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The sex biased litter *in utero* and its effect on post-natal health, development, and reproductive capacity of the commercial pig

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This thesis is dedicated to all past, present, and future pigs in our production systems worldwide.

Abstract

Environmental stressors *in-utero*, including extremes of steroid hormone exposure, can negatively affect reproductive function, the development of offspring, as well as life-course health trajectories in a myriad of species. However, few studies to date have investigated the mechanisms responsible. This thesis tested the hypothesis that a sex ratio biased uterine environment would influence the reproductive capacity of the gilt, and the health and production parameters of pigs within commercial production systems. An initial scoping review showed results from different in-utero sex ratios were highly variable both between and within species, but overarching themes of negative influences of androgenised uterine environments on reproductive outputs, physiological development, and maternal/paternal behaviour were established. Across species, the testosterone biased intra-uterine environment increased aggression whilst decreasing fearful behaviour. I subsequently investigated whether females gestated in biased uterine environments (65% or more of one sex) exhibited altered reproductive potential postnatally. I examined the ovarian reserve and endometrial morphology of these individuals, revealing significant effects to follicle profiles dependent on birth weights, but no effect on endometrial development. A collaborative project utilising commercial pigs investigated whether a hormonally biased environment influenced growth, health, and carcass quality parameters. This found that male and female pigs from a biased uterine environment were more likely to die or be culled before reaching slaughter weight, due to illness and injury, or due to behavioural vices. No effects on growth nor carcass parameters were identified based on uterine bias, but male pigs produced a higher quality carcass than females. In summary, I have provided evidence further supporting an altered reproductive capacity in female pigs and altered mortality causes in pigs from androgenised and oestrogenised litters compared to non-biased litters.

This thesis bridges the gap of how the hormonally biased uterine environment may mechanistically contribute to poor reproductive success in breeding sows. Further it is the first study to identify a higher life-course mortality rate in pigs originating from biased litters. It is recommended that the sex ratio of an originating litter, as well as an individual's birth weight, should be considered when selecting breeding sows.

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Abbreviations

AGD   anogenital distance.

CL   corpus lutea.

DHT   dihydrotestosterone propionate.
DOHaD   Developmental Origins of Health and Disease.

E   oestrogen.
E2   oestradiol.
EB   oestradiol benzoate.
ED   embryonic day.
EGF   epidermal growth factor.
ER   oestrogen receptor.

IHC   immunohistochemistry.
IUP   intra-uterine position.
IVF   in-vitro fertilization.

LH   luteinising hormone.
LSR   litter sex ratio.

PCNA   anti-proliferating cell nuclear antigen.
PGC   primordial germ cell.
PubMed   Publisher MEDLINE.

T   testosterone.
TOR   total ovarian reserve.
TP   testosterone proprionate.

UGKO   sheep uterine gland knock out.

WoS   Web of Science.

Preface - Impact of Covid-19

The Covid 19 pandemic had many substantial and severe practical effects on the execution of this thesis. This will be discussed regarding the three datasets that were planned, alongside laboratory access.

1. Reproductive tracts were collected in a longitudinal study from the National Pig Centre, University of Leeds farm. Tracts were harvested from both male and female pigs of each selected litter and tissues stored for future analyses. These data are presented in Chapters 3 and 4.

One batch of samples could not be collected from the pigs due to covid regulations. Research staff were not permitted access to the University farm during the first months of the UK lockdown in 2020. This limited the sample size that would otherwise have been achieved. Sample collection from some pigs were also missed at the abattoir due restrictions of access. Additionally, only one research member was allowed on site for the final collection time points. Due to the restricted lab access, there was no scope to analyse testicular samples.

2. Commercial life course data of pig health, growth and carcass traits were collected as part of a larger KTP-funded trial in collaboration with Karro Food Group Ltd. These data are presented in Chapter 5.

Participation in the trial was limited due to covid travel regulations in the UK. Due to this, individual weights at weaning could not be obtained. Furthermore, the number of litters that could be included were reduced. This is a direct result of fewer research staff being able to participate in the trial and measures being modified to allow fewer staff members to be able to collect the data. Hence, identification of the sex of both alive and dead born piglets could not be prioritised.

3. Farrowing data were being collected with CCTV footage of individually marked piglets of full litters. These data were collected to investigate the following;

   (a) Suckling behaviours and teat order development within litters, dependent on litter sex-bias.

   (b) Subsequent lactocrine effects on uterine horn development.

Two data sets aimed firstly to evaluate the effect on piglet behaviour during suckling events, and secondly to evaluate whether this held a knock-on effect on uterine gland development. These were mid-trial on farm but were cancelled mid-trial due to the Covid-19 pandemic. This resulted in all research trials on-farm being terminated, with only skeleton farm staff being allowed on site. This work could not be completed due to the physical, and legal restrictions resulting from the pandemic. It is important to note that the trials could not have easily been re-started as they relied on a different genetic combination from that currently used at the University farm. Consequently, it was not possible to have the pigs inseminated in such a way which would have accommodated the requirements of the trial.

Beyond the effects on husbandry and data collection, there was also substantial restrictions to research progression at the university due to the Covid-regulations within the faculty of medicine. Access to labs were the following;

- Labs fully closed on the 20th of March 2020 for 3.5 months.

- I was granted emergency and partial access in July 2020 for collection and processing of my final batches.

- September 2020 – March 2021. For seven months research students were granted only 12 hours per week in the laboratory

- June 2021 full access to labs resumed.

Chapter 1

Introduction

1.1   General background

The UK pig industry has faced many challenges over the past decade. Facing negative net margins, unpredictable markets, feed price volatility, and declining carcass values which have dropped by a third since 2013 [4]. One of the most important aspects for pig producers is to maximise the output from each individual animal whilst maintaining animal welfare [95, 101]. Between 2010 and 2016 the total number of pigs (*Sus scrofa domesticus*) farmed for food production in the UK increased [3]. During this period, the amount of meat produced increased from 772,000 to 919,000 tonnes, yet the number of pigs kept for breeding purposes decreased by 32% [3]. This was due to sows being more efficient/productive than previously, with more piglets per sow being weaned and a quicker turn-around time between litters [8, 136]. Although this is a positive change in terms of production efficiency, other factors such as breeding herd welfare, sow longevity, and piglet quality (one of good health, thrifty – good physicality, and good growth predictions), have by comparison been neglected [136, 156]. Despite some improvements in sow longevity and definite improvement in reproductive outputs, approximately 50-55% of sows still need to be replaced per annum in the UK breeding herd [55, 56]. Reducing replacement rates is one way to improve breeding efficiency. The main causes for culling of breeding stock include failure to display oestrus and failure of, or the need for, repeated inseminations for successful pregnancy [222].

1.1.1 Litter variability

Increased productivity of the breeding sow has resulted in an increase in variability [8, 136] both within and between litters in terms of growth and reproductive biology [252]. As the sows' reproductive capacity is pushed to its limits and litter sizes increase, several factors can begin to impact profitability including higher levels of within-litter variation in growth rates and health, as well as lower birth weights of the offspring [262]. Lower birth weights lead to increased mortality rates, reduced growth rates, along with lower carcass and meat quality [262]. Much of this variability remains unaccounted for. Factors which have been found to correlate to variability include genotype, disease, nutrition, hygiene, weight, age at weaning, management, group size, stocking density, mixing of batches, and animal behaviour [110]. However, Beaulieu et al. found no evidence that increased litter sizes are correlated to an increased variability in birth weight or performance in piglets [24]. This contradicts the general assumption that increased litter size is the main cause of variance in birth weight and other production and reproductive traits in pig production [24, 105, 184, 186]. One potential cause for variance in the development of post-natal pigs could be the environment to which they are exposed during the pre-natal period (gestation).

1.1.2 Intra-uterine environment

The intra-uterine environment has become of particular interest due to the paucity in knowledge about its effect on post-natal development of many species, including that of the domestic pig [262]. The influence that a sow exerts on her offspring extends beyond simple transmission of genetic information [229]. Some evidence suggests that higher prolificacy may exacerbate inconsistencies in ovulation rates and conceptus survival post-implantation [8]. Furthermore, variability in for example birth weights may contribute to higher pre-weaning mortality rates, slower growth rates, and decreased meat quality [60, 250, 259]. Modern production systems are now very controlled, with little within farm variation in the environment, management, and the substances that pigs are exposed to. Yet variability in piglet growth and development remains.

One potential explanatory factor for between-, and within-litter variation in responses shown to post-natal insult, is the sex ratio to which the offspring is exposed *in-utero*. This

could be particularly relevant when trying to understand variability between offspring born to sows that are kept in the same environment. In litter bearing species, the steroid hormone exposure, such as testosterone and oestrogen, to which offspring are subjected will differ depending on the sex ratio of the litter from which they originate [195]. There is little information regarding the natural bias of a sex in the commercial pig, although one study the European wild boar found a natural bias towards males in a litter versus females (1.3 : 1) [71]. For example, a foetus positioned between two males (2M) in the uterine horn will be exposed to significantly more testosterone and oestrogen than a foetus positioned between a male and a female (1M), or 2 females (0M) [195]. These sex ratios, or positions, are more likely to be extreme in larger litter sizes [76]. Further the crowded uterine horn enables for more hormone exposure than the smaller litters [236].

1.1.3   Sex steroid hormones

Steroid hormone synthesis is regulated by the hypothalamus-pituitary-gonadal (HPG) axis [251]. The specific synthesis pathway (Figure 1.1) of the steroid hormones is dependent on the sex of the offspring and the type of steroid that is being produced [193].

The sex steroid hormones are produced by the the gonads, with contribution from the adrenal cortex, and consist of androgens, oestrogens, and progestins [168]. Testosterone and androstenedione are the primary steroids produced in the male, and are predominantly synthesised by the testicular leydig cells [167]. From testosterone, dihydrotestosterone can then be synthesised in the prostate and seminiferous tubules of the testes via 5-alpha reductase. In females the ovary produces approximately 20% of the total androgen content, a similar amount is synthesised from androgenic precursors in other tissues, such as adipose tissues [12], and the remaining 50% originates from the adrenal cortex. Oestrogens make up the majority of sex steroids produced in the ovaries [12].

The ability of these sex hormones to exert an influence on systems once released into the bloodstream is dependent on the available receptors of target tissues. These receptors are generally found intracellularly, and are typically found in the nucleus, cytosol, and plasma membranes of the cells [168]. They are classically considered to pass through the cell membrane to bind to receptors in the cytoplasm and nucleus [72, 168]. Similarly, and particularly in the case of androgens, steroid hormone binding globulin (SHBG) -bound

androgens may interact with specific membrane-located SHBG receptors, before initiating their effect via a second messenger series. Typically a G-protein-linked increase in cyclic adenosine monophosphate, or inositol triphosphate [72]. From binding, the interval to gene expression within a targeted cell varies from hours to days [168]. Binding of the ligand (sex steroid) to the receptor causes receptor separation from cytoplasmic chaperone proteins, exposing nuclear localization sequences and thereby receptor-mediated activation, and the resulting biological action of the target cell can take place [50]. Oestrogen receptor (ER) α and β, androgen receptors, and progesterone receptors all belong to the steroid hormone receptor family, and function in a ligand-dependent manner [168]. Sex specific behaviours are modulated by steroid hormones binding to receptors in the brain. In female rodents, the highest density of oestrogen receptors are found in the anterior and ventromedial hypothalamus, the pituitary gland, amygdala, median eminence, and the medial preotic area [152, 153]. These areas are similar to those found for androgens in the female rodents' brain such as the ventromedial (and basomedial) hypothalamus, median eminence, and the pituitary gland [153]. In male rodents, the highest density of receptors for androgens are in the hippocampus, bed nucleus of the stria terminalis, medial preoptic area, anterior and lateral hypothalamus, amygdala, median eminence, and the pituitary gland [198].

Despite there being different receptors for oestrogens and androgens, it is important to note that testosterone may be aromatized into oestradiol (illustrated in Figure 1.1) and thereby testosterone can indirectly act on oestrogen receptors. This aromatase activity is at its highest in the foetal brain [150]. This is particularly important for the defeminisation and masculinization of the male brain. Defeminization ensures the ability to respond to the effects of oestradiol and progesterone in relation to female sexual behaviour (e.g. lordosis) is inhibited, and masculinization are the organizational events permissive to expression of male sexual behaviour [199]. Masculinization is dependent on neuronal oestradiol reaching critical levels whilst feminization is dependent on the absence of the critical levels [149, 199]. The male offspring will not only be exposed to the maternal and placental oestradiol, but the foetal synthesised testosterone is converted into oestradiol [150], boosting neuronal oestradiol levels. In rodents, there is a surge of testosterone production in the male at birth, whereas in the female the ovaries are quiescent, providing the sex specific hormonal exposure [257]. As described, these receptors are in many vital tissues (such as
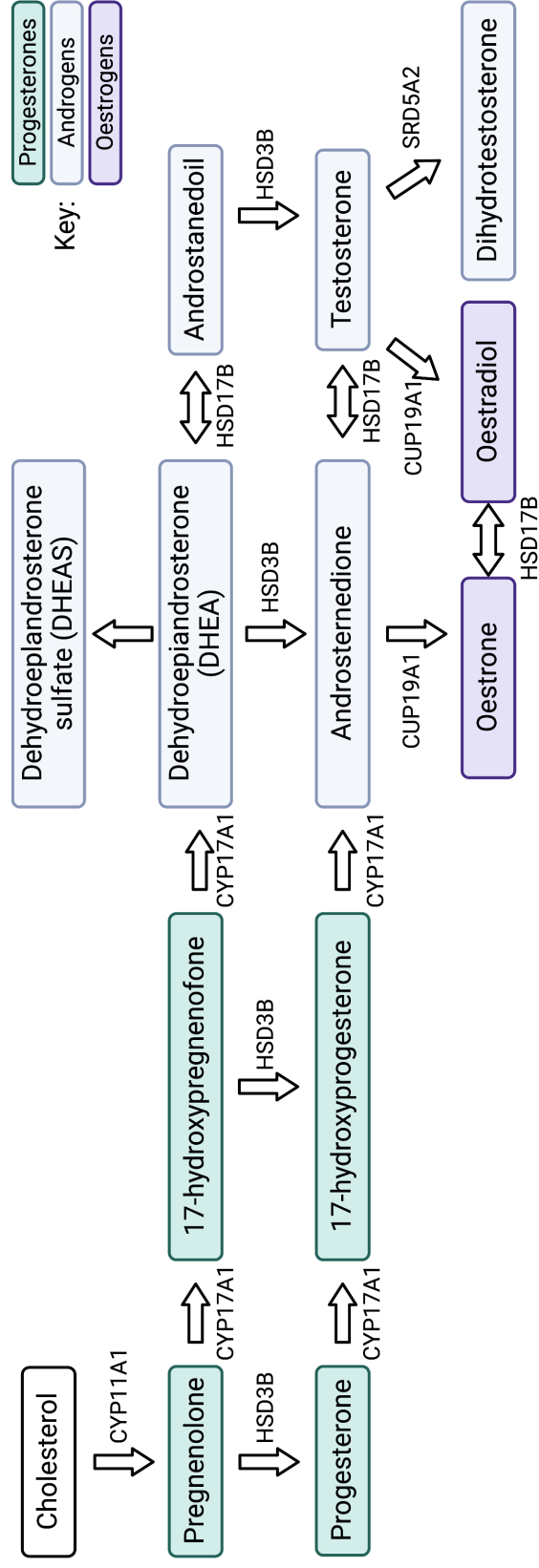
Figure 1.1: This illustration is adapted from that of [34] showing the major pathways in sex steroid biosynthesis. Created with BioRender.com.

reproductive and neural tissues) and the disruption of these receptor and steriodogenic genes can impact on the function of many different systems [167].

The sex steroids also hold implications on the pheromone production in many species. In pigs the salivary glands play a vital role on reproductive behaviour and oestrus [197]. 3-androstenol and 5α-androstenone, which combined have been identified in the submanibular glands and saliva of the boar, are considered important pheromones [176]. In pigs, gilts are known to have an earlier onset of puberty if exposed to boar pheromones, and may improve sow readiness for mating. Boar exposure is a commonly used commercial practice, often referred to as the boar effect [133]. This phenomenon is also seen in mice that are exposed to mature male urine, at which point there is an advancement in puberty onset, famously known as the 'Vandenbergh effect' [231], and the 'Whitten effect' where mature male urine can synchronise the oestrus cycles of females mice in the same colonies [248].

Progesterone and oestrogen are also the two primary steroid hormones produced by the placenta, and are necessary for a successful pregnancy to be established [208]. The placenta in the pig is not only able to synthesise oestrogen, but will also convert CL derived progesterone to oestrogens which is maintained throughout the pregnancy [135].

1.1.4 Foetal development

In the pig, embryos enter the uterus in a four-cell stage, by day 5 they reach the morula stage, and form into blastocysts by days 6-8 [9]. At day 11, blastocysts will undergo rapid elongation, typically completed at day 13 at which point embryos are in extremely elongated (60-100 cm) filamentous forms. Rapid growth (from 0.06 to 100g) occurs between embryonic day (ED) 20 to 100, a critical time period where most systems are developed [9]. The gonadal development of the pig will also begin at this time, with primordial germ cells appearing at ED 22-24 in both the male and [162]. At ED 26 testosterone production begins in the male offspring. In the female offspring the uterus, along with egg nests, begin to form at ED 30-35 [9, 162] with meiosis of germ cells beginning around ED 40-48 and primordial follicles established at ED 60 [9]. High levels of developmental plasticity during these embryonic developmental stages allows certain factors, including steroid hormone exposure, to hold significant influence on embryo development [179], potentially leading to long term physiological changes. This hypothesis is well established

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in the field of 'Developmental Origins of Health and Disease' (Developmental Origins of Health and Disease (DOHaD)), in which the contention is that developmental plasticity and *in-utero* programming of offspring could contribute to susceptibility of a range of adult diseases[242] with some of these being sexually dimorphic in nature, i.e. different outcomes between males and females. [179].

Different sex ratios within-litters are associated to individual variability, principally influenced by hormonal exposure during embryonic development [195]. Behavioural research around litter sex ratios has led to the suggestion that females originating from male-biased litters are 'masculinised'. Studies in which females have been treated with androgens or originated from androgenised environments have found that females display more aggressive behaviours than non-treated controls [81, 132, 147, 204], across many different species. Seyfang et al. [204] found that female pigs originating from male-biased litters displayed decreased fear or increased boldness, increased agonistic behaviours, and initiated a more fights. The influences of the biased litter may therefore have implications beyond behaviour as "knock-on" effects occur as an influence of aggression, e.g., affecting social ranking [51, 99]. It has also been hypothesised that the social rank of the pregnant sow will affect birth weights, growth, and behaviour of her offspring [137].

1.1.5 Reproductive traits and the sex biased litter

The sex ratio of a litter may not only affect production and behavioural traits in the offspring, but also alter the reproductive performance of the breeding herd, via gilt selection. There is some evidence that *in-utero* sex ratio can influence the age at first oestrus, litter sizes, interval timing between weaning and oestrus, and conception rates in pigs and several other species [58, 73, 139, 159, 229, 237, 240]. The reproductive efficacy of females from male-biased litters also seems to be affected; Drickamer et al. [61] found that females from male-biased litters have lower rates of successful breeding at the first attempt. Seyfang et al. [204] found that pigs in male-biased litters resulted in females that exhibited distorted luteinising hormone (LH) surge profiles, pre-ovulation. Although this influence of increased androgen levels in utero on the LH surge was supported by findings in rodents [240], it was not supported by earlier findings in the pig [183, 233]. The mechanisms by which a male-biased litter affects female reproductive traits remains unclear, and further

research is necessary to determine how these changes are realised. This is crucial in order to reduce sow wastage seeing as the main cause for culling breeding stock is failure to display oestrus behaviour or the need for repeated inseminations [233]. As outlined, understanding the effects of a biased litter, may help reduce gilt and sow wastage.

The effects seen from gestating in a hormonally biased uterine environment on litter bearing species is covered in detail in the scoping review in Chapter 2.

1.1.6   Conclusion

There is a clear gap in understanding how the uterine environment may be affecting both pre- and post- natal development in the commercial pig and how this impacts the development of health, behaviour, and production challenges throughout the life-course. The ultimate output of this project is to expand the knowledge of how the sex ratio of a litter may affect reproductive development and post-natal health and development in placental mammals.

1.2   Thesis format

1.2.1   Thesis aim

The aim for this PhD thesis is to investigate how the hormonal *in-utero* environment from which a pig originated may affect their subsequent reproductive potential and production parameters. This is met by evaluation of the three main objectives stated below.

1.2.2   Thesis objectives

There are three main objectives to fulfil the aims of this thesis. These are;

1. To understand the current research and literature available that has investigated the effect of a biased hormonal uterine environment on offspring development and success.

Chapter 2 aimed to provide this understanding. A scoping review was carried out with the aim of identifying the research, to date, that has investigated the *in-utero* hormonal effects on offspring development in any litter bearing species.

2. To investigate how a hormonally biased uterine environment may affect the reproductive potential of the sow.

This was investigated in Chapters 3 and 4. In Chapter 3, "Ovarian potential and influences of intra-uterine hormonal bias", the aim was to understand the impact on the ovarian reserve in the gilt by investigating not only the development of the primordial follicle pool, but also the recruitment and atresia of follicles, potentially influencing reproductive longevity and foetal development.

Chapter 4, "The influence of the intra-uterine hormonal bias on endometrial development", investigated how the development of the uterine horn of the gilt may be influenced, potentially affecting the capacity to establish and maintain pregnancy. This was done investigating differences in the gross morphology of uterine horns alongside the development of secretory structures and cell proliferation.

3. To investigate how the production parameters of the commercial pig originating from a hormonally biased uterine environment may be affected.

This was considered in Chapter 5, "The production parameters of the hormonally biased pig", where the effect of litter sex bias on the health, growth, and carcass traits of pigs in the commercial system investigated. Here individual pigs were tracked from birth through to slaughter with data collected for each point of interest through their life-course.

1.2.3 Thesis data structure

Three different sets of data are used in this thesis.

1. First, a comprehensive corpus of literature was created, filtered and assimilated for the scoping review in Chapter 2.

2. Reproductive tracts were collected in a longitudinal study from the National Pig Centre, University of Leeds farm. Tracts were harvested from both male and female pigs of each selected litter and tissues stored for future analyses. Not all collected samples were used in this thesis. These data are presented in Chapters 3 and 4.

3. Commercial life course data of pig health, growth and carcass traits were collected as part of a larger KTP-funded trial in collaboration with Karro Food Group Ltd. These data are presented in Chapter 5.

Two datasets were to be collected but were cancelled mid-trial due to the Covid-19 pandemic, which resulted in all research trials on-farm being terminated, and with only skeleton staff being allowed on site. This work could not be completed due to physical, and legal restrictions resulting from the pandemic. These were planned to contribute to an additional chapter between 3 and 4 and aimed to look at:

1. Effects of litter sex-bias onsuckling behaviours and teat order development

2. Subsequent lactocrine effects on uterine horn development

Chapter 2

Sex steroid hormone exposure *in-utero*; the consequences for post-natal development and health of the offspring - a scoping review

2.1 Introduction

The *in-utero* environment in which an individual develops can have a significant influence on the life-course health of that individual[242]. This *in-utero* environment can influence normal physiological processes such as establishing organ systems, as well as altering the likelihood of developing non-communicable diseases postnatally, such as infertility [92]. Termed the DOHaD, this hypothesis states that environmental stressors (including those in the maternal and/or paternal circulation) to which either the gamete (oocyte or sperm), embryo, or foetus is exposed, may affect a range of physiological functions later in life [242]. This exposure results in offspring becoming predisposed to certain diseases to which they otherwise would not have been as susceptible. The impact of this on an individual's life-course health is contingent on the nature of the stressor as well as the timing and duration of exposure in terms of development [92, 242]. One type of stressor is an individual's exposure to disproportionately high concentrations of steroid hormones originating from litter siblings.

Steroid hormones are a class of hormones that are derived from cholesterol, normally classed as either corticosteroids or sex steroids [193]. This review will only be considering sex steroids (e.g. oestrogens and androgens) which are typically derived from the gonads with synthesis being regulated by the hypothalamus-pituitary-gonadal (HPG) axis [251]. Sex hormones act as chemical messengers regulating many different physiological processes. During pregnancy in the pig, oestrogens in maternal circulation will rapidly decrease following ovulation, with short and small increases around embryonic day (ED) 12 and 25-30 [246]. It then remains low until parturition. The peak in maternal oestrogens at ED 12 coincide with embryo elongation and conceptus secretion of oestrogen and interleukin-$1\beta 2$, followed by foetal peak secretion of interferon-δ and interferon-γ at ED14-16 [246]. Progesterone concentrations will do the opposite, increasing from oestrus, peaking at ED 12-14, and then slowly declining until ED 30. In the foetus, a key role of steroid hormones lies in the development and function of reproductive systems [246]. While there is debate as to whether the androgens and oestrogens produced by offspring (beginning at ED 26) during *in-utero* development influence all of their littermates, there is evidence that there can be neighbour exposure *in-utero* which can impact on the development, and health of individual litter-mates later in life [236]. There is evidence in rats that these sex steroids may travel between offspring *in-utero* via intraluminal fluid, and also that testosterone diffuses between the foetal membranes to surrounding offspring [66, 236].

Since the 1980s research into how offspring may be affected by differing concentrations of steroid hormones has steadily increased, these studies have predominantly used two main methods to study this phenomena: (1) **intra-uterine position** which describes the exposure of steroids from foetal siblings during gestation in litter-bearing species; and (2) direct manipulation of **steroid hormones**.

1. First, the **intra-uterine position (IUP)** can be described by both an absolute and relative theorem. The absolute positioning defines where in the uterus the offspring was positioned, i.e. towards the uterine tubule junction, middle, or towards cervical body as reviewed [195]. In species such as the mouse that have a bidirectional uterine blood flow, those positioned in the medial parts of the uterine horns will receive the least nutrient rich blood, resulting in effects such as a lower birth weight than their

siblings [28, 236], whereas in rats where the bidirectional blood flow undergoes a shift in the heamodynamics resulting in a predominantly one directional blood flow during gestation. Hence, being located next to the cervix carries benefits, where the volume of blood per minute will be significantly higher than towards the uterine tubule junction [93, 145]. Accordingly, the morphological differences of uteri between species must be taken into consideration. Some studies investigated effects using the caudal male theory, in which they look at the number of males positioned upstream of the uterine blood flow, hypothesising that the offspring with more caudal males would be exposed to greater levels of as reviewed [195], and is dependent on the direction of the uterine blood flow in that species.

The IUP can also be studied via the relative uterine positioning which refers to where the foetus is located with reference to the sex of neighbouring littermates [195]. The definitions used for these positions varies between studies but will identify how many of each sex an individual offspring is surrounded by. These studies are dependent on the theory that the transfer of steroid hormones occurs via diffusion between the neighbouring foetal membranes.

In investigations on the effect of the IUP, there is often a high level of invasiveness towards both the mother and offspring as caesareans are the main method used to identify the IUP as reviewed [195], this entails slaughter endpoints to the mother and either euthanasia or surrogate mothers for the offspring. Due to this, studies often use the sex ratio of a litter as a lesser invasive method to study an androgenised, or oestrogenised uterine environment. The sex ratio of a litter is arguably easy to calculate immediately post parturition, as long as factors such as filial cannibalism or savaging is accounted and controlled for. Further to this, the anogenital distance (AGD) of animals has also been used as a proxy for the androgenisation of an animal.

2. Second, the manipulation of the uterine environment by administration of **steroid hormones**, or use of anti-androgens/oestrogens, is used to directly influence the uterine environment in which the embryos develop. Here, gestating mothers are often administered with testosterone or oestrogen in order to determine the influence that an androgenic vs oestrogenic uterine environment has on offspring.

There has been significant progress within this field since Ryan and Vandenbergh [195] published a comprehensive review of research investigating the IUP of litter bearing species. However, their review did not cover studies that had manipulated the hormonal uterine environment [195]. Summarising the current literature of both IUP and steroid hormone manipulations, alongside alternative methods, this study aims to cover a comprehensive array of research to date, which has investigated the effect of an androgenised or oestrogenised uterine environment on the reproduction, health, and development of litter bearing mammals. In regard to absolute uterine positions, this review will use the following terminology; 2M: individuals are those that were positioned between two males *in-utero*; 1M: animals are those that were positioned between one male and one female; 0M: animals were positioned between two females. This is to adhere to the most commonly used terminology whilst standardising it across different papers.

This review aims to summarise the most recent research investigating the effect of varying amounts of *in-utero* offspring derived sex steroid hormones, on life-course health in litter bearing mammals. Focusing on three main systems; physiological development, reproduction, and behaviour.

2.2 Materials and methods

2.2.1 Search strategy

An initial literature search was conducted in January 2018 using Web of Science (WoS) (https://www.webofscience.com/wos/woscc/basic-search) and Publisher MEDLINE (PubMed) (https://pubmed.ncbi.nlm.nih.gov). These two databases were considered sufficient to capture studies of both applied and mechanistic research. The search used key words relating to the uterine environment, sex steroid hormone secretion and litter bearing species (Table ??). A row represents the grouped terms between which the OR terms are used, and each row was combined using AND terms. NOT terms were deemed superfluous and were not used in this search in order to keep the search broad. Quotation marks were used to define exact terminology whereas an asterix was used where alternative endings to words were possible, e.g. Gestat* could include gestate, gestated, gestation. Default time searches were used for both search engines, with 1900-2022 and 1781-2022 for WoS

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and PubMed, respectively. No language, year of publication, or access restrictions were used.

To capture the papers published since the initial search, a secondary search was carried out on the 23rd of February 2022, using the same databases with the five-way combinations. The resulting references were transferred into Rayyan [129], a software designed to aid researchers in carrying out knowledge synthesis projects. Filtering (described in 2.3) of all papers was carried out using this software.

2.2.2 Search combinations

A traditional review will use a string of search terms and perform one search per database. It is possible that not all relevant scientific articles would be caught by this single search term due to use of differential terminology. To investigate whether this may be happening or not, especially during wide scoping reviews, the search was broken down into multiple searches, with different combinations of search term groups. Table 2.1 represents the different search term combinations used. Each of these searches were performed for both databases. As seen below, the traditional search including all search term groups was a five-way search, and will be referred to as the main search going forward. There are then additional and subsequent four-way breakdowns of these search term groups.

2.2.3 Eligibility requirements

A single corpus of all returned literature was compiled and duplicates were identified and removed using Rayyan. Papers were manually removed or retained based on relevance in three stages: (1) title; (2) content of the abstract; and (3) full text. At each stage inclusion and exclusion criteria (see below) were used in order to determine whether or not the papers were to be retained in the next stage corpus. A fourth stage was included where the retained papers were reference checked to identify any citations not captured by the search. See figure 2.1 for further details of the filtration process.

Table 2.1: Search terms combinations that were used for each data base. The four-way combination utilised four of the five groups used, each possible combination was in the data bases. The main search (five-way search) was every search term group combined as the traditional search.

		Combinations
4-way	**1**	(Uter* OR Gestat*) AND (Embryo* OR "pre-natal" OR Foet* OR "Fetus" OR "Fetal" OR Conceptus*) AND ("Sex" OR "Gender") AND (Develop* OR Reprod* OR ontogen*)
	2	(Uter* OR Gestat*) AND (Embryo* OR "pre-natal" OR Foet* OR "Fetus" OR "Fetal" OR Conceptus*) AND ("Sex" OR "Gender") AND (Litter*)
	3	(Uter* OR Gestat*) AND (Embryo* OR "pre-natal" OR Foet* OR "Fetus" OR "Fetal" OR Conceptus*) AND (Develop* OR Reprod* Ontogen*) AND (Litter*)
	4	(Uter* OR Gestat*) AND ("Sex" OR "Gender") AND (Develop* OR Reprod* Ontogen*) AND (Litter*)
	5	(Embryo* OR "pre-natal" OR Foet* OR "Fetus" OR "Fetal" OR Conceptus*) AND (Develop* OR Reprod* Ontogen*) AND ("Sex" OR "Gender") AND (Litter*)
5-way		(Uter* OR Gestat*) AND (Embryo* OR "pre-natal" OR Foet* OR "Fetus" OR "Fetal" OR Conceptus*) AND ("Sex" OR "Gender") AND (Develop* OR Reprod* OR Ontogen*) AND (Litter*)

The inclusion criteria were:

- The main aim of the paper was to investigate, describe, assess, or compare how the sex of the offspring *in-utero* affects the uterine environment and/or subsequent offspring development in litter bearing species;

- The study used either *in vivo* or *in vitro* methods;

- Contained pre-natal or post-natal developmental data points;

- The study was primary research.

Papers were excluded in concurrence with the following criteria:

- Not primary research – papers that followed a study design of a systematic review, meta-analysis or case report;

- Papers published in languages other than English.

The retained papers from these stages were included in the final review. The references of the retained papers were manually checked to ensure no relevant literature had been missed. Once this was complete the final corpus was reached.

