averaged $29.48 \pm 1.90\%$ and females from female-biased litters averaged $66.543 \pm 1.43\%$. Gilts from male-biased litters reached puberty at 183.20 ± 3.74 d and gilts from female-biased litters at 175.89 ± 3.77 d [$P = 0.188$, mean \pm standard error of the mean (SEM)]. At the time of LH sampling, there was no difference in weight between gilts from male-biased litters and gilts from female-biased litters with 157.90 ± 3.73 kg vs. 156.94 ± 2.58 kg for gilts from male- and female-biased litters, respectively ($P = 0.839$, mean \pm SEM). There was no difference in duration of altrenogest feeding between gilts from male-biased litters and gilts from female-biased litters with 30.50 ± 3.34 d vs. 38.56 ± 3.36 d, respectively ($P = 0.108$, mean \pm SEM). There was no difference in the interval from progesterone withdrawal to estrus with 6.20 ± 0.36 d vs. 5.78 ± 0.40 d for gilts from male- and female-biased litters, respectively ($P = 0.442$, mean \pm SEM). There was no difference in duration of estrus at the time of LH sampling. Gilts from male-biased litters stood for 3.10 ± 0.41 d and gilts from female-biased litters stood for 2.78 ± 0.43 d ($P = 0.595$, mean \pm SEM). All gilts ovulated during the LH sampling period as determined by presence of fresh corpora lutea on the ovaries at slaughter within 12 d of estrus and the concentration of progesterone in plasma. Both biases had a range of 13 to 19 corpora lutea with an average of 15.3.

Tonic Secretion of LH

There were no significant differences (mean \pm SEM) between gilts from male-biased litters and gilts from female-biased litters for overall LH concentrations during the 12 h sampling period (0.60 ± 0.03 ng/mL for both). Similarly, there were no differences in pulse frequency [0.81 ± 0.11 vs. 0.78 ± 0.05 (pulses/h); $P = 0.77$] or pulse amplitude (0.52 ± 0.03 vs. 0.53 ± 0.06 ng/mL; $P = 0.893$) for gilts from male-biased and female-biased litters, respectively. These results can be seen in Fig. 2. The individual profiles of LH during tonic secretion for gilts from female-biased litters and gilts from male-biased litters are shown in Fig. 3 and Fig. 4.

Surge Secretion of LH

As shown in Fig. 5, gilts from male-biased litters had a delayed onset of the LH surge ($P < 0.05$),

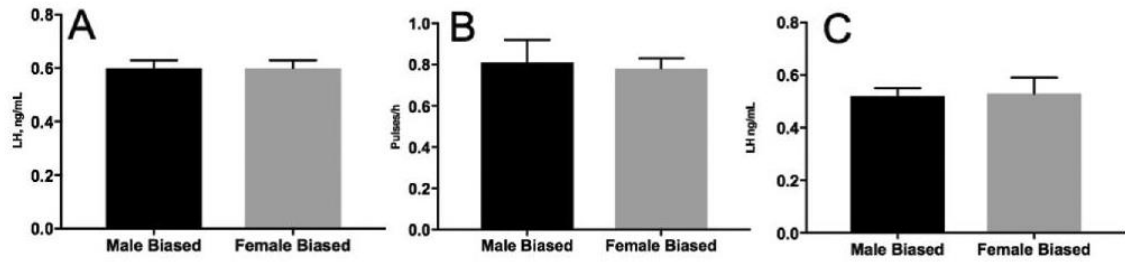


Figure 2. The overall concentration of luteinizing hormone (LH), number of LH pulses per hour and amplitude of LH pulse for pigs that developed in a male-biased or female-biased litter. Panel A depicts the overall concentration of LH for the 12 h sampling period, panel B depicts the number of pulses per h, and panel C depicts mean amplitude of LH for the 12 h sampling period. There were no significant differences between the groups.

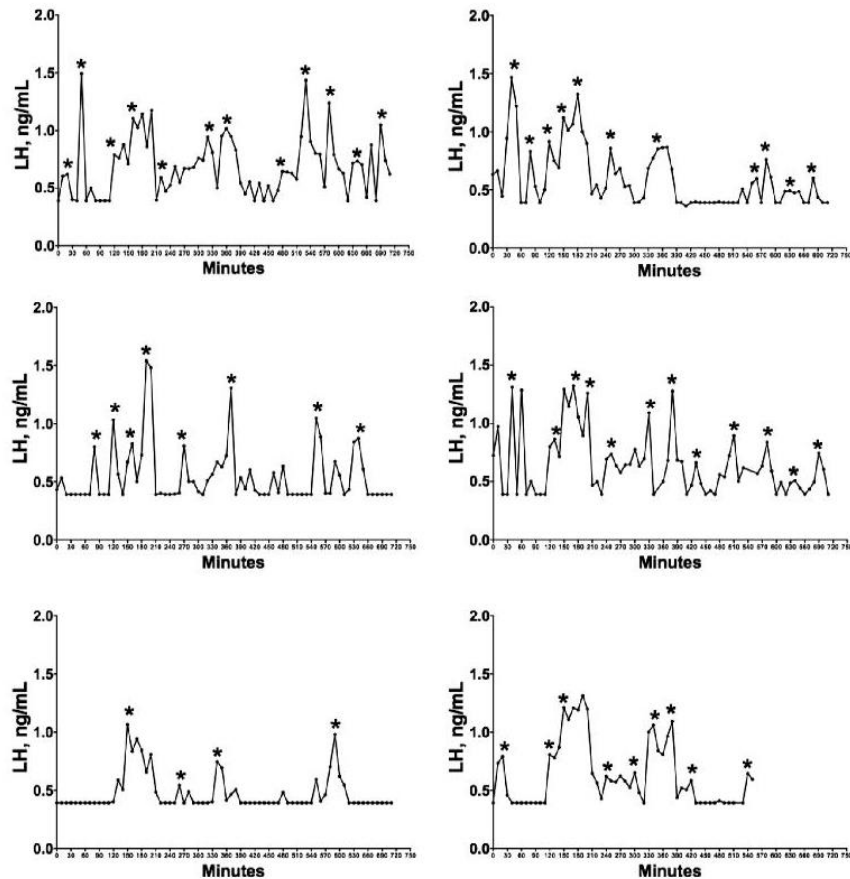


Figure 3. The tonic secretion of luteinizing hormone (LH) for pigs that developed in a male-biased litter. On day 3 after altrenogest withdrawal, blood samples were collected at 10 min intervals from 0900 to 2100 h and analyzed for the concentration of LH in ng/mL. Asterisk denotes pulse.

a reduced duration of the LH surge ($P < 0.05$), and a reduced area under the LH curve ($P < 0.05$) compared to gilts from female-biased litters. The amplitude of the LH surge was not significantly different ($P = 0.093$) between gilts from male-biased litters and gilts from female-biased litters. The individual profiles for LH during the preovulatory LH surge for gilts from male-biased litters are shown in Fig. 6 and the

gilts from male-biased litters are shown in Fig. 7. The change in mean LH for gilts from male-biased and gilts from female-biased litters is shown in Fig. 8.

DISCUSSION

Our data indicate that the LH surge of gilts that developed in a male-biased uterine environment was

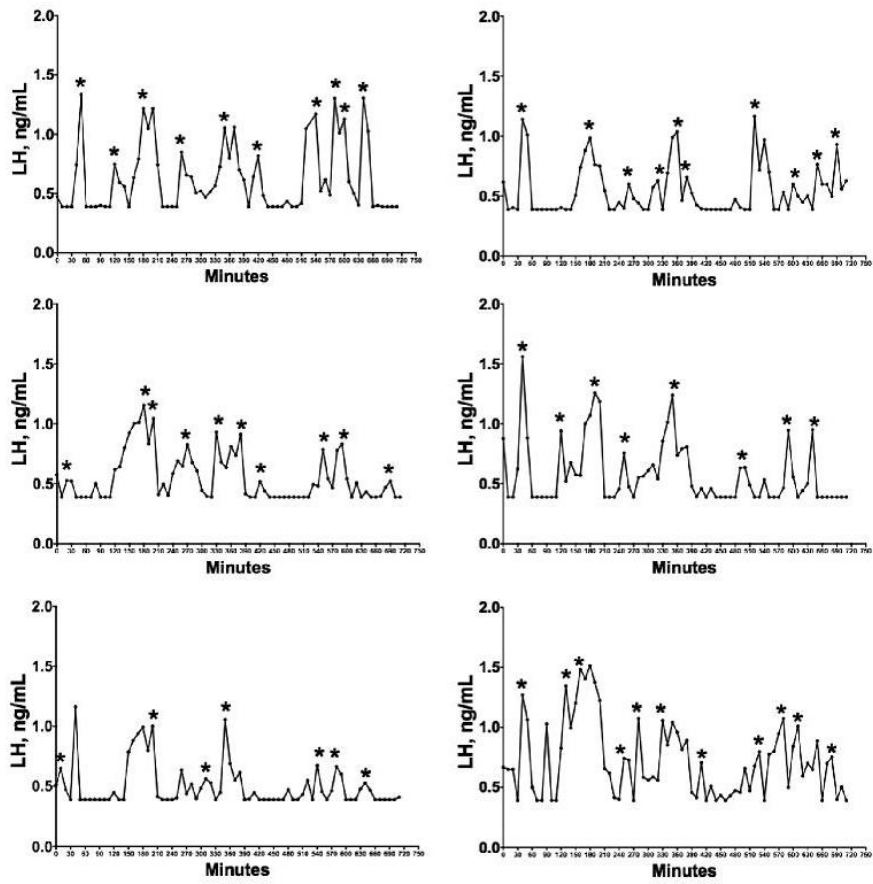


Figure 4. The tonic secretion of luteinizing hormone (LH) for pigs that developed in a female-biased litter. On day 3 after altrenogest withdrawal, blood samples were collected at 10 min intervals from 0900 to 2100 h and analyzed for the concentration of LH in ng/mL. Asterisk denotes a pulse.

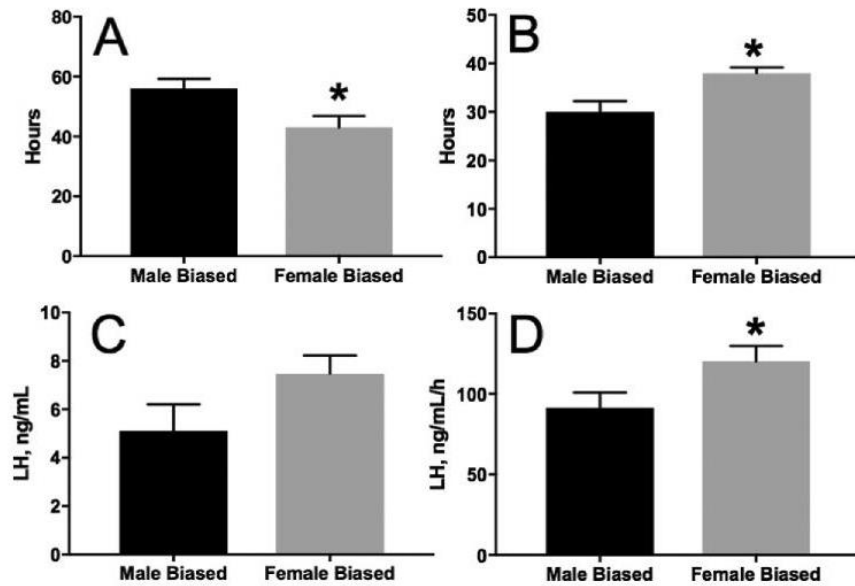


Figure 5. The characteristics of the preovulatory luteinizing hormone (LH) surge for gilts from male-biased and female-biased litters. The characteristics are the time of onset of the LH surge (panel A), the duration of the LH surge (panel B), the peak amplitude of the LH surge (panel C), and the area under curve of the LH surge (panel D). Asterisk denotes significant difference $P < 0.05$.

