The influence of low litter birth weight phenotype on placental and embryonic development at day 30 of gestation in multiparous sows

by

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ABSTRACT

The low litter birth weight phenotype (LLBWP) in sows represents a concern for the swine industry. The ability to predict this trait could be strategically directed toward selection for higher production efficiency. The aim of this study was to understand the biological processes associated with the LLBWP in order to improve overall breeding efficiency, lifetime productivity and number and quality of pigs weaned per sow in the breeding herd.

For this research, analyses were conducted on reproductive data from a purebred Large White maternal line (Hendrix Genetics), to identify sows (>2 parities) with repeatable high litter birth weight phenotype (HLBWP) or LLBWP (top 12% and bottom 12% of the population), with 7 to 22 total number born. A total of 40 sows were selected (n=20 HLBWP and n=20 LLBWP) and bred with semen from purebred Large White boars of proven fertility (Hendrix Genetics) on their second estrus following estrus synchronization with altrenogest (Matrix^{tm}, Merck AH, Kenilworth, NJ). Sows were euthanized on day 28-30 of gestation (mean ± sd; day 29.15 ± 0.6) and samples of placenta, and embryos collected. Total number of embryos (TNE), embryonic weight (EW), embryonic viability, and crown-rump length (CRL) measurements were recorded, along with the ovulation rate (OR) and allantochorionic fluid volume (AFV). The difference between TNE and OR was considered an indicator of early embryonic survivability, while the ratio between TNE and number of viable embryos was an indicator of late embryonic survivability.

No significant difference was detected ($P > 0.05$) in OR (LLBWP: 25.6 ± 1.06; HLBWP: 26.8 ± 1.06), TNE (LLBWP: 19.5 ± 1.19; HLBWP: 19.8 ± 1.12) and number of viable embryos (LLBWP: 16.4 ± 1.37; HLBWP: 16.6 ± 1.37) on day 30 of gestation. Consequently, no differences were found for early embryonic survivability (LLBWP: 0.78 ± 0.02; HLBWP: 0.76 ± 0.02, P=0.43), late embryonic survivability (LLBWP: 0.85 ± 0.01; HLBWP: 0.86 ± 0.01, P = 0.67) or

total embryonic survivability (LLBWP: 0.67 ± 0.02; HLBWP: 0.66 ± 0.02, P = 0.55). For embryonic and placental characteristics, there was no significant difference between LLBWP and HLBWP for EW (LLBWP: 0.80 ± 0.05 g; HLBWP: 0.88 ± 0.04 g, P=0.18) or CRL (LLBWP: 21.5 ± 0.7 mm; HLBWP: 21.9 ± 0.68 mm, P=0.46, Figure 3.9). However, placental development represented by the average AFV was significantly lower in the LLBWP compared to HLBWT group (LLBWP: 131 ± 9.82 mL; HLBWP: 149 ± 9.39 mL, P= 0.03). All viable embryos (n=610) were sex-typed by PCR. There was no significant effect of sex on these measures of embryonic development.

Within each litter birth weight phenotype (LBWP) group, 4 sows with the individual EW falling within the mean EW of the group ± SD were selected for embryonic and placental gene expression analyses (Illumina Next Generation Sequencing). Several differential expressed genes (DEGs) involved in biological pathways associated with ion and gas transportation, placental morphogenesis and hemodynamics, cellular metabolic process, detoxification process and regulation of cell proliferation were identified when comparing the transcriptome between the two LBWP groups. Therefore, in our study LLBWP sows showed unfavorable intrauterine environment at day 30 of development, which leads to impaired embryonic development and consequent lower BW of entire litters.

PREFACE

The research described in this Thesis was conducted to understand the effect of the low litter birth weight phenotype on embryonic and placental development at the physiological and molecular level. The Thesis consists of 5 chapters. Chapter 1 and 2 include a general introduction to the research topic, as well as an overview of the related literature and past research in this area. These chapters were written by myself with the assistance of my supervisor Dr. Michael Dyck and reviewed by Dr. Graham Plastow and Dr. Irene Wenger.

Chapter 3 of this Thesis describes the animal selection, animal work, data collection and analysis conducted to assess the effect of the litter birth weight phenotype on multiparous sows. The research concept and experimental design was conceived by Dr. Michael Dyck. The animal selection was performed by Chunyan Zhang and myself. Animal work and data collection was performed by myself, Dr. Michael Dyck, Dr. Stephen Tsoi, Dr. Irene Wenger, Chi Tran and Celine Dewit. Data analysis was conducted primarily by myself with the assistance of Dr. Michael Dyck and Jennifer Patterson. The research described on Chapter 3 is not yet published, but a manuscript is under preparation.

Chapter 4 of this Thesis describes analysis to determine the effect of the litter birth weight phenotype on embryonic and placental development through transcriptomic analysis. The experimental design for this study was conceived by Dr. Michael Dyck, Dr. Stephen Tsoi and myself. Execution of the molecular analysis was performed by myself and Chi Tran under the supervision of Dr. Stephen Tsoi. RNA seq analysis and bioinformatic analysis were performed by BGI. The research described on Chapter 4 is not yet published, but a manuscript is under preparation.

Chapter 5 of this Thesis reports the main findings and the impact of the low litter birth weight phenotype sows in the swine industry, as well as possible approaches to deal with this category of sows and their litters in pig production systems. This chapter was written by myself and reviewed by Dr. Michael Dyck, Dr. Graham Plastow and Dr. Irene Wenger.

Dedication

This thesis is dedicated to my mother Roseli, and my father Augusto. Thank you for your unconditional support, love, encouragement, and for always being by my side despite the distance.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABREVIATIONS

3b-HSD - Hydroxysteroid dehydrogenase

ADG - Average daily gain

AFV - Allantochorionic fluid volume

AQP1 - Aquaporins

BW - Birth weight

CDSN - Corneodesmosin

CL - Corpus luteum

CP - Crude protein

cPAS - Combinatorial probe-anchor synthesis

CRL - Crown-rump length

Ct - Threshold cycle

CXCL8 - C-X-C motif chemokine ligand 8

D30 - Day 30

D9 - Day 9

DEGs - Differential expressed genes

DNBs - DNA nanoballs

DSG3 - Desmoglein 3

EGFLAM - EGF like fibronectin type III and laminin G domains

EPGN - Epithelial mitogen

ERFE - Erythroferrone

EW - Embryo weight

FPKM - Fragments Per Kilobase of exon model per Million mapped reads

FSH - Follicle-stimulating hormone

GA# - GenBank accession numbers

GATA1 - GATA binding protein 1

GnRH - Gonadotropin releasing hormone

GO - Gene ontology

GS - Gene symbol

HBEGF - Heparin-binding epidermal growth factor

HLBWP - High litter birth weight phenotype

IFNG - Interferon-gamma

IL8 - Interleukin 8

KLF1 - Kruppel like factor 1

KLK11 - Kallikrein-related peptidase 11

KLK14 - Kallikrein-related peptidase 14

KRT17 - Keratin 17

LBWP - Litter birth weight phenotype

LH - Luteinizing hormone

LLBWP - Low litter birth weight phenotype

LW - Large White

MT - Metallothionein transport

MYADM - Myeloid associated differentiation marker

NGF - Nerve growth factor

NTN4 - Netrin-4

OR - Ovulation rate

P - Parity

PBS - Phosphate-buffered saline

PDPN - Podoplanin

PGF2α - Prostaglandin F2α

RCR - Rolling circle replication

RIN - RNA integrity number

RNA-seq - RNA-sequencing analysis

SEMA3B - Semaphorin 3B

SLC22A16 - Organic cation/carnitine transporter, member 16

SLC22A4 - Solute carrier family 22 (organic cation/zwitterion transporter, member 4)

SRY - Sex-determining Y region

SSC - Sus scrofa chromosomes

TAL1 - TAL bHLH transcription factor 1, erythroid differentiation factor

TBA - Total born alive

TNB - Total number born

TNE - Total number of embryos

TNV - Total number of viable embryos

TNW - Total number weaned

VEGF - Vascular endothelial growth factor

ZFX - Zinc finger protein X-linked

CHAPTER 1

1. General introduction

For the genetic improvement of commercial pigs, breeding goals change as the economic relevance of traits shifts and our ability to assess and measure traits evolves. As a result, the top genetic pig breeding systems have experienced significant advancements in traits that directly impact pork production efficiency. A specific example is the increase of the total number of piglets born (TNB) in the last few decades (Boulot et al, 2008). Litter size has been targeted in selection programs for generations through increases in ovulation rate (Schneider et al, 2014). This successful selection pressure has generated sows that are able to produce large litters and provide 30-35 piglets a year, considering 2.4 to 2.5 litters per sow per year. In Canada, sow litter size has increased from 12.71 in 2008 to 14.95 in 2018, an increase of 0.22 piglets born a year (PigChamp Benchmarking, 2018). A similar trend is seen in the US, where the TNB increased from 12.7 to 14.49 over the same period.

However, the selection pressure on TNB has resulted in a disproportional increase in ovulation rate, which was not accompanied by increased uterine capacity (Foxcroft et al, 2009). As an unintended consequence of impaired prenatal development, lower birth weight (BW) and higher within litter variation in BW has become more common. For this reason, inevitably, higher mortality rates are seen in all phases of production (Quiniou et al, 2002). For example, over the same time period mentioned above, the pre-weaning mortality has increased from 12.68% to 14.66% in Canada and from 12.19% to 14.45% in the US (PigChamp Benchmarking, 2018). Consequently, despite a considerable amount of research, low BW, poor piglet survival, and the consequent impaired piglet development slows down the progress expected from sows' hyper prolificacy (Boulot et al, 2008).

To overcome this problem, the average piglet BW has recently been included in genetic selection programs aiming to produce higher quality piglets. However, the result of years of genetic selection for highly prolific sows indirectly resulted in an extreme population of sows that gives birth to generalized low BW litters, independent of the litter size. This scenario is defined as the low litter birth weight phenotype (LLBWP). The LLBWP is a sow-related phenotype expressed in the piglets, in which sows repeatably produce low BW piglets across parities (Patterson & Foxcroft, 2019). Unlike individual low BW in a litter, the LLBWP is expected to affect the growth performance of entire litters, generalizing the problem. Nonetheless, the phenotype is passed through generations, perpetuating the problem in the production system (Zhang et al, 2018).

It is well known that the variation in BW and growth performance in pigs may be predominantly determined and established during embryonic and fetal development (Town et al, 2005; Foxcroft et al, 2009). Given the limited research on the root causes of the LLBWP, the primary objective of the research presented in this Thesis was to understand the physiological and genetic factors impacting the LLBWP in purebred Large White sows. In Chapter 3 of this Thesis, analyses were conducted to identify two extreme populations of sows related to the BW of their litters over at least two successive parities. The bottom 15% of the population represented the LLBWP, and top 15% of the population represented the high litter birth weight phenotype (HLBWP) sows, in which sows consistently produced high BW piglets over multiple parities. Therefore, in order to obtain a better understanding of the litter birth weight phenotype (LBWP) in sows, LLBWP and HLBWP were compared to elucidate the relationship between the LBWP extremes and key reproductive traits including ovulation rate, dynamics of embryonic mortality and their influence on embryonic and placental development at day 30 of gestation. In this scenario, the LLBWP is hypothesized to be driven by higher ovulation rates, a higher number of

embryos in utero and increased competition for uterine space during the early stages of development, in comparison to HLBWP sows. As a consequence, placental development and embryonic development would be negatively affected due to a limited uterine capacity. It is known that placental development, and its functional capacity to supply enough nutrients to conceptuses, are under the control of the embryonic and maternal genomes (Angioloni et al, 2006). Therefore, in Chapter 4, the effects of the LLBWP were compared to HLBWP placental and embryonic tissues at the molecular level through gene expression analysis and biological pathways evaluation. The ability to understand the physiological and molecular processes associated with the LBWP could be strategically directed toward selection to improve overall breeding efficiency and optimize litter quality performance in the pork industry.

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CHAPTER 2

2. Literature review

2.1 Prenatal development involved in piglet birth weight

It is well known that a considerable amount of the variation in birth weight (BW) and growth performance in pigs may be mostly determined and established during embryonic and fetal development (Town et al, 2005). This process is known as prenatal programming, and it can be influenced by events in utero such as uterine overcrowding and/or placental inefficiency (Foxcroft et al, 2006, 2009). One of the consequences of a negative environment in-uterus is low BW, which results in irreversibly compromised lifetime productivity. Nonetheless, events such as uterine overcrowding and placental inefficiency are not easily manipulated, especially after years of genetic selection targeting increased ovulation rates and a higher total number born (TNB). As reviewed by Zak et al (2017), reproductive traits are biologically complex and heritable within a population. Birth weight, for example, is a combination of ovulation, fertilization and implantation rates, conceptuses survival and development, the genetic quality of the conceptuses, placental efficiency, uterine capacity and the total number of piglets born.

This chapter will focus on the different prenatal processes involved in conceptus quality and piglet BW, including oogenesis and ovulation rate (OR), oocyte quality, fertilization, pre-implantation embryonic development, gestation signaling, placentation and formation of embryonic membranes. Essential events and timing of pig embryonic development are shown on Table 2.1, as well as the entire gestational period on Figure 2.1.

2.1.1 Oogenesis and ovulation rate

As reviewed by Hunter et al (2000), the beginning of oogenesis in gilts occurs prenatally. At birth, gilts have a pre-set number of primordial follicles for life, around 500,000 primordial follicles are present in both ovaries by 10 days after birth (Black & Erickson, 1968; Anderson, 2000; Wear et al, 2016). Oocytes develop from primordial germ cells, and their migration begins from the dorsal mesentery of the hindgut to the primordium of the gonad after migrating through the gut mesentery and the gonadal ridges of the mesonephros (Takagi et al, 1997). Once the primordial germ cells reach the developing ovary, the cells begin to differentiate into oogonia (Wear et al, 2016). Although visible at approximately days 24 to 26 of gestation, the gonads of a porcine embryo are already passing through mitotic divisions of the oogonia population around day 13 of development (Black & Erickson, 1968). During days 18 to 26 of gestation, there is a dramatic increase in the number of cells, which is concomitant with their arrival in the gonadal ridge (Takagi et al, 1997). Along with mitotic divisions, prenatal meiosis starts around day 40 of gestation (Hunter et al, 2000). Through this process, the number of chromosomes is divided, resulting in the creation of haploid oocytes (Wear et al, 2016). The process of primordial cell formation begins after meiosis when a single layer of flattened cells encloses the oocyte. The cells remain in the stage of nuclear arrest of the meiotic prophase I (known as germinal vesicle stage) until gilts reach puberty (Hunter et al, 2000; McLaughlin & McIver, 2009).

The initial growth of primordial follicles depends on endocrine activities such as the release of follicle-stimulating hormone (FSH) and regulatory effects from the somatic cells of the follicles (Dierich et al, 1998). At birth, the majority of the oocytes are surrounded by a single and flat layer of granulosa cells. However, the differentiation of a primordial cell into a primary follicle is first observed around day 70 - 90 of gestation (Hunter et al, 2000; Anderson, 2000). The growth of germ cells occurs in two phases: first, the growth of oocyte and the follicle is concomitant; second,

the size of the oocyte remains stable while the follicle continues to grow. Several stages of granulosa multiplication occur, resulting in up to 20 layers of cells (Morbeck et al, 1992). The increase in growth of granulosa finally culminates in the separation of cells, forming a fluid-filled cavity called antrum. Around 84 days are required for the transition from primordial follicles to the pre-ovulatory antral stage (Caárdenas & Pope, 2002) and nearly 100 days are necessary for ovulation to happen (Morbeck et al, 1992).

2.1.2 Oocyte quality

The development of follicles is controlled to regulate the time and number of follicles that reach maturity (Knox, 2015). According to Hunter et al (2000), oocyte quality is defined as the capability of an oocyte to become a viable offspring, and its quality is mostly influenced by the maturational process, which is initiated by a surge of luteinizing hormone (LH), also responsible for inducing ovulation at the end of follicular development.

By the time a gilt reaches puberty, during each estrous cycle, a pool of primordial cells is recruited to grow under the influence of hormonal action. According to Knox (2005), the pool of pre-ovulatory follicles present at the beginning of each follicular phase is developed during the previous luteal phase of the estrous cycle and consists of approximately 100 follicles. The follicular recruitment occurs between days 14 and 16 of estrous and by this time an average of 50 follicles are present in both ovaries. Many of them, however, degenerate through a process called atresia. The breakdown of the follicles occurs constantly during the sow's lifetime and at any stage of follicular development, but the majority disappear before they reach 6 mm in diameter (Foxcroft & Hunter, 1985). Therefore, only 30 to 40% of the recruited follicles are selected to complete the final maturation. Such a low-resolution rate is considered the result of a necessary selection process for oocyte quality (Grant et al, 1989; Foxcroft et al, 1987). The process of recruitment is dependent

on the release of FSH from the anterior pituitary, while the selection and follicular development for ovulation rely on LH (Knox, 2005). The oocytes only acquire the capacity to mature and complete the first meiotic division when follicles reach at least 1.8 mm in diameter. During this stage, however, only some oocytes are competent, whereas when they reach between 5 mm to 9 mm of follicular size, most oocytes are capable of passing through meiosis (Hunter, 2000). The synchronization of meiosis resumption of the oocyte and follicular rupture responsible for the release of the mature oocyte is decisive in facilitating subsequent fertilization and embryonic development (Espey, 1994).

The dynamics of follicle selection and growth are of utmost importance, especially in contemporaneous sows with high ovulations rates, since ovulatory follicle heterogeneity can result in compromised oocyte maturation and follicle response to the LH surge (Knox, 2015). This phenomenon, in turn, can result in reduced fertilization rates, the formation of cystic follicles, poorly formed corpora lutea and impaired posterior embryonic development and survival (Hunter, 2000; Da Silva et al, 2016; Knox, 2015). According to Ding and Foxcroft (1992), larger and more mature follicles at the time of ovulation produce better quality oocytes. The smaller follicles that ovulate later, develop into the smallest embryos and consequently, are more vulnerable to uterine environmental challenges (Pope et al, 1990). However, determining and controlling the quality of an oocyte is challenging, since even follicles of similar sizes can be morphologically and biochemically heterogeneous (Foxcroft & Hunter, 1985). In addition, the quality of the oocyte and its ovulation quality is influenced by many factors such as nutrition, seasonality, time of insemination relative to ovulation and the use of exogenous hormones (Ding & Foxcroft, 1992).

2.1.3 Estrous cycle

The estrous cycle in mature gilts and sows occurs in regular intervals of 18 to 24 days, and it is controlled by the hypothalamo-hypophyseal axis (Senger, 1997). In summary, gonadotropin releasing hormone (GnRH) is released from the hypothalamus into the hypothalamo-hypophyseal system and stimulates the production of Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) in the anterior pituitary, which in turn acts on tissues of the ovaries. Positive and negative feedback mechanisms within the entire axis, mediated by estradiol and progesterone, work to regulate the secretion of GnRH and other hormones. Ovaries, uterine, and placental hormones also play a role in the reproductive activity regulation (Senger, 1997).

The entire estrous cycle is divided into a follicular phase which lasts 4 to 6 days, and a luteal phase of 13 to 15 days. During the follicular phase, the secretion of estradiol by the ovarian follicles reaches the blood circulation, stimulates increased GnRH pulsatility and promotes FSH and LH release from the anterior pituitary. At the start of the follicular phase, FSH peaks for 24 to 36 hours before declining. The decline in FSH is a consequence of inhibin production by the follicles that acts on the pituitary to suppress FSH release. The selected follicles mature in size with an expanding fluid antrum. During this stage, small antral follicles (< 3mm) develop into large, pre-ovulatory follicles (> 6 mm) (Soede, Langendijk & Kemp, 2011). Estrus is the period around ovulation in which gilts and sows show a standing response for boars and are receptive to mating. In gilts and multiparous sows, the estrus period varies from 24 to 72 hours, and ovulation, in turn, occurs approximately around 44 hours after the onset of estrus (Bazer & Johnson, 2014) or on average 70% of the way through estrus (Soede & Kemp, 1997). After ovulation, the luteal phase takes place with the luteinization of the ovulatory follicles and formation of corpus luteum (CL), which are responsible for secreting progesterone and maintaining the pregnancy. In the case of

non-pregnancy, luteolysis (or luteal regression occurs – degradation of the CL) approximately 15 days after ovulation and a new cycle begins (Bazer & Thatcher, 1977).

2.1.4 Fertilization and pre-implantation embryonic development

Once gilts reach puberty, FSH secretion from the anterior pituitary stimulate some follicles to mature in a process called folliculogenesis. The selected maturing oocytes that were arrested at prophase of meiosis I (germinal vesicle stage), resume meiosis and continue until metaphase II of meiosis II, when they get arrested again before ovulation. If the oocyte is fertilized by a sperm, they resume and complete meiosis. Once fertilization occurs, the sperm stimuli causes the resumption of metaphase II (Sun & Nagai, 2003; Dyck & Ruvinsky, 2011).

In inseminated gilts or multiparous sows, fertilization occurs a few hours post-ovulation, near the ampullary-isthmic junction of the oviduct (Bazer & First, 1983). Approximately 48 hours after fertilization, the porcine embryo multiplies from the 2 to the 4-cell stage. Up until this point, the embryos rely on maternal genomic signaling, but once they reach the 4-cell stage, embryonic genome activation takes place during the 20 to 24 hours of development (Dyck & Ruvinsky, 2011; Oestrup et al, 2009; Brevini et al, 2007). Transport through the oviduct requires 72 hours and embryos of the 4 to 8-cell stage reach the uterine horn at the end of the third day after fertilization. Around day 5 of pregnancy, the embryo starts to develop into a 16-cell stage called a morula (Hunter, 1974). When the embryo reaches the 30-cells stage, a cavity called the blastocoel is formed. The blastocyst begins increasing in cell number, and to differentiate into two different cell types: the inner cell mass which will result in the embryo; and the trophectoderm, which is necessary for implantation. By this time, the embryo is around 0.5 to 1 mm in diameter and expands to 2 to 6 mm on day 10 of pregnancy (Bazer & Johnson, 2014).

The developing blastocyst hatches from the zona pellucida on day 7 of gestation and is exposed for the first time to the uterine epithelium. Direct contact with the maternal tissues and their secretions influences embryonic development (Hunter, 1977). Progesterone stimulates secretory activities of the uterus from days 0 to 5 of pregnancy, and on day 10 of gestation, progesterone levels decrease, and then increase again on day 12 (Geisert et al, 1994). At the same time, uterine growth factors are released by the uterine epithelium. This period marks the beginning of spacing, elongation and posterior attachment of the trophectoderm to the sow uterine epithelia (Bazer & Johnson, 2014). On day 9 of development, the embryonic sphere starts to develop a structure called the embryonic disk, which is fully formed by day 10 when the sphere is also enlarged. After day 10 the embryonic disk converts into an oval structure from which embryo elongation proceeds (Dyck & Ruvinsky, 2011). The elongation occurs during days 11 and 12 of gestation and is characterized by the transition from spherical, ovoid and tubular to filamentous stages (Bazer & Johnson, 2014). This process is based on the hypertrophy of the trophectoderm and endoderm (Kridli et al, 2016) and only occurs when the embryo reaches at least 10 mm in size (Geisert & Yelich, 1997). This period is marked by a complex pattern of gene expression (Ross et al, 2003). According to Bazer and Johnson (2014), the rate of embryonic development increases dramatically in a few hours. For example, during the initial stages, elongation occurs at 0.25 mm/hour and increases to 150 to 200mm/hour during the filament formation, which culminates with an embryo of 80 to 100 cm by 16 days of gestation. These intense transitions in morphology allows us to locate conceptus within the same uterus at different stages of development (spherical, tubular and filamentous) during this period (Bazer & Johnson, 2014). According to Ross et al (2003), 142 genes are differentially expressed among the three stages mentioned above.

The degree of elongation that the embryo achieves allows for maximum surface area of contact between the trophectoderm and uterine epithelia (Bazer, 2013) and determines the amount of uterine space that is occupied by each embryo during implantation. Therefore, embryos that experience a delayed development attach with only limited implantation surface area (Geisert et al, 1982), which in turn results in a smaller placental area (Stroband & Van der Lende, 1990) and a higher chance of embryonic mortality (Anderson, 2000). Along with elongation, the embryo starts to differentiate into germ layers in a process called gastrulation. Through this process, the endoderm, mesoderm and ectoderm are formed and will give rise to different types of tissues during continued development (Fléchon et al, 2004).

2.1.5 Spacing and gestation signaling

In order to signal their presence, the porcine conceptuses begin to secrete estrogen at the 10 mm spherical stage, between days 11 to 12 of gestation (Geisert et al, 1982). Then, as soon as the embryos develop from spherical to filamentous, estrogen production increases considerably (Pope, 1988). Concomitantly, the preimplantation embryos migrate and space themselves in both uterine horns, allowing the estrogen exposure to be distributed over the entire uterine surface (Pope et al, 1982). This process, known as spacing, appears to be random, and the equally spaced distribution of embryos throughout the uterine horns is facilitated by contractions of the myometrium (Dziuk, 1985). By day 15 of gestation, spacing of the conceptuses is complete, regardless of uterine size or number of embryos present (Dziuk, 1985).

It has been established that estrogen released by the preimplantation porcine embryo prevents prostaglandin F2α (PGF2α) produced by the uterine epithelia from being directed to the uterine venous drainage system (Bazer & Thatcher, 1977). Through this mechanism, estrogen inhibits the movement of PGF2α to the ovary and consequent regression of the CL, allowing for

the establishment and maintenance of pregnancy through the production of progesterone (Bazer et al, 1989). A second wave of estrogen production by the conceptuses occurs during days 15 and 30 of gestation, which is also considered important to the maintenance of the CL (Geisert et al, 1990; Anderson, 2000). The changes in the uterine environment in response to conceptus estrogen production at this time is important for nurturing the elongating blastocyst. However, it will also prompt less developed embryos to die (Roberts & Bazer, 1988), in a process called uterine-embryonic asynchrony (Pope, 1988). This occurs because, the most developed embryos influence the uterine environment in a way that is detrimental to the less developed embryos (Pope et al, 1990). An alternative mechanism is observed in the Chinese Meishan breed, known to have uniform BW piglets at term. This breed "employs" a unique physiological strategy that allows the embryos to develop at a more uniform rate (Langendijk et al, 2016). The Meishan embryos develop at a slower rate and are smaller compared to European breeds, which is possibly due to the lower secretion of estrogen compared to European breeds (Youngs et al, 1993). This scenario reduces the variation among embryos when the litter reaches the elongation period. Therefore, embryonic mortality is reduced, and the implantation spaces are more equally distributed (Langendijk et al, 2016).

2.1.6 Sex differentiation in development

As reviewed by Dyck & Ruvinsky (2011), the genetic differences between XX and XY in mammals is dependent upon the presence or absence of the Y chromosome. Embryos without the Y chromosome develop as females, while those carrying the Y chromosome develop as males. It is well accepted that the embryo's chromosomal constitution determines the migration of cells into the gonads and the final differentiation into a testis or an ovary. The first differences between male and female pig embryos start at day 26 of gestation (Hunter, 1995). The influence of chromosomal

constitution on the development induces sex differences in many organs and is initiated even before the gonads start to secrete steroid hormones (Arnold et al, 2004).

2.1.6.1 Male development

The differentiation of the biological male is mostly controlled by the SRY (sex-determining Y region) gene. The porcine SRY gene is located within the Y chromosome and is expressed in the cells of the genital ridge of the male embryo within days 21 to 26 of gestation, when the primitive gonads are still in a state of bipotential. The SRY drives the differentiation of testes, which is apparent at day 31 of development (Daneau et al, 1996) and is the gene with the strongest masculinization effect on the brain. Its action also triggers the testicular differentiation of Sertoli cells, evident around day 26 of development, and the production of anti-Müllerian hormone which starts at day 29. As a consequence, Wolffian ducts are converted into epididymis, vas deferens, seminal vesicles, the prostate and other structures; while the Müllerian ducts disappear. The production of hydroxysteroid dehydrogenase (3b-HSD) begins at days 30 to 35 of pregnancy, supporting the development of the Leydig cells which are responsible for the secretion of testosterone (Behringer, 1995). From the third trimester of gestation to 20 days of post-natal development, male germ cells are in constant rate of multiplication (Anderson, 2000).

2.1.6.2 Female development

In females, one of the two X chromosomes is transcriptionally silenced during early embryonic development in every somatic cell (Lyon, 1999). Preferential inactivation of the paternal X chromosome in pig XX embryos occurs around the trophoblastic stage of development (days 7 to 8 of gestation). After that, random inactivation follows during the formation of embryonic disc cells around day 12. With this inactivation, female tissues, including the brain, become natural mosaics, with one X randomly inactivated in each somatic cell (Lyon, 1961). As

a result, different X alleles are expressed in females, while in males a single variant is expressed at each locus. The X chromosome in the female, in the same way as Y chromosome in the male, is specialized for sex-specific functions. Furthermore, because of the lack of a Y chromosome in the female, there is no SRY gene expression. Therefore, there is no formation of Leydig cells and no production of testosterone. As a consequence, the gonadal development moves towards ovarian development. The Müllerian ducts develop into oviducts, uterus, cervix and the upper parts of the vagina, while Wolffian ducts disappear (Anderson, 2000).

2.1.7 Implantation and placentation

The placental function in pigs is strongly dependent on vascular development, area of placental exchange available for each embryo, litter size, stromal depth and uterine capacity (Knight et al, 1977). In this context, the initial phase of trophoblast expansion is responsible for establishing and limiting the perimeter for individual placental attachment (Geisert et al, 1997), and because of this, extensive angiogenesis occurs at the fetal-maternal interface to allow for adequate nutrient transfer between the mother and the conceptus (Stenhouse et al, 2019).

As early as day 13, the conceptuses start the process of placentation. The implantation process requires the adhesion and migration of specialized cells, so that the attachment of the embryo trophectoderm to the endometrium can occur (Bazer & Johnson, 2014). The complete attachment happens on day 18 of development (Friess et al, 1980) and from that point on, maternal and fetal blood are in apposition, although separated by six layers of cells, which is classified as Epitheliochorial placentation (Bazer et al, 2014). Appropriate regulation of uterine angiogenesis during this time is fundamental for the establishment and maintenance of pregnancy in pigs (Stenhouse et al, 2019). In order to increase maternal and fetal communication, invaginations or folds are formed by day 27 of gestation (Wright et al, 2016), decreasing the surface area for the

diffusion of nutrients (Enders, 1999). Even though it is not invasive, the interdigitation of the trophectoderm into the endometrium is progressive throughout gestation and tends to increase until the placenta is fully covered and is referred to as diffuse placentation (Bazer et al, 2014). The placental structure is divided into areolae, where the trophectoderm does not fuse with the luminal epithelium, and the interareolar area, where the maternal endometrium layer adheres to the fetal epithelium (trophectoderm) (Vallet et al, 2014). The individual placentas for each developing conceptus have approximately 2,500 areolae and their lumen are filled with secretions from uterine glands and histotrophs which include nutrient transport proteins, ions, cytokines, enzymes, hormones growth factors, proteases amino acids, glucose, among other substances (Knight et al, 1977).

Fetal and placenta weights tend to increase similarly throughout the gestation. However, Wright et al (2016) showed that placenta weight remains constant from days 32 to 37 of gestation, while fetal weight continues to increase. Similarly, by 42 days of pregnancy, fetal weight tends to double, while the placenta weight increases by a third. The findings of Stenhouse et al (2019) show that a wave of angiogenesis occurs during this interval in an attempt to increase the surface area and the vascular density available to support nutrient transfer, especially in lighter fetuses (Blomberg et al, 2010). According to the same author, the dynamic changes in placental structure and function in mid-gestation are reflected in remarkable changes in gene expression observed between day 45 to 60. During the entire gestational period, placental weight is always considerably heavier than fetal weight which supports fetal requirements during this period of accelerated growth (Vallet & Freking, 2007). The dynamics of fetal and placental weight are also consistent with the increase in the allantochorion volume, through which membrane expansion occurs, forcing contact with the maternal endometrium (Goldstein et al, 1980).

2.1.8 Formation of membranes and vesicles

The primary germ layers of the embryonic structure give rise to four membranes known as the vestigial yolk sac, allantois, amnion and chorion (Dyck & Ruvinsky, 2011). In early embryos, the yolk sac provides nutritive support to the embryo and it is fused with the chorion in areas that are richly vascularized, allowing quick absorption of nutrients (Hill, 2017). The amnion or amniotic sac is a protective vesicle that closely surrounds the embryo. It guarantees the symmetrical development of the embryo and does not allow adhesion to the external membranes. Its formation is complete by day 18 and as it is developing, the allantoic sac forms as an evagination of the hindgut and expands rapidly between days 18 and 30 (Friess et al, 1980). Lastly, the chorion originates from the trophoblastic capsule of the blastocyst and it is the exterior membrane. It encloses the embryo and other fetal membranes and is responsible for interacting with the maternal tract (Dyck & Ruvinsky, 2011).

The expansion and fusion of the chorion and allantois, through the accumulation of fluids, give rise to the chorioallantoic placentation around days 18 to 30 of gestation (Steven, 1975). On day 18, it accumulates approximately 1 ml of fluid, while around day 30 the accumulation is approximately 200 to 250 ml (Goldstein et al, 1980). By day 30, the chorion and the allantoic vessels are well fused, forming the allantochorion membrane. After this point the volume fluctuates, decreasing by day 45, increasing again on day 58 and once again decreasing on day 112 of gestation (Knight et al, 1977; Goldstein et al, 1980). By 70 days of gestation, the placental weight and surface area development is complete (Knight et al, 1977).

2.1.9 Influence of sex in placental development

The influence of sexual dimorphism in placental development has been described in humans and rats (Di Renzo et al, 2007). However, in pigs there is limited understanding of this

phenomena. Sexual dimorphism is evident in human placentas (Di Renzo et al, 2007; Ingemarsson, 2003), with sex influencing the expression of placental genes and the inflammatory response (Kraemer, 2000). It is proposed that male piglets have a survival disadvantage compared to their female littermates (Gabory et al, 2013). Male pig conceptuses have an increased growth rate compared to female conceptuses from day 10 of gestation onwards (Gabory et al, 2013). For that reason, it would be highly possible that sexual dimorphism in conceptus gene expression and protein secretion would be observed during the time of implantation in the pig. Along this line of thinking, Stenhouse et al (2019) hypothesized that male and female conceptuses communicate differently with the endometrium because of variations in hormone production throughout gestation. This may be related to the fact that male embryos produce testosterone at an earlier stage of gestation and in greater quantities than female embryos, whereas female fetuses produce large quantities of estradiol in late gestation (Vernunft et al, 2016).

2.1.10 Influence of uterine capacity in placental and embryonic growth

Uterine capacity is known as the number of the conceptuses that a uterus can carry through parturition (Ford et al, 2002). The increase in its capacity is considered to be a key factor in enhancing productivity in the swine industry by increasing prenatal survivability and quality of the piglets born (Freking et al, 2016). However, to select and determine uterine capacity is not an easy task. Usually it is measured based on piglet BW (Vallet et al, 2014) and the number of piglets born alive, assuming that the number of fetuses is not limiting (Lents et al, 2014).

Limited uterine capacity is responsible for a reduced litter size and piglet BW (Town et al, 2004; Vallet et al, 2014), as a consequence of compromised placental development and higher competition among embryos (Père et al, 1997). Since the porcine placentation is not invasive, it requires adequate surface area for nutrient exchange (Ford et al, 2002). Limitations in placental

size at the end of the embryonic period might not influence embryonic weight, but it contributes to a decrease in fetal development in later gestation as described by Town et al (2004). In their study, by limiting the number of embryos in the uterus by oviduct ligation, a higher placental weight at day 30 of gestation and no difference in the embryo weights in comparison to the control group (non-ligated sows) were observed. On the other hand, at day 90 of development, they observed a higher placental weight and fetal weight in the oviduct-ligated sows. These authors assumed that at day 30 of development the embryos are not as sensitive to compromised placental development as they are in the final gestational period. Therefore, an environment with limited uterine capacity depends on placental efficiency to "catch up" embryonic development.

Placental efficiency is defined as the weight of the conceptus per mass of placenta (Wilson & Ford, 2001; Wooding, 2008), or in other words, grams of fetus produced per grams of placenta. Placental efficiency is commonly used as a marker of placental function (Krombeen et al, 2019) and in general, highly efficient placentas have a greater nutrient transport capacity, while low efficient placentas have reduced nutrient support or don't have the same capability to support the developing conceptuses (Krombeen et al, 2019). However, within a litter, fetuses of the same size can come from placentas of dramatically different efficiencies, with up to 25% placental weight difference (Krombeen et al, 2019).

Highly efficient placentas tend to weigh less but have a denser vascular supply and a higher expression of vascular endothelial growth factor (VEGF) compared to less efficient placentas (Reynolds et al, 2009). As it is widely known, highly prolific pig breeds, such as the Chinese Meishan, have smaller placentas with an increased vascular density compared to less prolific European breeds (Foxcroft et al, 2006; Wootton et al, 1977). According to Mesa et al (2012), the vascular density of the Meishan endometrium doubles between days 70 and 90 of gestation, while

a Yorkshire conceptus doubles placental surface, but maintains constant placental and endometrial vascular density. This scenario illustrates a disadvantage for the European breeds, since uterine capacity becomes a limiting factor as a larger uterine space is required for proper nutritional exchange to occur.

As gestation progresses, the attachment area and the available vascular supply must increase to meet the requirements of the growing fetuses. According to Wright et al (2016), the folds that are present in the placenta tend to become narrower and deeper over time. In their study however, no difference in microfold length or depth among different sizes of fetuses were found. On the other hand, Vallet & Freking (2007) found that at day 105 of gestation, as a compensatory mechanism to increase total surface area, small fetuses showed deeper microfolds compared to large fetuses. Similarly, Stenhouse et al (2019) demonstrated that it is not only the width and remodeling of the bilayer that is important, but also the changes in the vascularity of the bilayer to compensate for the varying size of the fetus.

Stenhouse et al (2019) evaluated gene expression of placental and endometrium tissues and their findings support the concept that the tissues associated with smaller fetuses are attempting to improve fetal growth by transcriptionally altering the expression of candidate genes important in regulating placental and fetal development. Compensation mechanisms are present during development, especially during the second third of gestation. Specifically, the second phase of angiogenesis, which occurs around days 40 to 60 of gestation may be manipulated (Vonnahme et al, 2001) to attempt to increase the surface area and the vascular density (Stenhouse et al, 2018; Blomberg et al, 2010). It is not clear, however, if the attempt to support compensatory fetal growth by increasing placental efficiency is actually effective in producing uniform piglets within a litter.

Besides the attempt to increase placental efficiency by increasing vascular supply, Vallet et al (2011) and Vonnahme et al (2002) observed that the space made available in the uterus after embryonic or fetal mortality is taken up by adjacent conceptuses, as their placentas grew larger in weight and surface area. This, however, it not in agreement with other authors' observations that the size of the placenta is permanently established during early gestation (Knight et al, 1977; Vallet et al, 2014). Da Silva et al (2016) observed that sows with high pre-implantation mortality had a longer placental length and larger unoccupied spaces around each implantation site on day 35 of gestation. This scenario shows the potential for growth of the remaining vital embryos. On the other hand, sows with high post-implantation mortality culminated with a smaller area available around each embryonic-placental unit, smaller implantation area and placental size. Therefore, during this period, embryonic mortality is already a consequence of uterine overcrowding, in which the lack of space compromises placental development. According to Vallet et al (2011), although placental function is not fully determined at day 35 of gestation, approximately 90% of placental and conceptus weight is determined by their relationship around day 35 of gestation. As a consequence, even though the newly available space provided by embryonic mortality is occupied, and placental tissues expand, the benefits of this expansion seem to be small.

2.1.11 Fetal programming of muscle development

The process of myogenesis is characterized by two waves of development of the myofibers from myogenic precursor cells originating in the mesoderm (Rehfeldt et al, 2000). The first occurs around days 25 to 50 of gestation by the fusion of primary myoblasts, which act like a framework for the large population of secondary fibers (Miller et al, 1993). The second wave is responsible for the formation of secondary fibers between days 50 to 90 of pregnancy. The secondary fibers are disposed around the primary fibers, and it is estimated that in the final gestational period,

approximately 20 secondary fibers involve one primary fiber. The increase in the number of fibers ceases by day 90 of development; therefore, at the time of birth, the total number of fibers is already established. It is believed that the variation in primary myofiber number is genetically inherited, while secondary fibers may be modulated through nutrition and environment (Wigmore & Stickland, 1983).