2.2.4   Information extraction

Due to the large number of papers in the final corpus (115 papers), a structured data extraction was performed contrary to traditional scoping reviews which do not normally follow a systematic approach to data extraction . In this protocol the following information was extracted from the final corpus: (1) aim of study; (2) defined study population; (3) *intra-uterine* effect measured; (4) methodology; (5) outcome measures (i.e. weight); (6) results. Due to the diversity amongst the papers, consistent quality assessment was not possible. However, reporting of sample size and power calculations along with the appropriate use of control groups was noted for each paper. The level of invasiveness of experimental approaches used was also recorded. These were scored as: *low* - minimal manipulation of the animals by experienced handlers causing minimal distress; *moderate* - manipulation of the animals causing distress for short and defined periods of time, such as non-regular injections or blood sampling, including the euthanasia of mothers and

offspring; *high* – animals receive treatment or procedures considered to cause high levels of perceived stress or pain, such as major surgery, repeated injections or blood sampling, this also considers cumulative distress and includes the euthanasia of mothers and offspring [31]. This was used as an additional means to asses methods used in the studies, as to understand whether the less invasive means were sufficient in obtaining the understanding of the effect in question, and equally, where the level of invasiveness were justified.
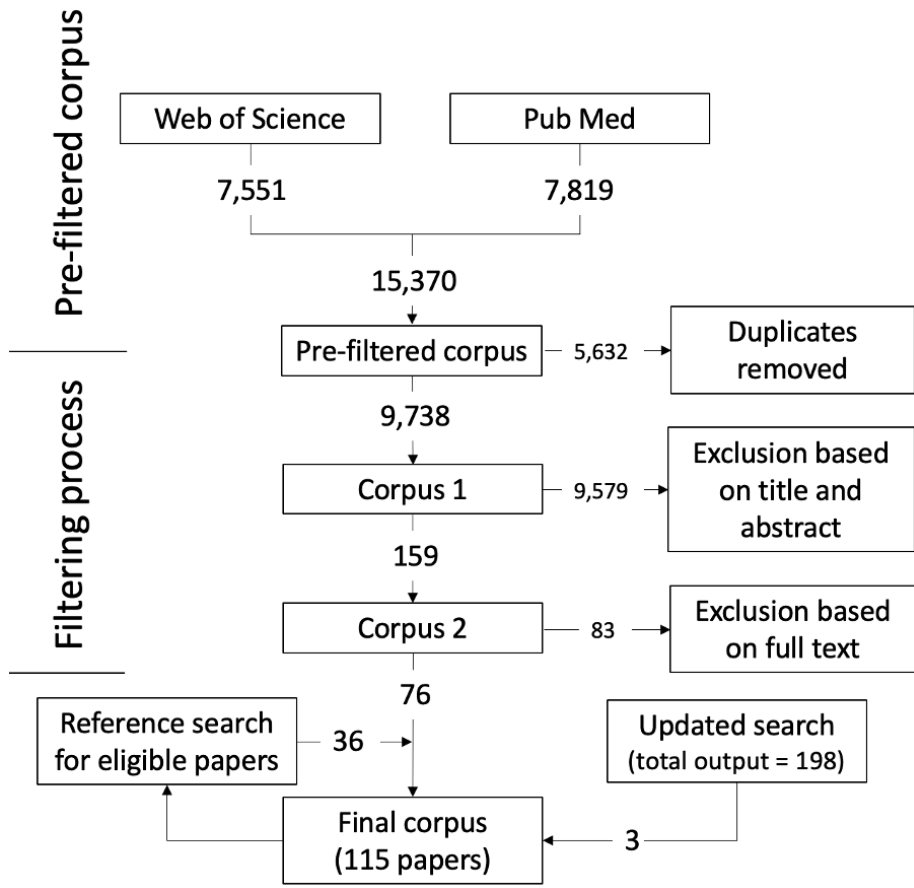
Figure 2.1: Number of papers included and excluded at the various stages of the literature search and refinement process. Illustrates the influx and removal of papers at each stage, resulting in the final corpus. The original search was conducted using the databases respective earliest year until January 2022, this was followed by an updated search on the 23rd of February 2022 using the main search terms.

2.3 Results

2.3.1 Search combination outputs

As seen in Figure 2.2, a large number of papers were missed by the main searches. Out of the 76 papers in the original search that were included, only 26 papers were captured by the main searches, whereas the 50 remaining papers were captured by the breakdown of the searches into four-way combinations. Out of these, there were also a large number of papers only found by one or the other database being either PubMed (n=39) or WoS (n=20), with only 17 papers being found by both databases.
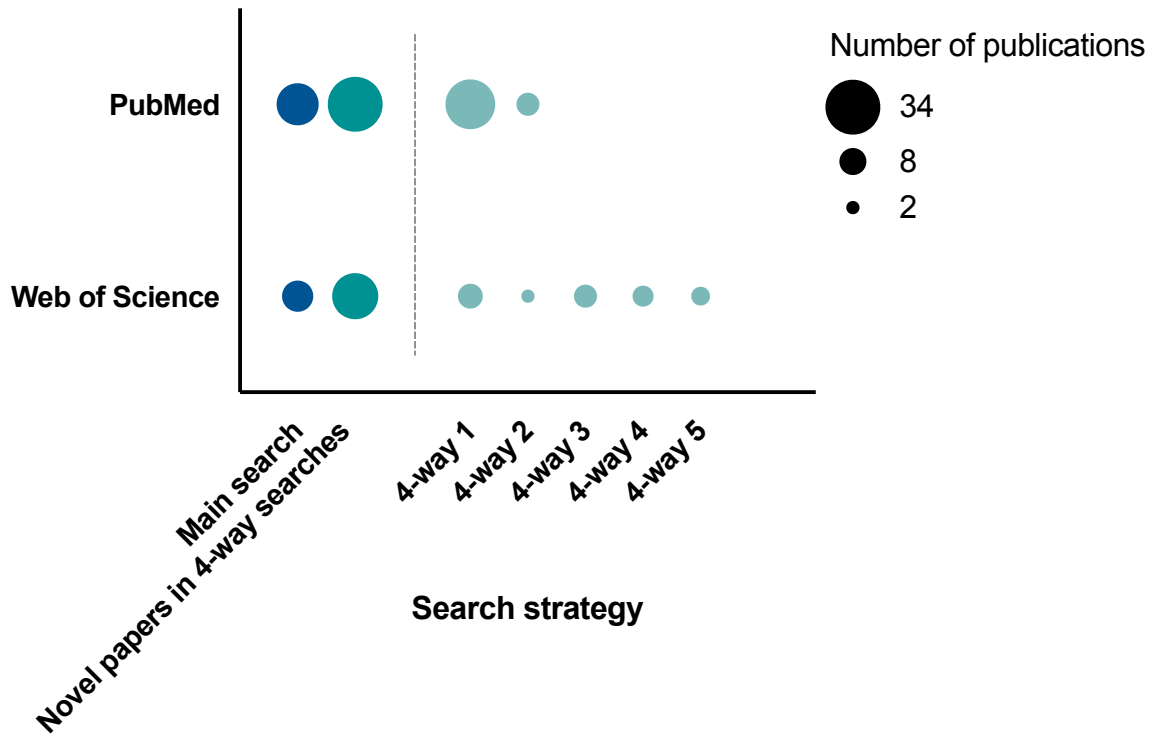
Figure 2.2: An illustration of the number of papers that were found in the main search (including all search term groups) as compared to the papers in the four-way combination searches that were not found by the main search, as per database. Following this is a breakdown of which four-way search the papers were found in. Main search is the use of five-way combination.

2.3.2 Characteristics of included studies

The initial searches identified a total of 15,370 papers, out of which 5,632 were duplicates and removed, leaving a pre-filtered corpus of 9,738 publications (Figure 2.1). Through

the filtering process a total of 76 papers were retained after stage three; from these, an additional 36 were added through a stage four reference check of the final papers. Three papers were included after the search was repeated in 2022. The final corpus included 115 papers in total (See Appendix A - Table 6.1).

As seen in Figure 2.3 the majority of studies were conducted in rodents (73 publications), including hamsters (1), degus (2), voles (2), nutria (2), guinea pigs (4), marmots (4), gerbils (13), but mainly in rats (32) and mice (34). There were 15 publications investigating the hormonal uterine environment in pigs, five in rabbits, three in ferrets and one in dogs. The majority of these were in captive animals, but a minority (5.22%, n=6) were carried out through observational or retrospective studies in wild populations of marmots. The papers were published during two main time periods — the late 1980/90s followed by a renewed interest in the topic around 2010.

Papers were assessed for their reporting of sample size, power calculations, and use of appropriate control groups where applicable to the study design. Reporting of sample size and power calculations, along with effect sizes was generally very low (<1%: Figure 2.4 C-E). Although appropriate control groups were used in most studies, those not using the appropriate controls (n = 27, 23.48%) mainly used the IUP as method of investigation. Hence 20 of the 77 papers using this method did not use the appropriate controls within their experimental design, i.e. no non-biased groups or 1M controls. These results can be seen in Figure 2.4 B.

The studies were invasive in terms of the intervention with 43.48% being considered moderately invasive, and 49.57 being highly invasive. Only 6.96% of the studies were considered to not cause the animals any significant stress or pain.
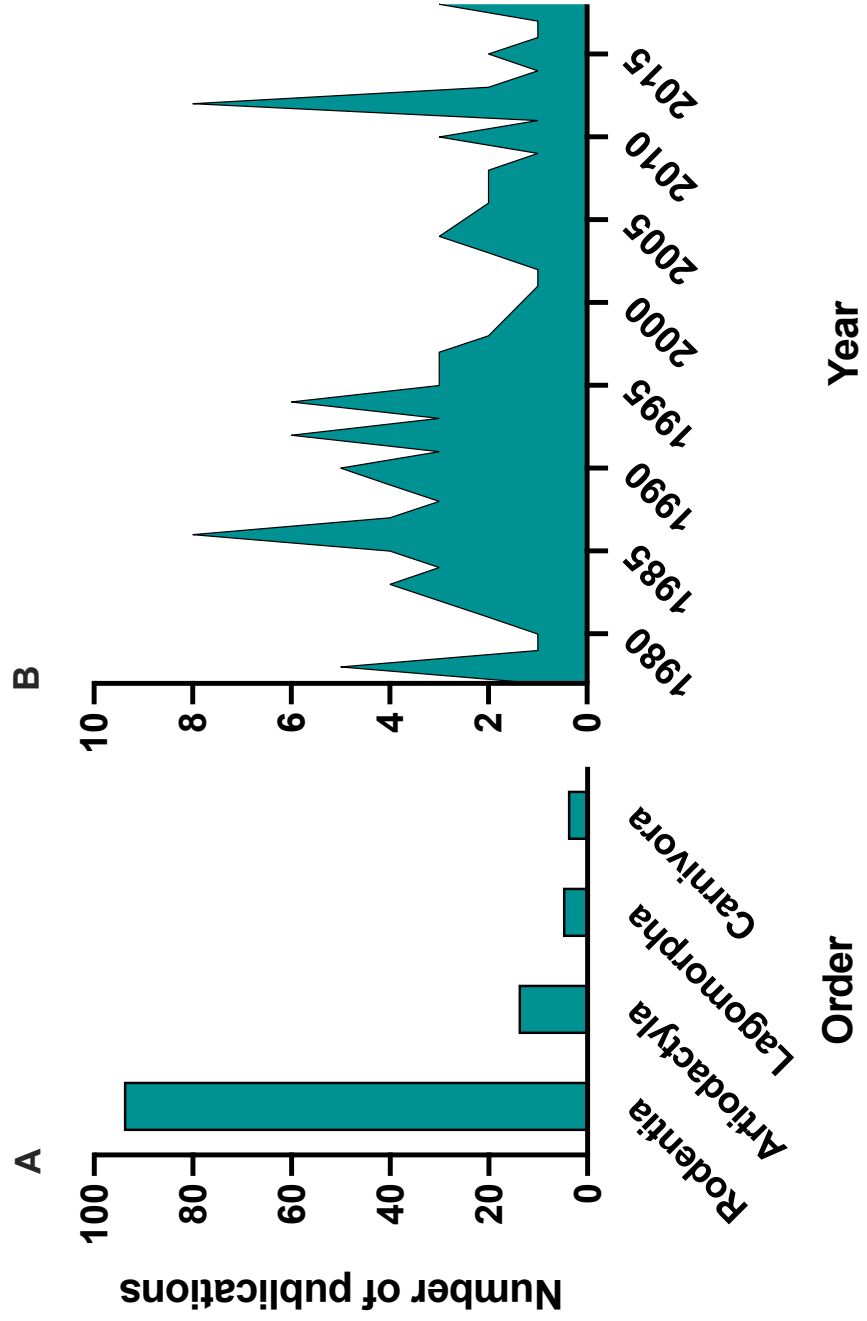
Figure 2.3: The number of publications retained in this scoping review based on the A) family to which the model species belonged to (Rodentia - rats, mice, voles, marmots, hamsters, guinea pigs, gerbils, and degus; artiodactyla - pigs; lagomorpha - rabbits; and, carnivora - dogs, and ferrets), and B) year of publication.
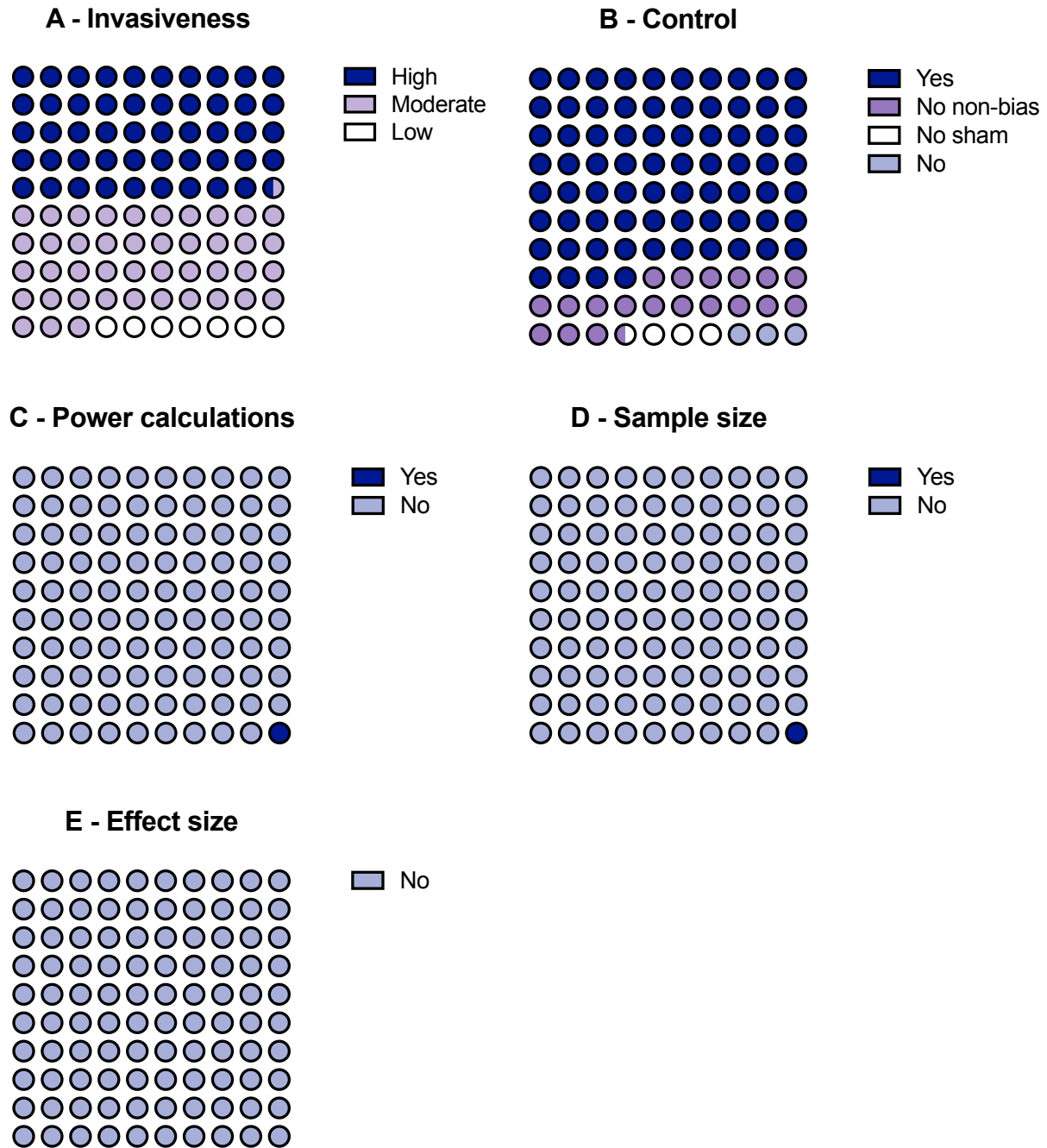
Figure 2.4: Quality assessment of the literature. A) The perceived invasiveness of the experimental design; Low being low levels of manipulation of animals causing minimal stress; Moderate being moderate manipulation of animals, euthanasia of mother and offspring included; High invasiveness was when manipulation probable in causing high levels of stress, pain, or adverse effects was used in combination with or excluding euthanasia. B) The inclusion of appropriate controls in the experimental design, these were dependent on the nature or experiment which was taken into consideration, in which some controls were not necessary. C) Number of papers reporting the use of a power calculation. D) Number of papers reporting the use of a sample size calculation. E) Number of papers reporting the effect size within their statistical analyses.

2.3.3 Models of hormonal uterine environment

2.3.3.1 Uterine positioning or litter sex bias

A common method to study the effect of a sex-biased litters is by performing a caesarean section before the estimated due date, in order to identify the absolute positioning of each foetus. The animals were manually removed from the mother and their uterine positions recorded, including the positions of their litter siblings. This method was used in ferrets [139], hamsters [241], gerbils [36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 75, 123, 207], guinea pigs [78], rabbits [20, 134], pigs [120, 143, 173, 191, 214, 253], mice [26, 29, 48, 79, 80, 100, 112, 125, 130, 131, 132, 148, 151, 166, 169, 170, 172, 183, 233, 234, 235, 237, 238, 239, 240, 245, 260], and rats [11, 103, 104, 107, 109, 144, 155, 165, 169, 178, 189, 216, 218, 219], to estimate the hormonal environment in which they developed. Two studies investigating IUP in nutria (a rodent) used government culled animals [68, 69].

Further studies used the bias of a litter as a proxy for the relative uterine positioning. These studies sexed the offspring at, or shortly after, birth resulting in a defined sex ratio of the litter. This was done in degus [51], wild populations of marmots using weaned litter sex ratios [99, 160, 161], rabbits [159], captive and wild voles [53, 77, 141], commercial pigs [61, 140, 157, 187, 201, 204, 205], and rats [46, 228]. Some studies used both manual and natural delivery [230] whilst others utilised hemi-hysterectomised rats to identify positioning based on birth order [108].

2.3.3.2 Testosterone and oestrogen exposure

Testosterone (T), or dihydrotestosterone propionate (DHT) was often used to mimic an androgenised uterine environment as seen in Table 2.2. This shows the array of different methodologies used to investigate the hormonal uterine environment, even within species. There are also studies using oestradiol administration, or synthesis blocking, to investigate the effect of siblings *in-utero*. For example, flutamide (4-nitro-3-trifluoromethylisobutyrylanilide) was used to block androgen synthesis in male and female rats [89].

Table 2.2: The different methodologies used within species to mimic a biased hormonal uterine environment. The different hormones, injection doses and frequencies along with injection sites have been summarised (I.m. = Intramuscular, S.c. = Subcutaneous, T = testosterone, TP = testosterone proprionate, DHTP = dihydrotestosterone proprionate, DHEA = dehydroepiandrosterone, DESDP = diethylstilbestrol dipropionate, P = progesterone, P5 = pregnenolone, EB = oestradiol benzoate).

Species	Testosterone	Gestational period (day)	Dosage	Frequency	Mode of Injection	Source
Mice	DHTP	12, 14, and 16	0.5, 1.0, 2.0 µg	Once daily	S.c.	(Mann and Svare, 1982)
Mice	DHTP	16.5-18.5	250 µg	Once daily	S.c.	(Witham et al., 2012)
Mice	T / DHEA / P / P5	11-17	1 or 500 mg	Once daily	S.c.	(Gandelman et al., 1979)
Guinea pig	EB / DESDP	29-68	1, 2, 3.3, 10 µg / 1, 3, 10 µg	6 days daily; then every other day	S.c.	(Hines and Goy et al., 1985)
Guinea pig	DESDP	29-65	3 µg	Once daily	Unknown	(Hines et al., 1987)
Guinea pig	DHTP / TP	(1) 30-39 / (2) 40-55 / (1) 30-39 / (2) 40-55	(1)10 mg; / (2) 1 mg / (1) 2.5, 5, or 10 mg; / (2) 1 mg	Once daily	Unknown	(Connolly & Resko, 1994)
Rabbit	TP	19-25	3µg OR 3 mg	Once daily	S.c.	(Banszegi et al., 2010)
Rabbit	TP	19-25	3µg OR 3 mg	Once daily	S.c.	(Banszegi et al., 2015)
Rat	DHTP	15-20	1 mg/kg	Once daily	I.m.	(Rodriguez et al., 2001)
Rat	DHTP	15.5-18.5 OR 18.5-21.5	1 OR 10 mg/kg	Once daily	S.c.	(Dean et al., 2012)
Rats	TP	16 or 19	1 mg	Once daily	I.m.	(Huffman and Hendricks, 1980)
Rat	Free T	16, 17, 18, 19, 20, 21, or 22	5 mg	Single dose	S.c.	(Rhees et al., 1997)
Rat	TP	15-19	0.5 mg/kg	Once daily	S.c.	(Chinnathambi et al., 2012)
Rat	TP	17-19	1 mg	Once daily	S.c.	(Dela Cruz & Pereira, 2012)
Rat	TP	14-19	2 mg	Once daily	I.m.	(Juarez et al., 1998)
Rat	TP	14-19	0, 0.1, 0.5, 1, 2, 5, 10 mg	Once daily	S.c.	(Wolf et al., 2002)
Rat	TP	16-20	2 mg	Once daily	I.m.	(Dunlap et al., 1978)
Rat	TP	(1) 15.5-16.5 / (2) 17.5-18.5 / (3)19.5-20.5	2 mg	Once daily	I.m.	(Ward et al., 1996)
Rats	TP	15-22 / 17 or 21	4 mg	Once daily or once	S.c.	(Ito et al., 1986)
Rats	TP	19 or 21	.25 mg	Once	I.m.	(Ichikawa and Fujii, 1982)
Dog	TP	24-43	1.1 mg/kg	Once daily	Unknown	(Beach et al., 1983)
Pig	TP	29,31,33, and 35	5, 10, or 20 mg/kg	Every two days	I.m.	(Ford and Christenson, 1987)
Pig	TP	30, 32, and 34 / 30, 32, 34, 40, 42, 44	25 mg/kg	Every two days	I.m.	(Petric et al., 2004)
Ferret	T	30-41	2-4 Pellets of 50 mg	Slow-release pellet	S.c.	(Tobet et al., 1986)

2.3.3.3   Anogenital distance

The AGD was also a used proxy for hormonal environment that an individual was exposed to *in utero*, often used in conjunction with, or as an outcome of the sex ratio of the litter from which they originated, such as in mice [126] and wild populations of degus [52].

2.3.3.4   Alternative methods

In some studies, same sex or mixed sex litters *in-utero* were created using in-vitro fertilization and blastocyst sexing [211]. Yet other studies used antiandrogens to investigate the role of androgens during gestation [89]. There have also been studies in rats blocking the synthesis of oestrogen using the aromatase inhibitor 1,4,6-androstatriene-3, 17-dione to investigate the effect of testosterone secretion and female uptake *in-utero* [119]). One study in mice investigated the effect of oestradiol (E2) on the testicular development of male siblings by utilising mice lacking oestrogenic receptors (ER) α or β [59].

2.3.3.5   Summary of models

In general, the main method used to investigate offspring hormonal influence on developmental outcome was the uterine positioning / litter sex bias (n=82), followed by administration of testosterone or oestrogen to skew *in-utero* steroid hormone exposure (n=26). Few studies used the AGD as a proxy of *in-utero* testosterone exposure (n=3), and some studies used alternative methods (n=4), being either skewing litter biases using in-vitro fertilization (IVF) sexing, or influencing hormone synthesis using; anti-androgens, synthesis blockers, or manipulating receptors.

2.3.4   Outcomes of studies

To assist classifying such a diverse array of studies we divided the studies into three outcome groups: (1) Physiological and systems development; (2) Behaviour; (3) Reproductive outcomes and development. The models used to investigate the hormonal impact were discussed collectively for each outcome.

2.3.4.1 Physiological/systems development

Influence on offspring weight and physiological traits

Studies using uterine position/sex bias demonstrated there was a clear influence on offspring weight. The body weight of male rats was found to be negatively correlated with increasing numbers of males *in-utero* until post-natal day 55, after which a higher body weight was associated with offspring originating from a male-biased litter [219]. In Mice, 2M females and males were significantly heavier than 0M females from day 6 post-partum and onwards [130], and in male prairie voles were also found to be significantly heavier at weaning if they originated from a male-only litter [53]. Male and female 2M pigs were also found to be heavier than their 0M counterparts [120, 214], however, Jang et al. [120] found that female 1M piglets were significantly heavier than both the 2M and 0M conspecifics [120]. Similarly, to that of female pigs, foetal piglets on ED 104 had significantly lower body weights if they were surrounded by two of the opposite sex [254]. In studies in male rabbits neither growth nor survivability was affected by adjacent males [20].

In contrast, external treatment with steroid hormones were less conclusive. The weight of commercial pig offspring from testosterone proprionate (TP) treated groups did not differ from control groups at birth, weaning, 4 and 6 months of age [181]. The weight of rat pups was significantly lower across both sexes, with T treatments as low as 0.5 mg, although the viability of these offspring remained unchanged [256]. Body weight and birth weight was not affected by 1 or 2 g TP treatment in mice [147].

Mice of 2M positions were found to have an increased 2D:4D ratio [112].

Effect on spinal nucleus of the bulbocavernosus

The spinal nucleus of the bulbocavernosus neurons, one of the superficial muscles of the perineum which participates in copulatory behaviour was affected by both the IUP and T treatment in both male and female gerbil offspring [75]. There was a significantly greater number of motoneurons in the spinal nucleus of the bulbocavernosus in 2M female gerbils than 0M females. In male gerbils, 2M males had a 50% larger bulbocavernosus muscle than 0M males [75]. This was also reflected in rats treated prenatally with testosterone

where the bulbocavernosus neurons in the spinal nucleus increased significantly, whilst neurons in the dorsolateral nucleus decreased [244].

Neurological influences

The hippocampal size in rodents is different between the sexes, with males having a larger hippocampal mass than females [84, 118] indicating development could be influenced by steroid hormones. However, it was found that 0M and 2M IUPs either reduced or eliminated the size difference between the sexes in the gerbil [207].

2M female gerbils have also been shown to have significantly increased cytochrome oxidase reactivity in the hypothalamus, demonstrating metabolic effects of the intra-uterine positioning in hypothalamic areas [123]. In the rat, ER α expression in the ventromedial nucleus of the hypothalamus is significantly higher in 2M females than 0M females, with the cytosine methylation in the ER promoter being greater in 0M females [165]. Hypothalamic GABA concentrations were found to be significantly higher in 0M than 1M and 2M rats [104]. The sexually dimorphic nucleus volume of the preoptic area in rats was greater in 2M males than 0M males [165]. This was not reflected in the female rat [178], nor was there any effect on the aromatase activity in the preoptic area in male rats dependent on IUP [218]. Female rats located caudally from two or more males had similar brain aromatase activity to those caudally located to 2 or fewer males [139]. Rats injected with 4 mg TP on ED 17 were found to have a higher volume of sexually dimorphic nucleus of the medial preoptic area [117]. Similarly, cells in the male nucleus of the preoptic area/anterior hypothalamus were significantly larger in TP treated female ferrets than control females although the brain weights were not affected [221]. Tobet et al. [218] found no correlation between the uterine positioning and aromatase activity in the hypothalamic, or temporal lobe tissues of female rat offspring. This was similarly unaffected in ferrets with differing numbers of caudal males *in-utero* [139].

Pei et al. [178] also found T and E concentrations of the brain to be significantly higher in 2M males compared to 0M males at ED 21. The midbrain of mice showed an increased density of μ-opioid receptors in females with 1 or more adjacent males *in-utero* [166].

Lung and ancillary systems

The submandibular gland concentration of epidermal growth factor (EGF) in mice was significantly higher in adult 2M females compared to 0M females, but not 1M females [26]. This was not replicated in males [26]. Chin glands in rabbits treated with 3 μg and 3 mg T were significantly bigger than those in controls [14], though this result was not replicated in a subsequent study [15].

Rats treated with T displayed a delayed functional maturation of the foetal lung, potentially via inhibition of the mesenchyme–type II cell interactions [190]. EGF binding sites in foetal lung tissue of rabbits was significantly lower in density of both male and female offspring treated with dihydrotestosterone [134].

Circulatory system and body hormonal content

An increased concentration of circulating T was found in androgenised female mice [255], female rats with caudal males [139], 2M female mice pups [235], and T treated male and female rats [35]. In accordance, female rats with caudal males [107] and 0M female and male gerbils [38] had a lower plasma androgen level than their non-male caudal and 2M counterparts. Female mice with longer AGDs (masculinised litters) had lower levels of calcium, cholesterol, phosphorus, iron, and protein in their blood chemistry profile [126]. T treatment in male and female rats did not seem to effect circulating E2 [35]. However, male hamsters who had two or more females located upstream displayed an increased level of oestrogen (E) and suppressed androgen in their blood [241]. Concurring, female ferrets with two or more males located caudally *in-utero* had a higher whole body androgen content than those with one or zero caudal males [139].

Contrary to these, another study in rats found pre-natal T treatment to significantly reduced level of circulating T in adulthood [58]. 0M male rats were also found to hold significantly higher levels of T, and lower levels of E in their blood serum than 1M and 2M pups [104].

Female mice foetuses had higher concentrations of T in their amniotic fluid if they came from a 2M position rather than 0M. This effect did not remain into adulthood [235].

In hamsters T levels in the amniotic fluid was not different between litters of different sex biases [78].

A greater capacity of 2M male mice to form DHT from T was suggested by approximately 60% greater activity levels of 5a-reductase compared to 0M males [170]. In rats, pre-natal T was found to increase arterial pressure [35]. 0M male mice were found to hold three times the concentration of androgen receptors in their prostates than 2M males, with no differences seen in ER concentrations [170]. Steroidal changes in rats due to T treatment was associated with defective expression of steroidogenic genes in both testes (upregulation of Star, Sf-1, and Hsd17b1) and ovaries (upregulation of Star and Cyp11a1, downregulation of Cyp19) [35].

2.3.4.2 Behaviour

Copulatory behaviours, attractiveness, and receptivity

Mounting behaviour and lordosis (body posture for sexual receptivity) was greatly affected by several models of an *in-utero* hormonal environment. Female with males either positioned caudally to them, or from male-biased litters, or exposed to pre-natal T treatment, displayed higher levels of mounting behaviour [46, 78, 109], and lower levels of lordosis [104, 109, 111, 188]. However, two studies found no effect of T treatment or a male-biased litter on lordosis (81,109). No differences were found in the latency to mount, number of mount bouts, or number of mount bouts with genital thrusting between 2M and 0M female mice [80]. Increased mounting behaviour was seen in male rats with caudal males *in-utero* [108]. Diethylstilbesterol dipropionate increased mounting behaviour in guinea pigs both in absence of, or in response to T. In response to oestradiol benzoate (EB) they exhibited an impaired display of both lordosis and mounting behaviour [89]. The behaviour of rats that had been treated with anti-androgens and subsequently treated with progesterone (females) or EB (males) showed increased lordosis [89].

In male gerbils, 2M positions resulted in shorter latencies to mount, to reach ejaculation, and between consecutive ejaculations. They were also more likely to ejaculate than 0M males [42]. Ito et al. [117] found a reduction in frequencies of mounting in all T treated groups of rats. Male ferrets exposed to a decreased level of E displayed reduced neck gripping and mounting when presented with females in heat [217].

29

Sexual receptivity scores in female rats were found to be lower in those treated with T on ED 19 compared with those treated on ED 16, or non-treated [111]. In pigs, immobilization at lower back pressure was used as a measure of receptivity that was displayed for fewer days in females treated with high levels of T during gestation, versus controls [73]. Similarly gonadectomised male and female rats demonstrated lower receptivity scores, with lordosis and darting between mounts being the assessed features [65].

2M gerbils were significantly more attractive to induced females then 0M males [43].

Scent marking occurred at a greater frequency in 2M male gerbils than 0M conspecifics [36]. Rabbits treated with 3 µg and 3 mg T and displayed higher levels of chin marking than control counterparts [14]. Male rats were found to spend significantly less time around females when treated with 1 mg of T (PD17-19, once daily) compared to their control counterparts [58]. Female mice were also significantly more attractive to males if they held an IUP of 0M rather than 2M [233]. Similarly, female prairie voles spent significantly less time with males from all-male litters vs female-biased or balanced litters [53].

Maternal and paternal behaviour

It was found that male and female gerbil pups that were located between males *in-utero* were given more maternal attention through anogenital licking [37]. It was also found that male gerbils positioned between two females were more attentive towards pups later in life than 2M males [45]. However, a study in spontaneous maternal behaviour and nest building found no differences between 2M and 0M female mice [132]. Maternal behaviour was negatively affected by T treatment by increasing self-grooming in mothers, whilst decreasing time spent near or interacting (sniffing and pawing) with rat pups [115, 124]. Sugawara et al. [211] found a higher level of maternal aggression during intruder tests when female mice originated from litters that had gestated in environments absent of male siblings.

Aggression, fear, and positive behaviours

Female 0M mice displayed higher avoidance behaviours in adulthood than did female 2M mice [100]. Male 2M mice were also found to display increased novelty seeking behaviour compared to 0M mice [172]. Female pigs were less likely to be fearful if from male-biased litters [204].

Aggressive behaviour was generally found to be increased in masculinised males and females, independent of model method used to study the uterine environment. Rats that had been treated with 1 and 2 g TP (PD12, 14, and 16) were found to be more aggressive by displaying significantly more attacks towards conspecifics than the control groups with oil injections or no injections [147]. Similarly to rats, mice would engage in fights sooner when treated with T versus control and vehicle groups [81]. 2M male and female mice were found to be significantly more aggressive [183, 238] and likely to display male sexual behaviour than 0M females [233], although, less likely to gain control over a food pellet [183]. In concordance, Gandelman et al. [82] found mice to display more male-like behaviours and display aggression if located between two males than if located between two females *in-utero*, this was also true for pregnant 2M mice [132]. Commercial pigs showed that 0M females were the recipients of the fewest bite attempts, with 2M females being pushed less often, although there was no difference in frequency of bites, attempted bites, threats, and pushes made by the two groups [173]. Contrary to evidence in mice, pigs were found to be more successful at gaining resources in a competitive environment when from a 2M position as they gained more weight than conspecifics when food availability was limited [191]. A study by Cologer-Clifford et al. [48] found that female mice who resided *in-utero* with any proportion of males were found to respond aggressively to an intruder when treated with a silastic 5 mg T implant (60-80 days of age), whereas female mice who were *in-utero* singly, did not hold this sensitivity and did not respond with aggression, but there was no significant difference between IUP and aggressive outcomes [79]. Masculinised female marmots were more likely to become dominant, and when they did, they were more likely to achieve this through fighting, defeating, and expelling the previous dominant female, rather than inheritance [99]. Masculinized female degus held more dominant ranks whereas feminized degus held subordinate ranks [51].
Masculinised juvenile marmots were more likely to initiate play and allogrooming [161].