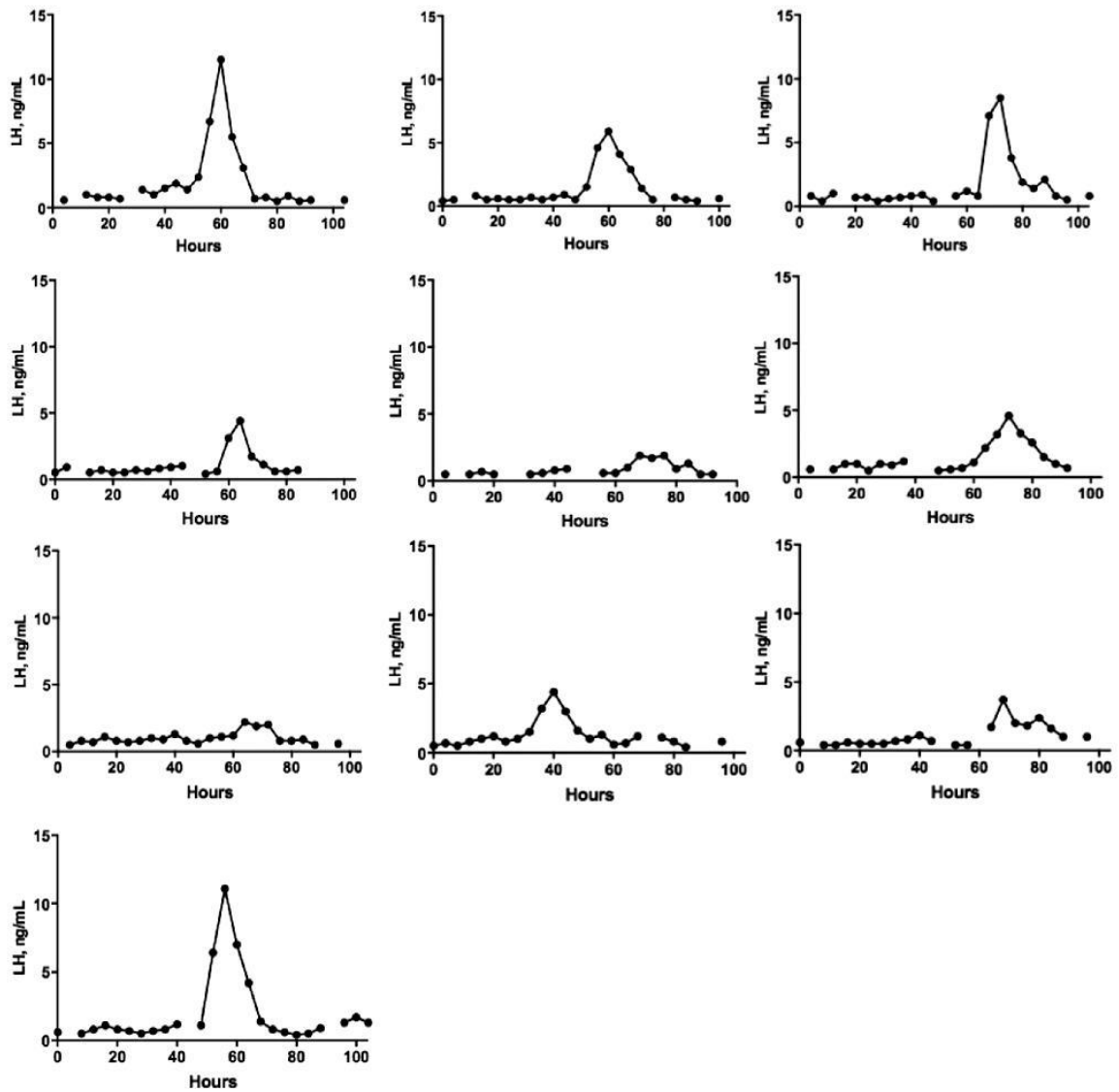


Figure 6. The profile of luteinizing hormone (LH) secretion during the LH surge for gilts that developed in a male-biased litter. To characterize the dynamics of the LH surge, starting at 0900 on day 4 after altrenogest withdrawal, blood was collected and analyzed for the concentration LH in ng/mL every 4 h until the end of estrus as determined by a failure to stand to back pressure in the presence of a boar. Hours on the x-axis denotes hours after commencement of sampling.

inhibited whereas the tonic secretion of LH was not affected. In gilts that developed in a male-biased uterine environment, the onset of the LH surge was delayed, the duration of the LH surge was reduced, and the plasma concentration of LH released during the surge was decreased. In contrast, none of the parameters of pulsatile secretion of LH differed between gilts that developed in a male-biased or female-biased uterine environment. A key point of difference between a male- and female-biased uterine environment in sheep and rodents is the predominance of sex steroids that are synthesized, with the

male-biased uterine environment being dominated by the synthesis of androgens. While this is a tempting explanation for our results in the pig, there is good evidence to suggest that steroids produced by male pigs in utero do not influence the concentration of testosterone in their female littermates (Framstad et al., 1990; Wise and Christenson, 1992). The mechanisms that cause the LH surge are complex and multifaceted and they are initiated by the positive feedback of estrogens on the hypothalamus and anterior pituitary gland (Goodman, 2014). Disruption of the LH surge will impact the onset of estrus, the timing of

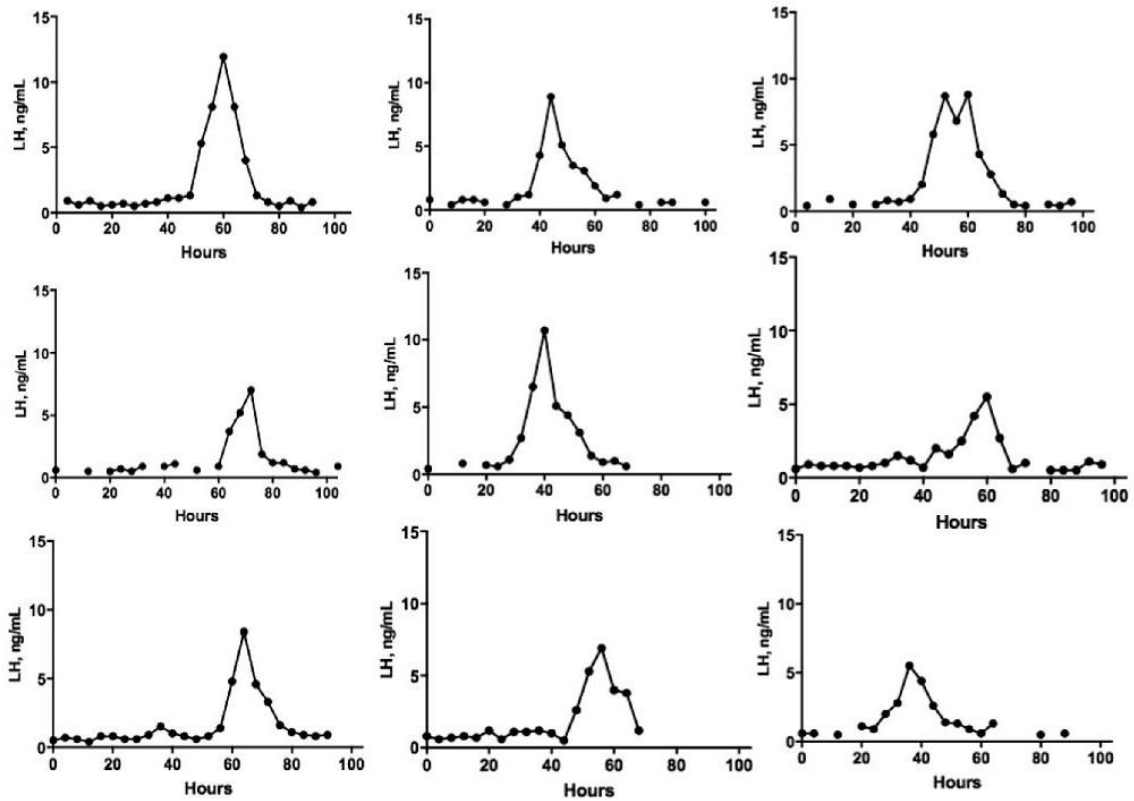


Figure 7. The profile of luteinizing hormone (LH) secretion during the LH surge for gilts that developed in a female-biased litter. To characterize the dynamics of the LH surge, starting at 0900 on day 4 after altrenogest withdrawal, blood was collected and analyzed for the concentration LH in ng/mL every 4 h until the end of estrus as determined by a failure to stand to back pressure in the presence of a boar. Hours on the x-axis denotes hours after commencement of sampling.

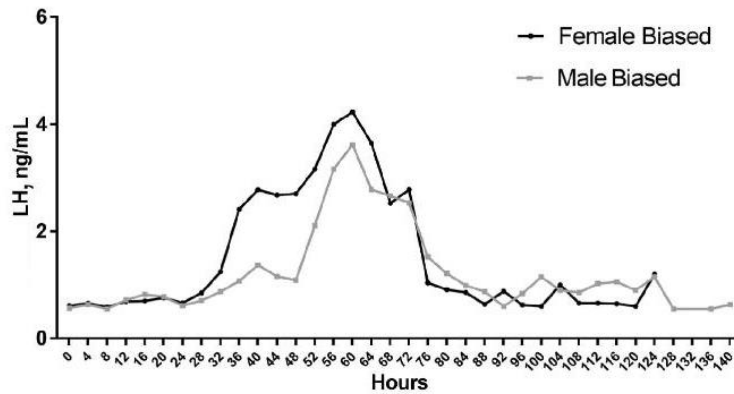


Figure 8. The mean luteinizing hormone (LH) during the LH surge for gilts that developed in a female-biased litter and gilts that developed in a male-biased litter. To characterize the dynamics of the LH surge, starting at 0900 on day 4 after altrenogest withdrawal, blood was collected and analyzed for the concentration LH in ng/mL every 4 h until the end of estrus as determined by a failure to stand to back pressure in the presence of a boar. Hours on the x-axis denotes hours after commencement of sampling. The within subjects standard error of the mean was 0.85.

ovulation, and the synchrony between these events which will, in turn, affect the chances of conception whether from natural or artificial insemination. Irrespective of the mechanisms involved, our data indicate that the sex bias of the uterine environment in

which a gilt develops has the potential to affect her reproductive success and this is consistent with much of the published literature in pigs (Lamberson et al., 1988; Parfet et al., 1990a, 1990b; Drickamer et al., 1997; Drickamer et al., 1999).

The mechanisms by which a male-biased uterine environment affects the LH surge in gilts cannot be determined from this study. Research in sheep that has unraveled the effects of the steroidal environment of the uterus in which females develop on the secretion of LH when the females have attained puberty (Robinson, 2006; Cheng et al., 2010; Abi Salloum et al., 2012) may provide some insight. When the circulating concentration of testosterone was increased in sows by injecting testosterone propionate, the stimulatory estrogen feedback on LH secretion was impaired in gilts at 160 d of age (Elsaesser and Parvizi, 1979; Petric et al., 2004). The concentration of testosterone in amniotic fluid and maternal and fetal circulation was increased in female fetuses when sows were injected with testosterone propionate (Ford and Christenson, 1987). This indicates that elevation of testosterone in maternal circulation via exogenous treatment can increase testosterone in the developing fetus. Female pigs that developed between 2 males, however, were not exposed to greater concentrations of testosterone than females that developed between 2 females (Framstad et al., 1990; Wise and Christenson, 1992). The deficits we have detected in the dynamics of the LH surge do resemble the effects of exposure to excessive concentrations of testosterone in utero and are consistent with previous research in the pig. This previous research indicated gilts from a male-biased litter were less likely to conceive at their first mating, were more sensitive to gonadotropins, were more aggressive and more males in a litter resulted in less teats (Drickamer et al., 1997; Drickamer et al., 1999, Seyfang et al., 2017, 2018). Gilts that developed in utero between 2 males reached puberty earlier than gilts that developed between 2 females while gilts that developed between 2 females showed enhanced receptivity (Parfet et al., 1990a, 1990b). Given the evidence in pigs that the gonadal steroids synthesized by males in utero did not influence the concentration of their female littermates and the epitheliochorial placenta of the pig (Framstad et al., 1990; Wise and Christenson, 1992) the pig may present a unique model. Exogenous testosterone treatment can affect the LH surge via the action of the androgens, however, the relevance of this in a natural situation is questionable and not yet clear. Further research is needed to better understand how a male-biased uterine environment affects a number of aspects of gilt reproduction including the effects on surge secretion of LH described in the current experiment.

