Relations between ovarian & embryonic traits in pigs

Effects of genetic selection for litter traits at birth

Carolina Lima Alvares Da Silva

Relations between ovarian & embryonic traits in pigs - Effects of genetic selection for litter traits at birth Carolina Lima Alvares Da Silva 2018

Propositions

1. A more crowded ovary with smaller corpora lutea results in a more crowded uterus with smaller piglets. (this thesis)

2. Genetic improvements in piglet birth weight without decreasing litter size can be done by the inclusion of corpora lutea weight and ovulation rate in the selection index. (this thesis)

3. Considering euthanasia as the humane endpoint of experimental animals in pain ignores the animals strongest instinct to survive.

4. The absence of references to old papers in scientific literature gives naive readers the idea that everything is new.

5. Pressure for a high number of publications creates doctors of philosophy that have no time to think.

6. Social media has decreased society's shame of ignorance.

Propositions belonging to the thesis entitled:
"Relations between ovarian & embryonic traits - Effects of genetic selection for litter traits at birth"

Carolina L.A. Da Silva
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To Artur and Eduarda, with love

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CHAPTER 1

General introduction

Pork meat is the most widely consumed meat around the world. In 2014, it amounted to 115.5 million tons (37% of the total amount of consumed meats), according to the Food and Agriculture Organization of the United Nations. The European Union (EU) is the second biggest producer of pork meat with 23,290 million tons produced in 2016, which is a record volume never earlier reported for the EU, behind China with 54,870 million tons (United States Department of Agriculture, 2016). The EU is also the second biggest consumer (20,974 million tons, 37 kg *per capita* in 2009), and in The Netherlands an average of 38 kg of pork meat *per capita* was consumed in 2015. The number of sows in the EU slightly declined from 12,300 to about 12,170 million heads from 2016 to 2017, but total slaughter increased by 1,050 million heads. This demonstrates that increased productivity of sows outbalances the lower sow numbers (United States Department of Agriculture, 2016). High productive and reproductive efficiencies contribute to the increase in productivity, and the largest contributing factor to this higher level of productivity is the increase in total number of piglets born per sow per year (Kridli et al. 2016).

Modern dam line sows are characterized by a large litter size, with an ability to produce up to 30 - 35 piglets per sow per year (Schneider et al. 2014) with 2.4 to 2.5 litters per sow per year. In The Netherlands, there was an increase of approximately 3 piglets born alive and of 2 piglets weaned per litter from 2000 to 2013, with an average total number of piglets born of 16.2 and 14.6 piglets born alive in 2016 (Agrovision BV, 2017). In the United States, the total number of piglets born and the number of piglets born alive increased by approximately 2 piglets from 2000 to 2015, reaching 13.6 and 12.4 per litter, respectively (United States Department of Agriculture, National Agricultural Statistics Service 2015). In Denmark, genetic selection for total number of piglets born from 1992 to 2004 led to a total increase of 3.8 piglets per litter (calculated as the difference in estimated breeding values for total number of piglets born between 1992 and 2004) in Danish Landrace lines (Su et al. 2007), and in 2011 the number of piglets produced per sow per year was 37.5 with 2.4 litters born per sow per year (Franke et al. 2013). However, genetic selection for sows with the ability to farrow a high number of piglets has led to a decrease in average piglet birth weight (Quiniou et al. 2002), and for each additional piglet born in a litter there is a decrease of 30 to 35 grams in the average piglet birth weight (van der Lende and de Jager 1991; Quiniou et al. 2002; Quesnel et al. 2008; Beaulieu et al. 2010). Additionally, genetic selection for high litter size also increased the within litter piglet birth weight variation (Quiniou et al. 2002; Wolf et al. 2008). Measurements of the coefficient of variation (CV) of birth weights of small litters (< 10 to 12 piglets born) and large litters (> 15 piglets born) revealed an increase in CV from approximately 15 to 17% in small litters to approximately 24% in large litters (Quiniou et al. 2002; Quesnel et al. 2008). Also, Milligan et al. (2002) observed, in a study with 416 litters (Yorkshire or Yorkshire x Landrace), that the number of piglets born alive had a negative phenotypic correlation with average piglet birth weight ($R = -0.46$, $P < 0.001$) and a positive phenotypic correlation with the coefficient of variation (CV) of litter birth weight ($R = + 0.39$; $P < 0.001$).

Piglet birth weight and within litter birth weight uniformity are important factors for piglet survival (Milligan et al. 2002; Quiniou et al. 2002). In organic crossbred sows (Yorkshire x Landrace) with an average litter size of 17.4 ± 0.3 total born piglets (16.2 ± 0.3 live born), piglet birth weight and litter uniformity were strongly related with piglet survival during lactation, as for every 100 g increase in average piglet birth weight there was an increase of 3.1% in piglet survival in the first 3 days after birth, and for every 1% reduction in the within litter birth weight coefficient of variation (CV), piglet survival increased with 1.1% (Wientjes et al. 2012b). Also, Milligan et al. (2002) analysed the percentage of survival during lactation in litters with a low CV (< 15 %), medium CV (15 to 20 %) and high CV (> 20 %) in within litter birth weight in litters with an low (< 1.3 kg), medium (1.3 to 1.5 kg) or high (> 1.5 kg) average piglet birth weight and observed that the lowest survival occurred in litters with both low average piglet birth weight and high birth weight variation (84% vs 97% for litters with both high average piglet birth weight and lower birth weight variation). Thus, a lower piglet survival in litters with lower birth weight uniformity was especially evident in litters with low average piglet birth weight. Piglets born with a low birth weight are at a particularly high risk for mortality due to their lower energy stores and higher susceptibility to hypothermia (Lay et al. 2002). Piglets born with a low birth weight have a delayed first suckle after birth and a lower ability to get to the best teats, since they are in disadvantage when competing with larger littermates at the udder (Lay et al. 2002). The resulting lower amount of colostrum and milk intake is associated with a poorer acquisition of passive immunity and low nutritional status, leading to higher postnatal mortality or reduced growth (Le Dividich 1999). Thus, phenotypically, the increase in pre weaning mortality that followed the increase in the number of piglets born is related with a decrease in the average piglet birth weight and with an increase in the within litter birth weight variation. The association between the increase in number of piglets born and the increase in pre-weaning mortality over the years is shown in Figure 1.1.

The causes for the negative relationships between the total number of piglets born, average piglet birth weight and within litter birth weight uniformity are known to be partly genetic. Table 1.1 shows that the phenotypic and genetic correlations between the total number of piglets born, average piglet birth weight and within litter piglet birth weight variation are similar. The number of piglets born alive in a litter have a negative genetic correlation with average piglet birth weight (-0.30 to -0.49), and a positive genetic correlation with within litter birth weight variation (0.21 to 0.25) and with pre-weaning mortality (0.25 to 0.45) (Lamberson and Johnson 1984; Knol 2001; Lund et al. 2002; Milligan et al. 2002; Damgaard et al. 2003; Su et al. 2007; Wolf et al. 2008). Moreover, litter traits have low to moderate heritabilities (Table 1.1), and on average, only 10%, 20% and 8% of the phenotypic variation in the total number of piglets born, average piglet birth weight and within litter piglet birth weight variation, respectively, is explained by genetic variation (Lamberson and Johnson 1984; Knol 2001; Lund et al. 2002; Milligan et al. 2002; Damgaard et al. 2003; Su et al. 2007; Wolf et al. 2008). However, these traits have a high phenotypic variation within and between populations. For example, in one population of Yorkshire x Landrace sows (n=101)

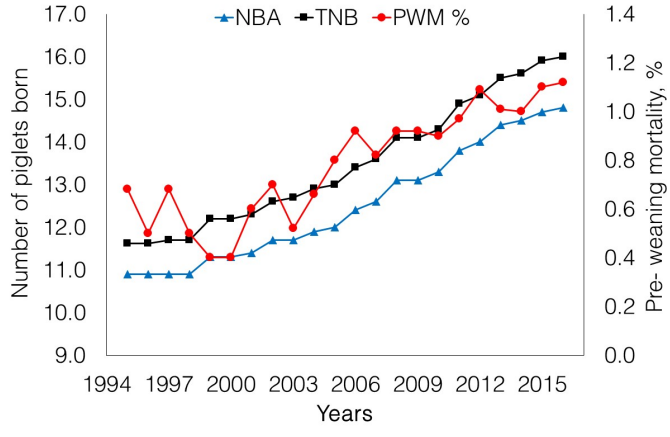

Figure 1.1 The increase in the number of piglets born and in pre-weaning mortality from 1995 until 2015 (Agrovision B.V.). NBA: number of piglets born alive, TNB: total number of piglets born, PWM: pre-weaning mortality (%).

in The Netherlands, total number of piglets born ranged from 9 up to 26, average piglet birth weight ranged from 914 g to 1618 g and within-litter birth weight variation ranged from 161 g to 555 g (Da Silva et al., 2017). Therefore, their additive genetic variability is far from negligible and genetic improvement is possible. However, the unfavourable phenotypic and genetic correlations between total number of piglets born, average piglets birth weight, within litter birth weight variation and pre weaning mortality (Table 1.1) limit their concomitant improvement by direct genetic selection (Spötter and Distl 2006). Consequently, modern genetic selection programs have focused on the use of selection index for piglet quality, taking into consideration the number of piglets born alive, average piglet birth weight adjusted for litter size and the standard deviation of piglets birth weight within a litter, using different weighing factors for each trait in the model, aiming to improve litter quality at birth (Brandt 1998). Recently, the use of genome wide association studies (GWAS) has allowed the identification of genes influencing litter characteristics at birth (Zhang et al. 2014;

Table 1.1 Summary of heritabilities (**diagonal, in bold**) and of genetic (*below diagonal, in italic*) and phenotypic correlations (above diagonal) between litter traits described on literature.

Traits[1]	**TNB**	**BW, Kg**	**BWV, Kg**	**PM, %**
TNB[2]	**0.09 to 0.13**	-0.46 to -0.48	0.14 to 0.39	0.14 to 0.27
BW[3]**, Kg**	*-0.30 to -0.49*	**0.16 to 0.39**	-0.05	-0.28 to -0.43
BWV[4]**, Kg**	*0.21 to 0.25*	*0.47 to 0.60*	**0.03 to 0.08**	0.11 to 0.34
PM [5]**, %**	*0.25 to 0.45*	*-0.24 to -0.26*	*0.25 to 0.28*	**0.01 to 0.06**

[1] Based on: (Lamberson and Johnson 1984; Knol 2001; Lund et al. 2002; Milligan et al. 2002; Damgaard et al. 2003; Su et al. 2007; Wolf et al. 2008).

[2] Total number of piglets born in a litter.

[3] Average piglet birth weight (Kg).

[4] Within litter piglet birth weight variation (Kg).

[5] Pre weaning piglet mortality (%).

Bergfelder-Drüing et al. 2015; Wang et al. 2016) or its components traits [such as ovulation rate (OR), (Schneider et al. 2014)]. This can be done either by linkage analyses to detect genomic regions harbouring genes responsible for the trait of interest or by identification of candidate genes via their physiological role in the reproduction process and/or their location in a genomic region linked with the phenotypic trait (Distl 2007). Successful selection on candidate genes requires a thorough understanding of the biological mechanisms by which the candidate gene affects litter traits. Therefore, more detailed physiological data on underlying traits and their relations is needed.

Litter characteristics at birth (total number of piglets born, number of piglets born alive, average piglet birth weight, within litter birth weight uniformity) are dependent on the interaction between many component traits, such as ovulation rate (OR), fertilization rate, early embryonic survival/mortality, embryonic development, uterine capacity and foetal survival and growth (Lund et al. 2002; Spötter and Distl 2006). Ovulation rate (OR), defined as the total number of oocytes shed by a sow in an estrous cycle, is the main component trait of total number of piglets born (Haley and Lee 1993; Bidanel et al. 1996; Schneider et al. 2014), and sets the maximum number of offspring a sow can produce in each pregnancy, since fertilization rates are considered optimal in pig production nowadays, i.e. close to 100 % (Spötter and Distl 2006). Ovulation rate is a heritable trait, with approximately 32% of its phenotypic variation explained by genetic variation (Schneider et al. 2014), and probably due to its high positive genetic correlation with the total number of piglets born [0.85 to 0.98, (Blasco et al. 1993)], increased sharply in the last decades in gilts and sows (Table 1.2), and values of 25 to 30 are relatively common nowadays (Patterson et al. 2008; Wientjes et al. 2013). According to Foxcroft et al. (2007) approximately 50% of the third parity sows in the study of Town et al. (2004) had 25 or more ovulations and 18% of these sows had 30 or more ovulations. Moreover, similar reports of OR were observed in the experiment of Patterson et al. (2008), in which over 40% of commercial dam line sows in parities four to six had OR of 25 or higher.

Despite the sharp increase in OR and its high genetic correlation with total number of piglets born, the phenotypic correlations between OR and total number of piglets born are much smaller, as for example 0.06 (Young et al. 1978), 0.21 (Haley and Lee 1992) and 0.34 (Christenson et al. 1987). In fact, King and Williams (1984) investigated the relationship between the increase in OR and the number of piglets born alive in 179 crossbred Large White x Landrace and 139 purebred Large White or Landrace primiparous sows and observed a significant positive linear relationship in which, in sows with OR from 8 up to 23, one extra ovulation led to an increase of 0.30 piglets born alive ($P < 0.001$).

Also, Blasco et al. (1996) observed an increase of only 0.21 piglets born with each extra ovulation in a population of Large White sows with OR ranging from 5 to 29. This limited increase in the number of piglets born alive in relation with an increase in OR is due to the positive genetic and phenotypic association between OR and pre-natal mortality (Young et al. 1977; Neal et al. 1989). The increase in pre-natal mortality with an increase in OR seems to be related with an increase in both pre-implantation mortality (early embryonic mortality)

Table 1.2 Examples of ovulation rate (OR) over decades in gilts and sows.