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Feeding and locomotory behaviour

2M male mice displayed a lower saccharin preference than 0M male mice [29]. In taste aversion tests, there were no differences in acquisition of an aversion in rats with or without caudal males, however, extinction of the aversion was delayed in rats that did have caudal males *in-utero* when treated with testosterone [11].

Masculinised marmots had lower survivability in their first hibernation and were more likely to disperse [160]. A difference in dispersal was however not found in the townsend vole [141]. Rod tests examine the coordination and balance of animals, and female rats with longer AGDs were found to perform better in the tests along with increased frequency of rearing [126]. General activity levels were found to be influenced, with 0M females being more active than 2M female mice, which was also reflected in males [130, 172]. Rats from 2M positions were also found to have higher levels of sniffing conspecifics; heads, bodies, and anogenital area [103]. Meadow voles were more successful and had shorter latency to completion of water-maze tasks when originating from male-biased litters [77]. Nagao et al. [169] found no effect of IUP on locomotor and general behaviour in mice nor rats.

2.3.4.3   Reproductive outcomes and development

Reproductive anatomical and morphological differences

T treatment and *in-utero* sex biases had a strong influence on the AGD of offspring. Being located between two female mice and not two males during gestation made offspring more susceptible to changes in AGD [240]. AGDs were found to be significantly longer in female rats when closer to males *in-utero* [46], in male-biased marmots, pigs and rats [61, 119, 160], when female and male rats were positioned caudally to males *in-utero* [144, 155] and finally when located between two males rather than one or two females in rats and mice [82, 151, 189, 219, 233, 260]. Female rats and mice exposed to T *in-utero* had longer AGDs than their control counterparts [57, 82, 188, 256], similarly, rabbits treated with 3 mg testosterone had longer AGDs and were heavier than females from lower T and control groups [14]. However this was not reflected in a later study by the same group [15]. Female ferrets displayed dose-dependent increases in AGDs [221] and longer AGD in offspring from mothers exposed to T on day 30, than control counterparts [217].

While 2M male gerbils, had larger ventral glands, and a higher relative testes weight than 0M conspecifics [42, 44]. In rats, caudal males *in-utero* also had increased testicular weight [144]. However, the prostatic epithelial buds, utricle, and seminal vesicles were significantly larger in 0M male rats than 2M males. Prostatic bud development was significantly more likely to occur in 0M females than 2M females [216].

Nagao et al. [169] found no effect of IUP on reproductive organ development in mice and rats. However, other research found that females exposed to 0.5 mg T or more *in-utero* held reduced nipple counts, cleft phallus, smaller vaginal orifices, and displayed a presence of prostate tissue [256]. This effect on the nipples and prostate tissue was corroborated by Dean et al.[57]. Commercial pigs also displayed masculinisation of external genitalia when treated with TP [181]. Likewise, external genitalia was altered in female rats treated with 4 mg TP on days 15-22 and single injection on day 17 with high masculinisation or lack of external vagina [117]. Genital external morphology was not affected by 1 or 2 g TP in mice [147]. It has been suggested that male rats exposed to excessive E during gestation may hold a higher chance of sperm abnormalities as males with inactivated ER β showed higher numbers of gonocytes through a decreased level of apoptosis [119]. 2M female mice held a significantly elevated level of β-glucuronidase activity in their preputial glands compared to 0M females [245].

Puberty and Oestrus

2M IUPs in female gerbils [39], male contiguity in mice [151], and T treatment in rats [188] resulted in delayed vaginal opening. Female guinea pigs exposed to diethylstilbestrol diproprionate also displayed delayed vaginal opening [106]. Although a study in mice by vom Saal in 1989 [196] found no difference in time of vaginal opening between 2M and 0M female offspring, they did find that 0M females displayed significantly shorter initial oestrous cycles than 2M females and mated at a younger age. Findings in the onset of puberty and breeding are conflicting both within and amongst species. In T treated male rats [58] and male-biased marmots and rabbits there was a delayed onset of puberty or breeding [159]. However, in other studies in female mice [255] and rats [256], androgenisation of the *in-utero* environment by T treatment led to early onset puberty but delayed parturition. Findings in pigs were equally controversial. Although

T treatment in pigs [73] was found to delay onset of puberty, sex bias studies weren't as conclusive. As the number of males in a litter increased, in combination with a smaller litter size, females reached puberty at an older age [140]. This was reflected where 0M females displayed earlier puberty and shorter post-pubertal cycles, however, this was also dependent on maternal stress where the opposite outcome was seen if the pregnant sow was stressed during gestation [237, 240]. Another study found that uterine position did not alter puberty onset in the commercial pig, but receptivity during the third oestrus was reduced in 2M females [173].

Luteinizing hormone (LH) surges in gilts (primiparous pigs) from male-biased litters were delayed and the surge the duration was decreased compared to those in female-biased groups [205]. However, no differences were found in mean plasma LH concentrations, nor in LH pulse frequencies, or amplitude, between control of TP treated offspring [181]. When challenged with EB following gonadectomy, guinea pigs exposed to T treatment whilst *in-utero* displayed decreased LH surges compared to their control counterparts, similar to that seen in pigs. The LH rise in the 5 and 10 mg T treated guinea pigs were 50 to 75% lower than control and lower T treated groups [49]. Gilts from male-biased litters were more likely to ovulate and had higher numbers of corpus lutea (CL) than those from female-biased litters [205]. Contrary to this, female guinea pigs exposed to 3 μg diethylstilbestrol diproprionate (PD 29-65) showed an absence of CL in response to EB [106].No effects were found in mice offspring developing in mixed or male-free litters on the length of the oestrous cycle, nor ovulation efficacy [211]. Nagao et al [169] found no effect of IUP on sexual maturation in mice and rats.

Reproductive success

There was a clear negative effect of originating from a male-biased litter on the reproductive success of female offspring. Female pigs had fewer failed breeding attempts during their first four breeding attempts if they originated from female biased litters [61]. The reproductive lifespan was longer in female mice, if not positioned between any males *in-utero*, producing more litters in total, containing live pups for a larger proportion of their lifespan [233].

Masculinised marmots were less likely to become pregnant and wean young [160], and

female degus with larger AGDs gave birth later in the breeding season compared to their female-biased counterparts [52]. Lactational performance, and litter size was not affected by 1 or 2 g TP in mice [147].

Litter characteristics

Findings in commercial pigs demonstrated different effects of litter sex ratio on offspring pre-weaning mortality dependent on breed, with the Polish Large White offspring showing increased mortality in female biased litters, and decreased mortality in the Polish Landrace offspring [187]. 2M male gerbils were found to sire on average 28% more offspring than their 0M counterparts [43]. 2M female gerbils from male-biased litters delivered a greater proportion of males during their reproductive lifetime than did 0M females from male-biased litters. The 2M females were also more likely to carry 2M female offspring [40, 41]. The first litters from mice originating from 2M, 1M and 0M *in-utero* positions consisted of 58%, 51%, and 42% males respectively, a trend that was also present in the second litter [230]. Rats also displayed significantly smaller litters with higher T treatments (5 and 10 mg) [256]. Masculinised female degus with longer AGDs gave birth to heavier offspring, with more male-biased litters [52]. There was no difference in litter size, success in raising offspring nor the sex ratio of subsequent progeny of female mice that had originated from mixed or male-free litters [211].

2.4   Discussion

This review aimed to summarise the research that investigated how the hormonally biased uterine environment affects offspring in mammalian litter bearing species. In total, 115 papers were included, and it identified three main methods that were used to investigate this. These included (1) the exact intrauterine position of offspring in relation to the sex of their neighbouring siblings, (2) the sex bias of a litter based on the proportion of each sex in the litter, and finally (3) direct manipulation of the hormonal environment through administration of steroid hormones.

2.4.1 Search methods

As visualised in figure 2.2, a large number of papers were missed by the main search. 50 out of the 76 papers (initial search) included were only captured one of the two data bases I used for this search. Further, a relatively large number of papers (n=36) were included after checking the references of included papers. It is evident that the main searches didn't capture a large proportion of the literature of interest for this review. The traditional format for a systematic literature search creates a search too focused for the purpose of a broader search. As this review aimed to include papers using any model, any litter bearing species, and any outcome, a structured search was found to be too rigorous to capture all relevant studies. However, including the extended searches reference checks seemed to capture the relevant literature. We further highlighted the necessity of using a broad range of databases, as few (n=17) papers were captured by both databases, and a majority only captured by one of the selected databases. This is likely due to papers being published in journals outside of each others remit, medical journals potentially outside of WoS remit.

2.4.2 Implications of method of investigation

There is a lot of variation in the methods used between studies, making direct comparison of results and conclusions within the field relatively difficult. Although this review has focused on litter bearing species only, there are significant biological differences to be considered when attempting to synthesise findings. For example, when looking at caudal males, the uterine blood flow between species differs, even between species which are morphologically similar such as the mouse and rat. There could possibly be within-species differences, such as between breeds or even individuals. This renders the caudal theory redundant. Similarly, the standard of reporting was not consistently high, and extracting information from a number of papers was difficult, with some information lacking, making further, true comparisons difficult, if not impossible. Due to these aspects, it is important that the discussion of findings below is read with this understanding, and that these issues must be taken into account especially if attempting to draw conclusions.

The different methods employed each hold their own limitations. Utilising the AGD of an animal is only a proxy measurement of the hormonal gestational environment in

which an animal is believed to have experienced. However, as the results reveal, the way in which the AGD of an individual is affected was not unanimous in the current research. Hence, the studies utilising only the AGD as a proxy of uterine hormonal environment may not be accurately estimating the gestational hormonal status of that individual.

For the sex bias and IUP studies, there was a high variation and/or no disclosure of the method utilised to determine sex ratios and sexing of offspring (especially important in early foetal subject). Similarly, litter sizes were seldomly reported. The litter size has been found to influence the passage of sex steroids between foetuses, specifically in rats, with minimal diffusion of testosterone between amniotic sacs when there is less than 6 offspring in the litter. This is due to the additional space within the uterine environment and between offspring [236]. Hence it has been hypothesised that the amount of steroid passing between siblings is greater the more crowded the uterus, i.e. larger litters.

There were also several different methods of anaesthetising both the mother and/or offspring. Especially when investigating physiological and blood chemistry content, different methods/substances used in anaesthetising the mother could interfere with the results obtained from the offspring [10].

As identified by Banszegi et al. [15], in studies utilising injections of for example hormones or anti-androgens/oestrogens, using not just a control group but also a sham injection is important. This is vital for the interpretation of findings, particularly those of behavioural nature, due to the stress associated with the procedure. Although we found very few studies that did not use a sham injection (Figure 2.4, B) there was nonetheless high variability between the days of injection, numbers of injections, concentrations used, and little to no reporting of how the used concentrations were established.

There was also poor reporting of power calculations (0.9%), sample size calculations (0.9%), or effect sizes (0%). Further appropriate controls were not used by 36.49% of the included papers.

2.4.3 Physiological and systems development

The weight off offspring, both birth weight and body weights at different life stages, were found to be significantly greater in male and female offspring that originated from both 2M positions and male-biased litters (rats; [219], mice; [130], praire voles; [53], pigs; [120, 214].

However, Wise and Christenson [254] found a drop in body weight of ED 104 piglets when surrounded by two of the opposite sex *in-utero*. Although results from IUP and litter sex ratio studies were overwhelmingly conclusive, the same was not found in those mimicking a biased environment using injected androgens. No difference was seen in weight in rabbits [20], pigs [147], or mice [181] when treated with T. However, in rats, body weight was found to be significantly reduced in both sexes with T treatments (PD14-19) as low as 0.5 mg [256]. There was also a strong suggestion that body weight and fat deposition may be regulated by prior *in-utero* location, influencing metabolic set points.

There is evidence that T treatment in both rabbits [134] and rats [190], male and female, affects the development of the respiratory system; with lower EGF binding sites in the lung plasma membrane and a delayed functional lung maturation. There is also evidence of an androgenised environment impacting on systems which only begin differentiation postnatally, such as the influence on EGF in the submandibular glands [26].

The 2D:4D ratio is commonly used as an indicative measure of excess androgen exposure in humans, where the ratio is reduced, and investigated in relation to behavioural outcomes [27]. Contrary to findings in humans, the 2D:4D ratio was greater in mice originating from a 2M IUP [112].

A significant body of work has investigated the hormonal effect on the neurological development of offspring [25]. The regions that were often studied were sexually dimorphic and/or known to participate in copulatory behaviour or synthesis of hormones.

Findings in the hippocampus showed that being in an extreme hormonal environment, both oestrogenised or androgenised, eliminated the sex differences between male and female hippocampal size [207]. An androgenised environment resulted in a larger sexually dimorphic nucleus of the medial preoptic area [117], whilst the cells in this area were also larger [221]. Androgenisation decreased hypothalamic GABA concentrations [104], this could have influences on the individuals reactivity towards stress, and could potentially impact on their exploratory behaviour. An androgenised environment led to suggested metabolic effects in the hypothalamic areas, although no alterations to potential androgen synthesis via aromatase activity was found [123, 139, 220]. There was also no effect of

androgenisation on the aromatase activity in the preoptic, or temporal lobe tissues in females [139, 220]. The sexually dimorphic nucleus volume of the preoptic area was greater in androgenised males [165, 178]. ERs in the anterior part of the hypothalamus were also found to be significantly higher in numbers in offspring from androgenised uterine environments with higher expression in receptors, and increased cytosine methylation in the receptor promotors in oestrogenised females [165]. An androgenised environment also increased the density of μ-opioid receptors in the midbrain [166], which is vital for the reward and motivation of an individual by blocking or upregulating nervous impulses.

In general, concentrations of T and E in the rodent brain were significantly higher in androgenised males [178], caudal males, neighbouring males (2M) and T treatment all seemed to influence circulating, and/or whole-body content of T and not E in both males and females [35, 38, 139, 255]. Changes in circulating steroids was also associated with defective expression of steroidogenic genes in both the testes and ovaries [35]. It is important to note that there were contradictory findings where decreased levels of circulating T was found in adults from an androgenised environment [58, 104, 107, 235]. In oestrogenised environments, there was a decreased level of T, and increased E in circulating blood [241]. Other aspects of the profile of circulating blood were affected, with levels of calcium, cholesterol, phosphorus, iron, and protein being decreased in androgenised offspring [126]. Similar to blood chemistry, the effect of androgen levels in the amniotic fluid was dependent on species. Mice from 2M IUPs had higher levels of androgens, whilst no differences were seen between positions in the hamster. This further indicates that the individual offspring uterine milieu will be influenced differently between species.

2.4.4 Behaviour

There was an overwhelming consensus amongst studies regarding the effect that an androgenised uterine environment held on the sexual behaviours displayed by both males and females. Not only did females show an increased level of what is typically considered a male behaviours, such as mounting behaviours [46, 78, 109, 233], but it also resulted in decreased female copulatory behaviours, such as lordosis [65, 103, 109, 111, 188]. This would suggest that the female brain may be exposed to higher levels of oestradiol, via diffused testosterone from neighbouring male foetuses during the sensitive period of brain

development. As a result, their copulatory behaviour is being skewed towards male typical behaviours which may reduce their potential reproductive receptivity, as females may not display oestrus, nor accept mounting when in oestrus. This may be what is resulting in the lower receptivity scores of females from an androgenised uterine environment [65, 73, 111]. Males also displayed this sensitivity to an androgenised uterine environment, where copulatory behaviour was further masculinised, with faster mounting, a higher likelihood to ejaculate, alongside a shorter time to and between ejaculations [42, 108]. Males from androgenised uterine environments may be considered to have a higher level of sexual efficienty.

Further to this, guinea pigs (females) and ferrets (males) that had been treated with anti-oestrogens during gestation displayed more female like behaviours [106, 217], mimicking the lack of aromatized testosterone influencing brain development. Hence, it can be taken that the level of testosterone that both male and female offspring are exposed to in late gestation has a direct influence on the development of the sexually dimorphic parts of their brain, and results in clear alterations to typical male or female sexual behaviours.

Despite the what could be perceived as a higher male reproductive efficiency from androgenised males, there are many other factors which affect mating success that must be considered. For example, the androgenised males were also found to be less attentive to females, but more attractive to induced females [43, 58]. Whilst androgenised males were more attractive to females, the androgenised female was less attractive to males [233]. Androgenised female mice held elevated levels of β-glucuronidase activity in their preputial glands [245]; this is a source of phermones which promote copulatory behvaiour. The increased enzymatic activity has been suggested to increase release of steroid metabolites in urine, potentially acting as pheromones. However, it has also been linked to inducing male aggression in male mice [116], one potentially explation of the mechanisms behind why androgenised females are less attractive to males. It may also be an indication to why androgenised gerbil offspring were found to recieve more maternal attention, via anogential licking, than their conspecifics [37].

Parental behaviour was affected differently between sexes. Paternal behaviour appeared to improve when males were from an oestrogenised uterine environment [45], and maternal intruder aggression in mice also increased when oestrogenised. The opposite, an

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androgenised uterine environment, had an overall negative impact on parental behaviour, with androgenised rats spending less time interacting with their pups [115, 124].

It is evident that the androgenised uterine environment held a large influence on the reproductive and parental behaviours of the gestating offspring. A more overarching finding was is that an androgenised environment leads to significantly higher levels of aggression across both genders [79, 81, 82, 132, 147, 183, 204, 238]. For example, it is suggested that pigs may be holding more dominant ranks, and part taking in more successful aggressive encounters than non-biased and oestrogenised individuals. Androgensied females are the recipients of the fewest push attempts whilst also pushing others significantly more, they were also the recipients of the most bites. Oestrogenised females received the most push attempts but the least bites, suggesting an avoidance of conflict [173]. This is supported in other species as well, where marmots and degus from androgenised litters were more likely to hold dominant positions [51, 99]. Not only were they more likely to hold dominant positions in the hierarchy, but they were also more likely than oestrogenised females to expel previous dominant females in aggressive encounters [51, 99].

Why animals are more aggressive is still poorly understood, and may be species specific. Increased aggression could be due to increased boldness or reduced fear. In mice the increase in aggression was interpreted as being due to reduced levels of experienced fear [204], potentially explaining why androgenised juvenile marmots initiate play and allogrooming more than oestrogenised juveniles [161], and why rats interacted more with conspecifics by sniffing [103]. The reduced fear would also be in accordance with androgenised male mice displaying increased novelty seeking behaviour [172]. Androgenised animals performed better in water-maze tasks (meadow voles) and rod tests (rats), with more rearing and displayed less avoidance behaviours [77, 100, 126], further suggesting that the androgenised uterine environment has either increased boldness, reduced fear, or influenced both in the androgenised individual.

General behaviour were also influenced, such as activity levels. Although the results were more contradictory. Both male and female mice were found to be less active if from an androgenised environment [130, 172], a recent study did not find any effect of androgenisation nor oestrogenised on the locomotor behaviour nor activity levels in rats nor mice [169]. Territorial behaviours were found to be altered by the intra-uterine environment

via increased ferequency of scent marking in both rabbits and gerbils when androgenised as compared to oestrogenised [14, 36, 42].

2.4.5 Reproductive success and development

Androgenised males held a greater capacity to form dihydrotestosterone from testosterone, whereas oestrogenised males a greater number of androgen receptors in their prostates [170], suggesting an enhancement of androdependent accessory organ development with small supplementations of E. Testes weights were also higher if androgenised [144] with the prostatic epithelial buds, utricle, and seminal vesicles being significantly larger in oestrogenised males [216], alongside a developmental inhibitory effect on testee development by endogenous oestrogens during foetal development [59]. Female external genitalia were masculinised when treated with T, [106, 117, 181, 188, 256]. Genital external morphology was not affected in mice [147]. Other body parts negatively affected by the androgenisation include reduced nipple counts, cleft phallus, and presence of prostate tissue in females [57, 256]. However, this was not investigated using the IUP or a litter sex bias.

The differential development of the AGD was overwhelmingly similar across species. In rats, mice, rabbits, ferrets, marmots and pigs, the AGD was significantly greater in the androgenised female, irrespective of model used [14, 42, 44, 57, 61, 81, 82, 119, 144, 151, 155, 160, 188, 189, 217, 219, 221, 233, 256, 260]. The contradictory results found by Banszegi et al. [15] might possibly have been due to the maternal stress during injections, which the authors suggest could cause under-masculinisation and therefore cancel out effects of injected T. This, along with the effect of androgenisation on piglet weights being reversed in litters that experienced maternal stress further supports the hypothesis that cortisol may be either interfering with, blocking, or causing changes that cancel out the effects of excess androgens. This indicates that those offspring that are developing in a non-biased uterine environment may be at a greater disadvantage if there is maternal stress during gestation. The androgenised uterine environment may buffer the negative impacts that increased cortisol could bear.

Physiological development of reproductive tracts and tissues was also influenced by the hormonal *in utero* environment. For example, androgenised males had larger ventral glands, and higher relative testes weight than oestrogenised males [42, 44], an increased development of male sexual organs. On the other hand androgenised females were found

to experience delayed vaginal opening by the majority of studies across all model types; in gerbils, mice, rats, guinea pigs, and pigs [39, 151]. Along with the delayed vaginal opening females reached puberty at an older age, and/or held a delayed first litter when from an androgenised gestation [52, 58, 73, 140, 159, 161]. It is important to note that there were some contradictory papers. For example in pigs, Lamberson et al. [140] found this delay puberty, but this was not reflected by Parfet et al. [173]. There were also contradictory findings in mice and rats where androgenisation led to early onset of puberty [255, 256]. Hence, although a majority of findings suggest a detrimental effect of androgenisation on puberty, there are still contradictory findings, not only between but within species. We suggest that there may be influence by external factors, such as cortisol influences mentioned above, that may be blocking the effect of an androgenised uterine environment.

Similar contradictions to those observed in the onset of puberty were seen in the oestrous cycle. Initial oestrous cycles were found to be shorter in oestrogenised mouse offspring [240], but likelihood of ovulating was either higher (pigs) or lower (guinnea pigs) in androgenised females [205]. Sugawara et al. [211] found no effects of androgenisation or oestrogenisation on the oestrous cycle nor ovulation in mice, whilst other findings in mice suggested that cyclicity became irregular and senescence was reached at an earlier age [255]. LH surges in androgenised female pigs and guinea pigs showed suppressed LH surges compared to oestrogenised females [49, 201]. A suppressed LH surge may lead to failure to ovulate, and therefore unsuccessful breeding attempts. As seen in puberty the effect on the oestrous cycle appears to be not only species dependent, but findings inconsistent even within species.

Litter characteristics of individuals was also influenced by the hormonal uterine environment in a similar fashion to the tract development. An androgenised uterine environment seemed to boost the male performance, but inhibit or reduce the performance of the female. Androgenised prairie voles were found to sire a greater number of offspring compared to oestrogenised males [53], potentially due to the enhanced sexual behaviour they display. Androgenised females on the other hand produce smaller or fewer litters in mice and rats [239, 256] with more male offspring in gerbils, degus, mice, and pigs [31, 65, 129, 141, 157]. The continuation of a male biased female going on to produce litters with more males stipulates a perpetuating effect. Developing in mixed or male-free litters

did not affect the litter size, success in raising offspring nor the sex ratio of subsequent progeny [211].

2.5   Conclusions

It is clear that the hormonal environment in which an individual gestated in may hold a significant impact on their health, development and reproductive potential. An androgenised environment impacts behaviour, weight, lung maturation, sexually dimorphic areas of the brain, along with areas linked to stress, reward and motivation. The results remain contradictory, there seems to be a lot of variability in the findings not only based on the model used to study the phenomenon of androgenisation, but also between- and within- species. The physiological and systems development of animals was often affected by an androgenised uterine environment. Animals from a 2M position of male biased litter were overwhelmingly heavier than their non-biased counterparts, whilst T treatment had more varied outcomes. It is however important to consider the difference between natural and pulsatile T release compared to supra-physiological T administration, which may account for variation seen between studies using the different models. The brain and lungs were also clearly affected by an androgenised environment along with both blood and amniotic fluid composition. Regarding behaviour, androgenisation conclusively led to a more aggressive, dominant and exploratory female. Although it improved maternal aggression, paternal and maternal attentiveness towards offspring decreased. The attractiveness and receptivity of females also decreased when androgenised and reproductive organs were often masculinised when treated with androgens. Females were less successful from a reproductive perspective with altered hormone profiles during oestrus, shorter reproductive lifespans, and producing smaller litters. Developmental differences to the AGD and vaginal opening where overwhelmingly conclusive in a range of species across all models, however, that is not the case for other reproductive outcomes. The onset of puberty was conflicting both between and within species. Most of the evidence supports the notion that an androgenised environment delays onset of puberty. Interestingly the earlier onset of puberty found in masculinised pigs occurred in those also experiencing maternal stress, this alongside shortened AGD in T treated females suggests that the maternal stress may be interfering with the effect that an androgenised uterine environment

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may bear on offspring, potentially through the influence of cortisol.

Despite evidence of a wide effect of the hormonal environment on several biological systems, there is little research attempting to understand why these changes are happening. The reason for the increased aggression is poorly understood, and why reproductive parameters of females are affected remains unknown. The gestational hormonal environment is clearly a source of variability in offspring originating from the same litter and it is evidently important that this environment be considered both when using animals in experimental designs, but it is also equally critical when selecting animals for breeding stock. Due to the differences seen both between and within species an understanding of the underlying mechanisms that are being affected is crucial. Further studies in this area are therefore critical, initially to facilitate and enable potential extrapolation of data and information, but to untie the complex effects seen in this review.

Chapter 3

Ovarian potential and influences of intra-uterine hormonal bias

3.1   Introduction

The maternal intra-uterine hormonal environment has been shown to influence many aspects of offspring development[91]. Studies across species specifically investigating the effects of a uterine environment biased by foetal hormones have indicated effects such as alterations to luteinising hormone profiles [201], altered physiological development such as delayed lung maturation [190], behavioural changes such as increased aggression [204], and reduced maternal and paternal behaviour [115]. These effects are observed following an androgenisation of the uterine environment in litter bearing species, often caused by disproportionate numbers of males *in-utero* [195]. This altered hormonal milieu that arises when there are proportionately more males in the uterus leads to siblings of that litter being exposed to a greater level of testosterone [236]. This may be a consequence of either; females positioned between males (which is proportionately more likely to happen in male-biased litters) or; the overall proportion of males excreting testosterone from the point of sexual differentiation [74]. A biased litter, one that skews toward a predominantly androgenised or oestrogenised environment, has often been defined as one consisting of >60% of one sex [195, 202]. In wild pig populations proportions of 1.3:1 males to females per litter have been reported [71]. However, the skew of sex ratios within litters are poorly investigated in commercial pig production systems as a whole, let alone between different

breeds and genetic combinations. The biased hormonal environment has been shown to affect several aspects of reproductive function in the offspring. Specifically, female pigs that originate from male-biased litters have fewer teats [62], a lower conception rate at first mating [61], increased sensitivity to gonadotropins [203], and altered LH surge profiles [201], in comparison to offspring originating from female-biased litters. The cause of these changes in reproductive efficacy remains unclear. Seyfang et al. [205] investigated the effect of litter bias on ovarian function in commercial pigs. Following stimulation of oestrus by gonadotrophin injection in 18-week-old pigs, two measurements were taken; likelihood to ovulate (proportion of females who ovulated vs non-ovulatory females) and number of ovulated follicles (determined via CL count), both proxy measures using Corpora Luteum (CL) presence as indicators. Female pigs from male-biased litters were found to be significantly more likely to ovulate than female-biased litters (86.0% vs 59.5%, respectively) and consequently have more corpora lutea (13.1 ± 1.5 vs 7.2 ± 1.7, respectively) than their female-biased counterparts. The authors suggested that organisational events during reproductive development may possibly be altered causing the observed differences between bias litters [205], although impacts on subsequent fertility were undetermined.

Arguably, one of the most vital organisational event in female reproductive development during gestation is the developing primordial germ cell (PGC)s. PGCs are originally derived from the proximal epiblast cells of pre-gastrulating embryos [142], i.e. prior to generation of the three primary germ layers. In pigs these have been identified in the dorsal mesentery at E18-20 (Embryonic day) which then migrate to colonise forming the genital ridge at E23-24 [212]. In the pig, meiosis, initiation, and formation of primordial follicles begins at E48 and continues until 25 days post parturition [162]. Differentiation of the Wolffian duct in the male begins at E26, at which point secretion of testosterone begins [9]. Hence, laying down and formation of the primordial follicles in female offspring occurs once offspring derived testosterone from male siblings is present in the uterine environment. The point at which the number of PGCs peaks is determined by morphogenesis and germ cell dynamics [128], further it is still not understood how the regulation of the pools survival and maintenance, maintained dormancy, and activation for folliculogenesis occurs [128]. These quiescent PGCs will begin to undergo folliculogenesis around 30-40 days postpartum; this marks the point at which the established primordial follicles are

recruited and develop to a pre-ovulatory stage [200]. Until recently, it was believed that the follicles present at birth were finite and not replenished, however, proliferative germ cells sustaining follicular numbers have since been discovered in the mammalian ovary [122, 171, 263]. Although these remain little understood, there is growing evidence that they could potentially sustain the primordial follicular population [96], and a murine study suggested this is vital to the maintenance of the primordial pool [122, 127]. There is at the point in time of this article no evidence for oogenic stem cells in the pig.

The total ovarian reserve (TOR) is a term used to describe the total number of healthy oocytes within the ovary. This is commonly used as a proxy to represent the reproductive capacity of a female [90]. Causes of a depleted or insufficient TOR can be described by exploring two main observations, decreased initial TOR or the accelerated recruitment of follicles and increased atresia. Female rodents from a hyper androgenised *in-utero* environments had lighter weight ovaries for their body size compared to similar sized females from a non-hyper androgenised environment, although a functional effect from this was not investigated [258]. Similarly, research in sheep has found that a prenatal hyper-androgenised environment leads to a decreased TOR and enhanced follicular recruitment with low birth weight observed in the same female offspring [210].

Data from other species indicates that an androgenised uterine environment effects the development of both gross morphology of reproductive tracts [46, 61, 117, 155, 160, 181, 216, 256], subsequent follicular recruitment [203]. It is also clear in the pig that there may be an effect on ovulation patterns [201, 203]. However, it is currently unclear whether an androgenised uterine environment affects PGC formation or follicular recruitment of the PGCs in the pig. This study was designed to investigate the influence of a sex-biased litter on the ovarian profile of the commercial pig. This was achieved through investigation of the two following objectives: I) Investigation of how the established primordial germ cell pool is affected by different *in-utero* hormonal biases; II) Identification of any differences in follicular recruitment, or follicular atresia profiles dependent on litter sex bias.

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3.2 Material and methods

After consultation with the University of Leeds Animal Welfare and Ethical Review Committee no ethical approval was sought. It was deemed that due to no interventions taking place, and the pigs remaining fully under the commercial management, ethics was not necessary. This complies with the 3Rs strategy of utilising animals which are already part of a process.

3.2.1 Animals

All animals were reared in a closed indoor herd at the National Pig Centre on a partially slatted floor system. The pigs had access to ad libitum feed and water. They were housed in pens of 2.45x1.55m with the sows in traditional farrowing crates from birth until 4 weeks of age. After this the pigs were mixed into groups of ten and placed in pens of 1.5x2.5m. At the age of 12 weeks the same ten pigs would be moved into pens of 1.7x4m where they remained until slaughter. All pigs across collection time points were reared in the same systems and buildings with no changes to diet or handlers. The pigs used were either not part of any other trials or used as control pigs in other dietary studies. Female pigs (JSR Large White x Landrace females JT dam-line x JSR Pietrain-based Geneconverter 900 sire-line) were identified and selected for tracking post-weaning based on the sex ratio of the litter from which they originated. They were subsequently tracked through the production system using RFID ear-tags. The pigs were weighed weekly in the month leading up to slaughter in order to optimise an accurate estimate of slaughter weight. At commercial slaughter weight (95-115 kg) pigs were delivered to the abattoir weekly over three collection time points per batch, a total of 10 time points. All animals were slaughtered at an average age of 168 days, ranging from the youngest being 147 and the oldest being 181 days of age.