We detected no difference in the frequency or amplitude of LH pulses between gilts that

developed in a male-biased litter and gilts that developed in a female-biased litter. This may be because the regions of the brain that control tonic and surge secretion of LH in pigs can be influenced at different periods of development in utero. Unlike sheep and rodents, little information on the precise mechanisms or brain regions that control surge and tonic secretion of LH exists in pigs, nor does information regarding the timing of development of these brain regions. If there are differences in the timing of development of the mechanisms that regulate tonic and surge secretion of LH in pigs, this would help explain our findings. We acknowledge that this is a hypothesis and requires testing, however, indirect evidence directly related to the gonadal steroids suggest that specific aspects of the reproductive axis can be affected at different times during gestation in gilts (Elsaesser and Parvizi, 1979; Petric et al., 2004). When pigs were treated with testosterone at day 30 of gestation the external genitalia of the female piglets were masculinized but when pigs were treated with testosterone at day 40 of gestation the external genitalia were unaffected (Petric et al., 2004). In addition, Petric et al. (2004) showed that the critical window for masculinizing the LH surge of female pigs was between days 35 and 39 of gestation and that the tonic release of LH was not affected by treatment with testosterone between days 30 and 36 of gestation or days 40 and 46 of gestation (Petric et al., 2004). Combined, these data suggest that the critical period for the organizational effects of the uterine environment on the mechanisms that regulate LH are unlikely to be one specific period during gestation but are likely to occur prior to day 90 of gestation. Our data support this view and support the likelihood that the mechanism which controls the LH surge in pigs may be influenced by the prenatal environment at a different time during gestation than the mechanism that controls tonic release of LH. Further research is needed to determine the mechanism and regions of the brain that regulate LH release in the pig and this may help explain the current data and advance our understanding of the critical period during gestation that the mechanism that regulates LH production in the pig can be affected.

The sex ratio of the birth litter affected surge secretion of LH but did not have an effect on tonic secretion of LH in female pigs. This partially supports our hypothesis. The current study has highlighted that there may be different periods during gestation where the prenatal environment can affect the control of the secretion of LH in female pigs

and reaffirms the need to better understand the neuroendocrine control of reproduction in the pig. This includes the mechanisms that regulate the effects of a male-biased litter on gilt reproduction. While the sex bias of a gilts birth litter can affect many aspects of the reproductive axis and this includes the surge secretion of LH, the mechanism remains elusive.

Conflict of interest statement. None declared.

LITERATURE CITED

- Abi Salloum, B., C. Herkimer, J. S. Lee, A. Veiga-Lopez, and V. Padmanabhan. 2012. Developmental programming: prenatal and postnatal contribution of androgens and insulin in the reprogramming of estradiol positive feedback disruptions in prenatal testosterone-treated sheep. *Endocrinology*. 153:2813–2822. doi:10.1210/en.2011-2074
- Barb, C. R., R. R. Kraeling, G. B. Rampacek, E. S. Fonda, and T. E. Kiser. 1982. Inhibition of ovulation and LH secretion in the gilt after treatment with ACTH or hydrocortisone. *J. Reprod. Fertil.* 64:85–92. doi:10.1530/jrf.0.0640085
- Cheng, G., L. M. Coolen, V. Padmanabhan, R. L. Goodman, and M. N. Lehman. 2010. The kisspeptin/neurokinin B/dynorphin (kndy) cell population of the arcuate nucleus: sex differences and effects of prenatal testosterone in sheep. *Endocrinology*. 151:301–311. doi:10.1210/en.2009-0541
- Drickamer, L. C., R. D. Arthur, and T. L. Rosenthal. 1997. Conception failure in swine: importance of the sex ratio of a female's birth litter and tests of other factors. *J. Anim. Sci.* 75:2192–2196. doi:10.2527/1997.7582192x
- Drickamer, L. C., T. L. Rosenthal, and R. D. Arthur. 1999. Factors affecting the number of teats in pigs. *J. Reprod. Fertil.* 115:97–100. doi:10.1530/jrf.0.1150097
- Elsaesser, F., and N. Parvizi. 1979. Estrogen feedback in the pig: sexual differentiation and the effect of prenatal testosterone treatment. *Biol. Reprod.* 20:1187–1193. doi:10.1095/biolreprod20.5.1187
- Ford, J. J., and R. K. Christenson. 1987. Influences of pre- and postnatal testosterone treatment on defeminization of sexual receptivity in pigs. *Biol. Reprod.* 36:581–587. doi:10.1095/biolreprod36.3.581
- Framstad, T., D. Grønset, N. Norman, and W. Velle. 1990. Androgens in fetal pigs in relation to sex of neighbour(s). *Zentralbl. Veterinarmed. A.* 37:45–50. doi:10.1111/j.1439-0442.1990.tb00874.x
- Goodman, R. L. 2014. Neuroendocrine control of gonadotropin secretion: comparative aspects. In: Knobil and Neill's *Physiology of Reproduction* London: Academic Press; 1537–1574.
- Lamberson, W. R., R. M. Blair, K. A. Rohde Parfet, B. N. Day, and R. K. Johnson. 1988. Effect of sex ratio of the birth litter on subsequent reproductive performance of gilts. *J. Anim. Sci.* 66:595–598. doi:10.2527/jas1988.663595x
- Niswender, G. D., L. E. Reichert, Jr, A. R. Midgley, Jr, and A. V. Nalbandov. 1969. Radioimmunoassay for bovine and ovine luteinizing hormone. *Endocrinology*. 84:1166–1173. doi:10.1210/endo-84-5-1166
- Parfet, K. R., V. K. Ganjam, W. R. Lamberson, A. R. Rieke, F. S. Vom Saal, and B. N. Day. 1990a. Intrauterine position effects in female swine: subsequent reproductive performance, and social and sexual behavior. *Appl. Anim. Behav. Sci.* 26:349–362. doi:10.1016/0168-1591(90)90034-B
- Parfet, K. A., W. R. Lamberson, A. R. Rieke, T. C. Cantley, V. K. Ganjam, F. S. vom Saal, and B. N. Day. 1990b. Intrauterine position effects in male and female swine: subsequent survivability, growth rate, morphology and semen characteristics. *J. Anim. Sci.* 68:179–185. doi:10.2527/1990.681179x
- Petric, N., Y. Kato, and F. Elsaesser. 2004. Influence of prenatal testosterone treatment on foetal and prepubertal lhbeta-subunit mRNA and plasma LH concentrations in the female pig. *Domest. Anim. Endocrinol.* 27:25–38. doi:10.1016/j.domaniend.2004.01.003
- Robinson, J. 2006. Prenatal programming of the female reproductive neuroendocrine system by androgens. *Reproduction*. 132:539–547. doi:10.1530/rep.1.00064
- Seyfang, J., C. R. Ralph, A. J. Tilbrook, and R. N. Kirkwood. 2017. Response to gonadotrophins differs for gilts from female- and male-biased litters. *Anim. Reprod. Sci.* 182:134–137. doi:10.1016/j.anireprosci.2017.05.012
- Seyfang, J., R. N. Kirkwood, A. J. Tilbrook, and C. R. Ralph. 2018. The sex ratio of a litter affects the behaviour of its female pigs until at least 16 weeks of age. *Appl. Anim. Behav. Sci.* 200:45–50. doi:10.1016/j.applanim.2017.12.001
- Turner, A. I., P. H. Hemsworth, B. J. Canny, and A. J. Tilbrook. 1999. Inhibition of the secretion of LH in ovariectomised pigs by sustained but not repeated acute elevation of cortisol in the absence but not the presence of oestradiol. *J. Endocrinol.* 163:477–486. doi:10.1677/joe.0.1630477
- Wise, T. H., and R. K. Christenson. 1992. Relationship of fetal position within the uterus to fetal weight, placental weight, testosterone, estrogens, and thymosin beta 4 concentrations at 70 and 104 days of gestation in swine. *J. Anim. Sci.* 70:2787–2793. doi:10.2527/1992.7092787x

Chapter 5:

Anogenital distance reflects the sex ratio of a gilt's birth litter and predicts her reproductive success

Statement of Authorship

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Contribution to the Paper	Assisted with the design of the experiment, managed and carried out the experimental trial, analysed statistics, drafted and edited manuscript.				
Overall percentage (%)	85				
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.				
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

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- iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Michelle Hebart				
Contribution to the Paper	Designed the statistical model.				
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Name of Co-Author	Cameron Ralph				
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Anogenital distance reflects the sex ratio of a gilt's birth litter and predicts her reproductive success.¹

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ABSTRACT: Anogenital distance (AGD) has been used to reflect masculinization in litter bearing species. As masculinization affects behavior and reproduction, AGD could be measured to assist in selecting gilts with a temperament more suited to commercial production and greater reproductive potential. We hypothesized that gilts from a male biased litter would have a longer AGD and poorer reproductive performance. In experiment one, AGD and weight were measured at d 1, d 21, and wk 16 of age for gilts from male biased ($\geq 60\%$ males; $n = 51$) and female biased ($\geq 60\%$ females; $n = 51$) litters. Sow AGD was measured 3 d after farrowing. In experiment two, AGD was measured at gilt selection at approximately 24 wks of age and gilts followed to second parity. Litter sex ratio affected AGD at 16 wks of age, with gilts from female biased litters having longer AGD (mean \pm SEM, 9.1 ± 0.7 mm vs 11.0 ± 0.6 mm $P = 0.013$). AGD was not different on d 1 or d 21. There was no effect of sex ratio on weight at any time and sow AGD was not associated with the sex ratio of her litter. Gilts with an AGD longer than the mean of 11.55 mm were heavier (mean \pm SEM, 118.8 ± 0.4 kg vs 117.7 ± 0.4 kg, $P = 0.023$), achieved puberty earlier (179.6 ± 0.6 d vs 182.2 ± 0.6 d, $P = 0.001$), were mated younger (200.6 ± 0.6 d vs 203.2 ± 0.6 d, $P = 0.001$), and were more likely to be mated (91% vs 83% , $P = 0.005$) than gilts with an AGD shorter than the mean. Gilts with an AGD greater than 11.55 mm had a greater born alive litter size (11.79 ± 0.20 vs 11.20 ± 0.19 , $P = 0.018$) compared to gilts with an AGD shorter than 11.55 mm. At 16 wks, AGD was associated with sex bias and could be used as a selection tool to predict reproductive success of the first parity, with a longer AGD being associated with gilts that had been born into a female biased litter and that had better reproductive performance.

Key words: gilts, litter sex bias, reproduction, selection.

INTRODUCTION

The proportion of males in a litter can affect the phenotype and physiology of gilts that develop in that litter. Gilts from male biased litters were more likely to fail to conceive on their first mating, were more sensitive to gonadotropins, had less teats than gilts from a female biased litter (Drickamer et al., 1997; Drickamer et al., 1999; Seyfang et al., 2017a). These traits coincide with what has been recorded in masculinized female rodents. Masculinized female rodents had an altered phenotype that included nipple retention, increased anogenital distance (AGD) and impaired reproductive potential (Hotchkiss et al., 2007). The AGD is measured from the anus to the genitals and can be used as an indicator of female masculinization (Clark et al., 1993; vom Saal et al., 1999; Correa et al., 2013). Males have longer AGD than females and there is variation within sex, with masculinized females having longer AGD than non-masculinized females (vom Saal et al., 1999). In rodents, masculinization can result from intrauterine position or litter sex ratio, with females developing between two males, or females from male biased litters, becoming masculinized (Clark et al., 1993; vom Saal et al., 1999). Sex biased litters are common in commercial pig production. Sex biased litters ($\geq 60\%$ males or females) can result for a variety of reasons including a sex biased conception, implantation, or fetal mortality (Clark et al., 1993; James, 2004; Grant et al., 2008; Rekiel et al., 2012). In the first of two experiments, we hypothesized that gilts from male biased litters will have longer AGD than gilts from female biased litters. The first experiment also aimed to determine an appropriate age to measure AGD to predict reproductive performance. In experiment two, we hypothesized that gilts with a longer AGD would have poorer reproductive performance than gilts with a shorter AGD. If AGD reflects the sex ratio of the birth litter and is reflective of future reproductive performance, then AGD could be a valuable selection tool.