After birth, myofiber size increases with the addition of nuclei from satellite cells and hypertrophy of existing myofibers (Rehfeldt et al, 2000). Therefore, muscle skeletal growth results from an increase in fiber size (hypertrophy). The fact that no fibers are formed after birth shows the importance of prenatal development, since factors that affect myofiber development can have a permanent and irreversible impact on muscle structure and postnatal growth (Du et al, 2010). Early uterine crowding is associated with lower number of muscle fibers, suggesting that this effect is related to limited placental development (Bérard et al, 2010; Town et al, 2004).

2.1.12 Early and late embryonic mortality

It is estimated that prenatal mortality in commercial genetic lines ranges from 30% to 50% (Pope et al, 1972; Geisert & Schmitt, 2002). The most significant portion occurs during the embryonic phase up to 35 days of gestation. The first wave with 20% to 30% of conceptus lost by day 21 and then a second wave of mortality with 10% to 15% lost to the end of the embryonic period (Ford et al, 2002).

Early embryonic mortality occurs before uterine implantation and contributes to the greatest rate of mortality during the embryonic period (Foxcroft et al, 2007). As reported by Youngs et al (1993), the majority of developing embryos (93% to 96%) survive until day 12 of gestation. After this stage, elongation, spacing and implantation occur, which means drastic morphological and endocrine changes. Studies report that embryonic mortality around this period

is not due to a lack of space, but asynchrony in conceptus-maternal communication and heterogeneity in early embryonic development (Pope et al, 1990; Langendijk et al, 2016). As already discussed in this review, the heterogeneity in embryonic development results in a non-favorable environment for less developed embryos when estrogen starts to be produced by more developed embryos in order to signal maternal recognition of pregnancy (Pope et al, 1990). Embryonic heterogeneity may also be associated with higher follicular and oocyte diversity (Pope et al, 1990) which may increase with higher OR and prolonged variation in ovulation time between oocytes (Soede & Kemp, 1993).

A recent study by Langendijk et al (2016) compared embryonic mortality between oviduct-ligated sows and control sows in three distinct stages of gestation (day 9, day 21, and day 35). Their results showed that regardless of sow treatment, 18% of embryos did not even implant, indicating early embryonic mortality during the period of spacing and some during implantation (day 12 to 14 of gestation). However, by increasing the available space in the oviduct-ligated sows, embryonic mortality from day 21 to day 35 was eliminated compared to embryo mortality of 14% during the same period in intact (control) sows. The results of Langendijk et al (2016) are in agreement with other researchers, emphasizing that unlike the first wave of mortality, the late embryonic mortality is due to lack of uterine space (Dziuk, 1968; Père et al, 1997; Town et al, 2004). It is clear that the area available for the placenta to expand is a limiting factor that decreases nutrient uptake. In the control group, the placentas were smaller, and nearly all the available space was occupied by implantations, leaving little space for the physical area of the implantations to increase (Langendijk et al, 2016).

2.1.13 Ovulation rate, embryonic mortality and embryonic development

The OR is characterized by the final number of follicles that passed through follicle recruitment and selection. Many studies have associated the number of ovulations to embryonic mortality and embryonic development (Vonnahme et al, 2002; Da Silva et al, 2016; Da Silva et al, 2017; Langendijk et al, 2016). According to these studies, higher OR leads to an increased embryonic mortality and fetal mortality due to a higher number of embryos being present in the uterus. According to Foxcroft et al (2009), sows with higher OR are inclined to have a greater number of embryos and therefore less space available for implantation. As a consequence, these sows may farrow litters with lower average BW. In addition, the heterogeneity of the surviving embryos during the early stages of development might lead to higher litter BW variation (Foxcroft et al, 2009; Pope et al, 1990; Xie et al, 1990). However, even though OR tends to be higher in contemporaneous sows, there is a limit to the number of embryos that can be supported.

The effects of uterine crowding were evaluated by the above-mentioned study of Langendijk et al (2016) and a negative correlation between OR and available space per embryo at day 35 of development was found. The same study observed that embryonic survival decreased with OR by 0.6% at day 21 for every ovulation, and by 1.3% at day 35 for every ovulation. The same phenomenon was verified by Da Silva et al (2016) where sows with a high OR showed a linear decrease in implantation and placental length and a decrease in uterine space around the viable embryos. Consequently, every extra ovulation represented an increase in early embryonic mortality of 0.49 and an increase of 0.24 on late embryonic mortality per extra ovulation. Vonnahme et al (2002) observed a correlation between OR (average 26.6) and the number of viable embryos at day 25 of pregnancy, but not at day 36 of pregnancy which can be explained by the loss of embryos in sows with higher OR.

The dynamics of OR and embryonic development tends to be different between gilts and sows. It is known that in comparison to multiparous sows, gilts have a lower OR (Da Silva et al, 2016; Da Silva et al, 2017; Town et al, 2005), lower litter size (Zindove et al, 2014) and lower average litter BW (Redmer et al, 2004). According to Da Silva et al (2016, 2017), in gilts and sows the increased OR results in early and late embryonic mortality and a limited increase in the number of vital embryos at day 35 of development. The authors verified that once the OR exceeds 26 ovulations in gilts and 22 in sows, the number of viable embryos does not increase linearly. For example, their findings show that multiparous sows with OR ranging from 22 to 38 ovulations had a plateau in the total number of viable embryos, corresponding to a maximum of 17 embryos. As a consequence, even though OR was higher in multiparous sows, the number of viable embryos was similar between gilts and sows in both studies (Da Silva et al, 2016, 2017).

2.2 Post-natal consequences of low litter birth weight phenotype

The prenatal development of porcine embryos and fetuses is a determinant of their post-natal performance. Additionally, low BW is a determinant of piglet mortality rates and growth performance in every phase of life. For terminal genetic lines, it is well known that low BW directly influences the efficiency of production and final carcass quality. On the other hand, the studies on replacement gilts still have not elucidated all the aspects related to development, but it is known that low BW gilts tend to have lower retention rates in the herd, decreasing their efficiency of production.

2.2.1 Low birth weight and pre-weaning mortality

In 2018, the pre-weaning mortality for Canadian and U.S herds was estimated to be 15.25% and 14.85%, respectively (PigCHAMP, 2018), suggesting that pre-weaning mortality plays a major role in reducing the overall productivity of the swine industry. Given that low BW

piglets have less body energy, poor passive immunity, are less competitive and have lower ability to maintain body temperature (Muns et al, 2016), these piglets represent the category with the highest risk for mortality (KilBride et al, 2014; Quiniou et al, 2002; Fix et al, 2010). According to Opschoor et al (2012), light piglets (< 0.7 kg) can experience up to 77% of pre-weaning mortality, while heavy piglets (> 1.8 kg) only experience 10% of losses. Zeng et al (2019) showed that low BW piglets (< 1 kg) contributed to 54.1% of the total pre-weaning losses, while heavy piglets divided into three categories (1.0 to 1.3 kg, 1.3 to 1.6 kg and >1.6) accounted for 24.2, 14.2 and 7.5% of mortality, respectively. Similar results were found by Smit et al (2013) when comparing average low BW (1.12 kg) and average high BW (1.79 kg) litters, which had 16.4% and 6.7% pre-weaning mortality rates, respectively. In addition, Zeng et al (2019) set a survivability threshold of 0.99 kg, where piglets below that weight showed an 8.5 times higher mortality rate compared with piglets with weights above that threshold. In similar studies, thresholds of 0.95 kg and 1.11 kg were set by Díaz et al (2017) and Feldpausch et al (2019), respectively. Magnabosco et al (2015), in a more detailed evaluation, established a cut-off point for assessing mortality rates from 24 hours after birth until 20 and 70 days of age. The threshold BW for survivability up to 24 hours after birth was 1.02 kg, 1.09 kg until 20 days and 1.10 g until 70 days of life.

Lighter piglets at the time of the birth tend to show lower vitality and are less efficient during suckling, making them less competitive and at a disadvantage compared to heavier littermates throughout the lactational period (Devillers et al, 2007). In addition, as reviewed by Zeng et al (2019), Quiniou et al (2002) and Canario et al (2006), the presence of low BW piglets increases the likelihood of stillborns in the litter. According to Milligan et al (2002), the most functional teats are occupied by heavier and dominant piglets who are better able to stimulate teats to produce milk. As a consequence, the colostrum intake in the first days of life in low BW piglets

may be limited, along with milk intake (Devillers et al, 2007). Even though the majority of pre-weaning mortality occurs in the first 4 days of life due to crushing and starvation, milk consumption is vital for long term survival and limited intake may induce another wave of mortality (Hartsock & Graves, 1976). Given all of the factors mentioned above, the difference in piglet BW tends to be maintained and even widened during the lactational period. In addition, there is evidence for impaired gut-immune development in low BW piglets during the post-natal period, which could increase disease susceptibility (Zhong et al, 2012). Similar results were found by Fouhse et al (2019) when comparing piglets originating from sows of low and high litter birth weight phenotype. Low BW piglets presented a reduced gut microbial diversity and differences in gene expression of metabolic and immune pathways genes, which may be associated with impaired growth and performance later in life.

2.2.2 Low birth weight and post-weaning mortality

Low BW piglets that survive the pre-weaning period tend to have a physical disadvantage during the weaning process when dealing with sudden environmental and nutritional changes (Muns et al, 2016). However, the reported relationship between BW and post-weaning mortality is not as evident as during the pre-weaning period. Fix et al (2010) found a significant effect of BW on nursery mortality; however, it was not associated with finishing mortality. Smith et al (2007) divided piglets into 2 groups based on BW and noted that the lightest BW group had the highest rate of mortality during the nursery phase. Larriestra et al (2006) report that weaning weight is a better estimator of nursery mortality, than BW. However, BW can be considered to be an effective indicator of future growth performance, especially weaning weight. Therefore, it is logical that the association between weaning weight and nursery mortality may be partially due to BW (Quiniou et al, 2002; Larriestra et al, 2006; Fix et al, 2010). In addition, the lack of correlation

between BW and mortality in finishing phases reported in these studies, might be due to the earlier waves of mortality. As a consequence, a limited number of light piglets reach the final stages of development and the association may be underestimated (Smit et al, 2013).

2.2.3 Low birth weight and subsequent growth performance

It is well reported that BW and weaning weight are the most important parameters that predict lifetime performance in pigs and that the profitability of the pig industry is highly associated with growth performance (Paredes et al, 2012; Douglas et al, 2013; Collins et al, 2017). Weaning weight influences subsequent performance, but more than that, influences the risk of diseases, post-weaning mortality and the efficiency of creating batches for all-in-all-out systems. Slow growing pigs are more costly to produce since they require extra space, are less feed efficient and tend to demand more labor due to cross-fostering, space allocation, increased chances of remixing animals and feed supplementation (Díaz et al, 2017).

Smit et al (2013) compared low, medium and high BW piglets and their lifetime performance. The average daily gain (ADG) during lactation was higher in high BW than in low BW litters, resulting in a significant higher weaning weight for high BW (6.49 kg) than low BW (5.56 kg) litters. The differences in body weight were maintained during the nursery and growing-to-finish phases where pigs were always lighter in the low BW group, compared to medium and high BW groups. Similarly, Nissen and Oksbjerg et al (2011) reported a reduced ADG by 30 g/d in low BW pigs during lactation. This trend remained from weaning to 150 days of age and showed a difference of by 87 g/d compared to high BW pigs. After weaning, feed intake was reduced by 160 g/d and feed conversion increased by 80 g per kg of gain in low BW pigs compared with high BW. Zeng et al (2019) divided piglets during the lactational and nursery period into two categories: slow or fast growing (Lactational slow: ADG $\leq$ 225 g/d; Lactational fast: ADG > 225 g/d; Nursery

slow: ADG ≤ 424 g/d; Nursery fast: ADG > 424 g/d). Their findings showed that slow growing piglets had 0.24 kg lower BW than fast growing piglets. Slow growing piglets weighed 1.7 kg less at weaning and 6.9 kg less during the finishing phase at day 167, compared to fast growing piglets. Similarly, Cabrera et al (2012) estimated that an increase of 1 kg in BW corresponded to an improvement of weaning weight from 1.5 to 1.9 kg, finishing weight from 6.6 to 12.5 kg, and a decrease in days to slaughter from 10.6 to 17.9 days. When the same author measured the differences between pigs of similar BW (1.43 kg) but different weaning weight (4.1 to 5.0 kg and 5.0 to 5.9 kg) the heavier category of pigs was able to reach 125 kg, 8 days sooner than the lighter category.

2.2.4 Birth weight and carcass quality

Extensive associations between myogenesis, BW, growth, carcass quality and comparisons of postnatal performance in low and high BW pigs have been studied in the last decades (Quiniou et al, 2002; Rehfeldt & Kuhn, 2006; Gondret et al, 2005; Fix et al, 2010). Muscle fiber development is mostly completed during the prenatal period. The reduced number of primary fibers at birth dictates subsequent secondary fiber growth and development causing long-term consequences for postnatal muscle growth and quality (Gondret et al, 2005; Rehfeldt & Kuhn, 2006; Liu et al, 2014). Concurring studies by Wigmore and Stickland (1983), as well as Rehfeldt and Kuhn (2006) estimated that low BW piglets formed fewer myofibers during fetal development when compared to high BW piglets, mainly due to a lower number of secondary fibers. As a consequence, these piglets grew slower during their lifetime. Dwyer et al (1993) suggest that while BW influences growth up to 70 days of age, muscle fiber number was important to determine the rate and efficiency of BW gain afterwards. Handel and Stickland (1987) suggest that a reduced number of fibers limit the capacity of postnatal lean growth.

As reported by Gondret et al (2005) and Rehfeldt and Kuhn (2006), low BW is known to affect lean growth performance after weaning. Rehfeldt and Kuhn (2006) found a tendency toward leaner carcasses in pigs with high BW (1.80 kg) compared with their lighter littermates (0.94 kg) at a similar slaughter age. Gondret et al (2005) compared pigs of low (0.75 to 1.25 kg) and high (1.75 to 2.05 kg) BW and reported a reduced lean meat content, more backfat, and a decreased proportion of ham, loin, and belly in the carcasses from low BW piglets. This tendency may be explained by the lower number and larger sizes of muscle fibers that in turn resulted in poorer carcass quality. Beaulieu et al (2010) found that the carcass crude protein content was similar regardless of BW; however, moisture retention increased, and intramuscular fat decreased as BW increased. It is known that postnatal muscular hypertrophy relies on the total number of muscle fibers and that the postnatal growth rate of the individual muscle fiber is higher when there are lower numbers of fibers which may cause the formation of large and giant fibers in low BW animals (Rehfeldt et al, 2000).

Beaulieu et al (2010) found that temperature, pH, objective color, shear force, soluble protein, and driploss as indicators of pork quality were unaffected by BW. There was no effect on initial tenderness or juiciness, flavor intensity, sustained juiciness, amount of connective tissue, overall tenderness, or overall palatability. However, flavor desirability was greatest for the lightest and the heaviest BW quartiles. In contrast, Gondret et al (2005) reported no impact of BW on drip loss or flavor, but lighter BW piglets produced less tender meat. Bérard et al (2008) reported decreased carcass yield and smaller kidneys and livers in pigs with low BW, but interestingly, also saw that pigs with medium BW produced more tender pork than light or heavy BW pigs. On the other hand, Nissen and Oksbjerg (2011) did not find any differences in meat content between low and heavy BW pigs.

The ultimate goal for the pig production industry is the sustainable production of high-quality pork. Given the impact that piglet BW has shown to have on muscle development and therefore meat quality, this trait is one that must be considered and managed.

2.2.5 Low birth weight and subsequent reproductive performance

The development and selection of gilts for future reproductive activities is the foundation of the production system and is still one of the main bottlenecks in pig production. Its impact can be verified by analyzing the Canadian sow herd data. In 2018, the Canadian sow herd was estimated to be 1.2 million animals. Considering a 35 to 50% replacement rate, up to 600,000 new gilts need to be incorporated into the sow population annually. To achieve this number, it is estimated that approximately 1 million gilts have to be born annually. Therefore, producing gilts in an efficient way should include the early identification of gilts that represent a balance between adequate future reproductive performance and genetic improvement.

It is well known that higher growth rates and body weight in gilts are associated with an earlier onset of puberty, which in turn can reduce the number of non-productive days and improve herd efficiency. Nonetheless, the impact of ADG is also reported to be associated with reproductive performance and longevity (Kummer et al, 2006; Filha et al, 2010; Kaneko & Koketsu, 2012). Magnabosco et al (2014) measured the development of gilts according to ADG (Low: 500–575 g/d; Intermediate: 580–625 g/d; and High: 630–790 g/d) and age of the first exposure to boar (140-155 and 156–170 days of age). The highest rate of estrus onset was in the group of gilts with the highest ADG (74.3%, compared to 65.5% and 64.3%, on Low and Intermediate, respectively) with an age of 156 to 170 days. In the same study, however, parity rate and total number born in the first parity were not affected by age at first estrus, nor ADG.

The importance of BW on ADG in gilts is reported by Magnabosco et al (2015), de Almeida et al (2014) and Almeida et al (2017). Magnabosco et al (2015) reported that gilts weighing less than 1kg at birth had a lower ADG at 20, 70 and 170 days of age and lower body weight at the time of selection for breeding (170 days). In addition, the total mortality up to 170 days was higher in the low BW group, therefore fewer gilts from this group reached the selection phase. Likewise, de Almeida et al (2014) observed that gilts born from purebred Landrace sows with a BW less than 1.2 kg, had a lower ADG during pre-weaning, nursery and selection at 155 days of age. In addition, a lower percentage of low BW gilts reached the selection phase. The chance of achieving selection was at least two times lower in the low BW group compared to heavier gilts at birth. Nonetheless, once selected for breeding, the age of puberty and anestrus rates were not different between low and high BW gilts. Therefore, the author proposed that once low BW gilts are able to achieve and pass selection, their individual performance tends to be similar to high BW gilts. Magnabosco et al (2016) evaluated the retention rate of low BW gilts, by analyzing the number of days they stayed in the herd from birth to third parity and found that low BW gilts stayed in the herd for a shorter period of time compared to high BW gilts. However, there was no difference between the two groups when they were compared from selection phase on, which is in agreement with de Almeida et al (2014).

The influence of BW on reproductive tract development and follicle dynamics was also studied by Almeida et al (2017a, 2017b). Almeida et al (2017a) demonstrated that BW did not affect the development of the genital tract or the total follicle population in the ovaries of gilts at 80 days of age. However, in subsequent work, Almeida et al (2017b) reported that low BW alters follicle dynamics in prepubertal gilts. In this work, gilts were classified into two categories of BW (Low BW: 0.8 to 1.2 kg and High BW: 1.8 to 2.2 kg) and evaluated the reproductive tract and

follicle dynamics at 150 days of age in the pre-pubertal gilts. High BW gilts had higher body weights and ADG compared to the low BW in every phase analyzed (24, 63, 107 and 150 days of age). Evaluation was also made on the length of the reproductive tract, and the only difference between categories was seen in the longer vaginal length in high BW gilts. The reproductive tract and ovarian weight were similar between the two groups, but the ovarian weight relative to body weight was higher in the low BW group. Although the ovaries were heavier related to body weight, low BW gilts presented an equal number of small antral follicles (<3 mm size) and lower number of medium antral follicles (3-5 mm size). According to the author, only 20.7% of low BW gilts presented more than 10 medium antral follicles, compared to 70.3% on high BW gilts. Additionally, morphometrical analyzes showed a lower number of primordial and pre-antral follicles and a higher number of atretic follicles per ovarian cortex area in low BW compared to high BW gilts (Almeida et al, 2017). Silva et al (2014) found differences in gene expression in CL vascularity and follicular development when low and high BW phenotype sows were compared, thus demonstrating a difference in reproductive tissues between sows of these two distinct groups. In a more extreme scenario, Da Silva-Buttkus et al (2003) evaluated the population of follicles in runt gilts (0.73 kg) at the time of the birth and a higher number of primordial follicles was detected, compared to high BW (1.53 kg). However, a reduction in the number of primary follicles and the absence of secondary follicles, in low BW gilts subjected to intrauterine growth retardation, demonstrated reduced follicular development already at the time of the birth. The combination of decreased ovarian activity and shorter vaginal length suggests lower OR (Knox et al, 2005) and a smaller litter size (Martin Rillo et al, 1998) could be expected from low BW gilts. In fact, Magnabosco et al (2016) pointed out that gilts born with a weight less than 1 kg produced 4.5 fewer piglets over three parities, an average of 1.6 fewer piglets than the mean of the heavier gilts.

2.2.6 Low birth weight phenotype in sows

After years of genetic selection intended to increase the number of total piglets born per litter, it is now possible to identify an extreme population of sows in every herd that consistently give birth to low BW piglets, independent of the litter size. It has been proposed that this sow-dependent phenotype is a result of the interaction among high OR, intra-uterine crowding of embryos and poor placental development (Foxcroft et al, 2006; Patterson & Foxcroft, 2019), which in turn affects fetal programming and consequently reduced post-natal potential of low BW piglets (Foxcroft et al, 2006; Rehfeldt & Kuhn, 2006). It is well known that there is a negative association between BW and total number of piglets born, however this association is limited to explain 20 to 25% of the variation in BW, which represents a 35 to 40 gram decrease in litter average BW for each additional piglet born (Smit et al, 2013). The same study demonstrated that, 16 total piglets born per litter was set as a threshold above which the average BW tended to decrease and cause an even more extreme low litter birth weight phenotype (LLBWP). Differing from individual low BW, the LLBWP is expected to affect the growth performance of entire litters, thereby generalizing the problem. It has been observed that 60% of piglets that originated from LLBWP sows have individual BW of 1.15 kg or less, and almost 90% born with 1.37 kg or less (Patterson & Foxcroft, 2016).

The LLBWP tends to remain constant during the entire productive life of the sow, which means it is highly repeatable (Smit et al, 2013). In the study by Smit et al (2013), it was reported that sows that produced an extremely low BW litter at the first parity defined as the bottom 15% of the population, continued to produce low BW litters in the subsequent parities. In subsequent work, Smit et al (2013) reported the correlation coefficient as r=0.49 of repeatability across successive parities. Patterson et al (2013) stated that the litter average BW shows 60% repeatability

with the majority of sows having consistently high litter birth weight phenotype (HLBWP) or LLBWP over multiple parities. Nonetheless, the phenotype is passed down through generations, perpetuating the problem in the production system. Zhang et al (2018) estimated the heritability for total litter BW and average individual BW were 0.36 ±0.06 and 0.39±0.06, while the repeatability was 56% for both litter BW and average individual BW. As a consequence, replacement gilts born from sows displaying LLBWP, have a greater chance to exhibit poor reproductive performance (Patterson et al, 2018). The retention rate of replacement gilts originated from sows classified in three distinct groups according to the BW phenotype on successive litter records: low (< 1.15 kg), low-medium (≥1.16 to ≤ 1.36 kg), medium-high (> 1.36 and ≤ 1.6 kg) or high (> 1.6 kg). Retention rate was found to be lower for low than for low-medium, medium-high and high sows in every period analyzed, at day 4 age (91.4, 94.1, 95.4, and 95.6 %, respectively), at day 24 of age (81.4, 84.5, 87.2, and 86.9 %, respectively), day (70 66.7, 75.4, 78.7, and 79.2%, respectively) and 190 days of age (42.6, 52.3, 55.3, and 56.2 %, respectively) (Patterson et al, 2018). Similar results were found by Flowers (2018), where 60% of gilts weighing less than 1.1 kg at birth failed to produce one litter and, for the ones that did, had reduced lifetime productivity whereby only 11% reached the sixth farrowing, compared to 32% of their heavier (1.58 kg) littermates. It is clear that sows expressing the LLBWP and the offspring they produce have negative consequences for the efficiency of genetic multiplication and reproductive potential in the herd. The detrimental effects of BW are not only restricted to small piglets within a litter but also extend to the entire litters that are prenatally programmed to have a lower than average BW.

2.3 The use of transcriptomics in animal studies

Powerful high-throughput genomic tools, such as microarray technology and RNA-seq have been used to conduct gene expression studies in domestic animal reproductive physiology.

Among other things, these technologies allow for a more in-depth analysis and better understanding of the relationship between the maternal reproductive tract and embryonic development in pigs and other domestic animals (Niemann et al, 2007; Tsoi et al, 2012; Dyck et al, 2014). Whether using microarray or RNA-seq, the shifts in mRNA abundance triggered by treatments of certain uterine conditions can be characterized in the conceptus.

The microarray technology allows for large-scale studies of gene expression. However, it has several limitations, that include issues with background hybridization (which limits the accuracy of expression measurements) probes with different hybridization properties, and the main issue being that the arrays are limited to analyzing only genes for which probes are designed and included in the array (Zhao et al, 2014). RNA-seq, on the other hand, is the direct sequencing of transcripts and it does not depend on genome annotation for prior probe selection, and avoids the related biases introduced during hybridization of microarrays. It also has advantages for detecting novel transcripts, allele-specific expression and splice junctions (Mortazavi et al, 2008; Zhao et al, 2014).

RNA-seq allows for the precise quantification of transcript levels in tissues and cells and enables scientists to explore how organisms respond to certain conditions (Gracey & Cossins, 2003). This technical capacity provides the ability for comprehensive gene expression profiling to identify active and inactive genes under certain experimental conditions, such as the use of nutrient additives (Dalto et al, 2015), bacterial infection (Fouhse et al, 2019) or different physiological or developmental stages in porcine breeding (Tsoi et al, 2012, 2016). These studies provide detailed gene expression profiles related to the underlying molecular pathways and gene interactions, in order to unravel the causal relationships between genes and particular physiological conditions. Therefore, the ability to understand how embryonic development is regulated, the central genes

and molecular markers that are related to appropriate development, and embryonic quality contribute to the understanding of basic reproduction and facilitates improved maternal management (Tsoi et al, 2012).

2.4 Conclusion

The TNB is considered to be a critical measure of success in the breeding herd in a pig production system, and to address this target, genetic selection for sow prolificacy and increased litter size received considerable focus for generations. In recent years, the selection focus has been shifting towards piglet quality, however the consequences of past selection related to TNB still influences the contemporary sow. This previous selection pressure resulted in a biological imbalance between OR and uterine capacity, which has contributed to a decrease in litter BW and higher within-litter variation in piglet BW. An additional, a consequence has been the establishment of a sub-population of sows that exhibit a repeatable LLBWP. Although TNB is critical to production efficiency, if BW and the survivability of piglets are low, the value of the increased TNB is minimal and limits the producer's productivity and profit potential.

In this scenario, the LLBWP plays a major role in the breeding herd. This phenotype tends to affect a portion of the of sows in every herd and besides the fact that the low BW is extended to the entire litter, the sows displaying the phenotype show the same reproductive behavior over parities, perpetuating the problem in the production system. Like other reproductive traits, BW is complex and heritable within a population, which makes this trait particularly important in sows at the multiplication level. The complexity of the trait makes it difficult to control, especially in litter bearing species, like pigs, where a variety of factors including ovulation, fertilization and implantation rates, conceptuses survival and development, the genetic quality of the conceptuses, placental efficiency, uterine capacity and the total number of piglets born affect prenatal

development. Therefore, the ability to identify this group of sows in a herd and to understand the mechanisms that drive the LLBWP will help to elucidate major aspects of the phenotype that can be directed towards overall improved breeding efficiency of a herd and the final production of high-quality litters.

2.5 Objective and hypothesis

As discussed, sows displaying the LLBWP repeatedly produce piglets with a generalized low BW during their productive lifetime. Although this phenomenon has been acknowledged and can be phenotypically identified at the farm level, the prenatal events that drive the low BW remains unknown. Therefore, the primary objective of our research was to understand the biological factors that underlie the LLBWP compared to sows with a HLBWP, namely, sows that consistently produce high BW piglets. This research involved three main steps. The first step was the determination and identification of LLBWP and HLBWP sows in a Large White nucleus unit population. Second, the physiological mechanisms that may be driving the low BW were evaluated at day 30 of gestation. Lastly, candidate genes and biological pathways from placental and embryonic tissues were analyzed. The overall hypothesis for this study was that the LLBWP sows have higher ovulation rates, a higher number of embryos in utero and increased competition for uterine space during the early stages of development compared to High LBWP sows. The consequence is a negative effect on placental development and embryonic development due to a limited uterine capacity that results in generalized low BW of piglets. In Chapter 3 of this thesis, ovulation rate, embryonic weight, size, viability and sex, together with placental measurements were assessed and compared between LLBWP and HLBWP sows. The effect of litter birth weight phenotype on conceptus and placental quality was evaluated through transcriptomics analyses in

order to understand the molecular mechanisms that may be impairing the prenatal development in LLBWP sows, as described in Chapter 4.

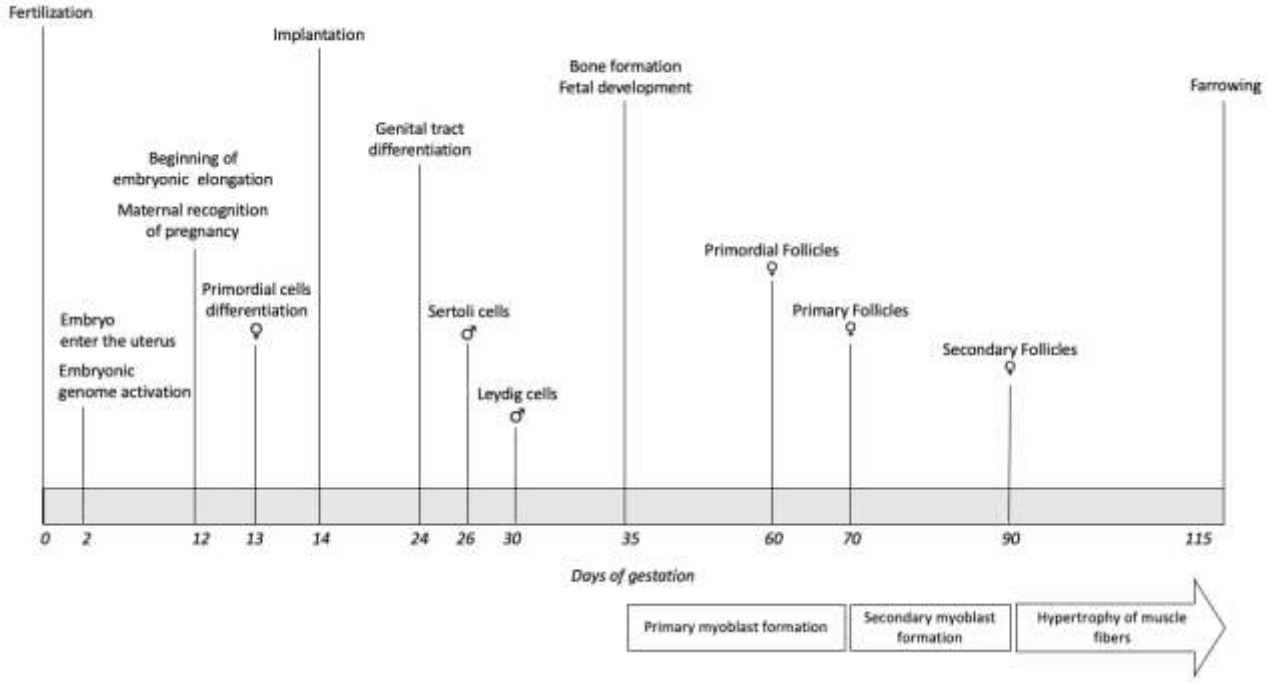
CHAPTER 2 - TABLES

Table 2.1 Essential events according to the day of development on pig embryonic development. Compiled from: Hunter et al (2000), Gandolfi et al (2005), Black and Erickson (1968), Dyck and Ruvinsky (2011), Oestrup et al (2009), Bazer and Johnson (2014), Pope (1988), Hunter (1995), Lyon (1999).

Day of development	Developmental event
Day 0	Fertilization
14 to 16 hours after fertilization	Cleavage to 2-cell stage
Day 1	Cleavage to 4-cell stage
Day 2	Cleavage to 8-cell stage
	Embryo genome activation
Day 2 to 3	Embryo enter the uterus
Day 3	Morula development
Day 5	Blastocoel development
	Spherical embryo with 0.5 to 1mm of diameter
Day 6	Blastocyst expansion
Day 7 to 8	Blastocyst hatches from zona pellucida
	Embryo exposed to the uterine epithelium
	Inactivation of one X chromosome in female embryos
Day 9 to 10	Formation of embryonic disc
	Spherical embryo with 2 to 6mm of diameter
Day 11 to 12	Maternal recognition of pregnancy
	Gastrulation begins
	Embryo starts to elongate and spacing throughout
	Primordial germ cell formation
Day 13	Female primordial cells begin to differentiate into oogonia
Day 14	Attachment of conceptus to uterine endometrium
	Implantation is accomplished
Day 16	Elongated embryo with 80 to 100cm
Day 17 to 18	Amnion completely formed
Day 19	Allantois fills and contacts the chorion
Day 24 to 26	Embryonic gonads visible
Day 26	Differentiation of Sertoli cells in male embryos
Day 27	Placental folding formation
Day 28	External genitalia differentiate
Day 29	Male embryos produce anti-Mullerian hormone
Day 30	Chorion fully vascularized by allantoic vessels
Day 35	Bone mineralization and beginning of fetal development

CHAPTER 2 – FIGURES

Figure 2.1 Chronological events of intrauterine embryonic and fetal development for female and male pig embryos. Compiled from: Hunter et al (2000), Gandolfi et al (2005), Black and Erickson (1968), Dyck and Ruvinsky (2011), Oestrup et al (2009), Bazer and Johnson (2014), Pope (1988), Hunter (1995), Lyon (1999).



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CHAPTER 3

Influence of litter birth weight phenotype in sows on ovulation rate, embryonic and placental development at day 30 of gestation

3.1 Introduction

Over the last few decades, the total number of piglets born per litter (TNB) has been considered to be one of the major components for measuring the efficiency and profitability of a pork production system. In order to meet production targets, genetic selection for sow prolificacy and litter size received a tremendous amount of focus. This selection pressure resulted in a disproportional increase in ovulation rate (OR) and prenatal development was consequently impaired (Vallet et al, 2014; Foxcroft et al, 2009). Lower piglet birth weight (BW) and higher within litter BW variation became common, and for this reason inevitably higher mortality rates and slower growth rates have been seen in all phases of production, causing economical losses to producers (Quiniou et al, 2002; Rehfeldt & Kuhn, 2006; Foxcroft et al, 2009). Furthermore, in the breeding herd, replacement gilts born with BW less than 1.0 to 1.2 kg have less chances of being selected for further reproductive activities, and the ones that are selected have lower retention rates compared to heavier littermates (Magnabosco et al, 2015; de Almeida et al, 2014).

Many studies have shed light on the negative association between BW and TNB. It is well known that a large proportion of the variation in BW and growth performance in pigs is mostly determined, and permanently established, during embryonic and fetal development in a process known as prenatal programming (Town et al, 2005; Foxcroft et al, 2009; Patterson & Harding, 2013). It is estimated that litters with 16 or more piglets, experience a 35 to 40 g decrease in average litter BW for each extra piglet born (Smit et al, 2013). This scenario is largely explained by the higher OR rates of contemporaneous sows, which in turn leads to a higher number of

embryos in-utero, inducing overcrowding and negatively affecting placental capacity (Wientjes et al, 2013; Da Silva et al, 2016). If embryonic mortality does not control the total number of embryos in the uterus in early gestation, these events may cause further reduced growth, increased fetal mortality in later stages of pregnancy and heterogeneity of the surviving fetuses, which generates irreversibly compromised lifetime productivity (Foxcroft et al, 2009). Notwithstanding, the TNB and BW association explains only 20 to 25% of the variation in BW (Smit et al, 2013). Therefore, a second scenario should be considered; the low litter birth weight phenotype (LLBWP).

After years of genetic selection intended to increase the TNB in maternal line sows, it is now possible to identify an extreme population of sows in every herd that gives birth to low BW piglets. The LLBWP is a sow-related phenotype whereby sows consistently produce low BW piglets across all parities, irrespective of the litter size (Patterson & Foxcroft, 2019). Unlike individual low BW, the LLBWP is expected to affect the growth performance of entire litters, thus generalizing the problem. It has been observed that 60% of piglets originating from LLBWP sows have individual BW ≤ 1.15 kg, and almost 90% are born with BW ≤ 1.37 kg (Patterson & Foxcroft, 2016). Recent studies have demonstrated that average litter BW shows a 40 to 60% repeatability with some sows consistently having high litter birth weight phenotype (HLBWP), where they consistently produce high BW piglets, or LLBWP over multiple parities (Smit et al, 2013; Patterson & Harding, 2013; Zhang et al, 2018). The phenotype is passed through generations, perpetuating the problem in the production system. Zhang et al (2018) estimated that the heritability for total litter BW and average individual BW were 36% and 39%, respectively. As a consequence, replacement gilts born from sows displaying the LLBWP, have a greater chance of having poor overall reproductive performance (Patterson et al, 2018; Patterson & Foxcroft, 2019).

In order to obtain a better understanding of the LBWP in sows, the objective of this study was to first identify a population of sows with repeatable LLBWP and HLBWP in a Large White nucleus population. Then secondly, to compare the LLBWP and HLBWP extreme groups, to elucidate the relationship between the LBWP and key reproductive traits including OR, dynamics of embryonic mortality and their influence on embryonic and placental development on day 30 (D30) of gestation and embryonic sex ratio. The LLBWP is hypothesized to be driven by higher ovulation rates, a higher number of embryos in utero and increased competition for uterine space during the early stages of development, in comparison to HLBWP sows. The consequence is a negative effect on placental development and embryonic development due to a limited uterine capacity. With that in mind, the ability to understand the physiological processes associated with the LBWP could be strategically directed toward selection to improve overall breeding efficiency and optimize litter quality performance in the pork industry.

3.2 Materials and Methods

3.2.1 General

The present study was conducted at the Swine Research and Technology Centre (SRTC), University of Alberta, between August 2018 and May 2019, in accordance with the Faculty Animal Policy and Welfare Committee-Livestock of the University of Alberta (Protocol - AUP00002650).

3.2.2 Sow selection

In this study, data was obtained from a nucleus farm from Hendrix Genetics (Tullymet Nucleus Unit, Balcarres, SK). The dataset on 459 Large White (LW) purebred sows and 10,349 individual piglets, included litter characteristics such as TNB, total born alive, number of piglets

weighed, total litter BW, average litter BW, number of piglets weighing < 800 g, number of piglets weaned (TNW), date of insemination, boar used and date of farrowing.

Within the Hendrix Genetics nucleus breeding system, a case-control study on reproductive history measurements of LW purebred sows was undertaken to identify sows that exhibited a LLBWP and HLBWP over at least two parities. Litters with less than 7 and over 22 piglets born were excluded from the selection. All analyses were performed using RCore Team (2017), "lme4" package (Bates et al, 2015). In order to analyze average litter BW by sow, a mixed effect model "lmer" was used with parity order, farrowing season and TNB as covariates, and the boar used in the last insemination as a random effect. The effect of parity order and TNB were found to be significant ($P<0.05$) and were used to adjust the raw average litter BW. A regression for individual sow average litter BW related to TNB was performed (Figure 3.1) and the repeatability of the trait analyzed (Figure 3.2). Only sows with limited variation in average litter BW (standard deviation within sow less than two times the average) were considered to have a repeatable phenotype and selected for LLBWP and HLBWP groups. Finally, 40 extreme sows (n=20 HLBWP and n=20 LLBWP) from the population (top 12% and bottom 12%) were selected. The mean average litter BW for LLBWP was 1.20 kg and for HLBWP was 1.50 kg (Figure 3.3, Table 3.1). The residuals analysis for each group are shown in Figure 3.4.

The comparisons between LLBWP and HLBWP reproductive data in Table 3.1 were obtained from the "lme4" package using a "lmer" to analyze average litter BW and total litter BW, with LBWP Group as the fixed effect and Sow ID and parity class as random effects (Figure 3.5). The TNB, total born alive, total number of piglets weighing less than 0.800 g were analyzed with a generalized linear mixed effect model "glmer" with LBWP Group as a fixed effect and Sow ID and parity as random effects.