Year	Average/decade	Mean OR	Genetic background	Authors[1]
Gilts				
1980	13.8	10.9±0.1	LW x LD	(Paterson et al.)
1982		14.2±2.5	Norwegian LD	(Blichfeldt and Almlid)
1984		14.6±0.2	LW, LD or LW x LD	(King and Williams)
1988		15.3	Crossbreds	(Kelly et al.)
1989		13.4±0.4	Not informed	(Rhodes et al.)
1989		14.5±0.4	Dutch LD	(van der Lende)
1993		14.0±0.6	LWxLD	(Irgang et al.)
1993		14.7±0.7	LD x LW	(Christenson)
1993		14.6±0.5	LWxLD	(Gama and Johnson)
1996	15.3	13.9±0.2	LW x French LD	(Bidanel et al.)
1997		17.4±2.4	LW	(Père et al.)
1997		19.9±1.6	Crossbreds	(Zak et al.)
1998		13.8±1.0	White Crossbred	(Pearson et al.)
1999		13.8±2.6	LD x LW	(Johnson et al.)
2005	16.3	20.4±3.4	LW x LD [2]	(Yen et al.)
2005		13.8±4.5	LW x LD [3]	(Yen et al.)
2006		16.1±0.6	LD x LW	(Ferguson et al.)
2007		15.0±0.3	Multiple cross[4]	(Freking et al.)
2010		15.2±0.01	Yorkshire x LD	(Li et al.)
2017	19.2	20.3±0.2	Yorkshire x LD	(Da Silva et al.)
2017		22.1±0.4	Landrace	(Da Silva et al.)
Sows				
1983	19.0	23.4±1.2	LWxLD	(Toplis et al.)
1987		14.5±0.6	Multiple cross	(Wu et al.)
1992		19.3±3.3	Yorkshire x LD	(Soede et al.)
1995		22.0±4.0	LD x LW	(Soede et al.)
1996	18.7	24.2	Hyperprolific line	(Driancourt and Terqui)
1996		17.2	LW	(Driancourt and Terqui)
1996		14.7±2.5	French LW	(Blasco et al.)
2000		18.2±1.2	Yorkshire x LD	(van den Brand et al.)
2002		26.6±0.4	LD x LW	(Vonnahme et al.; b)
2004	23.1	19.9±0.4	Crossbred	(Town et al.)
2005		22.7±0.2	LD x LW	(Town et al.)
2008		24.9±1.2	Not informed	(Gerritsen et al.)
2009		26±1.0	Topigs 40	(Langendijk et al.)
2012		24.3±1.2	Yorkshire x LD	(Wientjes et al.; a)
2016	23.9	22.0±0.9	LW x LD	(Langendijk et al.)
2016		25.5±5.0	Yorkshire x LD	(Da Silva et al.)

[1] From papers with different experimental groups, only data of the control groups is presented.

[2] Randomly selected control line.

[3] Selected for 10 generations for an index of ovulation rate and prenatal survival.

[4] Chester White x LD x LW x Yorkshire.

and post-implantation mortality (late embryonic mortality and foetal mortality) (van der Waaij et al. 2010).

Early embryonic mortality is the mortality occurring before uterine implantation at 13 days of pregnancy and makes up the largest proportion of prenatal losses in the pig (Foxcroft et al. 2007). Early embryonic mortality has been related with higher heterogeneity in early embryonic development (Pope et al. 1990). Just before uterine implantation, pig conceptuses undergo drastic morphological changes by developing from 1 to 2 mm sphere to a 9 to 10 mm long tubular shape between days 10 to 11 of pregnancy (Geisert et al. 2014). Rapid conceptus elongation occurs when they reach 10 mm in diameter at approximately day 11 of pregnancy. During elongation, between days 11 and 12 of pregnancy, conceptuses start producing estrogen, which is the primary signal for maternal recognition of pregnancy (Bazer and Thatcher 1977). However, due to heterogeneity in embryonic development, some pig conceptuses within a litter can still be spherical at day 11 of pregnancy, and spherical, tubular and elongated forms can be found within the same sow at 11 days of pregnancy (Stroband et al. 1984; Stroband and van der Lende 1990). Such embryonic heterogeneity results in mortality before implantation because the estrogen produced by the elongating conceptuses will stimulate uterine secretions to their own benefit, but will create a hostile environment for the less developed embryos (i.e. the ones that are still spherical or tubular) in a process named "uterine-embryonic asynchrony" (Pope et al. 1990). This heterogeneity in embryonic development has been linked with a higher follicular and oocyte diversity (Pope et al. 1990; Xie et al. 1990). Follicular and embryonic heterogeneity might increase with the increase in OR.

Late embryonic mortality, on the other hand, has been related with limitations in uterine capacity. Uterine capacity is defined as the number of conceptuses that the pig uterus can successfully carry to term (Ford et al. 2002), and previous studies have indicated that uterine capacity is exceeded when the number of embryos is above 14 (Dziuk 1968). The lack of sufficient space in the uterus compromises the uterine space acquired by each embryo at implantation. This smaller implantation site in the uterus will result in the development of a smaller placenta (Stroband and van der Lende 1990), which might lead to late embryonic mortality or compromised embryonic development due to insufficient placental supply of blood and nutrients (Vallet et al. 2014). Thus, as a consequence of the markedly increase in OR in modern dam line sows, the number of embryos surviving to the post-implantation period and therefore competing for a uterine implantation site in many cases greatly exceeds uterine capacity (Foxcroft et al. 2007).

Thus, an increase in OR leads to an increase in late embryonic mortality and in foetal mortality due to an increase in uterine crowding, i.e. increase in competition between embryos and foetuses for uterine space. Vonnahme et al. (2002a) observed a correlation between OR (average 26.6) and the number of vital embryos at day 25 of pregnancy ($r = 0.50$, $P < 0.0001$) but not at day 36 of pregnancy ($r = 0.02$; $P = 0.98$) which was due to the further loss of embryos in sows with higher OR. Also, van der Waaij et al. (2010), working with super-ovulated gilts (over 45 ovulations, ranging from 22 to 76) observed that

an increase in OR was related with a higher incidence of late embryonic mortality ($\beta = 0.36$; $P = 0.0003$), and the not vital foetuses at 40 days of pregnancy had also a smaller length of uterine implantation and smaller and lighter placentas in gilts with higher OR, indicating that the mortality was related with limited uterine space. Thus, the higher embryonic heterogeneity and the higher number of embryos entering the uterus and leading to uterine crowding are possible reasons for the increase in late embryonic mortality with the increase in OR.

The increase in OR can lead to a higher mortality throughout different processes, but the remaining question is if these processes may also influence average piglets birth weight and within litter piglet birth weight variation. van der Waaij et al. (2010) showed that in gilts with less than 30 ovulations the vital foetuses at 40 days of pregnancy had a placental weight which was on average 32% heavier than the vital foetuses in the gilts with more than 50 ovulations ($P= 0.004$). The effects of crowding are also present in non-super-ovulated sows, as sows with an average OR of 21.6 ± 0.9, available uterine space per embryo at 35 days of pregnancy was negatively correlated with OR ($r = -0.85$, $P \leq 0.05$) (Langendijk et al. 2016); and sows submitted to oviduct ligation to reduce the number of embryos entering the uterus had an 18% greater placenta area at 35 days of pregnancy (709 ± 23 vs 600 ± 54 cm^2) and a longer implantation length (19.0 vs 15.5 cm) than intact sows (Langendijk et al. 2012).

The limitation in the length of the uterine implantation site and the consequent limitation in placental growth that will occur (due to either heterogeneity in embryonic development or to uterine crowding), will compromise further foetal growth and consequently the piglets birth weight and survival. Low piglet survival results in large economic losses in pig production and has a detrimental impact on animal welfare. Thus, improvement of piglet survivability is of major importance for pig production systems (Knol 2001). Since sows with a higher OR are more likely to have embryos with lower implantation length, they might farrow litters with a lower average piglet birth weight (Foxcroft et al. 2007). A reduction in the available uterine space per ovulation (i.e. induced uterine crowding) in gilts at 35 days of pregnancy led to a reduction in the number of embryos (8.4 vs 12.0, $P < 0.001$) and to a lower placental weight (0.27 Kg vs 0.32 Kg, $P < 0.001$) and lower foetal weight (1.27 Kg vs 1.38 Kg, $P < 0.01$) at 112 days of pregnancy in comparison with control intact gilts (Père et al. 1997). The lower placental and foetal weight at later pregnancy observed by Père et al. (1997), indicates that the uterine crowding that occurred at early pregnancy compromised the embryonic survival (as observed by the lower number of embryos) but also the development of the placenta between days 20 and 30 of pregnancy, which is known to affect subsequent growth of the foetuses. Moreover, Père et al. (1997) showed that the increase in competition between littermates due to uterine crowding at the beginning of pregnancy leads to an increase in the variation in weight of the foetuses and of their placentas at 112 days of pregnancy. So, if sows with a higher OR have an increase in uterine crowding due to the higher number of embryos surviving to the post implantation period (Foxcroft et al. 2007), they are more likely to farrow litters with a higher within litter birth weight variation. Another possible mechanisms that links a higher OR with an increase in within litter birth weight

variation is, as discussed above, the increase in embryonic heterogeneity at early stages of development (Pope et al. 1990; Xie et al. 1990). If the embryos with delayed development did not die they might develop into the smaller foetuses. Thus, a higher OR might, throughout different mechanisms, influence piglet birth weight and therefore piglet survival.
It is important to increase the knowledge about these underlying traits, to better understand the physiological background of the negative associations between litter size and average piglet birth weight, piglet birth weight uniformity and piglet survival.

Thus, the objectives of this thesis are:

1) to understand the physiological consequences of increased number of corpora lutea (OR) for embryonic survival and development in multiparous sows (higher average OR) and in gilts (lower average OR) in early pregnancy, since sows and gilts have different litter characteristics;
2) to investigate the differences in OR and embryonic survival and development (underlying physiological components of litter characteristics) between gilts with low and high genetic potential (based on their estimated breeding values) for litter size, average piglet birth weight and within litter piglet birth weight variation,
3) to investigate the genetic variation of OR, corpora lutea characteristics and embryonic survival and development in gilts in early pregnancy;
4) to investigate the relationships between corpora lutea number (OR) and size and litter characteristics at birth in pregnant sows. A better understanding of the relationships between these underlying traits might reveal potential heritable traits to select for or to be taken into consideration in more balanced genetic selection programs aiming to improve piglet birth weight and survivability.

In **Chapter 2** of this thesis I provide a review of literature on follicular growth and quality, corpora lutea formation and factors that are related with a high or low ovulation rate. I also review the physiological mechanisms explaining embryonic mortality and development in the pig; which will clarify the pre-natal events that lead to litter characteristics at birth. In **Chapter 3** of this thesis I focus on the relationship between ovulation rate and embryonic survival and development at 35 days of pregnancy in a population of multiparous sows, and in **Chapter 4**, I focus on the relationship between ovulation rate and corpora lutea characteristics, embryonic survival and development at 35 days of pregnancy in a population of gilts. Due to the importance of OR for embryonic survival and development in early pregnancy, it is of great interest to understand the relationship between OR and litter characteristics at birth. So, in **Chapter 5** I checked the accuracy of transrectal ultrasonography to assess the number of corpora lutea (OR) and the average corpora lutea diameter measured by ultrasonography in sows at early pregnancy and investigate its relationship with their litter characteristics at birth. In **Chapter 6**, using pedigree information of gilts, I investigate the relationship between the estimated breeding values (EBV) for total number of piglets born, average piglets birth weight and within litter birth weight uniformity

of gilts and their phenotypic reproductive traits: OR, corpora lutea weight, total luteal mass and embryonic survival and development characteristics at 35 days of pregnancy. Moreover, I estimate the additive genetic variation of these reproductive traits and their genetic and phenotypic correlations.

In **Chapter 7** (the 'General discussion' of my PhD thesis), results of Chapters 3 to 6 are combined and discussed to further unravel the consequences of the increase in OR for embryonic survival and development. Moreover, I reflect on my work and evaluate if the objectives of my thesis have been achieved. I also place my work in a broader context, formulate recommendations for industry and define future research areas.

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CHAPTER 2

Literature review

Introduction

Total number of piglets born, average piglet birth weight and within litter piglet birth standard deviation are composite traits influenced by several underlying traits, such as ovulation rate (OR), fertilization rate, early embryonic survival and development and uterine capacity (Lund et al. 2002). Ovulation rate is the main component trait of total number of piglets born as it sets the maximum number of offspring a sow could carry throughout pregnancy (Spötter and Distl 2006). Due to its high genetic correlation with the total number of piglets born, OR has increased sharply in the last decades and this increase might have altered the relationship between OR and other underlying traits influencing litter characteristics at birth, such as embryonic survival and development, and uterine capacity. The altered relationship between OR and other underlying traits might be related with the lower piglet birth weight and higher within litter piglet birth weight standard deviation observed in big litters.
As ovulation rate (OR) it is the result of factors influencing the growth and development of follicles during early antral follicle development, the aim of this literature review is to provide the reader with information regarding follicular development, that will clarify not only how ovulation rate is determined but also how events occurring during follicular development might influence the quality of the follicles and oocytes, and the embryonic potential to develop into a live born piglet. Also, important events of early pregnancy in pigs, such as corpora lutea development, pregnancy recognition, early embryonic development and the importance of uterine capacity are approached.

Follicular development and ovulation

In mammals, oocytes develop from primordial germ cells (PGCs). There is a definite number of PGCs that are formed in the yolk sac epithelium of the embryo. These cells reach the primitive ovary after migrating through the gut mesentery and the gonadal ridges of the

Box 1. The estrous cycle

The estrous cycle in pigs spans a period of 18 to 24 days. It consists of a follicular phase of 4 to 6 days and a luteal phase of 13 to 15 days. During the follicular phase, small antral follicles (< 3mm) develop into large, pre-ovulatory follicles (> 6 mm) (Soede et al. 2011). Oestrus is the period around ovulation in which sows show a standing response for boars, thus allowing the boars to mate. The duration of oestrus (heat) may vary between sows from 24 h up to 96 h, but this variation is not related with systemic oestrogen concentrations. Ovulation may occur from 10 h to 85 h after the onset of oestrus, or on average 70% of the way through oestrus (Soede and Kemp 1997). After ovulation, the luteal phase will start with the luteinisation of the ovulatory follicles and formation of corpora lutea, which will secrete progesterone. In case of non-pregnancy, luteolysis occur around 15 days after ovulation (Bazer and Thatcher 1977).

mesonephros of the early embryo (Gandolfi et al. 2005). Once PGCs have reached the developing ovary the cells begin to differentiate into oogonia. The populations of oogonia proliferates until shortly before birth at which time the oogonia reach enter meiosis and are termed primary oocytes. The process of meiosis will halve the number of chromosomes resulting in the creation of haploid oocytes. Mammalian oocytes reach prophase of first meiosis (prophase I or germinal vesicle, GV) around the time of birth, and remain at this stage until the female reaches puberty (Wear et al. 2016). Fully grown follicular oocytes of most mammals are arrested at prophase I and resume meiosis at puberty. During and following the first meiosis resumption, chromatin starts to condense, germinal vesicle breakdown is initiated (GVBD), the metaphase I spindle is organized and the first polar body is extruded. Immediately thereafter, the oocytes enter meiosis II and are then arrested again at the metaphase II stage. Upon sperm stimuli oocytes resume meiosis II and complete maturation, emitting the second polar body. After sperm penetration, the oocytes develop a mechanism to block further penetration of surplus spermatozoa (polyspermy) (Sun and Nagai 2003). When oocytes enters meiosis I a single layer of flattened pregranulosa cells encloses it, thus forming the primordial follicle in the foetal or neonatal ovary (McLaughlin and McIver 2009). This is the first step of folliculogenesis, a process that leads to the formation and growth of the ovarian follicle. Primordial follicles form the stock from which all ovulatory follicles emerge. Approximately 500,000 primordial follicles are present in both ovaries by 10 days after birth (Wear et al. 2016).