3.2.2 Experimental design and tissue collections

Sows were monitored and following farrowing, litters were retrospectively assigned to one of three groups based on presumptive *in-utero* litter sex ratio. This was determined based on the sex of all piglets born (both live and dead). Mummified piglets were not included as accurate identification of sex was unreliable. The groups were; female-biased (65%
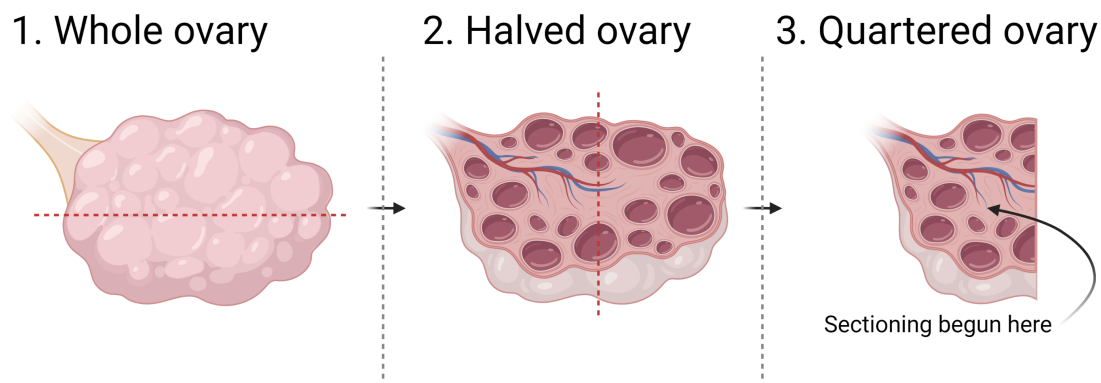
Figure 3.1: Schematic of how the ovaries were dissected, resulting in the final ovary sections. The place at which the first section was taken is marked out under 3. Quartered ovary. Created with Biorender.com.

females, n=15), non-biased (45.9-55% females, n=15), and male-biased (36% females, n=9). The groups used in this experiment (and Chapter 4) are more conservative than previous research. This allowed for the inclusion of a control group, or a "non-biased" group. This is beneficial as it allows for both extremes to be compared against a baseline litter rather than each other. By separating the groups I was more likely to identify effects from the extreme, whilst also allowing for the naturally skewed baseline sex ratio potentially present in commercial pigs as seen in the wild boar. Only litters with 10 or more live-born piglets were included.

Following group identification, in order to control for within-litter variability, two females were selected at random from each litter for reproductive tract collection at slaughter. Reproductive tract collections occurred in November 2018, June-August 2019, and July-August 2020. The tracts were collected on the abattoir line and transported to the laboratory on ice within 1.5 hours for tissue processing. The ovaries were dissected from the reproductive tracts and individually weighed. The right ovary was divided in half and placed in 10% neutral buffered formalin. Forty-eight hours later the ovaries were removed from formalin and further divided in half lengthways through the cortex, resulting in a quartered ovary (Figure 3.1). They were subsequently dehydrated through a series of ethanol washes (Table 3.1) and embedded in paraffin for histological examination. At this point, all samples were coded by an external researcher enabling me to work under blinded principles, not knowing which pig each sample originated from. Hence, all following work was conducted blind, including work for Chapter 4.

Table 3.1: The steps and associated reagents used in the embedding of all tissues used in this study.

Stage	Reagent	Time (hours)
1	70% EtOH	1+
2	70% EtOH	1
3	70% EtOH	2
4	90% EtOH	1.5
5	100% EtOH	1
6	100% EtOH	1.5
7	100% EtOH	2.5
8	Histoclear	1
9	Histoclear	1.5
10	Histoclear	2.5
11	Wax 1	2
12	Wax 2	2.5 (last 0.5 under vacuum)

The reproductive tracts that were suitable for this study were collected from a total of 39 gilts. These were from the following biases; male-biased=9, non-biased=15, and female-biased=15.

3.2.3 Histomorphometry and image analysis

For histomorphometry, 8 μm serial sections of the embedded ovary from an individual were mounted on polysine-coated Microslides (VWR International, Radnor, US). Slides were de-paraffinised through three changes of 10 minutes in Histo-clear. Slides were then hydrated through vigorous dipping in 100% EtOH (x3), 95% EtOH (x2), and 70% EtOH (x1) and finally rinsed in two to three changes of tap water. Following this a hematoxylin staining was carried out for five minutes and slides being rinsed in three to four changes of tap water, followed by a 5 second dip in 0.25% acid alcohol before being placed back into cold water. At this stage stain intensity was checked under a microscope before proceeding. The stain was blued in hot tap water for 1 minute and then rinsed in three to four changes of cold tap water. Slides were then placed in a Mordant of 95% EtOH. They were then stained in Eosin for 10 minutes. They were then dehydrated through two changes of 95% EtOH (10 dips each) and three changes of 100% EtOH (30-50 dips) and finally three changes of Histo-clear (10 minutes each). A full protocol can be seen in Appendix B - Ovary (solutions obtained from Thermo Fisher Scientific, Waltham, US). Ten sections per animal, each 160 μm apart, were selected serially with the initial section

being closest to the ovarian cortex, figure 3.1.

Due to the distance between sections, 160 µm, a cross section of an oocyte, <110 µm [164], could not be fully present in two analysed sections, ensuring that no follicle would be counted twice.These entire (quartered) ovarian sections were imaged using the Zeiss Axioscan Z1 slidescanner at 20x magnification. Total area was measured in µm2 and follicles counted using QuPath 0.2.0 [13]. The number of primordial, pre-antral, antral and atretic follicles were counted and classified according to Almeida et al. [6] (Figure 3.2).
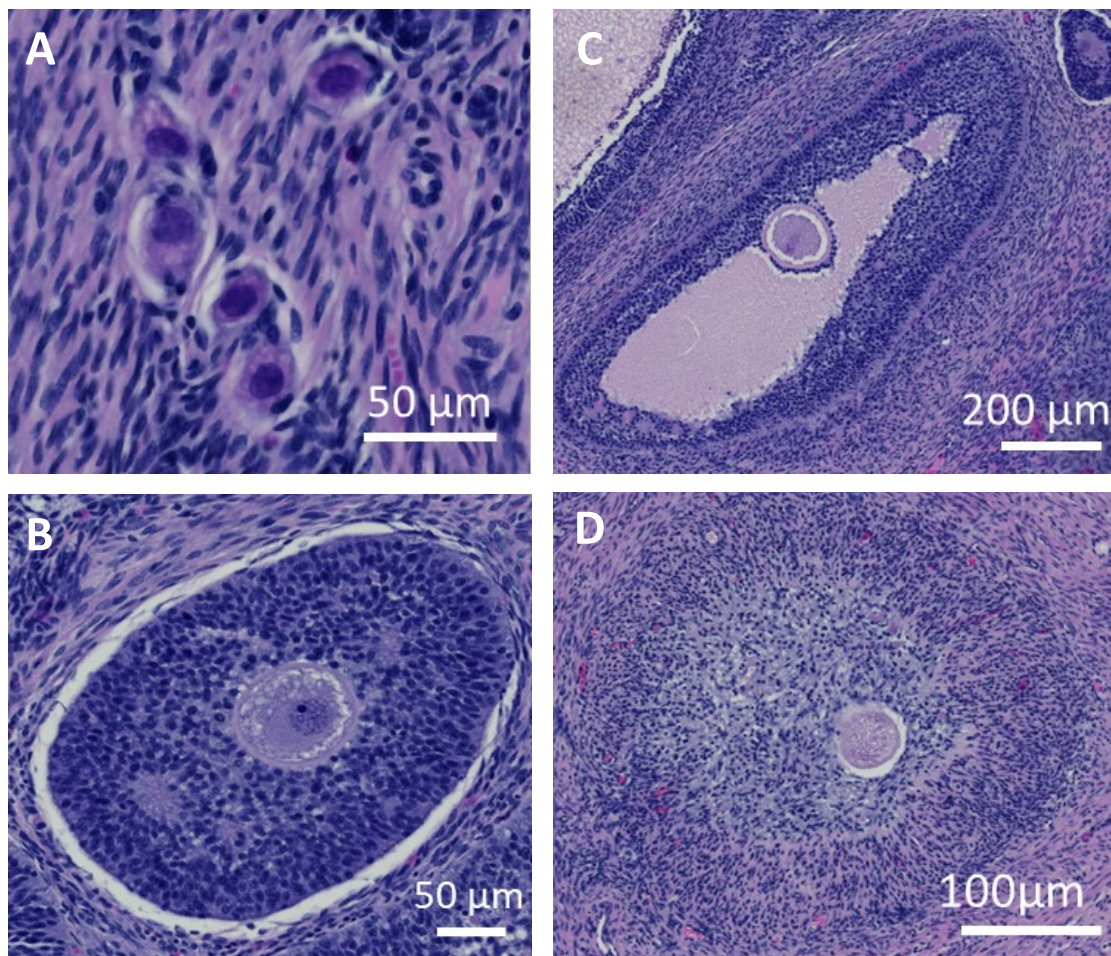
Figure 3.2: Images taken of follicles representing the different stages of development. A) A cluster of primordial follicles. B) Pre-antral follicle. C) Antral follicle. D) Atretic follicle.

Primordial follicles (Figure 3.2– A) were identified as an intact oocyte surrounded by a single layer of squamous (pre) granulosa cells. An enlarged oocyte that is surrounded by

a single, or multiple, layers of cuboidal granulosa cells was classified as pre-antral (Figure 3.2- B). Once the follicle had developed a clear antral cavity that was the same size as, or larger than, the oocyte it was classified as an antral follicle (Figure 3.2– C). The antral follicles have several layers of granulosa cells and have a well-developed thecal layer. All the above follicle types had intact oocytes with no signs of apoptosis or degradation. If degenerative changes had occurred including reduction of the oocyte or condensation of the nuclear chromatin, or changes to the antral cavity such as scattered granulosa cells, the follicle was identified as atretic (Figure 3.2- D).

3.2.4   Data processing and analyses

Data were analysed in two ways. Firstly, looking at follicle numbers in the ovary as a whole; and secondly controlling for variations in the manual dissection of the ovary by investigating the number of follicles per cm^2 of observed tissue.

Data normality was assessed using appropriate tests, histograms, and QQ-plots. Data considered to fit a Gamma distribution were tested using **"gamma_test"** in package "goft". Collinearity between predictor values was checked using the **"vif"** function in R package **"car"**. Ovary weight (g) and slaughter age (days) was removed due to collinearity (>3). Missing data was excluded from analyses, excluding a total of 5 animals. Critical alpha level was applied as p=0.05.

Variance of data points for the follicular counts within the sex ratio groups was measured using a levenes test, function **"leveneTest"**.

3.2.4.1   Analyses per ovary

Gamma regression models were used to test the effect of predictor values on the following response variables; (I) primordial follicle count, (II) recruited follicle count, (III) atretic follicle count, and (IV) total follicular count. All predictor and response variables are described in table 3.2.

Akaike Information Criterion (AIC) model selection was utilised to distinguish between a set of possible models, each describing the relationship between the predictor variables. Two different models were found to be the best fit for the response variables. Table 3.3

Table 3.2: All variables used in the statistical analyses with variable types, descriptions, and data type.

	Variable type	Description	Type
Primordial follicles	Response	Total primordial follicles	Continuous
Recruited follicles	Response	Total number of recruited follicles	Continuous
Atretic follicles	Response	Total number of recruited follicles	Continuous
Total follicles	Response	All follicle types combined	Continuous
Litter sex ratio	Predictor	Sex ratio of the litter of origin	Continuous
Birth weight	Predictor	Birth weight of the individual	Continuous
Slaughter weight	Predictor	Estimated slaughter weight (kg)	Continuous
Slaughter age	Predictor	Age at slaughter in days	Continuous
Litter	Random effect	Litter of origin	Categorical

describes each response variable used in the analyses, including details such as the specific model, the model type, and the AIC value of that model.

Model 1 - *<Response variable>* per ovary with predictor variables being *litter sex ratio* and *birth weight* as multiplicative, *litter* as random effect, and *slaughter weight* as additive.

Model 2 - *<Response variable>* per ovary with predictor variables *litter* as random effect, *litter sex ratio*, *slaughter weight*, and *birth weight* as additive.

Table 3.3: The specific model, and type of model used for each response variable as calculated per ovary, including the akaike information criterion value of the most compatible model.

Response variable	Model	Model type	AIC value
Primordial follicle count	Model 1	GLMM	561
Recruited follicle count	Model 1	GLMM	354
Atretic follicle count	Model 2	GLMM	377
Total follicle count	Model 1	GLMM	564

3.2.4.2 Analyses per cm^2

Gamma linear mixed effect models were used to test the effect of predictor values on the following response variables; (I) primordial follicle count; (II) recruited follicle count; (III) atretic follicle count; and (IV) total follicular count. All predictor and response variables are described in Table 3.2. All variables used in the statistical analyses with variable

types, descriptions, and data type. Analyses were carried out in R [215] using lme4 [19] with critical alpha level applied as p=.05.

AIC model selection was carried out to distinguish between a set of possible models, each describing the relationship between the predictor variables. The most appropriate model is described in Table 3.4.

Table 3.4: The specific model, and type of model used for each response variable as calculated per cm², including the AIC value of the most compatible model. Model 1 and 2 can be found above this figure.

Response variable	Model	Model type	AIC value
Primordial follicle count	Model 1	GLMM	369
Recruited follicle count	Model 1	GLMM	186
Atretic follicle count	Model 2	GLMM	191
Total follicle count	Model 1	GLMM	371

Models 1- *<Response variable>* per ovary with predictor variables being *litter sex ratio* and *birth weight* as multiplicative, *litter* as random effect, and *slaughter weight* as additive.

Models 2 - *<Response variable>* per ovary with predictor variables *litter* as random effect, *litter sex ratio*, *slaughter weight*, and *birth weight* as additive.

Pearson's correlations were calculated for birth weight, slaughter weight, and sex ratio **"cor.test"**.

3.3 Results

3.3.1 Data characteristics and variability

In total 34 pigs were used for analysis after exclusion of five animals due to missing data, they are detailed in Table 3.5. There 12 pigs from six female-biased litters, 13 from eight non-biased litters, and nine from five male-biased litters. Pigs were excluded from the trial if they

failed to reach commercial slaughter, no pigs needed to be excluded once reproductive tracts were collected. The mean birth weight and slaughter weight of the pigs from each

Table 3.5: Data collection including the sex ratio of litter of origin, total tissue area analysed per animal (cm^2), the weight of the analysed ovary, birth weight of the pig, and the estimated slaughter weight of the individual animals.

Animal tag	Litter	Sex ratio (%)	Tissue area (cm)	Ovary weight (g)	Birth weight (kg)	Slaughter weight (kg)
478	18	24	139052.78	4.72	1.61	110.30
479	18	24	155006.08	4.31	1.04	113.20
1928	1	27	144064.03	3.17	1.90	119.32
1912	1	27	102879.00	1.97	1.02	114.20
490	14	33	117079.41	3.57	1.64	117.90
484	13	35	201825.08	5.89	1.43	111.30
6627	13	35	176787.78	5.06	1.69	117.10
391	17	36	134389.03	3.13	1.49	112.80
403	17	36	182396.44	4.018	1.75	99.30
16	4	46	114098.43	2.18	1.47	108.64
17	4	46	112713.88	2.81	1.47	98.99
1920	7	46	157502.70	2.38.	1.81	117.45
312	6	47	212675.70	4.42	1.36	107.81
471	6	47	180158.08	4.28	1.43	106.13
482	10	47	168828.97	3.21	1.46	108.27
483	10	47	132076.37	2.18	1.43	109.53
3655	8	50	249770.67	3.68	2.07	117.50
5688	8	50	130138.86	2.56	1.34	108.12
1921	12	50	229535.21	5.35	1.36	116.76
1923	12	50	207720.18	5.96	1.37	107.66
Y862	20	50	225080.81	NA	1.25	NA
Y869	20	50	205176.66	4.63	1.45	NA
5620	9	53	191026.86	4.85	1.51	114.64
5650	9	53	152410.10	2.81	1.32	109.87
210	16	67	156474.29	3.29	1.71	113.80
212	16	67	209709.89	5.29	2.03	112.40
222	15	68	144583.20	4.30	1.05	112.90
250	15	68	148237.58	5.24	0.99	110.00
1137	3	70	155549.33	2.34	1.47	96.20
1140	3	70	176386.26	3.02	1.39	97.40
Y558	21	71	208819.30	NA	1.48	NA
Y649	21	71	128341.12	2.37	1.10	NA
502	22	73	154904.68	3.15	NA	97.70
1257	5	75	159583.15	3.24	1.63	96.10
1258	5	75	160193.16	3.04	1.67	102.60
1882	11	75	146780.46	2.46	2.40	106.60
1883	11	75	160521.92	2.81	2.18	109.00
22	19	83	196949.80	5.38	1.80	119.40
24	19	83	186800.15	5.76	2.24	116.20

bias group were as follows, respectively ($\pm$ SD); female-biased 1.65 ($\pm$ 0.446) and 107 ($\pm$ 8.140) kg, non-biased 1.47 ($\pm$ 0.207) and 110 ($\pm$ 5.275) kg, and male-biased 1.51 ($\pm$ 0.303) and 113 ($\pm$ 5.915) kg. The average ovarian tissue analysed was on average 16.6 ($\pm$ 3.197) cm^2 in female-biased pigs, 17.8 ($\pm$ 4.386) cm^2 in non-biased pigs, and 15.0 ($\pm$ 3.198) cm^2 in male-biased pigs. The ovarian weight was not significantly different between pigs from different bias groups (glmm GAMMA; t-value=0.035, n=34, p=0.973) with the average weight being 3.69 ($\pm$ 1.236) g in female-biased, 3.66 ($\pm$ 1.252) g in non-biased, and 3.98 ($\pm$ 1.176) g in male-biased pigs.

The variability of data points between the sex ratio groups were analysed as visualised in Figure 3.3. This revealed a bigger range in birth weights (F-value=4.073, df=35, p=.026) of females from both male-biased and female-biased litters compared to non-biased litters. There was a significantly larger variance of both primordial (F-value=4.801, df=36, p=.014) and total (F-value=5.381, df=36, p=.009) follicle numbers in females from male-biased compared to those that originated from non- or female-biased litters.

3.3.1.1 Relationship between sex ratio and body weights

A Pearson correlation test showed that the percentage of females in a litter and birth weight (Figure 3.4) were not strongly correlated (R=0.291, p=.077). Similarly, the slaughter weight and percentage of females in a litter were also not strongly correlated (R=0.301, p=.079).
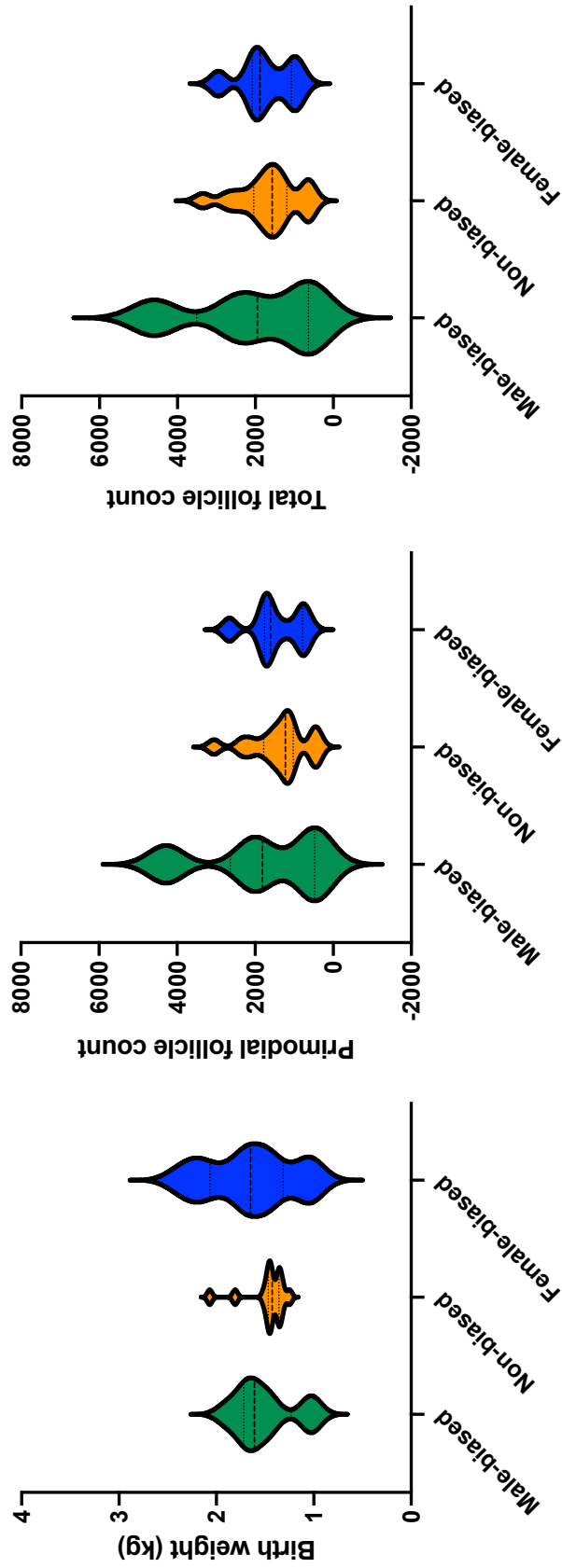
Figure 3.3: Variability of data represented by a violin plot of the birth weight (kg), primordial follicles, and total follicles between male-biased (n=9), non-biased (n=15), and female-biased (n=15) groups. Data were collected from hematoxylin & eosin stained ovarian sections and analysed using a Levenes test.
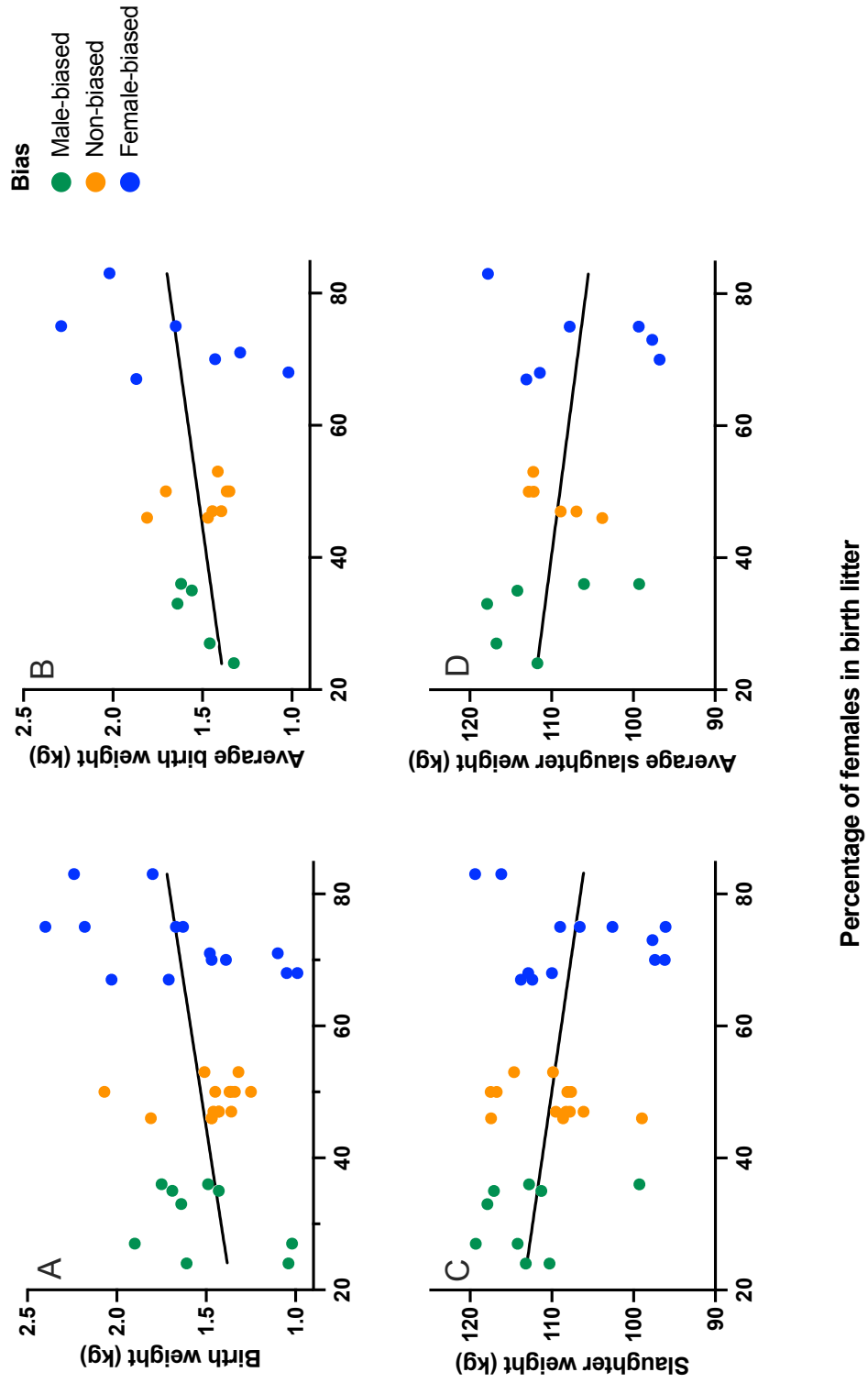
Figure 3.4: Changes in birth weight of individuals (A) or averaged per litter (B) and slaughter weight of individuals (C) or averaged per litter (D) dependent on the percentage of females from their litter of, n=34.

3.3.2 Ovarian sections
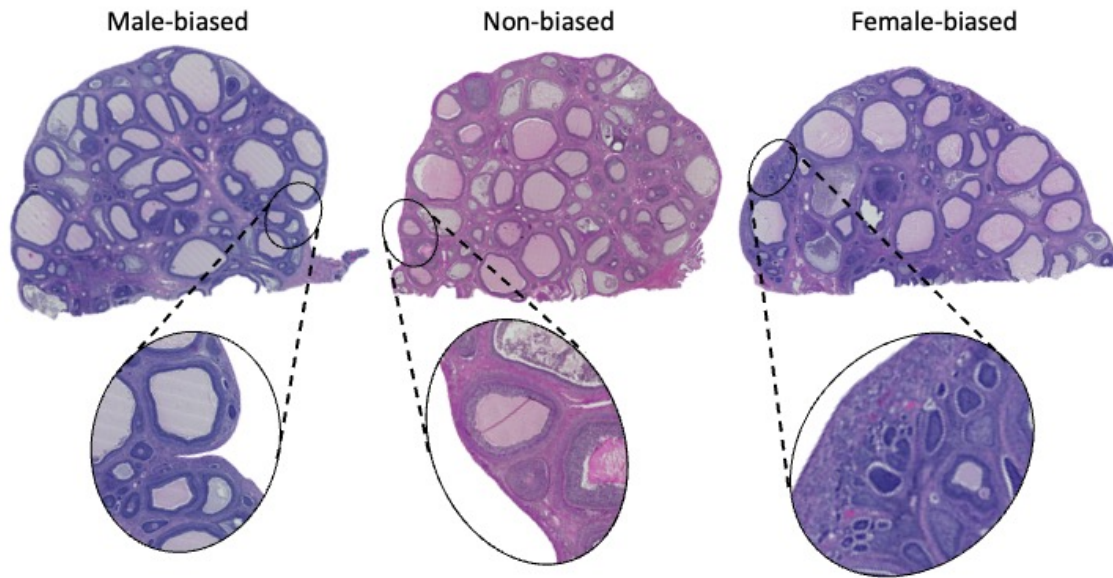
Figure 3.5: Representative figures of entire sections used for the collection of data points used in this study. Samples were collected from the male-biased (n=9), non-biased (n=15), and female-biased (n=15) litters and sections were stained using a hematoxylin and eosin stain. Images were captured using a Zeiss Axioscan Z1 slidescanner.

Sections were collected as described in Section *3.2.3 Histomorphometry and image analyses*. Below are representative images of what the sections looked like as whole cross sections. These were randomly selected from individuals of each litter bias, being male-biased, non-biased, and female-biased. Example ovarian sections from an animal from each litter sex bias is seen in Figure 3.5.

3.3.3 Analysis of follicular numbers per ovary

3.3.3.1 Interaction between birth weight and sex ratio on follicle numbers

There was a significant interaction in primordial (Gamma GLMM; t-value=-2.439, n=34, p=.015) and total (Gamma GLMM; t-value=-2.498, n=34, p=.013) follicle numbers per ovary between the birth weight of a pig and the sex ratio of the litter from which it originated. As seen in figure 3.6, follicle numbers were observed to have a negative correlation with increasing birth weight in individuals from male-biased litters. Contrary to this, female-biased and non-biased litters showed little differences in follicle numbers between

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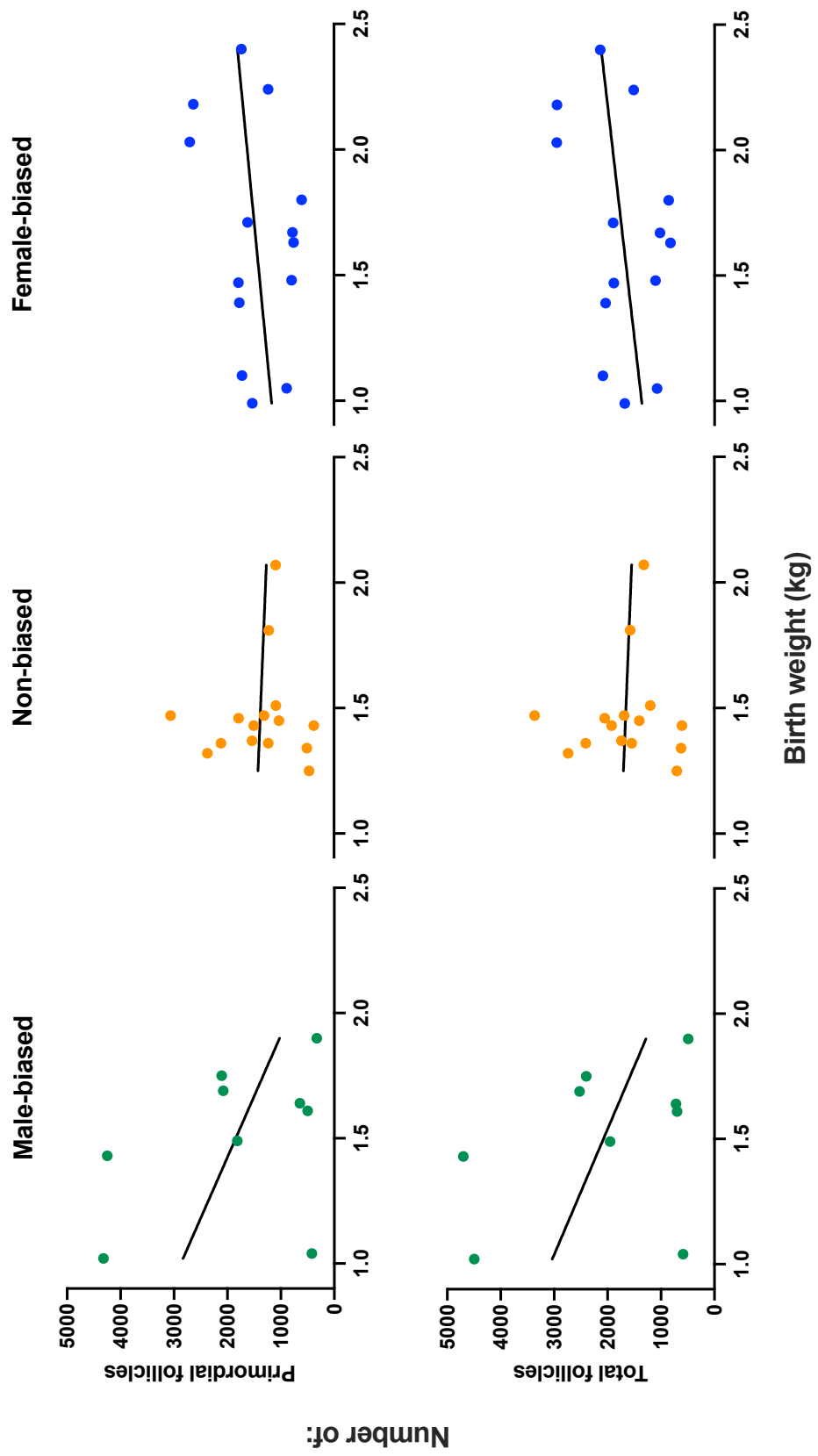
Figure 3.6: Birth weight of piglets for both primordial and total follicle numbers per ovary. Scatter plots hold fitted regression lines, individual points have been grouped according to the bias of the litter as either female-biased (>65% females), non-biased (45-50% females), or male-biased (<35% females), n=34.

different weights, with female-biased litters showing a slight increase in numbers with a higher birth weight.

No interaction was found between sex ratio and birth weight on the recruited follicles (GLMM; t-value=.238, n=34, p=.814).

3.3.3.2 Sex ratio and birth weight effects on recruited and atretic follicle profiles

The *in-utero* sex ratio of a litter held no effect (per ovary) for recruited (GLMM; t-value=-.008, n=34, p=.994) nor atretic (Gamma GLMM; z-value=.585, n=34, p=.558), follicle numbers. This was also the case for birth weight where no effects were seen for recruited (GLMM; t-value=.051, n=34, p=.960) nor atretic follicles (Gamma GLMM; t-value=-1.960, n=34, p=.0501).

3.3.3.3 Relationship between slaughter weight and follicle profiles

The slaughter weight had no effect on primordial (Gamma GLMM; t-value=.860, n=34, p=.390), recruited (GLMM; t-value=1.062, n=34, p=.297), atretic (Gamma GLMM; t-value=.1.282, n=34, p=.200), nor total (Gamma GLMM; t-value=-2.754, n=34, p=.433) follicle counts.

3.3.3.4 Sex ratio and birth weight effects on recruited and atretic follicle profiles

The *in-utero* sex ratio of a litter held no effect (per ovary) for recruited (GLMM; t-value=-.008, n=34, p=.994) nor atretic (Gamma GLMM; z-value=.585, n=34, p=.558), follicle numbers. This was also the case for birth weight where no effects were seen for recruited (GLMM; t-value=.051, n=34, p=.960) nor atretic follicles (Gamma GLMM; t-value=-1.960, n=34, p=.0501).

3.3.3.5 Relationship between slaughter weight and follicle profiles

The slaughter weight had no effect on primordial (Gamma GLMM; t-value=.860, n=34, p=.390), recruited (GLMM; t-value=1.062, n=34, p=.297), atretic (Gamma GLMM; t-value=.1.282, n=34, p=.200), nor total (Gamma GLMM; t-value=-2.754, n=34, p=.433) follicle counts.

3.3.4 Analysis of follicular numbers per cm²

Data were analysed based on follicles per cm^2 of tissue analysed to account for variation in the size of area analysed within and between individuals.