3.2.3 Synchronization and artificial insemination

All multiparous LW sows (parity 2 to 6) used in this study were previously selected for culling from the Tullymet Nucleus Unit (Balcarres, SK), due to Index (Estimated Breeding Value) or parity order (> 3 Parities). Only animals in good physical and health condition were considered suitable for this study. Sows were shipped to the SRTC and after 10 days of acclimatization, estrous cycles were synchronized using altrenogest (Matrix, Merck AH, Kenilworth, NJ) for 14 days (9 mL/sow/day). The sows were bred on their second estrus following altrenogest withdrawal at the time of estrus detection with an interval of 24h until the end of the estrus (0h, 24h, 48h) with homospermic semen doses stored up to 6 days. The semen was collected from pure LW boars of proven fertility (Hendrix Genetics) and extended with extra-long-term Duragen ® (Magapor S.L, Spain) commercial extender. To address any potential boar effects, each insemination was performed with semen from a different boar (total of 17 boars) with 80 mL and 2.5 billion cells, transported to the farm and stored at 17°C ± 2°C. Pregnancy was confirmed using real-time ultrasonography 24 to 26 days after insemination, and pregnant sows were euthanized on day 28 to 30 (day 29 ± 0.6) of gestation.

3.2.4 Feeding

At arrival to the SRTC, sows were individually weighed, and body condition score was assessed to determine feed allowance according to the NRC (2012) requirements. All sows were fed once daily with standard gestation diet (3.10 Mcal/kg, 14.77 % crude protein (CP) and 0.68% lysine). As a matter of control, feed refusals were weighed daily before the morning meal, and the feed intake was assumed to be equal to feed disappearance.

3.2.5 Collection of reproductive tissues

At day 28 to 30 of gestation (n = 20 HLBWP and n = 20 LLBWP) sows were euthanized, with the day of the first insemination being defined as day 1 of pregnancy. Euthanasia was conducted by qualified staff using approved necropsy procedures. The reproductive tracts were recovered from each sow immediately after euthanasia, and the number of corpora lutea (CL) on each ovary was counted as an assessment of OR. The uterine horns were separated from the broad ligament and opened at the antimesometrial side as embryo and placenta were removed one by one. Each embryo was collected and assessed individually for viability. The embryos were classified as viable, hemolyzed or underdeveloped (Figure 3.6). They were considered hemolyzed when there was hemolyzed amniotic fluid and the embryo was presenting a general redness appearance. Embryos were considered underdeveloped if there were resorbed embryonic membranes and evidence of implantation (e.g. loss of vascularity and/or tissue decay). After classification, crown-rump length (CRL, mm) and individual embryonic weight (EW, g) were measured. The total number of embryos (TNE) was calculated based on the number of viable (TNV), hemolyzed and underdeveloped embryos. The ratio between TNE and OR was considered early embryonic survivability; the TNE and TNV ratio was considered to be late embryonic survivability; and the ratio of TNV and OR was considered as the total embryonic survivability. The location of each embryo and allantochorionic fluid volumes (AFV, mL) of each placenta were recorded. Immediately after collection, embryos were snap frozen individually in liquid Nitrogen and stored at -80°C.

3.2.6 Sex typing D30 embryos

A total of 610 viable embryos were individually sex-typed for each treatment (LLBWP: 288 embryos; HLBWP: 329 embryos). An established DNA-based phenol-free embryo sex typing protocol was used as previously described (Blanes et al, 2016).

3.2.6.1 Sample preparation – grinding embryos

Each frozen embryo, previously stored at -80°C, was individually placed into a pre-frozen mortar on dry ice. Liquid Nitrogen was poured into the mortar to cover the embryo and it was ground into a fine powder using a pre-chilled pestle. The embryonic powder was then transferred to a pre-labeled sample tube with a micro-spatula and stored at -80°C until further manipulation. Examination gloves were changed after each embryo manipulation to avoid cross contamination between samples (McCulloch et al, 2012).

3.2.6.2 DNA preparation

Sample tubes containing the powdered embryos were transferred from -80°C storage into dry ice and 180 µl of 50 mM NaOH (sodium hydroxide) was pipetted into pre-labeled micro-centrifuge tubes. The embryo powder (about 5 to 10 mg) was transferred from the sample tube into a pre-labeled micro-centrifuge tube containing 50 mM NaOH solution. Once the sample was mixed with NaOH, DNA lysate was formed. Next, micro-centrifuge tubes with DNA lysate were transferred to a pre-heated incubator at 95°C for 5 minutes. Then, 20 µl of 1M Tris-HCl was added directly into the microcentrifuge tube and mixed by gently tapping the tube. The tubes with the DNA lysate were centrifuged at 2,000 g for two minutes at room temperature to remove undissolved tissue debris. The resulting DNA lysate was then ready to use as template in a PCR reaction.

3.2.6.3 Design sex-specific PCR primers

A modified HotSHOT method (Blanes et al, 2016) was used to obtain DNA for sex typing. The gene accession numbers for sex determining region Y (SRY) (NM_214452.3) and zinc finger protein X-linked (ZFX) gene (XM_005673501.1) were obtained from the NCBI website

www.ncbi.nml.nih.gov. The SRY and zinc finger protein X-linked (ZFX) genes were used for determining the sex of the embryos as described in Blanes et al (2016). Primers specifications are in Table 3.2.

3.2.6.4 Genomic DNA PCR condition and validation

In order to run the PCR reaction, 1µl of the embryo DNA lysate was used as a template for the 15µl PCR reaction. For quality control purposes, in each PCR run, a negative control with no template was prepared as well as two positive controls containing 0.5 ng of porcine genomic DNA, one from female and one from male embryo. A mixture of enzymes (Platinum II Hot-Start PCR Master Mix (2X), ThermoFisher Scientific ^{TM}) was used and a master mix prepared by adding primers and PCR water. The final volumes of Hot-Start and Master Mix were calculated based on the total number of PCR reactions to be performed. One microliter of the DNA lysate for each sample was added to a pre-prepared PCR tube with 14µl of the PCR Master Mix. The primers were added such that the final concentration of the primers from the two sex-specific genes was 0.2µM in a total of 15µl PCR reaction.

The PCR program was used in a thermal cycler as follows: 94°C for 2 minutes, 40 cycles each with a 15 sec melting step of 94°C, followed by 15 sec annealing step at 60°C, followed by a 15 sec elongation step at 68°C. PCR conditions were developed based on the primer's annealing temperatures and validated with pilot runs prior to the study. The reactions were incubated at 4°C until removal of the PCR tube for gel electrophoresis verification to assess amplicons and determine the sex. Amplicons were visualized on a 2% TBE agarose gel with an appropriate amount of non-toxic green fluorescent SYBR DNA gel stain added to visualize the bands. A volume of 1.5µl of loading dye (10X) was added into the PCR tube and mixed by pipetting the

PCR reaction buffer. Fifteen µl of each sample was loaded into the well and the agarose gel was run with appropriate voltage settings (e.g small apparatus at 100V, 96-well apparatus at 150V until the dye band runs halfway through the gel). A Typhon FLA 9500 laser scanner (GE Life Science) was used to capture the image of the gel (Figure 3.7).

3.2.7 Statistical analysis of data collected at D30 of gestation

All data analyses were performed with RCore Team (2017), with sow as the experimental unit for all variables tested. All data were checked for normality and homogeneity of variance using histograms, gplots and formal statistical tests (Studentized Residual) as part of the "lme4" package of RCore Team (2017). In order to account for any unbalanced effect, Kenward-Rogers test was used to test the degrees of freedom. Numerical data are expressed as LS means ± SEM or as percentages, according to the type of variable. Differences were considered significant at $P < 0.05$.

To analyze the effect of parity on EW, AFV, OR and survivability, parity was divided into two categories: Class 1 (Parities 2 and 3; 12 sows LLBWP, and 7 sows HLBWP) and Class 2 (Parities 4, 5 and 6; 9 sows LLBWP, and 10 sows HLBWP). In addition, to analyze the effect of TNE on embryonic and placental characteristics, four classes were created: Class 1 (10 to 14 embryos; 1 sow LLBWP, and 3 sows HLBWP); Class 2 (15 to 19 embryos; 7 sows LLBWP, and 6 sows HLBWP); Class 3 (20 to 23 embryos; 6 sows LLBWP, and 5 sows HLBWP) and Class 4 (24 to 28 embryos; 3 sows LLBWP and 5 sows HLBWP). Sex effects were also tested by splitting into Male (133 embryos LLBWP, and 181 embryos HLBWP) and Female (156 embryos LLBWP, and 149 embryos HLBWP) and day of collection was also tested (Day 28: 3 sows LLBWP, and 4 sows HLBWP; Day 29: 11 sows LLBWP, and 10 sows HLBWP; and Day 30: 4 sows LLBWP, and 5 sows HLBWP).

In order to analyze the sow's reproductive data, the fixed effect of parity on OR, early, late and total survivability was assessed using generalized linear model "glm" with binomial distribution in RCore Team (2017). Subsequently, a generalized linear mixed model ("glmer") with binomial distribution was used to analyze OR, early, late and total embryonic survivability. In these models, LBWP Group was considered as the fixed effect and parity class was included as a random effect. For early embryonic survivability, late embryonic survivability and total embryonic survivability the relationships considered were: TNE by OR; TNV by TNE; and TNV by OR, respectively.

The effect of parity class on EW, CRL and AFV was tested as a fixed effect together with LBWP Group, while Sow ID and day of collection were included as random effects. The effect of TNE was tested as a fixed effect together with LBWP Group, while Sow ID and day of collection were included as random effects. Subsequently, a mixed effect model ("lmer") was used to analyze EW, CRL and AFV. The models for EW and CRL included the fixed effect of LBWP Group (LLBWP or HLBWP) and Sow ID, day of collection and parity class as random effects. Total number of embryo categories and sex were tested, but no significance was found. Therefore, these were removed from the model. The TNE categories were found to be significant for AFV. As a consequence, the "lmer" model for AFV included LBWP as a fixed effect and Sow ID, day of collection, parity class and total number of embryos as random effects. Linear relationships were performed to determine if there was a correlation between reproductive parameters including OR and TNE and TNE viable, AFV, EW and CRL.

To obtain the sex distribution related to each LBWP Group, a generalized linear model "glm" was used. The ratio used was the number of males by total number of viable embryos (since

only viable embryos were sex typed). One sow (LLBWP) was excluded from the analyses due to an extreme low percentage of males in the litter (3 males out of 21 total viable embryos).

3.3 Results

Of the 40 bred sows selected, three were excluded from the analyses (2 sows from the LLBWP and 1 from the HLBWP) due to the presence of a low number of embryos at the time of euthanasia (less than 10 embryos). For the embryonic analyses, EW, CRL and AFV were adjusted for gestation length, (the day of gestation the measurements were collected), which ranged from 28 days to 30 days, with an average of 29.11 ± 0.74 days for the LLBWP Group and 29.09 ± 0.66 days for the HLBWP Group (Table 3.3).

3.3.1 Effect of parity classes on reproductive measurements

Effects of LBWP Group and parity class on OR, embryonic survival, embryonic and placental development are presented in Table 3.4. Ovulation rate was not affected by the parity of the sows, nor were embryonic survivability or embryonic development. The only variable affected by parity was AFV (Table 3.4) in which the lowest placental volume was found for LLBWP Parity Class 1 sows and the highest was found for HLBWP Class 2 sows (LLBWP Parity Class 1: 123.6 ± 10.5 mL and HLBWP Parity Class 2: 154.6 ± 10.04 mL, $P<0.05$).

3.3.2 Effect of day of collection on reproductive measurements

The effects of day of collection and LBWP group are presented in Table 3.5. The only significant difference was found on AFV in which LLBWP Day 28 Group had the lowest average placental volume while HLBWP Day 30 Group had the highest volume; all other groups had intermediate volumes (LLBWP Group Day 28: 114.4 ± 11.8 mL and HLBWP Day 30 Group: 160.1 ± 8.86 mL).

3.3.3 Effect of total number embryos on embryonic and placental development

The effect of TNE and LBWP Group is shown in Table 3.6. The effect on early and total embryonic survivability was significant whereby HLBWP Category 1 had the lowest rate of survival (Early survivability: 0.50 ± 0.05 and Total survivability: 0.47 ± 0.05); and LLBWP Category 4 had the highest survivability (Early survivability: 0.94 ± 0.01 and Total survivability: 0.84 ± 0.02, Figure 3.12). The number of embryos had no influence on embryonic development; however, it had a significant effect on AFV in which the lowest volume was verified in the LLBWP in the class with the greatest number of embryos in utero (Category 4: 115.5 mL ± 12.7) and it showed the greatest volume in the HLBWP in Category 2 with 15 to 19 embryos (163.3 mL ± 11.5). Although not significant, the EW was numerically greater in the HLBWP Category 2 (0.92 mm ± 0.06) and the CRL was also numerically greater (22.3 mm ± 0.5), which may be a consequence of the higher placental volume.

3.3.4 Effect of sex on embryonic and placental development

The sex ratio (%) was not significantly different between the two groups (LLBWP: 46%, HLBWP: 54%, P=0.11) and the effect of embryonic sex was tested on EW, AFV and CRL (Table 3.7) with no significance found.

3.3.5 Effect of LBWP on reproductive measurements, embryonic and placental development

Taking into consideration the final model to analyze the fixed effect of LBWP Group (Table 3.3), no significant difference was found for OR (LLBWP: 25.6 ± 1.06; HLBWP: 26.8 ± 1.06, P = 0.44). There were no significant differences in the TNE (LLBWP: 19.5 ± 1.19; HLBWP: 19.8 ± 1.12, P>0.05), or the TNV (LLBWP: 16.4 ± 1.37; HLBWP: 16.6 ± 1.37, P>0.05). Consequently, no differences were found for early embryonic survivability (LLBWP: 0.78;

HLBWP: 0.76, P=0.43), late embryonic survivability (LLBWP: 0.85; HLBWP: 0.86, P = 0.67) or total embryonic survivability (LLBWP: 0.67; HLBWP: 0.66, P = 0.55*,* Figure 3.8). For embryonic and placental characteristics, there was no significant difference between LLBWP and HLBWP for EW (LLBWP: 0.80 ± 0.05 g; HLBWP: 0.88 ± 0.04 g, P=0.18) or CRL (LLBWP: 21.5 ± 0.7 mm; HLBWP: 21.9 ± 0.68 mm, P=0.46*,* Figure 3.9). However, placental development represented by the average AFV was significantly lower in the LLBWP compared to HLBWT group (LLBWP: 131 ± 9.82 mL; HLBWP: 149 ± 9.39 mL, P= 0.03). Placental efficiency was calculated as the embryonic weight:placental weight ratio and there was no significant effect of LBWP on this measurement (P>0.05).

Ovulation rate was positively correlated with TNE (R^2 = 0.09; P < 0.03, n = 36), but it was not significantly correlated with TNV (R^2 = 0.03; P = 0.15, n = 36, Figure 3.10). On the other hand, TNE and TNV were found to be highly correlated (R^2 = 0.67; P < 0.001, n = 36). Average EW was positively related to AFV weight (R^2 = 0.46; P < 0.001, n = 617, Figure 3.11) and CRL (R^2 = 0.57; P < 0.001, n = 617). In addition, AFV and CRL were also significantly correlated (R^2 = 0.33; P < 0.001, n = 617). The analysis of placental efficiency showed a weak correlation between placental efficiency and EW (R^2 = 0.01; P < 0.05; n = 617), however it showed a stronger negative correlation with AFV (R^2 =0.35 P < 0.001; n = 617, Figure 3.13).

3.4 Discussion

The results of the present study provide unique insights into the biological processes involved in the LBWP of breeding sows. For this study, groups of sows exhibiting repeatable LLBWP and HLBWP were successfully identified within a breeding population. Analysis of the historical breeding data from these LLBWP and HLBWP groups showed that the mean TNB was similar between the two groups (LLBWP: 16.03 ± 0.74; HLBWP: 15.71 ± 0.73, P>0.05), as well

the total number of piglets born alive (LLBWP: 14.47 ± 0.35; HLBWP: 14.16 ± 0.35, P>0.05). However, the mean average BW (LLBWP: 1.21 ± 0.03; HLBWP: 1.50 ± 0.03, P<0.05) and the total litter BW (LLBWP: 18.94 ± 0.59; HLBWP: 22.21 ± 0.57, P<0.05) were significantly lower in the LLBWP, while the percent of piglets weighing less than 800 g (LLBWP: 0.11 ± 0.01; HLBWP: 0.03 ± 0.006, P<0.05) was significantly higher. Overall, this analysis suggests that in every population of sows, there is a subpopulation (corresponding to the bottom 10 to 15% of the herd) that consistently produce low BW piglets in consecutive parities. On the other extreme, there is also a group of sows representing the top 10 to 15% of the population, that within the same range of TNB are more efficient and produce repeatedly high BW piglets. As opposed to highly prolific sows, LLBWP sows do not produce extremely large litters, so the fact that they produce low BW piglets cannot be explained directly by the negative correlation between BW and TNB.

To date, many studies have investigated the detrimental effects of low BW in replacement gilts, replacement boars, and at the commercial level and it is clear that the production efficiency of low BW piglets is compromised at all levels (Smit et al, 2013; Magnabosco et al, 2015; Almeida et al, 2017; Patterson et al, 2018; Zeng et al, 2019). Besides the direct negative consequences observed in the pure-bred nucleus sows' litters, the trait is inherited by replacement gilts and the commercial lines tend to be affected, perpetuating the problem in the production system (Zhang et al, 2018). In our study, over consecutive parities, the LLBWP sows produced 50% of the litters (out of 114 litters analyzed) with an average individual BW of <1.2 kg, and there were no litters with ≥1.5 kg. While HLBWP sows produced only 5% of litters (out of 125 litters) with less than 1.2 kg and 43% with more than 1.5 kg. Therefore, the ability to identify the subpopulation of sows that may be preventing higher productivity and perpetuating the problem in the production system is of extreme importance. Besides, it is clear that producers can select against LLBWP without

missing out on possible future high-quality litters due to the repeatability of the trait (Patterson & Foxcroft, 2019). Therefore, to understand the differences at the physiological level that allow the selection of a top-performing subpopulation of sows to produce the same number of piglets with a higher BW, may help to select against LLBWP sows.

In our study, the overall OR was not different between the two groups (LLBWP: 25.6 ± 1.06 vs. HLBWP: 26.8 ± 1.06, P>0.05). Nevertheless, the upper range of OR recorded, with approximately 55% and 57% of LLBWP and HLBWP sows, respectively, having more than 20 ovulations and 16% and 26% having more than 25 ovulations or higher, confirms that the dynamics of OR was similar in both groups. In addition, OR was positively correlated with TNE ($R^2 = 0.09$; $P < 0.03$, Figure 3.8), but it was not significantly correlated with TNV ($R^2 = 0.03$; P = 0.15, Figure 3.8). These results are similar to what was found by Vonnahme et al (2002) in which a correlation between OR and number of viable conceptuses at day 25 of gestation was found ($R^2 = 0.50$; P < 0.0001), but not at day 36 ($R^2 = 0.02$; P = 0.98). Da Silva et al (2016) found that multiparous sows with OR ranging from 22 to 38 ovulations had a *plateau* in the total number of viable embryos corresponding to a maximum of 17, which is similar to our findings (LLBWP: 16.4 ± 1.37 and HLBWP: 16.6 ± 1.37). Overall, there appears to be a physiological limit to TNV in relation to an increase in OR. Both LLBWP and HLBWP sows exhibit a plateau in the TNV they can support in utero, which is controlled by embryonic mortality. Da Silva et al (2016) observed that an increased OR affected the development of embryonic-placental units at day 35 of gestation, which was related to a reduction in placental length and a reduction in embryonic spacing, implantation length and empty spaces around each vital embryonic-placental unit. In our study, however, there was no correlation between OR and embryonic and placental characteristics at D30 of gestation.

It is estimated that prenatal mortality in commercial genetic lines ranges from 30 to 50% (Pope et al, 1972; Geisert & Schmitt, 2002), with the most significant losses occurring during the embryonic phase, up to day 35 of gestation. Our results showed the same trend, independent of the LBWP group (total embryonic mortality: 33% and 34% for LLBWP and HLBWP, respectively). Within this range, around 20% to 30% of conceptuses are lost by day 21 and 10 to 15% are lost by the end of the embryonic period (Ford et al, 2002). As a convention, total embryonic mortality is the combination of early and late embryonic mortality. Early embryonic mortality is estimated as the difference between OR and the TNE (including underdeveloped, hemolyzed, and viable embryos) present at D30 of gestation and represents the losses that occur before implantation. Late embryonic mortality is estimated as the difference between TNE and TNV at D30 of gestation, representing the post-implantation mortality (Van der Waaij et al, 2010). In our study, the dynamics of early embryonic mortality and late embryonic mortality did not significantly differ between the two LBWP groups. Still, early embryonic mortality was found to be greater compared to late embryonic mortality in both groups (early embryonic mortality: 22% vs. 24% and late embryonic mortality: 15% vs. 14% for LLBWP and HLBWP, respectively). The higher early embryonic mortality may be supported by the drastic morphological changes both in the developing embryo and the sow's uterine environment, and also by the heterogeneity of development among embryos during the time of fertilization to the implantation period (Pope et al, 1990; Bazer & Johnson, 2014). On the other hand, late embryonic mortality is known to be associated with limited uterine capacity available for the post-implantation surviving embryos (Ford et al, 2002). As a consequence of compromised placental development and high competition among embryos, both litter size and piglet BW tend to decrease (Père et al, 1997; Town et al, 2004; Vallet et al, 2014). Da Silva et al (2016) observed that sows with high pre-implantation mortality

had a longer placental length and larger unoccupied spaces around each implantation site on day 35 of gestation. On the contrary, sows with high post-implantation mortality culminated with a smaller area available around each embryonic-placental unit, smaller implantation area, and placental size. Their results suggest that available space before the implantation can be taken up by the surviving embryos, while available space in the post-implantation period is limited. In our study, there was no association between the stage of embryonic mortality and embryonic and placental development at D30.

Although the number of embryos in-utero and all dynamics of embryonic survivability seem to be similar between the two LBWP groups, the mechanisms and efficiency of embryonic and placental development appear to be working in different ways. In our study, for the viable embryos, the embryonic size was not different between the two groups (LLBWP: 21.5 ± 0.71 mm; HLBWP: 21.9 ± 0.68 mm, P=0.46*)* which was expected, since CRL is a measure of gestational development and both groups were sampled at the same interval of development. In addition, there was no significant difference in EW between LLBWP and HLBWP (LLBWP: 0.80 ± 0.05 g; HLBWP: 0.88 ± 0.04 g, P=0.18). On the other hand, placental development or placental volume represented by the average AFV was significantly lower in the LLBWP compared to HLBWT group (LLBWP: 131 ± 9.82 mL; HLBWP: 149 ± 9.39 mL, P= 0.03). Placental volume was directly related to the uterine area occupied by each placenta. From this perspective, even though the number of embryonic-placental units were similar between LLBWP and HLBWP, the LLBWP sows had a significantly lower placental volume and a reduced uterine area available, which may limit subsequent prenatal development.

In addition, the average EW was positively correlated to AFV (R^2= 0.46; P < 0.001, n = 617, Figure 3.9) and CRL (R^2 = 0.57; P < 0.001, n = 617). According to Vallet et al (2011),

although the placental function is not completely developed at day 35 of gestation, approximately 90% of placental and conceptus weight are established by the relationship between them at this stage of gestation. In addition, during the course of pregnancy, embryonic and fetal development become more dependent on placental development (Wright et al, 2016). To study this dynamic, Town et al (2004), limited the number of embryos in utero by oviduct ligation, and found a higher placental weight at D30 of gestation but no effect on EW. However, at day 90 of development, in addition to higher placental weight, the fetal weight was also greater in the oviduct-ligated sows. Their results suggest that D30 embryos were less sensitive to the lack of space and nutrients than fetuses at day 90. Although the scope of our study did not allow the evaluation of fetal and placental development at the end of the gestational period, we hypothesize that the fetal weight would be significantly lower by the end of the gestational period in the LLBWP due to the lower placental development found on D30.

Placental efficiency is commonly used as a marker of placental function (Krombeen et al, 2019), and in general, highly efficient placentas have a denser vascular supply, while low-efficiency placentas have reduced nutrient support or a failure to adapt to a particular condition (Reynolds et al, 2009; Krombeen et al, 2019). The placental efficiency calculation, defined as the weight of the conceptus per mass of placenta (in our study defined as volume), showed a significant positive correlation with EW and a negative correlation with AFV, similar to what was found by Town et al (2005). However, this was not significantly different between LBWP groups when sow was considered as the experimental unit. When each embryonic-placental unit is considered as an experimental unit, the placental efficiency was significantly lower in the LLBWP sows. Although the sow must be considered the experimental unit in this case, this context reveals a trend that possibly would be significant if the number of sows in this study was greater.

The impact of sexual differentiation on embryonic and placental development, as well as embryonic mortality, has been studied previously and it is well accepted that male piglets tend to be heavier than females at birth (Baxter et al, 2012). Stenhouse et al (2019) stated that male and female pig conceptuses communicate differently with the endometrium as a result of the different hormonal production throughout gestation. While male embryos produce testosterone at an earlier stage of gestation and in greater quantities than female embryos, female fetuses produce large quantities of estradiol in late gestation (Vernunft et al, 2016). These dynamics cause male pig conceptuses to have an increased growth rate compared to female conceptuses from day 10 of gestation onwards. In our study, no significant differences in EW or placental development were observed between D30 female and male embryos. In addition, the LLBWP embryos (female and male) were always lighter than HLBWP embryos, independent of the sex. This lack of difference could be related to the early stage of gestation analyzed. Although generally heavier, higher rates of growth and development make male embryos more susceptible to a limited uterine environment since their nutritional requirement is increased (Gabory et al, 2013). In addition to that, the sex ratio is believed to be influenced by genomic imprinting. The present study showed a non-significant difference in sex ratio between LLBWP and HLBWP (LLBWP: 47% vs HLBWP: 54%). Our results are not different from others that evaluated lower uterine capacity or nutrient restrictions during the gestational period (Smit et al, 2007; Vinsky et al, 2006). Presumably, the uterine crowding and lower nutrient exchange between mother and conceptuses were not enough to cause a biased sex ratio in our study.

Unlike other studies (Town et al, 2005; Da Silva et al, 2016), in the present study OR was not significantly affected by the parity of the sow nor embryonic survivability or embryonic development. Our results may be related to the limited number of sows sampled in each parity

class. The only variable affected by parity was AFV (Table 3.3) in which the lowest volume was found on LLBWP Parity Class 1 sows, and the highest was found on HLBWP Class 2 sows. However, lower placental volume was always found for the LLBWP sows, irrespective of parity, or OR class. The AFV was also significantly affected by the TNE in which the lowest volume was verified on the LLBWP with 24 to 28 embryos (115.5 ± 12.7 mL) and the highest for HLBWP sows with 15-19 embryos (163.3 ± 11.5 mL). Considering the broader picture, the results suggest that a higher number of embryos has a greater negative impact on LLBWP sows than in the HLBWP sows. In addition, when each TNE categories were compared between the two groups, the LLBWP sows always had a lower volume. Interestingly, the Category 1 (10 to 14 embryos) within the two LBWP did not show the highest AFV or EW, contrary to what was expected, as stated by Da Silva et al (2016). We propose that this result is due to the unusually high rates of embryonic mortality that sows from Category 1 suffered (LLBWP: 48% and HLBWP: 53% of total embryonic mortality). Despite the effects of TNE and parity on the reproductive characteristics, there were limited effects of these factors on the embryonic and placental development at D30 of gestation. However, these results should be carefully interpreted due to the reduced number of sows in each category. In addition, the limited effects of OR and TNE in our results may be explained by the fact that sows with historically extreme litters (<7 or >22 TNB) were not selected for this study. Therefore, in both groups there is a consistency related to these traits. And despite being different, they are not as extreme as it is commonly reported in studies involving OR, TNE and its consequences on embryonic development.

3.5 Conclusion

Initially, we hypothesized that the LLBWP group would have a higher OR and a higher number of embryos in the early stages of development, which would affect the dynamics of

embryonic survivability, spacing, and negatively affect the placental development (Foxcroft et al, 2009). Instead, we observed a similar OR and number of embryos present in both LBWP groups during the pre-implantation and post-implantation period. Interestingly, even though the number of embryos was similar between LBWP groups, the LLBWP showed impaired placental development, suggesting that the events that are driving the low BW of piglets in the LLBWP sows are not simply related to the number of embryos in the early gestational period. It is clear that the LLBWP sows had a lower individual placental space available for the embryos, which may be explained by a lower uterine capacity.

In addition, even though the TNE and TNV were similar between the two groups, sows from the LLBWP group appear to be more affected when the number of embryos present increases. These findings could be related to the lower placental efficiency that the LLBWP sows express during the gestational period. Altogether, these findings led us to consider that the uterine space and the efficiency by which nutrients are exchanged between mother and conceptus is the major cause of low BW in the LLBWP sows. As a consequence, these sows have their fetal programming affected, and consequently, the post-natal potential of low BW piglets is established. In conclusion, the identification and strategic culling of LLBWP sows in a herd can help producers to achieve higher breeding efficiency performance and avoid the adverse effects of generalized low BW in downstream commercial units.

CHAPTER 3 – FIGURES

Figure 3.1. Correlation between total number born (TNB) and average litter birth weight (kg) for Large White purebred sows.

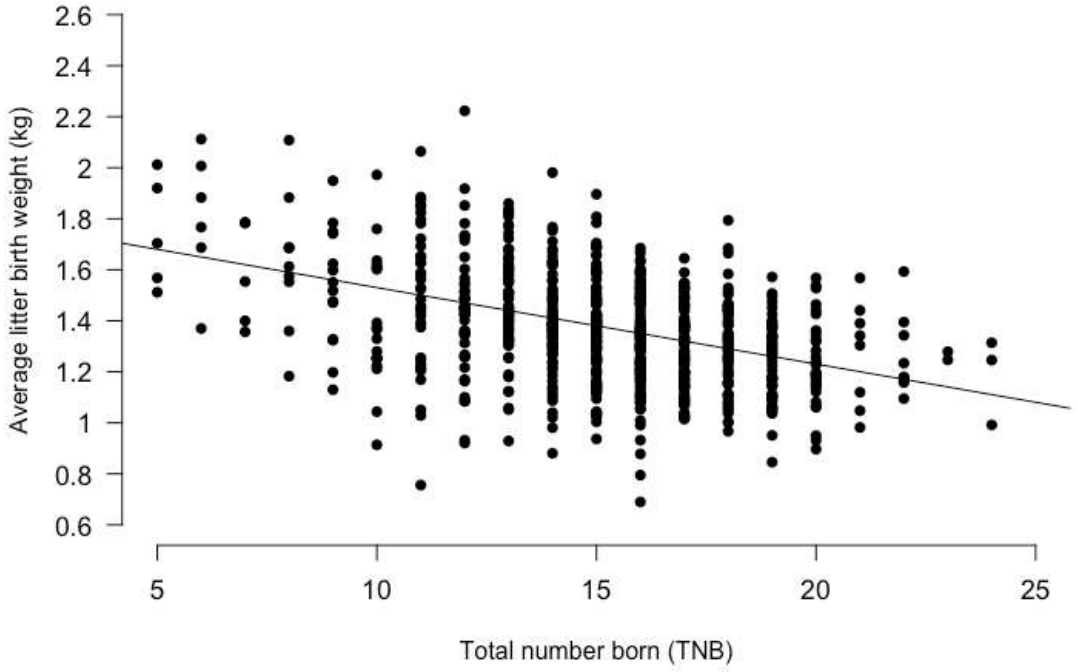


Figure 3.2. Repeatability of LBWP analysis. **Graphic (A):** Sows selected for HLBWP; **Graphic (B):** Sows selected for LLBWP. The graphics below show the mean residuals (•) and the SD of the residuals of individual litters (gray bars) for each sow. The red lines represent the mean of SD of the residuals ± SD of SD of the residuals and the green shadow represents mean of the SD of residuals. Sows with the SD of the residuals within the red lines were considered to exhibit a consistent phenotype (highly repeatable litter BW).

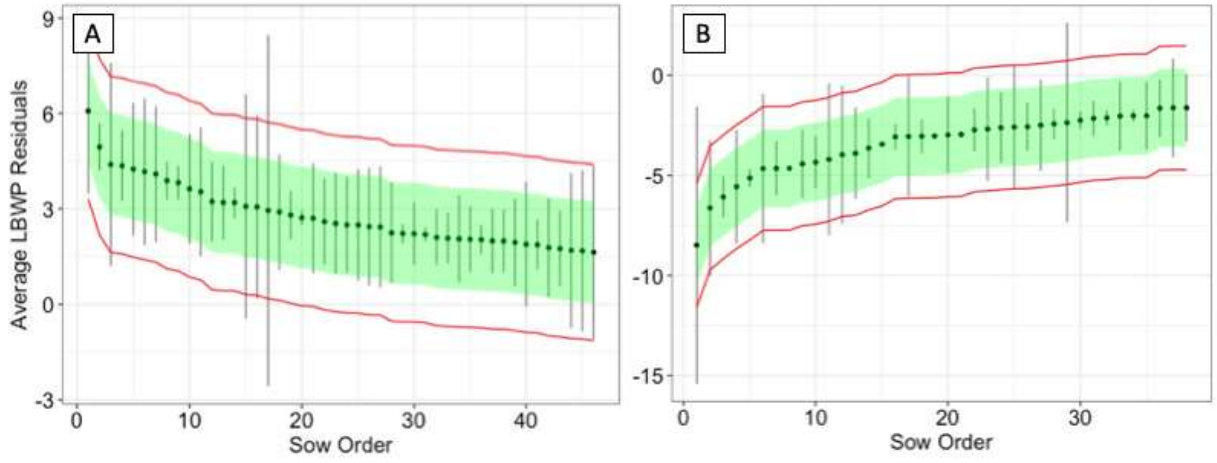


Figure 3.3. Relationship between litter size (total number of piglets born) and BW (average birth weight per litter) for Large White purebred sows where average litter birth weight >1.56 kg represents HLBWP (●), 1.55 to 1.21 kg represents Medium LBWP (■) and <1.2 kg represents LLBWP (▲).

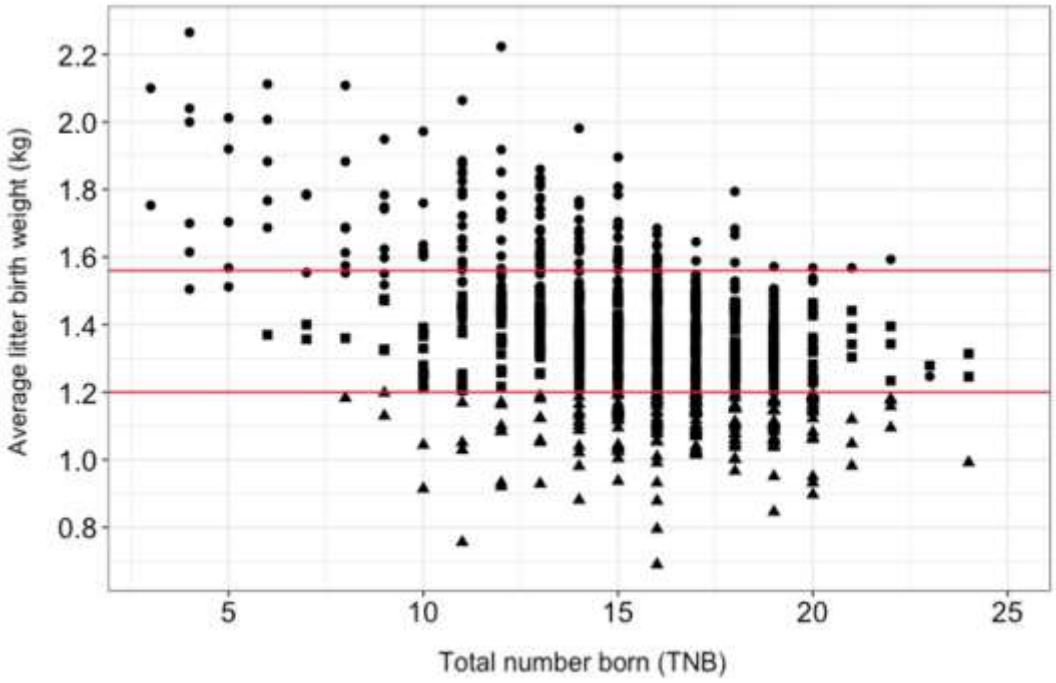


Figure 3.4. Results of the selection for LBWP sows, with HLBWP (•) and LLBWP (▲). **Graphic (A):** Mean LBWP residuals for the selected sows; **Graphic (B):** LBWP repeatability for the selected sows; **Graphic (C):** LBWP residuals for TNB categories; **Graphic (D):** LBWP repeatability for TNB categories.

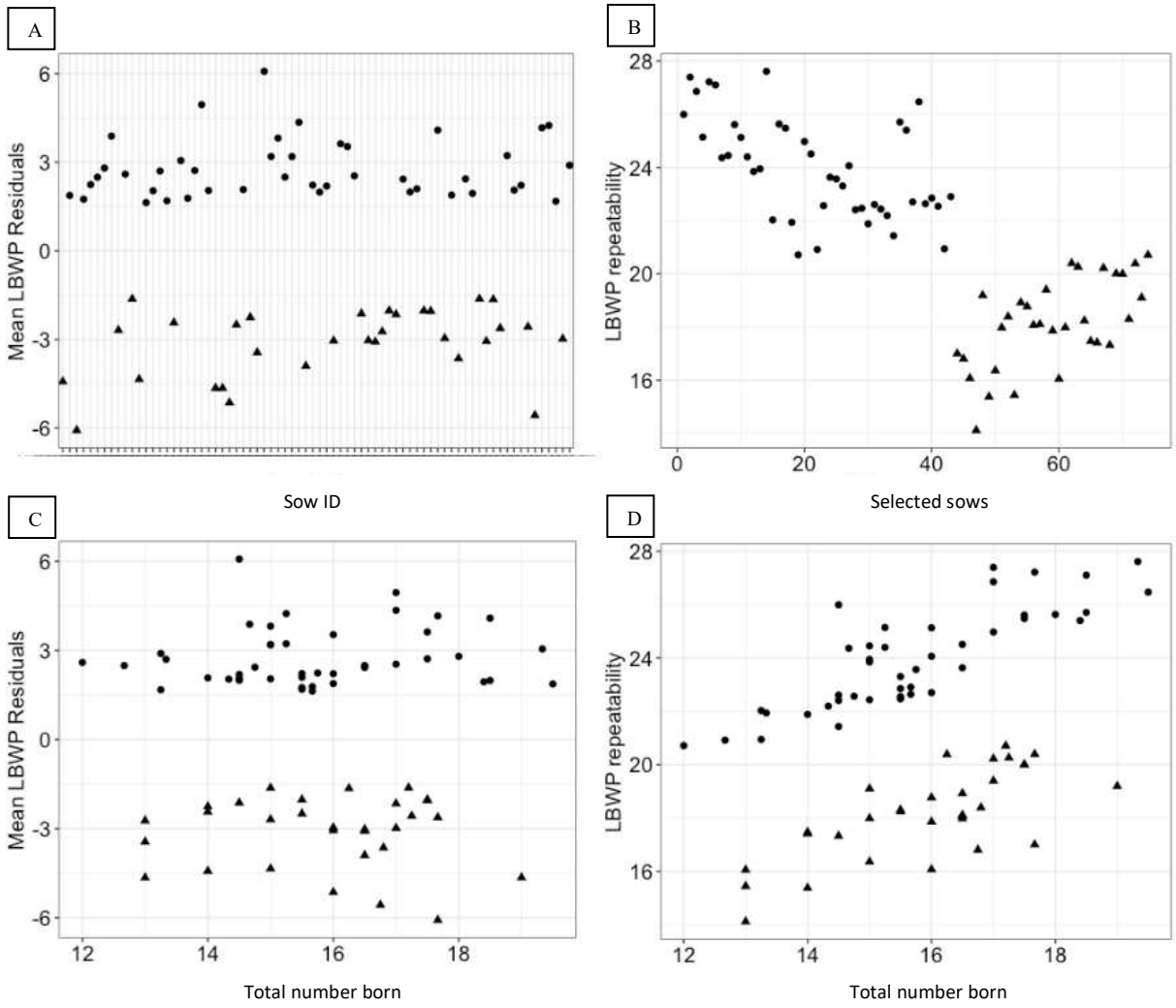


Figure 3.5. Reproductive measurements of the selected sows, with HLBWP (•), LLBWP (▲). **Graphic (A):** Association between total litter birth weight (kg) and mean individual birth weight; **Graphic (B):** Association between mean individual birth weight (kg) and TNB; **Graphic (C):** Repeatability of average litter birth weight phenotype over the parities by Group of LBWP.