Primordial ovarian follicles are not stimulated to grow at the same time, and only a small number begin their development while the rest remain quiescent. Initiation of growth of primordial follicles involves endocrine actions and regulatory effects of local factors from the somatic cells of the follicle, such as transforming growth factor-beta (TGF-β), growth differentiation factor 9 (GDF9), bone morphogenic protein 15 (BMP15) and by the oocytes (Kim 2012), follicle-stimulating hormone (FSH), and probably from the growing oocyte (Dierich et al. 1998). Primordial follicles contain an immature-sized oocyte, surrounded by one to two layers of fattened cells. They grow to primary follicular stage (approximately 120 μm), when the oocyte becomes surrounded by one to three layers of granulosa cells. The follicle than enters the secondary stage with 3 to 20 layers of granulosa cells (Christenson et al. 1985), with a diameter of 140 to 400 μm (Morbeck et al. 1992). Further growth past the 400 μm stage is characterised by an increase in the number of layers of granulosa cells, which subsequently separate from each other resulting in formation of the antrum, a fluid filled cavity (Caárdenas and Pope 2002). In pigs, activated primary follicles require approximately 84 days for growing to the antral stage (Caárdenas and Pope 2002). Antral follicles emerge from the primordial population at approximately 90 days of age in pigs (Guthrie et al. 1995). In the antral class, most follicles range in size from 0.4 (400 μm) to above 1.5 mm with external and internal theca cell layers and 10 to 30 layers of granulosa cells (Knox 2005).

The time required from early antral follicles of approximately 400 μm to grow to up to 3 mm was estimated to be 2 weeks, and the growth of follicles from 3 mm to pre-ovulatory size of 10 mm was estimated to take 5 days (Morbeck et al. 1992). The rate of growth of porcine

follicles from 3 to 10 mm in diameter has been estimated to be 1.14 mm per day (Dailey et al. 1976). The pool of antral follicles that is present at the onset of the follicular phase has developed during the late luteal phase of the oestrous cycle or during lactation and may consist of approximately 100 follicles, on average (Knox 2005). Antral follicular growth is controlled by a system of positive and negative feedback of reproductive hormones (Guthrie 2005), produced and released from the hypothalamus (gonadotropin-releasing hormone, GnRH), the pituitary [follicle-stimulating hormone (FSH); luteinizing hormone (LH); oxytocin and prolactin), the ovaries [progesterone (P4); 17β-oestradiol (E2); inhibin, folistatin, relaxin] and the uterus [prostaglandin F2α (PGF2α)]. Two terms are used to describe the follicular processes leading up to ovulation: follicular recruitment and selection. Recruitment refers to the population of small and medium follicles (< 3 mm and 4 to 6 mm, respectively) present at the surface of the ovary that may be selected as ovulatory follicles, while selection refers to those follicles that escape atresia and ovulate (> 6 mm; Knox, 2005).

Follicular recruitment occurs between 14 and 16 of the estrous cycle or shortly after weaning (Clark et al. 1982). On day 16, approximately 40 to 50 follicles form 2 to 6 mm in diameter are present in both ovaries (Grant et al. 1989). Most follicles, and the oocytes they contain, degenerate and disappear from the ovaries throughout the process of atresia. Atresia is the means by which follicles that contain oocytes in an inappropriate stage of development are eliminated from advancing to ovulatory status (Guthrie and Garrett 2001). Although atresia may occur any time during development of antral follicles, most follicles are lost during the transition from the small to large size, i.e. around 3 up to 10 mm (Guthrie et al. 1995), mostly before reaching 6 mm in diameter (Dailey et al. 1976; Grant et al. 1989). Therefore, only around 30 to 40% of the recruited follicles are selected to complete final maturation and ovulate. Ovulatory follicles are not readily identifiable until the day 20 of the estrous cycle (Grant et al. 1989), which suggests that selection is not completed until near ovulation (Foxcroft et al. 1987).

Recruitment of follicles occurs when pulsatile GnRH/LH release shifts from a lesser frequency/greater amplitude pattern to a greater frequency/lesser amplitude pattern (Soede et al. 2011). Pulsatile GnRH release induces the release of FSH and LH (Sesti and Britt 1993). FSH and LH exert their function by activating membrane receptors of target cells, which activate the membrane-bound adenylate cyclase (AC), leading to the production of cyclic adenosine 5'-monophosphate (cAMP), that will act as the intracellular messenger. The production of cAMP leads to the phosphorylation of cellular proteins and the induction of specific cellular events (Andersen 1995). During follicular growth, LH binds to LH specific membrane receptors located at the theca interna cells, thus activating a cascade of intracellular events to convert cholesterol to androstenedione and/or testosterone (Young and McNeilly 2010). First cholesterol will be transported to the mitochondria where it will be converted to pregnenolone by P450 cholesterol side chain cleavage enzyme (P450ssc or CYP11AI). Pregnenolone will be converted to progesterone at the smooth endoplasmatic reticulum by Δ^5 -3β Hydroxysteroid dehydrogenase (3β-HSD), which will be converted to

androstenedione or testosterone by 17α- hydroxylase (CYP17) and 3β- hydroxysteroid dehydrogenase, respectively. Androstenedione and/or testosterone diffuses out of the theca interna cells and enters the granulosa cells, which contain receptors for FSH. When FSH binds to its receptor, it causes the conversion of testosterone to 17β- oestradiol (Picton et al. 1999) throughout the actions of the enzymes P450 aromatase and 17β- hydroxysteroid dehydrogenase (Young and McNeilly 2010). Follicle-stimulating hormone (FSH) is responsible for the increase in the number of follicles that are recruited [reaching the medium (4 to 6 mm) to large (> 6 mm) category]. Once the follicles are recruited, they begin to produce oestradiol and small quantities of inhibin. As inhibin levels increases, its negative feedback on FSH increases (Noguchi et al. 2010). Thus, FSH begins to decline and LH becomes more important for follicular development. With decreasing levels of FSH, recruitment will stop and part of the growing follicles in the pool will become atretic. However, part of the follicles will continue to grow even though FSH levels are reduced because they start to synthesize LH receptors also in the granulosa cells (Hillier et al. 1994). Selection of follicles for ovulation occurs from the recruited pool and is marked by a shift of follicular growth dependence from FSH to LH. So, LH is necessary for the further growth of the follicles to pre-ovulatory size during selection (Knox 2005). Increased peripheral concentrations of 17β-oestradiol induce the pre ovulatory LH surge and a small FSH surge via positive feedback causing an immediate decrease in peripheral concentrations of 17β-oestradiol. An postovulatory elevation, or secondary surge, in FSH 24 hours after the pre-ovulatory discharge of FSH and LH occurs, and is important for recruitment of follicles for the next ovulation (Knox et al. 1991). The LH surge initiates the follicular changes that result in ovulation and luteinisation of the follicular wall, thus triggering corpora lutea formation and progesterone production (Soede et al. 2011).

During folliculogenesis, the growth of oocytes will first occur coincident and correlated in a positive linear manner with the follicular growth. During this phase, pig oocytes will growth from 30 μm in primordial follicles to 100 μm in antral follicles of 700 μm in diameter. Oocytes will reach 115 μm in 1.8 mm antral follicles and reach 120 μm in follicles ≥ 5 mm, after which the size of the oocyte remains constant despite continuation of follicular growth (Motlík and Fulka). Pig oocytes originated from follicles with less than 700 μm are incapable of completing meiotic resumption (Motlík and Fulka 1986), and the rate of oocyte growth is related directly to the number of granulosa cells coupled it and the granulosa cells effectively increase the surface area of the oocyte. The oocyte does not acquire the competence to complete the first meiotic division until it has reached almost the maximum size (Moor and Dai 2001). The surface to volume ratio of the oocytes increases together with the rate of entry of small molecules (i.e. c-kit, c-kit ligand and stem cell factor, GDF-9) crucial for oocyte growth and development (Hunter 2000). Increased *in vitro* developmental competence of oocytes correlates with increased oocyte diameter and follicle diameter in the pig (Liu et al. 2002; Lucas et al. 2002). This appears to be related with the increased ability of oocytes from larger follicles to complete meiotic and cytoplasmic maturation. In fact, a greater proportion of pig oocytes from follicles of 3 to 8 mm reach metaphase II (MII) in comparison with those

from follicles smaller than 3 mm (Motlík and Fulka 1986; Liu et al. 2002; Lucas et al. 2002). Moreover, the ability of spermatozoa to penetrate the oocyte, an indicator of sufficient cytoplasmic components for fertilization, also increases with follicle size (Lucas et al. 2003). In addition to providing metabolic support, the pre-ovulatory follicles generates signals required for the completion of oocyte maturation, in response to the LH surge. The pre-ovulatory LH surge results in the elimination of one or more inhibitory substances such as oocyte maturation inhibitor (OMI) and the elimination of these inhibitory substances leads to the activation of cyclins, phosphatases and kinases, which are required for nuclear maturation to be achieved (Fulka et al. 1994). Oocyte nuclear maturation includes: (1) the acquisition of meiotic competence. Growing pig oocytes (diameter ≤ 90 μm) are unable to resume meiosis *in vitro*. The inability of growing oocytes to resume meiosis is related with their inability to activate the maturation promotion factor (MPF) and/or mitogen activated protein (MAP) kinase, two important signal molecules that control meiosis resumption; (2) resumption of first meiosis. Full meiotic competence is reached in ovarian follicles of 3 mm in diameter or more; (3) assembly of meiotic apparatus and (4) metaphase II arrest, which is related with higher levels of MPF (Sun and Nagai 2003). Oocyte cytoplasmic maturation includes those events that instill upon the oocyte a capacity to complete nuclear maturation, fertilization, and early embryogenesis and thus provide a foundation for implantation, initiation of pregnancy, and normal foetal development (Watson 2007). In general terms, this process involves the accumulation of mRNA, proteins, substrates, and nutrients that are required to achieve oocyte developmental competence that fosters embryonic developmental competence (Watson 2007).

The pre-ovulatory LH surge will also trigger the initiation of the ovulatory process itself, with degradation of the follicular wall and expulsion of the cumulus-oocyte complex from the follicle. The tight synchronization of the resumption of oocyte meiosis and follicular rupture insures the release of mature oocytes, which can be fertilized and undergo embryonic development (Espey and Lipner 1994). Ovulation takes place on average 27 to 33 hours after the peak of LH surge, which occurs 41 to 47 hours after the onset of the oestradiol surge (Soede et al. 1994), with follicle diameter ranging from 6 to 8 mm (Soede et al. 2011). The period between rupture of the first and last follicle is on average 1.8 ± 0.6 hours, ranging from less than 1 to 3 hours in sows (Soede et al. 1992). However it is important to consider that these estimations of duration of ovulation were done 25 years ago, using sows with an average ovulation rate of 18.6 (based on corpora lutea counting after slaughter), and that both the average and the variation in this period might have changed in modern sows with ovulation rates above 25. The local events within the ovary itself that lead to follicular rupture are not clearly understood and the precise mechanisms of ovulation has been related to a number of factors, like proteolytic enzyme activity on the follicular wall (Cajander and Bjersing 1975), morphologic changes in the stigma that favours follicular rupture (Parr 1975), perifollicular ovarian smooth muscle contractions, changes in the ovarian intercellular collagen bundles such as increased distensibility and plasticity (Weiner et al. 1975), and vascular alterations in the perifollicular vessels (Migone et al. 2016). Up to now, not one

specific hypothesis completely explains the entire mechanism of follicular rupture but it is likely that many factors complement one another in the mechanics necessary to achieve ovulation. The recognition of smooth muscles and autonomic nerves within the ovarian stroma and the demonstration of contractions in the ovaries suggests that smooth muscle contraction is indeed involved in the ovulatory process (Stefenson et al. 1981). Also, vasoconstriction of thecal vessels at the apex of the pre-ovulatory follicle is necessary for follicle rupture at ovulation (Migone et al. 2016). The relationship between these factors might be different in sows with high and low ovulation rate inducing differences in the duration of ovulation. Although research in the past did not find a relationship between the duration of ovulation and embryonic development heterogeneity (Soede et al. 1992), an increase in duration of ovulation in sows with much higher number of pre ovulatory follicles cannot be ruled out.

The release of gonadotropins might be influenced by nutrition, with a clear inhibition of GnRH and of the release of LH and FSH by undernutrition (Prunier and Quesnel 2000). Studies have shown that growth of the surface follicles was influenced by feeding regimen. For instance, the mean diameter and volume of the 10 biggest follicles were decreased in underfed females (Quesnel et al. 1998). Moreover, primiparous sows submitted to feed restriction during their last week of lactation and inseminated at their first oestrus after weaning had lower ovulation rate (15.4 ± 2.3 vs 19.9 ± 1.6, $P < 0.05$) and embryonic survival (64.4 ± 6.1 % vs 87.5 ± 6.4%, $P < 0.05$) than control sows fed "*to appetite*" during lactation (Zak et al. 1997a), which was later associated with impaired oocyte quality as a result of lower follicular quality (Zak et al. 1997b). In cyclic gilts, feed restriction during the luteal phase seems to do not affect ovulation rate at the subsequent cycle. On the other hand, feed restriction starting during the luteal phase or at luteolysis and maintained during the follicular phase reduced ovulation rate at next oestrus, since it occurred during recruitment and selection of the pre-ovulatory follicles (Prunier and Quesnel 2000). The effects of nutrition on folliculogenesis might occur throughout metabolic mediators acting directly at the ovarian level, like for example insulin-like growth factor –I (IGF-I), leptin, growth hormone (GH) and insulin (Prunier and Quesnel 2000).

Physiological background leading to low or high ovulation rate

Ovulation rate (OR) is determined by the final number of ovulatory follicles, which is determined by how many follicles are recruited and by the ability of recruited follicles to continue to grow and avoid atresia during selection. A high OR, can therefore occur throughout an increase in the number of recruited follicles (Zimmerman and Cunningham 1975; Vatzias 1993; Knox et al. 2003), a decrease in the incidence of follicular atresia (Dailey et al. 1975), and throughout an extended selection period throughout the follicular phase or selection of greater numbers of follicles over an extended follicular phase (Kelly et al. 1988b), which will all lead to an increase in the number of selected follicles that will reach ovulation.