MATERIALS AND METHODS

Experiment one

This experiment was conducted at the Roseworthy Piggery, The University of Adelaide (South Australia) in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC 2013). It was approved by the University of Adelaide Animal Ethics Committee (approval number S-2015-060B).

A total of 102 terminal line gilts from Large White x Landrace sows were selected from 40 litters consisting of $\geq 60\%$ male (male biased; $n = 51$) or $\geq 60\%$ female (female biased; $n = 51$) piglets, including stillborn piglets. Up to three gilts per litter were selected and were raised under standard Australian commercial conditions; born in farrowing crates in a batch farrow system and weaned at approximately d 28 into a straw-based ecoshelter. The study began in May 2015 and was completed in December 2015 over four farrowing batches. Pigs were allowed free access to feed and water at all times.

Using digital calipers, the AGD (mm) was measured from the anus to the start of vulva opening (AGD1), to the end of the vulva opening (AGD2), and to the ventral tip of the vulva (AGD3) of 40 sows three days after farrowing at which time they were also weighed. Four sows were excluded from the analysis due to injury to the vulva. The AGD of three female offspring from each litter were also measured at d 1 and d 21 after birth, and at wk 16. The same animals were used at each timepoint with a maximum sample size at d 1. Due to management practices not all animals could be used at each of the subsequent timepoints. Mortalities were recorded throughout, however, no animals that died prior to wk 16 were included in this analysis. The piglets were weighed at each time. Day 21 was used as a measure of weaning AGD. The AGD was measured at wk 16 in experiment one as this is when gilt selection occurs at the Roseworthy piggery and we wanted to determine whether AGD measurement at

the time of selection could be included in the selection criteria. The birth sow AGD was used to determine if there was a relationship between sow AGD and the sex ratio of her litter.

Experiment two

From November to December 2016 on a commercial SunPork facility in South Australia, 747 Landrace, and Landrace x Large White gilts were selected at approximately 24 wks of age. All procedures complied with the farm's internal animal welfare standards. Gilts weighed from 105 to 130 kg, had at least 14 teats and had good leg conformation. The AGD from the anus to the start of the vulva opening was measured at selection using digital calipers. Boar exposure for 20 min/d began the day after selection in a detection-mating area. A boar was housed with approximately 20 gilts and with fenceline contact with a further 3 boars until the gilts exhibited their pubertal estrus. Gilts were considered estrous if they stood to backpressure in the presence of a boar. If estrus was not detected within 6 wks of exposure the gilts were culled. After estrus detection, gilts were housed in pens with other gilts achieving estrus and checked daily for second estrus from 18 d in the detection mating area. Gilts were inseminated with 3×10^9 sperm in 80 mL extender at detection of their second estrus and again 24 h later if still in estrus. After mating gilts were transferred to a group housed straw based system with 50 - 100 gilts per group. Animals were fed a standard commercial diet and had free access to water at all times. Pregnancy status was confirmed at 4 wks by ultrasound and non-pregnant gilts were removed. Gilts were re-housed into conventional farrowing crates 3 d before due date. Immediately after farrowing the litter size born alive, stillborn, and mummified fetuses was recorded, as was total number of females (born alive and stillborns). The total litter size was calculated as born alive, stillborns, and mummified fetuses combined. The percentage of females was calculated as [total number of females (born alive + stillborn)/ litter size

(born alive + stillborn)]. Retrospective data mining of Metafarms was used to collect remating and second parity data. Culled gilts were recorded throughout the experimental period from selection, with reasons for culling being anestrus, negative pregnancy test, not in pig, lameness, illness, injury, death, or unknown reason. Culling results were then separated into first and second parity, with first parity being from selection until weaning of their first litter, and second parity from weaning of their first litter until weaning of the second litter.

Statistical methods

To determine whether there was a difference in AGD in gilts from male biased litter compared to gilts from a female biased litter in experiment one, AGD 1, 2 and 3 were analyzed using a linear mixed model (IBM SPSS Statistics 22) for each time point. The fixed factors included in the model were weight, parity (0 to 5), and skew ($\geq 60\%$ male = male biased or $\geq 60\%$ female = female biased), with litter ID as a random factor and weight included as a covariate. Gilt body weight at each time was also run as the dependent variable with fixed factors sow parity and skew, with litter ID as a random factor. The same method was employed to analyze sow AGD 1, 2 and 3, with sow weight as a covariate, to determine if there was any relationship with the sex ratio of their offspring litter. To determine if there was a difference in reproductive performance between gilts with short AGDs compared with longer ADGs, experiment two data were used. The AGD measurements were divided into short and long AGDs; based on the mean AGD (11.55 ± 2.68 mm). Short AGDs were defined as those that were shorter than the mean (5.81 mm to 11.55 mm) and long AGDs as those longer than the mean (11.56 mm to 17.36 mm). A general linear model was used for weight at selection, age at puberty, age at first mating, born alive litter size, total born litter size, proportion of females in the litter, and the wean to estrus interval with weight at selection included as a covariate and breed (Landrace, Landrace X Large White), date selected, and AGD

category as fixed factors. Due to the distribution of the data, a generalized linear model with a Poisson distribution was used for the stillborn and mummified litter size. A generalized linear model with a binomial distribution was used for the percentage of gilts mated, positive pregnancy check, percentage that did not farrow after a positive pregnancy check (NIPs), farrowing rate, and culled percentage. Again, these tests included weight as a covariate and breed, date selected, and AGD category as fixed factors. A chi-square test was used for the cull reason classifications. The threshold for significance was $P < 0.05$. All AGD data was analyzed and is presented in millimeters.

RESULTS

Experiment one

At d 1 of age, the AGD (AGD1, AGD2, or AGD3; mean \pm SEM) of gilts from female biased litters was not different to the AGD of gilts from male biased litters. At d 21, AGD2 was longer for gilts from female biased litters than gilts from male biased litters ($P = 0.036$, Table 1). At wk 16, AGD1 and AGD2 were longer for gilts from female biased litters than gilts from male biased litters ($P = 0.013$ and $P = 0.019$ respectively, Table 1). The body weight of gilts from female biased litters was not different to the body weight of gilts from male biased litters at any time. The AGD of the birth sow (AGD1, AGD2, or AGD3) was not associated with the sex ratio of her litter (Table 2).

Experiment two

Gilts with long AGDs were heavier at selection at 24 wks of age ($P = 0.023$) and, with weight as a covariate, they achieved puberty earlier and were mated earlier than those having shorter than average AGD ($P = 0.001$, Table 3) and were more likely to be mated ($P = 0.005$, Table 3). Gilts with a short AGD were predominantly culled for anestrus (40 % of culls), whereas those with a long AGD were more likely to be culled for a negative pregnancy check (35 % of culls). There was an increase in total born alive for gilts with long AGDs compared to short AGDs in their first parity ($P = 0.018$, Table 3). There were no other differences in reproductive performance in first or second parity.

DISCUSSION

The current research provides evidence that gilts with an AGD greater than 11.55 mm at 24 wks of age performed more effectively in a commercial breeding herd than gilts with an AGD less than 11.55 mm. In addition, our research indicates that gilts with a greater AGD at 16 wks of age were likely from a female biased litter. Females from female biased litters are generally better suited as potential replacement females as they are reproductively more efficient and productive than females from male biased litters and our results align with these findings (Edgerton and Cromwell 1987). Our result that gilts from a female biased litter had a longer AGD than gilts from a male biased litter is inconsistent with previous research conducted in rodents and pigs (McDermott et al., 1978; Drickamer et al., 1997; vom Saal et al., 1999; Nagao et al., 2004; Correa et al., 2013). Nonetheless, we provide evidence that AGD can be used to predict reproductive success in pigs and that the AGD of pigs from female biased litters was greater than the AGD of pigs from male biased litters.

That gilts from male biased litters had shorter AGD than gilts from female biased litters at 16 weeks of age suggests species differences accounting in AGD determinations. Rodents have a different endocrine profile during pregnancy than sows and this difference may result in greater aromatisation of testosterone to estrogen at the time when the external genitalia are being formed. The progesterone levels in the maternal environment of rabbits, rats and mice are low during sexual differentiation and external genitalia development, which occurs in early gestation (Garland et al., 1987). In the pig, however, progesterone reaches its peak concentration by early pregnancy (Robertson and King 1974). When the concentration of progesterone is high aromatase activity is high, therefore, testosterone is more likely to be aromatised to estrogen. This means that although testosterone concentrations may be increased in male biased litters around the time of external genitalia development, it is more likely

to be aromatized to estrogen in pigs as they have higher maternal levels of progesterone at this time than in other species. This would then lead to an increase in estrogen exposure during the development of external genitalia. In rodents increased AGD is usually reflective of exposure to excessive concentrations of androgens in utero (McDermott et al., 1978; vom Saal et al., 1999; Nagao et al., 2004) while excessive levels of estrogen would result in a decreased AGD (Mitchell et al., 2015). This may mean that in our study the AGDs were reduced in male biased litters and that AGDs from female biased litters were normal, not increased. Mack et al. (2014) found that males from a female biased litter had an increased AGD compared to those from a male biased litter, highlighting that the sex ratio of a litter can alter the AGD in pigs, and that a female biased litter may result in phenotypic masculinization. In pigs, this may be due to increasing the number of females in a litter decreasing the amount of estrogen, which may result in masculinization of the offspring in female biased litters (Mack et al., 2014). Mitchell et al. (2015) also highlighted that AGD can change over time depending on the balance of estrogen and testosterone in the animal. This may be why our results at 16 weeks of age differ from those of Drickamer et al. (1997) who found that day old gilts from male biased litters did exhibit a masculine phenotype with an increased AGD. The timing of AGD development may occur during a short window of time, as indicated by Petric et al. (2004), who found that female pigs from sows treated with testosterone at d 30 of gestation would exhibit masculinized external genitalia but the same treatment at d 40 resulted in no change. Female biased litters may have lower levels of estrogens in utero at day 30 of gestation compared to male biased litters as female fetuses produce estrogens later in development than do males (Parma et al., 1999; McCoard et al., 2002). Further, testes also release estrogens and in pigs the testes produce more estrogen than many other species (Booth 1983; Raeside et al., 1993), again suggesting a species difference in the hormone profile of the uterine environment at the time of genitalia development, which could impact AGD.

Boars also have higher levels of plasma estrogens than gilts both pre- and postnatally (Choong and Raeside 1974; Booth 1983; Raeside et al., 1993; Tarraf and Knight 1995). This contributes to the theory that AGD are feminized in male biased litters, but that these females are masculinized in other aspects such as their reproduction due to the difference in timing of development relative to the AGD. The effects of a male biased litter on reproduction were consistent with the literature, suggesting that gilts from a male biased litter have been masculinized in utero (Clark et al., 1993; vom Saal et al., 1999; Bánszegi et al., 2012). Being able to determine the birth litter sex ratio using AGD would be beneficial in a gilt selection scheme as the sex ratio of the birth litter can affect behavior and elements of reproduction in pigs (Seyfang et al., 2017a; Seyfang et al., 2017b).

Experiment two showed that gilts that had a longer AGD achieved puberty earlier and had larger litter sizes than gilts with shorter AGD, which, based on data from experiment one, suggests these females were from female biased litters and presumably not masculinized. It is interesting that the effect on litter size was limited to the first parity. The reason for the parity effect is not known but may reflect additional management factors masking potential improvements in reproduction, particularly lactation management. Previous studies in pigs have found that gilts from female biased litters were less aggressive and less likely to ovulate at 18 wks of age in response to exogenous gonadotrophins (Seyfang et al., 2017a; Seyfang et al., 2017b), and Drickamer et al. (1997) found that gilts from male biased litters were less likely to become pregnant after their first mating than gilts from female biased litters. This again highlights that the sex ratio of the birth litter can affect masculinization and reproductive performance.

The AGD of female pigs was associated with birth litter sex ratio, with the current work showing that at 16 wks of age AGD could reflect the sex bias of the birth litter.