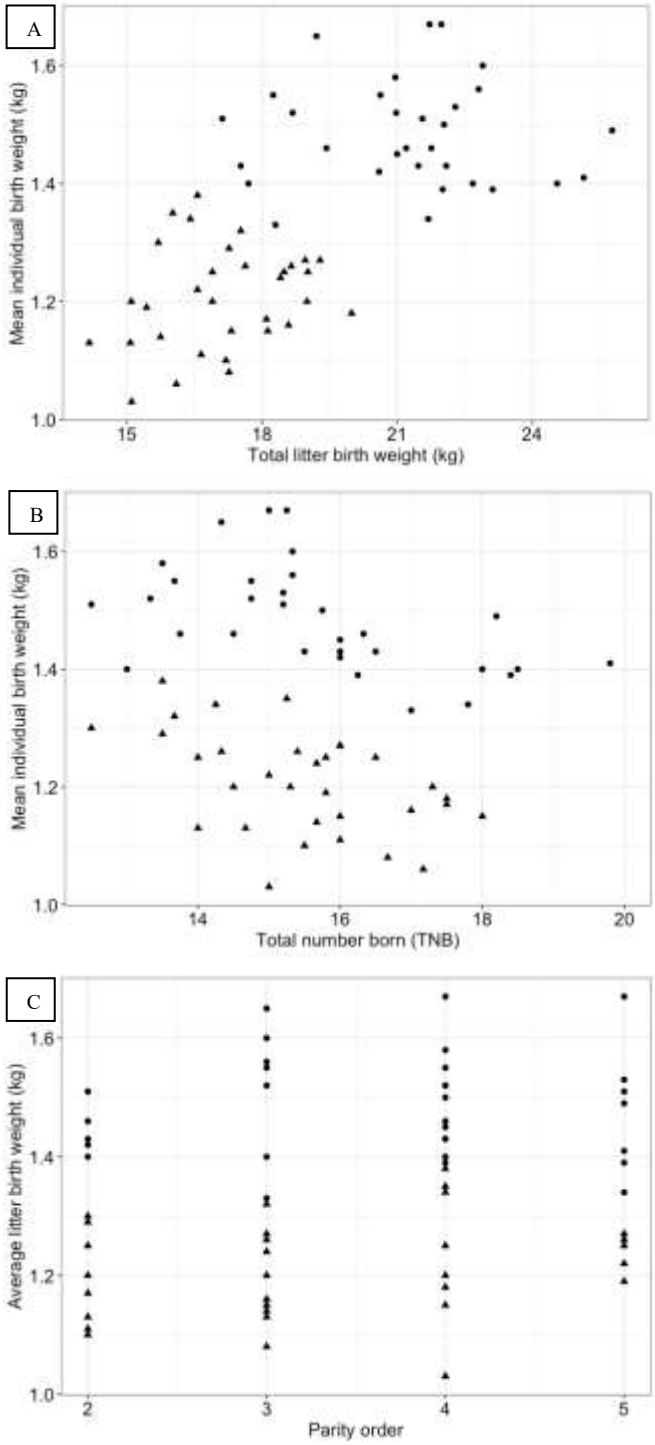


Figure 3.6. Embryonic viability classification. **Figure (A):** viable embryo; **Figure (B):** hemolyzed embryo; **Figure (C):** underdeveloped embryo.

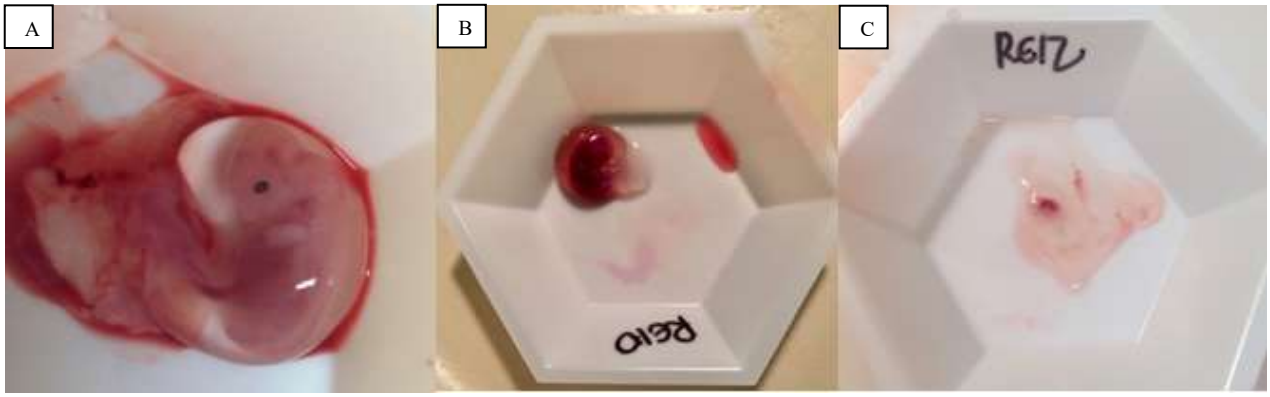


Figure 3.7. Example of embryonic sexing results. The embryonic sex was determined by identifying embryos with one band as female and two bands as male. In every run a known sample of female and male embryos were added as controls. PCR products represented by a lower band (400bp) and upper band (506bp).

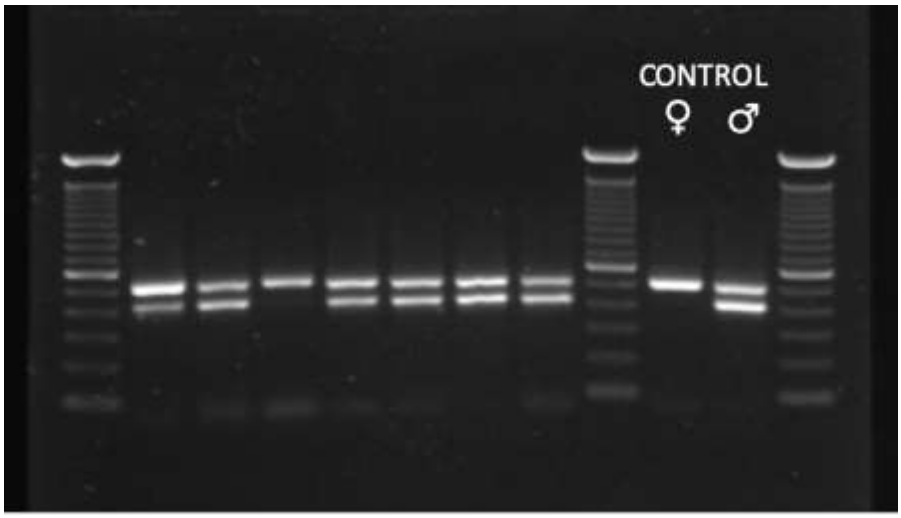


Figure 3.8. Graphic (A): Results of ovulation rate (OR), total number of embryos (TNE) and total number of viable embryos (TNV)**; Graphic (B):** Results of early, late and total embryonic survivability for LLBWP and HLBWP. Where early survivability was assessed by (TNE/OR), late survivability (TNV/TNE) and total survivability (TNV).

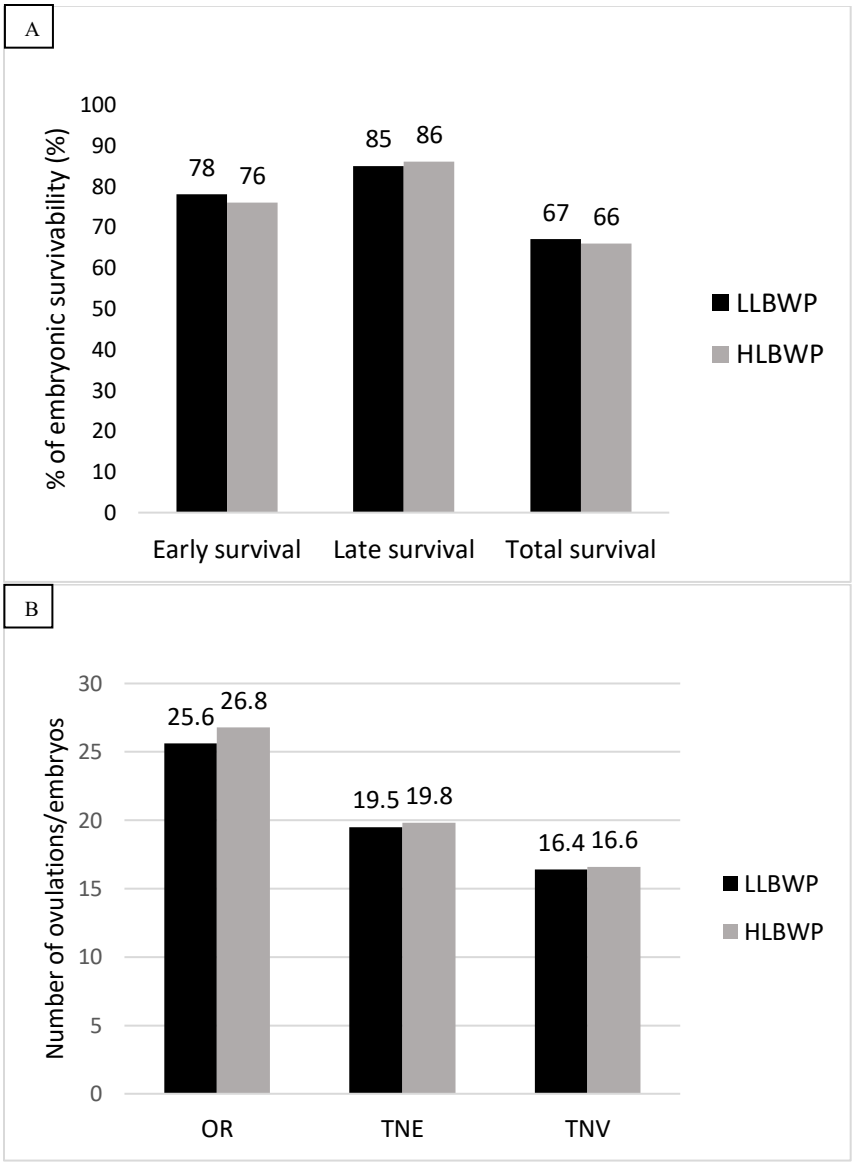


Figure 3.9. Results of EW, CRL and AFV for LLBWP and HLBWP. There was no significant difference between LBWP groups (P>0.05) for EW and CRL. AFV had a significant difference (P<0.05).

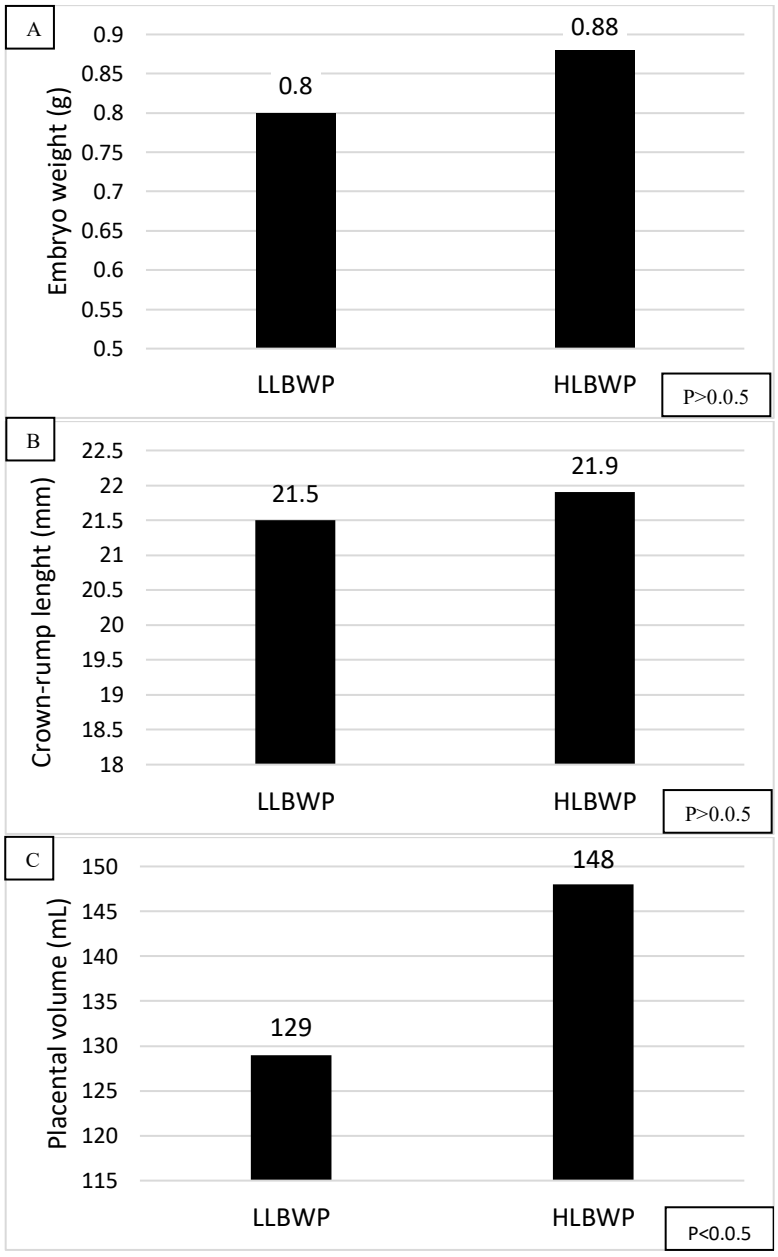


Figure 3.10. Graphic (A): Relationship between OR and TNE at D30 of gestation (Ovulation rate = 11.59 + 0.32 R^2 = 0.09; P = 0.003; n = 36, LLBWP and HLBWP sows); **Graphic (B):** Relationship between OR and TNV (Ovulation rate = 11.62 + 0.21; R^2 = 0.03; P =0.15; n = 36); **Graphic (C):** Relationship between TNE and TNV (TNE = 1.09 + 0.80 R^2 = 0.67; P <0.001; n = 36, LLBWP and HLBWP sows).

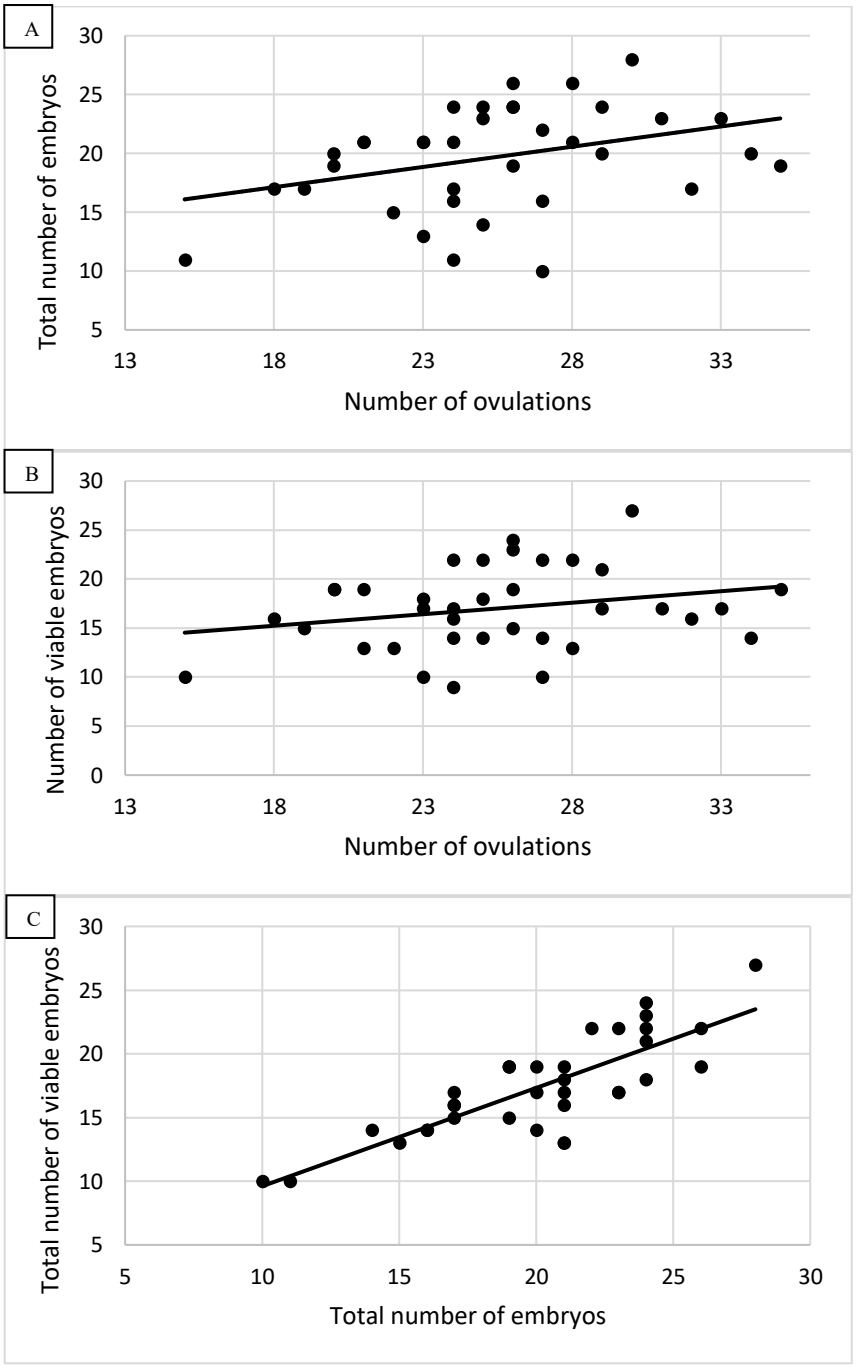


Figure 3.11. Graphic (A): Relationship between AFV and EW at D30 of gestation (average placental weight = 0.39 + 0.003 (average embryonic weight); R^2 = 0.46; P < 0.001; n = 617, LLBWP and HLBWP embryos and placenta); **Graphic (B):** Relationship between AFV and CRL at D30 of gestation (average placental weight = 17.67 + 0.02; R^2 =0.33 P < 0.0001; n = 617, LLBWP and HLBWP embryos and placenta). **Graphic (C):** Relationship between CRL and EW at D30 of gestation (average placental weight = 15.17 + 7.57; R^2 = 0.57 P < 0.001; n = 617, LLBWP and HLBWP embryos and placenta).

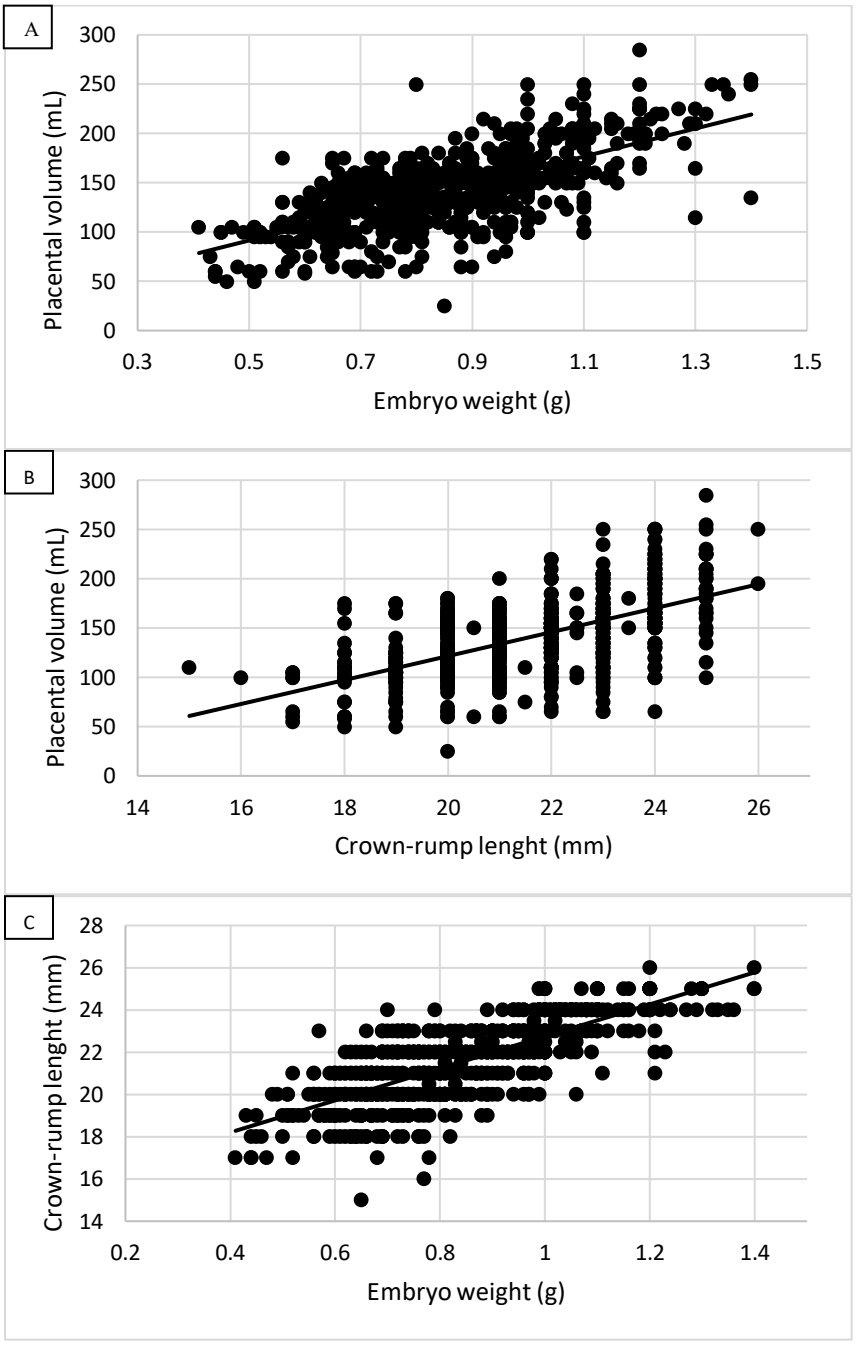


Figure 3.12. Results of early survivability **(Graphic A)** , late survivability **(Graphic B)** and total survivability **(Graphic C)** when LBWP and TNE Categories were considered covarietes and their interaction was analyzed. Values without a common superscript (a, b) differed significantly ($P < 0.05$).

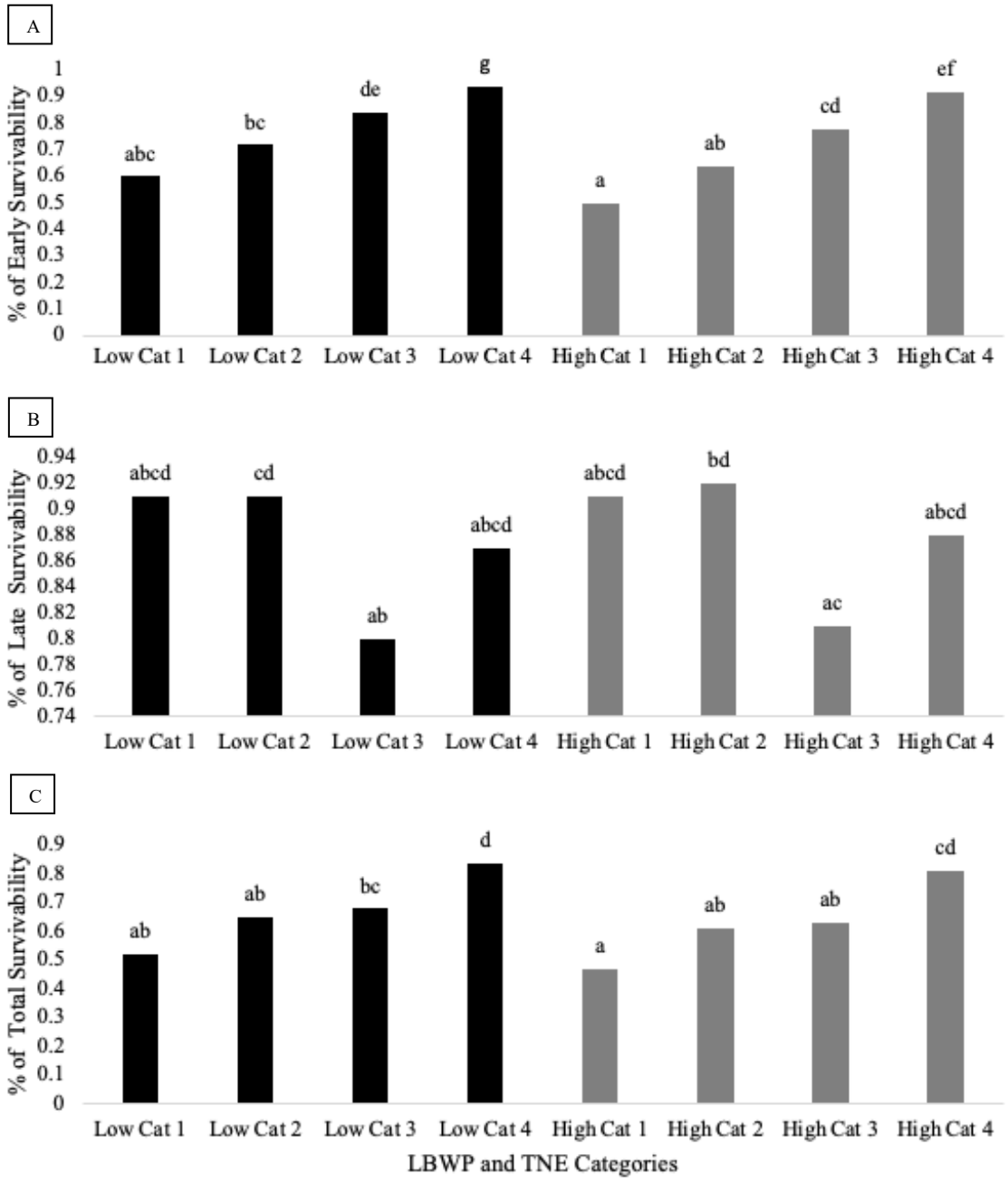
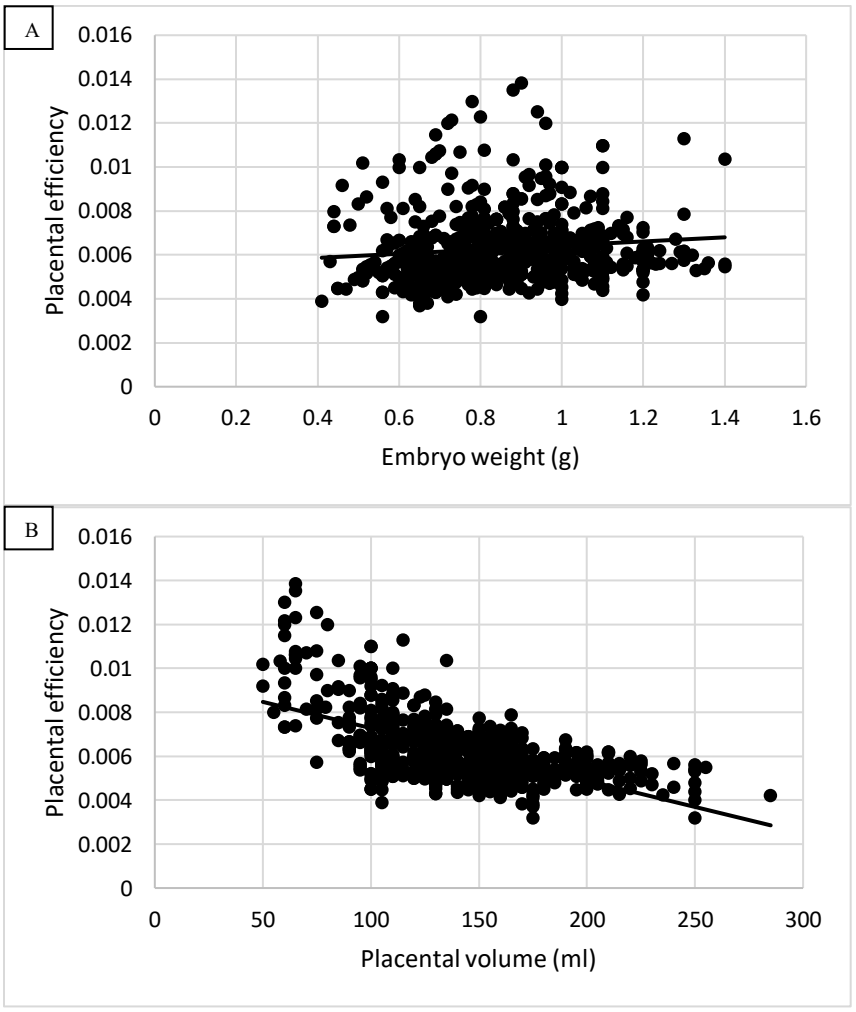


Figure 3.13. Graphic (A): Relationship between placental efficiency and EW at D30 of gestation (average placental efficiency= 0.76 + 13.71 (average embryonic weight); $R^2 = 0.01$; $P < 0.05$; n = 617); **Graphic (B):** Relationship between placental efficiency and placental volume (AFV) at D30 of gestation (average placental weight = 236.286 - 15080; $R^2 = 0.35$ $P < 0.0001$; n = 617).



CHAPTER 3 - TABLES

Table 3.1. Historical reproductive data for the selected LLBWP and HLBWP sows. Least square means ± s.e.m for historical reproductive data for the selected LLBWP and HLBWP sows.

	LLBWP (n=20)	HLBWP (n=20)
Mean parity order	3.4 ± 0.19	3.74 ± 0.20
Number of litters	109	116
Average birth weight, kg	1.21 ± 0.03[a]	1.50 ± 0.03[b]
Total litter birth weight, kg	18.94 ± 0.59[a]	22.21 ± 0.57[b]
Total number of piglets born	16.03 ± 0.74	15.71 ± 0.73
Total number of piglets born alive	14.47 ± 0.35	14.16 ± 0.35
TBA/TNB	0.92 ± 0.01	0.91 ± 0.01
Total number of piglets weaned	12.27 ± 1.32	12.30 ± 1.31
Piglets weighing <800 g, %	0.11 ± 0.01[a]	0.03 ± 0.006[b]

Within rows, values without a common superscript (a, b) differed significantly (P < 0.05)

Table 3.2. PCR sexing primer information.

Primer names	Sequence	Start	Stop	Length	GC content	Melt temp[a]
SRY Forward	GGGAAAGGCTCCTCACTATTT	91	112	21	47.6%	62°C
SRY Reverse	AGGGATACATCCTCTCCTCTAC	469	491	22	50%	62°C
ZFX Forward	GTGCTGCTTTGTCTTGGAATG	3291	3312	21	47.6%	62°C
ZFX Reverse	GAGGGAGTTAGGTCTGGATACT	3775	3797	22	50%	62°C

[a] Melting temperature calculation according to the Tm requirement of Phire hot Start II DNA Polymerase from thermo-scientific-web-tools/tm-calculator

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Table 3.3. Results of reproductive characteristics of sows (least square means ± SEM) for LLBWP and HLBWP.

Variable	LLBWP (n=18)	HLBWP (n=19)
Day of gestation at slaughter	29.11 ± 0.74	29.09 ± 0.66
Breeding rate (%)	100 (18/18)	100 (19/19)
Pregnancy rate (%)	100 (18/18)	100 (19/19)
Ovulation Rate	25.6 ± 1.06	26.8 ± 1.06
Total Number of Embryos	351	357
Mean Total Number of Embryos	19.5 ± 1.19	19.8 ± 1.12
Total Number of Viable Embryos	306	318
Mean Total Number of Viable Embryos	16.4 ± 1.37	16.6 ± 1.37
Early Embryonic Survival (%)	0.78 ± 0.02	0.76 ± 0.02
Late Embryonic Survival (%)	0.85 ± 0.01	0.86 ± 0.01
Total Embryonic Survival	0.67 ± 0.02	0.66 ± 0.02
Embryonic Weight (g)	0.80 ± 0.05	0.88 ± 0.04
Crown-rump Length (mm)	21.5 ± 0.71	21.9 ± 0.68
Allantochorionic Fluid Volume (mL)	129 ± 10.71[a]	148 ± 10.31[b]

Within rows, values without a common superscript (a, b) differed significantly ($P < 0.05$)

Table 3.4. Effect of parity classes (P) and LBWP Groups on OR, embryonic and placental measurements.

	LLBWP		HLBWP	
	P1	P2	P1	P2
Ovulation Rate	24.6 ± 1.18	26.4 ± 1.47	25.8 ± 1.43	27.6 ± 1.16
Early Embryonic Survival (%)	0.80 ± 0.02	0.75 ± 0.02	0.79 ± 0.02	0.73 ± 0.02
Late Embryonic Survival (%)	0.85 ± 0.02	0.86 ± 0.02	0.86 ± 0.02	0.86 ± 0.02
Total Embryonic Survival	0.69 ± 0.02	0.64 ± 0.03	0.68 ± 0.02	0.64 ± 0.02
Embryonic Weight (g)	0.77 ± 0.05	0.85 ± 0.05	0.82 ± 0.05	0.91 ± 0.05
Crown-rump Length (mm)	20.8 ± 0.42	22.0 ± 0.48	21.2 ± 0.46	22.4 ± 0.40
Allantochorionic Fluid Volume (mL)	123.6 ± 10.5[a]	138.1 ± 11[ab]	140.2 ± 10.8[ab]	154.6 ± 10.04[b]

Table 3.5. Effect of LBWP and day of collection categories on embryonic and placental measurements.

Variable	LLBWP			HLBWP		
	Day of Gestation (Euthanasia)					
	28	29	30	28	29	30
Embryo Weight, g	0.71±0.06	0.82±0.04	0.84±0.05	0.79±0.06	0.89±0.04	0.91±0.05
Crown-Rump Length, mm	20.85±0.59	21.19±0.39	22.03±0.52	21.40±0.57	21.74±0.41	22.59±0.44
Allantochorionic Fluid Volume, mL	114.4±11.8^{a}	134.7±7.59^{ab}	143.7±9.9^{ab}	130.8±10.8^{ab}	151.2±7.9^{ab}	160.1±8.86^{b}

Within rows, values without a common superscript (a, b) differed significantly ($P < 0.05$)

Table 3.6. Effect of LBWP and TNE categories on embryonic and placental measurements.

Variable	LLBWP				HLBWP			
	Cat 1	Cat 2	Cat 3	Cat 4	Cat 1	Cat 2	Cat 3	Cat 4
	(10-14)	(15-19)	(20-23)	(24-28)	(10-14)	(15-19)	(20-23)	(24-28)
Early E. Survivability	0.60 ± 0.05^{abc}	0.72 ± 0.03^{bc}	0.84 ± 0.02^{de}	0.94 ± 0.01^{g}	0.50 ± 0.05^{a}	0.64 ± 0.03^{ab}	0.78± 0.02^{cd}	0.92 ± 0.01^{ef}
Late Embryonic Survivability	0.91 ± 0.04^{abcd}	0.91 ± 0.02^{cd}	0.80± 0.02 ^{ab}	0.87 ± 0.02^{abcd}	0.91± 0.03^{abcd}	0.92 ± 0.02^{bd}	0.81 ± 0.02^{ac}	0.88 ± 0.02^{abcd}
Total Survivability	0.52 ± 0.05^{ab}	0.65 ± 0.03^{ab}	0.68 ± 0.03^{bc}	0.84 ±0.02^{d}	0.47 ± 0.05^{a}	0.61 ± 0.03^{ab}	0.63 ± 0.03^{ab}	0.81 ± 0.02^{cd}
Embryo weight (g)	0.74 ± 0.1	0.83 ± 0.06	0.79 ± 0.06	0.78 ± 0.07	0.83 ± 0.09	0.92 ± 0.06	0.88 ± 0.06	0.86 ± 0.06
Crown-rump length (mm)	21.3 ± 0.9	21.6 ± 0.5	21.2 ± 0.5	20.8 ±0.7	22.0 ± 0.8	22.3 ± 0.5	21.9 ± 0.6	21.5 ± 0.6
Allantochorionic Fluid Volume (mL)	131.2 ± 15.7^{ab}	145.2 ± 11.5^{ab}	127.4 ± 11.6^{ab}	115.5 ± 12.7^{a}	149.2 ± 14.9^{ab}	163.3 ± 11.5^{b}	145.4 ± 11.8^{ab}	133.5 ± 12.0^{ab}

Within rows, values without a common superscript (a, b) differed significantly ($P < 0.05$).

Table 3.7. Influence of embryo sex on embryonic and placental characteristics and embryonic.

Variable	LLBWP		HLBWP	
Sex ratio (%)	0.47		0.54	
	Female	Male	Female	Male
Total Number (n)	156	133	149	181
Embryonic Weight (g)	0.80 ± 0.05	0.81 ± 0.05	0.87 ± 0.05	0.88 ± 0.05
Crown-rump length (mm)	21.4 ± 0.48	21.4 ± 0.48	22.0 ± 0.44	22.0 ± 0.44
Placental Volume (mL)	131 ± 9.58	133 ± 9.57	148 ± 9.10	150± 9.07

Within rows, values without a common superscript (a, b) differed significantly ($P < 0.05$)

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CHAPTER 4

RNA sequencing identifies differentially expressed genes from transcriptome of porcine embryos and placental tissues from high and low litter birth weight phenotype sows

4.1 Introduction

As outlined in the previous chapters of this Thesis, after years of genetic selection to increase the total number of piglets born per litter (TNB), it is possible to identify an extreme population of sows in every herd that give birth to low birth weight (BW) piglets, independent of litter size. The low litter birth weight phenotype (LLBWP) is a sow-related phenotype expressed in the piglets, in which sows consistently produce low BW litters across parities (Patterson & Foxcroft, 2019). Different from individual low BW, the LLBWP affects the growth performance of entire litters. Recent research has demonstrated that litter average BW shows between 40 to 60% repeatability, with some sows consistently producing low BW piglets over multiple parities (Smit et al, 2013; Patterson et al, 2011; Zhang et al, 2018). Nonetheless, the phenotype is passed down through generations, perpetuating the problem in the production system (Zhang et al, 2018).

The BW phenotypic outcome of the litter is dependent on adequate placental morphogenesis and intrauterine environment (Foxcroft et al, 2009; Reynolds et al, 2009). The conceptuses are dependent upon the placenta for the regulation of nutrients, gases, and waste exchanges between them and the mother. In turn, the functional capacity of the placenta to supply the above-mentioned demands is under the control of the fetal and maternal genomes (Angioloni et al, 2006). Furthermore, the establishment of pregnancy requires a close physical and molecular communication between the conceptuses and the maternal reproductive tract that begins during implantation and continues until the placenta is fully formed (Jessmon et al, 2009). In Chapter 3, the physiological data showed that the uterine capacity in LLBWP sows affected optimal placental

development, which in turn affects the placental volume and embryonic development. However, there is clearly more to placental development than changes in placental volume during gestation. With that in mind, the ability to identify candidate genes and biological pathways, together with the physiological data previously analyzed, is expected to facilitate a better understanding of how the litter birth weight phenotype (LBWP) affects these traits.

Usually two different strategies are used to detect the effect of specific genes on a trait, such as BW (Snelling et al, 2013; Karisa et al, 2013). The first uses linkage analyses to detect regions harboring the genes related to the trait (Steibel et al, 2011). The second identifies candidate genes via their physiological role in reproduction or locates the genomic region linked with a phenotype (Spotter & Distl, 2006). Significant association has been shown for litter BW and individual BW with regions on *Sus scrofa* chromosomes (SSC) 1, 4, 5, 6, 7 (Zhang et al, 2014). Also, Zhang et al (2018) analyzed the genotypes of sows from the same population used in this study and found associations with both individual and litter BW on SSC1, SSC9, and SSC19. In the present project, to obtain a better understanding of the manifestation of the LBWP phenotypic sows, RNA-sequencing analysis (RNA-seq) was used to identify genes differentially expressed by LLBWP and high litter birth weight phenotype (HLBWP) sows in embryonic and placental tissues. RNA-seq allows for the precise quantification of transcript levels in tissues and cells and enables scientists to explore how organisms respond to certain conditions (Gracey & Cossins, 2003). This technical capacity provides the ability for comprehensive gene expression profiling to identify active and inactive genes under certain experimental conditions, such as the use of nutrient additives (Dalto et al, 2015), microbiome changes (Fouhse et al, 2019) or different physiological and developmental stages in porcine breeding (Tsoi et al, 2012; Tsoi et al, 2016). These studies provide detailed gene expression profiles related to the underlying molecular pathways and gene

interactions in order to unravel the causal relationships between genes and particular physiological conditions. Therefore, the ability to understand how embryonic development is regulated, the central genes, and molecular markers that are related to appropriate development and embryonic quality, contributes to the understanding of basic reproduction and facilitates improved maternal management (Tsoi et al, 2012).