In the pig, FSH maintain a population of medium follicles from which the ovulatory follicles are selected. The number of medium follicles and the concentration of plasma FSH remain fairly constant during the luteal phase of the estrous cycle or with feeding of altrenogest (a progesterone agonist). However, during maturation of large follicles, the decrease in number of medium follicles is accompanied by a 60% decrease in plasma FSH concentrations (Guthrie et al. 1988). Thus, FSH is the main hormone controlling follicular growth in pigs and its secretion is in turn controlled via negative feedback done by the main secretory products of large dominant follicles: oestradiol and inhibin A (Hunter et al. 2004). Because of its importance on follicular recruitment, differences in FSH levels are believed to be the main factor determining a low or a high ovulation rate in pigs. For example, at the time of major follicular depletion, FSH decreases abruptly, and at the time of follicle reappearance, FSH increases in a large surge (Knox et al. 2003; Guthrie 2005). Knox et al. (2003) observed higher FSH levels in gilts selected for high OR (for 9 generations) than in the control line gilts during the ovulatory period (1.6 vs 1.1 ng/ml), and during mid (3.1 vs 2.6 ng/ml) and late (3.0 vs 2.4 ng/ml) luteal phase. Pregnant mare serum gonadotropin (eCG), an hormone retrieved from the pregnant mare that provokes an FSH activity in pigs, was given to sexually mature crossbred gilts (6 to 7 months, 100 to 120 Kg) on day 11 of the estrous cycle, and the ovaries were examined at 24, 48, 72 or 96 hours after the treatment (Liu et al. 2003). The number of small (< 3mm, 31.5) and medium (3-5 mm, 23.0) follicles 24 hours after the immunization was higher in treated gilts than in control gilts at the same stage of the estrous cycle (20 small and 6.5 medium follicles at day 12, $P < 0.05$), and the number of large follicles ($\geq$ 5 mm) was higher ($P < 0.05$) at 72 h (8.5 vs 2.5) and 96 h (7.0 vs 3.0) in treated than in control gilts (Liu et al. 2003). These results indicates that eCG/FSH treatment on day 11 of the estrous cycle promotes the development of antral follicles in mature gilts. Selection of ovulatory follicles occurs from the proliferating pool of 1 to 6 mm follicles present at days 14 to 16 of the estrous cycle (end of the luteal phase). Suppression of FSH with porcine follicular fluid (pFF), that contain inhibin as it main component, for 36 hours around the time of luteolysis reduced the number of small, medium and even large follicles before the onset of estrus (Knox and Zimmerman 1993) and at estrus (Knox 1989). Additionally, immunization of crossbred pre-pubertal gilts against bovine inhibin alpha (inhibin A) changed the pattern of secretion of FSH before and after estrus, with an 27% increase in the area under the curve for FSH in gilts immunized against inhibin during the 5 days that preceded the LH surge. This, resulted in a 39% increase in OR ($P < 0.01$), with an average of 17.8 (16 to 22) for the immunized gilts against an average of 12.8 (11 to 17) for the control gilts (King et al. 1993). Therefore, we can conclude that FSH levels just prior to and following luteolysis influences the number of medium follicles at later stages of the follicular development (Knox 2005), and therefore ovulation rate.

Alterations in the amount of atresia during the follicular phase of the estrous cycle could play an important role in the determination of the final OR (Dailey et al. 1975). A reduction in granulosa cell apoptosis in small (< 3mm) and medium (3 – 5 mm) follicles 24, 48, 72 and 96 hours after immunization of gilts against inhibin A, thus favouring FSH, indicates that the

FSH activity can rescue early atretic follicles (Liu et al. 2003). Follicles can be atretic or healthy based on their 17β-oestradiol and progesterone production by granulosa cells (Henderson et al. 1987). Gilts selected for high OR maintained more estrogen-active follicles of 5 to 6.9 mm in diameter during the mid- to late- follicular phase, and they continue to select and mature ovulatory follicles from this pool of larger estrogen active follicles (Vatzias 1993). Driancourt and Terqui (1996) compared follicular maturation in high and low OR sows [23.1 vs 18.8 pre-ovulatory follicles] at 1, 3 or 5 days of the follicular phase and observed an higher estradiol concentrations in sows with high OR at days 3 (12.2 vs 9.9 ng/ml) and 5 (33.4 vs 22.1 ng/ml) in comparison with sows with low OR. Despite the higher estradiol, sows with high OR had smaller follicular size at days 3 (5.6 vs 5.9 mm) and 5 (6.8 vs 7.5 mm) than sows with low OR. These results indicate a greater ability of follicles from high ovulation females to aromatize androgens to estrogens. Additionally, the total number of estrogen active follicles, defined as the number of follicles 5 to ≥ 7 mm in diameter with 17β-oestradiol production ≥ 100 ng/ml of follicular fluid, was greater in gilts with higher OR (20.4) than for gilts with lower OR (13.8) (Yen et al. 2005). What triggers this increased aromatization capacity is not known, but one hypothesis could be an altered initiation of the LH surge. The relationship between LH levels at different stages of the estrous cycle and OR is not clear. Although Knox et al. (2003) observed that LH was higher during the ovulatory period in gilts with high OR (18.8 corpora lutea, 2.3 vs 1.7 ng/ml) in comparison with gilts with low OR (14.3 corpora lutea), Kelly et al. (1988b), Hunter et al. (1993) and Mariscal et al. (1998) did not find an statistically significant difference in LH levels during ovulation in high and low OR animals. Also, despite the higher LH surge during ovulation, Knox et al. (2003), did not observe different systemic levels of 17β- estradiol in gilts with high (20.9 CL) and low (14.7 CL) OR around the pre-ovulatory LH surge (approximately 65 pg/ml for gilts with high and low OR, respectively; $P > 0.05$). Thus, taking into consideration that the LH surge is triggered by the peak of estradiol surge, this raises the question of what other factors could explain the higher LH surge in gilts with higher OR.

The pattern of growth of the ovulatory follicles seems to be different between animals with high or low ovulation rate. The difference in OR between Chester White and Poland China gilts (13.4 vs 10.8 corpora lutea, respectively) was traceable to greater numbers of antral follicles with more than 1 mm in diameter in Chester White gilts on days 3, 7, 11, 15 and 19 of the estrous cycle (Kirkpatrick et al. 1967). Although, Kelly et al. (1988a) observed a similar number of small (< 3 mm), medium (3 to 6.9 mm) and large (7 to 12 mm) follicles at days 3, 15 and 19 of the estrous cycle (day 0 was the first day of standing heat) in gilts selected for high OR (18.5 corpora lutea) and in gilts of a control line (15.3 corpora lutea). Driancourt and Terqui (1996) compared follicular growth in high and low OR sows [23.1 vs 18.8 pre-ovulatory follicles] at 1, 3 or 5 of the follicular phase and observed that in low OR sows, already at day 3, the sum of the number of large (5.1 to 7.0 mm) and extra-large follicles (≥ 7.1 mm) was close to the number of ovulations (18.2 vs 18.8), while in high OR sows seven additional ovulatory follicles developed between days 3 (17.2 large and 0 extra-large follicles) and 5 (18.2 large and 6.0 extra-large follicles). Hence, recruitment of ovulatory

follicles extends later during the follicular phase in sows with higher OR, a finding that was previously reported by Vatzias et al. (1991). Moreover, a high ovulating line of gilts that ovulated on average 3.2 more follicles than the control line (18.5 vs 15.3), had a longer follicular phase, as suggested by a 1.5 day longer interestrous interval in comparison with the control line (21.9 vs 20.4 days) keeping similar patterns of luteal regression. This longer follicular phase may be used to recruit additional follicles for ovulation (Kelly et al. 1988a). Moreover, there appears to be considerable variation within and between animals in selected pre-ovulatory large follicles size (7-11 mm), but also morphology and steroidogenic activity (Hunter and Wiesak 1990). This may indicate that not all follicles are at the same stage of development at the time of selection (Knox 2005); a variation that can be higher in animals with a higher OR and therefore a higher window of selection.
The changes in the conditions that control follicle development and selection for ovulation may control not only OR but also the quality of the follicles and, consequently of the oocytes that are released. Oocyte quality impacts early embryonic survival, the establishment and maintenance of pregnancy, embryonic and foetal survival and development and even adult life diseases (Krisher 2004). Moreover, follicular and oocyte quality might influence the development of corpora lutea.

Corpora lutea development and regression

The pre-ovulatory surge of LH initiates the differentiation of residual follicular cells into luteinized cells that will form the corpus luteum (i.e. corpora lutea in plural, Figure 2.1), switching the main ovarian steroid production to progesterone. The corpus luteum (CL) consists of different cell populations, which includes small luteal cells, large luteal cells, distinct morphological, endocrinological and biochemical properties. It is generally believed fibroblast, capillary endothelial cells and pericytes (Farin et al. 1986). These cells have

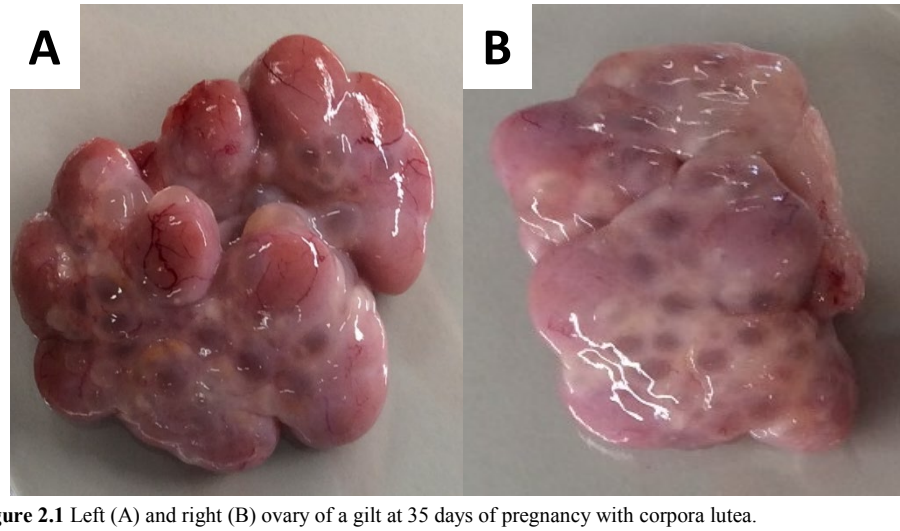

Figure 2.1 Left (A) and right (B) ovary of a gilt at 35 days of pregnancy with corpora lutea.

distinct morphological, endocrinological and biochemical properties. It is generally believed that large luteal cells differentiate from granulosal cells and that small luteal cells differentiate from thecal cells (O'Shea 1987). However, some studies also describes the differentiation of small luteal cells into large luteal cells (Cran 1983; Fritz and Fitz 1991). Small and large luteal cells are the only steroidogenic cells in the corpora lutea of the pig (Lemon and Loir 1977), but with different capabilities: small luteal cells have a smaller basal progesterone secretion *in vitro* than large luteal cells, while having a greater magnitude in progesterone secretion when stimulated by LH.

After the pre-ovulatory gonadotropin surge and before follicular rupture follicular diameter increases and the follicular wall becomes slightly folded (McClellan et al. 1975). Also, gap junctions among the granulosa cells decrease and the granulosa cells become dispersed. In the theca interna cells, morphological changes in the microcirculatory system follows the LH surge, leading to 1) vasodilatation and hyperaemia, which is probably the result of histamine release and production of prostaglandin E2 (PGE2) causing the contraction of the endothelium and relaxation of the smooth muscles, resulting in vasodilatation and 2) increased vascular permeability; 3) tissue edema; 4) congestion; 5) ischemia; 6) vascular injury; 7) angiogenesis (Cavender and Murdoch 1988). After follicular rupture, the follicular wall infolds thus facilitating the migration of fibroblasts, endothelial cells and theca interna cells into the central regions of the developing corpora lutea. Luteinisation involves changes in the cellular organelles that participate in steroid production, as an increase smooth endoplasmic reticulum, increased size of the Golgi apparatus and an increased number and complexity of mitochondria (McClellan et al. 1975). Another aspect of early luteal development is the rate of tissue growth and cellular proliferation during this time. In the pig, the corpora lutea (CL) area increases from approximately 0.35 cm^2 at 24 hours after mating to 1.0 cm^2 six days after mating (Tast et al. 2002). This growth in domestic animals is believed to be the result of an increase in the size of large luteal cells, which either do not proliferate or undergo minimal proliferation only at the beginning of the oestrous cycle (Zheng et al. 1994), and an increase in the number of small luteal cells, fibroblasts and endothelial cells, that appear to proliferate much more rapidly throughout the oestrus cycle and even into early pregnancy (Reynolds and Redmer 1998) .The factors regulating the proliferation of small luteal cells and fibroblasts are not well characterized but some studies have suggested the participation of fibroblast growth factors (Redmer and Reynolds 1996), growth hormone (Juengel et al. 1997) and LH (Grazul-Bilska et al. 1995). Vascular endothelial growth factor (VEGF), a mitogen specific for endothelial cells which expression is induced by LH, is probably a primary regulator of proliferation of luteal endothelial cells. Also, fibroblast growth factor 2 (FGF-2) has been detected in porcine luteal tissues and the angiogenic activities of the corpora lutea could be partially immunoneutralized with antibody against FGF-2, indicating its action in endothelial proliferation (Redmer and Reynolds 1996). Proliferation of endothelia cells is crucial for the neovascularization during luteal development that results in the corpus luteum's extensive capillary network (Redmer and Reynolds 1996). In fact, the mature CL is highly vascular and receives one of the greatest

rates of blood flow, per unit of tissue mass, of any organ (Redmer and Reynolds 1996). The capillary network of the mature CL is so extensive that the majority of parenchymal (steroidogenic) cells are adjacent to one or more capillaries (Zheng et al. 1994), and up to 85% of the cells that proliferate during CL growth are endothelial cells (Redmer and Reynolds 1996). Porcine CL reaches its full size between days 10 to 12 after ovulation (Langendijk and Peltoniemi 2013), and the size of a cyclic CL varies from 5 to 10 mm, reaching 6 to 11 mm in pregnant sows (Kähn et al. 2004). According to McEntee (2012) the corpus luteum of the sow can reach approximately 12 mm.

Despite the morphological changes described above, the pre-ovulatory gonadotropin surge initiates distinct changes in both expression and regulation of steroidogenic enzymes switching the mainly steroid production to progesterone (Smith et al. 1994). This is accomplished by increased expression of enzymes necessary for conversion of cholesterol to progesterone [cholesterol side-chain cleavage cytochrome P-450 complex (P-450scc) and 3β-hydroxysteroid dehydrogenase/Δ^5,Δ^4 isomerase (3β-HSD)], together with a reduction in the expression of enzymes that convert progesterone to estrogens (Tomac et al. 2011).