Whilst we have not tested the mechanism controlling the AGD in pigs, it is possible that the physiological mechanism in pigs is different to that in rodents. In pigs, the steroids produced by males in utero does not influence the concentration of testosterone in the neighbouring females (Framstad et al., 1990; Wise and Christenson 1992). However, rodents have hemochorial placentation whereas pigs have an epitheliochorial placenta. This may change the mode of transfer of androgens or other male hormones that could influence the AGD, therefore resulting in a species difference in AGD. This also supports the theory that the effects of males on their littermates is litter-wide in pigs, rather than an effect of the intrauterine position, as the male biased uterine environment does not affect testosterone levels between gilts within a litter, but it could still affect the testosterone levels of the litter compared to a litter with a different bias. Alternatively, it could be a different effect of the male biased uterine environment that affects the AGD in pigs. Measurement of AGD when gilts are selected for the breeding herd could enable producers to determine if the gilt was from a male or female biased birth litter, with a longer AGD being associated with better reproductive performance as shown by a decreased age at puberty, increased mating rate, and increased born alive in parity 1. Importantly, since the AGD at 16 wks of age reflected the birth sex ratio and the AGD was also positively associated with reproductive performance of gilts, it has the potential to be included in the selection procedure to increase reproductive uniformity and production.

LITERATURE CITED

Bánszegi, O., Szenczi, P., Dombay, K., Bilkó, Á., Altbäcker, V. 2012. Anogenital distance as a predictor of attractiveness, litter size and sex ratio of rabbit does. *Physiol. Behav.* 105(5), 1226-1230. [doi: 10.1016/j.physbeh.2012.01.002](https://doi.org/10.1016/j.physbeh.2012.01.002)

Booth, W. D. 1983. Development of some male characteristics supported by oestrone but not dehydroepiandrosterone in the boar. *J. Reprod. Fert.* 68(1), 9-16. doi: 10.1530/jrf.0.0590155

Choong, C. H., Raeside, J. I. 1974. Chemical determination of oestrogen distribution in the foetus and placenta of the domestic pig. *Acta endocrinologica*, 77(1), 171-185. doi: 10.1530/acta.0.0770171

Clark, M. M., Karpiuk, P., Galef, B. G. 1993. Hormonally mediated inheritance of acquired characteristics in Mongolian gerbils. *Nature* 364(6439), 712-712.

Correa, L. A., Frugone, M. J., Soto-Gamboa, M. 2013. Social dominance and behavioral consequences of intrauterine position in female groups of the social rodent *Octodon degus*. *Physiol. Beh.* 119, 161-167. [doi: 10.1016/j.physbeh.2013.06.002](https://doi.org/10.1016/j.physbeh.2013.06.002)

Drickamer, L. C., Arthur, R. D., Rosenthal, T. L. 1997. Conception failure in swine: importance of the sex ratio of a female's birth litter and tests of other factors. *J. Anim. Sci.* 75(8), 2192-2196. doi:10.2527/1997.7582192x

Drickamer, L. C., Rosenthal, T. L., Arthur, R. D. 1999. Factors affecting the number of teats in pigs. *J. Reprod. Fertil.* 115(1), 97-100. doi: 10.1530/jrf.0.1150097

Edgerton, L. A., Cromwell, G. L. 1987. Sex of siblings may influence reproductive performance in sows. *J. Anim. Sci.* 63(1), 365.

- Garland, H. O., Atherton, J. C., Baylis, C., Morgan, M. R. A., Milne, C. M. 1987. Hormone profiles for progesterone, oestradiol, prolactin, plasma renin activity, aldosterone and corticosterone during pregnancy and pseudopregnancy in two strains of rat: correlation with renal studies. *J. Endocrinol.* 113(3), 435-444. doi: 10.1677/joe.0.1130435
- Grant, V. J., Irwin, R. J., Standley, N. T., Shelling, A. N., Chamley, L. W. 2008. Sex of bovine embryos may be related to mothers' preovulatory follicular testosterone. *Biol. Reprod.* 78(5), 812-815. doi: [10.1095/biolreprod.107.066050](https://doi.org/10.1095/biolreprod.107.066050)
- Hotchkiss, A. K., Lambright, C. S., Ostby, J. S., Parks-Saldutti, L., Vandenberg, J. G., Gray, L. E. 2007. Prenatal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague-Dawley rats. *Toxicol. Sci.* 96(2), 335-345. doi: [10.1093/toxsci/kfm002](https://doi.org/10.1093/toxsci/kfm002)
- James, W. H. 2004. Further evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels around the time of conception. *Hum. Reprod.* 19(6), 1250-1256. doi: [10.1093/humrep/deh245](https://doi.org/10.1093/humrep/deh245)
- Mack, L. A., Lay Jr, D. C., Eicher, S. D., Johnson, A. K., Richert, B. T., Pajor, E. A. 2014. Growth and reproductive development of male piglets are more vulnerable to midgestation maternal stress than that of female piglets. *J. Anim. Sci.* 92(2), 530-548. doi: [10.2527/jas.2013-6773](https://doi.org/10.2527/jas.2013-6773)
- McCoard, S. A., Wise, T. H., Ford, J. J. 2002. Expression levels of Mullerian-inhibiting substance, GATA4 and 17alpha-hydroxylase/17, 20-lyase cytochrome P450 during embryonic gonadal development in two diverse breeds of swine. *J. Endocrinol.* 175(2), 365-374. doi: [10.1677/joe.0.1750365](https://doi.org/10.1677/joe.0.1750365)

McDermott, N.J., Gandelman, R., Reinisch, J. M. 1978. Contiguity to male fetuses influences ano-genital distance and time of vaginal opening in mice. *Physiol. Behav.* 20(5), 661-663. [doi: 10.1016/0031-9384\(78\)90261-5](https://doi.org/10.1016/0031-9384(78)90261-5)

Mitchell, R. T., Mungall, W., McKinnell, C., Sharpe, R. M., Cruickshanks, L., Milne, L., Smith, L. B. 2015. Anogenital distance plasticity in adulthood: implications for its use as a biomarker of fetal androgen action. *Endocrinology*, 156(1), 24-31. [doi: 10.1210/en.2014-1534](https://doi.org/10.1210/en.2014-1534)

Nagao, T., Wada, K., Kuwagata, M., Nakagomi, M., Watanabe, C., Yoshimura, S., Saito, Y., Usumi, K., Kanno, J. 2004. Intrauterine position and postnatal growth in Sprague–Dawley rats and ICR mice. *Reprod. Toxicol.* 18(1), 109-120. [doi: 10.1016/j.reprotox.2003.10.009](https://doi.org/10.1016/j.reprotox.2003.10.009)

Parma, P., Pailhoux, E., Cotinot, C. 1999. Reverse transcription-polymerase chain reaction analysis of genes involved in gonadal differentiation in pigs. *Biol. Reprod.* 61(3), 741-748. [doi: 10.1095/biolreprod61.3.741](https://doi.org/10.1095/biolreprod61.3.741)

Petric, N., Kato, Y., Elsaesser, F. 2004. Influence of prenatal testosterone treatment on foetal and prepubertal LH β -subunit mRNA and plasma LH concentrations in the female pig. *Domest. Anim. Endocrinol.* 27(1), 25-38. [doi: 10.1016/j.domaniend.2004.01.003](https://doi.org/10.1016/j.domaniend.2004.01.003)

Raeside, J. I., Sigman, D. M. 1975. Testosterone levels in early fetal testes of domestic pigs. *Biol. Reprod.* 13(3), 318-321. [doi: 10.1095/biolreprod13.3.318](https://doi.org/10.1095/biolreprod13.3.318)

Rekiel, A., Więcek, J., Wojtasik, M., Ptak, J., Blicharski, T., Mroczko, L. 2012. Effect of sex ratio in the litter in which Polish Large White and Polish Landrace sows were born on the number of piglets born and reared. *Ann. Anim. Sci.* 12(2), 179-185. [doi: 10.2478/v10220-012-0015-5](https://doi.org/10.2478/v10220-012-0015-5)

Robertson, H. A., King, G. J. 1974. Plasma concentrations of progesterone, oestrone, oestradiol-17 β and of oestrone sulphate in the pig at implantation, during pregnancy and at parturition. *J. Reprod. Fertil.* 40(1), 133-141.

Seyfang, J., Ralph, C. R., Tilbrook, A. J., Kirkwood, R. N. 2017a. Response to gonadotrophins differs for gilts from female- and male-biased litters. *Anim. Reprod. Sci.* 182, 134-137. doi: [10.1016/j.anireprosci.2017.05.012](https://doi.org/10.1016/j.anireprosci.2017.05.012)

Seyfang, J., Kirkwood, R. N., Tilbrook, A. J., Ralph, C. R. 2017b. The sex ratio of a litter affects the behaviour of its female pigs until at least 16 weeks of age. *Appl. Anim. Behav. Sci.* In press. doi: [10.1016/j.applanim.2017.12.001](https://doi.org/10.1016/j.applanim.2017.12.001)

Tarraf, C. G., Knight, J. W. 1995. Effect of uterine space and fetal sex on conceptus development and in vitro release of progesterone and estrone from regions of the porcine placenta throughout gestation. *Domest. Anim. Endocrinol.* 12(1), 63-71. doi: [10.1016/0739-7240\(94\)00009-P](https://doi.org/10.1016/0739-7240(94)00009-P)

vom Saal, F. S., Clark, M. M., Galef, B. G., Drickamer, L. C., Vandenberg, J. G. 1999. Intrauterine position phenomenon. *Encyclopedia of Reproduction* 2, 893-900.

Tables

1.

	Male biased	Female biased	P value
D1	<i>n</i> = 51	<i>n</i> = 51	
Weight (kg)	1.77 ± 0.07	1.70 ± 0.08	0.406
AGD1 (mm)	4.62 ± 0.25	5.26 ± 0.25	0.051
AGD2 (mm)	11.26 ± 0.55	11.73 ± 0.57	0.514
AGD3 (mm)	17.09 ± 0.72	17.14 ± 0.76	0.963
D21	<i>n</i> = 26	<i>n</i> = 33	
Weight (kg)	7.01 ± 0.32	7.59 ± 0.27	0.107
AGD1 (mm)	5.73 ± 0.41	6.48 ± 0.34	0.105
AGD2 (mm)	10.33 ± 2.60	11.63 ± 2.58	0.036
AGD3 (mm)	16.46 ± 0.65	17.27 ± 0.54	0.265
Wk 16	<i>n</i> = 26	<i>n</i> = 35	
Weight (kg)	70.40 ± 2.24	71.20 ± 1.71	0.735
AGD1 (mm)	9.09 ± 0.72	11.01 ± 0.55	0.013
AGD2 (mm)	21.87 ± 1.22	25.17 ± 0.93	0.019
AGD3 (mm)	32.73 ± 1.61	34.94 ± 1.22	0.199

2.

	Male biased	Female biased	P value
Sow	<i>n</i> = 21	<i>n</i> = 15	
Weight (kg)	224.10 ± 8.03	218.75 ± 9.80	0.687
AGD1 (mm)	25.53 ± 2.01	26.96 ± 2.44	0.596
AGD2 (mm)	50.13 ± 3.78	51.55 ± 4.60	0.779
AGD3 (mm)	68.25 ± 4.26	66.92 ± 5.17	0.816

3.

	Short AGD <i>n</i> = 398	Long AGD <i>n</i> = 349	P value
Weight at selection (kg)	117.68 ± 0.36	118.78 ± 0.38	0.023
Age at puberty (days)	182.17 ± 0.61	179.56 ± 0.62	0.001
Age at first mating (days)	203.17 ± 0.61	200.56 ± 0.62	0.001
Mated (%)	83	91	0.005
Positive pregnancy check (%)	91	91	0.871
NIPS (%)	9	8	0.761
Parity 1			
Farrowing rate (%)	79	80	0.732
Born alive	11.20 ± 0.19	11.79 ± 0.20	0.018
Stillborn	0.77 ± 0.06	0.76 ± 0.06	0.948
Mummified	0.18 ± 0.03	0.14 ± 0.03	0.244
Total litter size	12.21 ± 0.20	12.70 ± 0.21	0.060
Females in offspring litter (%)	45.84 ± 1.13	46.76 ± 1.15	0.513
Culled (%)	33	27	0.096
Top cull reason	Anestrus (40%)	Negative pregnancy check (35%)	0.011
Remated (%)	90	92	0.602
Wean to estrus interval (days)	5.92 ± 0.38	6.24 ± 0.39	0.509
Positive pregnancy check (%)	84	88	0.137

Parity 2

Subsequent farrowing rate (%)	84	88	0.205
Born alive	11.99 ± 0.25	11.99 ± 0.26	0.996
Stillborn	0.50 ± 0.06	0.64 ± 0.07	0.054
Mummified	0.13 ± 0.03	0.13 ± 0.03	0.930
Total litter size	12.70 ± 0.26	12.85 ± 0.27	0.651
Culled (%)	23	19	0.284
Top cull reason	Negative pregnancy check (36%)	Negative pregnancy check (27%)	0.433

Table 1. Mean \pm SEM weight (kg) of gilts and mean \pm SEM anogenital distance (mm) as measured from the anus to the start of vulval opening (AGD1), to the end of the vulval opening (AGD2), and to the ventral tip of the vulva (AGD3) at d 1 after birth, d 21, and wk 16 for gilts from male biased litters and gilts from female biased litters. The maximum sample size was reached at d 1 and a subset of those animals were recorded at the subsequent timepoints due to management restraints.