4.2 Materials and Methods

4.2.1 General

All animal procedures were conducted at the University of Alberta, Swine Research and Technology Centre (SRTC, Edmonton, Alberta, Canada) with the approval of the Faculty Animal Policy and Welfare Committee – Livestock (Protocol - AUP00002650). As previously described in Chapter 3 of this thesis, 40 (n=20 LLBWP and n=20 HLBWP) pure bred Large White sows from a nucleus farm from Hendrix Genetics (Tullymet Nucleus Unit, Balcarres, SK) were selected based on their reproductive and BW history as their expression of a clear LLBWP and HLBWP over at least 2 successive parities. The selection concentrated on the bottom 12% and top 12% of the population, from which the average litter BW was 1.20 kg for LLBWP and 1.50 kg for HLBWP. The sows were received at the SRTC and were bred with semen from purebred Large White boars of proven fertility (Hendrix Genetics) on their second estrus following altrenogest withdrawal (Matrix^{tm}, Merck AH, Kenilworth, NJ). Sows were euthanized on day 28 to 30 of gestation (mean ± s.d; day 29.15 ± 0.6) and samples of placenta and embryos were individually collected. Total number of embryos (TNE), embryo weight (EW), embryonic viability, and crown-rump length (CRL) measurements were recorded, along with the ovulation rate (OR) and allantochorionic fluid volume (AFV) as described in Chapter 3 of this Thesis.

4.2.2 Sow selection for genomic purposes

Data analysis on EW was conducted using "lme4" package (Bates et al, 2018). The EW was adjusted for the random effects: day of collection, parity class, sow ID and TNE. The fixed effect was Group (LLBWP and HLBWP). The mean EW for LLBWP and HLBWP was not significantly different (LLBWP: 0.80 ± 0.05; HLBWP: 0.88 ± 0.04, P=0.18). Within each LBWP group, 4 sows with the individual EW falling within the mean EW of the group ± s.d and with at least 10 embryos were selected for gene expression analyses. Within each sow, 4 male and 4 female embryos were pooled separately. Finally, 4 placental tissues from each of the selected sows were also selected for genomic purposes.

4.2.3 Total RNA extraction from D30 embryos and placenta

Each frozen embryo was individually placed into a pre-frozen mortar on dry ice. Liquid nitrogen was poured into the mortar to cover the embryo and it was ground into a fine powder using a pre-chilled pestle. The embryonic powder was then transferred to a pre-labeled sample tube with a micro-spatula and stored at -80°C until further manipulation. Examination gloves were changed after each embryo manipulation to avoid cross contamination between samples (Blanes et al, 2016). Approximately 0.05 mg of powdered embryo (n=64) or placental tissues (n=32) was transferred into a 2 ml micro-centrifuge tube containing 500 µl of Nucleozol® Reagent (MACHEREY-NAGEL, Bethlehem, PA 18020, USA) and homogenized into lysates. Total RNA was purified from lysates using NucleoSpin® RNA silica spin columns (MACHEREY-NAGEL, Bethlehem, PA 18020, USA) as per the manufacturer's instructions. Total RNA QC was performed using Agilent 2200 TapeStation System (Agilent, USA) and NanoDrop (Thermo, USA). At least 1 µg of high quality total RNA (RNA integrity number (RIN) > 8.0) from each sample was shipped to BGI (Shenzhen, China) for RNA sequencing (RNA-seq) service including library construction and bioinformatics analysis.

4.2.4 Gene expression, library template construction and sequencing

Total RNA samples were purified for mRNA isolation performed using poly-T-oligo attached magnetic beads from total RNA and cleaved into small fragments using divalent cations under elevated temperature. Double-stranded cDNA was created using random priming with reverse transcriptase, DNA Polymerase I and RNase H. After adaptor ligation to both ends of cDNA molecules, cDNA enrichment was done by PCR. The double stranded PCR products were heat denatured and circularized by the splint oligo to create a final library of single strand DNA circle (ssDNA circle). Finally, these ssDNA circle molecules were used to create DNA nanoballs (DNBs) by rolling circle replication (RCR) and loaded into the patterned nanoarrays with pair-end reads of 100 bp to read through on the BGISEQ-500 platform (BGI, Shenzhen, China). DNBSeq™ is a recent Next Generation Sequence platform and the library templates construction is different from Illumina. It uses DNA nanoballs (DNB) and combinatorial probe-anchor synthesis (cPAS), giving a higher signal-to-noise ratio, higher spot densities, and faster sequencing times (Fehlmann et al, 2016).

4.2.5 Bioinformatics workflow

4.2.5.1 Read mapping and gene quantification

In this project, the BGISEQ-500 platform was used to generate raw sequence reads. After using SOAPnuke software (GigaScience, https://doi.org/10.1093/gigascience/gix120), reads from adaptors, unknown bases (N) and low-quality sequences were removed. Finally, clean reads were stored in FASTQ format. HISAT (Kim & Salzberg, 2015) was used to map clean reads to the reference genome (Sscrofa 11.1) and Bowtie2 (Langmead & Salzberg, 2012) was used to map clean reads to reference transcripts. All sequences data were deposited to the NCBI SRA (Sequence Read Archive) database at BioProject with accession number: PRJNA608736. Gene

expression levels were calculated using the Fragments Per Kilobase of exon model per Million mapped reads (FPKM) method provided by RSEM software (Li & Dewey, 2011).

4.2.5.2 Differentially expressed gene detection

DEGs detection was performed by the comparison between LLBWP and HLBWP directly from the placental tissues and D30 embryos with the same sex shown in Figure 4.1. DEseq2 algorithm (Love et al, 2014) was applied to measure the gene expression levels using FPKM value and filter DEGs, respectively. The criteria for filtering DEGs were as follows: Log2 Fold Change ≥ 0.7 (up-regulated genes) or ≤ 0.7 (down-regulated genes) and Adjusted P-value ≤ 0.05.

4.2.5.3 Functional enrichment analysis for DEG

Using gene ID from the DEGs of D30 embryo and placental tissues to identify gene symbol (GS) and GenBank accession numbers (GA#) for the pig, the correct identification of human GS for the same GS in pig was performed by Blast search. Gene ontology (GO) and pathway analysis of DEGs were conducted using *GOnet* (https://tools.dice-database.org/GOnet/) (Pomaznov & Peters, 2018). Fisher's exact test was applied to identify the significant GO categories or biological process, selecting the analysis type for GO term enrichment. Corrected P-values calculated according to FDR control procedure were considered significant when lower than 0.05 calculated according to FDR control procedure (Benjamini & Hochberg, 1995).

4.2.6 Quantitative real-time RT-PCR validation

A two-step quantitative real-time RT-PCR (RT-qPCR) was performed. Eight RNA samples from embryos (LLBWP, n = 4 and HLBWP, n = 4) and six placental tissues (LLBWP, n = 3 and HLBWP, n = 3) were selected using the same samples as for RNA-seq. The first-strand of cDNA synthesis started with 500 ng of total RNA after ezDNase treatment to remove DNA before

using it in 20 µl of the SuperScript™ IV VILO™ Master Mix (Invitrogen, USA). After synthesis, 2 µl of the total cDNA reaction mixture was taken as a template to perform the real-time qPCR with PrimeTime® RT-PCR Kit (IDT, USA) in 10 µl total volume of qPCR according to the instruction manual on QuantStudio Flex 6 system (Thermo Fisher Scientific, CA) all samples were assayed in duplicate wells. Fast program was used as follows: 95°C for 3min, followed by 45 cycles of 95°C for 5s, 60°C for 30s. Ct values and primer efficiencies were obtained from SDS2.3 Software (Applied Biosystems) installed in the system by performing auto-setting for threshold cycle (Ct) and baseline calculation.

Seven DE genes (*HMBS*, *MT1A*, *RHAG*, *SLC22A16* from D30 embryo; *CDSN*, *HBEGF*, *PDPN* from placenta tissues) were validated by RT-qPCR. PrimerQuest tool from IDT (https://www.idtdna.com/Primerquest/Home/Index) was used to design primers and probes for both DE and reference genes (*HPRT1*, *PGF*). Validation of the final ideal primer and probe sequences was conducted to ensure they were not located within the same exon using Primer-Blast from NCBI (Ye et al, 2012). The sequence information for the primers is given in Table 4.1. For RT-qPCR analysis, the Relative Expression Software Tool 2009 (REST; http://rest.gene-quantification.info/) was used to implement a randomized test (Pfaffl et al, 2004) and to assess statistical significance of the up- or down-regulation of the target genes after normalization to the reference gene. Statistical analyses were considered significant when $P \leq 0.05$.

4.3 Results

4.3.1 Transcripts mapping and annotation

After mapping clean reads to the pig reference genome and transcripts, the average genome and uniquely gene mapping rate was 91.26% and 62.17% respectively. In D30 embryos, 18,018 genes were identified in which 17,256 were known genes and 763 were novel genes. In placental

tissues, 17,140 were identified in which 16,407 were known genes and 733 were novel genes. To show the number of common and unique genes between embryo and placenta, we counted the average gene quantity from individual samples in each LBWP group and the result is shown in Table 4.1S.

4.3.2 DEG analyses in D30 embryos and placental tissues

The level of gene expression (FPKM) on D30 embryos from sows with LLBWP and HLBWP are shown in Table 4.2S and Table 4.3S for female and male embryos, respectively. The overall expression levels between the LLBWP and HLBWP group from female (Figure 4.2A) and male (Figure 4.2B) embryos were nearly identical. Therefore, there was no embryonic sex difference in gene expression from sows between the two groups. DEseq2 algorithm was used to identify DEG of D30 embryos from LLBWP and HLBWP. A total of 160 genes were down-regulated and 4 genes were up-regulated in LLBWP as compared to HLBWP (Table 4.4S) and (Figure 4.3). Among all the 160 down-regulated genes, two gene families *MYADM* and *SLC7A3* (Table 4.5S) were found at multiple loci located on chromosome 7 and 6 (Figure 4.4) respectively.

A similar approach was applied to find the level of gene expression (FPKM) in placenta from sows with LLBWP and HLBWP, as shown in Table 4.6S. A scatter plot was generated to visualize the quantitative differences in gene expression between LLBWP to HLBWP, a total of 82 DEGs in placental tissues (Table 4.7S) with 43 down-regulated genes (green dots) and 39 up-regulated genes (red dots) in LLBWP compared to HLBWP (Figure 4.5).

4.3.3 Functional enrichment analysis for DEGs in embryonic and placental tissues

GO term enrichment was performed first using ***GOnet*** on DEGs in D30 embryo and GO entries with corresponding gene numbers ≥2 in the three classifications were screened, including 75 biological processes (Table 4.8S), 2 cellular components (Table 4.9S) and 9 molecular

functions (Table 4.10S). Two biological processes were found in placental tissues, both related to anatomical structure morphogenesis and tissue development (Table 4.11S). No statistically significant cellular components and molecular functions were found in placental tissues. Most annotated genes in biological processes category in D30 embryo were also related to the anatomical structure morphogenesis (57 genes) and regulation of biological quality (49 genes), while the least annotated genes were related to the carnitine transmembrane transport (*SLC22A16*, *SLC22A4*) and peptidyl-arginine ADP-ribosylation (*ART4*, *ART5*).

The more detailed analysis using *GOnet* specifically aims to construct and display interactive graphs that include GO terms and genes while retaining term-gene relationships. A graphical network output shows how some DEGs interact with each other from different biological processes in D30 embryos (Figure 4.6) and placental tissues (Figure 4.7) in LLBWP compared to HLBWP. For example, in LLBWP D30 embryos down-regulated genes such as *CCR1*, *CCRL2*, *CCR3* and *XCR1* involved in chemokine-mediated are taking part in multiple biological processes: immune system, cellular divalent inorganic cation and cellular metal ion homeostasis. Also, metallothioneins such as *MT1A, MT2A, MT1E* and *MT1X* are involved in cellular response of copper and zinc ions, detoxification of copper ion, cellular and transition ion homeostasis. Two GO terms, erythrocyte differentiation (*AHSP, ALAS2, DMTN, DYRK3, EPB42, GATA1, IKZF1, KLF1, RHAG, SLC4A1, TAL1, TRIM10*) and ammonium transmembrane transport (*AQP1, RHAG, RHCE, SLC22A16, SLC22A4*) were most significantly enriched (in dark green) (Figure 4.6). In placental tissues from LLBWP compared to HLBWP, 7 down-regulated genes (*COBL, CRABP2, FREM2, ISL1, NTN4, SEMA3B, SEMA3E*) and 9 up-regulated genes (*BMP4, CDSN, EGFLAM, GCNT3, HBEGF, KLK14, KRT17, PDPN, RIPOR2*) are both involved in anatomical structure morphogenesis and tissue development (Figure 4.7).

4.3.4 Validation of DEG data by RT-qPCR

The LBWP (LLBWP and HLBWP) effect on the expression of some genes related with the main GO terms from D30 embryos and placental tissues confirmed by RT-qPCR is the same result as found in RNA-seq (Table 4.2). The expression of *HMBS, MT1A, RHAG* and *SLC22A16* confirmed down regulation in D30 embryos whereas *CDSN, HBEGF* and *PDPN* were up regulated in placental tissues in LLBWP when compared to HLBWP.

4.4 Discussion

Sows displaying the LLBWP tend to produce litters with generalized low BW (Foxcroft et al, 2009). In the present population of sows, for example, 50% of the litters produced by LLBWP sows had an average individual BW of <1.2 kg, and there were no litters with BW ≥1.5 kg. Therefore, the group of sows displaying this phenotype is believed to make the most substantial contribution to the low BW of piglets and in the variation in postnatal growth performance, independent of the TNB (Foxcroft et al, 2009). Knowing that this trait is repeatable over parities and passed to the next generation of replacement gilts in the breeding herd, the ability to understand the biological and molecular mechanisms that are driving this scenario can be directed toward selection to increase the efficiency of the pig breeding herd (Foxcroft et al, 2009; Smit et al, 2013; Patterson et al, 2011; Zhang et al, 2018). As described in Chapter 3, the physiological data showed that the uterine capacity in LLBWP influenced placental development, which in turn affects placental volume and embryonic development. Therefore, the main goal of the work in this chapter was to understand the molecular mechanisms and biological pathways that are driving the LLBWP at the placental and embryonic level. The ability to understand normal maternal-embryonic dialogue at the molecular level is critical to developing breeding strategies that improve fetal development.

4.4.1 Genes differentially expressed in placental tissues from LLBWP and HLBWP

The placental interface mediates the interaction between the mother and the conceptuses. Gene expression studies have been performed in several species to examine how changes in gene expression participate in the crosstalk between the maternal and embryonic tissues (Pavlicˇev et al, 2017). Placental efficiency is known to be regulated by a variety of factors, including the surface area of exchange, the thickness of the exchange barrier, blood flow at both the maternal, and conceptuses sides and the number and efficiency of transporters (Angioloni et al, 2006). According to our molecular data results, several DEGs involved in placental biological pathways such as tissue development, anatomical structure and morphogenesis were negatively affected down regulated in the LLBWP sows.

In order to analyze the gene expression patterns of the LBWP groups, the DEGs were obtained from the comparison between LLBWP placentas versus HLBWP placentas. The comparison revealed 15 genes upregulated (*DSG3, SOX15, KRT17, PDPN, HBEGF, CDSN, KLK14, NGF, EPGN, GCNT3, RIRPOR2, EGFLAM, BMP4, ARHGAP22, FOXD2, CXCL8*), 13 downregulated (*PYY, MYADM, MYH15, SULT1B1, SEMA3E, CRABP2, SEMA3B, NTN4, COBL, FREM2, ISL1, NCMAP, TNFAIP2, TMEM176B*) and significantly associated with tissue development and anatomical structure morphogenesis pathways in LLBWP sows. Collectively, based on the action of these genes, biological pathways involved in tissue morphogenesis, angiogenesis, and nutrient transport activity and the immune function seem to be unbalanced and negatively affected in the LLBWP placental tissues. The mechanism by which each one of the genes acts, however, changes.

4.4.1.1 Placental angiogenesis and nutrient transport activity in LLBWP

In pigs, throughout gestation, the fetuses' increased demand for nutrients is met by the remodeling of placental folds and increased blood flow (Reynolds et al, 2009; Wright et al, 2016). Angiogenesis occurs mainly during two waves; the first one is during the post-implantation period to day 20 of gestation, and the second from days 50 to 70 (Tayade et al, 2007). As a result, proper regulation of placental angiogenesis seems to influence the efficiency through which the establishment and maintenance of pregnancy occurs (Stenhouse et al, 2019a,b). The process is regulated by a complex range of genes that have the ability to improve placental angiogenesis and consequently support placental development at critical stages of gestation. According to our findings, LLBWP expressed a group of genes that appear to be related to deficient angiogenesis processes and nutrient support.

Among them, PDPN (Podoplanin) is recognized to be part of the regulation of cell proliferation and wound healing (Freitag et al, 2013). This gene affects the mucin-type transmembrane protein, and in humans, it is known to play an essential role in a variety of physiological and pathological processes such as angiogenesis, inflammation, thrombus formation, and cancer progression, as well as in cellular adhesion, migration and chemotaxis (Freitag et al, 2013; Kandemir et al, 2019). PDPN is believed to be related to fetal vessel angiogenesis during the placental development. Altered expression of this gene may be related to impaired fetal interstitial fluid homeostasis and impaired angiogenesis (Wang et al, 2011). Nonetheless, *PDPN* expression was found to be upregulated during ischemia-hypoxia, inflammation, and in cases of pre-eclampsia in humans (Kandemir et al, 2019). Similarly, EGFLAM (EGF like fibronectin type III and laminin G domains) is known to be involved in cellular growth, differentiation, and proliferation, including angiogenesis. Any alteration of the expression pattern of this gene can

contribute to a reduction or modification on fetal development as a reduced ability on tissue development (Vicente et al, 2013).

Two of the placental DEGs, *NTN4* (*Netrin-4*), and *SEMA3B* (*Semaphorin 3B*), found in LLBWP are known to have anti-angiogenic properties. NTN4 functions in biological processes such as angiogenesis, morphogenesis, and differentiation (Dakouane-Giudicelli et al, 2014). The same author first reported an anti-angiogenic activity of NTN4 in the human placenta and suggested a possible involvement of NTN4 in angiogenesis-related pathologies as intrauterine growth retardation. Additionally, Zhou et al (2013) and Samara et al (2019) mentioned that SEMA3B is considered as a marker for pre-eclampsia in humans and there is evidence that its action may downregulate VEGF (Vascular endothelial growth factor) action. The gene action of VEGF is widely studied, and it is known to mediate the progressive increase in placental vascularity in pigs (Vonnahme et al, 2001, 2002).

Another pathway that directly influences adequate blood flow and nutrient transport is the cornification of placentas from LLBWP. Cornification is a natural process that drives toward programmed cell death, which results in corneocytes and lipids, essential to the cornified skin layer to have resistance, elasticity and water repellence (Levine & Kroemer et al, 2019). However, in the case of placental tissue, if exacerbated, this process can create a barrier and interfere in the crossing of nutrients and gases (Ishida-Yamamoto & Igawa, 2015). Among our findings, *CDSN* (Corneodesmosin) expression was upregulated in LLBWP placental tissues. Garrido-Gomez et al (2017) found an upregulation of this gene in human chorion with reduced blood perfusion. The reduced uterine perfusion is explained by the fact that the CDSN molecule is the major component related to the cornification of epithelial layers. In our research, other genes associated with cornification were up regulated in LLBWP sows, *KRT17* (*Keratin 17*) and *KLK14* (*Kallikrein-*

related peptidase 14), *KLK11* (*Kallikrein-related peptidase 11*), *EPGN* (*Epithelial mitogen*) and *DSG3* (*Desmoglein 3*). *DSG3* is also known to be related to cellular apoptosis, programmed cell death, cleavage of cellular proteins, cornification and keratinization (Eckhart et al, 2013).

4.4.1.2 Placental chemokine and immune system in LLBWP

Chemokines' primary function is to command immune cell migration into infected or inflamed tissue to initiate an effective immune response (Charo & Ransohoff, 2006, Du et al, 2014). They also play a role in angiogenesis, hematopoiesis and regulate activation, proliferation, differentiation, and apoptosis in the cells they attract (Drake et al, 2002; Hannan et al, 2007). Functional chemokines and their receptors are widely expressed in maternal-conceptus tissues and are a major player in tissue communication and pregnancy success (Du et al, 2014). Reproductive success relies on the ability of the maternal tract to remain tolerant to the fetuses and at the same time to protect them from infections (Warning et al, 2011). To achieve this goal, appropriate communication has to be established and maintained. The adverse effects of pro-inflammatory cytokines during human and mouse pregnancy are already well known (Polgar & Hill, 2002; Dent, 2002; Patrick & Smith, 2002). In women, these pathways can lead to endothelial cell injury, reduced blood supply, and subsequent embryonic death, in addition to deficient angiogenesis (Stemmer, 2000). Croy et al (2009) suggested that an elevated concentration of pro-inflammatory cytokines attack maternal endothelial cells, ultimately restricting blood supply to an already stressed conceptus.

Within our findings, the chemokine mediated signaling pathway and immune system processes in LLBWP are hypothesized to be working together. *CXCL8 (C-X-C motif chemokine ligand 8)* was found to be upregulated in LLBWP. Also known as *IL8 (interleukin 8)*, it is a member of the chemokine family, and it is a major mediator of inflammatory response. The

encoded protein is secreted by neutrophils, where it serves as chemotactic factor by conducting the neutrophils to the site of infection (Russo et al, 2014). *CXCL8* was found to be elevated during pre-eclampsia in women and pre-term labor as a result of an overall pro-inflammatory reproductive environment (Sakai et al, 2004; Szarka et al, 2010). Another finding was related to the upregulation of *IFNG (Interferon-gamma)*, which is a proinflammatory cytokine secreted in the uterus during early pregnancy, and it is produced by uterine natural killer cells in maternal endometrium (Murphy et al, 2009). Porcine embryonic and fetal loss has been associated with an elevation in *IFGN* expression (Tayade et al, 2007). Tayade et al (2007) found highly elevated expression of *IFGN* in biopsies of days 15 to 23 of gestation on attachment sites of viable retarded conceptuses compared to healthy littermate sites. According to the authors, IFNG through immune-mediated mechanisms, may compromise the first wave of angiogenesis immediately after the implantation period causing conceptus stress and subsequent growth retardation and loss. In addition, *NGF (Nerve growth factor)*, which is a neurotrophin associated with diseases of the immune system and inflammation, was also upregulated in LLBWP. Jana et al (2012) found the expression of significantly increased in gilts with induced endometritis compared to sows with normal uterine environment. Altogether, based on the mentioned studies, chemokines and immune responses at the maternal-embryonic interface may be overacting and, instead of protecting the embryos, creates an unsuitable environment for adequate embryonic development.

4.4.1.3 Placental compensation mechanisms in LLBWP

Researchers have shown that placentas linked to low weight fetuses tend to exert compensatory mechanisms mainly during the second third of the gestational period to get over the growth development (Vallet & Freking, 2007; Stenhouse et al, 2019a,b). In pigs, even though it is not invasive, the interdigitation of the trophectoderm into the endometrium is progressive

throughout gestation and tends to increase until the placenta is entirely covered (Bazer et al, 2014). Following this finding, Vallet and Freking (2007) mentioned that as a compensatory mechanism to increase total surface area, lighter fetuses showed deeper placental microfolds compared to heavier fetuses. In our study, this trend was observed in LLBWP sows through the upregulation of *HBEGF* (*Heparin-binding epidermal growth factor*). HBEGF is a molecular mediator of blastocyst implantation, which signals between the endometrium and implanting trophoblast cells to synchronize their corresponding developmental stages. Also, in pigs, *HBEGF* expression by trophoblast cells of the developing placenta appears to regulate extra villous differentiation and provide cytoprotection as a manner of compensation (Kennedy et al, 1994; Iwamoto et al, 2003). In addition, Stenhouse et al (2019a) demonstrated that it is not only the width and remodeling of the bilayer but also its vascularity that changes to compensate for and rescue the size of the fetus. Specifically, key genes related to placental and embryo development altered their expression during the second phase of angiogenesis (Vonnahme et al, 2001; Stenhouse et al, 2019a; Blomberg et al, 2010; Stenhouse et al, 2019b). The absence of other genes related to compensatory mechanisms in our study may be justified by the fact that the D30 of gestation lies in between the two waves of angiogenesis, and therefore the period of greater compensatory activity had not yet been reached. Also, differently from other studies, we are not evaluating extreme low weight conceptuses caused by intrauterine growth retardation, so the mechanisms of compensation were not expected to be as dramatic as in these other cases.

4.4.2 Genes differentially expressed in embryonic tissues from LLBWP and HLBWP

When present, the effectiveness through which the compensation mechanisms recover the weight of fetuses, is not yet clear (Stenhouse et al, 2019a). However, based on our findings and analyzing the historical reproductive performance of the selected sows (Chapter 3), it is clear that

even though other compensation mechanisms may be activated later on gestation, they are unlikely to recover the growth rate of the conceptuses. These compensatory mechanisms need not be confined only to the placenta, but also within the uterus and the fetuses themselves. In current study, besides the lower uterine capacity of LLBWP sows, another fact that corroborates with the hypothesis that those embryos would not catch up on growth, is that not only the gene expression of placental tissues is affected, but the embryonic gene expression and biological pathways is affected as well. The embryonic tissues also presented an impaired molecular transport mechanism and the inability to catch up nutrients and excrete toxic substances, reducing their developmental potential, possibly as a response to the placental insufficiency. Moreover, the results from the embryonic transcriptomes showed that embryonic tissues from LLBWP sows have impaired pathways related to the immune system, cellular divalent inorganic cation, cellular metal ion homeostasis, amino acid transfer and uptake, and erythrocyte development and differentiation.

4.4.2.1 Embryonic erythrocyte differentiation and development

One of the two most significant pathways affected in LLBWP sows was the erythrocyte differentiation and development (*AHSP, ALAS2, DMTN, DYRK3, EPB42, GATA1, IKZF1, KLF1, RHAG, SLC4A1, TAL1, TRIM10*). Erythropoiesis is the process through which the red blood cells (erythrocytes) are produced from an erythropoietic stem cell to a mature red blood cell. In pigs, this maturation process occurs during the late embryonic and early fetal development and it is associated with an improvement in the oxygenation of conceptuses (Vallet et al, 2000). Early in development, erythropoiesis occurs in the mesodermal cells of the yolk sac, and it is known that later on, it is dependent on the secretion of appropriate substrates by the uterus (Klemcke et al, 1998; Pearson et al, 1998). Vallet et al (2000) evaluated the detrimental effects of the intrauterine crowding on fetal weight and erythropoiesis from day 24 to 40 of gestation and found a positive

correlation between hemoglobin and fetal weight. Therefore, low weight fetuses tend to have their erythropoiesis process negatively affected, meaning that the ability to carry oxygen is reduced. A later finding of Vallet et al (2003) showed that the maturation of the fetal blood supply occurs earlier in sows with greater uterine capacity probably because of their placental function efficiency. Our results are directly related to this finding. Among the downregulated genes, *TAL1* (*TAL bHLH transcription factor 1, erythroid differentiation factor*) is a major regulator at multiple stages of hematopoiesis and it is required for establishing hematopoietic stem cells during embryogenesis and during the differentiation process (Aplan et al, 1992; Porcher et al, 1999). GATA1 (GATA binding protein 1) is essential for the early stage of hematopoiesis (Tsai & Orkin, 1997). And KLF1 (Kruppel like factor 1), is a transcriptional factor required for erythroid differentiation, and is associated with hematopoiesis and angiogenesis. Therefore, embryonic erythropoiesis is affected in embryos from LLBWP sows, which might be a consequence of their lower placental function and uterine capacity.

Within our findings, seven genes with independent loci spread across 16.8 Kb size range were located on chromosome 7. *MYADM* (*myeloid associated differentiation marker*) is considered to be a hematopoietic-associated marker gene. A large number of hematopoietic cytokines and their receptors, as well as transcription factors, have been shown to be involved in the maturation of blood cells (Pettersson et al, 2000). The *MYADM* gene family members play roles in membrane organization and formation of myeloid cells (blood cells). Therefore, this gene is believed to play a role in red blood cell morphology. It is known that erythrocytes with altered morphological structure often exhibit increased structural fragility causing osmotic imbalance and increased membrane rigidity (Paszty et al, 1997; Gonzales et al, 2013). The gene products from the *MYADM* family are also widely expressed in a number of cell lines, including up-regulation in

pluripotent stem cells destined to complete erythropoiesis (Petersson et al, 2000). Gonzales et al (2013) linked normal red blood cell function to increased lamb weights, and therefore suggests that marker-assisted selection could be performed towards this gene in ewes.

4.4.2.2 Embryonic amino acid transport and uptake

Normal embryonic and fetal development depends on a continuous supply of amino acids, glucose, and minerals from the mother via the placenta, and a reduced concentration in fetal circulation is associated with compromised prenatal growth (Cetin et al, 1996; Economides et al, 1989). The embryonic uptake of amino acids is affected by the maternal amino acid supply to the placenta, placental blood flow, the capacity of the placenta transport systems and placental and fetal amino acids metabolism (Regnault et al, 2002). In our study, besides the compromised placental transport systems, the ammonium trans-membrane transport pathway was significantly downregulated in embryos from LLBWP (*SLC22A4, SLC22A16, RHCE, AQP1, RHAG*). *SLC22A4* (*solute carrier family 22* (*organic cation/zwitterion transporter, member 4*)) and *SLC22A16* (*organic cation/carnitine transporter, member 16*) were downregulated in the LLBWP embryos, indicating a possible dysfunctional transport system. The solute carrier 22 family has a high affinity for amino acids and specially carnitine transport (Xu & You, 2017). Carnitine is a quaternary ammonium compound and a critical cofactor in the metabolism of lipids and, therefore, in the production of cellular energy. The availability of carnitine has been reported to be essential for developing fetuses in processes underlying fetal maturation (Arenas et al, 1998). Interestingly, many recent studies have shown that the supplementation of L-carnitine during pregnancy and lactation increase BW and weaning weight of piglets (Ramanau et al, 2004; Eder et al, 2005). The same pathway was also influenced by the downregulation of *AQP1* (*aquaporins*). AQP channels are extensively distributed in animals and plants where they mediate the transmembrane movement

of water to maintain cellular fluid and osmotic balance (Shiels & Griffin, 1993). Altered *AQP1* expression could disturb water transport, resulting in suppression of cell proliferation and, eventually, apoptosis. It is known that when *AQP1* expression is downregulated, the permeability to water is significantly reduced, cell volume is also reduced, and apoptosis, finally, occurs. The same gene appears to also influence the metabolism of cellular response to metals (Zheng et al, 2017).

Among the genes found down regulated in LLBWP embryos, *SLC7A3* was found at multiple loci and there are 8 genes spreading across 22.5 Kb size range located at chromosome 6 (Figure 4.4). *SLC7A3* belongs to a family of cationic amino acid transporters that uptake metabolites such as arginine, ornithine, and lysine (Hosokawa et al, 1997). As an arginine transporter, *SLC7A3* upregulation is known to be promoted by glutamine deprivation (Lowman et al, 2019). The *SLC7A3* upregulation promotes arginine cellular uptake which, in turn, promotes cell growth and proliferation (Gao et al, 2009, Lowman et al, 2019). The fact that *SLC7A3* was down regulated in LLBWP embryos may be related to the fact that LLBWP conceptuses were in need of glutamine compared to HLBWP embryos, which experience a higher or more efficient growth rate.

4.4.2.3 Embryonic cellular response to inorganic substances

The cellular responses to inorganic substances such as Cadmium, Copper, and Zinc were significantly downregulated in the LLBWP embryos. *ERFE (erythroferrone)* gene is also related to ion homeostasis, positive regulation of glucose import, and control of fatty acid metabolic process. The metallothionein transport (*MT*) family *(MT1X, MT1A, MT1H)* has been associated with metal micronutrients homeostasis and heavy metals detoxification (Bremner et al, 1987). The downregulation of this gene family might be related to the reduced cellular response to metals and

ions and also to the reduced detoxification of copper ion observed in our results. The physiological function of the *MT* genes is known to help protect against oxidative stress and stress of dietary Zinc and other micronutrient deficiency (Cousins, 1985; Andrews & Geiser, 1999; Song et al, 2009). The mineral Zinc, for example, is involved in several cellular processes, such as proliferation, immune function, antioxidant defense, gene expression, and RNA polymerase activity (Onagbesan et al, 2006; Falchuk & Montorzi, 2001). Its homeostasis is essential for optimal metabolic functions. During the gestational period, Zinc regulates the expression of many developmental genes crucial for fetal growth, and in rodents, the maternal Zinc deficiency consistently causes reduced BW (Vallee & Falchuk, 1993; Tian et al, 2013). The inefficient uptake and lack of Zinc homeostasis may cause long-lasting detrimental effects on embryos, with important implications for postnatal health (Tian et al, 2013). In addition, the essential yet toxic nature of copper demands tight regulation of the copper homeostatic machinery to ensure that sufficient copper is present in the cell to drive critical biochemical processes while preventing the accumulation to toxic levels (Solomons, 1985). Toxic levels of copper can generate cellular damage such as the oxidation of proteins, cleavage of DNA and RNA molecules, and membrane damage due to lipid peroxidation (Halliwell & Gutteridge, 1986).

4.5 Conclusions

Based on our findings, it is clear that the placental and embryonic molecular pathways related to appropriate morphogenesis act different in the LLBWP sows at D30 of gestation. The inefficiency through which nutrients are transferred from the mother to the embryos caused by an inefficiency of placental angiogenesis, exacerbated immune function and cornification process directly affects the ability of the embryos to develop. The reduced ability for crosstalk between mother and embryos from LLBWP is believed to cause a negative impact on embryonic

erythrocyte differentiation, amino acid uptake and cellular response to inorganic substances; all pathways known to directly affect proper morphogenesis and prenatal growth. In conclusion, our study suggests that LLBWP sows show an impaired prenatal programming at the molecular level which manifests in this undesirable generalized phenotype in the population of sows analyzed. Our findings aim to help the development of breeding strategies that improve embryonic and fetal development.

CHAPTER 4 - FIGURES

Figure 4.1. Differentially expressed genes (DEGs) comparison design for D30 embryos and placental tissues. **A.** Comparison among D30 embryos from both sexes according to the LBWP Group. **B.** Comparison between placental tissues from LLBWP and HLBWP.

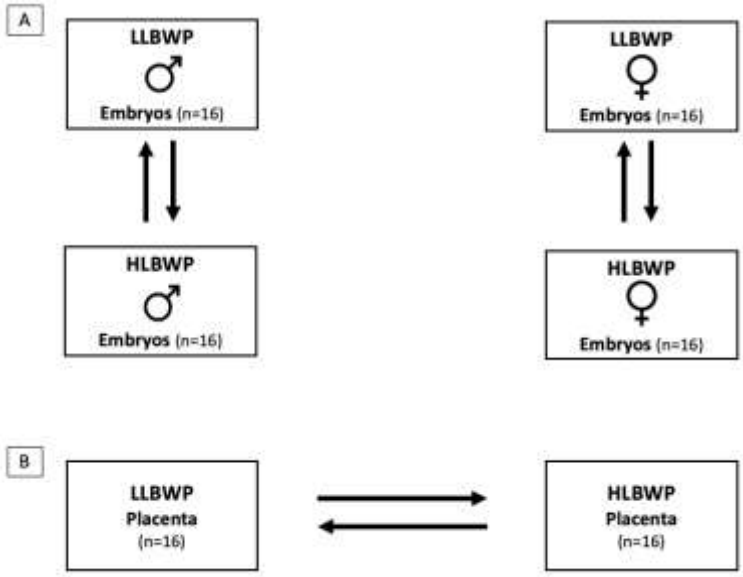


Figure 4. 2. Level of gene expression from LLBWP and HLBWP in (A) female D30 embryos and (B) male D30 embryos.

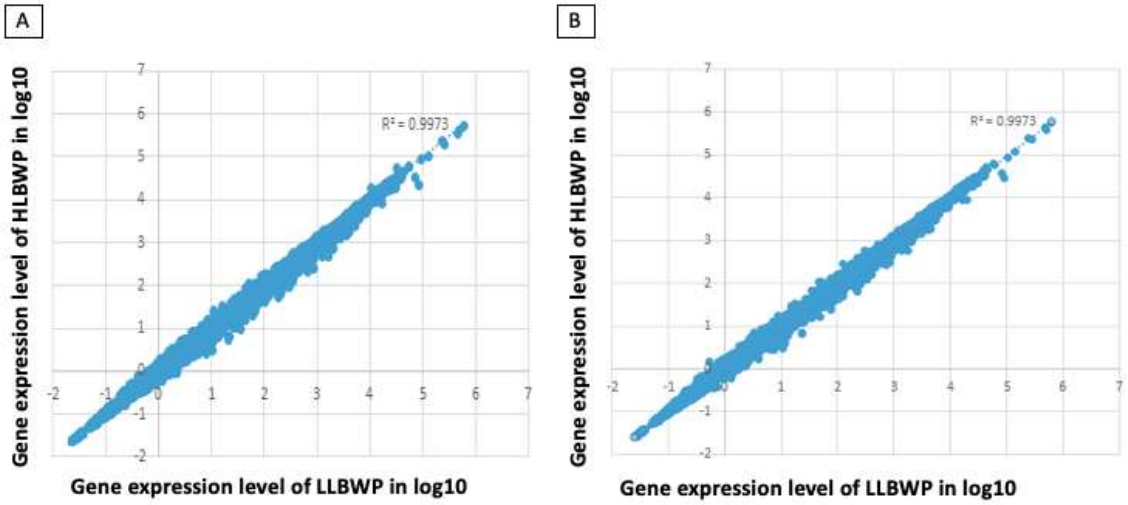


Figure 4.3. Gene expression level of embryos from HLBWP and LLBWP sows in log10. Genes involved in response to LBWP regarding D30 embryos was performed using DEseq2 algorithm.

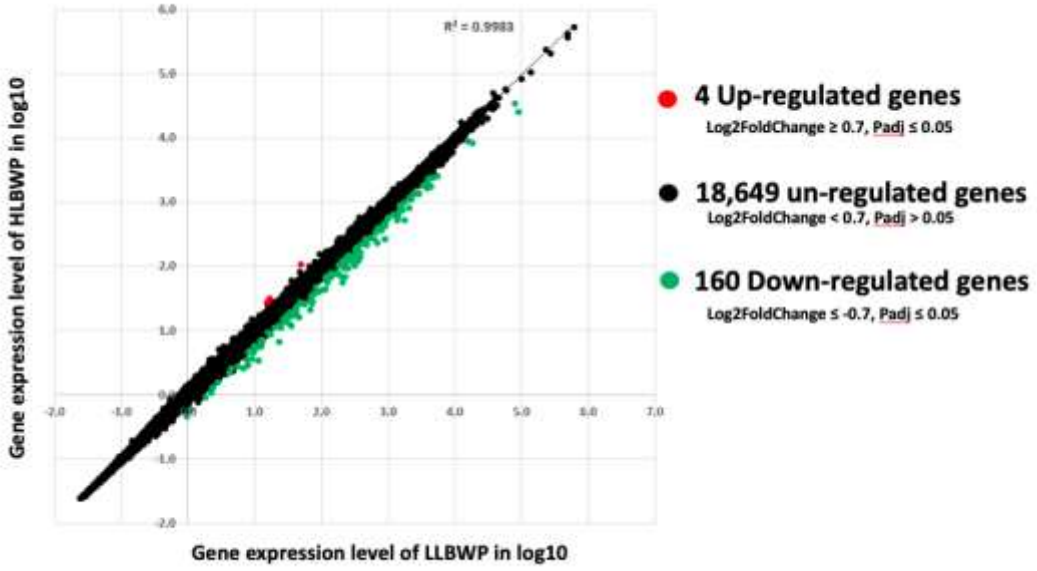
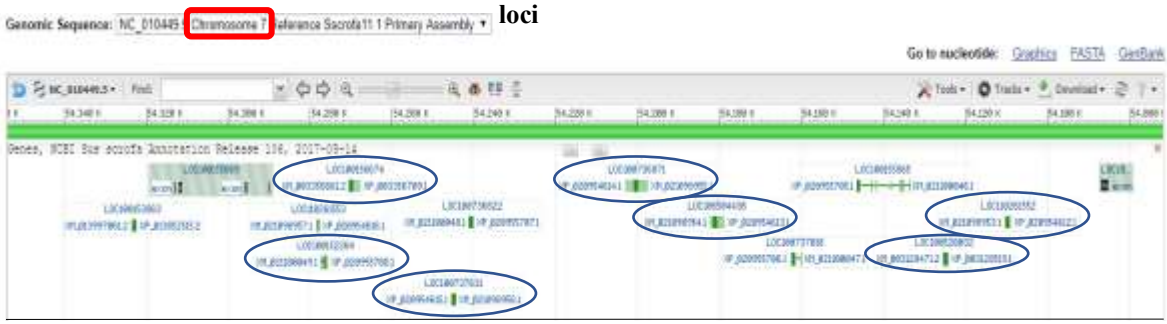


Figure 4.4. From the gene expression level analysis (Figure 4.3), among all the 160 down-regulated genes, two gene families *MYADM* and *SLC7A3* were found at multiple loci located at chromosome 7 & 6, respectively.