Progesterone, as all steroid hormones, is lipophilic and therefore pass through the target cell membrane by normal diffusion. Within the target cells, steroid hormones bind to their receptors in the nucleus. After binding to the target gene, the steroid-receptor complex initiates synthesis of specific messenger ribonucleic acid (mRNA) molecules from deoxyribonucleic acid (DNA) in the chromatin. The mRNA is then translocated to the cytoplasm where synthesis of new proteins occurs, which are actively transported to the nucleus where they are responsible for the biological activities of a steroid hormone (Tsai and O'Malley 1994). In the case of progesterone, main actions are to prepare the reproductive tract for pregnancy. Synthesis of progesterone receptors is dependent on the previous exposure of the tissue to estrogens. In the uterus, progesterone will inhibit mitosis of the endometrium, induce stromal differentiation, stimulate glandular secretion in association with the accumulation of basal vacuoles in the glandular epithelium, change the pattern of proteins secreted by endometrial cells and induce quiescence of the myometrium (Niswender et al. 2000).

Two main sources of cholesterol are used by the porcine CL to produce progesterone: low-density lipoproteins (LDL) and high-density lipoprotein (HDL) (Tomac et al. 2011). Once free cholesterol is present in the cytosol of the cell, it can be used for steroidogenesis or formation of cell membranes, or it can be esterified with fatty acids to form cholesterol esters by cholesterol ester synthetase, which could also be used for progesterone production (Tomac et al. 2011). Moreover, under conditions of lipid deprivation (reduced lipoprotein synthesis or in most *in vitro* conditions), porcine luteal cells are capable of synthesizing cholesterol from acetate (Cook et al. 1967). There appears to be some species differences in their preference for LDL or HDL (Niswender et al. 2000), and the porcine corpora lutea uses primarily LDL (Brannian and Stouffer 1993).

The major mechanisms for obtaining cholesterol are endocytosis of LDL or selective uptake of HDL, and while luteal cells can produce cholesterol by the *de novo synthesis*, this method

plays a minor role in the normal functioning tissue, as evidenced by the low levels of limiting enzymes in the cholesterol biosynthetic pathway (Christenson and Devoto 2003). Cholesterol is then transported to the outer membrane of the mitochondria by the cytoskeleton (microtubules and microfilaments) and steroid binding proteins; and from the outer membrane to the inner membrane, an rate-limiting stage on the steroid synthesis, by Steroidogenic acute regulatory proteins (StAR), through phosphorylation by protein kinase A (PKA) (Ikonen 1997). Once transported to the mitochondrial matrix, the enzyme P450scc (cholesterol side chain cleavage cytochrome P450) will convert cholesterol to pregnenolone by cleavage of cholesterol side chain, in a rate-limiting step in progesterone production (Smith et al. 1994; Niswender et al. 2000). The second key step in progesterone production is the transport of pregnenolone to the smooth endoplasmic reticulum to be converted to progesterone by 3β-hydroxysteroid-dehydrogenase/Δ5, Δ4 isomerase (3β-HSD) (Smith et al. 1994; Niswender et al. 2000). In relation with the LH surge, systemic progesterone levels will rise and reach a maximum value of 40 ng/ml at days 11 to 15 of the oestrus cycle (Knox et al. 2003), which coincides with the maximum CL area of around 0.9 to 1.0 cm^2 as observed in pregnant sows (Langendijk and Peltoniemi 2013). In both pregnant and non-pregnant sows systemic concentrations of progesterone rises up to 14 days after the onset of oestrus. In non-pregnant sows systemic progesterone levels decreases drastically due to luteolysis after 16 days, and in pregnant sows a decrease of 30 to 70% will occurs between days 14 and 30 (Ziecik et al. 1986), following a decrease in luteal tissue mass which reasons are unknown (Langendijk and Peltoniemi 2013).

In the pig development of CL and progesterone secretion will occur independent of LH input from the pituitary until 10 to 12 days after ovulation (Peltoniemi et al. 1995), but beyond 12 days of pregnancy support of CL by LH does become important. Hypophysectomy on the day after heat or mating does not prevent the development of normal-sized, progesterone-secreting CL up to 12 days, but regression of the CL occur between days 16 and 20 in mated hypophysectomised sows. Beyond days 10 to 12 of the luteal phase, support of the CL by LH becomes important, and chronic treatment with a gonadotropin-releasing hormone (GnRH) agonist from days 14 or 21 of pregnancy interrupted LH secretion and lead to a decline in progesterone level and loss of pregnancy in all sows approximately 15 h after the beginning of the treatment (Peltoniemi et al. 1995). Luteinizing hormone (LH) secretion during the luteal phase of the oestrous cycle and during early pregnancy is characterized by a lesser frequency of greater amplitude LH pulses (Langendijk et al. 2007). Progesterone secretion by the ovaries also occurs in pulses, and some of these pulses are temporally associated with the LH pulses (Brüssow et al. 2011). Progesterone is also produced by the ovulatory follicles before ovulation occur, as following the LH surge the theca interna cells stop their steroid production at progesterone; and this local elevation of progesterone (follicular level) is essential for ovulation because progesterone stimulate synthesis of collagenase by theca interna cells, that will break collagen, a major component of the connective tissue (Salehnia and Zavareh 2013). Moreover, LH in a time and dose dependent manner increased secretion of progesterone by cultured luteal slices collected at mid luteal

phase (Przygrodzka et al. 2014) indicating that LH have an important if not decisive function in the maintenance of porcine CL function.

In the pig, small and large luteal cells of the CL, collected on days 8 and 9 of the oestrus cycle, have been shown to be regulated in different ways not only by LH but also by prostaglandin E2 (PGE2) (Richards et al. 1994). The physiological concentrations of LH increases secretion of progesterone from small luteal cells (theca origin) but not from large luteal cells (granulosa origin) (Tekpetey and Armstrong 1991). Conversely, PGE2 stimulates progesterone secretion by the large luteal cells but not by the small luteal cells (Richards et al. 1994). A part of the potential mechanism by which the pig conceptus prevents luteolysis is changing prostaglandin synthesis in favour of the luteoprotective PGE2. The porcine conceptus and endometrium synthesize large amounts of PGE2 before implantation, and the PGE2:PGF2α ration is increased in the uterine lumen and vein, as well as in the trophoblastic tissue on days 10 to 13 of gestation. Ford and Christenson (1991) observed a direct effect of exogenous PGE2 delivered in implants to luteal tissue in protecting porcine CL from the luteolytic dose of PGF2α.

The supportive role of 17β-estradiol in maintenance of porcine luteal function has also been documented. Kidder et al. (1955) was the first to report that exogenous oestrogen extended the luteal function in pigs. Later, it was shown that oestrogens decreases the release of prostaglandin F-2α into the uterine vein, thus preventing the luteolytic signal and prolonging the lifespan of the pig CL throughout pregnancy (Bazer and Thatcher 1977; Bazer et al. 1986). Exogenous administration of 17β- estradiol in the mid luteal phase prolongs the luteal function in sows (Ford et al. 1982). But it was only in 1989, that Conleyt and Ford (1989) demonstrated *in vivo* a direct luteotrophic effect of oestrogen in the pig CL by implanting oestradiol in the 3 randomly choose CL 11 days after heat in gilts. The implants contained 150 (group 1), 500 (group 2) and 1500 μg (group 3) of oestradiol and released hormones at 4.4, 15 and 22.4 μg/day respectively. The authors then observed that oestradiol implanted CL were heavier (345 vs 276.4 mg) and had a higher progesterone content (23.0 vs 16.5 μg) and concentration (66.2 vs 58.7 ng/mg) than non-implanted CL in group 3 gilts, which had also higher values than gilts in groups 2 and 1. It is possible that the influence of estradiol on the CL is at least partly due to an increase in the expression of LH receptors (Garverick et al. 1982), thus increasing the CL sensitivity to LH and consequently its response.

Regression of porcine CL, or luteolysis, is a one to three day process that occurs on the end of the luteal phase as a result of increased pulsatile release of endometrial prostaglandin F2α (PGF2α) (Moeljono et al. 1977), which leads to a decrease in progesterone production and a structural regression of the CL involving widespread cell apoptosis (Bacci et al. 1996). The pig CL are only sensitive to luteolytic effect of PGF2α after day 12 of the estrous cycle, even though receptors for PGF are abundantly expressed in the CL (Gadsby et al. 1993), and the mechanism that prevent the PGF2α induced luteolysis before day 12 and then allow luteolysis to occur after day 12 (acquisition of luteolytic sensitivity) is unknown. Recently, it was shown that elimination of the protective effect of intraluteal progesterone does not directly cause luteolysis of the porcine CL, but allows PGF2α to induce luteolytic responses (favoured

apoptotic pathways) even in CL at day 9 of the estrous cycle, which have not acquired the luteolytic capacity (Diaz et al. 2011). This indicates that the loss of steroidogenic capacity might participate in the luteolytic capacity acquisition. Prostaglandin F2α induces different molecular pathways in porcine CL with and without acquired luteolytic sensitivity. Experiments with *in vitro* incubation of luteal tissue showed an increased progesterone secretion after PGF2α treatment during the mid-luteal phase of the estrous cycle (Przygrodzka et al. 2014). The endometrium is the source of luteolytic PGF pulses, because it expresses oxytocin receptors and cyclooxygenase 2 (COX-2), a rate limiting enzyme in the synthesis of prostaglandins. The luteolytic mechanism that develops in the endometrium requires sequential effects of progesterone, estrogen and oxytocin, acting through their respective receptors. At heat (day 0), estrogens from the pre-ovulatory follicles stimulates an increase in the expression of uterine estrogen receptor alpha (ERα), progesterone receptor (PR) and oxytocin receptor (OTR) expression. However, PGF2α is not secreted because there are no CL to secrete oxytocin. During early luteal phase, progesterone from the CL stimulates accumulation of phospholipids in the endometrium that can liberate arachidonic acid for synthesis and secretion of PGF. Progesterone levels will increase during the luteal phase and will block expression of ERα and OTR in the endometrium. However, continuous exposure of the uterus to progesterone for 8 to 10 days down-regulates expression of PR in the endometrium after days 11 to 12, allowing for rapid increases in expression of ERα on day 13 followed by OTR on day 14. Oxytocin, secreted from the posterior pituitary and from the CL will then induce secretion of luteolytic pulses of PGF2α from the endometrium, causing the regression of the CL (Spencer and Bazer 2004a). It is important to notice that the role of oxytocin in controlling PGF2α is not so well stablished in the pig (Waclawik et al. 2010), and oxytocin might not be responsible for the initiation of luteolysis but is more likely to be involved in the control of pulsatile release of PGF2α, especially the height and frequency of the peaks of this hormone during luteolysis (Waclawik 2011).

Pregnancy recognition and early embryonic and placental development

Fertilization of oocytes occurs in the oviduct, at the ampullary-isthimic junction, and the first 2 to 3 cleavage divisions of the fertilized oocytes take place in the oviduct (Hunter 1977). Approximately 20 hours post ovulation, porcine embryos reach the 2 cell stage and in 48 hours the 4 cell stage. Transport through the fallopian tubes lasts 72 hours; and embryos of 3 to 8 cell stage of development will than reach the tip of the uterine horn at the end of the third day post ovulation (Oxenreider and Day 1965). While migrating through the uterus, embryos will reach the morula stage at day 5. When they reach the 30 cell stage, a cavity named blastocoel is formed, leading to the formation of a blastocyst consisting of a trophoblast and inner cell mass, both still covered by the zona pellucida (a glycoprotein layer that surrounds the plasma membrane of mammalian oocytes within the follicles). Just before the day 7, the embryos hatch from the zona pellucida (Hunter 1977). During this time, metabolism of the developing embryos is influenced by the nature and volume of the tubal

secretions, these in turn being dependent upon the balance of ovarian hormones (Hunter 1977). Progesterone is involved in the stimulation of the secretory activity of the uterus, and its receptors are expressed at high levels at days 0 to 5 of pregnancy decreasing after day 10, and reaching a plateau at day 12 in the uterus (Geisert et al. 1994).

Between days 7 and 12 of pregnancy, the embryos migrate from the oviductal to the cervical end of the uterine horns to redistribute themselves subsequently over the full length of both uterine horns in a process named spacing (Dziuk 1985). During this passive trans uterine migration, embryos are distributed randomly by contractions of the myometrium (Dziuk 1985). The embryos do not seem to have a specific designated destination, and mixing of embryos from different horns regularly takes place (Dziuk et al. 1964). In fact, when allowed to enter the uterus from only one oviduct, porcine embryos migrate from one horn to the other during days 8 and 9 of pregnancy and are completely spaced within the uterus by day 15, regardless of the number of embryos or uterine size (Pope et al. 1982). Moreover, the number of embryos does not affect the rate or distance of migration (Dziuk 1985). On the other hand, spacing is influenced by the presence of other embryos. Pig embryos are positioned in a relatively equal distance to each other, regardless of the total space available (Anderson and Parker 1976; Dziuk 1985).