3.3.4.1 Interaction between birth weight and sex ratio on follicle numbers (cm²)

For an individual here was no significant interaction with *in-utero* sex ratio of that pigs litter and their birth weight for the number of recruited follicles (GLMM; t-value=.789, n=34, p=.436). There was a significant interaction in primordial (Gamma GLMM; t-value=-2.637, n=34, p=.008) and total (Gamma GLMM; t-value=-2.754, n=34, p=.006) follicle numbers per cm^2 between these two predictor variables i.e. sex ratio *in-utero* and birth weight. As seen in Figure 3.7, increasing birth weight was negatively correlated with primordial and total follicle numbers in females from male biased litters. Contrary to this, female-biased and non-biased litters showed no difference in follicle numbers between different weights.

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For an individual here was no significant interaction with *in-utero* sex ratio of that pigs litter and their birth weight for the number of recruited follicles (GLMM; t-value=.789, n=34, p=.436). There was a significant interaction in primordial (Gamma GLMM; t-value=-2.637, n=34, p=.008) and total (Gamma GLMM; t-value=-2.754, n=34, p=.006) follicle numbers per cm^2 between these two predictor variables i.e. sex ratio *in-utero* and birth weight. As seen in Figure 3.7, increasing birth weight was negatively correlated with primordial and total follicle numbers in females from male biased litters. Contrary to this, female-biased and non-biased litters showed no difference in follicle numbers between different weights.

Figure 3.7: Birth weight of piglets for both primordial and total follicle numbers per cm2. Scatter plots hold fitted regression lines, individual points have been grouped according to the bias of the litter as either female-biased (>65% females), non-biased (45-54.9% females), and male-biased (<35% females), n=34.

3.3.5.2 Sex ratio and birth weight effects on recruited and atretic follicle profiles

No significant difference in numbers were found across sex ratios for recruited (GLMM; t-value=.442, n=34, p=.679), nor atretic (Gamma GLMM; t-value=.817, n=34, p=.414) follicles. Similarly, both recruited (GLMM; t-value=669, n=34, p=.509) and atretic (Gamma GLMM; t-value=1.775, n=34, p=.0759) follicle numbers were not affected by the birth weight of an individual.

3.3.5.3 Slaughter weight and follicle profiles

The slaughter weight of an individual had no effect on follicular numbers per cm2 for primordial (Gamma GLMM; t-value=860, n=34, p=.589), recruited (GLMM; t-value=.995, n=34, p=.330), atretic (Gamma GLMM; t-value=1.468, n=34, p=.142), nor total (Gamma GLMM; t-value=2.754, n=34, p=.611).

3.4 Discussion

The aim of this study was to investigate if the ovarian reserve was adversely affected in females gestated in a predominantly male *in-utero* environments, and whether this could account for the detrimental effects seen in female reproductive success from male-biased litters. Hypothesising that there would be a reduction in TOR and/or a potential increase in follicular recruitment and atresia.

Previous research in various species suggests an important role of androgens on follicular and CL development. For example, in gilts this is illustrated by increased ovulation rates in gilts treated with dihydrotestosterone [30], and CL dysfunction marked by decreased progesterone production when flutamide was used to block androgenic actions [97]. Overall, this study found no effects of sex ratio on the recruitment, nor atresia of follicles, suggesting that an androgenised uterine environment does not interfere with folliculogenesis, nor the breakdown of follicles in the pre-pubertal commercial pig. Under the premise of this study pigs were expected to, at slaughter, not yet have become cyclic. Some pigs may however have reached puberty earlier than the norm, skewing results. Therefore, this suggests that to truly understand the effects of a biased litter on

the recruitment and atresia of follicles, future work should utilise synchronised gilts, and these current findings should be cautiously interpreted. Androgens in sheep were found to increase follicular recruitment when offspring were in a hyper-androgenised maternal circulation. This resulted in detrimental effects such as multi-follicular ovaries or early cessation of cyclicity [210]. However, it is important to note that sheep normally only bear one to three offspring per gestation and hence aren't litter bearing. Thereby, they are less likely to be exposed to a biased uterine environment as the pig is, potentially leading to a higher sensitivity to hyper-androgenisation of the uterine environment. This, alongside the differences in placentation and uterine vascular systems between sheep and pigs could potentially explaining the species differences.

Piglets that are below 1.3 kg at birth have been found to exhibit less competent post-natal development and reduced survivability [185], suggesting a detrimental effect of low birth weight on offspring. Compensatory growth has been found to lead to delayed puberty onset in mice [163], although there is evidence that piglets do not display this "catch-up" growth, like other species [113]. Low birth weight piglets have also been found to grow less than normal compared to high birth weight piglets throughout their life course [223]. Gilts of a low birth weight (<1kg) were found to produce less piglets alongside holding a lower longevity in the breeding herd [146]. Although they were found to hold similar farrowing rates and age of puberty [146]. The birth weight of gilts has also previously been shown to alter their follicular profile at 150 days of age. Low birth weight females were found to hold fewer antral (medium sized; 3-5mm) and pre-antral follicles, whilst displaying more atretic follicles [6]. Contrary to this previous study by Almeeida et al., an earlier paper identified no difference in follicular numbers, nor the ovarian size [224]. Despite no effects seen in the number of follicles recruited, females from male-biased litters had a higher count of primordial and total follicles both per ovary, and per cm2 when the individual pig had a low birth weight (Figure 3.6 and Figure 3.7). However, in females from a female-biased litter with a greater birth weight had a higher number of primordial and total follicles. This suggests that the effect of an androgenised environment and an oestrogenised uterine environment seem to have opposite influences on the development of the primordial follicle pool, further confounded by the birth weight of the particular individual. An androgenised uterine environment may increase primordial germ cell pro-

66

liferation, resulting in a larger TOR. Seyfang et al. [205] found that androgenised female pigs were more likely to ovulate and had higher CL counts when from a male-biased litter compared to female-biased litters when treated with gonadotrophins at 18 weeks. This could be due two reasons, firstly increased ovulation per cycle, or in combination with a higher TOR as I demonstrate in these findings. Research in non-litter bearing species, who will have different timings of PGC establishment, conflicts with that in pigs; findings in sheep in which individuals from a hyper-androgenised uterine environment displayed lower TORs than their control counterparts [210]. This may be due to the pre-mentioned species differences, or due to the study design mimicking maternal testosterone via intramuscular testosterone propionate in pregnant ewes, rather than uterine hyper-androgenisation.

Research, particularly in rodents and sheep, has found that the fine tuning of two specific pathways (PTEN/PI3K/PDPK1/AKT1 and BMP/AMH/SMAD pathways) are responsible for the relationship between the PGC pool and recruited follicular pools [162]. They also found that the mutation or deletion of genes encoding the receptors, ligands, or signalling effectors of the pathways leads to accelerated exhaustion, dramatic activation, or premature loss of primordial follicles [162]. There could potentially be interactions between the intra-uterine hormonal environment and genes crucial to these key pathways during formation of the primordial follicles and subsequent postnatal ovarian function.

The interaction between birth weight of an individual and sex bias of the litter from which they originated identified a higher number of follicles in lower body weights when litters were male-biased. Despite this, there is no correlation between the percentage of females in a litter and body weight, however, birth weight was found to be more variable within litters that were either oestrogenised or androgenised.

The TOR has been found to be greatly variable within individuals of the same species, with reports of up to 20-fold differences in individuals through the neonatal period and puberty [67, 243]. Even the strain of mice has shown to account for major variation with regard to follicular profiles [127]. This would partially clarify the high variation seen in follicular numbers of pigs from male-biased litters in our data, however, it is interesting to note that the variation is considerably smaller in both non- and female-biased litters. There may be an underlying cause of the increased variation of TOR in male biased litters

that hold functional consequences not yet understood. Further to this, it is important to note that this data is mainly driven by a few individuals. The fore-mentioned evidence that follicle numbers can be highly variable between individuals implies that there is no suggestion that these data points would be outliers or irregular. A larger sample size could provide more confidence in the results. There is little available information on follicular counts in the domesticated, nor non-domesticated, pig for comparisons to be made. Therefore my recommendation is that future studies obtain a larger male-biased group to provide a higher confidence of the results.

The results were the same whether analysing the data per ovary, or whether controlling for the total tissue size analysed. This suggests that the method of sampling partial sections should not hold an effect on outcomes when investigating the ovarian profile of ovaries. However, the subjective nature of manual quantification of the TOR must be taken into consideration when interpreting the results from this study. There is a lack of validated and objective measures of the TOR and although measures were taken to minimise effects of handler bias, including blind counting by one researcher, there may be unintentional introduction of bias. Future research should investigate the TOR of naturally synchronised (i.e. using heat detection and opportunistic end points) second parity pigs in order to further understand the effects of a sex biased litter on the follicular profile of commercial pigs. Synchronisation would allow for a detailed understanding of deviating recruitment patterns observed in previous studies, which this experimental design doesn't hold sensitivity to fully investigate. Further to this study, investigating the depletion of the ovarian reserve over time would help understand the long-term effects that a bias may hold on reproductive longevity. There may be an increase in the depletion of TOR in older pigs, which would normally see a 70% decrease from E50 to 300 days after birth [98]. A major limitation to this study is that there is no identification of the absolute uterine position, hence there isn't any way to identify the level of androgenisation that an animal has been exposed to. In future studies, I suggest selecting piglets from the biased litters based on the AGD of individuals rather than randomly as in this experimental design. This would allow selection of pigs that were likely in specific absolute uterine positions. Finally, albeit currently a very novel avenue, there may be an effect of an androgenised uterine environment on the development of oogonial stem cells, if they functionally exist

within the pig. Potentially inhibiting a maintenance of the TOR.

In conclusion, originating from an androgenised uterine environment, and having a low birth weight increases the primordial ovarian reserve in the commercial pig, but it also increases the variability of primordial follicle numbers as compared to pigs that originate from non- and female-biased litters. Conversely, a higher birth weight resulted in a greater primordial ovarian reserve if the female pig originated from an oestrogenised uterine environment. Pigs from either litter bias (male or female) were found to have a significantly higher variation in birth weight than if a pig originated from a non-biased litter. With the prior understanding of an androgenised uterine environment holding detrimental influences on female reproductive success, further investigation into how the increased primordial follicle numbers may be influencing recruitment, atresia, and fertility is crucial to understand how this may be impacting reproduction.

Chapter 4

The influence of the intra-uterine hormonal bias on the development of the uterine endometrium

4.1 Introduction

The ovulation rate of an individual imposes a clear and direct limitation on potential litter sizes in the pig. However, despite ovulation rates having improved via selective pressure this hasn't been reflected in any significant increases to litter sizes (1). With high ovulation, and conception rates in the breeding sow of greater than 95% (2), the major limiting factor to maximized litter sizes is embryonic death. Only 30-50% of fertilised ova survive through gestation [182]. This loss is predominantly seen during the pre- and peri-implantation period defined as Embryonic Days (ED) 12-18 [86, 114, 182]. As described by Ross et al. [192], this coincides with conceptus elongation, synthesis and release of oestrogen for maternal pregnancy recognition, and trophectoderm differentiation, which is followed by foetal and epithelial attachment [192]. There is then a secondary wave of embryonic loss at ED 30-40 due to crowded *in-utero* conditions [226]. It is therefore clear that litter sizes, and conceptus survival, is contingent on uterine capacity. This is determined by three main factors; uterine length, uterine blood flow, and uterine gland development [226].

The uterine capacity of a pig is critical as it contributes to the reproductive potential

of the female i.e. even if there are large numbers of developmentally competent embryos present there is a physical limit on the numbers that can implant. It is vital that the uterine tissues can recognise and respond to maternal and conceptus signals crucial for a successful and established pregnancy [18, 94, 226]. Not only is this vital for pregnancy recognition, but the uterine capacity will define the environment in which foetal development occurs [18]. The Developmental Origins of Health and Disease (DOHaD) describes how the developmental plasticity and *in-utero* programming of offspring could contribute to susceptibility of a range of adult disease with some of these being sexually dimorphic in nature i.e. different outcomes between males and females [179]. The functional layer of the uterus that is especially crucial for successful pregnancy outcomes, the endometrium, may be influenced.

The uterine endometrium is a heterogenous tissue comprised of several different cell types including various secretory cells such as the luminal and glandular epithelium [114]. The endometrial surface of the pig is folded, which at conceptus attachment (ED 14) undergoes a conceptus localised increase in endometrial surface folding [85]. The opposite folds interlock, reducing the luminal space throughout pregnancy, maximising luminal epithelial and foetal contact [114], this is crucial to facilitate maternal and foetal communication. Endometrial glands along with the luminal epithelium secrete uterine luminal fluid, a complex array of proteins and related substances [22]. The uterine luminal fluid is critical for endometrial function and conceptus survival as they contain enzymes, growth factors, cytokines, nutrients, transport proteins, and other regulatory molecules (see review by Bazer and Johnson 2014) [23]. Maternal endometrial gland hyperplasia and hypertrophy is extensive during gestation (12,13) with large amounts of granular, acid phosphate-positive material within the glands, indicative of a high level of secretory activity [94]. Sheep with blocked uterine horn gland development (sheep uterine gland knock out (UGKO)) indicate a failure of conceptus elongation at ED14 and are rendered infertile [23]. This evidences the fact that uterine glands are critical mediators for the uterine ability to support a successful pregnancy. Although it is evident that attachment/adhesion factors of the uterus are essential for elongation, as blastocysts must be transferred from culture to the uterine lumen for elongation to occur in both sheep [209] and pigs [87], it is important to recognise the differences in conceptus elongation between

the species. Wherein the sheep elongation is uterine mediated, in pigs it is also embryo derived. Uniquely to all other large farm animals the elongation of the pig conceptus is reliant on conceptus expression of interleukin-1B2 as demonstrated by interleukin-1B2 null embryos failing to rapidly elongate [249].

Formation of the uterine glands occur in the neonatal piglet by branching and budding of the luminal epithelium [94], reaching histoarchitectural maturity by 120 days of age [180]. Although the glands form neonatally, the histogenesis of the initial uterine horn development, and luminal epithelium, takes place *in-utero* [94, 213] making this process vulnerable to environmental effects to which the foetus is exposed to. The understanding of the impact disruptions of uterine development may hold on endometrial structure and function in adult mammals is crucial for unravelling the high rates of peri-implantation embryonic loss in both livestock and humans [94], as this may render the uterine unable to support the individuals that are especially small for their gestational age. For example, adult cows exposed to progesterone and oestradiol benzoate *in-utero* demonstrate a reduced number of endometrial glands [16, 17, 70]. Furthermore, in pigs, neonatal progesterone treatment initially accelerated gland development, but reduced adult glandular development [225]. Impairment, as described above, has been indicative of a reduced fertile capacity [213].

This study aims to investigate how a sex biased *in-utero* environment may influence the development of endometrial glands a component of the endometrium that is critical for successful pregnancy. To do this we investigated the influence of *in-utero* sex ratio a female was gestated in on uterine morphology and endometrial gland proliferation.

4.2 Methods

4.2.1 Experimental design and animal collections

Refer to Chapter 3 Sections *3.2.1 Animals* and *3.2.2 Experimental design and tissue collections* for the experimental design, selection, and collection of animals. Successfully collected reproductive tracts suitable for this study were from a total of 38 sows, which originated from 22 litters. These were from the following biases; male-biased = 8, non-biased = 15, and female-biased = 15. One pig was removed due to an active infection of

the uterine horns.

4.2.2 Tissue dissections

Both the left and right uterine horns were dissected away from underlying tissue and a 1cm cross section from the top 1/3rd of the horns were collected (Figure 4.1) and placed in neutral buffered formalin for 48 hours. Sections were subsequently embedded in paraffin for histological examination; see Chapter 3, section *3.2.2 Experimental design and tissue collections* - Table 3.1 for detailed protocol.

Figure 4.1: Example reproductive tract of the gilt at collection. Arrows indicate where a 1 cm cross section of the uterine horn was taken from both left, and right, uterine horns.

4.2.3 Histomorphometry

For histomorphometry, uterine horns were serially cross-sectioned at a thickness of 8 µm and mounted on polysine-coated Microslides (VWR International, Radnor, US). For morphological examinations one section per horn per animal were selected and stained using hematoxylin and eosin (H&E) with standard techniques (solutions obtained from Thermo Fisher Scientific, US). For detailed H&E stain protocol please see Chapter 3 *Section 3.2.3* and appendix B - H&E protocol. An entire section per animal, per uterine horn, was then imaged using the Zeiss Axioscan Z1 slidescanner at 20x magnification. QuPath 0.3.0 [13]
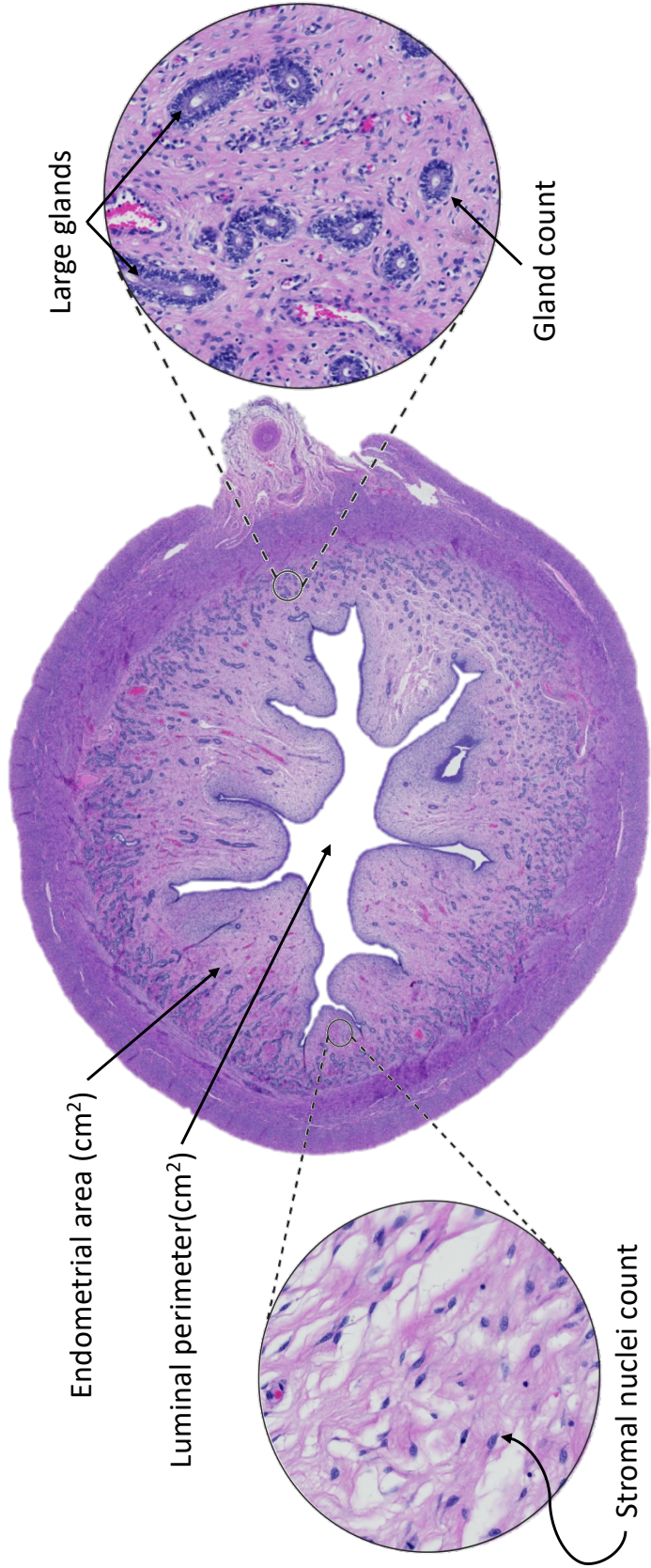
Figure 4.2: Uterine cross section with an hematoxylin & eosin stain and indicators of the morphological structures investigated.

Large glands

Gland count

Endometrial area (cm^2)

Luminal perimeter(cm^2)

Stromal nuclei count

was used for the subsequent analyses. For both uterine horns of each pig (n=38) the endometrial area was measured (cm^2) along with luminal perimeter (cm^2). Manual counts were made of the glands in the endometrium of total sections, with the number of large glands also counted. An automated cell nuclei detection within QuPath was used to count the stromal cells within an area of 20,000 μm. These structures are visualised in Figure 4.2.

4.2.4 Immunohistochemistry

For investigating uterine gland proliferation one section per uterine horn were stained using an immunohistochemistry (IHC) technique using the Vector Lab VectaStain Elite ABC-HRP kit. Overnight incubations (4°C) of the sections were carried out with mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) (1:200, Invitrogen). Control sections were incubated with a mouse IgG Isotype Control (1:200, Invitrogen). The sections were then incubated with the secondary antibody and ABC reagent. Development of sections was carried out using diaminobenzidine substrate (ThermoFisher). Please see Appendix C - IHC protocol for the detailed IHC protocol.

An automated DAB stain analysis was used (QuPath) for the entire sections of the uterine horns to measure stain intensity. This allowed for identification of cells with a negative or positive stain, along with division of positively stained cells into a low, moderate, or high stain intensity. Parameters used for the detection were refined using three sections previously stained in the stain optimisation process. The parameters used for stain detection were as follows; pixel size, 0.5µm with thresholds being Low; 0.1-0.3, Moderate; 0.3-0.5, High; 0.8-1.

Entire IHC sections were imaged using the Zeiss Axioscan Z1 slidescanner at 40x magnification (Figure 4.3).

4.2.5 Data processing and analyses

The following analyses were performed to determine if there was an effect of litter sex bias on the uterine development of gilts, and are summarised in Table 4.1.
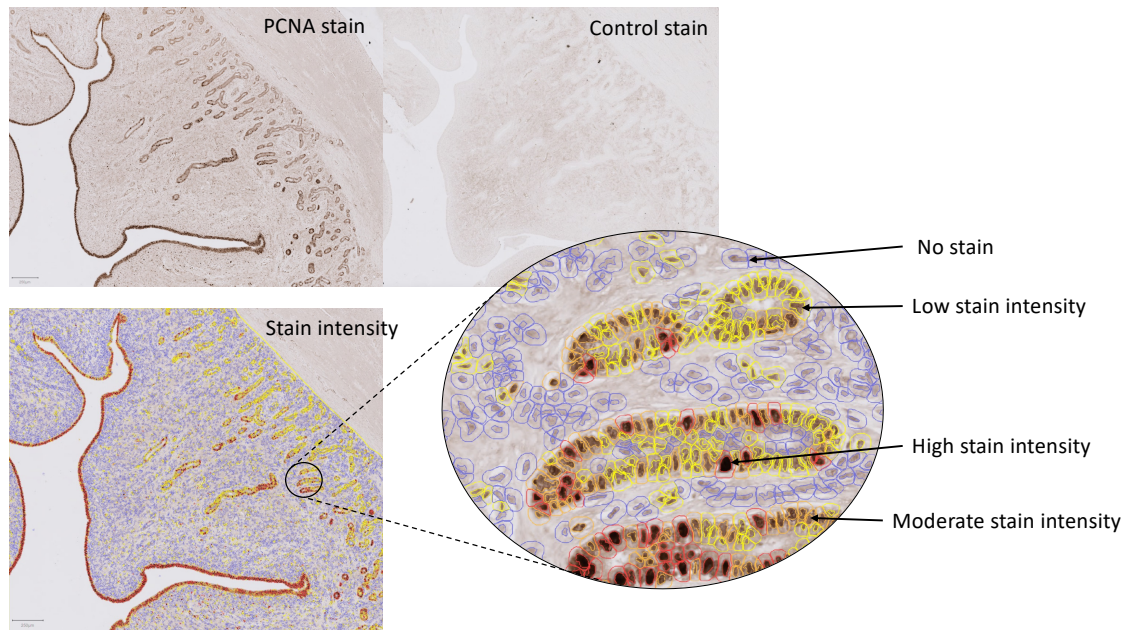
Figure 4.3: Images of immunohistochemistry stained sections (8 μm) using a proliferating cell nuclear antigen antibody, including the identified stain intensity and the control section. The automated stain detection output can be seen including visual representations of cells considered to have no stain, low stain, moderate stain, and high stain intensities.

Generalised linear mixed effect models were used to test the effect of predictor variables for both the morphological and proliferative analyses. Analyses were performed in R [215] using lme4 [19] with the critical alpha level applied p=.05. Normality of data were checked using function **"shapiro.test"** and by observing the variance of residuals. Non-normal data were either of a Poisson distribution or checked for fit of a Gamma distribution using function **"gamma_test"** in R package **"goft"**. Collinearity between predictor values was checked using the **"vif"** function in R package **"car"**. Any values resulting from the collinearity test lower than three were considered not collinear. Collinearity was found between predictor values Slaughter age and Slaughter, so slaughter age was removed from the models. All predictor and response variables are described in Table 4.1.

Akaike Information Criterion (AIC) model selection was used to distinguish between a set of possible models, each describing the relationship between the predictor variables. The AIC value of the best fitting model is included in Table 4.2. Transformations were required for certain variables to fit their distributions. This was with either a square root transformation, function **"sqrt"**, or a log transformation, function **"log"**. The models used in analyses were either;

Table 4.1: All variables used in the statistical analyses with variable types, descriptions, and data type.

	Variable type	Description	Type
Endometrial area (EA)	Response	Endometrial area (µm2) of entire section	Continuous
Luminal perimeter (LP)	Response	Luminal Perimeter (µm) - representing surface area available to foetal communication	Continuous
Stromal nuclei (SN)	Response	Number of stromal nuclei within 20,000 µm2	Count
Gland count (GC)	Response	Number of uterine glands within the whole section	Count
Large gland count (LGC)	Response	Large gland count in the whole section	Count
Positively stained cells (PS)	Response	Proportion of positively stained cells vs no stain	Continuous
Low stain intensity (LS)	Response	Number of cells with a low stain divided by all detected nuclei	Continuous
Moderate stain intensity (MS)	Response	Number of cells with a moderate stain divided by all detected nuclei	Continuous
High stain intensity (HS)	Response	Number of cells with a high stain divided by all detected nuclei	Continuous
LP:EA	Response	Luminal perimeter in proportion to endometrial area	Continuous
SN:EA	Response	Stromal nuclei as a proportion of endometrial area	Continuous
GC:EA	Response	Glandular count as a proportion of endometrial area	Continuous
SN:LP	Response	Stromal nuclei as a proportion of luminal perimeter	Continuous
GC:LP	Response	Glandular count as a proportion of luminal perimeter	Continuous
GC:SN	Response	Glandular count as a proportion of stromal nuclei	Continuous
Litter sex ratio	Predictor	Sex ratio of the litter of origin	Continuous
Birth weight	Predictor	Birth weight (kg) of the individual	Continuous
Slaughter weight	Predictor	Estimated slaughter weight (kg)	Continuous
Litter	Random effect	Litter of origin	Categorical

Model 1 - *<Response variable>* with predictor variables being *litter sex ratio* and *birth weight* as multiplicative, *slaughter weight* as additive, and *animal ID* nested within *litter* as random effect.

Model 2 - *<Response variable>* with predictor variables being *litter sex ratio*, *birth weight*, and *slaughter weight* as additive, with *animal ID* nested within *litter* as random effect.

Variance of data points for the response variables within the sex ratio groups were measured using **"leveneTest"**. Similarly, this was done to investigate the difference between right and left uterine horns of individuals from different litter sex biases for each response variable.

4.2.6   Morphological analyses

Analyses of data were performed to investigate difference of rudimentary morphology and is detailed in Table 4.2. Endometrial area and luminal perimeter (log transformed) were both of a gamma distribution and used Model 1. Counts of stromal nuclei, gland count, and large gland count all fitted a poisson distribution with Model 2 being the best fit.

Proportional analyses were conducted to investigate whether there was a difference in the proportion of secretory structures between bias litters. Model 2 held the best fit and they all held a gamma distribution.

The following comparisons were made, luminal perimeter, stromal cells and endometrial glands in relation to endometrial area:

- Luminal perimeter divided by endometrial area.

- Stromal cell count divided by endometrial area.

- Endometrial gland count divided by endometrial area.

It was then important to compare whether these secretory cells differed in proportion to each other between individuals from different *in-utero* sex ratios.

- Stromal cells vs luminal perimeter (square root transformation).

- Gland count vs luminal perimeter.

- Gland count vs stromal cells.

4.2.7 Proliferation analyses

The stained cells were analysed in the same manner as the morphological analyses. However, low stain intensity was found to hold a normal distribution and was analysed using Model 2, and high stain intensity required a square root transformation (Table 4.2). Similarly to the morphological analyses, the total number of positively stained cells were analysed as a proportion of secretory structures, all which required a Log transformation and held a Gamma distribution. This analyses is described in 4.2. The following comparisons were made;

- Total number of positively stained cells divided by endometrial area.

- Total number of positively stained cells divided by luminal perimeter.

- Total number of positively stained cells divided by total gland count.

Table 4.2: Description of predictor variables and model characteristics, including data type, distribution, transformations if applicable and the model function used in R. The AIC values from the best fitting models are included.

Predictor variable	Data type	Distribution	Transformation	Function	Model type	AIC value
Morphological data						
Endometrial area	Numeric	Gamma		GLMER	Multiplicative	-126
Luminal perimeter	Numeric	Gamma	Log transformed	GLMER	Multiplicative	-10
Stromal cells	Count	Poisson		GLMER	Additive	573
Gland count	Count	Poisson		GLMER	Additive	1400
Large gland count	Count	Poisson		GLMER	Additive	791
Proportional data						
LP:EA	Numeric	Gamma		GLMER	Additive	1478
SN:EA	Numeric	Gamma		GLMER	Additive	531
GC:EA	Numeric	Gamma		GLMER	Additive	1015
SN:LP	Numeric	Gamma	Square root transformed	GLMER	Additive	117
GC:LP	Numeric	Gamma		GLMER	Additive	751
GC:SN	Numeric	Gamma		GLMER	Additive	444
Proliferation data						
Positively stained cells	Numeric	Gamma		GLMER	Multiplicative	-68
Low stain intensity	Numeric	Gaussian		LMER	Multiplicative	-119
Moderate stain intensity	Numeric	Gamma		GLMER	Multiplicative	-216
High stain intensity	Numeric	Gamma	Square root transformed	GLMER	Multiplicative	-149
Proportional proliferation data						
POS:EA	Numeric	Gamma	Log transformed	GLMER	Additive	91
POS:LP	Numeric	Gamma	Log transformed	GLMER	Additive	140
POS:GC	Numeric	Gamma	Log transformed	GLMER	Additive	98

4.3 Results

4.3.1 Data description and variability

Table 4.3 illustrates the total number of tracts that were collected for this study.

In total 38 pigs were used for analysis, as detailed in Table 4.3. Per bias group there were 15 pigs from female-biased litters, 15 from non-biased litters, and 8 from male-biased litters. Pigs were excluded from the trial if they failed to reach commercial slaughter, and one pig was excluded due to an active infection in the reproductive tract. The mean birth weight and slaughter weight of the pigs from each bias group were as follows, respectively ($\pm$ SD); female-biased 1.653 ($\pm$ 0.446) and 107 ($\pm$ 8.140) kg, non-biased 1.50 ($\pm$ 0.218) and 109 ($\pm$ 5.554) kg, and male-biased 1.608 ($\pm$ 0.299) and 112 ($\pm$ 6.111) kg.

The variability of data points between the sex ratio groups were analysed using the Levene's test (Figure 4.4). This revealed that there was an increased range of stromal cells in endometria recovered from females from sex-biased litters compared to non-biased (F-value=3.8134, df=71, p=.027). There was also significantly more variability in birth weights (kg) from individuals born to sex ratio extremes compared to non-biased litters (F-value=7.909, df=71, p<.001). The variability did not differ between litter sex bias in endometrial area (F-value=.2472, df=71, p=.7817), luminal perimeter (F-value=2.3527, df=71, p=.1025), nor total gland (F-value=2.4973, df=71, p=.08951) or large endometrial gland counts (F-value=2.0419, df=71, p=.1373). Further to this, the variability between the left and right uterine horn of the same animal for response variables was analysed. There was no higher level of variability within animal uterine horns between different sex ratio litters for endometrial area (F-value=.078, df=2, p=.925), stromal nuclei (F-value=1.407, df=2, p=0.259), glandular count (F-value=1.123, df=2, p=0.337), positively stained cells (F-value=2.499, df=2, p=0.098). We did however identify a higher level of variability between an individuals right and left uterine horn for the luminal perimeter when originating from a male-biased litter (F-value=4.558, df=2, p=0.018).

Table 4.3: Summary of the data for each animal used in uterine tissue analyses. These data include sex ratio of litter of origin, birth weight of the pig, and the estimated slaughter weight of the animal.