7 MYADM (myeloid-associated differentiation marker-like) loci



8 SLC7A3 (cationic amino acid transporter 3-like)

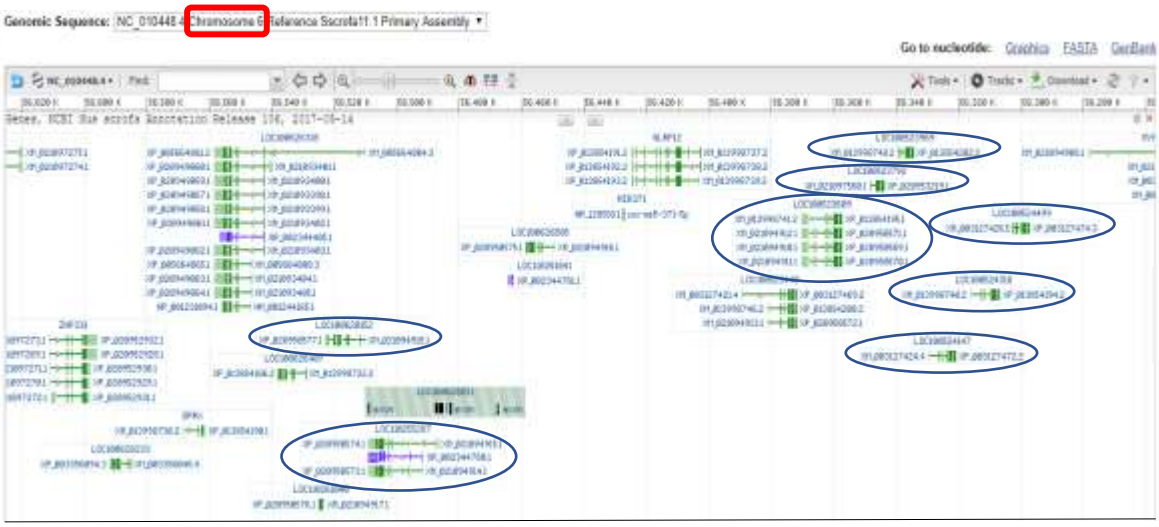


Figure 4.5. Gene expression level of embryos placental tissues from HLBWP and LLBWP sows in log10. A total of 82 genes were differentially expressed in placental tissues with 43 down-regulated and 39 up-regulated.

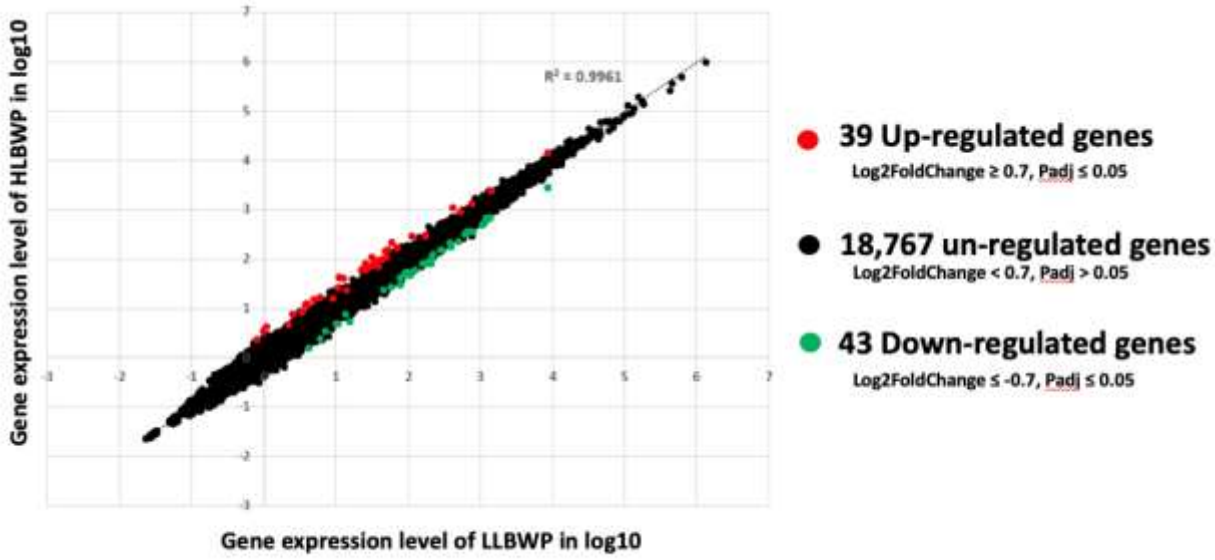
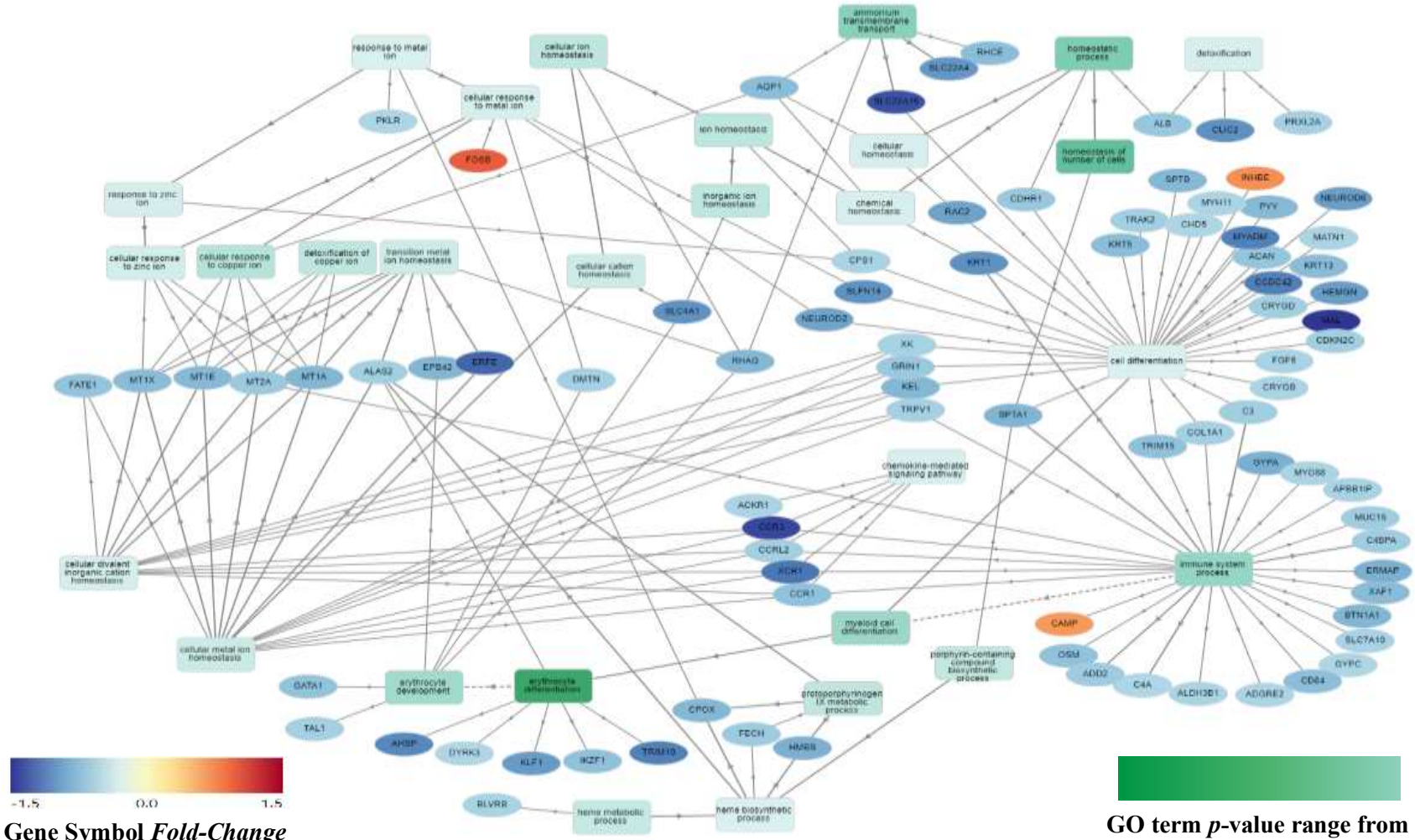


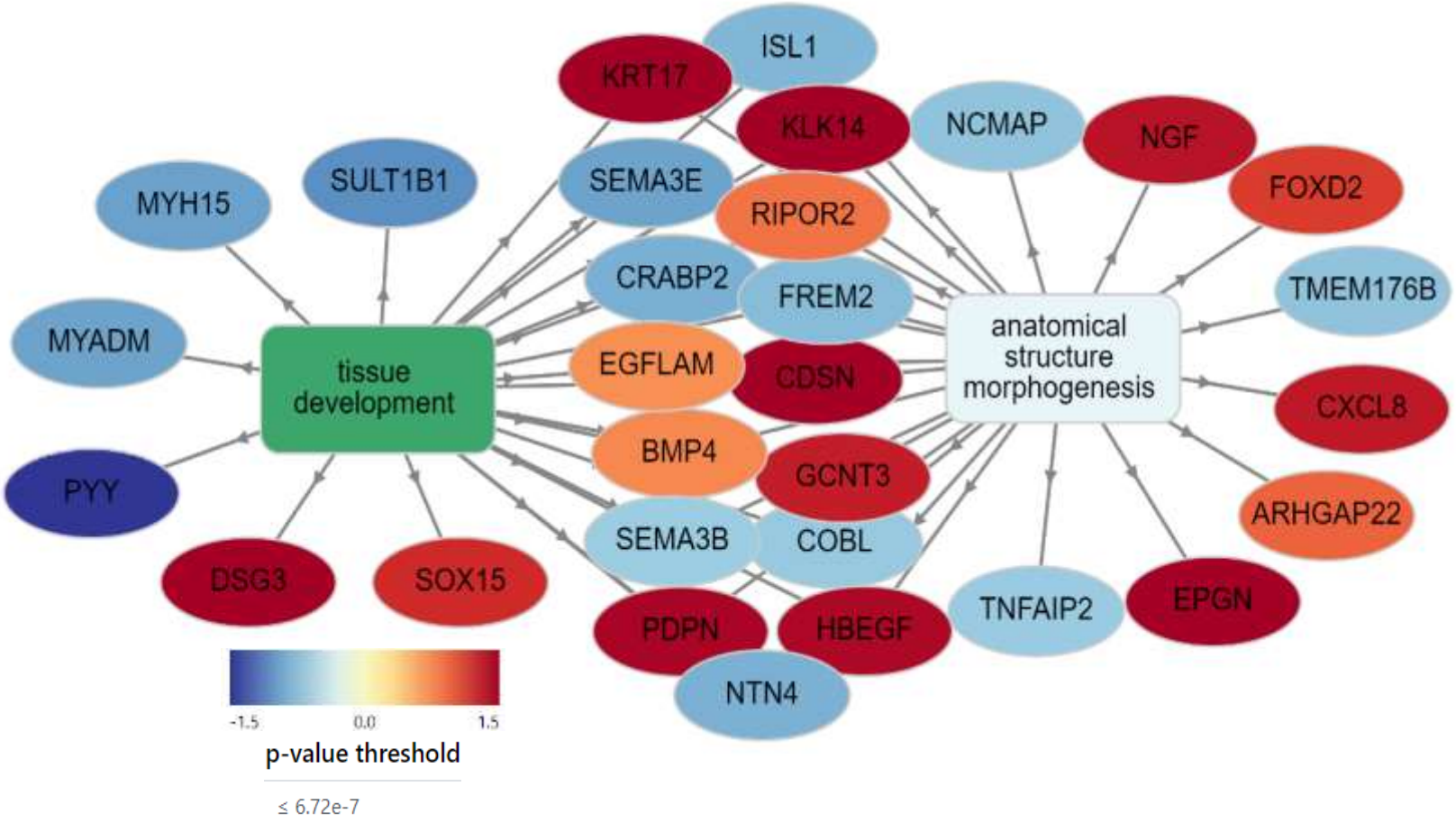
Figure 4.6. Graphic network output with DEGs interaction from different biological pathways in D30 embryos.



Gene Symbol *Fold-Change values*

GO term *p*-value range from 4.46e-12 to 5.0e-5

Figure 4.7. Graphic network output with DEGs interaction from different biological pathways in placental tissue.



CHAPTER – TABLES

Table 4.1 Sequence information for the quantitative real-time RT-PCR validation.

	Sequence information
HMBS	
FWD	CAG GAG TTC AGT GCC ATC AT
REV	CCT GAC CCA CAG CAT ACA TAC
PRB	/56-FAM/AC TCC TCA G/Zen/G GTG CAG GAT CTG TC/3IABkFQ/
MT1A	
FWD	TCA CCT GCC TCC ACT CAT
REV	GGA GCA GCA GCT CTT CTT G
PRB	/56-FAM/AA AGC CTG C/Zen/A GAT GCA CCT CCT /3IABkFQ/
RHAG	
FWD	GCA ATT GTT GGA GGG CTA ATC
REV	CTC TTA GGA ACC TCC CAG TAA AC
PRB	/56-FAM/CA GTT CTC G/Zen/T CAG ATG GCT GTC CC/3IABkFQ/
SLC22A16	
FWD	TTT CTG TGT GGC ATA GGA GTG
REV	ACA AAC ACC ACC ACG AGA TAG
PRB	/56-FAM/CC AAC CAT A/Zen/G CAA GAA GAA AGC GTG C/3IABkFQ/
CDSN	
FWD	TGG GCA GGT GTC TCA ATA ATC
REV	CAA GGC GTA GGA GAG CAT TAC
PRB	/56-FAM/TT GGT GTC C/Zen/T GGG TCT CTT CTT AGG A/3IABkFQ/
HBEGF	
FWD	GGC AAA GGG TTA GGG AAG AA
REV	ACA CCT CTC TCC GTG GTA A
PRB	/56-FAM/AT TTC TGC A/Zen/T CCA CGG AGA GTG CA/3IABkFQ/
PDPN	
FWD	CAG TCC CAC GAT AAA GGA GAT G
REV	ACC AAT GAA TCC AAT GGC TAG TA
PRB	/56-FAM/AC GGT GAC C/Zen/C TAG TTG GAA TCA CA/3IABkFQ/
HPRT1	
FWD	ACCTAATCATTATGCCGAGGATTT
REV	GCCTCCCATCTCTTTCATCAC
PRB	/56-FAM/TATGGACAG/ZEN/GACTGAACGGCTTGC/3IABkFQ/
PGF	
FWD	CTACGTGGAGCTGACATTCT
REV	CTGCTTCTCTCTCTTCCTCTTC
PRB	/56-FAM/TGCGGGAGA/ZEN/AGATGAAGCCAGAAA/3IABkFQ/

Table 4.2 Relative expression between LLBWP and HLBWP in D30 placental and embryonic tissues obtained from the RNA-seq and RT-qPCR studies.

	RNAseq		**RT-qPCR**	
Genes	**log2FoldChange (LLBWP/HLBWP)**	**P adj**	**log2FoldChange (LLBWP/HLBWP)**	**P-value**
D30 Embryos				
HMBS	-1.04	8.86889E-13	-0.91	0.0038
MT1A	-1.00	0.007991104	-1.59	0.0045
RHAG	-0.99	4.67467E-11	-1.4	0.0047
SLC22A16	-1.64	9.94284E-10	-1.81	0.0008
Placenta				
CDSN	1.62	0.016555081	3.55	0.0057
HBEGF	1.48	0.011060742	3.33	0.002
PDPN	1.50	0.003	2.14	0.0018

4.6 References

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CHAPTER 5

5. General discussion and conclusions

5.1 The origin of litter birth weight phenotype

In order to obtain high reproductive efficiency in a herd, a balance between optimal litter size and piglet quality must be achieved. To meet this objective, breeding goals have changed, transitioning from an emphasis on sow prolificacy and TNB, to sows' lifetime productivity, production of quality piglets weaned, robustness, and meat quality. Therefore, in recent years, traits of medium and low heritability that require extensive data recording became crucial. Litter size, indeed, is a critical measure of success in a pig breeding herd farm and must be maintained. Still, if BW and piglet survivability are low, the value of a litter size increment is minimal.

Even though the traits of interest for selection pressure have changed, the consequences of past selection efforts still negatively influence the contemporaneous sows. These negative consequences are observed at a direct and indirect level. The direct consequences are already well recognized and are related to highly prolific sows producing large litters with high within litter variation and individual low BW piglets. However, the indirect consequence, represented by the low litter birth weight phenotype (LLBWP), requires further understanding and extensive consideration. This subpopulation is believed to make the most substantial contribution to the low birth weight (BW) of piglets and variation in postnatal growth performance, independent of the total number born (TNB) (Foxcroft et al, 2009). Previous studies have already described the postnatal growth performance (from birth to slaughter) of progeny from specific litter average BW phenotypes and also on the reproductive performance of replacement gilts (Smit et al, 2013; Patterson & Harding, 2013; Patterson et

al, 2018; Zhang et al, 2018; Patterson & Foxcroft, 2019). However, the present study was the first to investigate the origin and prenatal mechanisms that lead to the LLBWP in multiparous sows.

In this study, when the Large White sows were selected from a Nucleus Unit breeding population, a similar range of TNB was one of the criteria used to select sows from a repeatable LLBWP and high litter birth weight phenotype (HLBWP), which was designed to exclude the effect of hyper prolific sows (>22) or sows producing small litters (<8) on BW. So, instead of focusing on the individual BW of piglets, our study aimed to understand the causes of variation in BW when the litter size was more balanced and BW was uniform within a litter. The principal concept driving this research was that changing patterns of ovulation rate (OR) and embryonic losses would limit placental development, causing generalized low BW litters. Therefore, it was hypothesized that the LLBWP sows described in this Thesis would show higher OR and a higher number of embryos *in utero* by day 30 (D30) of gestation, when compared to HLBWP sows. However, based on the results presented in this Thesis, our hypothesis was rejected. LLBWP and HLBWP sows presented a similar OR, similar total number of embryos (TNE) and total number of viable embryos (TNV), showing that the dynamics of embryonic mortality is comparable in the two extreme populations of sows at D30 of gestation. However, despite showing a similar number of embryos *in utero*, the uterine capacity in LLBWP sows was below the threshold for optimal placental development. The physiological results support that, independent of litter size, the LLBWP in a substantial population of multiparous nucleus sows is due to a cascade of events, primarily caused by a lower uterine capacity, which generates lower placental development and efficiency on D30 of gestation onwards, negatively affecting embryonic development.

However, there is undoubtedly more to placental development than differences in placental volume during gestation. With that understanding, normal maternal-embryonic dialogue at the molecular level is crucial to develop breeding strategies that improve prenatal development. At the molecular level, the transcriptomics analysis allowed us to investigate and determine candidate genes and biological pathways that may be involved in the lower placental efficiency in LLBWP sows. According to our RNA sequence results, several differentially expressed genes (DEGs) involved in placental biological pathways as tissue development, anatomical structure, and morphogenesis were differentially expressed in the LLBWP sows. A total of 89 DEGs were found in placental tissues, with 43 down-regulated and 39 up-regulated in the LLBWP sows compared to HLBWP. Collectively, based on the action of these genes specified on Chapter 4, we can hypothesize that deficient angiogenesis, impaired nutrient transport activity, and an unbalanced placental immune function manifest in the LLBWP.

A total of 160 genes were down-regulated, and only 4 genes were up-regulated in LLBWP as compared to HLBWP in embryonic tissues. Possibly, as a response to the placental insufficiency, the embryonic tissues also presented an impaired molecular transport mechanism and the inability to absorb minerals and amino acids and excrete toxic substances, reducing their developmental potential. Moreover, the results from the embryonic transcriptomes showed that embryonic tissues from LLBWP sows have DEGs related to the immune system, cellular divalent inorganic cation, cellular metal ion homeostasis, amino acid transfer and uptake, and erythrocyte development and differentiation. Based on our findings, it is evident that placental and embryonic molecular pathways related to appropriate morphogenesis work differently in the LLBWP sows at D30 of gestation. The inefficiency

through which nutrients are transferred from the mother to the embryos directly affects the ability of the embryos to take up nutrients essential for adequate growth. In conclusion, the prenatal programming of this undesirable phenotype at the nucleus level impacts the lifetime performance of piglets born from LLBWP sows.

5.1.1 Prenatal programming and epigenetics

Post-natal performance in swine is determined by both genetic and environmental factors, including the prenatal environment and health status. Based on our results, it is clear that the prenatal programming of sows displaying the LLBWP is impairing embryonic development. In this case, epigenetic changes drive prenatal programming, culminating in impaired conceptus development and generalized low BW, causing lifelong changes in the performance of the offspring. However, besides the fact that the sow individual genotype influences prenatal programming, it is likely to be affected by numerous factors other than the reproductive physiological dynamics.

In our study, for example, a population of purebred Large White sows was analyzed. Therefore, proposing or deducing that our results could be applied to any other population of sows from other genetic lines or crossbreed sows at the commercial level has to be carefully evaluated. Nucleus or high genetic farms have a high health status, higher amounts of labor and care per pig, and again, the reproductive herd is purebred, targeted for selection purposes. In addition, the environment in Nucleus farms is different from commercial production systems where the main focus is on low cost production, and the health status is often compromised by diseases that reduce the efficiency of production. Related to that, it is known that animals that perform best in Nucleus units might not necessarily perform optimlly at the commercial level. Also, reproductive processes such as ovulation, implantation rates, and

embryonic mortality are the most sensitive reproductive pathways influenced by stress, nutritional status of the sow, and environmental pressure. Therefore, beyond the work being done to understand the underlying physiology and molecular pathways associated with the LLBWP in this population of sows, there is a need for further studies on how environment regulates gene expression and influences the regulatory components of the biological pathways and significant candidate genes we found in our study.

5.2 Management strategies for LLBWP sows

It is not an easy task to determine selection pressures and to find a balance to manage reproductive traits such as ovulation rate, uterine capacity and placental efficiency. In this scenario, the LLBWP is also included. As discussed before, genetic programs include BW as a component of their selection pressure, and in every production system, there is a threshold for the minimum BW of a gilt to be considered a future breeding replacement. However, even with this pressure, the presence of sows carrying this unwanted phenotype remains. Knowing that the presence of this subpopulation of sows is a reality in production systems, the ability to strategically introduce management practices to lessen the impact of this phenotype should be considered.

5.2.1 Sow identification and phenotype measurement

From a practical point of view, a particularity of this phenotype is that LLBWP sows produce a reasonable litter size and do not produce a high percentage of extremely low BW piglets. As a consequence, the LLBWP sows do not attract much attention of attendants in a farrowing room and usually are not considered “problematic” sows. Consequently, they will rarely be considered for culling in cases where the phenotype is not actively measured. Knowing that the foundation of every genetic program is the phenotypic data collected within

the production system, the only possible way to measure this trait is to weigh piglets at the time of birth, over consecutive parities. For that, the data collected on-farm must be accurate and consistent. Only in this way, it can be considered a strong candidate for selection. Moreover, the statistical analysis performed in order to identify LLBWP and HLBWP sows in this study (explained in detail in Chapter 3 of this Thesis) may serve as an example for herd analysis and LLBWP sows' identification in a farm population.

5.2.2. Strategic culling

Once identified, and considering the repeatability of the LLBWP trait, producers can effectively select against LLBWP sows after measuring two or three consecutive parities (Patterson & Harding, 2013; Smit et al, 2013; Zhang et al, 2018). Particularly at the multiplication level, the ability to predict LLBWP can be directed to strategic culling decisions to increase the efficiency of a genetic improvement program. The possibility of culling these sows allows producers to intervene on a cascade of events that reduce the efficiency of production globally in the system, reducing the risk of passing this unfavorable low BW trait to the downstream commercial units. However, if it is not possible to cull these sows, due to strategic decisions or other problems occurring concomitantly in the farm, the identification process can be used in favor of further strategic management practices on-farm.

5.2.3. Pre-weaning management practices

The identification of LLBWP sows in a herd allows for the segregation of these sows in farrowing rooms, for example. As the expected BW is known, special assistance could be provided during farrowing and first days of life, reducing piglet losses due to neonatal mortality and also assisting to guarantee adequate colostrum and milk intake in the first hours of life. Newborn piglets have high energy requirements and are in a negative energy balance

shortly after birth since their body fat represents less than 2% of body mass (Theil et al, 2014). Furthermore, when low BW piglets are being considered, this scenario becomes even more extreme (Theil et al, 2014). The colostrum intake, therefore, is necessary for increasing body energy and warmth, and in cases where ingestion is not adequate, mortality rates increase considerably (Le Dividich & Noblet, 1981). In addition to impacting survival, Vallet et al (2015) reported an increased age at puberty and a reduction in the number of piglets born when the estimated colostrum intake was low.

Another practice that could be performed more effectively with the application of sow segregation, is cross-fostering. According to Flowers et al (2018), light BW replacement gilts recovered, or at least improved their performance later in life, when kept in smaller litters. According to the author, gilts raised in smaller litters had higher weaning weights and had greater lifetime productivity. It is also known that small piglets have greater growth when they are grouped together with piglets of similar sizes since this category tend to miss more nursing episodes when they are in the same litter as medium or high BW littermates (Huting et al, 2017; Marcatti, 1986; Deen & Bilkei, 2004). In general, small piglets coming from LLBWP should be fostered to a sow with other small individuals. Additionally, when this is not possible, the litter size should be reduced with the removal of the larger pigs, in order that the colostrum and milk intake can be optimized.

There is also some discussion at the production level as to whether small piglets require too much time investment from farrowing attendants. The probability of survival of lightweight piglets is low, and if they survive, feed and medication costs may become uneconomical during grower and finishing stages. Thus, the teat space would be better

allocated to a larger piglet with increased viability. However, any decision involving euthanasia of smaller piglets would give rise to an ethical debate.

5.2.4 Weaning age of low BW piglets

It is known that low BW piglets have impaired development of intestinal defenses compared to high BW piglets and are more vulnerable to pathologies associated with the weaning period (De Vos et al, 2014; Lessard et al, 2018; Fouhse et al, 2019). With this in mind, if the farm workflow allows, one option is to increase the weaning age of these litters. Piglets with older ages at weaning tend to be less compromised by the stressors generated by this event (Worobec et al, 1999). In addition, in a recent study, a positive effect of increasing weaning age from 19 to 28 days was found on weight gain in the first week in the nursery (Faccin et al, 2020). Since weaning weight is one of the essential traits that determine lifetime growth performance (Collins et al, 2017), this prospect should be considered.

5.2.5 Sow nutrition during gestation and lactation

The identification and segregation of LLBWP sows may also allow for special attention to their nutritional management. Many studies have tried to elucidate the influence of energetic balance, supplementation of a range of amino acids, minerals, fatty acids, among others, during the gestational period as a way to increase piglet BW (Gonçalves et al, 2016; Mateo et al, 2008; Quesnel et al, 2014; Tanghe & Smet, 2013; Smit et al. 2013). Most of them, however, had minimal or no effect when trying to overcome low BW piglets. A reasonable explanation is that a gestating sow prioritizes nutrient needs towards fetal growth, independent of her energetic balance or nutrient supply (Theil et al, 2014). For example, Rehfeld and Kuhn (2006) mentioned that only severe long-lasting maternal undernutrition had negative impacts on BW, while temporary feed restrictions showed no effect.

In addition, there is a standard practice in the pig production industry, known as “bump feeding”, in which the amount of feed is significantly increased in the last third of gestation in order to compensate for exponential fetal growth. However, this feeding approach was recently shown to not be beneficial for increasing piglet BW in the contemporaneous multiparous sow (Gonçalves et al, 2016; Soto et al, 2011; Mallmann et al, 2018). It is believed that the leaner phenotype of current sows, makes them more efficient in prioritizing fetal growth. Consequently, an extra amount of feed is not required to achieve optimal fetal development, and also does not improve the programmed fetal growth (Theil et al, 2014). However, more studies need to be conducted in order to elucidate if any nutritional management is effective in sows exhibiting the LLBWP.

Maintaining a balanced body condition of sows in the breeding herd is still believed to be the single most critical practice to increase the quality of the piglets born, piglet survivability, and lifetime performance of the sows (Wientjes et al, 2013). Fat sows at farrowing have more stillborn piglets, eat less and lose more weight during the lactational period, which can negatively affect piglet weaning weight, wean-to-estrus interval and subsequent litter size (Rozeboom, 2010; Mallmann et al, 2018). Keeping the herd in optimal body condition during gestation allows producers to maximize sow feed intake during the lactational period, which is one of the most important practices to improve piglet weaning weight (Sulabo et al, 2010). Therefore, the impact of a poorly managed body condition in LLBWP sows could cause additional severe consequences to the production unit.

5.3 Future research perspectives

One of the main findings of our study was that LLBWP purebred Large White sows have an impaired placental efficiency, which was assessed by the lower placental volume and

through the DEGs found for placental tissues when comparing LLBWP and HLBWP. Collectively, based on the action of the DEGs specified in Chapter 4 of this thesis, we hypothesized that deficient angiogenesis, impaired nutrient transport activity, and an unbalanced placental immune function manifest in generating the LLBWP. However, in order to prove that these pathways have a direct relation to a physiological outcome, additional detailed physiological studies need to be conducted to confirm our hypothesis.

Another limitation of our study is related to the limited genetic background of the sows analyzed. The strict use of a purebred Large White sows limits the understanding of how this phenotype acts in other purebred lines and, particularly, how the phenotype performs at the commercial level in crossbred sows. Therefore, beyond the work being conducted to understand the underlying physiology and molecular pathways associated with the LLBWP in purebred of sows, there is a need for further related studies on commercial crossbred lines. Additionally, the manner in which environment regulates gene expression and influences the regulatory components of the biological pathways and significant candidate genes we found in our study, also need to be clarify.

5.4 Overall conclusions

The present study provided unique insights into the biological and molecular processes related to the LBWP in contemporaneous sows. Overall, the physiological results of this research did not support our initial hypothesis. Therefore, the generalized low BW is not caused directly by higher ovulation rates and uterine overcrowding at the beginning of the gestational period, but presumably by limited uterine capacity and lower placental efficiency. Based on our findings, we suggested that the placental and embryonic molecular pathways related to appropriate morphogenesis are impaired in the LLBWP sows at D30 of gestation.

The need for a better understanding of how quality piglets are efficiently produced follows the current global pig production scenario of increased concern for animal welfare and desire on antibiotic reduction. In light of this, piglet BW represents a major relevance. It is clear that the swine industry should strive to decrease the percentage of LLBWP litters as the greatest opportunity to increase piglet quality and weight at birth. The new insights provided by this study on how the LLBWP negatively influences sows at the reproductive level can lead to future selection for more efficient sows. Until these have been established, management strategies to deal with LLBWP sows and their litters should be acknowledged.

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Appendix 1

Influence of litter birth weight phenotype on embryonic development at day 9 of gestation

Introduction

The embryonic development in pigs is distinguished in two main periods, pre-implantation and post-implantation. The pre-implantation period is characterized by a dramatic rate of morphological changes from the 2-cell stage after fertilization, to an elongated morphology (around days 11 to 12 of gestation) just prior to when implantation begins at day 14 of gestation (Pollard, 1990; Wilson & Ford, 1997). As reviewed in detail in Chapter 2 of this Thesis, at approximately day 9 to 10 of development the porcine embryonic disc is fully formed, and the blastocyst enlarges reaching a diameter of around 1 centimeter. During days 11 and 12 the embryonic disc develops into an oval shape and it is associated with the beginning of elongation that, over a couple of hours, results in the transformation of the spherical blastocyst of about 1cm in diameter to an approximately 1m long thin filamentous structure (Oestrup et al, 2009). The degree of elongation the embryos reach is correlated with the uterine surface area that is taken up by each embryo and, therefore, delimits the space occupied by them during implantation (Bazer et al, 2013). In this scenario, embryos experiencing delayed development have access to restricted uterine area and develop smaller placentas, which affects their growth rate during the entire gestational period (Geisert et al, 1982).

As mentioned previously in Chapter 3, the low litter birth weight phenotype (LLBWP) was hypothesized to be driven by imbalances between sow's reproductive traits ovulation rate (OR) and uterine capacity. As a consequence, uterine overcrowding would negatively affect

embryonic survivability and development. Based on this, we hypothesized that the pre-implantation embryonic development would be already impaired at the molecular level at day 9 (D9) of gestation in LLBWP. The main objective of collecting embryos at D9 of gestation was to evaluate the influence of LLBWP and high litter birth weight phenotype (HLBWP) on the molecular development of pre-implantation embryos. The choice of sampling at D9 of development aimed to guarantee the embryo collection at the spherical stage, which would greatly facilitate sampling compared to elongated embryos.

Sample collection

The sow selection, estrous synchronization and artificial insemination for the D9 of gestation sampling from LLBWP and HLBWP sows, were performed as the protocol described in Chapter 3 (*3.3.2 Sow selection; 3.2.3 Synchronization and artificial insemination*). At day 8, 9 or 10 of gestation (9.0 ± 0.6; n = 9 LLBWP and n = 8 HLBWP) sows were euthanized on-site, with the day of the first insemination being defined as day 1 of pregnancy. The sample collection procedure for D9 is shown in Figure 6.2. The reproductive tracts were recovered from each sow immediately after euthanasia, and the number of corpus luteum (CL) on each ovary was assessed. After removing the broad ligament, the uterine horns were clamped, and each horn was evaluated separately. The embryos were flushed from each uterine horn with 25 mL of Phosphate-buffered saline (PBS) solution injected into the uterine lumen with a blunt needle syringe inserted through the uterine horn at the utero-tubal junction. The flushing was collected into a 50 mL sterile tube through another modified syringe inserted through an incision by the end of the uterine horn. Each uterine horn was flushed twice in attempt to harvest as many embryos as possible. The flush was transferred to a pre-warmed Petri dish and using a stereomicroscope (LEICA MZ125), the number of pre-implantation

embryos for each uterine horn was recorded, along with their relative stage of development through a visual approach (small spherical, medium spherical, large spherical, medium collapsed and large collapsed; Figure 6.3). Immediately after embryonic assessment and collection, the embryos were transferred and stored individually in 1.5 ml microcentrifuge tubes (Eppendorf), centrifuged briefly and 300 µl of DNA/RNA shield solution was added (Zymo Research). Finally, the samples were stored in -80°C for further genomic analysis.

Embryonic sexing and RNA extraction

The DNA and RNA extraction of D9 embryos was performed according to the manufacturer's protocol (Zymo Research) and the embryonic sex was determined using the same protocol as described in Chapter 3 (*3.2.6.2 DNA preparation; 3.2.6.3 Design sex-specific PCR primers; 3.2.6.4 Genomic DNA PCR condition and validation).* Based on the embryonic morphology (stage of development) 4 sows were selected from each LBWP group. Only sows with embryos at the spherical stage were selected in order to standardize the analysis.

Results

Similar to the results found in Chapter 3, there was no significant difference in OR between sows from the two LBWP groups (LLBWP: 24.84; HLBWP 25.91). In total, 267 embryos were recovered, with the recovery rate (%; number of embryos recovered/OR, considering fertilization rate as 100%) of the flushing method being 58.7% for LLBWP and 78.6% for HLBWP (Table 6.1). The embryonic development assessment showed a lack of uniformity between LLBWP and HLBWP embryos. In addition to that, there was also a variability among sows of the same LBWP group as well (Figure 6.1). Generically, the LLBWP sows showed a higher percentage of embryos in earlier stages of development (small

spherical), when compared to HLBWP embryos. However, the variation in embryonic size among sows in LLBWP and HLBWP was also high.

The embryonic sexing methodology applied was not as successful as described in Chapter 3 with D30 embryos (Table 6.2). In total, 101 embryos were sexed (n = 61 LLBWP; n = 40 HLBWP), 49.5% were found to be female and 35.64% were male embryos. The remaining 17% of the embryos were not able to be identified as male or female. This result is possibly due to a reduced cellular content of D9 embryos. Due to this, the PCR amplification was not efficient and resulted in no bands or bands that were too faint to distinguish between male and female amplicon patterns. Besides, 52% of the embryos failed the RNA integrity number (RIN) assessment, in which values ≤ 6.0 were considered not a good fit for further gene expression analysis. This may be due to the RNA becoming degraded during the embryo selection process before adding the DNA/RNA shield solution.

Discussion

As previously mentioned, this aspect of the project aimed to evaluate the influence of LBWP on the pre-implantation development of LLBWP and HLBWP sows. Initially, we hypothesized that LLBWP sows would show an impaired and delayed embryonic development during the early stages of development affecting the rate of embryonic elongation resulting in smaller implantation areas and negatively affecting placentation. As observed for the D30 of gestation sows results, there was no difference in OR between LLBWP and HLBWP. The embryonic recovery rate was similar to other studies that conducted sample collection at the same stage of development (Silva et al, 2013). However, it is important to keep in mind that since the embryos were flushed from the uterine horns, the total number of embryos found does not correspond to the real total number of embryos

present within the reproductive tract, since some may have been lost during the process. Consequently, this portion of the project did not aim to evaluate uterine overcrowding.

Even though the sampling occurred from days 8 to 10 of development in both groups, a high variability on embryonic morphology was found (Figure 6.1), where LLBWP sows showed a higher percentage of embryos on earlier stages of development compared to HLBWP sows. In our study, as a matter of standardization, the day the sow received the first dose of semen was considered the first day of gestation. However, it is known that the estrus length and time of ovulation in sows varies individually (Soede and Kemp, 1997). Therefore, since the exact time of ovulation was not determined individually by ultrasonography, the exact day or time of development was not accurately accounted for. Based on our findings and due to the limitation in our experimental design, we cannot conclude that the delayed embryonic development observed in LLBWP is influenced by the LBWP or if it is simply a technical limitation of determining the exact time of ovulation. Therefore, even though the percentage of smaller and less developed embryos were seen in the LLBWP embryos, it cannot be concluded that the LBWP is influencing these findings or if it is simply due to a later ovulation timing of those sows.

The lack of uniformity of the embryos collected negatively affected the standardization of selection for functional genomic purposes. We were able to select 4 sows from each LBWP group that were in the same stage of development (spherical stage). However, the sexing and RNA quality approaches performed were not successful. A total 52% of the embryos failed the RIN assessment, in which values ≤ 6.0 were considered not a good fit for further gene expression analysis. Besides, 17% of the embryos were not able to be sexed.

Conclusion

Due to the heterogeneity of embryonic morphology, inefficient sexing procedures and low RNA quality of embryos collected from LLBWP and HLBWP at D9 of gestation, the decision was made not to include in the larger study presented in this Thesis. For a subsequent study, the assessment of the exact time of ovulation should be considered, so the stage of development is accurately accounted.

Figures

Figure 6.1 Percent of embryos in each category of development for each LBWP Group.