Table 2. Mean \pm SEM weight (kg) of sows and mean \pm SEM anogenital distance (mm) as measured from the anus to the start of vulva opening (AGD1), to the end of the vulva opening (AGD2), and to the tip of the vulva (AGD3) 3 d after farrowing for male biased and female biased offspring.

Table 3. The anogenital distance (AGD) as measured from the anus to the opening of the vulva in millimeters separated into two AGDs; short and long (short = 5.81 mm to 11.55 mm and long = 11.56 mm to 17.36 mm) with (mean \pm SEM) reproductive data from selection until second parity as well as weight to assess the effect of the AGD on reproductive performance.

Chapter 6: Discussion

General Discussion

Gilts from male biased litters had smaller AGDs which was associated with poorer reproductive performance in the first parity, they had an impaired LH surge, were more responsive to exogenous gonadotrophins, and were more aggressive than gilts from female biased litters. Throughout this thesis we found evidence that gilts from a male biased litter are likely masculinised as aggression and poorer reproductive performance is consistent with the findings in masculinised rodents (Hauser and Gandelman 1983; Ryan and Vandenberg 2002; Hotchkiss et al., 2007). The unifying hypothesis of this thesis that females that developed in a male biased litter will be masculinised and therefore have impaired reproductive performance and be more aggressive than those born into female biased litters is supported. The four experimental chapters cover behaviour, reproduction, and commercial application.

Chapter 2 covers the first experiment that tested the difference in behaviour of gilts from male biased litters compared to those from female biased litters. In this experiment, we found that the sex ratio of the birth litter can affect behaviour and these effects last until at least 16 weeks of age. Gilts from female biased litters took longer to explore a new environment than those from male biased litters, indicating that these gilts were more fearful. Gilts from male biased litters were shown to be more aggressive as indicated by a greater number of scratches at weaning and an increased likelihood of fighting in the resident intruder test. This supports the unifying hypothesis that gilts from a male biased litter are more aggressive than gilts from female biased litters. These experiments highlight a difference in behaviour for gilts from male biased litters compared to female biased litters, although the commercial implications for production are still unclear. They also contribute to the hypothesis that gilts from male biased litters have been masculinised *in utero*, as masculinised animals behaved differently to non-masculinised animals (Hauser and Gandelman 1983; Ryan and

Vandenbergh 2002). While these findings do not have direct commercial implications they do highlight a difference in behaviour between gilts from male biased and female biased litters. We also do not know if these differences will continue to have an effect on behaviour past 16 weeks of age although this seems likely. If the behavioural differences continue throughout life and affect behaviour in group housing systems then it could have an impact on welfare and production. Research should be continued in this area to determine if selecting against gilts from male biased litters could decrease aggression in group housing systems.

The effect of a gilt's birth litter sex ratio on her reproductive potential was explored in both experiments 2 and 3, which are covered in chapters 3 and 4, to address the hypothesis that gilts from a male biased litter will have poorer reproductive performance than gilts from female biased litters. Chapter 3 was a proof of concept experiment that supplied evidence that responses to gonadotropins are different in gilts from male biased compared to female biased litters. Gilts from a male biased litter had a higher ovulation rate than those from a female biased litter in response to exogenous gonadotrophins administered at 18 weeks of age. This means that there was a difference in the reproductive axis that controls ovulation and, therefore, the sex bias of a gilt's birth litter has the potential to impact commercial productivity. Increased ovulation rates have also been shown in gilts treated with dihydrotestosterone (Cardenas et al., 2002). The dihydrotestosterone levels are likely to be higher in the uterine environment of a male biased litter and this may have an effect on the development of the ovarian cells. As the neonatal plasma testosterone levels were not different, this suggests that any androgenic effects are permanent organisational effects that occur during development. This supports the hypothesis that gilts from a male biased litter have been masculinised *in utero*. This highlights a different response to exogenous gonadotrophins for gilts from male biased litters compared to female

biased litters but there is still a need to understand what happens to ovulation and the endocrine profile of these gilts during a natural oestrous event. This is explored in chapter 4 which highlights again there are differences between gilts from male biased and female biased litters, this time with gilts from a male biased litter having a delayed LH surge and a decreased overall concentration of LH during the surge. However, the tonic secretion of LH was not affected. Ovulation rate was also not affected, however, as the overall concentration of the LH surge was affected there may still be effects on luteal quality and pregnancy maintenance. Disruption of the LH surge may also affect the timing of ovulation during oestrus, which can then affect the chances of conception from either natural or artificial insemination. Therefore, it is possible that the differences seen during a natural oestrus could have a commercial impact but it needs to be researched further. The differences in this chapter are consistent with the oestrogenic effects of testosterone (Abi Salloum et al., 2012; Padmanabhan et al., 2012; Padmanabhan et al., 2015) rather than the androgenic effects (Masek et al., 1999; Robinson et al., 1999; Sarma et al., 2005; Padmanabhan and Veiga-Lopez 2013), which gives us some insight into the possible mechanism responsible for the differences. Again, it supports the hypothesis that gilts from a male biased litter have been masculinised compared to gilts from female biased litters, and that they may have poorer reproductive performance as a result.

Chapter 2, 3, and 4 combined indicate that the breeding value of a female, as determined by her reproductive performance and behaviour, can be affected by the sex ratio of her birth litter. Gilts from male biased litters did not perform as well as gilts from female biased litters as they were more aggressive and had a delayed LH surge with a lower overall concentration of LH. This supports the unifying hypothesis of this thesis. However, female biased gilts may be more prone to anxious behaviour such as delayed exploration of a new environment and were less responsive to exogenous

gonadotrophins. It is still unclear which is better suited to the commercial breeding industry and how we may implement the selection of these females.

The effect of a gilt's birth litter sex ratio on her phenotype and the associations this has with reproductive performance at a commercial facility were investigated in chapter 5, through experiments 4 and 5. The anogenital distance was an effective marker for a gilt's birth litter sex ratio from 16 weeks of age. This is beneficial for commercial piggeries as the birth sex ratio cannot always be used on farms for various reasons such as different sites for farrowing and gilt selection. Many farms also do not record the sex at birth, including stillborns, and instead record the total number of males at 24 hours of age. Although this may affect rearing environment and the number of females that can be selected, it is not an accurate measure of the sex ratio *in utero*. Therefore, an objective phenotypic measure is more appropriate. The AGD can be measured on gilts at selection from 16 weeks of age, and a longer AGD was associated with being born into a female biased litter. An above average AGD was also associated with better reproductive performance in the first parity and these gilts were likely from female biased litters. These reproductive results, combined with the AGD data show that gilts from a female biased litter are likely to be better reproductive performers, which again supports the unifying hypothesis of this thesis.

By combining the results from all of the experiments we can deduce that there are significant differences between gilts from male biased and female biased litters and that selecting gilts from both biases may be increasing variability within our breeding herds. These studies suggest that females from a female biased litter, or those with a longer than average AGD, are more suited to commercial breeding systems as they are less aggressive (chapter 2), have a more stable neuroendocrine pathway (chapter 3), have higher levels of LH around ovulation (chapter 4), have an increased AGD which is associated with younger puberty age, increased likelihood of being mated,

and a higher litter size born in their first parity (chapter 5). This supports the unifying hypothesis that gilts from male biased litters have impaired reproduction and are more aggressive than gilts from female biased litters, and it is likely that this is due to masculinisation of gilts from male biased litters. This leaves us with implications for industry, as well as paving the way for future studies.

Further Research

We need to understand more about the mechanism that causes the reproductive, behavioural, and endocrine differences between gilts from male biased and female biased litters. This could be done by conducting experiments to look at hormone levels and hormone transfer *in utero*, as well as the effects of exogenous androgens and androgen antagonists. This would help us understand why we see differences in gilts from male and female biased litters and also how these changes occur. There is a need to determine the mechanisms and regions of the brain that regulate LH release in the pig as this may help explain the current data and advance our understanding of the effect of gonadal steroids or other male-derived compounds, as well as the timing of their effects during gestation, that then affect the mechanisms regulating LH activity in the pig. KNDy cells are directly involved in the HPG axis and may regulate LH production in pigs and could explain how a male biased uterine environment was able to impair surge secretion of LH but have no effect on its pulsatile secretion. This could be due to a change in the structure, number or function of KNDy cells. Currently, we understand very little about KNDy cells in pigs. However, there is a sexually dimorphic difference with male sheep having fewer KNDy neurons than females (Cheng et al., 2010) which could then mean that the sex bias of the birth litter of a gilt can affect their KNDy cell populations. Further, in some species, exposure to gonadal steroids during fetal development has been shown to affect KNDy cell number and function (Van

Leeuwen et al., 1995; Goodman et al., 2007; Cheng et al., 2010; Lehman et al., 2010b; Merkley 2013). The brains of the gilts used in experiment 3, chapter 4, were collected at the conclusion on the experiment and will be analyzed for their KNDy cell populations.

Conclusions

This dissertation provides evidence that a gilt's birth litter sex ratio can impact behaviour, reproduction, and phenotype and support for the unifying hypothesis. We have contributed fundamental knowledge that highlights differences between gilts from male biased and females biased litters. There is further research required into the mechanisms responsible for the differences seen between gilts from male biased and female biased litters. However, the current studies contribute to the evidence that gilts from male biased litters are likely to be masculinised by androgens *in utero*, most likely due to the effects of a male biased uterine environment. Gilts from a female biased litters are likely to be better suited to the breeding herd as they are less aggressive and have an increased AGD, which was associated with better reproductive performance in the first parity.

Collated References

- Abi Salloum B, Herkimer C, Lee JS, Veiga-Lopez A, Padmanabhan V (2012) Developmental programming: prenatal and postnatal contribution of androgens and insulin in the reprogramming of estradiol positive feedback disruptions in prenatal testosterone-treated sheep. *Endocrinology* **153**, 2813-2822.
- Arnold AP (2004) Sex chromosomes and brain gender. *Nature Reviews Neuroscience* **5**, 701–708.
- Baidoo SK, Aherne FX, Kirkwood RN, Foxcroft GR (1992) Effect of feed intake during lactation and after weaning on sow reproductive performance. *Canadian Journal of Animal Science* **72**, 911–917.
- Bánszegi O, Altbäcker V, Bilkó Á (2009) Intrauterine position influences anatomy and behavior in domestic rabbits. *Physiology and Behavior* **98**, 258–262.
- Bánszegi O, Altbäcker V, Dúcs A, Bilkó Á (2010) Testosterone treatment of pregnant rabbits affects sexual development of their daughters. *Physiology and Behavior* **101**, 422–427.
- Bánszegi O, Szenczi P, Dombay K, Bilkó Á, Altbäcker V (2012) Anogenital distance as a predictor of attractiveness, litter size and sex ratio of rabbit does. *Physiology and Behavior* **105**, 1226–1230.
- Barb CR, Kraeling RR, Rampacek GB, Fonda ES, Kiser TE (1982) Inhibition of ovulation and LH secretion in the gilt after treatment with ACTH or hydrocortisone. *Journal of Reproduction and Fertility* **64**, 85-92.
- Bate LA, Hacker R, Kreukniet M (1985) The relationship between serum testosterone levels, sex and teat-seeking ability of newborn piglets. *Canadian Journal of Animal Science* **65**, 627–630.