Animal tag	Litter	Sex ratio (%)	Birth weight (kg)	Slaughter weight (kg)
478	18	24	1.61	110.30
479	18	24	1.04	113.20
1928	1	27	1.90	119.32
488	14	33	2.00	114.70
490	14	33	1.64	117.90
484	13	35	1.43	111.30
391	17	36	1.49	112.80
403	17	36	1.75	99.30
16	4	46	1.47	108.64
17	4	46	1.47	98.99
1920	7	46	1.81	117.45
1345	2	47	1.77	100.00
312	6	47	1.36	107.81
471	6	47	1.43	106.13
482	10	47	1.46	108.27
483	10	47	1.43	109.53
3655	8	50	2.07	117.50
5688	8	50	1.34	108.12
1923	12	50	1.37	107.66
Y862	20	50	1.25	NA
Y869	20	50	1.45	NA
5620	9	53	1.51	114.64
5650	9	53	1.32	109.87
210	16	67	1.71	113.80
212	16	67	2.03	112.40
222	15	68	1.05	112.90
250	15	68	0.99	110.00
1137	3	70	1.47	96.20
1140	3	70	1.39	97.40
Y558	21	71	1.48	NA
Y649	21	71	1.10	NA
502	22	73	NA	97.70
1257	5	75	1.63	96.10
1258	5	75	1.67	102.60
1882	11	75	2.40	106.60
1883	11	75	2.18	109.00
22	19	83	1.80	119.40
24	19	83	2.24	116.20
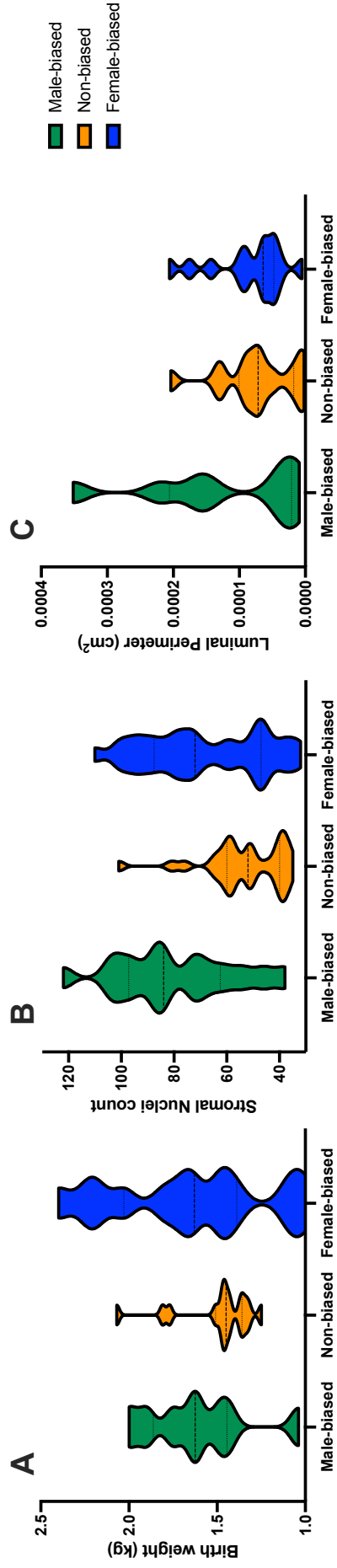
Figure 4.4: Variability of data as assessed by a Levenes test, represented by a violin plot of the number of (A) birth weight (kg), (B) stromal nuclei counts and (C) the difference between between the luminal perimeter (cm²) of the right and left uterine horn of individuals, from male-biased (n=8), non-biased (n=15), and female biased (n=15) groups.

4.3.2   Morphological data

4.3.2.1   Analyses of structural features

There was no interaction observed between the sex ratio and birth weight for endometrial area (Gamma GLMM; t-value=-1.530, n= 64, p=.126), nor luminal perimeter (Gamma GLMM; t-value=-1.595, n=64, p=.1107). The sex ratio of the litter from which a pig originated, birth weight and slaughter weight did not affect the total cross section of the endometrial area (Gamma GLMM, n=64; t-value=.140, p=.889; t-value=-1.289, p=.197; and t-value=-1.289, p=.197; respectively), size of the luminal area as measured by luminal perimeter (Gamma GLMM, n=64; t-value=1.096, p=.2731; t-value=-1.915, p=.055; t-value=-.151, p=.880; respectively), nor number of stromal cells as measured by nuclear staining (Poisson GLMM, n=64: t-value=-.346, p=.729; t-value=-.742, p=.458; t-value=.559, p=.576; respectively). These results all represented in figure 4.5.

The total endometrial gland numbers (Poisson GLMM, n=64; z-value=.117, p=.907; z-value=.058, p=.954; z-value=.054, p=.957; respectively) and larger glands alone (Poisson GLMM, n=64; z-value=-1.033, p=.302; z-value=.432, p=.666; z-value=-.908, p=.364; respectively) were not significantly different between individuals of different *in-utero* sex ratios, birth weights, or slaughter weights.

4.3.2.2   Proportional analyses

The proportion of the luminal perimeter (cm^2), glandular count, and stromal nuclei in relation to the endometrial area was analysed. No effects were seen for the ratio of luminal perimeter nor stromal cell number nuclei to endometrial area for females from different sex ratio litters (Gamma GLMM, n=64; t-value=.843, p=.399; t-value=.371, p=.710; respectively), birth weight (Gamma GLMM, n=64; t-value=-.099, p=.921; t-value=-.099, p=.921; respectively), slaughter weight (Gamma GLMM, n=64; t-value=-1.387, p=.166; t-value=-1.004, p=.3152; respectively). However, although the ratio of the total number of glands and endometrial area for females from different sex ratios (Gamma GLMM; t-value=-0.420, n=64, p=.675), and different slaughter weights (Gamma GLMM; t-value=-0.420, n=64, p=.675) were not significantly different. There was a significantly higher
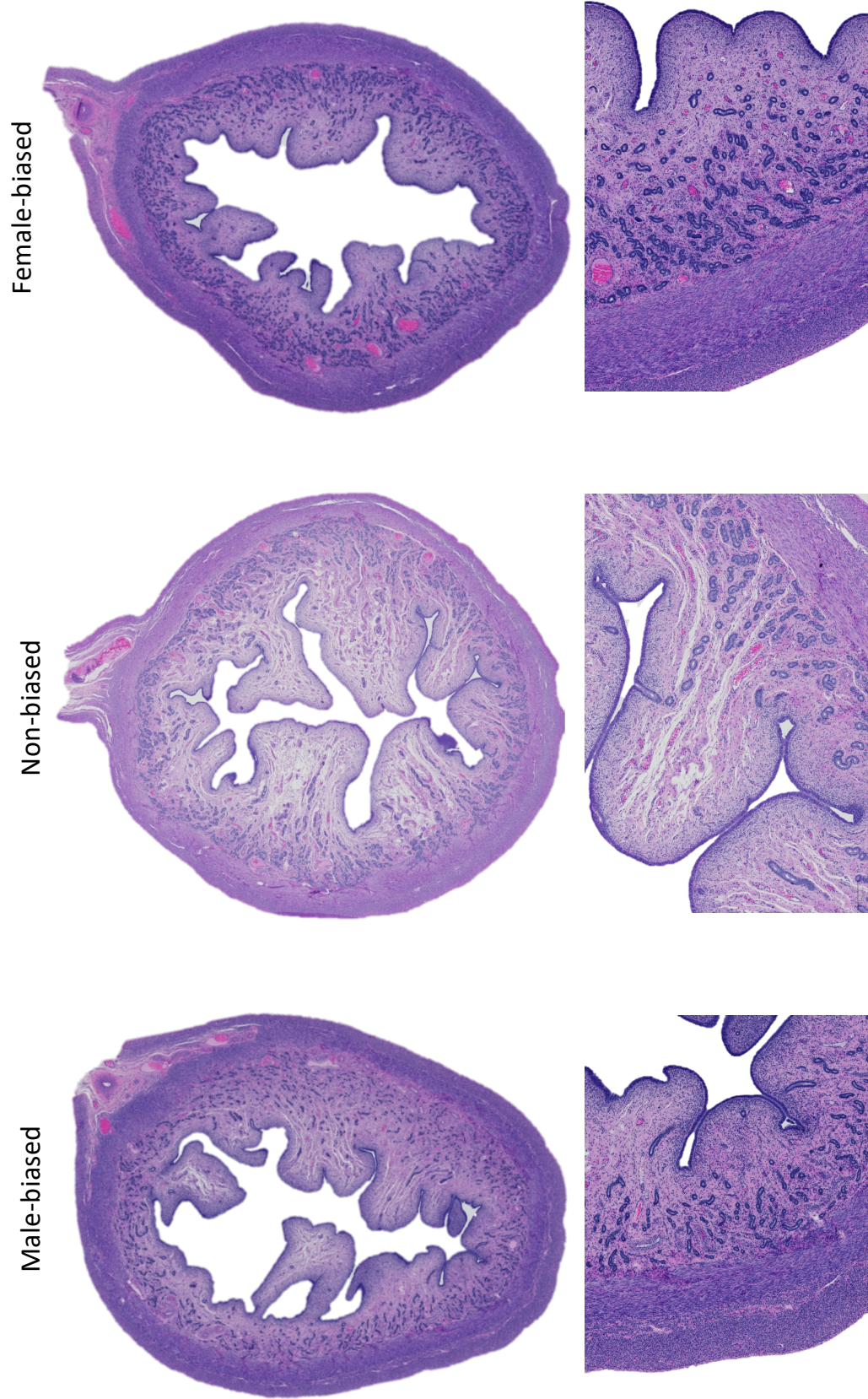
Figure 4.5: Example images of hematoxylin & eosin stained cross sections ($8~\mu m$) selected at random from each litter bias; male-biased, non-biased, and female-biased.

proportion of glands in the endometrial area the higher the birth weight (Gamma GLMM; t-value=2.005, n= 64, p=.045), Figure 4.6.
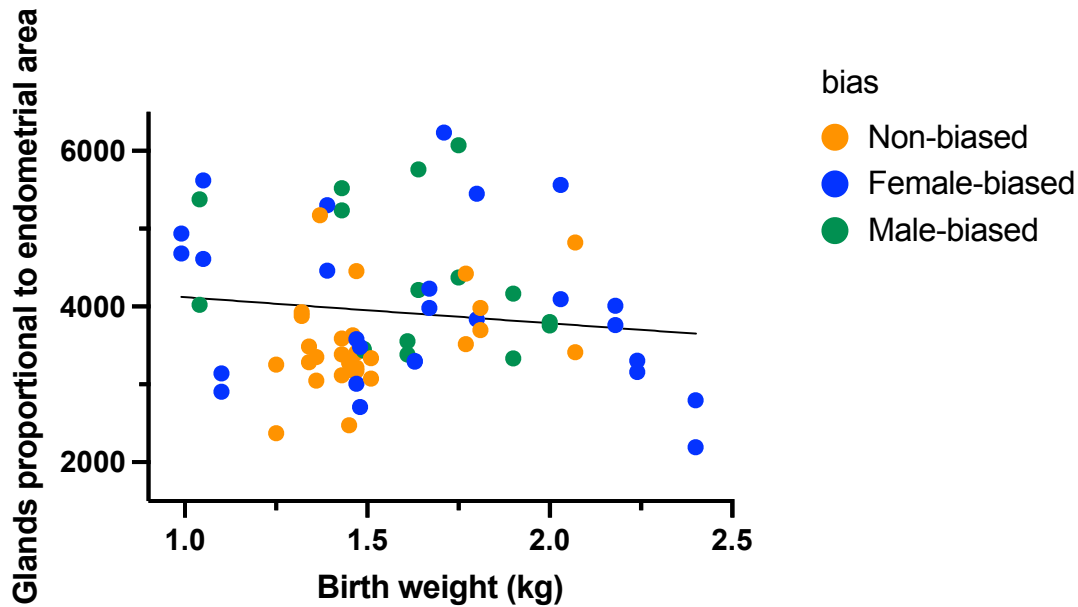
Figure 4.6: The ratio of glands to endometrial area in female pigs (n=38) in relation to their birth weight (kg).

The analyses investigating the ratio of different secretory tissues within the uterine horn demonstrated no significant differences in ratios between stromal nuclei and luminal perimeter (Gamma GLMM, n=64; Sex ratio - t-value=-0.294, p=.769; Birth weight - t-value=1.674, p=.094; Slaughter weight - t-value=-.552, p=.581), gland counts and luminal perimeter (Gamma GLMM, n=64; Sex ratio - t-value=-1.315, p=.1884; Birth weight - t-value=1.709, p=.087; Slaughter weight - t-value=.045, p=.964), nor gland counts and stromal nuclei (Gamma GLMM, n=64; Sex ratio - t-value=-.434, p=.664; Birth weight - t-value=-.265, p=.791; Slaughter weight - t-value=.314, p=.754).

4.3.3 Immunohistochemistry
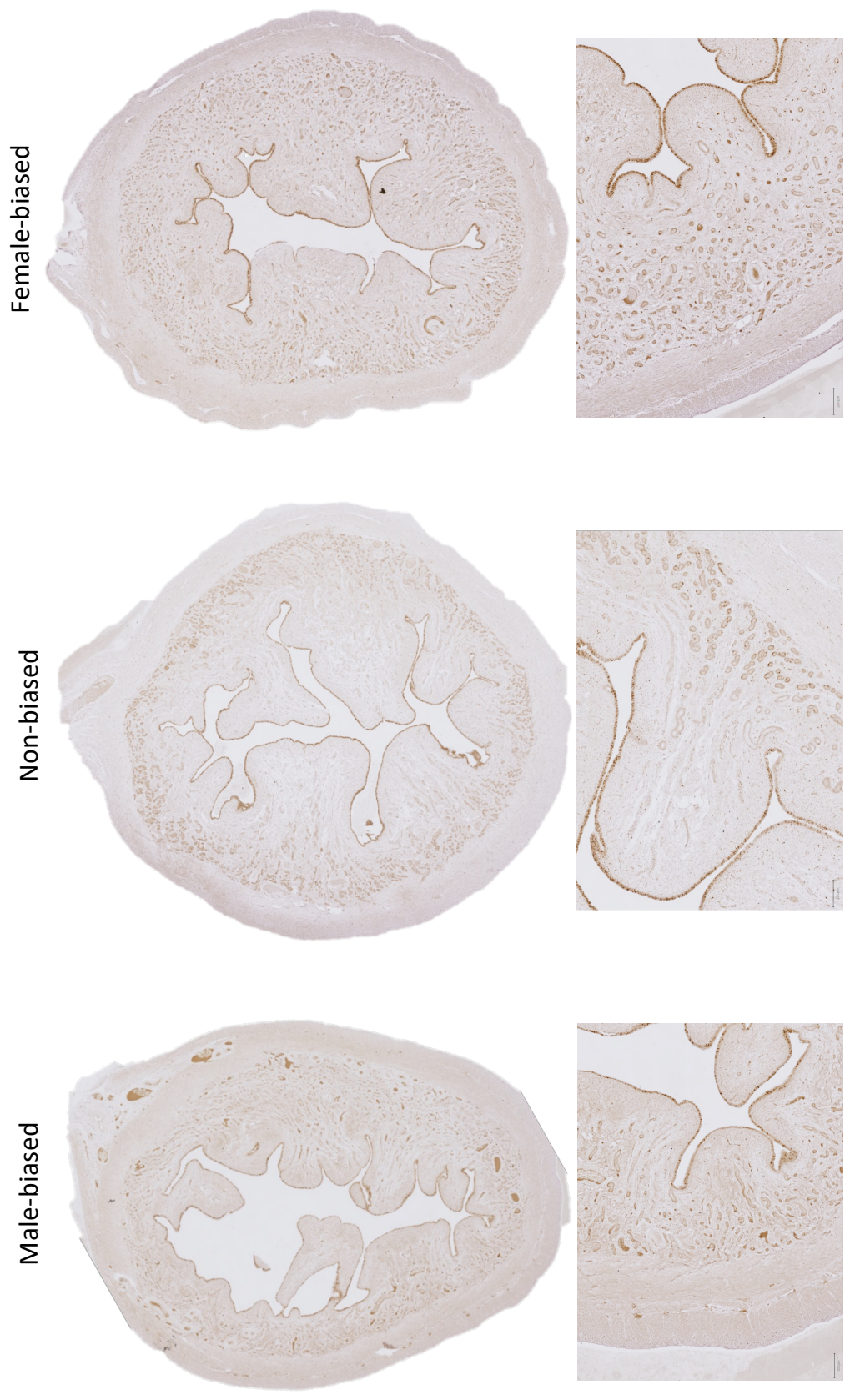
Figure 4.7: Example images of proliferating cell nuclear antigen immunohistochemistry stained cross sections (8 μm) selected at random from each litter bias; male-biased, non-biased, and female-biased.

No significant interaction was found between birth weight nor sex ratio for the proportion of positively PCNA-stained nuclei (Gamma GLMM; t-value=-1.266, n=63, p=.206), nuclei with low stain intensity (GLMM; t-value=.713, n=63, p=.482), nuclei with a moderate stain intensity (Gamma GLMM; t-value=-.819, n=63, p=.413), nor the nuclei with a high stain intensity (Gamma GLMM; t-value=-1.435, n=63, p=.151).

The sex ratio of the litter from which a pig originated, birth weight and slaughter weight did not show a significant difference in the effect on the proportion of positively stained cells (Gamma GLMM, n=63; t-value=.015, p=.988; t-value=.922, p=.357; t-value=-.934, p=.350; respectively), nuclei with a low stain intensity (GLMM, n=63; t-value=.255, p=.800; t-value=-496, p=.624; t-value=.020, p=.984; respectively), moderate stain intensity (Gamma GLMM, n=63; t-value=-.518, p=.604; t-value=1.065, p=.287; t-value=-.565, p=.572; respectively), and high stain intensity (Gamma GLMM, n=63; t-value=.023, p=.982; t-value=1.043, p=.297; t-value=-.162, p=.871; respectively). Further to this, litter sex ratio, birth weight (kg), nor slaughter weight (kg) significantly influenced the total number of positively stained cells as a proportion of the endometrial area (cm^2) (Gamma GLMM, n=61; t-value=-.252, p=.801; t-value=-.712, p=.477; t-value=-.185, p=.853; respectively), luminal perimeter (gamma GLM, n=61; t-value=-.264, p=.792; t-value=-.556, p=.571; t-value=-.370, p=.711; respectively) nor total glandular count (gamma GLMM, n=61; t-value=.013, p=.990; t-value=-.849, p=.396; t-value=-.187, p=.852; respectively).

4.4 Discussion

The initial hypothesis was that a male-biased uterine environment would have a detrimental effect on uterine morphology, and lead to a lower level of endometrial gland proliferation in the female pig. This study did not find any evidence suggesting that this was the case, hence, it cannot be concluded that gestation of a female in a male-biased uterine environment is detrimental to uterine development. In turn, there is no suggestion of an effect on the ability of the female pig to establish or maintain pregnancy. However, it is important to note that there is a higher variability in the number of stromal nuclei found in the endometrial tissue, for pigs from either litter bias as compared to gilts from non-biased litters.

This study did not demonstrate an effect of litter sex ratio on any of the defined uterine measures, their relative ratios, or the endometrial cell proliferation. However, it did find that the number of stromal cell nuclei within the endometrium were significantly more variable in females that came from either extreme, male- and female-biased litters. This could potentially impact on stromal-derived growth factors that enhance secretion from the luminal and glandular epithelium [33]. The stromal cells are also key in supporting the underlying implantation structures and hence [33], variability within their numbers could impact on the uterine implantation capabilities, and thus survival of the embryo. As anticipated, we did not identify any significant effect of the sex bias of a litter on the variation of the parameters between the two horns of an individual for most of the studied parameters. However, we did identify that pigs from male-biased litters has a significantly higher variation in the luminal perimeter (cm^2) between the left and the right horn. This may be a symptom of the delayed reproductive development and maturation and puberty seen in male-biased individual pigs [140] and gerbils [39]. Variability is known to be a major issue for producers for the reasons discussed in the thesis introduction and is commonly found in many different aspects of production. Placental efficiency was found to be highly variable in the large white at an approximate level of three-fold [252], with two-fold variation within litters. As indicated in these findings, higher levels of variation were seen in both male-, and female- biased litters, compared to the non-biased litters. This suggests that a biased litter may be a contributor to the variability of reproductive output commonly reported in female pigs. Furthermore, such biased litters were found to lead to more variation in the birth weight of offspring. Selection for larger litter sizes over time has resulted in litters of higher numbers but with low and greatly variable birth weights [262]. Low birth weight piglets from large litters are often cross fostered or euthanized as they will not be able to compete with their larger siblings for teats and have poor pre-weaning survival rates [226]. What is also evident is that the birth weight of a female pig had a significant effect on the proportion of glands in the uterus, relative to the uterine size. In this instance the higher the birth weight, the lower the proportion of glands. There is suggestion that the birth weight of an individual impacts on their future reproductive capacity. For example, pigs with a birth weight lower than 1 kg produced less piglets and held a shorter reproductive lifespan than their higher birth weight counterparts [146]. This reduced reproductive efficacy has been identified in low

birth weight piglets in several other studies [175]. Despite this, there is little investigation into the effects on, for example, uterine glands. Although the birth weight of pigs was found to not influence uterine gland development [224], most research has investigated more rudimentary measures of reproductive tract morphology, such as uterine and ovarian length or weight, and follicle counts [7] with no effects seen in low birth weight pigs in some studies [6] or negative impacts on follicular numbers and increased atretic follicles in others [6].

Despite there being, to our knowledge, no previous research specifically investigating the effect of an *in-utero* sex bias on the uterine morphology or efficiency, uterine glands have been shown to be affected by post-natal treatments, such as lactocrine effects influencing morphogenic aspects [158] and oestrogenic influences leading to reduced uterine responsiveness to embryotropic signals [213]. The animals used for this study were assumed to not be cycling animals and were not synchronised. There is a possibility that some animals may have become cyclic or other additional factors may be at play which we were unable to control for. Future investigations should used cycling, and synchronised animals in order to control for variability between individuals that may be caused by these factors.

We acknowledge that the current study holds some limitations and recommend that future research should investigate the differences across several sections of the uterine horn rather than a single location as in this research. This would allow for replication of the proliferation analysis across the uterine horn for a more representative sample. We would also recommend a separate investigation into whether there are any differences to both the morphological structures and cell proliferation of the left vs right uterine horn of individual pigs. Further, we recommend that more sections are stained for the proliferation analysis to allow for replication within individuals.

Future investigations could assess whether there is a difference in the presence of secreted phosphoprotein 1 between pigs of different litter sex ratios as this plays an important many aspects of gestation. It holds key roles in implantation [121], and foetal and placental development of pigs [83, 121, 247]. Secreted phosphoprotein 1 protein levels have been identified as higher in the more prolific meishan breed than the large white, in both glandular epithelium, luminal epithelium, and placenta [102]. Therefore, I sug-

gest investigating this protein expression may elucidate the reduced reproductive capacity previously seen in pigs from male biased litters which we haven't identified in this study. Finally, as per chapter 3, we recommend that further studies aiming to investigate the effect of a sex biased litter on reproductive development, or outcome, should utilise a naturally synchronised parity 2 population. This could be done by performing oestrus checks on the pigs to detect ovulation and culling the pigs at the point of detection. This would avoid any influence of exogenous hormones used for artificial synchronisation, whilst removing the variation of non-synchronised animals that may have masked differences in the phenomena which I have studied.

This study did not identify any mechanisms by which the reduced reproductive capacity may be realised. There was no influence of the litter sex ratio on the rudimentary morphology of the uterine horn by analysis of endometrial area, luminal perimeter, stromal nuclei, nor gland count. Nor was there any differences in the ratio of these secretory structures in relation to tissue size, or each other, apart from the increased proportion of glands in relation to the endometrial area of pigs with a lower birth weight. There was also a more variable stromal nuclei count in pigs from a male-, and female- biased litter, and higher individual variation between uterine luminal perimeter in male-biased females. Further research is needed to understand the impact that a biased litter is having on the female offspring as suggested above.

Chapter 5

Investigating the effect of litter sex ratio on health, development and production quality in the commercial pig

5.1 Introduction

In recent years, commercial pig production has faced unprecedented hardship. Continual pressure has been placed on modern pig production for the last decade. It has faced negative net margins, unpredictable markets, feed price volatility, and a loss of carcass value by a third since 2013 [4]. Furthermore, serious challenges are compounding, with; CO_2 shortages (necessary for slaughter), removal of zinc from weaner diets, a lack of skilled workforce [206], recent increases in oil and gas prices, alongside the dramatic rise in wheat prices as a result of the Russian invasion of Ukraine [63].

With these added pressures, producers must ensure maximised production efficiency to minimise any loss in profit. For example, breeding stock now produce larger litters than ever with approximately 12.5 piglets born alive per litter in 2010, and about 15.5 in 2021 [2]. As a result of breeding pigs for increased reproductive performance and production value; herd welfare, sow longevity, and piglet quality have been neglected [136, 156], and

more pigs born dead than previously (0.75 per litter in 2010 vs 1 per litter in 2021) [2].

Increased productivity has also resulted in an increased variation within herds [8, 136]. For example, larger litter sizes have been correlated with several negative factors impacting profitability, including higher levels of within-litter variation in growth and health parameters, and lower birth weights [262]. Factors such as low birth weight have been found to have significant effects on mortality rates, growth rates, and the carcass and meat quality of these pigs [262]. Poor health and growth, a negative impact on profitability, can be caused by several factors including poor nutrition and/or housing, inadequate husbandry, or genetics [227]. However, today's production systems are highly homogenous wihtin farms [1, 177]. Pigs at a specific producer will often be of the same genetics, fed the same diets, be exposed to the same environment, and be reared by the same stockpersons [1, 177]. Yet, there remains a considerable variability of pig parameters even within batches [110]. This has been shown to result in additional costs to the farmer by poor matching of diet specifications, grading losses, additional time required to meet market specifications, and challenges associated with health management [227]. Much of this variation is unaccounted for and any steps taken to reduce variability of the growth, health, and production parameters of the production pig would potentially improve profitability and overall efficiency of pig production [227].

One avenue that remains largely unexplored as a potential cause of variability between individual pigs is the sex ratio of the litter from which a pig originated. Although there has been recent interest in the sex ratio and its effect on the reproductive capacity of females, as revealed in Chapter 2, there has been limited investigation into its effect on production traits and health across the life-course in pigs reared under otherwise similar conditions. For a detailed discussion about the impacts of the sex-biased uterine environment, please see chapter 2 (scoping review). In pigs, the sex-biased uterine environment compromises reproductive efficacy [140, 201, 202, 205], alters behaviour [173, 204], and influences growth traits [173]. Female pigs become masculinised when gestated with predominantly male siblings [173, 204], displaying higher levels of aggression and agonistic behaviours, whilst displaying lower levels of fearful behaviours [204]. These pigs also initiate more fights than female-biased counterparts [204]. Pigs positioned between two males *in-utero* have greater weights than if positioned between two females when later fed on restrictive diets,

but not on unrestricted diets [120, 191]. This suggests that the effect could be due to a behavioural difference between the pigs, or physiological difference through a re-direction of investment into other aspects of homeostasis.

This study aims to determine whether the sex ratio of the litter that a pig has originated from influences production parameters including, (i) health - disease, injury prevalence, and medical intervention; (ii) development – growth and feed conversion ratios; and (iii) carcass traits - grading, age at slaughter, and carcass value.

5.2   Methods

The data were collected as part of the 'Optimal Pig' project, a KTP-funded project between University of Leeds and KARRO food group. The purpose of the Optimal pig trial was to investigate whether there was any effect of the sire-lines used within the company on production parameters. Collaborative work was agreed pre-trial for the data to be used in this thesis, separately investigating the effect of the litter sex ratio (LSR) on production parameters.

Due to the nature of this collaboration under the movement restrictions of Covid-19 in 2020, I was unable to assist with the collection of these data as originally planned. Prior to collections I was part of discussions on how to best collect data from abattoirs, including the data that would be necessary for the subsequent calculations of litter sex ratios. This defined the sexing of each piglet at birth, including dead born piglets, but excluding mummified piglets as standard.

Prior to receiving any data, the following research questions and hypotheses were formulated. Variables included in the data were at that point unknown, but the aims and objectives of the project were known. Hence, everything within **research questions and hypotheses** was planned prior to receiving the data set. Following this, the trial methods are described, and finally the analyses performed on the data.

5.2.1 Research questions and hypotheses

5.2.1.1 Specific research quesitons

(i) Does the LSR of a pig impact on life course health through the production system?

This will optimally be measured through: (a) disease, or (b) injury the pig experiences during its life course, along with (c) any medical interventions that are used to treat the individual throughout the various production stages.

(ii) Does the LSR of a pig impact on the development of the production pig?

This will optimally be measured through: (a) growth of the pig with weight measurements at birth, weaning, grower and finisher stages, (b) feed conversion ratios (FCR) to indicate the efficiency of the pigs for converting feed consumed into body weight.

(iii) Does the LSR of a pig impact on the carcass traits in the production pig?

This will optimally be measured through: (a) carcass warm weights; (b) grade conformation and fat coverage (scoring system; SEUROP) using one or more of the following measurements: intra-scope (optical probe), Fat-O-Meater (FOM), Hennessy Grading Probe (HGP II), CSB Ultra-Meater, AutoFom (Fully automatic ultrasonic carcase grading) OR the lean meat content; (c) disease prevalence leading to carcass rejection, e.g. pyaemia/generalised abscessation/septic pleurisy, fever/septicaemia/toxaemia, polyarthritis, oedema/emaciation.

5.2.1.2 Hypotheses

(i) Does the LSR of a pig impact on life course health through the production system?

a.

HA1. A male-biased LSR leads to a pig being more susceptible to disease during its life course.

H01. A male-biased LSR does not influence susceptibility to disease during its life course

b.

HA2. A male-biased LSR leads to a pig obtaining more injuries over its life course

H02. A male-biased LSR does not lead to a pig obtaining more injuries over its life course

c.

HA3. A pig originating from a biased LSR requires more medical intervention than other biased litters.

H03. A pig originating from a biased LSR does not require more medical intervention than other biased litters.

(ii) Does the LSR of a pig impact on the development of the production pig?

a.

HA4. A male-biased pig will have a higher weight gain than pigs from other biased litters.

H04. A male-biased pig will have not have a higher weight gain than pigs from other biased litters.

b.

HA5. A male-biased pig will have a lower FCR than pigs from other biased
litters.

H05. There is no difference in FCR between pigs of different LSRs.

**(iii) Does the LSR of a pig impact on the carcass traits in the production
pig?**

a.

HA6. Carcass warm weights will be significantly higher in pigs from male-
biased litters.

H06. Carcass warm weights will not be significantly higher in pigs from male-
biased litters.

b.

HA7. Carcasses of pigs originating from male-biased litters will obtain a higher
overall score more than pigs of other LSRs.

H07. Carcasses of pigs originating from male-biased litters will not obtain a
higher overall score more than pigs of other LSRs.

c.

HA8. Carcasses of pigs originating from biased LSRs will more frequently be
rejected due to disease prevalence.

H08. Carcasses of pigs originating from biased LSRs will not be more fre-
quently rejected due to disease prevalence.

5.2.2 Pre-collection plan for statistical analysis

Mixed linear models were to be used to analyse the predictor variables in R [215]. Dis-
tribution analyses would be performed for each predictor variable to define the specific

linear model used. Where appropriate outliers would be removed, or data transformed using natural log or square root transformations. Optimal models would be calculated using step-wise regression. Random factors were expected to be Batch with Sire-line nested. Sex would optimally be included as an interaction of the litter sex ratio. Incomplete or missing data would be treated as NA and be excluded from analyses.

Exploratory data analyses were to be performed if data were available on the LSR of the litter bearing sow. This would aim to investigate whether this held any effect on the litter that she carried; e.g. piglets born alive, piglets born dead, piglet weights, ratio of her litter, interventions (creep feed, milk let down issues, savaging

5.2.3 Data transfer and changes to analyses plan

Once data collection and the initial data plan was complete, the data were shared with me for analyses. Few alterations needed to be made in consideration to the available data. The necessary changes are described in Table 5.1.

Table 5.1: Alterations made to the defined research questions and hypotheses outlined in section 5.2.1.

	Research questions	
	Unavailable data	*Added data*
i	Death	
	Cause of death	
ii	Feed conversion ratios	Weaning weights
iii	Carcass disease prevalence	
	Hypotheses	
Unaltered hypotheses	*Altered hypotheses*	*Removed hypotheses*
1, 2, 3, 4, and 7	6 – Carcass warm weights changed to carcass cold weights	5 and 8

5.2.4 Optimal pig - trial methods

The pigs used for the Optimal Pig trial were not part of any other trial or research investigation.

Four batches of sows at Nether Bogside farm (Aberdeenshire, Scotland) were included in the Optimal Pig trial, with the batches being served at 6-week intervals with three

different sire-lines selected for the trial (Table 5.2). Out of the total 519 sows that were serviced for the trial, 445 farrowed and a total of 5930 piglets were individually tagged. Data collection was completed in March 2021.

Once farrowed, both live and dead born piglets were visually sexed based on external genitalia, weighed, and tagged (only live born) using RFID for individual identification at day 0. The piglet and sow ID's were linked, along with the tag colour referring to sire-line genetics. No further data was collected for dead born piglets.

At weaning, when the piglets were 4 weeks of age, each litter was weighed as a group (individual measurements were not taken). Pigs were then transported for four hours to the nursery farm South Slipperfield (Scottish borders, Scotland) via Karro's standard haulage provider. At the point of weaning any pigs that weighed 4 kgs and under, and any ill piglets were removed from the trial. These pigs were sent to a specialist unit for small pig rearing. An average of 65 pigs per batch were removed in this way. The nursery farm kept the trial pigs for five weeks, after which they were moved onto three different finishing farms. During their life-course, pigs were reared under normal management procedures and were all on the same diets through each stage.

Table 5.2: Table describing genetics and the dates of artificial insemination used for each batch. This also includes which farm the subsequent offspring of each batch went to through breeders, nursery, and the grower-finisher stages.

Batch number	Sirelines used	Serve date (2020)	Breeder	Nursery	Grower-Finisher
1	PIC 327￼PIC 337￼Rattlerow Tendershire	13 January	Nether Bogside	South Slipperfield	East Common
2	PIC 327￼PIC 337￼Rattlerow EBX	24 February	Nether Bogside	South Slipperfield	Boythorpe Cottage
3	PIC 327￼Rattlerow Tendershire￼Rattlerow EBX	6 April	Nether Bogside	South Slipperfield	Burnhouse Quarry
4	PIC 337￼Rattlerow Tendershire￼Rattlerow EBX	18 May	Netherbogside	South Slipperfield	East Common

5.2.4.1 Weights

Individual weights of all pigs from batches 2 - 4 were collected at the following timepoints (dead born piglets were not weighed);

- 0 days old

- at weaning - average litter weight

- 9 weeks old – when moved to finishing unit

- 23 weeks old or at first draw (pre-slaughter weights)

Due to COVID-19 restrictions, batch 1 were group weighed and day 0 information was collected by farm staff rather than by the researcher.

The weights at 9 weeks and first draw (for slaughter) or aged 23 weeks old were recorded on a digital system called Pig Expert (AgriSyst) for all batches.