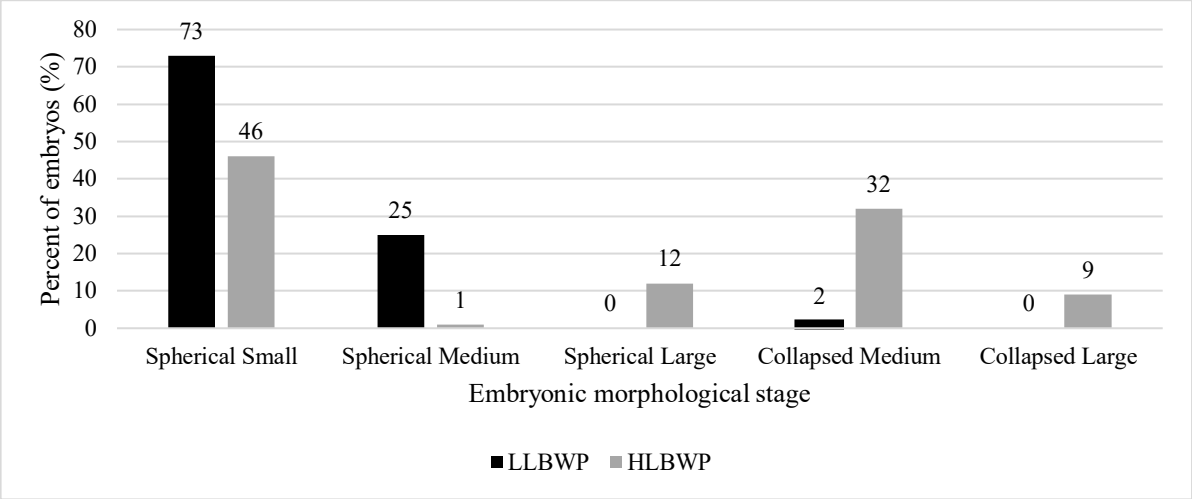
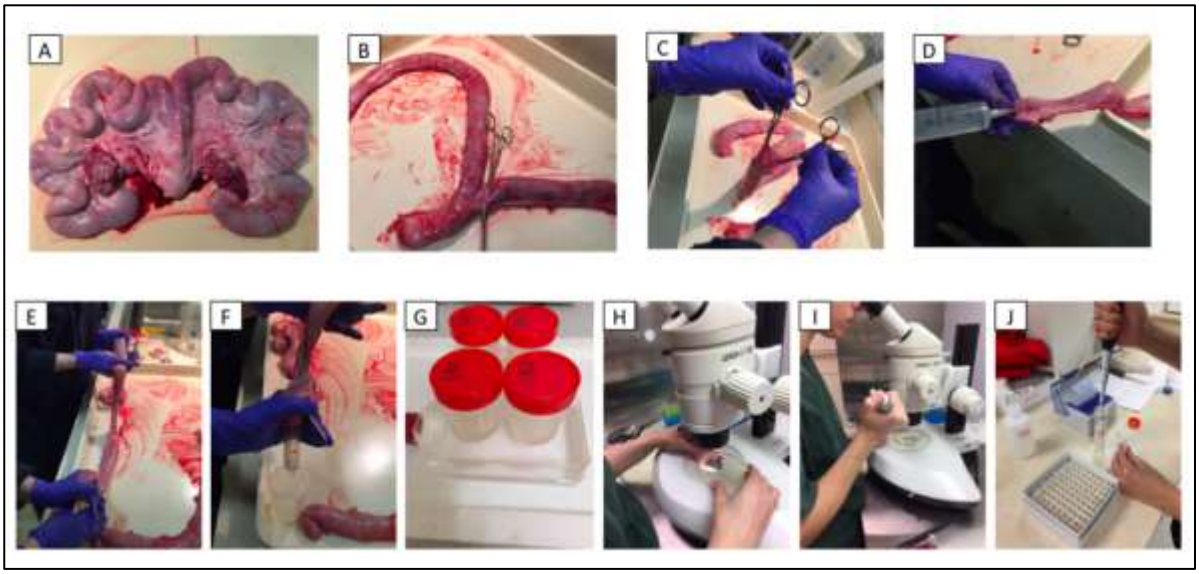
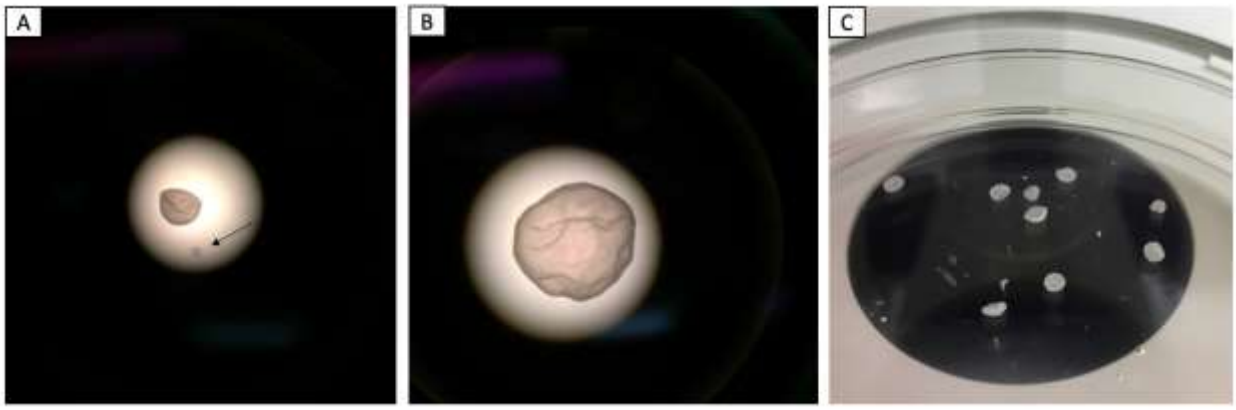


Figure 6.2 Overall sample collection process.



A. Reproductive tract from a sows euthanized at D9 of gestation; **B.** After removing the broad ligament, the extreme of one uterine horn was clamped; **C.** The two uterine horns were separated and a modified syringe was inserted one extreme of the uterine horn; **D.** From the opposite extreme 25 mL of BPS was flushed from the oviduct towards the uterine horn; **E.** The uterine horn was slowly massaged, in order to direct the fluid and embryos towards the syringe; **G.** Once the liquid was collected in an individual container, the process was repeated; **H.** In the lab, the material from each container was evaluated individually. The embryos were classified in relation to developmental stage; **I.** The embryos were collected individually; **J.** 20 µl of DNA/RNA shield was added in each tube and stored in -20°C.

Figure 6.2 Embryonic development (morphology) assessment. Example of embryonic development assessment. **A.** Medium collapsed embryo and small spherical embryo (black arrow); **B.** Medium collapsed embryo zoomed in; **C.** Large collapsed embryos on the plate.



Tables

Table 6.1 Average ovulation rate, average number of embryos and total number of embryos recovered from LLBWP and HLBWP sows.

	LLBWP (n = 9 sows)	HLBWP (n=8 sows)
Average Ovulation Rate	24.84	25.91
Average Number of Embryos Recovered	14.59	20.37
Total Number of Embryos Recovered	129	138
Percent of embryos recovered	58.7%	78.6%

Table 6.2 Embryonic sex determination and RNA integrity assessment for the selected embryos from LLBWP and HLBWP sows.

	LLBWP (n = 4 sows; n = 63 embryos)	HLBWP (n = 4 sows; n = 41 embryos)
Embryonic sex determination		
Female	25 (40.9%)	25 (62.5%)
Male	23 (37.7%)	13 (32.5%)
Unknown	15 (24.5%)	3 (0.75%)
RNA Integrity Assessment		
Failed (RIN ≤ 6)	19 (30%)	30 (73%)
Passed (RIN > 6)	44 (69%)	11 (26%))

*Only sows with the majority of embryos on spherical stage of development (Spherical small, spherical medium) were selected.

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Appendix 2

Table 4.1S Gene statistics from D30 embryos and placenta tissues.

Sample	Group N	Tissue type	Total Clean Reads	Total Mapping Ratio	Uniquely Mapping Ratio	Total Gene N	Known Gene N	Novel Gene N	Av total gene N	Av Known Gene N	Av Novel Gene N
H1FE1	HF	D30 embryo (female)	41990492	89.09%	58.58%	17964	17206	758			
H1FE2	HF	D30 embryo (female)	43049466	89.03%	58.22%	17952	17194	758			
H1FE3	HF	D30 embryo (female)	45643616	88.89%	57.65%	18051	17282	769			
H1FE4	HF	D30 embryo (female)	43319362	89.04%	58.03%	18011	17240	771			
H2FE1	HF	D30 embryo (female)	60240252	89.14%	58.42%	18213	17420	793			
H2FE2	HF	D30 embryo (female)	41265138	89.15%	58.16%	17902	17150	752			
H2FE3	HF	D30 embryo (female)	49028048	88.91%	58.15%	18039	17259	780			
H2FE4	HF	D30 embryo (female)	48839674	88.99%	58.05%	18039	17272	767			
H3FE1	HF	D30 embryo (female)	50531172	89.18%	58.53%	17970	17222	748			
H3FE2	HF	D30 embryo (female)	57769096	89.70%	59.88%	18033	17262	771			
H3FE3	HF	D30 embryo (female)	43966972	89.71%	59.83%	17910	17159	751			
H3FE4	HF	D30 embryo (female)	56100256	89.76%	58.81%	18045	17290	755			
H3FE5	HF	D30 embryo (female)	51724752	89.67%	58.51%	17959	17195	764			
H4FE1	HF	D30 embryo (female)	49324456	89.57%	59.42%	18062	17275	787			
H4FE2	HF	D30 embryo (female)	50414730	89.47%	58.04%	18063	17295	768			
H4FE3	HF	D30 embryo (female)	53327604	89.63%	59.53%	18094	17327	767			
L1FE1	LF	D30 embryo (female)	42189492	91.13%	61.74%	17929	17163	766			
L1FE2	LF	D30 embryo (female)	62898668	91.11%	61.36%	18176	17394	782			
L1FE3	LF	D30 embryo (female)	47016658	90.93%	61.21%	18036	17271	765			
L1FE4	LF	D30 embryo (female)	43037194	91.08%	62.48%	17936	17191	745			
L1FE5	LF	D30 embryo (female)	45971514	91.09%	62.99%	18002	17234	768			
L2FE1	LF	D30 embryo (female)	42575514	91.33%	63.53%	17950	17202	748			
L2FE2	LF	D30 embryo (female)	41133572	92.52%	65.90%	17908	17155	753			
L2FE3	LF	D30 embryo (female)	40120642	92.11%	64.53%	17902	17152	750			
L2FE4	LF	D30 embryo (female)	42880700	92.43%	65.76%	17979	17237	742			

L2FE5	LF	D30 embryo (female)	41905186	92.68%	66.58%	17963	17200	763			
L2FE6	LF	D30 embryo (female)	43959516	92.21%	64.95%	17988	17222	766			
L3FE1	LF	D30 embryo (female)	44406876	92.30%	65.11%	17981	17227	754			
L3FE2	LF	D30 embryo (female)	40953710	92.38%	65.53%	17835	17097	738			
L4FE1	LF	D30 embryo (female)	63346838	92.11%	63.59%	18201	17442	759			
L4FE2	LF	D30 embryo (female)	55790738	92.43%	64.28%	18050	17284	766			
L4FE3	LF	D30 embryo (female)	54245368	92.18%	64.42%	18092	17322	770			
H1ME1	HM	D30 embryo (male)	48999010	88.77%	56.70%	18029	17271	758			
H1ME2	HM	D30 embryo (male)	40640812	88.91%	57.87%	17913	17162	751			
H2ME1	HM	D30 embryo (male)	42195326	89.42%	59.34%	17988	17234	754			
H2ME2	HM	D30 embryo (male)	55191802	88.99%	57.71%	18177	17415	762			
H2ME3	HM	D30 embryo (male)	55660564	89.33%	59.00%	18131	17351	780			
H2ME4	HM	D30 embryo (male)	58580658	89.72%	58.72%	18130	17350	780			
H3ME1	HM	D30 embryo (male)	48436418	89.57%	58.70%	17916	17166	750			
H3ME2	HM	D30 embryo (male)	49887468	89.69%	58.51%	17906	17151	755			
H3ME3	HM	D30 embryo (male)	44388176	89.51%	57.79%	17876	17138	738			
H3ME4	HM	D30 embryo (male)	48607198	89.57%	57.86%	17993	17247	746			
H3ME5	HM	D30 embryo (male)	53072284	89.63%	58.11%	18002	17260	742			
H4ME1	HM	D30 embryo (male)	51316142	89.68%	59.08%	18055	17286	769			
H4ME2	HM	D30 embryo (male)	52326612	89.49%	58.32%	18113	17339	774			
H4ME3	HM	D30 embryo (male)	54651130	89.64%	58.67%	18074	17301	773			
H4ME4	HM	D30 embryo (male)	48750238	89.40%	59.50%	18026	17269	757			
H4ME5	HM	D30 embryo (male)	40296516	90.61%	60.45%	17942	17197	745			
L1ME1	LM	D30 embryo (male)	46295886	91.27%	60.84%	18018	17245	773			
L1ME2	LM	D30 embryo (male)	49107546	91.16%	62.03%	18079	17311	768			
L1ME3	LM	D30 embryo (male)	40484716	91.18%	61.94%	17935	17179	756			
L1ME4	LM	D30 embryo (male)	43694124	91.16%	62.71%	18014	17251	763			
L2ME1	LM	D30 embryo (male)	44453934	92.50%	65.84%	17935	17191	744			
L2ME2	LM	D30 embryo (male)	43501724	92.68%	66.24%	17932	17165	767			
L2ME3	LM	D30 embryo (male)	63223122	91.73%	63.50%	18147	17374	773			

L2ME4	LM	D30 embryo (male)	46046846	92.22%	65.33%	18067	17298	769			
L2ME5	LM	D30 embryo (male)	50345520	91.74%	63.79%	18038	17274	764			
L3ME1	LM	D30 embryo (male)	44199792	92.34%	65.92%	17999	17234	765			
L3ME2	LM	D30 embryo (male)	49770964	92.64%	65.31%	18089	17316	773			
L3ME3	LM	D30 embryo (male)	57518110	92.29%	64.02%	18125	17353	772			
L3ME4	LM	D30 embryo (male)	55421088	92.50%	64.44%	18110	17339	771			
L4ME1	LM	D30 embryo (male)	61714546	92.53%	64.12%	18159	17369	790			
L4ME3	LM	D30 embryo (male)	52773612	92.52%	64.64%	18008	17238	770	18018	17256	763
H1P1	HP	Placenta	54625708	92.07%	61.18%	17370	16619	751			
H1P2	HP	Placenta	55631584	91.79%	59.35%	17133	16423	710			
H1P3	HP	Placenta	56573692	91.89%	59.21%	16891	16183	708			
H1P4	HP	Placenta	61427972	92.07%	58.58%	17272	16551	721			
H2P1	HP	Placenta	55821022	91.92%	62.05%	17102	16361	741			
H2P2	HP	Placenta	54311262	92.11%	62.56%	17386	16632	754			
H2P3	HP	Placenta	53549068	91.64%	59.70%	16890	16177	713			
H2P4	HP	Placenta	60818826	91.47%	57.76%	17687	16941	746			
H3P1	HP	Placenta	63765292	91.02%	61.63%	17295	16539	756			
H3P2	HP	Placenta	64989772	91.27%	61.06%	17352	16583	769			
H3P3	HP	Placenta	54878994	91.78%	60.70%	16987	16251	736			
H3P4	HP	Placenta	52841040	91.82%	60.69%	17116	16380	736			
H3P5	HP	Placenta	54850602	92.55%	64.47%	17337	16584	753			
H4P1	HP	Placenta	56099958	92.96%	64.06%	16696	15982	714			
H4P2	HP	Placenta	57435900	92.82%	62.68%	16648	15958	690			
H4P3	HP	Placenta	50093204	92.82%	63.38%	16797	16073	724			
H4P4	HP	Placenta	54655802	92.57%	63.04%	17050	16322	728			
L1P1	LP	Placenta	55652172	92.28%	64.55%	17270	16513	757			
L1P2	LP	Placenta	52730600	92.48%	65.81%	17370	16611	759			
L1P3	LP	Placenta	49922634	92.37%	62.57%	17177	16438	739			
L1P4	LP	Placenta	49380930	92.45%	65.01%	17055	16321	734			
L1P5	LP	Placenta	54113650	92.30%	65.32%	17163	16415	748			

L1P6	LP	Placenta	57550076	92.16%	64.69%	17317	16571	746			
L2P1	LP	Placenta	53260174	92.94%	66.59%	17060	16333	727			
L2P2	LP	Placenta	60990258	92.50%	67.73%	17417	16667	750			
L2P3	LP	Placenta	51515040	92.61%	68.08%	17276	16530	746			
L2P4	LP	Placenta	48773882	92.45%	67.65%	17130	16390	740			
L3P1	LP	Placenta	46614686	93.06%	64.71%	16479	15809	670			
L3P2	LP	Placenta	61553626	92.79%	66.33%	17353	16610	743			
L3P3	LP	Placenta	51244050	92.99%	66.36%	17066	16335	731			
L3P4	LP	Placenta	53420862	92.37%	68.09%	17332	16586	746			
L4P1	LP	Placenta	49495322	93.21%	68.21%	17265	16536	729			
L4P2	LP	Placenta	57476448	92.91%	67.16%	17266	16542	724			
L4P3	LP	Placenta	52555520	92.84%	66.71%	17336	16604	732			
L4P4	LP	Placenta	51347058	93.15%	66.17%	16552	15869	683	17140	16407	733
Average			51025366	91.26%	62.17%	17139.8	16406.83	732.9714			
SD				0.014105	0.032511						

Table 4.2S Level of gene expression (FPKM) for female D30 embryos from LLBWP and HLBWP sows.

Table 4.3S Level of gene expression (FPKM) for male D30 embryos from LLBWP and HLBWP sows.

Table 4.4S List of down and up-regulated genes in LLBWP as compared to HLBWP.

Table 4.5S List of genes differentially expressed in LLBWP embryonic tissues compared to HLBWP.

Gene ID	Symbol	log2Fold Change	P adj	Chr	Genomic NC	Start position	End position	Orientation	Exon N	Description
100518860	ABCC4	-0.73	3.9E-06	11						multidrug resistance-associated protein 4-like; PREDICTED: Homo sapiens ATP binding cassette subfamily C member 4 (ABCC4)
110255846	ABCC4	-0.71	0.01	11	NC_010453.5	64435609	64460309	plus	4	multidrug resistance-associated protein 4-like; PREDICTED: Homo sapiens ATP binding cassette subfamily C member 4 (ABCC4)
110259013	ABCC4	-0.86	0.02	Un						multidrug resistance-associated protein 4-like; PREDICTED: Homo sapiens ATP binding cassette subfamily C member 4 (ABCC4)
397255	ACAN	-0.87	4.5E-08	7	NC_010449.5	54449830	54520606	plus	18	aggrecan
100154447	ACKR1	-0.73	4.2E-07	4	NC_010446.5	91224089	91225610	minus	2	atypical chemokine receptor 1 (Duffy blood group)
100624449	ADD2	-0.81	3.8E-14	3	NC_010445.4	71729882	71845761	plus	18	adducin 2
100516957	ADGRE2	-0.72	6.4E-03	2	NC_010444.4	63853304	63900460	plus	26	adhesion G protein-coupled receptor E2-like
102164536	AHSP	-1.32	2.1E-10	3	NC_010445.4	17059340	17060343	minus	3	alpha hemoglobin stabilizing protein
100518817	ALAS2	-0.78	9.6E-07	X	NC_010461.5	47871519	47896041	minus	11	5'-aminolevulinate synthase 2
396960	ALB	-0.86	5.9E-04	8	NC_010450.4	69643427	69663152	plus	14	albumin
100739347	ALDH3B1	-0.73	1.3E-04	2	NC_010444.4	4868561	4889179	minus	12	aldehyde dehydrogenase 3 family member B1
100157679	ANK1	-1.15	1.8E-14	17	NC_010459.5	10752258	10985129	minus	42	ankyrin 1
100514179	APBB1IP	-0.74	9.8E-09	10	NC_010452.4	49125074	49242864	minus	14	amyloid beta precursor protein binding family B member 1 interacting protein
407773	AQP1	-0.94	6.3E-06	18	NC_010460.4	42063482	42076741	minus	4	aquaporin 1 (Colton blood group)
100519298	ARRDC2	-0.74	2.4E-11	2	NC_010444.4	59771104	59778500	minus	9	arrestin domain containing 2
100152653	ART4	-1.07	1.9E-09	5	NC_010447.5	57708463	57727678	plus	4	ADP-ribosyltransferase 4 (Dombrock blood group)
100522939	ART5	-1.75	8.1E-11	9	NC_010451.4	6443768	6449203	plus	7	ADP-ribosyltransferase 5
100511749	ASPN	-1.03	1.3E-21	3	NC_010445.4	42165553	42192902	minus	9	asporin
100738802	BCAN	-1.12	1.3E-06	4	NC_010446.5	93432331	93450895	minus	14	brevican
100625219	BLVRB	-0.74	6.8E-10	6	NC_010448.4	48715540	48728433	minus	5	biliverdin reductase B
100626139	BTN1A1	-1.00	0.04	7	NC_010449.5	20977940	20985300	plus	7	butyrophilin subfamily 1 member A1
397072	C3	-0.79	8.2E-03	2	NC_010444.4	72431470	72471622	plus	40	complement C3
445467	C4A	-0.70	4.1E-06	7	NC_010449.5	24068019	24083060	plus	41	complement C4A (Rodgers blood group)

396982	C4BPA	-0.80	0.05	9	NC_010451.4	67815544	67827509	plus	4	complement component 4 binding protein, alpha
100170138	CAMP	0.83	0.03	13	NC_010455.5	30959802	30961519	plus	4	antibacterial protein
100156753	CAPN11	-0.72	0.02	7	NC_010449.5	39168991	39191934	plus	22	calpain 11
100514007	CCDC42	-1.38	2.7E-11	12	NC_010454.4	53869559	53883929	minus	7	coiled-coil domain containing 42
414374	CCR1	-0.90	3.6E-07	13	NC_010455.5	29227218	29233862	minus	2	C-C motif chemokine receptor 1
414373	CCR3	-1.71	3.6E-07	13	NC_010455.5	29251950	29286470	plus	4	C-C motif chemokine receptor 3
414370	CCRL2	-0.78	4.6E-09	13	NC_010455.5	29402488	29410508	plus	10	C-C motif chemokine receptor like 2
100152851	CD84	-0.93	1.9E-10	4	NC_010446.5	89932020	89980476	plus	12	CD84 molecule
100512562	CDHR1	-0.85	3.7E-04	14	NC_010456.5	85429506	85455823	plus	17	cadherin related family member 1
100515210	CDKN2C	-0.79	5.6E-13	6	NC_010448.4	1.61E+08	1.61E+08	minus	3	cyclin dependent kinase inhibitor 2C
100738053	CHD5	-0.71	5.8E-13	6	NC_010448.4	67017410	67086685	minus	42	chromodomain helicase DNA binding protein 5
100153817	CLDN20	-0.75	1.8E-03	1	NC_010443.5	11617807	11618550	minus	1	claudin 20
100516055	CLIC2	-1.26	1.8E-10	X	NC_010461.5	1.26E+08	1.26E+08	minus	7	chloride intracellular channel 2
100738123	COL1A1	-0.77	4.9E-14	12	NC_010454.4	26379087	26397180	minus	51	collagen type I alpha 1 chain
100516642	COL6A6	-1.05	2.7E-11	13	NC_010455.5	1817475	1985968	minus	37	collagen type VI alpha 6 chain
100511474	CPOX	-0.99	1.6E-13	13	NC_010455.5	1.6E+08	1.6E+08	plus	7	coproporphyrinogen oxidase
100157716	CPS1	-0.73	1.7E-03	15	NC_010457.5	1.13E+08	1.13E+08	plus	40	carbamoyl-phosphate synthase 1
100156107	CRYGB	-0.73	2.4E-03	15	NC_010457.5	1.11E+08	1.11E+08	minus	4	gamma-crystallin B
110257071	CRYGC	-0.89	2.6E-05	15	NC_010457.5	1.11E+08	1.11E+08	minus	3	gamma-crystallin C-like
100519628	CRYGD	-0.79	6.7E-03	15	NC_010457.5	1.11E+08	1.11E+08	minus	3	gamma-crystallin D
100523909	CYP4B1	-0.75	0.02	6	NC_010448.4	1.65E+08	1.65E+08	minus	12	cytochrome P450 4B1
102167481	CYP4F3	-0.73	0.04	2	NC_010444.4	62007022	62030545	minus	13	cytochrome P450 4F6-like
100157295	DENND4A	-0.77	2.1E-12	1	NC_010443.5	1.64E+08	1.64E+08	minus	33	DENN domain containing 4A
100157264	DMTN	-0.77	2.2E-12	14	NC_010456.5	6315643	6346722	plus	19	dematin actin binding protein
100739107	DYRK3	-0.70	1.3E-09	9	NC_010451.4	67268600	67279617	plus	3	dual specificity tyrosine phosphorylation regulated kinase 3
110261113	E2F2	-1.03	5.7E-15	6	NC_010448.4	81377922	81398862	minus	7	E2F transcription factor 2
100512958	ECRG4	-0.77	4.6E-06	3	NC_010445.4	48960413	48971878	minus	5	ECRG4 augurin precursor
100518056	EHBP1L1	-0.83	1.9E-09	2	NC_010444.4	6662460	6678721	minus	22	EH domain binding protein 1 like 1
100152957	EPB42	-1.00	1.5E-10	1	NC_010443.5	1.28E+08	1.28E+08	plus	14	erythrocyte membrane protein band 4.2

100737148	ERFE	-1.53	4.3E-07	15	NC_010457.5	1.38E+08	1.38E+08	plus	8	erythroferrone
100620314	ERMAP	-1.04	1.1E-10	6	NC_010448.4	1.69E+08	1.69E+08	minus	13	erythroblast membrane associated protein (Scianna blood group)
396705	FATE1	-0.88	0.02	X	NC_010461.5	1.23E+08	1.23E+08	plus	5	fetal and adult testis expressed 1
100322873	FECH	-0.82	1.7E-12	1	NC_010443.5	1.07E+08	1.07E+08	minus	11	ferrochelatase
100152116	FGF6	-0.74	9.2E-05	5	NC_010447.5	65975807	65990035	plus	3	fibroblast growth factor 6
110261009	FOSB	1.18	2.7E-05	6	NC_010448.4	51836780	51844531	plus	4	FosB proto-oncogene, AP-1 transcription factor subunit
100520836	GALNT6	-0.97	3.9E-09	5						polypeptide N-acetylgalactosaminyltransferase 6
100620659	GATA1	-0.89	6.8E-12	X	NC_010461.5	42898431	42905939	plus	6	GATA binding protein 1
100514698	GFI1B	-0.80	1.2E-08	1	NC_010443.5	2.73E+08	2.73E+08	plus	8	growth factor independent 1B transcriptional repressor
397440	GRIN1	-0.84	1.5E-07	Un						glutamate ionotropic receptor NMDA type subunit 1
100525591	GYPA	-1.05	6.5E-11	8	NC_010450.4	84037574	84067096	plus	7	glycophorin A (MNS blood group)
100518277	GYPC	-0.71	3.5E-10	15	NC_010457.5	25576343	25606913	minus	3	glycophorin C (Gerbich blood group)
595122	H1-3	-0.77	2.5E-12	7	NC_010449.5	20881642	20882307	minus	1	histone H1.3-like protein
110259958	HBA2	-1.10	1.8E-10	3	NC_010445.4	41482353	41483564	minus	3	hemoglobin subunit alpha; Homo sapiens hemoglobin subunit alpha 2 (HBA2)
100737768	HBA2	-1.10	2.4E-10	3	NC_010445.4	41486699	41487559	minus	3	hemoglobin subunit alpha; Homo sapiens hemoglobin subunit alpha 2 (HBA2)
407066	HBB	-1.76	1.0E-09	9	NC_010451.4	4800683	4801941	minus	3	hemoglobin, beta
100737727	HBM	-1.52	2.5E-08	3	NC_010445.4	41489312	41490110	minus	3	hemoglobin subunit mu
100155492	HEMGN	-1.18	3.2E-12	1	NC_010443.5	2.4E+08	2.4E+08	minus	8	hemogen
396581	HMBS	-1.04	8.9E-13	9	NC_010451.4	46300039	46308681	plus	16	hydroxymethylbilane synthase
397443	HPD	-1.04	7.5E-04	14	NC_010456.5	30845608	30857658	plus	14	4-hydroxyphenylpyruvate dioxygenase
110255862	HRCT1	-0.84	1.7E-03	1	NC_010443.5	2.37E+08	2.37E+08	plus	1	histidine rich carboxyl terminus 1
100515333	IKZF1	-0.91	2.3E-11	9	NC_010451.4	1.36E+08	1.36E+08	plus	13	IKAROS family zinc finger 1
100518554	INHBE	0.89	0.04	5	NC_010447.5	22732774	22734716	plus	2	inhibin subunit beta E
100511542	KEL	-0.99	3.8E-06	18	NC_010460.4	7242378	7279345	plus	22	Kell metallo-endopeptidase (Kell blood group)
100174958	KLF1	-1.19	1.7E-11	2	NC_010444.4	66144767	66148500	plus	3	Kruppel like factor 1
100049664	KRT1	-1.26	9.2E-05	5	NC_010447.5	18012922	18018582	minus	9	keratin 1
100515166	KRT13	-0.95	4.9E-03	12	NC_010454.4	21139296	21143556	plus	8	keratin, type I cytoskeletal 13

100511564	KRT5	-0.94	9.8E-11	5	NC_010447.5	17862047	17868397	minus	9	keratin 5
100627608	LOC100627608	-1.02	0.04	x	NC_010461.5	42559714	42567979	plus	7	protein SSX1-like
102161780	LOC102161780	-1.30	1.1E-07	4	NC_010446.5	92808788	92821202	minus	7	SLAM family member 9-like
106504658	LOC106504658	-0.73	0.02	8	NC_010450.4	51534820	51576623	plus	8	uncharacterized LOC106504658
106505794	LOC106505794	-0.95	1.3E-06	13	NC_010455.5	1.42E+08	1.42E+08	minus	1	uncharacterized LOC106505794
106506534	LOC106506534	-0.82	0.02	11	NC_010453.5	38188894	38207206	plus	7	uncharacterized LOC106506534
110256914	LOC110256914	-0.86	0.02	15	NC_010457.5	66870667	66872288	minus	3	uncharacterized LOC110256914
110257477	LOC110257477	-0.77	2.4E-03	17	NC_010459.5	3801146	3801970	minus	1	uncharacterized LOC110257477
110259374	LOC110259374	-1.03	2.0E-08	2	NC_010444.4	76648079	76651850	minus	1	uncharacterized LOC110259374
110259514	LOC110259514	-0.90	4.5E-05	1	NC_010443.5	1.32E+08	1.32E+08	plus	3	uncharacterized LOC110259514
100738720	MAL	-1.81	2.2E-09	3	NC_010445.4	46181554	46196723	minus	4	myelin and lymphocyte protein; Homo sapiens mal, T cell differentiation protein (MAL)
100624277	MATN1	-0.73	1.5E-04	6	NC_010448.4	87342030	87351483	minus	8	matrilin 1
100152017	MBOAT1	-0.73	8.4E-10	7	NC_010449.5	15532641	15644332	minus	17	membrane bound O-acyltransferase domain containing 1
100627859	MS4A12	-0.80	0.02	2	NC_010444.4	11177406	11193744	minus	8	membrane spanning 4-domains A12
397417	MT1A	-1.00	8.0E-03	6	NC_010448.4	18672016	18673673	plus	3	metallothionein 1A
100037920	MT1D	-0.87	9.4E-03	6	NC_010448.4	18650985	18652317	plus	4	metallothionein-1E
102166944	MT1E	-0.97	8.8E-03	6	NC_010448.4	18660665	18662000	plus	3	metallothionein-1E-like
100739663	MT1X	-0.95	0.02	6	NC_010448.4	18655972	18657515	plus	3	metallothionein-1C
396827	MT2A	-0.84	0.02	6	NC_010448.4	18645125	18646034	plus	3	metallothionein-2A
100624460	MUC16	-0.80	0.01	2	NC_010444.4	66901635	67017040	minus	60	mucin-16
106504436	MYADM	-1.48	2.6E-12	7	NC_010449.5	54178736	54182408	minus	1	myeloid-associated differentiation marker-like; Homo sapiens myeloid associated differentiation marker (MYADM)
100736871	MYADM	-1.65	3.5E-12	7	NC_010449.5	54197396	54203024	plus	1	myeloid-associated differentiation marker-like; Homo sapiens myeloid associated differentiation marker (MYADM)
110261552	MYADM	-1.10	2.4E-07	7	NC_010449.5	54108754	54110398	minus	1	myeloid-associated differentiation marker-like; Homo sapiens myeloid associated differentiation marker (MYADM)

100520032	MYADM	-1.33	3.2E-05	7	NC_010449.5	54124213	54125511	minus	1	myeloid-associated differentiation marker-like; Homo sapiens myeloid associated differentiation marker (MYADM)
100737631	MYADM	-1.24	5.4E-03	7	NC_010449.5	54244429	54245507	plus	1	myeloid-associated differentiation marker-like; Homo sapiens myeloid associated differentiation marker (MYADM)
100156674	MYADM	-1.36	0.01	7	NC_010449.5	54267572	54270462	minus	1	myeloid-associated differentiation marker-like; Homo sapiens myeloid associated differentiation marker (MYADM)
100152264	MYADM	-0.77	0.03	7	NC_010449.5	54275607	54277300	minus	2	myeloid-associated differentiation marker-like; Homo sapiens myeloid associated differentiation marker (MYADM)
396646	MYD88	-0.73	9.9E-10	13	NC_010455.5	22976951	22979568	plus	5	MYD88 innate immune signal transduction adaptor
100627924	MYH11	-0.73	5.6E-08	3	NC_010445.4	7002667	7143093	plus	42	myosin heavy chain 11
100739447	NALCN	-0.97	7.7E-04	11	NC_010453.5	69710355	70023020	minus	45	sodium leak channel, non-selective
102167934	NEUROD2	-1.13	1.4E-12	12	NC_010454.4	22709473	22713785	plus	2	neuronal differentiation 2
110257619	NEUROD6	-1.17	3.0E-12	18	NC_010460.4	41757356	41763837	plus	2	neuronal differentiation 6
100157948	NFE2	-0.88	3.9E-11	5	NC_010447.5	19494672	19501810	minus	4	nuclear factor, erythroid 2
100523536	NFIX	-0.79	6.8E-16	2	NC_010444.4	65954808	66056591	minus	14	nuclear factor I X
100515982	NPC1L1	-0.86	1.3E-03	18	NC_010460.4	50726854	50757676	plus	19	NPC1 like intracellular cholesterol transporter 1
110259901	OR7A10	-0.80	0.05	3	NC_010445.4	17054075	17055386	plus	1	olfactory receptor 7A17-like
100152038	OSM	-0.90	4.8E-04	14	NC_010456.5	47242767	47246836	minus	3	oncostatin M
100621260	PCTP	-1.02	1.6E-03	12	NC_010454.4	32121343	32156021	plus	6	phosphatidylcholine transfer protein; Homo sapiens phosphatidylcholine transfer protein (PCTP)
100621753	PHOSPHO1	-1.23	4.0E-06	12	NC_010454.4	25441564	25449267	minus	5	phosphoethanolamine/phosphocholine phosphatase 1
102158609	PHOSPHO1	-1.11	8.7E-03	12	NC_010454.4	25315628	25321976	plus	3	phosphoethanolamine/phosphocholine phosphatase
100738344	PKLR	-0.71	1.6E-12	4	NC_010446.5	94536978	94546561	plus	12	pyruvate kinase L/R
100155717	PRXL2A	-0.77	1.2E-13	14	NC_010456.5	82249450	82271227	plus	9	peroxiredoxin like 2A
100512433	PYY	-0.97	4.5E-04	12	NC_010454.4	19190703	19208373	plus	7	peptide YY
100621675	RAC2	-1.14	6.9E-13	5	NC_010447.5	10553986	10571082	plus	9	Rac family small GTPase 2
100513761	RASL10A	-1.10	2.4E-08	14	NC_010456.5	46455441	46457977	minus	4	RAS like family 10 member A
100516620	RHAG	-0.99	4.7E-11	7	NC_010449.5	43704152	43730944	minus	10	Rh associated glycoprotein

397670	RHCE	-0.94	1.4E-08	6	NC_010448.4	82880585	82925238	minus	10	Rh blood group CcEe antigens
100521485	RTL8C	0.79	0.04	X	NC_010461.5	1.11E+08	1.11E+08	minus	1	protein FAM127
100625708	SAMD7	-0.97	8.9E-03	13	NC_010455.5	1.09E+08	1.09E+08	plus	7	sterile alpha motif domain containing 7
100157861	SLC14A1	-1.14	9.0E-06	1	NC_010443.5	95354848	95381492	plus	13	solute carrier family 14 member 1 (Kidd blood group)
100525793	SLC22A16	-1.64	9.9E-10	1	NC_010443.5	76423386	76468267	minus	9	solute carrier family 22 member 16
100271724	SLC22A4	-1.21	6.2E-08	2	NC_010444.4	1.35E+08	1.35E+08	plus	12	solute carrier family 22 member 4
100514249	SLC4A1	-1.25	1.8E-13	12	NC_010454.4	18957813	18975266	plus	21	solute carrier family 4 member 1 (Diego blood group)
100525049	SLC7A10	-0.74	0.04	6	NC_010448.4	43002427	43017605	minus	11	solute carrier family 7 member 10
100524318	SLC7A3	-1.46	2.3E-09	6	NC_010448.4	56306715	56316274	minus	12	cationic amino acid transporter 3-like; Homo sapiens solute carrier family 7 member 3 (SLC7A3)
100523609	SLC7A3	-1.37	1.8E-07	6	NC_010448.4	56357056	56370491	minus	13	cationic amino acid transporter 3-like; Homo sapiens solute carrier family 7 member 3 (SLC7A3)
100524499	SLC7A3	-1.24	5.7E-06	6	NC_010448.4	56297284	56302622	minus	12	cationic amino acid transporter 3-like; Homo sapiens solute carrier family 7 member 3 (SLC7A3)
100524147	SLC7A3	-1.38	2.7E-05	6	NC_010448.4	56320868	56329710	minus	12	cationic amino acid transporter 3-like; Homo sapiens solute carrier family 7 member 3 (SLC7A3)
100523969	SLC7A3	-1.30	2.5E-04	6	NC_010448.4	56334357	56339410	minus	11	cationic amino acid transporter 3-like; Homo sapiens solute carrier family 7 member 3 (SLC7A3)
100620152	SLC7A3	-1.05	5.4E-04	6	NC_010448.4	56510063	56522955	plus	15	cationic amino acid transporter 3-like; Homo sapiens solute carrier family 7 member 3 (SLC7A3)
100523792	SLC7A3	-1.01	7.8E-03	6	NC_010448.4	56343859	56348350	minus	10	cationic amino acid transporter 3-like; Homo sapiens solute carrier family 7 member 3 (SLC7A3)
110255287	SLC7A3	-1.16	0.03	6	NC_010448.4	56486342	56509078	plus	18	cationic amino acid transporter 3-like; Homo sapiens solute carrier family 7 member 3 (SLC7A3)
110256053	SLFN14	-1.25	6.1E-14	12	NC_010454.4	39947791	39962823	plus	5	schlafen family member 14
100157430	SNORC	-0.78	4.6E-03	15	NC_010457.5	1.33E+08	1.33E+08	plus	3	secondary ossification center associated regulator of chondrocyte maturation
100624664	SNX22	-1.11	2.5E-11	1	NC_010443.5	1.08E+08	1.08E+08	minus	7	sorting nexin 22
396872	SPINK4	-0.97	6.8E-06	10	NC_010452.4	33246260	33255617	minus	4	serine peptidase inhibitor Kazal type 4

100152068	SPTA1	-1.01	7.6E-10	4	NC_010446.5	91573470	91640057	plus	52	spectrin alpha, erythrocytic 1
100154659	SPTB	-0.98	1.3E-12	7	NC_010449.5	88812717	88954471	minus	39	spectrin beta, erythrocytic
100521731	TAL1	-0.77	6.1E-11	6	NC_010448.4	1.64E+08	1.64E+08	plus	8	TAL bHLH transcription factor 1, erythroid differentiation factor
100525341	TENT5C	-0.92	7.2E-10	4	NC_010446.5	1.03E+08	1.03E+08	minus	3	terminal nucleotidyltransferase 5C
100737915	TEX14	-0.88	1.9E-08	12	NC_010454.4	34815472	34975901	minus	33	testis expressed 14, intercellular bridge forming factor
100153940	THBS2	-0.75	2.5E-14	1	NC_010443.5	854473	882467	plus	23	thrombospondin 2
106507461	TLCD4	-0.70	7.0E-12	4	NC_010446.5	1.22E+08	1.22E+08	minus	18	TLC domain containing 4
100627220	TMCC2	-1.69	2.5E-14	9	NC_010451.4	65910175	65952606	plus	8	transmembrane and coiled-coil domain family 2
100511477	TRAK2	-0.78	1.2E-13	15	NC_010457.5	1.05E+08	1.05E+08	minus	17	trafficking kinesin protein 2
100144459	TRIM10	-1.37	8.4E-14	7	NC_010449.5	22718000	22731445	minus	8	tripartite motif containing 10
100144461	TRIM15	-0.91	6.4E-04	7	NC_010449.5	22730372	22742941	plus	9	tripartite motif containing 15
100294687	TRIM40	-1.31	3.4E-12	7	NC_010449.5	22702891	22717850	plus	6	tripartite motif containing 40
100519825	TRIM58	-1.68	3.8E-11	2	NC_010444.4	55822361	55843923	minus	7	tripartite motif containing 58
100519212	TRPV1	-0.77	0.04	12	NC_010454.4	49676536	49709634	minus	17	transient receptor potential cation channel subfamily V member 1
396592	TSPO	-1.04	3.9E-10	5	NC_010447.5	5723190	5735097	minus	4	translocator protein
100155551	TSPO2	-0.79	2.0E-03	7	NC_010449.5	36347027	36352497	plus	5	translocator protein 2
102159947	XAF1	-0.95	0.01	12	NC_010454.4	50644783	50659807	minus	8	XIAP associated factor 1
414375	XCR1	-1.43	7.4E-06	13	NC_010455.5	29150376	29233802	minus	6	X-C motif chemokine receptor 1
100511169	XK	-0.75	5.0E-11	X	NC_010461.5	33544287	33602576	plus	3	X-linked Kx blood group

Table 4.6S Level of gene expression (FPKM) in placental tissues from LLBWP sows compared to HLBWP sows.