While migrating and spacing through the uterus porcine embryos undergo drastic morphological changes. Release of uterine growth factors is clearly involved with the growth and differentiation of the pig conceptuses following hatching from the zona pellucida on days 6 and 7 of pregnancy. After hatching, peri-implantation development in the pig is unique in that conceptuses develop from 1 to 2 mm sphere to 9 to 10 mm long ovoid shape conceptuses between days 8 to 10 of pregnancy (Geisert et al. 2014). Pig conceptuses can still be spherical blastocysts at 10 days of pregnancy, with a diameter that varies from 0.3 to 2 mm on day 8, from 0.5 to 2 mm on day 9 and from 1 to 8 mm on day 10. Also at 11 days of pregnancy, blastocysts can still be spherical with a diameter ranging from 1.4 to 10 mm, and in some sows conceptuses on spherical, tubular and filamentous forms can be found (Stroband et al. 1984). Rapid blastocyst elongation of the trophectoderm occurs between days 10 to 12, to allow maximum surface area of contact between the trophectoderm and uterine luminal epithelia and superficial glandular epithelia (Bazer 2013). During elongation the conceptuses change from spherical forms of 3 to 10 mm in diameter, as stated above, to tubular (10 to 50 mm long) and to filamentous (> 100 mm long) forms , achieving lengths of 700 to 1,000 mm by 16 days of pregnancy (Anderson 1978). During blastocyst elongation between days 10 and 12 of pregnancy they start producing estrogen which signals for maternal recognition of pregnancy in swine (Bazer and Thatcher 1977). The specific factor or factors involved with triggering the rapid morphological transformation of the ovoid conceptus to its filamentous shape are currently unknown. Although endometrial release of growth factors is involved in conceptus growth and development, variation in stages of development prior to and during the time of trophoblast elongation (spherical, ovoid, tubular and filamentous conceptuses present within the same litter) indicate that elongation is not

necessarily triggered by a uterine-stimulated event but rather a specific stage of conceptus differentiation and development (Geisert et al. 1982a; Stroband and van der Lende 1990).
Rapid conceptuses elongation provides the mechanism for delivery of estrogen across the uterine surface to maintain corpora lutea function. In pigs, the maintenance and establishment of pregnancy requires a biphasic pattern of estrogen secretion, predominantly 17β-estradiol, on days 11 and 12 and again between days 15 and 25-30 of pregnancy (Geisert et al. 1990). Secretion of 17β-estradiol by the elongating embryos on days 11 and 12 also leads to changes in the uterine environment that benefit the development of the elongating embryos. The uterine environment changes are due to the release of secretory material from uterine glandular epithelial cells into the lumen of the glands in response to the conceptuses estradiol (Geisert et al. 1982b; Fazleabas et al. 1985). This dumping of secretions, which can also be mimicked by exogenous oestrogen administration (Geisert et al. 1982c), results in marked change in both the amount and the composition of the uterine fluids [for example, before day 11 the amount of protein in the pig uterine flushes is lower] (Geisert et al. 1982b). This sudden change in the uterine milieu, although important in the nurturing of the elongating blastocysts at this critical stage of the pregnancy, it will cause the less developed embryos to die (Roberts and Bazer 1988), i.e. uterine-embryonic asynchrony (Pope 1988).
Also, secretion of 17β-estradiol by the elongating conceptuses does not inhibit the secretion of PGF2α by the uterine endometrium, rather it activates a mechanism whereby secretion of PGF2α is into the uterine lumen (exocrine secretion) rather than into the uterine vasculature (endocrine secretion) which would facilitate the counter-current exchange to the ovarian artery, and therefore the reach into the CL (Bazer and Thatcher 1977). An additional antiluteolytic mechanism could involve the retrograde transfer of PGF2α from the venous blood and uterine lymph into the uterine lumen and the ability of uterine veins and arterial walls to accumulate PGF2α (Krzymowski and Stefańczyk-Krzymowska 2004). The conceptus estrogen on day 12 also modulate expression of genes responsible for endometrial remodelling for implantation between days 13 and 25 of pregnancy (Joyce et al. 2007b). For example *STAT1* (signal transducer and activator of transcription 1) expression in the luminal epithelium (Joyce et al. 2007b), which is a transcription factor that can be activated by several ligands such as Interferon alpha (IFNa), Interferon gamma (IFNg), Epidermal Growth Factor (EGF) and Interleukin 6 (IL-6) (Joyce et al. 2007a). Additionally, a supporting role of estrogen is played by increased amounts of prostaglandin E2 originating from the conceptuses and from the endometrium prior to implantation (Waclawik and Ziecik 2007). Greater PGE2 secretion in the gravid uterine horn of unilaterally pregnant pigs is associated with increased luteal weights and progesterone concentrations of ipsilateral corpora lutea (Christenson et al. 1994), and the infusion of PGE2 into the ovarian artery elevates the concentration of progesterone in the ovarian venous blood on days 13 and 14 of pregnancy (Waclawik 2011). The trophectoderm also secretes interleukin 1 beta (IL1β) during this period and estrogen appears to modulate uterine responses to IL1β (Bazer 2013). Pig conceptus trophectoderm secretes both interferon γ (IFNG) and interferon δ (IFND) during the peri-implantation period of pregnancy (11 to 12 days). IFNG mRNA is abundant in

trophectoderm between days 13 and 20 of pregnancy, whereas IFND mRNA is detectable in day 14 conceptuses (Bazer 2013). Although there is no evidence that either IFNG or IFND have antiluteolytic effects preventing regression of corpora lutea or altering concentrations of progesterone in plasma, they do stimulate secretion of PGE2 by uterine cells, which may enhance structural integrity of corpora lutea and their secretion of progesterone (Harney and Bazer 1989). Another event associated with blastocyst elongation is the sequestering of histotroph, the endometrial epithelial cell secretions in the uterine lumen made it to provide nourishment for the developing blastocysts, which is crucial for embryonic survival before complete attachment of the trophectoderm to the endometrial epithelium (Bazer and Thatcher 1977; Geisert et al. 1982b).

Placentation in the pig relies on conceptuses elongation to increase available surface area for gas and nutrient exchange (Kridli et al. 2016). Elongation (days 10 to 12) occurs primarily as a result of hypertrophy of the trophectoderm and endoderm (Geisert et al. 1982a). The rate of elongation changes from 0.25mm / hour during the initial stages to 150 to 200 mm / hour during the ensuing stages of filament formation. Elongation allows the embryos to reach 80 to 100 cm by day 16 of pregnancy (Bazer and Johnson 2014). After elongation, implantation (initial attachment of the trophoblast to the maternal uterine epithelium) will start (Geisert et al. 1982a). The initial attachment of the pig blastocysts is always mesometrial and begins with a loose contact between elongated trophoblast and uterine epithelium near the embryoblast around days 13 to 14 of pregnancy, and progresses to the tips of the blastocyst during the following days. During the peri-implantation period of pregnancy, uterine luminal epithelium and the conceptuses trophectoderm develop adhesion competency in synchrony to initiate an attachment cascade within a restricted period of the uterine cycle termed the “window of receptivity” (Bazer and Johnson 2014). Uterine endometrial responses to implantation are complex. In addition to remodelling of the uterine luminal epithelium (Aboagye-Mathiesen et al. 1994), both luminal and superficial glandular epithelium secrete histotroph to nourish and support development of the conceptuses before implantation (Spencer and Bazer 2004b). Uterine stroma transforms to control movement of the conceptus through the uterine wall during implantation while generating a cytokine-rich environment that directly promotes angiogenesis to ensure sufficient blood flow to the placenta for hematotrophic nourishment of foetal development (Bany and Cross 2006). Conceptuses implantation first requires the loss of anti-adhesive molecules in the glycocalyx of luminal epithelium, comprised largely of mucins that inhibit attachment. This results in the “unmasking” of molecules, including selectins and galectins, which will contribute for the initial attachment of the conceptuses (non-adhesive of pre-contact phase of implantation) (Bazer and Johnson 2014). These low affinity contacts are then replaced by more stable and adhesive interactions between integrins and osteopontin from the maternal extra-cellular matrix (ECM), at day 18 of pregnancy when the completion of attachment will occur by interlocking microvilli to form the epitheliochorial placenta (Geisert et al. 1982a). The attachment to the maternal uterine surface (days 13 to 18) is essential for establishing

sufficient placental uterine area for subsequent nutrient transport for piglet survival to term (Geisert et al. 2014).

Once the conceptuses attach to the uterine epithelium, maternal and foetal blood must be brought into close apposition to allow for transplacental exchange of molecules while maintaining separation of the maternal and foetal circulatory systems (Bazer and Johnson 2014). Although the pig trophoblast possesses invasive properties (Samuel and Perry 1972), placentation is non-invasive, whereby the uterine luminal epithelium remains intact throughout pregnancy (King 1993; Geisert et al. 2014). Although non-invasive, the place where conceptuses attach to the uterine epithelium is clearly visible after removal of the conceptus from days 13 onwards, since the mucosa facing the elongated blastocyst is slightly hyperaemic along the line of the mesometrium (Perry and Rowlands 1962). After dilation and shortening of the blastocyst, the uterine mucosa facing the chorioallantois remains strongly hyperaemic throughout pregnancy, resulting in clearly reddened "implantation sites" alternating with white bands (Perry and Rowlands 1962). Placental trophectoderm directly attaches to the luminal epithelium, which serves as the conduit for maternal hematotrophic and histotrophic support for conceptus growth and development (Bazer and Johnson 2014). Extensive remodelling to form chorionic or placental ridges and the corresponding invaginations will result in folding that will increase the area of uterine-placental association. There is progressive interdigitating of microvilli on trophectoderm and luminal epithelium that eventually covers the entire placenta, except at the openings of the uterine glands; where the trophectoderm never fuses with the luminal epithelium forming the areola. The placenta of each embryo in a litter has about 2,500 areolae (Knight et al. 1977), that are formed by days 25 to 30 of pregnancy reaching maximal numbers at 70 days of pregnancy (Knight et al. 1977; Friess et al. 1980). The lumen of the areolae is filled with secretions from the uterine glands and histotroph, and consequently the number of areolae influences embryonic growth and foetal weight during pregnancy (Knight et al. 1977). Thus, the physical structure of the placenta is divided into areolae (with the histotrophic nutrition) and interareolar area (maternal endometrial epithelium layer tightly adhered to a foetal epithelium layer, the trophectoderm) (Vallet et al. 2014a). The interareolar area can be divided in two different areas with different structures and function. The first one, top and lateral side of foetal ridges, seem to be predestined for transmission of more diffusible substances like Oxygen and Carbone Dioxide; while the second area, the depths of the chorionic troughs, seems to be predestined for the transport of blood-borne nutrients and less diffusible substances secreted by the uterine epithelium (Friess et al. 1980).

Four different membranous structures are involved in placental development: chorion, amnion, vitelline sac or yolk sac, and the allantoic sac. The chorion is the outermost layer of the foetal membranes and a direct descendant of the outer blastocyst wall, the trophoblast. The chorion is crucial for placental development, as is represents the decisive exchange barrier between the maternal and the foetal organisms (Patten 1948). The yolk sac epithelium is of endodermal origin together with the foetal vascularized mesenchyme, and in the pig shows local fusion with the chorion, in areas where the vitelline vessels connect chorionic

and foetal vessels. In early porcine embryos, the yolk sac serves as an organ providing nutritive material to the embryo, since its abundant vessels are in a position that makes absorption from the uterus readily possible (Patten 1948; Hill 2017). The allantois is the extra-embryonic urinary bladder, originating from the embryonic endoderm, surrounded by mesenchyme and is richly vascularized (allantois vessels). These vessels fuse with the chorionic capillary bed and connect the chorionic capillaries with the capillary bed of the foetus forming the chorioallantoic placenta. Later in embryonic development, the allantois becomes highly developed and takes over the yolk sac function of embryo nutrition. It first appears at 14 days of pregnancy and grows very rapidly (Patten 1948). Around day 17 of pregnancy it is as large as the embryo itself and its crescent shaped horns extend towards opposite ends of the conceptus. At this time, large branches of the caudal end of the aorta (allantoic arteries and umbilical arteries) already have broken up into a maze of thin-walled vessels in the allantoic wall. The amnion derives from the embryonic ectoderm, and surrounds the embryo acting as an maternal-foetal barrier. The amniotic sac is completely formed at 18 days of pregnancy, when it becomes filled with a watery fluid in which the embryo is suspended, and it protects the embryos from mechanical injuries (Patten 1948). Rapid expansion and development of the chorion (trophectoderm) and allantois occurs between days 18 and 30 of gestation. Fusion of the chorion and allantois then takes place between day 30 to 60 and by days 60 to 70 placenta development is complete in terms of surface area and number of areolae (Knight et al. 1977).

Placental development, and therefore, the efficiency of nutrient exchange between the maternal and foetal blood supplies affects the development of the pig foetuses and thus influences litter size, piglet birth weight and therefore, piglet survival after birth (Vallet and Freking 2007). Placental length increases in the most rapid rate between days 20 and 30 of pregnancy, but length growth goes on until 60 days of pregnancy. From days 60 up to term, there is little change in the length of placenta (Knight et al. 1977). The increase in placental length precedes the increase in placental weight, that reaches a maximum at 65 days of pregnancy. Interestingly, the most rapid increase in foetal growth occurs after 50 days of pregnancy, period in which placental development remains rather static (Knight et al. 1977). Thus, the extent of placental development between days 20 to 30 of pregnancy has a significant impact on subsequent foetal growth (Knight et al. 1977; Vallet and Freking 2007).

Influence of uterine capacity on placental and embryonic growth

The concept of uterine capacity in pigs has been researched by different authors (Bazer et al. 1969; Webel and Dziuk 1974; Knight et al. 1977; Christenson et al. 1987; Bennett and Leymaster 1989). Uterine capacity (UC) is defined as the number of conceptuses that the pig uterus can successfully carry until farrowing (Ford et al. 2002) or as the ability of the uterus to provide the necessary nutrients to maintain embryonic and foetal growth until farrowing (Vallet et al. 2014a). Although direct measurements of UC are difficult to obtain (Lents et al. 2014), typically UC has been measured as the number of piglets born alive and it assumes that the number of embryos is not limiting (Lents et al. 2014). However, according to Vallet

et al. (2014a) estimations of uterine capacity have to take into consideration not only the number of piglets born alive but also any component reflecting differences in piglets birth weight, since an uterus that can carry ten piglets with 2 Kg each has a higher capacity than a uterus that can carry ten piglets with 1 Kg each.

One of the methods developed to measure UC is the surgical removal of one uterine horn and one ovary (unilateral hysterectomy-ovariectomy, UHO) to reduce the uterine space available per potential embryo (i.e. per ovulation, as the remaining ovary compensates the absence of the other ovary) (Vallet et al. 2014a). Christenson et al. (1987) compared gilts submitted to UHO (n = 110) at 8 to 12 days after their first estrus with control gilts (n = 142) at farrow and observed that control gilts (n=110) had higher litter size and higher piglet birth weight than UHO gilts (9.0 ± 0.3 vs 5.7 ± 0.3 and 1387 ± 21 g vs 1,162 ± 37 g, $P < 0.001$). The authors concluded that UHO gilts that had the largest litters at farrowing may have a higher uterine capacity. This study shows that as the uterine space available per embryo decreases the embryonic and foetal mortality increases and placental development is compromised, thus compromising piglet birth weight.

Limited UC will decrease both litter size and piglet birth weight (Town et al. 2004; Vallet et al. 2014b). The proposed mechanism involved in this reductions are compromised placental development together with the higher competition between embryos for biochemical factors and nutrients (Père et al. 1997). Pigs have epitheliochorial and diffuse placentation, in which the chorionic epithelium is in direct contact with the uterine luminal epithelium (MacDonald and Bosma 1985). This type of placentation is appositional rather than invasive and relies largely on simple diffusion, and consequently, it requires an adequate surface area for nutrient exchange from maternal to foetal blood streams (Ford et al. 2002). Thus, as the UC is reduced so it will be the uterine space available for placental development.