5.2.4.2 Health

Deaths prior to slaughter along with date and weight, and any medical treatments were recorded. For all batches at South Slipperfield farm, and Batches 2 and 3 at Boythorpe Cottage and Burnhouses Quarry farms, data on deaths and treatments were recorded by farm staff. At East Common (Batches 1 and 4) data on deaths and treatments were recorded on the Pig Expert (AgriSyst) app by a single member of the farm staff. These data were downloaded at the conclusion of each batch.

5.2.4.3 Carcass traits

Each pig was assigned sire-line level slap-mark numbers (rudimentary tattoos routinely used by law for pig identification), meaning if the ear tag (personal ID) was lost, information could be aggregated to sire-line level, but not the individual pig. Hence, any pigs whose ID could not be identified on site were not included for analyses of carcass data as the aggregated data did not contain litter sex ratio detail.

The tag was scanned and linked with the kill number that is assigned to each individual pig. Kill data from the abattoir could then be matched with the individual pig

IDs and data linked to individual litter origin. At slaughter, dead weight, back fat and condemnations were recorded against the individual pig ID.

5.2.5   Data processing and analyses

The following analyses were performed on the response variables that are summarised in Table 5.3.

Sex ratio of the litter from which an individual originated was calculated as % of females per litter, including both live and dead born pigs. Any litters that didn't have complete data for the sex of all offspring in a litter were excluded from analyses. Following this, a total of 313 litters remained to be included in the analyses. It is important to note that the criteria for the sex-bias litter did not follow the methods used in the two previous data chapters. Due to the nature of the study, the sex ratios were collected as continuous percentages through 0-100% rather than grouped biases (<35%, 45-54.9%, and >65% females) as the large data set allowed for this without needing to take group balances into account.

Generalised linear mixed effect models were used to test the effect of predictor variables. Analyses were performed using lme4 [19], and MASS [232], with critical alpha level applied as p=.05. Normality of continuous data was assumed checked by observing the variance of residuals. Data of a categorical data were coded for binomial analyses. These were analysed using **glm (family = binomial)**.

Categorical data that was multinomial were analysed by grouping the sex ratio of a litter into female-biased, non-biased, and male-biased. These were then analysed using the Pearsons chi-squared test, and where appropriate using a simulated p-value based on 2000 replicates. Collinearity between predictor variables were checked using the **"vif"** function in R package **"car"** where any values lower than three were considered not collinear. There was no collinearity between predictor values.

AIC model selection was performed to establish which model had the best fit between a set of possible models. The AIC of the used models can be found in Table 5.3. Models with interactive elements were re-run as an additive model if no significant interactive effects were found.

Table 5.3: Description of predictor variables and model characteristics. The AIC values from the best fitting models are included.

Predictor variable	Data type	Distribution	Data level	AIC value
Growth analyses (kg)				
Birth weight	Continuous	Normal	Individual	4052
Total litter birth weights	Continuous	Normal	Group	1787
Average pig weight from different sex ratios	Continuous	Normal	Group	51
The litter weight at weaning	Continuous	Normal	Group	2528
Week 9 weights	Continuous	Normal	Individual	18274
Week 23 weights	Continuous	Normal	Individual	21691
Health analyses				
Pigs removed from trial	Categorical	Binomial	Individual	384
Mortality	Categorical	Binomial	Individual	2726
Growth stage at death	Categorical	Binomial	Individual	
Cause of death	Categorical	Binomial	Individual	
Treated pigs	Categorical	Binomial	Individual	1993
Carcass analyses				
Age at slaughter (days)	Continuous	Normal	Individual	14364
Back fat depth (mm)	Continuous	Normal	Individual	9882
Weight of cold carcass with trimmings (kg)	Continuous	Normal	Individual	14314
Grade	Categorical	Binomial	Individual	
Lean meat content (kg)	Continuous	Normal	Individual	9340
Final carcass value (£)	Continuous	Normal	Individual	17251

5.2.5.1 Health analyses

All health data, except stage of death and cause of death, consisted of categorical data and hence were analysed following binomial assumptions. The models for all analysed data were as follows:

> *<Response variable>* with predictor variables being *Litter sex ratio* and *Sex* as multiplicative, with random effects being *Parity* nested within *Sow ID*, and *Sire line* nested within *Batch*.

Stage and cause of death were both analysed using a chi-squared test. Cause of death was analysed using a simulated p-value based on 2000 replicates (as part of the R function **chisq.test(, simulate.p.value = TRUE)** this was used due to the unbalanced number of pigs from each cause (Injury or Illness = 17, Unthrifty = 17, Behaviour = 20, Unknown = 50).

5.2.5.2 Growth analyses

Two different models were used to analyse the growth data dependent on data structure; individual level data or group level data:

Individual level data

> *<Response variable>* with predictor variables being *Litter sex ratio* and *Sex* as additive, with *Parity* nested within *Sow ID*, and *Sire line* nested within *Batch* as random effects.

Group level data

> *<Response variable>* with predictor variable being *Litter sex ratio*, with *Parity*, and *Sire line* nested within *Batch*, as random effects.

Litter wean weight was analysed without nesting *Sire line* in *Batch*. Weights at week 9 and slaughter weight were analysed without nesting *Parity* within *Sow ID* The birth weight group, i.e. low, medium or high birth weights, were analysed using a chi-squared test.

5.2.5.3 Carcass analyses

Analyses of data collected at the abattoir were analysed using the following model:

> *<Response variable>* with predictor variables being *Litter sex ratio* and *Sex* as multiplicative, with *Parity* nested within *Sow ID*, and *Sire line* nested within *Batch*, as random effects.

Only the net cold carcass weight was analysed without nesting *Parity* and *Sow ID*.

The grade allocated to the carcass was analysed using a chi-squared test with a simulated p-value.

5.3 Results

The number of animals used for each analysis was affected by the number of missing values and pigs that either survived through the system, or due to the data not being collected for various reasons. A summary of the total number of animals available for each analysed variable, alongside the number of animals per batch and sire line has been included to aid the readers understanding of the power behind each individual model (Table 5.4).

Table 5.4: The number of total animals included in a model, with the number of litters represented in brackets. These numbers have then been broken down to the number of animals that were part of each batch, and also the number from each sire line. This has been done for each predictor variable that was studied.

	Total	Batch number				Sire line			
	# pigs (# litters)	1	2	3	4	EBX	PIC 327	PIC 337	Tendershire
Growth analyses (kg)									
Birth weight	3823 (306)	0 (0)	1029 (81)	1383 (115)	1412 (110)	1313 (103)	484 (65)	814 (67)	848 (71)
Litter birth weights	3919 (313)	80 (7)	1043 (81)	1383 (115)	1414 (110)	1318 (103)	872 (67)	855 (70)	874 (73)
Average pig weight	3919 (313)	80 (7)	1043 (81)	1384 (115)	1415 (110)	1319 (103)	873 (67)	856 (70)	875 (73)
Litter weight at weaning	3774 (306)	80 (7)	1029 (80)	1372 (114)	1294 (105)	1224 (98)	872 (67)	829 (69)	849 (72)
Week 9 weights	3274 (313)	80 (7)	879 (81)	1139 (115)	1176 (110)	1087 (103)	714 (67)	729 (70)	743 (73)
Week 23 weights	2777 (313)	75 (7)	807 (81)	971 (115)	924 (110)	902 (103)	624 (67)	634 (70)	617 (73)
Health analyses									
Pigs removed from trial	1067 (88)	79 (7)	990 (81)	0 (0)	0 (0)	278 (22)	359 (30)	406 (34)	24 (2)
Mortality	3919 (313)	80 (7)	1043 (81)	1383 (115)	1414 (110)	1318 (103)	872 (67)	855 (70)	874 (73)
Growth stage at death	432 (187)	3 (3)	89 (51)	93 (58)	247 (75)	182 (67)	72 (40)	90 (42)	88 (38)
Cause of death	239 (100)	2 (2)	16 (14)	41 (32)	180 (52)	106 (38)	21 (16)	53 (22)	79 (26)
Treated pigs	3919 (313)	80 (7)	1043 (81)	1383 (115)	1414 (110)	1318 (103)	872 (67)	855 (70)	874 (73)
Carcass analyses									
Age at slaughter (days)	2055 (312)	47 (7)	581 (81)	817 (114)	611 (110)	632 (102)	463 (67)	484 (70)	476 (73)
Back fat depth (mm)	1985 (312)	42 (7)	581 (81)	764 (114)	598 (110)	599 (102)	445 (67)	482 (70)	459 (73)
Cold carcass (kg)	1986 (312)	43 (7)	582 (81)	765 (114)	599 (110)	600 (102)	446 (67)	483 (70)	460 (73)
Grade	1987 (312)	44 (7)	583 (81)	766 (114)	600 (110)	601 (102)	447 (67)	484 (70)	461 (73)
Lean meat content	1988 (312)	45 (7)	584 (81)	767 (114)	601 (110)	602 (102)	448 (67)	485 (70)	462 (73)
Final carcass value	1989 (312)	46 (7)	585 (81)	768 (114)	602 (110)	603 (102)	449 (67)	486 (70)	463 (73)

5.3.1 Health data

There was no interactive effect of the litter sex bias and offspring sex on the number of pigs removed from the trial for being 'smalls' (being too small or ill) (binomial GLMM; z=.187, n=1067, p=.852), the number of pigs that needed to be treated (binomial GLMM; z=-.645, n=3919, p=.519), nor on the number of pigs that died pre-slaughter (binomial GLMM, z=-1.659, n=3919, p=.097), Figure 5.1. The stage at which the pigs died was not significantly different between pigs from female-, non- or male-biased litters (X^2=7.346, p=.1187). However, the cause of death was significantly different between pigs from different *in-utero* sex biases (X^2=17.19, p=.009) (Figure 5.2). Pigs from a female-biased litters (std residual = 1.277) were more likely to die from behavioural causes than non-biased (std residual = 0.031) and male-biased litters, whilst pigs from male-biased litters (std residual = -1.582) were significantly less likely to die from behavioural causes than non-biased litters. Pigs from both male-biased (std residual = 0.977) and female-biased (std residual = 0.726) litters were more likely than those from non-biased litters (std residual = -0.903) to die due to injury or illness, but less likely to die from unknown causes (std residuals; -1.430, -1.917 and 1.723 respectively). Pigs from male-biased litters (std residual = 1.246) were also more likely to die from being unthrifty than pigs from female-biased (std residual = -0.269) and non-biased litters (std residual = -0.590).
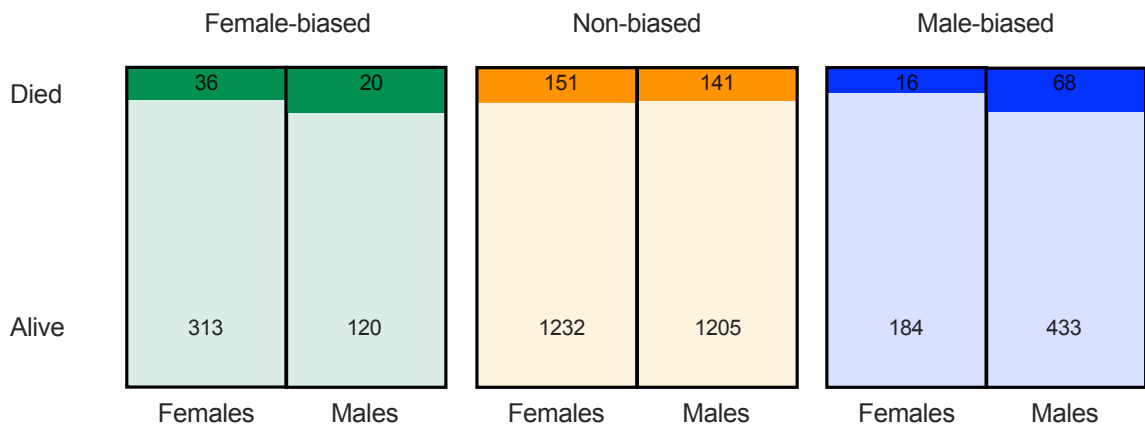
Figure 5.1: The proportional mortality of pigs based on their sex and whether they originated from a female-, non-, or male-biased litter. The numbers at the top of each stack represents the number of pigs that died prior to slaughter (culls), with the numbers below representing the pigs that were alive until commercial slaughter. This figure illustrates non significant data at critical alpha level of 0.05.
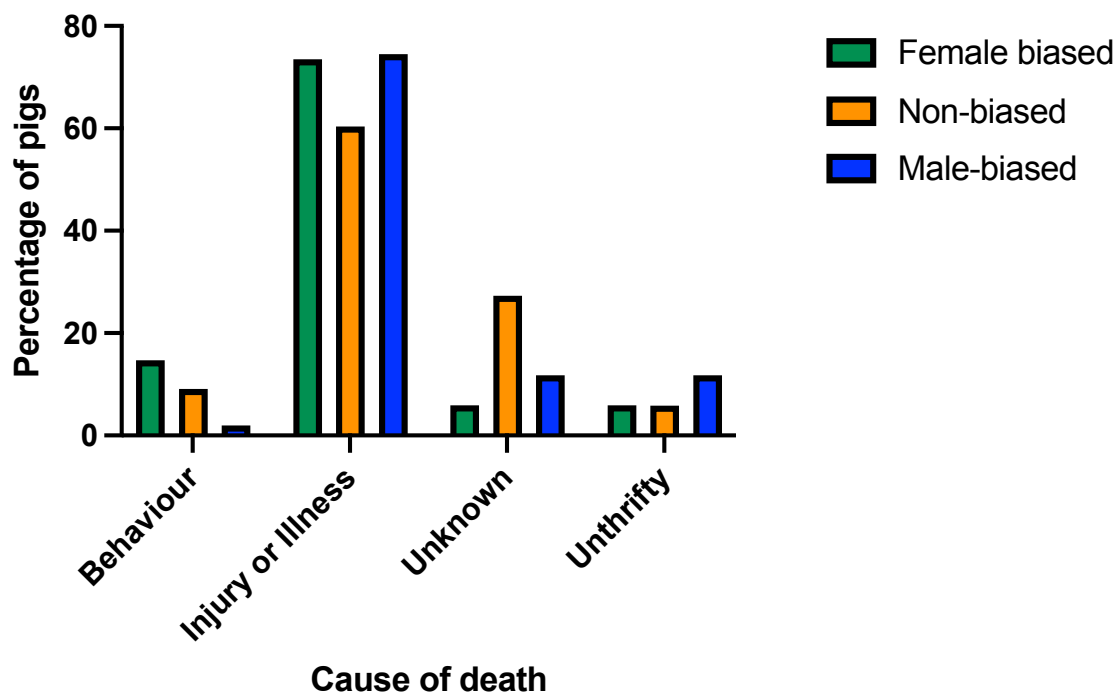
Figure 5.2: The cause of mortality in pigs from different *in-utero* sex ratios represented as a percentage of total pigs per group. Behavioural causes included fighting and vice behaviours. Injury or Illness included: Hernias, lameness, skin conditions, swollen abdomens, glasses, meningitis, pneumonia, streptococcus and non-fighting related wounds. Unknown was any unknown cause of death. Unthrifty: pigs culled because that were not meeting production targets and creating an excessive cost to production. The number of pigs per male- (n=51), non- (n=154), and female-biased (n=34) litters for each mortality cause were respectively; behaviour 1, 14, and 4; injury and illness 38, 89, 25; unthrifty 6, 9, 2; unknown 6, 42, 2.

There was no significant difference in the number of pigs taken off trial at weaning, pigs requiring treatment, nor the number of pigs that died between different *in-utero* sex ratios (binomial GLMM, z-value=-.769, n=1067, p=.442; z-value=-.776, n=3919, p=438; z-value=.923, n=3919, p=.356, respectively), or sex (binomial GLMM; z-value=1.195, n=1067, p=.232; z-value=.254, n=3919, p=.799; z-value=1.394, n=3919, p=.163, respectively).

5.3.2 Growth data

There was no interaction effect between the *in-utero* sex ratio of a pig and its sex for the weight at any point across the life course (GLMM; at birth – t=-.817, n=3823, p=.414; week 9 – t=-.543, n=3273, p=.587; week 23 – t=.014, n=2777, p=0.989).

Table 5.5: Analysis results of the weight data from pigs. Analyses were performed using a GLMM in R.

	Litter sex ratio		Sex	
	t-value	*p-value*	*t-value*	*p-value*
Birth weight	-5.565	0.573	3.350	0.0008
Total litter birth weights	0.437	0.662	NA	
Average piglet weight per litter sex ratio	-1.570	0.115	NA	
Litter wean weights	1.268	0.206	NA	
Week 9 weights	0.898	0.370	1.322	0.186
Week 23 weights	0.273	0.785	0.025	0.980

No significant difference was found between weights (kg) of pigs born from litters of different *in-utero* sex ratios, at any point across the life course (Table 5.5). There was also no difference in the proportion of pigs of high, medium, or low birth weights between the different *in-utero* sex ratios ($X^2(4)$=3.051, p=.5494). The sex of an individual pig held no effect on the weight of a pig at week 9 and 23, but male pigs were significantly heavier at birth than female counterparts (Figure 5.3).
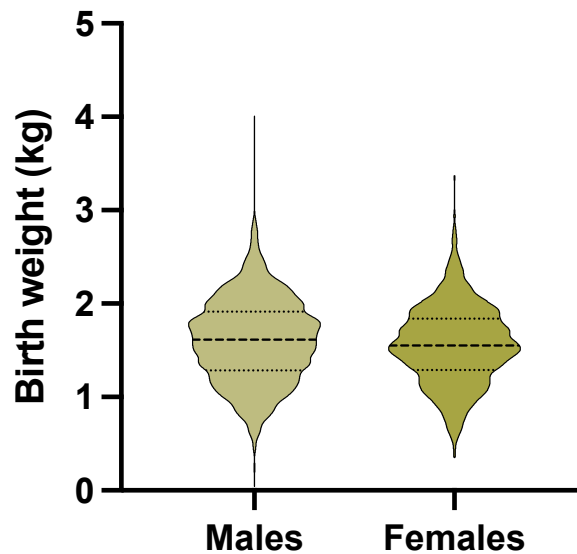
Figure 5.3: The weight of both males and females at birth (kg) with a significantly higher weight in male than female pigs (GLMM; t-value=3.348, n=3823, p<0.001).

5.3.3 Carcass data

Table 5.6: Analysis results of the carcass data from pigs. Analyses were performed using a GLMM in R.

	Litter sex ratio		Sex	
	t-value	*p-value*	*t-value*	*p-value*
Slaughter age	0.502	0.616	0.797	0.426
Probe depth (mm)	-0.098	0.922	-12.517	<0.001
Net cold weight (kg)	-0.160	0.873	-6.58	<0.001
Carcass lean meat	0.09	0.929	12.11	<0.001
Carcass value (£)	0.519	0.604	3.646	<0.001
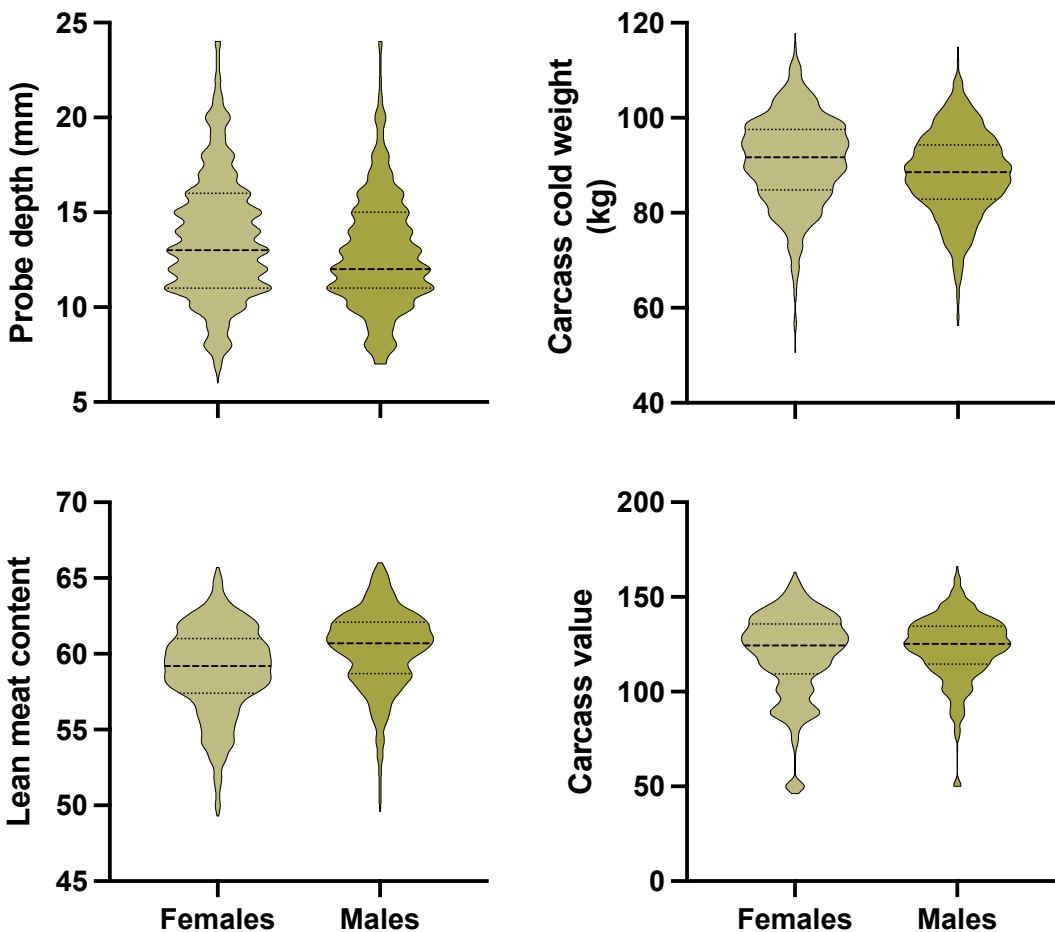
Figure 5.4: Represents the results from the carcass data analyses. Male pigs held an increased lean meat content and carcass value compared to females. Whilst females held an increased probe depth and cold carcass weight as compared to males (GLMM; Probe depth (mm) t-value=-12.517, n=1985, p<.0001; Carcass cold weight (kg) t-value=-6.58, n=1985, p<.001; Carcass lean meat t-value=12.11, n=1985, p<.001; Carcass value (£) t-value=3.646, n=1985, p<.001)

There were no interactions found for the *in-utero* sex ratio and sex of an individual on any of the analysed carcass data (p>0.05).

The litter sex ratio from which an individual originated did not affect the age at which the animal was sent to slaughter, nor any of the carcass quality traits (Table 5.6). The carcass grade was also unaffected by litter sex bias (X^2 = 22.918, p=.864).

The sex of the individual however did impact on carcass quality traits (Table 5.6). With the probe depth (-1.646 mm) and net carcass cold weights (-2.653 kg) being lower in males than females, increasing both the lean meat content (1.389) and the overall value (£3.098) of the carcass in the male pig (Figure 5.4).

5.4 Discussion

This chapter aimed to investigate whether the sex ratio of a litter that pigs originated from had any bearing on their subsequent health, development, and production quality. We found an effect on the unplanned death of pigs. Although there was no higher likely-hood for pigs to die as a result of gestating in any specific litter bias, there was a significant difference in their cause of death prior to slaughter. Hence Hypotheses i A and B were accepted, pigs from male- and female- biased litters were more susceptible to succumb to disease, injury, and behavioural vices during their life course than pigs from non-biased litters. Contrary to most of the hypotheses, the results of this study uncovered an overall small effect of originating from a biased litter on the production parameters of pigs. Hypotheses i C, ii A, iii A and B, were all rejected. There were no effects found of the litter sex bias on the medical intervention, weights, or carcass weights and quality of an individual pig.

Weights during the life course of pigs were unaffected by both the sex ratio from which they originated and individual sex. This is also reflected in the litter weights, suggesting that sows which gestate differently biased litters still produce similar number of piglets to one another. These findings aren't in accordance with previous research that has identified contradictory effects of sex ratio on the subsequent growth of the offspring in pigs. One study found male and female pigs were heavier if from 2M positions than 0M [214]. Jang et al. [120] found 1M pigs heavier than 2M or 0M conspecifics. Whilst

Rohde Parfet et al. [191] found a greater weight gain in males (but not females) who had been positioned between two males *in-utero* when fed restrictively, compared to males positioned between two females. This suggests it is potentially advantageous being a male from a male-biased litter from a behavioural perspective, or physiological through animals not androgenised holding a reduced feed conversion ratio. As the current study did not know the absolute uterine positions, but the litter sex ratio, this may explain why no effects were found. Unexpectedly, males held a heavier weight than females at birth. This is a widely confirmed phenomena and in accordance with many studies both in pigs [5, 21, 261].

There was no overall effect of the sex bias of a litter on the offspring carcass classifications. Associated hypotheses (iii A and B) were rejected. We did however identify a significant effect of the offspring sex on the carcass measurements. Female pigs were found to have deeper back fat (probe measurements mm) than their male counterparts [154]. They were also found to have a higher net cold carcass weight (kg) than males. Whilst there was a higher level of fat in the female pigs, male pigs were shown to have a higher lean meat content, and hence, a higher value (GBP) carcass than the female carcass points. This is a well-known difference between the sexes in commercial pigs [154]. Hence, there does not seem to be a penalty to the farmer if the pig that reaches commercial slaughter originated from a litter that was biased.

There was no penalty on the pigs that reached slaughter from different litter sex biases, and no difference in the number of pigs that faced premature mortality. Similarly, the stage at which the pigs died did not differ between different litter sex ratios. There was however a difference in why pigs died prior to slaughter. Firstly, pigs from either litter bias were more likely to die from injury or illness before reaching commercial slaughter. Hence, Hypotheses i A and B were accepted, pigs from a male-biased, and female-biased litter, succumbed due to disease or injury more than pigs from non-biased litters. It is also in accordance with the theory of DOHaD, that *in-utero* programming of offspring could contribute to susceptibility of adult disease and mortality, with a sexually dimorphic in nature [179]. Sex steroids hold an important role in the immune system, with endocrine gland-secreted steroid hormones regulating many types of immune-cell function [32, 54]. Immune cells also hold the ability to synthesise self regulating steroids [32, 194]. The

pathology of the immune system, alongside normal development of the immune response may be highly dependent on correct steroid signalling [32]. Disruption of this, by for example a biased hormonal environment *in utero* may hold epigenetic consequences on the development of the immune system, the ability for steroids to correctly cross-talk with the immune cells, or even the immune cells own ability to synthesise steroid hormones. Any minor change in sex steroid expression could hold substantial impacts on disease susceptibility and illness as the influence that a steroid hormone can have on an immune cell is based not only on the microenvironment and steroid type, but also on the steroid concentration and time of exposure [32].

Pre-natal survivability has previously been found unaffected by litter bias in the pig [191], and these are the first indications of bias affecting post-natal survivability. This provides a potential avenue to minimise profit losses, if we can further understand why pre-slaughter mortality is occuring it provides opportunity to develop interventions. For example, this study suggests that potential behavioural aspects may be at play, and consideration when grouping pigs may reduce the behavioural vices that increased the mortality rate of pigs from female-biased litters. Alongside being more likely to succumb to disease or illness, female-biased pigs were also more likely to die due to behavioural vices, i.e. as a result of fighting or vice behaviours. There are indications that the cause of death may be due to the well-established behavioural changes that are seen in pigs from biased litters [173, 204]. Pigs from male-biased litters have shown to have higher resource-guarding tendencies [173] along with initiating more fights, being less fearful whilst also being bolder than their non- and female-biased counterparts [204]. A higher number of female-biased pigs died due to behavioural causes, whilst less non-biased pigs died from behavioural causes, even fewer male-biased pigs died than non- and female- biased pigs. Overall, there were a very low number of pigs from male-biased litters that died due to this (n=1). This suggests that the increased aggression may lead the male-biased pigs to be less vulnerable targets to fighting and/or bullying, and vice behaviours such as tail biting, than the other pigs. The effect of a male-bias on fight initiation and tail biting would be an avenue to study within the production systems, as male-biased pigs may be initiating such behaviours and thereby increasing mortality levels on farm. Pigs from female-biased litters seemed to be more susceptible to becoming a target of fighting or vice behaviours.

This suggests they may have lower aggression levels or be more fearful than their non- and male-biased conspecifics, potentially leading to them being targeted within their groups. However, although this cause of death may be behaviourally related, it may be due to other causes entirely. There is a chance that the female-biased pigs have a poorer physiological composition and therefore subcoming to the behavioural vices. Although we cannot identify what the cause was in this study a poor physiological composition was considered the less likely explanation. Accounting for the lack of differences seen between pigs of different litter biases in both growth and carcass composition the evidence suggests that this is more likely due to a behavioural cause. Although it is possible that they may benefit from a behavioural advantage, male-biased pigs were also more frequently culled for being unthrifty compared with female-biased and non-biased pigs. Both male- and female-biased pigs were more likely to succumb to illness and injuries, which is supported by the notion that the *in-utero* programming of offspring could contribute to susceptibility of a range of adult disease [179]. It should however be noted that there were significantly more non-biased pigs than female- and male-biased pigs that died from unknown causes. Hence, these numbers, if the cause were known, may have balanced out some of the differences seen in death causes, injury and illness, or behaviour.

Overall, being from a female- or male-biased litter did not directly affect the growth or carcass quality of pigs within modern production systems. Hence, the evidence suggests that litter sex ratio does not significantly contribute to the variation seen in growth and carcass quality within the UK production systems. Male pigs held a higher quality and leaner carcass than their female counterparts. We identified a that the cause of pre-slaughter mortality differed between pigs of different litter biases, with both biases leading to more deaths caused by disease and illness. Female-biased litters leading to more deaths due to behavioural vices, and male-biased litters leading to more deaths from being unthrifty.

Despite being the first study of its kind, with such a large data set available to analyse, there are certain limitations that the size held on the study design. Primarily, this includes the fact that pigs were reared on different finisher farms. Although this is inevitable with such a large sample size, it may introduce variability that could be masking effects that would otherwise be seen. The farms were all managed by the same parent company, and

run using the same systems and feeds. Despite this, there will be uncontrollable differences in the environment that were introduced outside of our control. Similarly, this means that the data reported on the health of the pigs throughout the system may vary between farms alongside the accuracy of the reporting, despite handler training. Further to the

issues of using different finisher farms, there were also several different sire lines used in this study. Taken together the different farms and sire lines may be introducing to much noise into the data set, rendering prediction any meaningful information from the study impossible. To overcome these limitations we recommend that future research utilises pigs reared in the same conditions, from the same sire lines with as few semen donors as possible. This way, the genetics of an individual may be accounted for and controlled for in a more specific manner.As any study utilising animals within a commercial setting,

there are environmental influences that can only be mitigated to a certain extent. There is however also a big strength resulting from this. This commercial study paradigm creates a stressful environment in comparison to the alternative controlled lab environment. This stress may be allowing for the effects of originating from a sex biased litter to become apparent, i.e. the environmental stressor of being reared under commercial settings may exacerbate symptoms or traits that these individuals hold as a result of their litter sex ratios, which may otherwise not have been affecting them to a notable extent.

We have identified and provided insight into how behavioural changes in pigs from male- and female- biased litters may be causing a real effect amongst pig herds and could potentially be causing a real loss in profit for the producer. A further study into how the male-biased increase in aggression may be influencing the mortality rates on farm would give us significantly more insight into the true costs that they may be adding to the system. Balancing groups according to the effects seen from biased litters may help reduce mortality.

Chapter 6

General Discussion

6.1 Sex biased litter effects on commercial pig health, development, and reproductive capacity

The aim of this PhD thesis was to investigate the impact of biased *in-utero* hormonal environments on offspring reproductive potential and production parameters. To meet this aim, the studies conducted within this thesis have (i) synthesised the existing evidence for the effects of hormonally biased uterine environments on offspring, and (ii) provided novel insights into the effects of hormonally biased uterine environments on the development of reproductive tracts in gilts, and life-course mortality of postnatal offspring. Although previous research indicates there is a negative effect of extreme hormonal uterine milieu on reproductive outcomes in commercial pigs [202], the underlying mechanisms are poorly understood. It is vital that we improve our understanding of the mechanisms at play which would allow us to develop interventions, maximize commercial production, but also mitigate the potential associated welfare concerns linked to the behavioural effects. With increasing pressures on sustainable farming systems to minimise waste through multiple means, then minimising the numbers of failed breeding sows, could greatly contribute to a more sustainable system. The work presented in this thesis addresses this evidence gap, by contributing to our understanding of the mechanistic and physiological alterations that may result in reduced reproductive outputs. This work also improves our understanding of the effects of androgenisation or oestrogenisation on pig production costs.

6.1.1 Existing evidence for the impact of the *in-utero* environment on offspring development

Previously, a review in 2002 [195] synthesised the effect of the intrauterine position on developmental outcomes in a range of species. A later review by Seyfang et al. 2018 [202] reviewed these effects in rodents, sheep and pigs with a focus on knowledge gaps in the commercial pig. However, these reviews did not consider alternative methods of investigating the hormonal bias and its effects on offspring. This review is important as it is the first to consolidate findings across a broad range of species, whilst also considering studies that have utilised proxies to investigate the effect on offspring development. I have further subdivided the effects by physiological systems. There is still little research studying the mechanistic effects of how changes occur, rather the published literature to date focuses on the changes that can be found.

Research investigating offspring gonadal hormones and its effect on siblings in pigs has been published in two waves, with the majority before 1995 and a subsequent, more recent increase since 2012, evidencing a renewed interest in this topic. It is challenging to synthesise offspring gonadal hormone effects across species, as research is often conducted using different methodologies (e.g. absolute *intra-uterine* position, proxies of androgenisation, or hormonal manipulation) and report a wide range of outcome parameters (e.g. from physiological to behavioural). Chapter Two (scoping review), to the best of our knowledge, is the first study to systematically synthesise the existing literature investigating the effect of a hormonally biased uterine environment on offspring development in all litter bearing mammalian species. Chapter Two is also the first review to include the findings of research using proxy measures of the hormonal environment, such as the anogenital distance and hormonal administration, instead of solely focusing on research using sex ratio or *intra-uterine* position as a measure of the hormonal environment. It is vital to include papers using proxy measures of the hormonal environment in the evaluation of *intra-uterine* hormonal effects. Not only are they valid, but they help advance out understanding of which systems may be impacted when offspring develop in a uterine environment skewed by the natural occurrence of sex biases.