- Boissy A, Bouissou MF (1994) Effects of androgen treatment on behavioral and physiological responses of heifers to fear-eliciting situations. *Hormones and Behaviour* **28**(1), 66–83.
- Boissy A, Bouissou MF (1995) Assessment of individual differences in behavioural reactions of heifers exposed to various fear-eliciting situations. *Applied Animal Behaviour Science* **46**(1-2), 17–31.
- Booth WD (1983) Development of some male characteristics supported by oestrone but not dehydroepiandrosterone in the boar. *Journal of Reproduction and Fertility* **68**(1), 9-16.
- Bouissou MF, Vandenheede M (1996) Long-term effects of androgen treatment on fear reactions in ewes. *Hormones and Behaviour* **30**(1), 93–99.
- Braastad BO (1998) Effects of prenatal stress on behaviour of offspring of laboratory and farmed mammals. *Applied Animal Behaviour Science* **61**, 159–180.
- Broom DM (1981) 'Biology of behaviour.' (Cambridge University Press: Cambridge).
- Cardenas H, Herrick JR, Pope WF (2002) Increased ovulation rate in gilts treated with dihydrotestosterone. *Reproduction* **123**, 527–533.
- Carlson NR (1998) 'Physiology of behaviour.' (Pearson: Boston, MA).
- Cheng G, Coolen LM, Padmanabhan V, Goodman RL, Lehman MN (2010) The kisspeptin/neurokinin B/dynorphin (KNDy) cell population of the arcuate nucleus: sex differences and effects of prenatal testosterone in sheep. *Endocrinology* **151**, 301–311.
- Choong CH, Raeside JI (1974) Chemical determination of oestrogen distribution in the foetus and placenta of the domestic pig. *Acta endocrinologica* **77**(1), 171-185.

Clark MM, Karpiuk P, Galef BG (1993) Hormonally mediated inheritance of acquired characteristics in Mongolian gerbils. *Nature* **364**, 712.

Clemens LG, Gladue BA, Coniglio LP (1978) Prenatal endogenous androgenic influences on masculine sexual behavior and genital morphology in male and female rats. *Hormones and Behavior* **10**, 40–53.

Colenbrander B, De Jong FH, Wensing CJ (1978) Changes in serum testosterone concentrations in the male pig during development. *Journal of Reproduction and Fertility* **53**, 377–380.

Correa LA, Frugone MJ, Soto-Gamboa M (2013) Social dominance and behavioral consequences of intrauterine position in female groups of the social rodent *Octodon degus*. *Physiology and Behavior* **119**, 161–167.

Cruz CD, Pereira OC (2012) Prenatal testosterone supplementation alters puberty onset, aggressive behavior, and partner preference in adult male rats. *The Journal of Physiological Sciences* **62**, 123–131.

D'Eath RB (2002) Individual aggressiveness measured in a resident-intruder test predicts the persistence of aggressive behaviour and weight gain of young pigs after mixing. *Applied Animal Behaviour Science* **77**, 267–283.

D'Eath RB, Lawrence AB (2004) Early life predictors of the development of aggressive behaviour in the domestic pig. *Animal Behaviour* **67**, 501–509.

D'Eath RB, Pickup HE (2002) Behaviour of young growing pigs in a resident-Intruder test designed to measure aggressiveness. *Aggressive Behavior* **28**(5), 401–415.

- Drickamer LC, Arthur RD, Rosenthal TL (1997) Conception failure in swine: importance of the sex ratio of a female's birth litter and tests of other factors. *Journal of Animal Science* **75**, 2192–2196.
- Drickamer LC, Rosenthal TL, Arthur RD (1999a) Factors affecting the number of teats in pigs. *Journal of Reproduction and Fertility* **115**, 97–100.
- Drickamer LC, Arthur RD, Rosenthal TL (1999b) Predictors of social dominance and aggression in gilts. *Applied Animal Behaviour Science* **63**, 121–129.
- Edgerton LA, Cromwell GL (1987) Sex of siblings may influence reproductive performance in sows. *Journal of Animal Science* **63**, 365
- Elsaesser F, Parvizi N (1979) Estrogen feedback in the pig: sexual differentiation and the effect of prenatal testosterone treatment. *Biology of Reproduction* **20**, 1187–1193.
- Erhard HW, Mendl M, Ashley DD (1997) Individual aggressiveness of pigs can be measured and used to reduce aggression after mixing. *Applied Animal Behaviour Science* **54**, 137–151.
- Ford JJ, Christenson RK (1987) Influences of pre-and postnatal testosterone treatment on defeminization of sexual receptivity in pigs. *Biology of Reproduction* **36**, 581–587.
- Foxcroft G, Patterson J, Dyck M (2010) Improving production efficiency in a competitive industry. In '24th Manitoba swine seminar. Sharing ideas and information for efficient pork production', 3–4 February 2010, Manitoba, Canada. pp. 81–98. (Manitoba Pork Council: Winnipeg, Manitoba, Canada)

Framstad T, Grønset D, Norman N, Velle W (1990) Androgens in Fetal Pigs in Relation to Sex of Neighbour (s). *Transboundary and Emerging Diseases* **37**(1-10), 45-50.

Fraser D (1974) The vocalizations and other behaviour of growing pigs in an open field test. *Applied Animal Ethology* **1**(1), 3–16.

Garland HO, Atherton JC, Baylis C, Morgan MRA, Milne CM (1987) Hormone profiles for progesterone, oestradiol, prolactin, plasma renin activity, aldosterone and corticosterone during pregnancy and pseudopregnancy in two strains of rat: correlation with renal studies. *Journal of Endocrinology* **113**(3), 435-444.

Goodman RL (2014) Neuroendocrine control of gonadotropin secretion: comparative aspects. Knobil and Neill's Physiology of Reproduction: Two-Volume Set; 1537-1574.

Goodman RL, Lehman MN, Smith JT, Coolen LM, De Oliveira CV, Jafarzadehshirazi MR, Pereira A, Iqbal J, Caraty A, Ciofi P, Clarke IJ (2007) Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. *Endocrinology* **148**, 5752–5760.

Graham KM, Mylniczenko ND, Burns CM, Bettinger TL, Wheaton CJ (2015) Examining factors that may influence accurate measurement of testosterone in sea turtles. *Journal of Veterinary Diagnostic Investigation* **28**, 12–19.

Grant VJ (2007) Could maternal testosterone levels govern mammalian sex ratio deviations? *Journal of Theoretical Biology* **246**, 708–719.

Grant VJ, Irwin RJ (2005) Follicular fluid steroid levels and subsequent sex of bovine embryos. *Journal of Experimental Zoology. Part A, Comparative Experimental Biology* **303A**, 1120–1125.

Grant VJ, Irwin RJ, Standley NT, Shelling AN, Chamley LW (2008) Sex of bovine embryos may be related to mothers' preovulatory follicular testosterone. *Biology of Reproduction* **78**, 812–815.

Gray GD, Wexler BC (1980) Estrogen and testosterone sensitivity of middle-aged female rats in the regulation of LH. *Experimental Gerontology* **15**, 201–207.

Grzesiak M, Knapczyk-Stwora K, Ciereszko RE, Golas A, Wiecech I, Slomczynska M (2014) Androgen deficiency during mid-and late pregnancy alters progesterone production and metabolism in the porcine corpus luteum. *Reproduction Science* **21**, 778–790.

Hauser H, Gandelman R (1983) Contiguity to males in utero affects avoidance responding in adult female mice. *Science* **220**, 437–438.

Hernández-Tristán R, Arevalo C, Canals S (1999) Effect of prenatal uterine position on male and female rats sexual behavior. *Physiology and Behavior* **67**, 401–408.

Hogan JA (1965) An experimental study of conflict and fear: an analysis of behavior of young chicks toward a mealworm. Part I. The behavior of chicks which do not eat the mealworm. *Behaviour* **25**(1), 45–96.

Hohenbrink S, Meinecke-Tillmann S (2012) Influence of social dominance on the secondary sex ratio and factors affecting hierarchy in Holstein dairy cows. *Journal of Dairy Science* **95**, 5694–5701.

Hotchkiss AK, Lambright CS, Ostby JS, Parks-Saldutti L, Vandenberg JG, Gray LE (2007) Prenatal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague-Dawley rats. *Toxicological Sciences* **96**, 335–345.

- Hughes IA (2001) Minireview: sex differentiation. *Endocrinology* **142**, 3281–3287.
- Jackson LM, Timmer KM, Foster DL (2008) Sexual differentiation of the external genitalia and the timing of puberty in the presence of an antiandrogen in sheep. *Endocrinology* **149**, 4200–4208.
- James WH (1996) Evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels at the time of conception. *Journal of Theoretical Biology* **180**, 271–286.
- James WH (2004) Further evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels around the time of conception. *Human Reproduction (Oxford, England)* **19**, 1250–1256.
- Jones RB (1996) Fear and adaptability in poultry: insights, implications and imperatives. *World's Poultry Science Journal* **52**(02), 131–174.
- Jones RB, Satterlee DG, Cadd GG (1999). Timidity in Japanese quail: effects of vitamin C and divergent selection for adrenocortical response. *Physiology and Behavior* **67**(1), 117–120.
- Jubilan BM, Nyby JG (1992) The intrauterine position phenomenon and precopulatory behaviors of house mice. *Physiology and Behavior* **51**, 857–872.
- Kanitz E, Puppe B, Tuchscherer M, Heberer M, Viergutz T, Tuchscherer A (2009) A single exposure to social isolation in domestic piglets activates behavioural arousal, neuroendocrine stress hormones, and stress-related gene expression in the brain. *Physiology and Behavior* **98**, 176–185.

Karniychuk UU, Van Breedam W, Van Roy N, Rogel-Gaillard C, Nauwynck HJ (2012) Demonstration of microchimerism in pregnant sows and effects of congenital PRRSV infection. *Veterinary Research* **43**, 19

Kirkwood RN, Aherne FX (1985) Energy intake, body composition and reproductive performance of the gilt. *Journal of Animal Science* **60**, 1518–1529.

Kirkwood RN, Lapwood KR, Smith WC, Anderson IL (1984) Post weaning plasma levels of LH, prolactin, oestradiol 17 β and progesterone in sows following lactations of 10 or 35 days. *Journal of Reproduction and Fertility* **70**, 95–102.

Krackow SV (1992) Sex ratio manipulation in wild house mice: the effect of fetal resorption in relation to the mode of reproduction. *Biology of Reproduction* **47**, 541–548.

Kratochwil K (1971) *In vitro* analysis of the hormonal basis for the sexual dimorphism in the embryonic development of the mouse mammary gland. *Development* **25**, 141–153.

Lamberson WR, Blair RM, Rohde Parfet KA, Day BN, Johnson RK (1988) Effect of sex ratio of the birth litter on subsequent reproductive performance of gilts. *Journal of Animal Science* **66**, 595–598.

Lane EA, Hyde TS (1973) Effect of maternal stress on fertility and sex ratio: a pilot study with rats. *Journal of Abnormal Psychology* **82**, 78–80.

Lehman MN, Coolen LM, Goodman RL (2010) Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology* **151**, 3479–3489.

- Lehman MN, Merkley CM, Coolen LM, Goodman RL (2010b) Anatomy of the kisspeptin neural network in mammals. *Brain Research* **1364**, 90-102.
- Mack LA, Lay DC, Eicher SD, Johnson AK, Richert BT, Pajor EA (2014) Growth and reproductive development of male piglets are more vulnerable to midgestation maternal stress than that of female piglets. *Journal of Animal Science* **92**, 530–548.
- Masek KS, Wood RI, Foster DL (1999) Prenatal Dihydrotestosterone Differentially Masculinizes Tonic and Surge Modes of Luteinizing Hormone Secretion in Sheep 1. *Endocrinology* **140**(8), 3459-3466.
- McClearn GE, Meredith W (1964) Dimensional analysis of activity and elimination in a genetically heterogeneous group of mice (*Mus musculus*). *Animal Behavior* **12**(1), 1–10.
- McCoard SA, Wise TH, Ford JJ (2002) Expression levels of Mullerian-inhibiting substance, GATA4 and 17 α -hydroxylase/17, 20-lyase cytochrome P450 during embryonic gonadal development in two diverse breeds of swine. *Journal of Endocrinology* **175**(2), 365-374.
- McDermott NJ, Gandelman R, Reinisch JM (1978) Contiguity to male fetuses influences ano-genital distance and time of vaginal opening in mice. *Physiology and Behavior* **20**, 661–663.
- Meikle DB, Drickamer LC, Vessey SH, Arthur RD, Rosenthal TL (1996) Dominance rank and parental investment in swine (*Sus scrofa domesticus*). *Ethology* **102**, 969–978.
- Mendl M, Paul ES (1991) Litter composition affects parental care, offspring growth and the development of aggressive behaviour in wild house mice. *Behaviour* **116**, 90–108.