Limitations of UC are set to start at the time of conceptuses elongation (days 10 to 12 of pregnancy), but most of embryonic and foetal mortality occurring due to limited UC occurs between days 25 to 45 of pregnancy (Ford et al. 2002). However, Wu et al. (1989), by restricting the uterine space available to each potential embryo (i.e. per ovulation) to 10, 20 or 50 cm at 13 days of pregnancy, showed that the more limited the space (i.e. the uterine capacity) the lower the foetal survival at 50 days of pregnancy, and the losses occurred mainly after 20 days of pregnancy. Also, a reduction in the uterine space available per embryo increased the number of mummified foetuses (Wu et al. 1988). Père et al. (1997), observed that in UHO gilts mean weight of the foetuses at 112 days of pregnancy was significantly smaller than in gilts submitted to oviduct ligation (LIG) to decrease the number of embryos competing for uterine space. The increased competition between foetuses due to uterine crowding at the beginning of pregnancy in the UHO gilts led to a higher variation in placental and foetal weight at 112 days of pregnancy (Père et al. 1997).

It seems like there is an upper limit on the number of conceptuses that can be accommodated by the uterus, regardless of ovulation rate. Vonnahme et al. (2002) slaughtered sows (n = 244) at days 25, 36 and 44 of pregnancy and observed that there was a high positive correlation between ovulation rate (26.6 ± 0.4) and the number of vital conceptuses present

in the uterus at 25 days of pregnancy ($r = + 0.50$, $P < 0.001$), but this association was no longer significant on days 36 and 44 of pregnancy ($P > 0.05$). So, there was a decrease ($P < 0.05$) in conceptuses survival (number of vital conceptuses/ovulation rate) from 60.2% at 25 days of pregnancy, to 46.3% at 44 days of pregnancy. Moreover, the authors observed that placental weight was negatively correlated with the number of vital conceptuses in the uterus on days 25 ($r = -0.36$, $P < 0.005$), 36 ($r = -0.27$, $P < 0.05$) and 44 ($r = - 0.33$, $P < 0.01$) of pregnancy. Thus, due to the high OR and the high number of conceptuses reaching the uterus in this population of sows, uterine horn length and uterine space available per embryo seems to have restricted the number of conceptuses surviving up to term.

Town et al. (2004) used unilateral oviduct ligation (LIG) in 30 sows to reduce the number of embryos reaching the uterus and compared the conceptuses survival and development at 30 and 90 days of pregnancy with intact control (CTR) sows. The authors observed that LIG sows had less vital embryos in comparison with CTR sows (9.3 vs 15.1, $P < 0.01$) and also higher placental weight (26.2 vs 19.2 g, $P < 0.001$) at 30 days of pregnancy, but found no influence on the weight of the embryos ($P > 0.05$). Also, at 90 days of pregnancy placental weight was higher in the LIG group (274 vs 219 g, $P = 0.003$) and at this stage of pregnancy also the weight of the vital foetuses was higher (679 vs 588 g, $P = 0.002$). Moreover, average placental weight was negatively correlated with the number of vital embryos at 30 days of pregnancy ($r = - 0.61$; $P < 0.01$) and with the number of vital foetuses at 90 days of pregnancy ($r = -0.67$, $P < 0.01$) in both groups of sows. The authors concluded that day 30 embryos are less sensitive to nutrient limitations imposed by compromised placental development due to limited uterine capacity than foetuses in later pregnancy, and that early limitations in placental size at 30 days of pregnancy limit foetal development later in gestation. Similar results were described by Knight et al. (1977), whom showed that insufficient placental development between days 20 and 30 of pregnancy had significant negative influence on foetal growth and survival in a crowded uterine environment. Thus, limitations in UC at early pregnancy will compromise placental development and future foetal growth, which then decreases the piglets birth weight.

Freking et al. (2007) investigated conceptus survival and growth in gilts from genetic lines selected for a higher ovulation rate or for a higher uterine capacity during 11 generations. They observed that in comparison with unselected control lines, selection for OR increased OR by 3.2 ova, decreased UC by 0.97 pigs, decreased prenatal survival by 10.3% and increased litter size by 0.30 pigs only. On the other hand, gilts selected for UC increased OR by 1.3 ova, increased UC by 2.2 pigs, increased prenatal survival by 3.5% and increased litter size by 0.62 pigs. From these populations of gilts, ~500 gilts were selected and submitted to UHO, mated and slaughtered at different stages of pregnancy. The authors reported that gilts from the OR line had less uterine space available per embryo at implantation and due to uterine crowding they had lower average placental weight and higher foetal mortality. Gilts from the UC line, however, had more embryos with sufficient placental development and survived to and beyond 45 days of pregnancy. Despite the smallest number of foetuses at

45 days of pregnancy, gilts of the OR line had the lowest average foetal and placental weight. Thus, improvements of uterine capacity increases survival of foetuses to term.

Improvements of UC can be done through direct selection for litter size, litter birth weight or live piglets at day 5 after birth, however, direct measurements of UC are difficult to obtain. Young et al. (1996) reported that UC is genetically correlated with prepuberal uterine length and weight, and a multiple trait animal model that uses these measurements provides with the possibility to indirectly select for higher UC. However, measurements of uterine horn length and weight have to be done through the slaughter of the animals, which makes it unpractical. Recent research has been done trying to assess uterine and ovarian characteristics in prepuberal gilts (130 to 170 days of age) selected for UC with the use of transrectal ultrasonography, aiming to identify measurable characteristics related with uterine length or uterine weight, that could estimate gilts UC (Lents et al. 2014). The authors found that uterine diameter (measured as the diameter of the cross-section of the uterine horn) tended to be correlated with uterine horn weight ($r = 0.23$, $P = 0.08$). Moreover, although measurements of uterine diameter by ultrasound were not capable of identifying significant differences in UC in this gilts, the authors also found that ovarian weight after slaughter was correlated with uterine horn weight ($r = 0.40$, $P < 0.001$) and length ($r = 0.28$, $P < 0.05$), and it could be used as an measurement of UC. Thus, future investigations on the ovarian characteristics measured by ultrasonography and their relationships with UC are worthwhile.

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CHAPTER 3

Relationship between ovulation rate and embryonic and placental characteristics in multiparous sows at 35 days of pregnancy

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Abstract

The objective of this study was to investigate relationships between ovulation rate (OR) and embryonic and placental development in sows. Topigs Norsvin® sows (n = 91, parity 2 to17) from three different genetic backgrounds were slaughtered at 35 days of pregnancy and the reproductive tract was collected. The corpora lutea (CL) were counted and the number of vital and non-vital embryos, embryonic spacing (distance between two embryos), implantation length, placental length, placental weight and embryonic weight were assessed. The difference between number of CL and total number of embryos was considered as early embryonic mortality. The number of non-vital embryos was considered as late mortality. Relationships between OR and all other variables were investigated using two models: the first considered parity as class effect (n = 91) and the second used a subset of sows with parities 4-10 (n = 47) to analyse the genetic background as class effect. OR was significantly affected by parity ($P < 0.0001$), but was not affected by the genetic background of the sows. Parity and genetic background did not affect embryonic and placental characteristics at 35 days of pregnancy. OR (varying from 17 to 38 CL) was positively related with early embryonic mortality ($\beta = 0.49 \pm 0.1$ n/ovulations, $P < 0.0001$), with late embryonic mortality or number of non-vital embryos ($\beta = 0.24 \pm 0.1$ n/ovulations, $P = 0.001$) and with the number of vital embryos ($\beta = 0.26 \pm 0.1$ n/ovulations, $P = 0.01$). However, dividing OR in 4 classes, showed that the number of vital embryos was lowest in OR class 1 (17 to 21 CL), but not different for the other OR classes, suggesting a plateau for number of vital embryos for OR above 22. There was a negative linear relationship between OR and vital embryonic spacing ($\beta = -0.45 \pm 0.1$ cm/ovulation, $P = 0.001$), implantation length ($\beta = -0.35 \pm 0.1$ cm/ovulation, $P = 0.003$), placental length ($\beta = -0.38 \pm 0.2$ cm/ovulation, $P = 0.05$) and empty space around embryonic-placental unit ($\beta = -0.4 \pm 0.2$ cm/ovulation, $P = 0.02$), indicating uterine crowding. Further analyses showed that effects of OR on embryonic and uterine parameters were related with the increase in late mortality and not early embryonic mortality. Therefore, we conclude that a high OR results in an moderate increase in the number of vital embryos at day 35 of pregnancy, but compromises development in the surviving embryonic/placental units, suggesting that the future growth and survival of the embryos might be further compromised.

Keywords: ovulation rate, early embryonic mortality, late embryonic mortality, embryo development, placental development.

Implications

Selection for increased litter size in sows has also resulted in a disproportional increase in ovulation rate. The larger litter size has been associated with a lower birth weight and higher within litter variation in birth weight and consequently higher mortality rates during lactation, raising economic and welfare concerns. This paper shows that in multiparous sows, high ovulation rates results in an moderate increase in the number of viable embryos at day 35 of pregnancy, but compromise the placental development of the embryos surviving to day 35 and thus, may contribute to lower piglet birth weight.

Introduction

In pork production systems, an important aspect of profitability is a high number of piglets produced per sow per year. To accomplish this, pig breeding programs have been focusing on components such as litter size and pre-weaning mortality (Johnson et al. 1999). Selection for litter size has been shown to disproportionally increase ovulation rate (OR) and prenatal mortality (Johnson et al. 1999; van der Waaij et al. 2010; Vallet et al. 2014) and OR of 25 to 30 are relatively common nowadays (Patterson et al. 2008; Wientjes et al. 2013). The increase in prenatal mortality with an increase in OR seems due to both an increase in pre-implantation and post-implantation mortality (van der Waaij et al. 2010). Pre-implantation mortality has been associated with embryonic heterogeneity within a litter; less developed embryos cannot develop further in a uterine environment that is advanced by the more developed embryos (Pope et al. 1990). As embryonic heterogeneity has been largely attributed to follicle heterogeneity (Pope et al. 1990), possibly sows with a high OR have a more heterogeneous follicle pool. Post-implantation mortality related with a high ovulation rate seems due to effects of intra-uterine crowding and associated competition for space and/or nutrients, both early post-implantation (Geisert and Schmitt 2002) and throughout the remainder of pregnancy (Foxcroft et al. 2007). Several authors have found a relationship between placental weight and foetal weight at the end of gestation, indicating that foetal development is dependent on placental size (van der Lende 1989; Freking et al. 2007) and Père et al. (1997) found that a high number of embryos at day 35 of pregnancy not only resulted in a higher foetal loss, but also resulted in a lower placental weight and foetal weight of the surviving embryos at 112 days of pregnancy. Thus, in a crowded uterus, placental growth is compromised, which subsequently limits foetal development. This intra-uterine crowding is apparent in high prolific sows as shown by the lower average piglet birth weight and increased variation in piglet birth weight in sows with a high litter size (Milligan et al. 2002; Quesnel et al. 2008). Therefore, if selection for litter size results in a substantial increase in ovulation rate, the associated high numbers of embryos in early gestation may negatively impact the growth of the surviving embryos and thereby result in a low piglet birth weight. As piglet birth weight and birth weight variation are important characteristics for piglet survival and further development (Milligan et al. 2002), a further insight in

relationships between OR and foetal development and survival is warranted. Therefore, the aim of this experiment was to investigate the relationships between OR and embryonic mortality and embryonic and placental characteristics of multiparous sows at 35 days of pregnancy, to better understand mechanisms that lead to prenatal losses and reduced piglet birth weight in highly prolific sows.

Material and methods

Animals

Multiparous sows (parity 2 to 17, n = 91), from one commercial farm and three different genetic backgrounds (sire line cross, n = 46; purebred Landrace, n = 17 and crossbred Yorkshire x Landrace, n = 28; Topigs Norsvin, Vught, The Netherlands), were used.

Measurements

Sows were slaughtered at a local abattoir at day 35.0 ± 0.1 (mean ± SEM) of pregnancy and the uterus and ovaries of each sow were collected. Ovulation rate was assessed by counting the number of corpora lutea on both ovaries. Both uterine horns were separated from the mesometrium and opened at the anti-mesometrial side. After opening the uterus, embryos were separated from their placentas and counted. Embryos were classified as vital or non-vital according to their visual appearance and were considered non-vital when there was a presence of haemolysed amniotic fluid, resorbed embryonic membranes, or both (van der Waaij et al., 2010); and when there was evidence of implantation, combined with placental or embryonic remnants. The difference between OR and total number of embryos was considered as early embryonic mortality. The number of non-vital embryos was considered as late embryonic mortality.

The embryonic-placental units were separated from the uterine wall and all vital embryos and their placentas were individually weighed. In addition, placental length between the necrotic tips of the placenta was measured on a wet surface and also the length of the uterine horns was measured on a wet surface, from the utero-tubal junction to the uterine body. The length of each implantation site on the uterine wall was measured. Implantation sites were recognized by the reddening of the endometrium, compared to the whiter area (‘unoccupied space’) in between. The middle of the implantation site was considered to be the embryonic position within the uterine horn. Embryonic spacing was determined as the distance between two embryonic positions. For the first embryo at the ovarian end, embryonic spacing was defined as the distance from the embryonic position to the utero-tubal junction. For the first embryo at the cervical end, embryonic spacing was defined as the length from embryonic position to the cervix. Furthermore, the length of the whiter, ‘unoccupied’, areas of the uterine wall on both sides of a embryonic-placental unit site were defined as the ‘empty space’ surrounding that embryo.

Statistical Analyses

To analyse parity effects on several embryonic, placental and uterine characteristics, parity was divided into 3 categories: class 1 (parities 2 and 3, n = 25), class 2 (parities 4 to 10, n = 47) and class 3 (parities 11 to 17, n = 19). Parity and genetic background were confounded as all second, third and fourth parity sows were from the same genetic background (sire line cross). Therefore, the effect of parity and genetic backgrounds could not be assessed simultaneously in the same model. To analyse effects of genetic background, only sows from parity class 2 (parities 4 to 10, n = 47) were used. The fixed class effects of parity or genetic background on OR were each assessed using PROC GLM in SAS 9.3 (Proc. GLM, SAS Inst. Inc., Cary, NC). Subsequently, to assess effects of parity or genetic background and ovulation rate on embryonic, placental and uterine characteristics, the fixed class effects of parity or genetic background and the fixed continuous effect of OR, and their interaction were assessed. The analysed characteristics were: embryo numbers (total, vital, non-vital, early embryonic mortality), average and standard deviation of vital embryonic and placental characteristics of the vital embryos (embryonic weight, embryonic spacing, implantation length, placental weight and length and empty spaces around each implantation site), and total uterine length. All characteristics were normally distributed, based on skewness and kurtosis analyses of variables and model residuals. Preliminary analyses demonstrated that the interaction between parity class and OR, and the interaction between genetic background and OR were never significant. Therefore, these interaction effects were excluded from the models. Additionally, to check the linearity of the OR effects in the earlier models, OR was analysed as class variable [class 1 (range 17 to 21, n = 20), class 2 (range 22 to 24, n = 23), class 3 (range 25 to 28, n = 24) and class 4 (range 29 to 38, n = 24)], The interaction between OR classes and parity classes were never significant and were therefore excluded from the model.