Chapter Two incorporated studies investigating a wide variety of litter-bearing species, allowing evaluation of reported effects both within- and between-species. The majority of

studies investigated the impact of the *intra-uterine* environment on rodents, and nearly all studies were conducted in captive species. The results of Chapter Two evidenced that *intra-uterine* environmental effects differ both within- and between-species. The methodology used within the same model of the uterine environment could vary quite significantly. I also elucidated considerable disagreement both between and within species. For example, the findings of the scoping review highlight the need for standardised methodology when investigating the effect of the *intra-uterine* environment on litter bearing species. There are several confounding factors to consider when making between species comparisons. The different placental types and uterine blood supply between species will influence the subsequent interactions with hormonal transport between foetal units *in-utero*. These findings also highlight the danger of extrapolating findings across species, and even breeds, within this field.

Despite a wide range of studies investigating intra-uterine environmental effects, several studies (n = 27, 23.48%) failed to report the use of sample size calculations or appropriate controls. This was particularly evident in papers using sex ratio as a proxy for the hormonal uterine environment. This limits our confidence in the findings as it may be an effect of mode of treatment.

Scoping reviews are exploratory, and typically address a broad question. To allow for this broader scope, as compared to a traditional systematic review, they typically have a more expansive inclusion criterion. I conducted a scoping review to ensure the findings of all relevant studies were included and synthesised in this analysis. As the search terms used in literature reviews can bias the resulting bodies of literature, I investigated the impact of search strategies on resulting papers and research findings (see Chapter 2 *section 2*). The search strategy analysis revealed that only 26 of the original papers were captured by the main search strings, the remaining 50 papers were only captured by the breakdown of the searches in four-way combinations. These results highlight the importance of carefully considering the search terms used in scoping or systematic literature reviews. By using the four-way search strings and reference checks of included papers, the results of this study provide a thorough synthesis of the existing evidence of the effects of the intra-uterine environment on offspring development.

This review provides a snapshot into the literature at the point of the review search.

As a scoping review, it has allowed the reader to quickly understand what the scientific field has been focusing on, alongside an understanding of the more uncommon approaches taken to study the effect of a hormonally biased uterine environment. With the apparent increased interest in the field within the recent years, I recommend that an updated and systematic review be performed for the different models used to study the phenomenon as identified here, focusing on either the physiological or behavioural development. Further, there are many unexplored avenues following effects that were identified by these studies. For example, understanding the driver behind increased aggression of androgenised females would be beneficial to understand how to better mitigate any negative outcomes that this may hold on rearing domesticated species. As I have attempted to further explore in this thesis, there is still little understanding as to why the reduced reproductive success that is widely observed is seen in the androgenised female, and even less is known about the potential multi-generational effects that these may be incurring. There is also very little work being done to understand the effects of an androgenised or oestrogenised males reproductive success.

This is yet a young field with abundant hypotheses to be answered. With the growing understanding of the epigenetic landscape this topic has a plefora of avenues yet to be explored.

6.1.2 The influence of the intra-uterine environment on reproductive development

Chapter Three (ovarian profiles) and Chapter Four (uterine development) are the first to quantify sex-biased litter effects on female pig follicular profiles and endometrial development. Previous findings have identified negative effects of a male-biased uterus in female pig reproduction development [202]. For example, females that gestate in a male-biased uterus have reduced reproductive success as gilts compared to females that gestate in a non- or female-biased uterus [202]. Previous research has, however, failed to identify the mechanistic changes that may be leading to the reduction in gilt reproductive success. For example, Seyfang et al. [201] explored these effects on the luteinizing hormone profile of gilts from male- and female- biased litters, revealing an altered pre-ovulatory LH pulse, but no effect on ovulation patterns. This thesis contributes to filling this evidence gap and understanding of how reproductive success is affected by exploring how hormonally biased

uterine environments effect the follicular pools of prepubertal gilts. I found no evidence of a difference in follicular recruitment, nor atretic follicle counts between pigs originating from extreme-biased and non-biased litters. Chapter three reveals that the number of primordial follicles is affected by (i) an individual's originating litter sex ratio, and (ii) by individual birth weight. This may be an explanatory factor as to why findings in previous research have been highly variable, even within species, seeing as birth weight is often not controlled for, nor included in the analysis.

The findings of Chapter four identified the importance of considering the interactive effect of birth weight on an individual's susceptibility to extreme uterine hormonal environment effects. A low birth weight is a recognised negative impact on the subsequent reproductive capacity of not only the female pig [224], but also in several other species such as the human [64, 174]. What is clear in the literature, is that the effect of birth weight often interacts with other factors. For example, the age of puberty was delayed in gilts of higher birth weights if they also held a slower growth rate [224]. We further illustrate interactive effects, as Chapter four revealed that individuals of lower birth weight are more susceptible to the influences of an androgenised uterine environment, whereas higher birth weight individuals are more susceptible to the influences of an oestrogenised uterine environment. Current recommendations suggest that breeding sows should be selected based on the anogenital distance at 16 weeks of age, as sows with a longer anogenital distance are more likely to have increased reproductive longevity and success. This thesis supports this as a valuable selection tool. The findings of this thesis suggest that individual birth weight may be interfering with an oestrogenised environment and may be able to further inform on an individual's reproductive potential.

No effects of litter sex ratio on any of the defined uterine measures, structural ratios, or the cell proliferation within the endometrium. I did however find that pigs with a higher birth weight held a higher proportion of glands within the endometrial area. This is important due to the function of the glands. These have a vital role of supporting the growth and in-utero development of the foetus through secretion of the luminal fluid. The luminal fluid is critical for conceptus survival as they contain enzymes, growth factors, cytokines, nutrients, transport proteins, and other regulatory molecules [23].

Taken together, the findings of Chapters Three and Four suggest that the reproductive

capacity of female pigs is influenced by the in-utero sex ratio uterine environment, by altering the number and potentially also the quality of the follicles, but not endometrial morphology. The results of this thesis also highlight the interactive influence of individual birth weight and the uterine hormonal bias on how individuals are affected by their litter bias.

6.1.3 The influence of the uterine environment on production parameters and health

Although previous studies have investigated the effects of a hormonally biased uterus on pig growth, no previous research has been conducted to investigate the effect of litter sex biases on pig production parameters. Furthermore, this thesis is the first to analyse the effects of litter sex biases on pig production parameters using structured big data. Having access to this large data set provided further confidence in the results. The results reported in Chapter Two (review) highlights variable effects of litter sex bias, both within- and between-species, on growth parameters (weight) at pre-natal, and early post-natal time points. Building on these findings, Chapter Five (production parameters) reports on the effect of the hormonally biased uterine environment on the production parameters of the UK commercial pig. This was assessed in three main categories; (i) health parameters, (ii) growth parameters, and (iii) carcass quality. This thesis provides the first evidence of the practical effects of litter sex biases on producer profits through assessment of growth parameters and carcass quality. Although prenatal and early life mortality in the pig as affected by biased litters has previously been assessed, this chapter is the first — to the best of our knowledge — to investigate life course mortality in the commercial pig. These results have important implications for maximising production profits, by potentially improving production efficiency, reducing costs, and reducing loss in profits.

By investigating pigs of different genetics, we found that the sex ratio of the litter from which a pig originated held little effect on the production outcomes commonly measured in UK production systems. There was no penalty to the producer regarding the litter sex ratio from which a pig originated. Overall, the growth and age at which the pigs reached target slaughter weights, alongside overall carcass value, was unaffected. However, the cause of pigs dying prior to slaughter was different for pigs of different litter sex ratios.

The increase in mortality for both female- and male-biased pigs was predominantly due to illness or injury. Pigs from sex-biased, compared to non-biased litters, were more susceptible to injury and disease. These findings further support the DoHAD theory which suggests a sexually dimorphic effect of uterine stressors, and a predisposure of certain diseases and illnesses to individuals who gestated in a sub-optimal *in utero* environment [179]. Pigs from sex-biased litters also held a higher probability of mortality due to behavioural vices, such as injury from fights or stereotypic behaviour such as tail biting. Pigs from female- biased litters were significantly more likely to succumb to behavioural vices compared to pigs from non- or male-biased litters. Androgenised pigs were however less likely than non-biased litters to die due to behavioural vices. This suggests that pigs from an oestrogenised uterine environment were more likely to be targets of aggressive and/or bullying behaviour, whereas pigs from an androgenised uterine environment were less likely to be the targets. There is an overarching consensus that an androgenised uterine environment increases aggressive behaviour whilst decreasing fearful behaviour in pigs. Extrapolating from this, one interpretation could be that an androgenised male pig may not only be targeted less due to a more dominant position in the hierarchy, but may also be the animals targeting the more fearful and less dominant in the herd (i.e. oestrogenised individuals). These findings could inform farmers on group dynamics which may benefit group cohesion, thereby reducing stress. Grouping animals according to the litter bias they originated from may help mitigate the increased mortality seen in males from androgenised litters. However, further investigation is needed into the specific changes that may be influencing group dynamics in order to untease the applied influences and to develop commercial interventions.

As anticipated, this chapter found that the sex of a pig held a significant influence on the carcass composition at slaughter. This thesis was in accordance with the notion that an entire male pig will result in a leaner and higher value carcass then their female counterparts [88, 138].

6.2 Future work and recommendations

This thesis takes important steps in understanding the impact of a sex-biased uterine environment on pig reproductive capacity, production, and health. This information is

vital as it brings us closer to understanding how the negative effects on reproductive outputs previously seen in pigs may be taking effect. Within the available literature to date, there is still little evidence available on the mechanistic causes of the reproductive differences seen in females from androgenised uterine environments. More work is required to understand these mechanistic causes.

6.2.1 Reproductive capacity

It is clear that sex-biased litters influence both pig behaviour and female pig reproductive outputs [202]. Endocrine alterations have been identified, however, these did not seem to influence the recruitment and ovulation of follicles. Following the evidence presented in this thesis that the primordial pool is influenced by both androgenised and oestrogenised uterine environments, I suggest investigating the quality of the primordial pool, alongside the developmental capacity from pigs of different litter sex biases. Follicular quality may explain why a male-biased female has reduced reproductive capacity. Although there are no available, validated, assessments of oocyte or embryo quality as of yet, there are a few options. collected oocytes could be assessed by two methods, molecular or functional. For the former I suggest utilising single cell RNA sequencing tools (such as the Q^2ChIP or µChIP assays) to assess differences in gene expression of the oocytes between animals of different in-utero environments. Functional assessment of the oocyte quality could utilise IVF or ICSI to assess the initial stages of embryonic development and the rate of any developmental abnormalities that may occur. Although I did not find any evidence implying that the follicular profiles or atresia of follicles are altered by litter bias extremes, investigating the follicular profile and atresia or apoptosis of follicles of each stage in a naturally synchronised group of females may be important. Taking the limitations of this thesis into consideration, I recommend that future work should utilise naturally synchronised pubertal females (utilising heat detection and opportunistic end points) in order to control for between-individual variation. Further I suggest that an individuals birth weight must either be considered in the analysis, as in this thesis, or controlled for in the experimental design. This is to mitigate the clear influence that birth weight held on how an individual was affected by the sex ratio of the litter from which they originated.

6.2.2 Effects on the male offspring

Alongside the work that was conducted in this thesis to investigate how the female pigs reproductive tracts were affected by litter sex ratio, male testicles from the same litters were also collected. I propose that these testicles should be used to determine whether oestrogenised or androgenised uterine environments may be influencing reproductive success of sire-lines alongside the maternal-lines. This could be done by assessing the semen quality of these individuals in a similar manor to the oocyte quality. Morphological assessments should be made on the sperm quality, as standard. Further, IVF and/or ICSI could be used in order to assess the fertilization capacity of the semen. I suggest this be done using naturally collected semen as the progression through the epididymal is vital for the semen to obtain the correct RNA payload enabling successful embryonic development [47].

There was suggestion that the male pig from a biased uterine environment may suffer higher mortality than their female and non-biased male counterparts. I suggest that there should be investigation into the cause of this as these may be due to either physiological aspects, or behavioural outcomes. For this I suggest using sexed litters, creating the skewed litter sex biases. Behavioural assessments should be conducted (such as intruder tests and novel object tests) in combination with behavioural observations at key time points during their life-course (i.e. weaning and moving). Further to this detailed analyses of death causes throughout the system should be performed at batch level, with retrospective use of CCTV footage to improve the estimation of the cause of death.

6.2.3 Sex bias and group cohesion

Beyond exploring the reproductive potential of the female, which could greatly benefit the producer through better breeding herd selection, I suggest considering an individual's litter sex ratio when forming groups in commercial production settings. This may reduce the stress experienced by individuals, thereby improving welfare, as well as reducing pig mortality rates on farm. Following from this, I suggest two future studies. Firstly, investigating how different combinations of individuals of different litter sex ratios may affect hierarchy and thereby group dynamic stability. This could also investigate how different group dynamics would lead to different stress levels amongst the individuals of those

groups. I strongly believe that positive welfare measures such as cognitive bias should be included, as regulations are moving further towards positive welfare rather than purely an absence of negative welfare. Secondly, I recommend exploring whether the androgenised pigs within the herd may be causing a majority of vice and/or negative behaviours. I recommend that the scope of this would include positive interactions such as allo-grooming, and play, alongside the negative behaviours such as tail biting, fighting, and bullying. This is since androgenised pigs may not only be contributing to the aggressive interactions, but may also be contributing to many of the positive behaviours, such as play, due to the boldness that has been recognised as a trait of these individuals.

Current measures to mitigate fighting include the mixing of pigs of different sizes allowing for easier establishment of a hierarchy within the group, with the least amount of physical confrontation. However, this represents a penalty to the producer as maintaining homologous groups allows for a better tailored diet, treatment, and flow through the production system. Conducting these suggested studies may reveal that a combination of "personalities" of pigs from different sex bias groups may be better equipped to reduce bullying and create a more stable hierarchy as compared to grouping based on pig size.

6.3   Limitations

There are limitations in the studies presented in this thesis due to logistical and economic constraints. This project was also impacted by the COVID-19 pandemic, which disrupted research between 2020 and 2022.

I firstly acknowledge that the pigs used for Chapters Three and Four were collected with a year between collection time points, due to financial constraints. Whilst this is a limitation, potential negative impacts were mitigated by ensuring all pigs included in this study were undergoing similar points of development during the same seasonal time points, i.e. the pigs were born, reared, and slaughtered at similar seasonal time points over the three years. This reduced any potential impact of different data collection time points on research findings. I was also limited to collecting the reproductive tracts at commercial slaughter age. Although the pigs should not yet be cyclic at this age, I was unable to ensure this by either slaughtering the pigs at a younger age or waiting until the pigs were pubertal and synchronising them, to ensure that they were at the same follicular

and endometrial stages. To this end, we recommend that the analyses on recruited and atretic follicles should be interpreted with caution, and we recommend further research utilising synchronised animals to ensure appropriate comparisons can be made. However, I am confident that this will not have affected the primordial follicle counts.

Due to financial constraints, this project was unable to acquire full batches of pigs as part of paid research trials. Instead, pigs were opportunistically included in this study from available litters at the National Pig Centre. Due to the opportunistic nature of these data collections, this project experienced complications and delays in data collection when the commercial unit's sire line genetics was changed midway through the trial. Fortunately, insemination with the original genetics was quickly performed to ensure sufficient sample sizes could be obtained and the project was delayed but unaffected.

I acknowledge that the lack of insight into the absolute uterine position is a major limitation to this study. Due to this, there is no way to identify the level of androgenisation or oestrogenisation that the individuals used were exposed to *in utero*. In future studies I suggest that rather than random selection of piglets from the biased litters piglets should be selected based on their AGD (longest for androgenised litters, median for the non-biased litters, and shortest for oestrogenised litters). Although there would still be an unknown absolute positioning, this would give a stronger indication to the levels of androgens they were exposed to throughout their gestation. Further to this, it is important to note that the non-biased group used in this study may not perfectly represent the true non-biased litter. Without any previous research investigating the natural sex bias of a litter, there was no way to adjust our groups accordingly. However, extrapolating the information from the wild boar I can confidently make the assumption that the natural non-biased group will fall within the scope of 45-54.9% females.
One option to circumvent the forementioned issues would be to use sexed semen and/or IVF to create litters of only female, mixed, and only male. Although this would loose the strengths of using a commercially relevant population, it would avoid confounding factors previously mentioned and give a clear distinction between groups. In such a study the hormonal environment would be better understood by the researcher.

Unfortunately, the trial planned with Karro Food Group Ltd began in March 2020, when travel and work restrictions were enforced to control the COVID-19 pandemic in

the UK. Although I had contributed to the trial design, I was unable to take part in the data collection due to travel restrictions and university COVID regulations. I was therefore unable to collect individual level weight data at weaning and to determine litter sex ratios. Instead, research staff at Karro were guided in how to define litter sex ratios. Although the research staff were guided as to how the sex ratio of a litter was to be defined, I was personally unable to ensure that pigs were classified and identified as for my two previous data chapters. Furthermore, there were a limited number of staff available to assist in data collection, resulting in many litters lacking sex identification for every litter member. Only litters where all individuals were sexed (both live-, still-born, and crushed piglets) were included in these analyses. Although this reduced the number of litters that could be included, we were still left with a large number of individuals, 3921 pigs from 313 litters (from 5650 individuals). As there was no underlying cause for missing data, the omissions had nothing to do with the studied phenomenon, the exclusion remain completely unbiased. Due to this, the results of the statistical models still provide information regarding the impact of litter sex ratios on pig production parameters, and I can confidently conclude that the missing data are unlikely to have impacted results.

6.4 Conclusions

The work presented in this thesis advances our understanding on how individual offspring may be impacted by a hormonally biased uterine environment. I have provided a comprehensive synthesis of the reported findings within this field from a wide range of species and utilising a breadth of models and methodologies. The results of this work have identified a high variation within outcomes of different systems, not only between species but also between individuals of the same species. This highlights the importance of thorough reporting of methodologies, alongside appropriate use of controls. This work also emphasises the importance of considering between-species differences when interpreting results from published papers. The findings in this thesis have laid out evidence that the reproductive capacity of the female pig from an extreme hormonal uterine environment may be reduced due to altered follicular profiles. This evidence suggests that both individual birth weight and litter sex ratios impact female pig follicular pools, dependent on which bias they gestated in.

Furthermore, this thesis has identified a higher proportion of male pigs from a hormonally biased uterine environment will die prematurely, compared to pigs from non-biased uterine environments. This increased level of mortality may be due to behavioural alterations and increased susceptibility to illness and disease. Thereby, I have provided evidence that further supports an altered reproductive capacity in female pigs from hormonally biased litters, coupled with higher mortality levels in male pigs from hormonally biased litters compared to non-biased litters. This contributes to filling the gap in evidence on how the hormonally biased uterine environment may mechanistically be contributing to a poor reproductive success in the breeding sow. Further it is the first study to identify a higher life-course mortality rate in pigs originating from biased litters, and the first to directly identify how a hormonally biased uterine environment may have a direct impact on producer profits.

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Appendix A

Scoping review

Table 6.1: The final corpus included in the scoping review (Chapter 2). This includes the corpus from which the papers were included, along with the author of the paper. Further information on which species the study involved and which effect on the uterine horn that was measured.

Corpus	Author	Species	Effect measured
Original search	Correa, et al., 2016	Degus	AGD
Original search	Correa, et al., 2013	Degus	AGD
Original search	Beach, et al., 1983	Dog	Testosterone
Original search	Tobet and Baum, 1987	Ferrets	Estrogen
Original search	Tober et al, 1986	Ferrets	Testosterone
Original search	Krohmer and Baum, 1988	Ferrets	Uterine position
Original search	Clark, et al., 1998	Gerbil	Uterine position
Original search	Clark, et al., 1993	Gerbil	Uterine position
Original search	Clark and Galef, 1995	Gerbil	Uterine position
Original search	Forger, et al., 1996	Gerbil	Uterine position
Original search	Sherry, et al., 1996	Gerbil	Uterine position
Original search	Jones, et al., 1997	Gerbil	Uterine position
Original search	Clark, et al., 1991	Gerbil	Uterine position
Reference checks	Clark et al, 1989	Gerbils	Uterine position
Reference checks	Clark et al, 1993	Gerbils	Uterine position
Reference checks	Clark et al, 1990	Gerbils	Uterine position
Reference checks	Clark et al, 1992	Gerbils	Uterine position
Reference checks	Clark and galef, 1988	Gerbils	Uterine position
Reference checks	Clark et al, 1992	Gerbils	Uterine position
Original search	Hines and Goy, 1985	Guinea Pig	Estrogen
Original search	Hines, et al., 1987	Guinea Pigs	Oestradiol
Original search	Connolly and Resko, 1994	Guinea Pigs	Testosterone
Original search	Gandelman, 1986	Guinea Pigs	Uterine position
Reference checks	Vomachka, 1986	Hamsters	Uterine position
Original search	Monclus, et al., 2011	Marmots	Litter sex bias
Original search	Hacklander and Arnold, 2012	Marmots	Litter sex bias
Original search	Monclus and Blumstein, 2012	Marmots	Litter sex bias
Original search	Monclus, et al., 2013	Marmots; Rabbits	Litter sex bias
Original search	Kerin, et al., 2003	Mice	AGD
Original search	Sugawara, et al., 2012	Mice	IVF controlled bias
Original search	Vandenbergh and Huggett, 1994	Mice	Mixed
Original search	Delbes, et al., 2004	Mice	Oestradoil

Corpus	Author	Species	Effect measured
Original search	Mann and Svare, 1983	Mice	Testosterone
Original search	Witham, et al., 2012	Mice	Testosterone
Original search	Gandelman et al, 1979	Mice	Testosterone
Original search	Cologer-Clifford, 1992	Mice	Uterine position
Original search	Vom Saal, et al., 1983	Mice	Uterine position
Reference checks	Hauser and Gandelman, 1983	Mice	Uterine position
Original search	Gandelman and Graham, 1986	Mice	Uterine position
Reference checks	Kinsley et al, 1986	Mice	Uterine position
Reference checks	Quadagno et al, 1987	Mice	Uterine position
Reference checks	Gandelman and Kozak, 1988	Mice	Uterine position
Reference checks	Bushong and Mann, 1994	Mice	Uterine position
Original search	Palanza, et al., 2001	Mice	Uterine position
Original search	Jubilan and Nyby, 1992	Mice	Uterine position
Reference checks	Gandelman, 1977	Mice	Uterine position
Original search	Vom Saal, et al., 1990	Mice	Uterine position
Original search	Kinsley, et al., 1986	Mice	Uterine position
Original search	Morley-Fletcher, et al, 2003	Mice	Uterine position
Reference checks	Vom Saal, 1980	Mice	Uterine position
Reference checks	Brown et al, 1984	Mice	Uterine position
Reference checks	Kinsley, 1986	Mice	Uterine position
Reference checks	Hurd, et al., 2008	Mice	Uterine position
Original search	Nonneman, et al., 1992	Mice	Uterine position
Reference checks	Mcdermott et al, 1978	Mice	Uterine position
Reference checks	Vom saal, 1989	Mice	Uterine position
Reference checks	Zielinski, et al, 1991	Mice	Uterine position
Reference checks	Wechman, et al., 1985	Mice	Uterine position
Original search	Vom Saal and Bronson, 1978	Mice	Uterine position
Reference checks	Vom Saal and Moyer, 1985	Mice	Uterine position
Original search	Vom Saal, et al., 1991	Mice	Uterine position
Updated search	Fishman et al., 2018	Nutria	Uterine position
Updated search	Fishman et al., 2019	Nutria	Uterine position
Original search	Seyfang, et al., 2018	Pigs	Litter sex bias
Original search	Seyfang, et al., 2018	Pigs	Litter sex bias
Original search	Rekiel et al, 2012	Pigs	Litter sex bias
Original search	Drickamer et al, 1997	Pigs	Litter sex bias
Original search	Seyfang, et al., 2017	Pigs	Litter sex bias
Original search	Lamberson and Blair, 1988	Pigs	Littter sex bias
Original search	Ford, 1987	Pigs	Testosterone
Original search	Petric et al, 2004	Pigs	Testosterone
Reference checks	parfet, 1990	Pigs	Uterine position
Original search	Tarraf and Knight, 1995	Pigs	Uterine position
Original search	Wise and Christenson, 1992	Pigs	Uterine position
Original search	Jang, et al, 2014	Pigs	Uterine position
Updated search	Lents and Freking, 2019	Pigs	Uterine position
Original search	Rohde Parfet, et al, 1990	Pigs	Uterine position

Corpus	Author	Species	Effect measured
Original search	Klein and Nielsen, 1993	Rabbits	Testosterone
Original search	Banszegi, et al., 2015	Rabbits	Testosterone
Original search	Banszegi, et al., 2010	Rabbits	Testosterone
Original search	Bautista, et al., 2015	Rabbits	Uterine position
Original search	Jahagirdar, et al., 2008	Rats	Androgens/Oestradiols
Original search	Gladue and Clemens, 1978	Rats	IVF Anti-androgens
Original search	van de Poll, et al., 1982	Rats	Litter sex bias
Original search	Clemens et al, 1978	Rats	Litter sex bias / Sex ratio
Original search	Houtsmuller, et al., 1994	Rats	Mixed
Original search	Dunlap, 1978	Rats	Testosterone
Original search	Huffman and Hendricks. 1981	Rats	Testosterone
Original search	Ichikawa and Fujii, 1982	Rats	Testosterone
Original search	Juarez, et al., 1998	Rats	Testosterone
Original search	Chinnathambi, et al., 2012	Rats	Testosterone
Original search	Ward, et al., 1996	Rats	Testosterone
Original search	Ito et al, 1986	Rats	Testosterone
Original search	Rodriguez, et al, 2009	Rats	Testosterone
Original search	Dela Cruz and Pereira, 2012	Rats	Testosterone
Original search	Wolf, et al., 2002	Rats	Testosterone
Original search	Dean, et al., 2012	Rats	Testosterone
Original search	Rhees, et al., 1997	Rats	Testosterone
Reference checks	Houtsmuller and Slob, 1990	Rats	Uterine position
Reference checks	Babine and Smotherman, 1984	Rats	Uterine position
Reference checks	Meisel and Ward, 1981	Rats	Uterine position
Reference checks	Richmond and Sachs, 1984	Rats	Uterine position
Reference checks	Lephart, et al., 1989	Rats	Uterine position
Reference checks	Tobet, et al., 1985	Rats	Uterine position
Reference checks	Pei, et al., 2006	Rats	Uterine position
Reference checks	Mori, et al., 2010	Rats	Uterine position
Reference checks	Timms, et al., 1999	Rats	Uterine position
Reference checks	Hernandez-Tristan, et al., 2006	Rats	Uterine position
Reference checks	Houtsmuller, et al., 1995	Rats	Uterine position
Reference checks	Hernandez-Tristan, et al., 1999	Rats	Uterine position
Reference checks	Tobet and Baum, 1982	Rats	Uterine position/Testosterone
Original search	Nagao, et al., 2004	Rats; Mice	Uterine position
Original search	Galea, et al., 1994	Vole (meadow)	Litter sex bias
Original search	Curtis, 2010	Vole (prairie)	Litter sex bias
Original search	Lambin, 1994	Voles	Litter sex bias

Appendix B

Hematoxylin and Eosin stain for paraffin sections - protocol

This protocol was developed under the supervision of the Bioimaging Facility at the University of Leeds.

1. De-paraffinize slides through three changes of Histo-clear, incubating slides 10 min in each change.

2. Hydrate slides to water by dipping them (vigorously) 20-40 times in each of three changes of 100% ethanol, two changes of 95% ethanol, one of 70% ethanol.

3. Rinse with 2-3 changes of tap water.

4. Stain with <u>Hematoxylin</u> for 5 minutes.

5. Rinse in tap water using silver vat under a slow rate,   3-4 changes water.

6. Dip once in 0.25% acid alcohol and remove straight back into cold water.

7. Blue in hot tap water for 1 min.

8. Rinse in tap water using silver vat under a slow rate,   3-4 changes water.

9. Mordant in 95% ethanol for 15-20 dips.

10. Stain in <u>Eosin</u> for 10 minutes.

11. Dehydrate through 2 changes of 95% ethanol (10 dips each) followed by 3 changes of 100% ethanol (30-50 dips each).

12. Clear through three changes of Histo-clear, 10 minutes each.

13. If slides are clear go to step 14, if not, leave in Histo-clear overnight and coverslip the following morning.

14. Mount coverslip with DTP.

Substrates

1. Dewaxing histo-clear (10 min)

2. Dewaxing histo-clear (10 min)

3. Dewaxing histo-clear (10 min)

4. 100% EtOH, hydrating (20-40dips)

5. 100% EtOH, hydrating (20-40dips)

6. 100% EtOH, hydrating (20-40dips)

7. 95% EtOH, hydrating (20-40dips)

8. 95% EtOH, hydrating (20-40dips)

9. 70% EtOH, hydrating (20-40dips)

10. Hematoxylin (5min)

11. 0.25% AcidAlcohol (Five seconds)

12. 95% EtOH, Mordant (15-20dips)

13. Eosin (10 min)

14. 95% EtOH, dehydratingg (20-40dips)

15. 95% EtOH, dehydrating (20-40dips)

16. 100% EtOH, dehydrating (20-40dips)

17. 100% EtOH, dehydrating (20-40dips)

18. 100% EtOH, dehydrating (20-40dips)

19. Mounting histo-clear (10 min)

20. Mounting histo-clear (10 min)

21. Mounting histo-clear (10 min)

Appendix C

IHC protocol

Note all washes with the buffer Phosphate Buffered Saline (PBS) use 1xPBS.

Day 1

Load the slides into the grey holder warm is 60 degrees for 10-20 minutes

1. Deparaffinise slides and rehydrate (In fume hood):

    (a) Dip in slides up and down 3-5 times and then leave in Histoclear for 10 min

    (b) Dip in Histoclear for 10 min

    (c) Dip in 100% EtOH 30-40 times

    (d) Dip in 100% EtOH 30-40 times

    (e) Dip in 75% EtOH 30-40 times

    (f) Dip in 75% EtOH 30-40 times

    (g) Leave in PBS for 5 min

2. Antigen retrieval

    (a) Place grey racks sideways in container

    (b) Pour citrate buffer (citrate buffer 0.1M, pH 6.0) into container and wrap in clingfilm (pierce a few times)

    (c) Set microwave on high and put slides in for 20 min

    (d) Remove clingfilm and allow slides to cool for 20 min

    (e) Dip in PBS for 5 min

3. Destroy endogenous peroxidase activity

    (a) Create 1% H_2O_2 (hydrogen peroxide) with 250 ml PBS just before use

    (b) Immerse slides in this for 15 min at room temperature (RT)

(c) Dip with ddH2O(double distilled water) with agitation

(d) Dip with ddH2O(double distilled water) with agitation

(e) Dip with ddH2O(double distilled water) with agitation

(f) Dip in PBS for 5 min

(g) Dip in PBS for 5 min

4. Blocking

(a) Remove excess liquid from slide by tapping on tissue paper

(b) Delineate (circle) the sections on the slide with PAP pen

(c) Make up blocking solution; add 150µl (3 drops) of yellow labelled Vectastain kit to 10 ml of buffer. Add to each section on the slide (Mix well but do not vortex)

(d) Incubate in humid chamber for 20 min at RT

(e) Leave in PBS for 5 minutes.

(f) Remove liquid by flicking/tapping.

5. Primary Ab (1º Ab) incubation

Add 60 µl of predetermined 1ºAb concentration (made up in 1xPBS) to the bottom section on the slide and add 60 µl of IgG negative control (made up to same concentration as 1º Ab in 1xPBS) to the top section.

(a) Incubate in humid chamber at 4°C overnight.

Day 2

Remove slides from chamber and tap off liquid onto paper towel. Dip in PBS 2x 5 min.

1. Secondary Ab (2º Antibody) incubation

(a) Remove excess liquid from slides by tapping on tissue.

(b) Add 150ul of yellow bottle (2 drops) to 10ml of buffer, and 50ul (1 drop) of 2° Ab. Add this to each section on slide.

(c) Incubate in humidified chamber for 60 mins at 37°C

(d) Remove from chamber and tap off excess liquid

(e) Dip in PBS for 5 min

(f) Dip in PBS for 5 min

2. Detector reagent

   (a) Mix detector reagent well before use (Grey bottle: Do not vortex)

       i. Add 2 drops (100ul gray label) of Reagent A to 5 ml of buffer

       ii. Add 2 drops (100ul of gray label) of reagent B to the same container

       iii. Mix immediately and let stand for 15-20 minutes

   (b) Remove excess liquid from slides by tapping on tissue and add detector reagent to each section on slide

   (c) Incubate for 30 min at 37°C in humid chamber

   (d) Remove from chamber and tap off excess liquid

   (e) Dip in PBS for 5 min

   (f) Dip in PBS for 5 min

3. Visualisation

   (a) Dilute the stock solution of DAB (kept in aliquots at -20°C in the dark as it is light sensitive) 1:10 with PBS to a final working concentration of 1 mg/ml

   (b) Add H2O2 to working stock of DAB in 1:1000

   (c) Add this to each section on your slide to visualise

   (d) Allow slides to sit in DAB for 30 sec, 1 min, 2 min, 5 min or for as long as is required to visualise staining

   To stop DAB reaction immerse slides in tap water

4. Mounting

   (a) Immerse slides in 75% EtOH for 2 min

   (b) Immerse slides in 75% EtOH for 2 min

(c) 100% EtOH

(d) 100% EtOH

(e) Dip in histoclear for 2 min.

(f) Dip in histoclear for 2 min.

(g) Add DPX and coverslide then allow to dry.

Store in dark as DAB is light sensitive