Mendl M, Zanella AJ, Broom DM, Whittemore CT (1995) Maternal social status and birth sex ratio in domestic pigs: an analysis of mechanisms. *Animal Behaviour* **50**, 1361–1370.

Merkley CM (2013) The role of kisspeptin and KNDy cells in the reproductive neuroendocrine system. PhD thesis, The University of Western Ontario, London, Ontario, Canada.

Mitchell RT, Mungall W, McKinnell C, Sharpe RM, Cruickshanks L, Milne L, Smith LB (2015) Anogenital distance plasticity in adulthood: implications for its use as a biomarker of fetal androgen action. *Endocrinology* **156**(1), 24-31.

Nagao T, Wada K, Kuwagata M, Nakagomi M, Watanabe C, Yoshimura S, Saito Y, Usumi K, Kanno J (2004) Intrauterine position and postnatal growth in Sprague–Dawley rats and ICR mice. *Reproductive Toxicology (Elmsford, N.Y.)* **18**, 109–120.

Navara KJ, Nelson RJ (2009) Prenatal environmental influences on the production of sex-specific traits in mammals. *Seminars in Cell and Developmental Biology* **20**, 313–319.

Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, Steiner RA (2009) Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *The Journal of Neuroscience* **29**, 11859–11866.

Niswender GD, Reichert Jr LE, Midgley Jr AR, Nalbandov AV (1969) Radioimmunoassay for Bovine and Ovine Luteinizing Hormone 1. *Endocrinology* **84**(5), 1166-1173.

Padmanabhan V, Smith P, Veiga-Lopez A (2012) Developmental programming: impact of prenatal testosterone treatment and postnatal obesity on ovarian follicular dynamics. *Journal of Developmental Origins of Health and Disease* **3**(4), 276-286.

Padmanabhan V, Veiga-Lopez A (2013) Sheep models of polycystic ovary syndrome phenotype. *Molecular and Cellular Endocrinology* **373**, 8–20.

Padmanabhan V, Veiga-Lopez A, Herkimer C, Abi Salloum B, Moeller J, Beckett E, Sreedharan R (2015) Developmental programming: prenatal and postnatal androgen antagonist and insulin sensitizer interventions prevent advancement of puberty and improve LH surge dynamics in prenatal testosterone-treated sheep. *Endocrinology* **156**(7), 2678-2692.

Parfet KR, Ganjam VK, Lamberson WR, Rieke AR, vom Saal FS, Day BN (1990) Intrauterine position effects in female swine: subsequent reproductive performance, and social and sexual behavior. *Applied Animal Behaviour Science* **26**, 349–362.

Parfet KR, Lamberson WR, Rieke AR, Cantley TC, Ganjam VK, Vom Saal FS, Day BN (1990b) Intrauterine position effects in male and female swine: subsequent survivability, growth rate, morphology and semen characteristics. *Journal of Animal Science* **68**(1), 179-185.

Parma P, Pailhoux E, Cotinot C (1999) Reverse transcription-polymerase chain reaction analysis of genes involved in gonadal differentiation in pigs. *Biology of Reproduction* **61**(3), 741-748.

Petric N, Kato Y, Elsaesser F (2004) Influence of prenatal testosterone treatment on foetal and prepubertal LH β -subunit mRNA and plasma LH concentrations in the female pig. *Domestic Animal Endocrinology* **27**(1), 25-38.

Pond WG, Boleman SL, Fiorotto ML, Ho H, Knabe DA, Mersmann HJ, Savell JW, Su DR (2000) Perinatal ontogeny of brain growth in the domestic pig. *Experimental Biology and Medicine* **223**, 102–108.

Pope WF, Cardenas H (2006) Androgens in female pig reproduction: actions mediated by the androgen receptor. *Society of Reproduction and Fertility Supplement* **62**, 55–67.

Pratt NC, Lisk RD (1989) Effects of social stress during early pregnancy on litter size and sex ratio in the golden hamster (*Mesocricetus auratus*). *Journal of Reproduction and Fertility* **87**, 763–769.

Raeside JI, Sigman DM (1975) Testosterone levels in early fetal testes of domestic pigs. *Biology of Reproduction* **13**, 318–321.

Ralph CR, Staveley LM, Burnard CL, Plush KJ (2015) Piglets born with a high degree of meconium staining display altered behaviour throughout lactation. *Animal Production Science* **55**(12), 1475.

Rekiel A, Więcek J, Wojtasik M, Ptak J, Blicharski T, Mroczko L (2012) Effect of sex ratio in the litter in which Polish Large White and Polish Landrace sows were born on the number of piglets born and reared. *Annals of Animal Science* **12**, 179–185.

Rines JP, vom Saal FS (1984) Fetal effects on sexual behavior and aggression in young and old female mice treated with estrogen and testosterone. *Hormones and Behavior* **18**, 117–129.

Robertson HA, King GJ (1997) Plasma concentrations of progesterone, oestrone, oestradiol-17 β and of oestrone sulphate in the pig at implantation, during pregnancy and at parturition. *Journal of Reproduction and Fertility* **40**(1), 133-141.

Robinson J (2006) Prenatal programming of the female reproductive neuroendocrine system by androgens. *Reproduction* **132**, 539-547.

Robinson JE, Forsdike RA, Taylor JA (1999) In utero exposure of female lambs to testosterone reduces the sensitivity of the GnRH neuronal network to inhibition by progesterone. *Endocrinology* **140**(12), 5797-5805.

Roeling TA, Veening JG, Kruk MR, Peters JP, Vermelis MEJ, Nieuwenhuys R (1994) Efferent connections of the hypothalamic 'aggression area' in the rat. *Neuroscience* **59**, 1001–1024.

Romeyer A, Bouissou MF (1992) Assessment of fear reactions in domestic sheep, and influence of breed and rearing conditions. *Applied Animal Behaviour Science* **34**(1), 93–119.

Roussel S, Hemsworth PH, Boissy A, Duvaux-Ponter C (2004) Effects of repeated stress during pregnancy in ewes on the behavioural and physiological responses to stressful events and birth weight of their offspring. *Applied Animal Behaviour Science* **85**(3), 259–276.

Ryan BC, Vandenberg JG (2002) Intrauterine position effects. *Neuroscience and Biobehavioral Reviews* **26**, 665–678.

Sarma HN, Manikkam M, Herkimer C, Dell'Orco J, Welch KB, Foster DL, Padmanabhan V (2005) Fetal programming: excess prenatal testosterone reduces postnatal luteinizing hormone, but not follicle-stimulating hormone responsiveness, to estradiol negative feedback in the female. *Endocrinology* **146**(10), 4281-4291.

Seyfang J, Ralph CR, Tilbrook AJ, Kirkwood RN (2017a) Response to gonadotrophins differs for gilts from female- and male-biased litters. *Animal Reproduction Science* **182**, 134-137.

Seyfang J, Kirkwood RN, Tilbrook AJ, Ralph CR (2017b) The sex ratio of a litter affects the behaviour of its female pigs until at least 16 weeks of age. *Applied Animal Behaviour Science*. In press.

Sheldon BC, West SA (2004) Maternal dominance, maternal condition, and offspring sex ratio in ungulate mammals. *American Naturalist* **163**, 40–54.

Stiles J, Jernigan TL (2010) The basics of brain development. *Neuropsychology Review* **20**, 327–348.

Tapp AL, Maybery MT, Whitehouse AJ (2011) Evaluating the twin testosterone transfer hypothesis: a review of the empirical evidence. *Hormones and Behavior* **60**, 713–722.

Tarraff CG, Knight JW (1995) Effect of uterine space and fetal sex on conceptus development and in vitro release of progesterone and estrone from regions of the porcine placenta throughout gestation. *Domestic Animal Endocrinology* **12**(1), 63-71.

Thiessen DD, Zolman JF, Rodgers DA (1962) Relation between adrenal weight, brain cholinesterase activity, and hole-in-wall behavior of mice under different living conditions. *Journal of Comparative and Physiological Psychology* **55**(2), 186.

Tilbrook AJ, Clarke IJ (2001) Negative feedback regulation of the secretion and actions of GnRH in Males. *Biology of Reproduction* **64**, 735–742.

Trivers RL, Willard DE (1973) Natural selection of parental ability to vary the sex ratio of offspring. *Science* **179**, 90–92.

Turner AI, Hemsworth PH, Canny BJ, Tilbrook AJ (1999) Inhibition of the secretion of LH in ovariectomised pigs by sustained but not repeated acute elevation of cortisol in

the absence but not the presence of oestradiol. *Journal of Endocrinology* **163**, 477-486.

Uller T, Meylan S, De Fraipont M, Clobert J (2005) Is sexual dimorphism affected by the combined action of prenatal stress and sex ratio? *Journal of Experimental Zoology. Part A, Comparative Experimental Biology* **303A**, 1110–1114.

Van Leeuwen FW, Chouham S, Axelson JF, Swaab DF, Van Eerdenburg FJ (1995) Sex differences in the distribution of estrogen receptors in the septal area and hypothalamus of the domestic pig (*Sus scrofa*). *Neuroscience* **64**(1):261-275.

Van Reenen CG, O'Connell NE, Van der Werf JT, Korte SM, Hopster H, Jones RB, Blokhuis HJ (2005) Responses of calves to acute stress: individual consistency and relations between behavioral and physiological measures. *Physiology and Behavior* **85**(5), 557–570.

Vandenheede M, Bouissou MF (1993) Effect of androgen treatment on fear reactions in ewes. *Hormones and Behavior* **27**(4), 435–448.

Veiga-Lopez A, Astapova OI, Aizenberg EF, Lee JS, Padmanabhan V (2009) Developmental programming: contribution of prenatal androgen and estrogen to estradiol feedback systems and periovulatory hormonal dynamics in sheep. *Biology of Reproduction* **80**, 718–725.

Verdon M, Morrison RS, Hemsworth PH (2016) Rearing piglets in multi-litter group lactation systems: effects on piglet aggression and injuries post-weaning. *Applied Animal Behaviour Science* **183**, 35–41.

vom Saal FS (1989a) The production of and sensitivity to cues that delay puberty and prolong subsequent oestrous cycles in female mice are influenced by prior intrauterine position. *Journal of Reproduction and Fertility* **86**, 457–471.

vom Saal FS (1989b) Sexual differentiation in litter-bearing mammals: influence of sex of adjacent fetuses *in utero*. *Journal of Animal Science* **67**, 1824–1840.

vom Saal FS, Bronson FH (1978) *In utero* proximity of female mouse fetuses to males: effect on reproductive performance during later life. *Biology of Reproduction* **19**, 842–853.

vom Saal FS, Clark MM, Galef BG, Drickamer LC, Vandenberg JG (1999) Intrauterine position phenomenon. *Encyclopedia of Reproduction* **2**, 893–900.

von Borell E, Ladewig J (1992) Relationship between behaviour and adrenocortical response pattern in domestic pigs. *Applied Animal Behaviour Science* **34**(3), 195–206.

Wakabayashi Y, Nakada T, Murata K, Ohkura S, Mogi K, Navarro VM, Clifton DK, Mori Y, Tsukamura H, Maeda KI, Steiner RA (2010) Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. *The Journal of Neuroscience* **30**, 3124–3132.

Widowski TM, Cottrell T, Dewey CE, Friendship RM (2003) Observations of piglet directed behavior patterns and skin lesions in eleven commercial swine herds. *Journal of Swine Health and Production* **11**(4), 181–185.

Wise TH, Christenson RK (1992) Relationship of fetal position within the uterus to fetal weight, placental weight, testosterone, estrogens, and thymosin beta 4 concentrations at 70 and 104 days of gestation in swine. *Journal of Animal Science* **70**, 2787–2793.