Gene ID	Length	HP-Expression	LP-Expression	Symbol	HP-Expression in log10	LP-Expression in log10
100512433	986	37.14226	12.849	PYY	1.569868	1.108869
407066	644	8366.645	2947.777	HBB	3.922551	3.469495
100154934	3341	14.99093	5.448277	VENTX	1.175829	0.736259
100524028	1297	4.119619	1.624064	ZMYND12	0.614857	0.210603
100736832	12574	202.1752	83.06936	GRIN2A	2.305728	1.919441

100621200	4015	75.26393	30.97948	SYT8	1.876587	1.491074
100624742	1938	5.837315	2.567573	FAM57B	0.766213	0.409523
100519534	6152	489.9822	230.3385	ANO3	2.69018	2.362367
100620752	3220	103.4764	48.64691	ZNF385B	2.014841	1.687055
100624541	3944	384.0205	181.4537	LOC100624541	2.584354	2.258766
100157079	4784	776.4913	371.1233	SLC16A12	2.890137	2.569518
100525144	958	10.44547	5.007435	SLC51B	1.018928	0.699615
100623412	2851	1062.476	518.5942	CDH16	3.026319	2.714828
100154368	1793	202.2931	100.0961	PLA1A	2.305981	2.000417
100156168	2771	1130.178	562.4009	PRSS35	3.053147	2.750046
492314	885	170.5531	85.04779	TAC3	2.23186	1.929663
100621079	6356	6.98221	3.548633	MYH15	0.843993	0.550061
110257526	11122	56.37542	28.8231	LOC110257526	1.75109	1.459741
100518506	2621	209.5698	107.7023	FAM177B	2.321329	2.032225
100524100	5735	1329.466	685.2609	SEMA3E	3.123677	2.835856
106504436	3673	9.788487	5.04554	LOC106504436	0.990716	0.702908
100511641	9230	120.603	62.30611	GRIK3	2.081358	1.794531
100521274	1809	169.5057	89.19619	FAM131C	2.229184	1.950346
100155151	585	45.00214	24.20415	CRABP2	1.653233	1.38389
100516554	3348	691.6317	375.2197	NTN4	2.839875	2.574286
106506286	6269	152.6067	83.71646	LOC106506286	2.183574	1.922811
100522480	5017	74.87157	41.50868	MCTP1	1.874317	1.618139
100511417	3834	77.21499	43.27445	ISL1	1.887702	1.636232
100520347	4617	287.587	161.4195	TSPYL4	2.458769	2.207956
102157778	7438	631.0438	355.5268	LOC102157778	2.800059	2.550872
100156351	4089	101.966	57.69159	GARNL3	2.008456	1.761112
106508661	13500	1304.129	743.0498	FREM2	3.11532	2.871018
100519410	2598	630.6105	361.5427	GALNT18	2.799761	2.55816
641343	4532	269.0888	154.701	SLC34A3	2.429896	2.189493
100156934	3499	582.384	335.8478	PLEKHD1	2.765209	2.526142

397456	822	80.07852	46.46436	PTGDS	1.903516	1.66712
100737047	4612	13.26981	7.786152	SDR42E2	1.122865	0.891323
100521800	3919	91.41357	53.97384	NCMAP	1.961011	1.732183
102164898	6080	371.4198	219.8582	SEC16B	2.569865	2.342143
100517619	1199	92.11993	54.54331	TMEM176B	1.964354	1.736741
100516568	5482	217.2161	131.7658	COBL	2.336892	2.119803
110258335	3990	90.09795	55.5794	TNFAIP2	1.954715	1.744914
100153200	3059	1149.908	710.5288	SEMA3B	3.060663	2.851582
100518875	4152	1416.614	2377.427	EGFLAM	3.151252	3.376107
100113425	2096	522.9968	906.581	BMP4	2.718499	2.957407
100515686	1113	169.9721	299.5988	TMEM92	2.230378	2.47654
110258214	4174	8316.654	14660.77	LOC110258214	3.919949	4.166157
100627107	1369	13.59232	24.76775	KATNAL2	1.133294	1.393887
100622968	2838	759.6889	1385.781	DUSP6	2.880636	3.141694
100154661	5128	171.4625	317.708	RIPOR2	2.234169	2.502028
100155621	429	8.83707	16.54895	SEM1	0.946308	1.21877
102159729	2834	37.17975	72.7122	ARHGAP22	1.570306	1.861607
100627907	4267	35.86779	78.62855	DTNA	1.554705	1.89558
110261233	1682	2.116936	4.757721	FOXD2	0.325708	0.677399
100525205	1714	48.89124	110.8295	FOSL1	1.689231	2.044655
100522397	2307	25.90568	62.32208	SOX15	1.413395	1.794642
397103	5044	30.55766	75.03188	PCSK1	1.48512	1.875246
100517850	3538	40.88499	102.4334	LOC100517850	1.611564	2.010442
100522112	3815	69.22522	176.4438	GCNT3	1.840264	2.246606
396880	1491	10.36735	26.75181	CXCL8	1.015668	1.427353
100738968	1118	3.296645	8.740052	NGF	0.518072	0.941514
100516906	2484	3.233608	8.61603	EGR4	0.509687	0.935307
397564	2643	109.6649	305.3242	HBEGF	2.040068	2.484761
100738269	2312	403.7211	1141.804	PDPN	2.606081	3.057591
110258353	1980	34.35694	97.48321	ASPG	1.536014	1.98893

397563	1173	21.98574	62.6085	HTR1B	1.342141	1.796633
110261033	1956	5.953644	17.41729	KLK14	0.774783	1.240981
106504366	2360	0.771877	2.350847	MUC21	-0.11245	0.371224
100144482	2478	51.3833	157.8388	CDSN	1.710822	2.198214
110258215	735	2.444949	7.698349	LOC110258215	0.38827	0.886398
100512412	1567	3.945417	12.42835	APOBEC1	0.596093	1.094414
100519286	1960	47.38062	153.8254	SERPINB2	1.675601	2.187028
396991	717	25.41181	83.83129	IFNG	1.405036	1.923406
100625733	5437	3.726629	12.34187	DSG3	0.571316	1.091381
100626168	506	12.38349	41.16796	S100A5	1.092843	1.614559
102161388	2749	4.76583	15.89905	EPGN	0.678139	1.201371
110261028	1392	3.829065	13.05911	KLK9	0.583093	1.115913
100525099	2490	30.99649	110.5827	LOC100525099	1.491313	2.043687
110261030	1493	0.956643	3.609351	KLK11	-0.01925	0.557429
110261495	1118	58.7397	224.5585	LY6G6C	1.768932	2.351329
397469	694	10.69021	44.90372	SPRP	1.028986	1.652282
100737113	1558	1.044223	4.446427	LOC100737113	0.018793	0.648011

Table 4.7S List of genes differentially expressed in LLBWP placental tissues compared to HLBWP.

GeneID	Symbol	log2 FoldChange (LP/HP)	Padj	Chr	Genomic NC	Start Position	End Position	Orientation	Exon N	description
100517850	ABCC4	1.33	0.022649	18	NC_010460.4	6334420	6348887	plus	9	multidrug resistance-associated protein 4-like [Sus scrofa (pig)]; Homo sapiens ATP binding cassette subfamily C member 4 (ABCC4), transcript variant 1, mRNA
100519534	ANO3	-1.09	0.01338	2	NC_010444.4	33618740	34048973	minus	30	anoctamin 3
100512412	APOBEC1	1.66	0.015861	5	NC_010447.5	62811819	62820532	minus	5	apolipoprotein B mRNA editing enzyme catalytic subunit 1
102159729	ARHGAP22	0.97	0.049409	14	NC_010456.5	89113774	89331629	minus	16	Rho GTPase activating protein 22
110258353	ASPG	1.50	0.007625	Un						asparaginase
100113425	BMP4	0.79	0.048027	1	NC_010443.5	1.83E+08	1.83E+08	minus	4	bone morphogenetic protein 4

100623412	CDH16	-1.03	0.025475	6	NC_010448.4	27589183	27599002	minus	18	cadherin 16
100144482	CDSN	1.62	0.016555	7	NC_010449.5	23520142	23524372	minus	2	corneodesmosin
100516568	COBL	-0.72	0.048833	9	NC_010451.4	1.37E+08	1.37E+08	minus	14	cordon-bleu WH2 repeat protein
100155151	CRABP2	-0.89	0.03177	4	NC_010446.5	93388198	93393861	plus	4	cellular retinoic acid binding protein 2
396880	CXCL8	1.37	0.022417	8	NC_010450.4	69932646	69935861	plus	4	C-X-C motif chemokine ligand 8
100625733	DSG3	1.73	0.010956	6	NC_010448.4	1.15E+08	1.15E+08	plus	16	desmoglein 3
100627907	DTNA	1.13	0.035314	6	NC_010448.4	1.18E+08	1.19E+08	plus	29	dystrobrevin alpha
100622968	DUSP6	0.87	0.027688	5	NC_010447.5	93303058	93307510	plus	3	dual specificity phosphatase 6
100518875	EGFLAM	0.75	0.026725	16	NC_010458.4	23402937	23606234	plus	22	EGF like, fibronectin type III and laminin G domains
100516906	EGR4	1.41	0.043154	3	NC_010445.4	69485919	69488788	plus	2	early growth response 4
102161388	EPGN	1.74	0.019503	8	NC_010450.4	70266867	70301250	plus	6	epithelial mitogen
100521274	FAM131C	-0.93	0.049336	6	NC_010448.4	75193397	75213081	minus	9	family with sequence similarity 131 member C
100518506	FAM177B	-0.96	0.014135	10	NC_010452.4	11491364	11501865	plus	6	family with sequence similarity 177 member B
100525205	FOSL1	1.18	0.027741	2	NC_010444.4	6430158	6436173	plus	4	FOS like 1, AP-1 transcription factor subunit
110261233	FOXD2	1.17	0.022501	6	NC_010448.4	1.64E+08	1.64E+08	minus	1	forkhead box D2
106508661	FREM2	-0.81	0.018179	11	NC_010453.5	13775267	14116377	plus	30	FRAS1 related extracellular matrix 2
100519410	GALNT18	-0.80	0.025738	2	NC_010444.4	47863233	48231113	plus	11	polypeptide N-acetylgalactosaminyltransferase 18
100156351	GARNL3	-0.82	0.022501	1	NC_010443.5	2.68E+08	2.68E+08	plus	31	GTPase activating Rap/RanGAP domain like 3
100522112	GCNT3	1.35	0.04707	1	NC_010443.5	1.12E+08	1.13E+08	minus	10	glucosaminyl (N-acetyl) transferase 3, mucin type
100511641	GRIK3	-0.95	0.021355	6	NC_010448.4	92880297	93128048	minus	17	glutamate ionotropic receptor kainate type subunit 3
100736832	GRIN2A	-1.28	0.011061	3	NC_010445.4	32749329	33149350	plus	14	glutamate ionotropic receptor NMDA type subunit 2A
407066	HBB	-1.51	0.003299	9	NC_010451.4	4800683	4801941	minus	3	hemoglobin, beta
397564	HBEGF	1.48	0.011061	2	NC_010444.4	1.42E+08	1.42E+08	minus	6	heparin binding EGF like growth factor
397563	HTR1B	1.51	0.019315	1	NC_010443.5	88597510	88598682	plus	1	5-hydroxytryptamine receptor 1B
396991	IFNG	1.72	0.023166	5	NC_010447.5	32477906	32482670	minus	4	interferon gamma

100511417	ISL1	-0.84	0.045583	16	NC_010458.4	30962994	30974201	plus	6	ISL LIM homeobox 1
100627107	KATNAL2	0.87	0.022501	1	NC_010443.5	96566852	96676184	plus	17	katanin catalytic subunit A1 like 2
110261030	KLK11	1.92	0.011061	6	NC_010448.4	55679862	55685688	minus	6	kallikrein related peptidase 11
110261033	KLK14	1.55	0.019978	6	NC_010448.4	55719698	55726099	minus	5	kallikrein related peptidase 14
110261028	KLK9	1.77	0.018179	6	NC_010448.4	55662108	55669463	minus	5	kallikrein related peptidase 9
100737113	KRT17	2.09	0.007625	12	NC_010454.4	21024395	21029875	plus	8	keratin, type I cytoskeletal 17
100525099	LOC10052 5099	1.83	0.002544	2	NC_010444.4	52247737	52255792	plus	6	L-amino-acid oxidase-like
102157778	LOC10215 7778	-0.83	0.01338	1	NC_010443.5	51369470	51429036	minus	6	collagen alpha-1(I) chain-like
106506286	LOC10650 6286	-0.87	0.033099	1	NC_010443.5	1816683	1835173	plus	5	uncharacterized LOC106506286
110257526	LOC11025 7526	-0.97	0.014198	18	NC_010460.4	45451266	45460418	minus	4	ena/VASP-like protein
110258214	LOC11025 8214	0.82	0.011061	Un						basic salivary proline-rich protein 4-like
110258215	LOC11025 8215	1.65	0.013985	Un						progesterone receptor-like
110261495	LY6G6C	1.93	0.002544	7	NC_010449.5	23829790	23833610	minus	3	lymphocyte antigen 6 family member G6C
100522480	MCTP1	-0.85	0.048027	2	NC_010444.4	1.01E+08	1.02E+08	minus	25	multiple C2 and transmembrane domain containing 1
106504366	MUC21	1.61	0.049336	7	NC_010449.5	23452510	23456682	plus	3	mucin 21, cell surface associated
106504436	MYADM	-0.96	0.037488	7	NC_010449.5	54178736	54182408	minus	1	myeloid-associated differentiation marker-like; Homo sapiens myeloid associated differentiation marker (MYADM), transcript variant 2, mRNA
100621079	MYH15	-0.98	0.035314	13	NC_010455.5	1.51E+08	1.51E+08	plus	40	myosin heavy chain 15
100521800	NCMAP	-0.76	0.022501	6	NC_010448.4	82242371	82283887	plus	5	non-compact myelin associated protein
100738968	NGF	1.41	0.011061	4	NC_010446.5	1.05E+08	1.05E+08	plus	4	nerve growth factor
100516554	NTN4	-0.88	0.022068	5	NC_010447.5	87671153	87784704	plus	12	netrin 4
397103	PCSK1	1.30	0.019978	2	NC_010444.4	1.03E+08	1.03E+08	minus	14	proprotein convertase subtilisin/kexin type 1
100738269	PDPN	1.50	0.002544	6	NC_010448.4	73019063	73050000	plus	6	podoplanin
100154368	PLA1A	-1.02	0.033621	13	NC_010455.5	1.41E+08	1.41E+08	minus	11	phospholipase A1 member A

100156934	PLEKHD1	-0.79	0.024433	7	NC_010449.5	93152946	93187730	plus	15	pleckstrin homology and coiled-coil domain containing D1
100156168	PRSS35	-1.01	0.023157	1	NC_010443.5	82714880	82734883	minus	2	serine protease 35
397456	PTGDS	-0.79	0.039634	Un						prostaglandin D2 synthase
100512433	PYY	-1.53	0.002544	12	NC_010454.4	19190703	19208373	plus	7	peptide YY
100154661	RIPOR2	0.89	0.022501	7	NC_010449.5	19609358	19842345	minus	27	RHO family interacting cell polarization regulator 2
100626168	S100A5	1.73	0.010601	4	NC_010446.5	96093204	96096870	plus	3	S100 calcium binding protein A5
100737047	SDR42E2	-0.77	0.027695	3	NC_010445.4	23989671	24026548	minus	12	short chain dehydrogenase/reductase family 42E, member 2
102164898	SEC16B	-0.76	0.049336	9	NC_010451.4	1.2E+08	1.2E+08	minus	30	SEC16 homolog B, endoplasmic reticulum export factor
100155621	SEM1	0.91	0.03177	13	NC_010455.5	1.45E+08	1.45E+08	plus	1	SEM1, 26S proteasome complex subunit
100153200	SEMA3B	-0.69	0.048027	13	NC_010455.5	32737100	32749633	plus	21	semaphorin 3B
100524100	SEMA3E	-0.96	0.033427	9	NC_010451.4	96682178	96941074	plus	19	semaphorin 3E
100519286	SERPINB2	1.70	0.013985	1	NC_010443.5	1.58E+08	1.58E+08	minus	8	serpin family B member 2
100157079	SLC16A12	-1.07	0.015106	14	NC_010456.5	1.01E+08	1.01E+08	minus	12	solute carrier family 16 member 12
641343	SLC34A3	-0.80	0.022106	1						solute carrier family 34 member 3
100525144	SLC51B	-1.06	0.048027	1	NC_010443.5	1.63E+08	1.63E+08	plus	4	solute carrier family 51 subunit beta
100522397	SOX15	1.27	0.017913	12	NC_010454.4	52882840	52885785	minus	2	SRY-box transcription factor 15
397469	SPRP	2.07	0.002826	4	NC_010446.5	96588991	96590696	minus	2	small proline-rich protein
100624541	SULT1B1	-1.08	0.011061	8	NC_010450.4	66727047	66748761	minus	8	sulfotransferase family cytosolic 1B member 1
100621200	SYT8	-1.28	0.006782	2	NC_010444.4	1245988	1250422	plus	10	synaptotagmin 8
492314	TAC3	-1.00	0.03526	5	NC_010447.5	22324927	22336464	minus	7	tachykinin precursor 3
100624742	TLCD3B	-1.18	0.049336	3	NC_010445.4	18224652	18231416	minus	5	TLCD3B TLC domain containing 3B
100517619	TMEM176 B	-0.76	0.039579	18	NC_010460.4	6334420	6348887	plus	9	transmembrane protein 176B
100515686	TMEM92	0.82	0.018179	12	NC_010454.4	26466613	26472865	plus	5	transmembrane protein 92
110258335	TNFAIP2	-0.70	0.010956	Un						TNF alpha induced protein 2
100520347	TSPYL4	-0.83	0.006782	1	NC_010443.5	81888946	81893643	minus	1	TSPY like 4
100154934	VENTX	-1.46	0.005671	14	NC_010456.5	1.41E+08	1.41E+08	minus	3	VENT homeobox

100524028	ZMYND12	-1.34	0.032168	6	NC_010448.4	1.69E+08	1.69E+08	plus	9	zinc finger MYND-type containing 12
100620752	ZNF385B	-1.09	0.015106	15	NC_010457.5	85041947	85469112	minus	17	zinc finger protein 385B

Table 4.8S GO term enrichment IDs on DEGs in D30 embryos on biological processes pathway.

Go term ID	P	FDR adj	Nof Genes	Genes	Go term Definition
GO:0034101	0.00E+00	6.20E-08	12	AHSP|ALAS2|DMTN|DYRK3|EPB42|GATA1|IKZF1|KLF1|RHAG|SLC4A1|TAL1|TRIM10	erythrocyte homeostasis
GO:0030218	0.00E+00	3.88E-08	12	AHSP|ALAS2|DMTN|DYRK3|EPB42|GATA1|IKZF1|KLF1|RHAG|SLC4A1|TAL1|TRIM10	erythrocyte differentiation
GO:0002262	1.00E-10	3.08E-07	12	AHSP|ALAS2|DMTN|DYRK3|EPB42|GATA1|IKZF1|KLF1|RHAG|SLC4A1|TAL1|TRIM10	myeloid cell homeostasis
GO:0048872	3.90E-09	9.72E-06	13	AHSP|ALAS2|DMTN|DYRK3|EPB42|GATA1|IKZF1|KLF1|RHAG|SLC4A1|SPTA1| TAL1|TRIM10	homeostasis of number of cells
GO:0042592	3.55E-08	7.13E-05	33	AHSP|ALAS2|ALB|AQP1|CCR1|CCR3|CCRL2|CDHR1|CPS1|DMTN|DYRK3|EPB42| ERFE|FATE1|GATA1|GRIN1|IKZF1|KEL|KLF1|KRT1|MT1A|MT1E|MT1X| MT2A|RAC2|RHAG|SLC4A1|SPTA1|TAL1|TRIM10|TRPV1|XCR1|XK	homeostatic process
GO:0072488	6.38E-08	1.07E-04	5	AQP1|RHAG|RHCE|SLC22A16|SLC22A4	ammonium transmembrane transport
GO:0002376	1.57E-07	2.26E-04	40	ADD2|ADGRE2|AHSP|ALAS2|ALDH3B1|APBB1IP|BTN1A1|C3|C4A|C4BPA|CAMP|CCR1| CCR3|CCRL2|CD84|COL1A1|DMTN|DYRK3|EPB42|ERMAP|GATA1|GYPA|GYPC|IKZF1| KLF1|KRT1|MT2A|MUC16|MYD88|OSM|RHAG|SLC4A1|SLC7A10|SPTA1|TAL1|TRIM10| TRIM15|TRPV1|XAF1|XCR1	immune system process
GO:0030099	2.15E-07	2.70E-04	12	AHSP|ALAS2|DMTN|DYRK3|EPB42|GATA1|IKZF1|KLF1|RHAG|SLC4A1|TAL1|TRIM10	myeloid cell differentiation
GO:0048821	3.18E-07	3.55E-04	6	DMTN|EPB42|GATA1|RHAG|SLC4A1|TAL1	erythrocyte development
GO:0006778	4.85E-07	4.88E-04	6	ALAS2|BLVRB|CPOX|FECH|HMBS|SPTA1	porphyrin-containing compound metabolic process
GO:0071280	1.53E-06	1.36E-03	5	AQP1|MT1A|MT1E|MT1X|MT2A	cellular response to copper ion
GO:0055065	1.63E-06	1.36E-03	17	ALAS2|CCR1|CCR3|CCRL2|EPB42|ERFE|FATE1|GRIN1|KEL|MT1A|MT1E|MT1X| MT2A|RHAG|TRPV1|XCR1|XK	metal ion homeostasis
GO:0055080	2.43E-06	1.48E-03	18	ALAS2|CCR1|CCR3|CCRL2|EPB42|ERFE|FATE1|GRIN1|KEL|MT1A|MT1E|MT1X|MT2A| RHAG|SLC4A1|TRPV1|XCR1|XK	cation homeostasis
GO:0050801	2.48E-06	1.48E-03	19	ALAS2|CCR1|CCR3|CCRL2|CPS1|EPB42|ERFE|FATE1|GRIN1|KEL|MT1A|MT1E|MT1X| MT2A|RHAG|SLC4A1|TRPV1|XCR1|XK	ion homeostasis
GO:0098771	2.72E-06	1.48E-03	18	ALAS2|CCR1|CCR3|CCRL2|EPB42|ERFE|FATE1|GRIN1|KEL|MT1A|MT1E| MT1X|MT2A|RHAG|SLC4A1|TRPV1|XCR1|XK	inorganic ion homeostasis
GO:0006873	2.79E-06	1.48E-03	17	ALAS2|CCR1|CCR3|CCRL2|ERFE|FATE1|GRIN1|KEL|MT1A|MT1E|MT1X|MT2A|RHAG| SLC4A1|TRPV1|XCR1|XK	cellular ion homeostasis
GO:1990169	2.79E-06	1.48E-03	4	MT1A|MT1E|MT1X|MT2A	stress response to copper ion
GO:0046501	2.79E-06	1.48E-03	4	ALAS2|CPOX|FECH|HMBS	protoporphyrinogen IX metabolic process
GO:0010273	2.79E-06	1.48E-03	4	MT1A|MT1E|MT1X|MT2A	detoxification of copper ion

GO:0006779	3.86E-06	1.91E-03	5	ALAS2|CPOX|FECH|HMBS|SPTA1	porphyrin-containing compound biosynthetic process
GO:0061687	4.16E-06	1.91E-03	4	MT1A|MT1E|MT1X|MT2A	detoxification of inorganic compound
GO:0033013	4.19E-06	1.91E-03	6	ALAS2|BLVRB|CPOX|FECH|HMBS|SPTA1	tetrapyrrole metabolic process
GO:0042168	4.75E-06	2.07E-03	5	ALAS2|BLVRB|CPOX|FECH|HMBS	heme metabolic process
GO:0097501	5.96E-06	2.50E-03	4	MT1A|MT1E|MT1X|MT2A	stress response to metal ion
GO:0033014	6.98E-06	2.81E-03	5	ALAS2|CPOX|FECH|HMBS|SPTA1	tetrapyrrole biosynthetic process
GO:0006875	7.95E-06	3.07E-03	15	ALAS2|CCR1|CCR3|CCRL2|ERFE|FATE1|GRIN1|KEL|MT1A|MT1E|MT1X|MT2A|TRPV1|XCR1|XK	cellular metal ion homeostasis
GO:0055076	1.02E-05	3.66E-03	8	ALAS2|EPB42|ERFE|MT1A|MT1E|MT1X|MT2A|RHAG	transition metal ion homeostasis
GO:0030003	1.02E-05	3.66E-03	16	ALAS2|CCR1|CCR3|CCRL2|ERFE|FATE1|GRIN1|KEL|MT1A|MT1E|MT1X|MT2A|SLC4A1|TRPV1|XCR1|XK	cellular cation homeostasis
GO:0061515	1.13E-05	3.93E-03	6	DMTN|EPB42|GATA1|RHAG|SLC4A1|TAL1	myeloid cell development
GO:0055082	1.92E-05	6.24E-03	17	ALAS2|CCR1|CCR3|CCRL2|ERFE|FATE1|GRIN1|KEL|MT1A|MT1E|MT1X|MT2A|RHAG|SLC4A1|TRPV1|XCR1|XK	cellular chemical homeostasis
GO:0071294	1.92E-05	6.24E-03	4	MT1A|MT1E|MT1X|MT2A	cellular response to zinc ion
GO:0072503	2.00E-05	6.28E-03	13	CCR1|CCR3|CCRL2|FATE1|GRIN1|KEL|MT1A|MT1E|MT1X|MT2A|TRPV1|XCR1|XK	cellular divalent inorganic cation homeostasis
GO:0015696	2.18E-05	6.45E-03	5	AQP1|RHAG|RHCE|SLC22A16|SLC22A4	ammonium transport
GO:0046688	2.18E-05	6.45E-03	5	AQP1|MT1A|MT1E|MT1X|MT2A	response to copper ion
GO:0010043	2.51E-05	7.22E-03	5	CPS1|MT1A|MT1E|MT1X|MT2A	response to zinc ion
GO:0071248	2.82E-05	7.68E-03	9	AQP1|DMTN|FOSB|MT1A|MT1E|MT1X|MT2A|NEUROD2|SLFN14	cellular response to metal ion
GO:0070098	2.88E-05	7.68E-03	5	ACKR1|CCR1|CCR3|CCRL2|XCR1	chemokine-mediated signaling pathway
GO:0048878	2.90E-05	7.68E-03	21	ALAS2|AQP1|CCR1|CCR3|CCRL2|CPS1|EPB42|ERFE|FATE1|GRIN1|KEL|KRT1|MT1A|MT1E|MT1X|MT2A|RHAG|SLC4A1|TRPV1|XCR1|XK	chemical homeostasis
GO:0098754	3.09E-05	7.83E-03	7	ALB|CLIC2|MT1A|MT1E|MT1X|MT2A|PRXL2A	detoxification
GO:0019725	3.12E-05	7.83E-03	19	ALAS2|AQP1|CCR1|CCR3|CCRL2|ERFE|FATE1|GRIN1|KEL|MT1A|MT1E|MT1X|MT2A|RAC2|RHAG|SLC4A1|TRPV1|XCR1|XK	cellular homeostasis
GO:0072507	3.19E-05	7.83E-03	13	CCR1|CCR3|CCRL2|FATE1|GRIN1|KEL|MT1A|MT1E|MT1X|MT2A|TRPV1|XCR1|XK	divalent inorganic cation homeostasis
GO:0030154	3.66E-05	8.76E-03	45	ACAN|AHSP|ALAS2|C3|CCDC42|CDHR1|CDKN2C|CHD5|COL1A1|CPS1|CRYGB|CRYGD|DMTN|DYRK3|	cell differentiation

				EPB42|FGF6|GATA1|GRIN1|HEMGN|IKZF1|INHBE|KEL|KLF1|KRT1|KRT13|KRT5| MAL|MATN1|MYADM|MYH11|NEUROD2|NEUROD6|PYY|RAC2|RHAG|SLC22A16| SLC4A1|SLFN14|SPTA1|SPTB|TAL1|TRAK2|TRIM10|TRIM15|XK	
GO:0048869	3.78E-05	8.84E-03	46	ACAN|AHSP|ALAS2|C3|CCDC42|CDHR1|CDKN2C|CHD5|COL1A1|CPS1|CRYGB|CRYGD| DMTN|DYRK3|ECRG4|EPB42|FGF6|GATA1|GRIN1|HEMGN|IKZF1|INHBE|KEL| KLF1|KRT1|KRT13|KRT5|MAL|MATN1|MYADM|MYH11NEUROD2|NEUROD6| PYY|RAC2|RHAG|SLC22A16|SLC4A1|SLFN14|SPTA1|SPTB|TAL1|TRAK2| TRIM10|TRIM15|XK	cellular developmental process
GO:0010038	4.11E-05	9.40E-03	12	AQP1|CPOX|CPS1|DMTN|FOSB|MT1A|MT1E|MT1X|MT2A|NEUROD2|PKLR|SLFN14	response to metal ion
GO:0006783	4.69E-05	1.05E-02	4	ALAS2|CPOX|FECH|HMBS	heme biosynthetic process
GO:0030097	4.95E-05	1.07E-02	14	ADD2|AHSP|ALAS2|DMTN|DYRK3|EPB42|GATA1|IKZF1|KLF1|RHAG|SLC4A1| SPTA1|TAL1|TRIM10	hemopoiesis
GO:0020027	5.02E-05	1.07E-02	3	AHSP|ALAS2|EPB42	hemoglobin metabolic process
GO:0030837	5.41E-05	1.13E-02	5	ADD2|DMTN|MYADM|SPTA1|SPTB	negative regulation of actin filament polymerization
GO:0019722	6.44E-05	1.32E-02	7	CCR1|CCR3|CCRL2|CLIC2|DMTN|GRIN1|XCR1	calcium-mediated signaling
GO:0046683	7.29E-05	1.41E-02	7	AQP1|COL1A1|CPS1|DMTN|FOSB|PKLR|TRPV1	response to organophosphorus
GO:0006782	7.47E-05	1.41E-02	3	ALAS2|CPOX|HMBS	protoporphyrinogen IX biosynthetic process
GO:0019755	7.47E-05	1.41E-02	3	AQP1|RHAG|SLC14A1	one-carbon compound transport
GO:1990868	7.59E-05	1.41E-02	5	ACKR1|CCR1|CCR3|CCRL2|XCR1	response to chemokine
GO:1990869	7.59E-05	1.41E-02	5	ACKR1|CCR1|CCR3|CCRL2|XCR1	cellular response to chemokine
GO:0019932	7.80E-05	1.43E-02	10	ADGRE2|AQP1|CCR1|CCR3|CCRL2|CLIC2|DMTN|GRIN1|MT2A|XCR1	second-messenger-mediated signaling
GO:0071241	8.38E-05	1.50E-02	9	AQP1|DMTN|FOSB|MT1A|MT1E|MT1X|MT2A|NEUROD2|SLFN14	cellular response to inorganic substance
GO:0051591	8.64E-05	1.52E-02	6	AQP1|COL1A1|CPS1|DMTN|FOSB|PKLR	response to cAMP
GO:0006882	9.62E-05	1.67E-02	4	MT1A|MT1E|MT1X|MT2A	cellular zinc ion homeostasis
GO:0048534	1.08E-04	1.84E-02	14	ADD2|AHSP|ALAS2|DMTN|DYRK3|EPB42|GATA1|IKZF1|KLF1|RHAG|SLC4A1|SPTA1| TAL1|TRIM10	hematopoietic or lymphoid organ development
GO:0051693	1.13E-04	1.89E-02	4	ADD2|DMTN|SPTA1|SPTB	actin filament capping
GO:0042440	1.39E-04	2.29E-02	5	ALAS2|BLVRB|CPOX|FECH|HMBS	pigment metabolic process

GO:0014074	1.45E-04	2.35E-02	7	AQP1|COL1A1|CPS1|DMTN|FOSB|PKLR|TRPV1	response to purine-containing compound
GO:0055069	1.52E-04	2.43E-02	4	MT1A|MT1E|MT1X|MT2A	zinc ion homeostasis
GO:0030835	1.75E-04	2.75E-02	4	ADD2|DMTN|SPTA1|SPTB	negative regulation of actin filament depolymerization
GO:0065008	1.83E-04	2.83E-02	49	ADD2|AHSP|ALAS2|ALB|AQP1|C3|CCR1|CCR3|CCRL2|CDHR1|CLIC2|COL1A1| CPS1|DMTN|DYRK3|EPB42|ERFE|FATE1|GATA1|GRIN1|IKZF1|KEL|KLF1|KRT1| MT1A|MT1E|MT1X|MT2A|MYADM|MYD88|NALCN|NEUROD2|NFE2|OSM|PCTP| RAC2|RHAG|SLC22A4|SLC4A1|SPTA1|SPTB|TAL1|TENT5C|THBS2|TRIM10|TRIM58| TRPV1|XCR1|XK	regulation of biological quality
GO:0046916	1.87E-04	2.84E-02	6	ALAS2|ERFE|MT1A|MT1E|MT1X|MT2A	cellular transition metal ion homeostasis
GO:0006952	1.89E-04	2.84E-02	19	ACKR1|ADGRE2|C3|C4A|C4BPA|CAMP|CCR1|CCR3|CCRL2|CD84|KRT1|MT2A| MYD88|NEUROD2|TRIM10|TRIM15|TRPV1|XAF1|XCR1	defense response
GO:0002520	2.06E-04	3.04E-02	14	ADD2|AHSP|ALAS2|DMTN|DYRK3|EPB42|GATA1|IKZF1|KLF1|RHAG|SLC4A1| SPTA1|TAL1|TRIM10	immune system development
GO:0032272	2.18E-04	3.17E-02	5	ADD2|DMTN|MYADM|SPTA1|SPTB	negative regulation of protein polymerization
GO:0071276	2.29E-04	3.24E-02	4	MT1A|MT1E|MT1X|MT2A	cellular response to cadmium ion
GO:0006956	2.29E-04	3.24E-02	4	C3|C4A|C4BPA|KRT1	complement activation
GO:1902603	2.86E-04	3.94E-02	2	SLC22A16|SLC22A4	carnitine transmembrane transport
GO:0018120	2.86E-04	3.94E-02	2	ART4|ART5	peptidyl-arginine ADP-ribosylation
GO:0048856	3.27E-04	4.44E-02	57	ACAN|ADD2|AHSP|ALAS2|AQP1|ASPN|BCAN|C3|CCDC42|CDHR1|CDKN2C|CHD5| COL1A1|CPS1|CRYGB|CRYGC|CRYGD|DMTN|DYRK3|ECRG4|EPB42|FGF6|GATA1| GFI1B|GRIN1|HEMGN|IKZF1|INHBE|KEL|KLF1|KRT1|KRT13|KRT5|MAL| MATN1|MYADM|MYH11|NEUROD2|NEUROD6|NFE2|OSM|PHOSPHO1|PYY|RAC2| RHAG|SLC22A16|SLC4A1|SLFN14|SNORC|SPTA1|SPTB|TAL1|TENT5C|TRAK2|TRIM10| TRIM15|XK	anatomical structure development
GO:0010035	3.69E-04	4.94E-02	13	AQP1|COL1A1|CPOX|CPS1|DMTN|FOSB|MT1A|MT1E|MT1X|MT2A|NEUROD2| PKLR|SLFN14	response to inorganic substance

Table 4.9S GO term enrichment IDs on DEGs in D30 embryos on biological cellular components pathway.

Go term ID	Go term definition	Pvalue	PFDR adj	Nof Genes	Genes
GO:0014731	spectrin-associated cytoskeleton	2.85E-07	4.29E-04	4	ANK1|DMTN|SPTA1|SPTB
GO:0030863	cortical cytoskeleton	1.88E-05	1.41E-02	7	DMTN|EPB42|GYPC|MYADM|SLC4A1|SPTA1|SPTB

Table 4.10S GO term enrichment IDs on DEGs in D30 embryos on molecular functions pathway.

Go term ID	Pvalue	PFDR_adj	Nof Genes	GO term definition	Genes
GO:0005198	1.99E-08	5.08E-05	21	structural molecule activity	ACAN|ADD2|ANK1|ASPN|CLDN20|COL1A1|COL6A6 |CPOX|CRYGB|CRYGC|CRYGD|EPB42|KRT1|KRT13| KRT5|MAL|MATN1|MYH11|SPTA1|SPTB|THBS2
GO:0019957	6.06E-07	7.74E-04	5	C-C chemokine binding	ACKR1|CCR1|CCR3|CCRL2|XCR1
GO:0008519	1.96E-06	1.67E-03	5	ammonium transmembrane transporter activity	AQP1|RHAG|RHCE|SLC22A16|SLC22A4
GO:0019956	2.74E-06	1.75E-03	5	chemokine binding	ACKR1|CCR1|CCR3|CCRL2|XCR1
GO:0005212	1.86E-05	7.91E-03	4	structural constituent of eye lens	CPOX|CRYGB|CRYGC|CRYGD
GO:0016493	1.86E-05	7.91E-03	4	C-C chemokine receptor activity	CCR1|CCR3|CCRL2|XCR1
GO:0004950	3.09E-05	9.86E-03	4	chemokine receptor activity	CCR1|CCR3|CCRL2|XCR1
GO:0001637	3.09E-05	9.86E-03	4	G protein-coupled chemoattractant receptor activity	CCR1|CCR3|CCRL2|XCR1
GO:0005200	8.32E-05	2.36E-02	6	structural constituent of cytoskeleton	ADD2|ANK1|EPB42|KRT5|SPTA1|SPTB
GO:0015226	1.46E-04	3.74E-02	2	carnitine transmembrane transporter activity	SLC22A16|SLC22A4

Table 4.11S GO term enrichment IDs on DEGs in D30 placental tissues on anatomical structure morphogenesis and tissue development.

Go term ID	GO term definition	P	PFDR adj	Nof Genes	Genes
GO:0009653	anatomical structure morphogenesis	6.72E-07	3.70E-03	24	ARHGAP22|BMP4|CDSN|COBL|CRABP2|CXCL8|EGFLAM|EPGN| FOXD2|FREM2|GCNT3|HBEGF|ISL1|KLK14|KRT17|NCMAP|NGF| NTN4|PDPN|RIPOR2|SEMA3B|SEMA3E|TMEM176B|TNFAIP2
GO:0009888	tissue development	3.77E-07	3.70E-03	22	BMP4|CDSN|COBL|CRABP2|DSG3|EGFLAM|FREM2|GCNT3| HBEGF|ISL1|KLK14|KRT17|MYADM|MYH15|NTN4|PDPN|PYY| RIPOR2|SEMA3B|SEMA3E|SOX15|SULT1B1