To study if the effects on embryonic and placental characteristics were related to early or late embryonic mortality, early and late mortality were classified into 4 classes. For early embryonic mortality these classes were: class 1 (range -4 to 1, n = 18), class 2 (range 2 to 3, n = 21), class 3 (4 to 6, n = 24) and class 4 (7 to 25, n = 28) and for late embryonic mortality: class 1 (range 0 to 1, n = 22), class 2 (range 2 to 3, n = 27), class 3 (range 4 to 5, n = 24) and class 4 (range 6 to 15, n = 18). Early or late embryonic mortality class were included in a model together with the fixed class effect of parity and their interaction, and analysed in relation to embryonic spacing, implantation length, vital placental length and the empty spaces around embryos. Preliminary analyses demonstrated that the interaction between early embryonic mortality or late embryonic mortality classes with parity class were never significant. Therefore, the interactions were excluded from the models. Results are presented as LSMeans ± SEM, and are considered significant at $P \leq 0.05$.

Results

Descriptive statistics

Averages and standard deviations of the measured variables are presented in Table 3.1. Average OR was 25.5 ± 5.0. The average total number of embryos was 20.1 ± 5.2 and that of vital embryos was 16.4 ± 3.9. The average number of non-vital embryos was 3.7 ± 3.0. Average implantation length was 19.2 ± 4.8 cm and the space between two embryos, or embryonic spacing, was 25.0 ± 6.0 cm. The empty uterine space around each embryonic-placental unit was 15.4 ± 7.1 cm and average placental length was 44.0 ± 8.0 cm.

Parity and genetic background effects on OR

Ovulation rate was lower in parity class 1 than in both other parity classes ($P < 0.001$) and parity class 2 had a lower OR than parity class 3 ($P = 0.05$, Table 3.2). Ovulation rate was not affected by genetic background of the sows ($P = 0.93$; Table 3.3).

Effects of parity and OR on embryonic and placental characteristics

Parity class did not significantly affect any of the embryonic, placental or uterine characteristics when OR was included in the statistical model (Table 3.2). An increase in OR was significantly associated with an increase in number of vital embryos

Table 3.1 Summary statistics for vital embryonic, placental and uterine variables from sows at 35 days of pregnancy.

Variables	n	Mean	SD	CV%	Min	Max
Sows						
Ovulation Rate	91	25.5	5.0	19.8	17	38
Number of embryos	91	20.1	5.2	25.9	9	33
Number of vital embryos	91	16.4	3.9	24.4	8	29
Number of non-vital embryos	91	3.7	3.0	81.1	0	15
Early embryonic mortality[1]	91	5.4	5.4	100.0	-4.0	25.0
Uterine length (cm)	91	529.0	88.6	16.7	318.0	761.0
Average						
Embryo Weight (g)	91	4.9	1.0	20.4	3.1	8.0
Embryo Spacing (cm)[2]	91	25.0	6.0	24.0	12.2	36.9
Implantation Length (cm)	91	19.2	4.8	25.0	8.6	31.4
Placental Length (cm)	91	44.0	8.0	18.2	25.0	67.4
Placental Weight (g)	91	46.0	11.0	23.9	19.2	76.7
Empty Space (cm)[3]	91	15.4	7.1	46.0	5.6	48.1
Standard Deviations						
Embryo Weight (g)	91	0.55	0.20	36.53	0.22	1.41
Embryo Spacing (cm)[2]	91	8.4	3.1	36.9	3.4	20.3
Implantation Length (cm)	91	7.2	2.2	30.4	3.3	16.1
Placental Length (cm)	91	10.7	3.0	28.0	5.0	19.0
Placental Weight (g)	91	14.5	4.2	29.0	6.4	24.6
Empty Space (cm)[3]	91	9.0	6.0	66.7	2.3	27.5

[1] Number of corpora lutea that do not account for an embryo.

[2] Distance between two embryos.

[3] Total empty uterine space around each vital embryo-placental unit.

Table 3.2 Effect of parity class and the ovulation rate (OR) on vital embryonic, placental and uterine characteristics in sows at 35 days of pregnancy.

Variables	Parity class (Model 1)			OR	SEM	P values	
	2 to 3	4 to 10	11 to 17	β		OR	Parity
n	25	47	19				
OR (SEM)[1]	21.8 (0.88)	26.0 (0.64)	28.9 (1.01)				<.0001
Number of embryos	20.1	20.9	18.0	0.51	0.11	<.0001	0.09
Vital embryos	16.4	17.0	15.1	0.26	0.09	0.01	0.20
Non-vital embryos	3.8	3.9	2.9	0.24	0.07	0.001	0.48
Early embryonic mortality[2]	5.3	4.6	7.4	0.49	0.11	<.0001	0.09
Uterine length (cm)	538.1	543.1	482.1	2.15	2.10	0.031	0.05
Averages							
Embryo Weight (g)	5.2	4.8	4.6	-0.02	0.02	0.42	0.14
Embryo Spacing (cm)[3]	24.3	24.6	26.0	-0.45	0.14	0.0013	0.63
Implantation Length (cm)	18.9	19.3	19.6	-0.35	0.11	0.003	0.92
Placental Length (cm)	42.6	43.4	46.8	-0.38	0.19	0.05	0.25
Placental Weight (g)	49.3	44.7	44.6	-0.27	0.26	0.30	0.26
Empty Space (cm)[4]	15.1	14.7	17.5	-0.40	0.17	0.02	0.37
Standard Deviations							
Embryo Weight (g)	0.54	0.57	0.53	0.0002	0.005	0.96	0.73
Embryo Spacing (cm)[3]	8.4	8.1	9.1	-0.15	0.07	0.05	0.49
Implantation Length (cm)	7.4	7.5	6.4	-0.05	0.05	0.34	0.21
Placental Length (cm)	10.9	10.3	11.4	0.02	0.07	0.81	0.44
Placental Weight (g)	15.7	13.9	14.5	0.15	0.10	0.14	0.27
Empty Space (cm)[4]	8.4	8.9	9.9	-0.25	0.14	0.08	0.76

[1] Effect of parity classes on ovulation rate (OR).

[2] Number of corpora lutea that do not account for an embryo.

[3] Distance between two embryos.

[4] Total empty uterine space around each vital embryo-placental unit.

(β = 0.26 ± 0.09 n/ovulation, P = 0.01), non-vital embryos (β = 0.24 ± 0.07 n/ovulation, P = 0.001) and early embryonic mortality rate (β = 0.49 ± 0.11 n/ovulation, P < 0.0001), as presented in Table 3.2. Figure 3.1 confirms the linearity of the relationships between OR class and number of vital embryos (Figure 3.1A), non-vital embryos or late embryonic mortality (Figure 3.1B) and early embryonic mortality (Figure 3.1C). The within-class relationships between OR and number of vital embryos were 1.24 ± 0.5 n/ovulation (P = 0.02), 0.10 ± 0.4 n/ovulation (P = 0.8), -0.15 ± 0.3 n/ovulation (P = 0.6) and 0.00 ± 0.1 n/ovulation (P = 0.9), for OR classes 1, 2, 3 and 4, respectively, indicating a relatively weak relationship between OR and number of vital embryos above an OR of 22.

An increase in OR was linearly associated with a decrease in vital embryonic spacing (β = -0.45 ± 0.14 cm/ovulation; P = 0.001), vital implantation length (β = -0.35 ± 0.11 cm/ovulation; P = 0.003), vital placental length (β = -0.38 ± 0.19 cm/ovulation; P = 0.05) and in the empty space around the implantation sites of the vital embryos (β = -0.40 cm/ovulation, P = 0.02). However, there was no relationship between OR and average embryonic weight (P = 0.42) or embryonic weight standard deviation (P = 0.96) at day 35 of pregnancy. Figures 3.2A, D, G and J confirms the linearity of the OR relations with vital embryonic spacing,

Table 3.3 Effect of genetic background and the ovulation rate (OR) on embryonic, placental and uterine characteristics in sows at 35 days of pregnancy.

Variables	Genetic Background (Model 2)			OR	SEM	P values	
	Sire Line	Landrace	Y*L[1]	β		OR	GB
n	20	10	17				
OR (SEM)[2]	25.8 (1.07)	26.4 (1.51)	26.1 (1.16)				0.93
Number of embryos	21.0	22.7	20.5	0.58	0.15	0.001	0.52
Vital embryos	16.7	19.0	16.6	0.29	0.14	0.05	0.34
Non-vital embryos	4.3	3.7	3.9	0.29	0.10	0.004	0.84
Early embryonic mortality[3]	5.0	3.3	5.5	0.42	0.15	0.01	0.52
Uterine length (cm)	536.0	528.9	563.2	2.19	2.77	0.43	0.51
Embryo Weight (g)	4.8	4.7	4.9	-0.03	0.03	0.36	0.85
Embryo Spacing (cm)[4]	24.3	21.8	25.9	-0.44	0.16	0.01	0.16
Implantation Length (cm)	18.7	17.9	20.1	-0.36	0.13	0.01	0.39
Placental Length (cm)	42.1	43.6	44.2	-0.39	0.25	0.12	0.72
Placental Weight (g)	44.7	44.5	44.5	-0.49	0.35	0.17	0.99
Empty Space (cm)[5]	15.3	10.9	15.7	-0.34	0.20	0.09	0.13
Embryo Weight (g)	0.59	0.55	0.55	-0.001	0.01	0.83	0.81
Embryo Spacing (cm)[4]	8.8	6.6	7.9	-0.20	0.08	0.01	0.08
Implantation Length (cm)	7.8	6.7	7.5	-0.08	0.08	0.28	0.54
Placental Length (cm)	10.1	11.2	10.2	0.03	0.09	0.75	0.62
Placental Weight (g)	13.7	14.4	14.0	0.09	0.13	0.51	0.92
Empty Space (cm)[5]	9.4	5.9	9.6	-0.34	0.18	0.06	0.21

[1] Yorkshire * Landrace sows.

[2] Effect of genetic background classes on ovulation rate.

[3] Number of corpora lutea that do not account for an embryo.

[4] Distance between two vital embryos.

[5] Total empty uterine space around each vital embryo-placental unit.

vital implantation length, vital placental length and empty uterine space around each vital embryo.

Effects of genetic background and OR on embryonic and placental characteristics

For sows with parity 4 to 10, genetic background did not significantly affect any of the measured embryonic, placental or uterine characteristics, when OR was included in the statistical analyses (Table 3.3). This limited dataset, therefore, shows similar relationships between OR and the number of vital embryos, non-vital embryos, total number of embryos and early embryonic mortality as in the model with parity class effect. In addition, a comparable negative relationship between the OR and average implantation length, average embryonic spacing and embryonic spacing standard deviation were found.

Relationships between early and late embryonic mortality with vital embryonic and placental characteristics

A higher incidence of early embryonic mortality tended to increase vital embryonic space (Figure 3.2B) and vital implantation length (Figure 3.2H) and increased vital placental length (Figure 3.2K) and empty uterine space around the vital embryos (Figure 3.2E). Figure 3.2

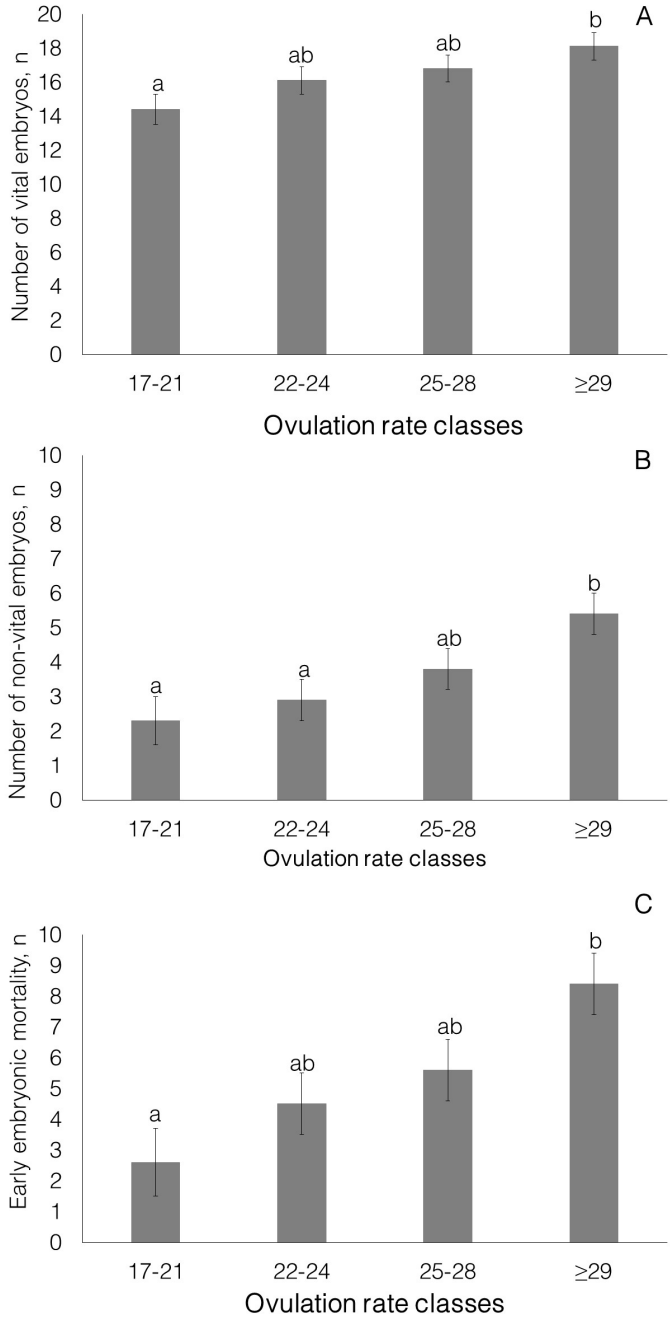

Figure 3.1 Estimated least square means for the effect of ovulation rate (OR) classes [number of corpora lutea, (class 1, range 17 to 21, n = 20; class 2, range 22 to 24, n = 23, class 3, range 25 to 28, n = 24 and class 4, range 29 to 38, n = 24)] on number of vital embryos (panel a; P = 0.05); number of non-vital embryos (panel b; P = 0.01) and on early embryonic mortality incidence (panel c; P = 0.01) at 35 days of pregnancy. Significant differences between classes are indicated by letters above the columns and the error bars indicated a single SE of the estimates.

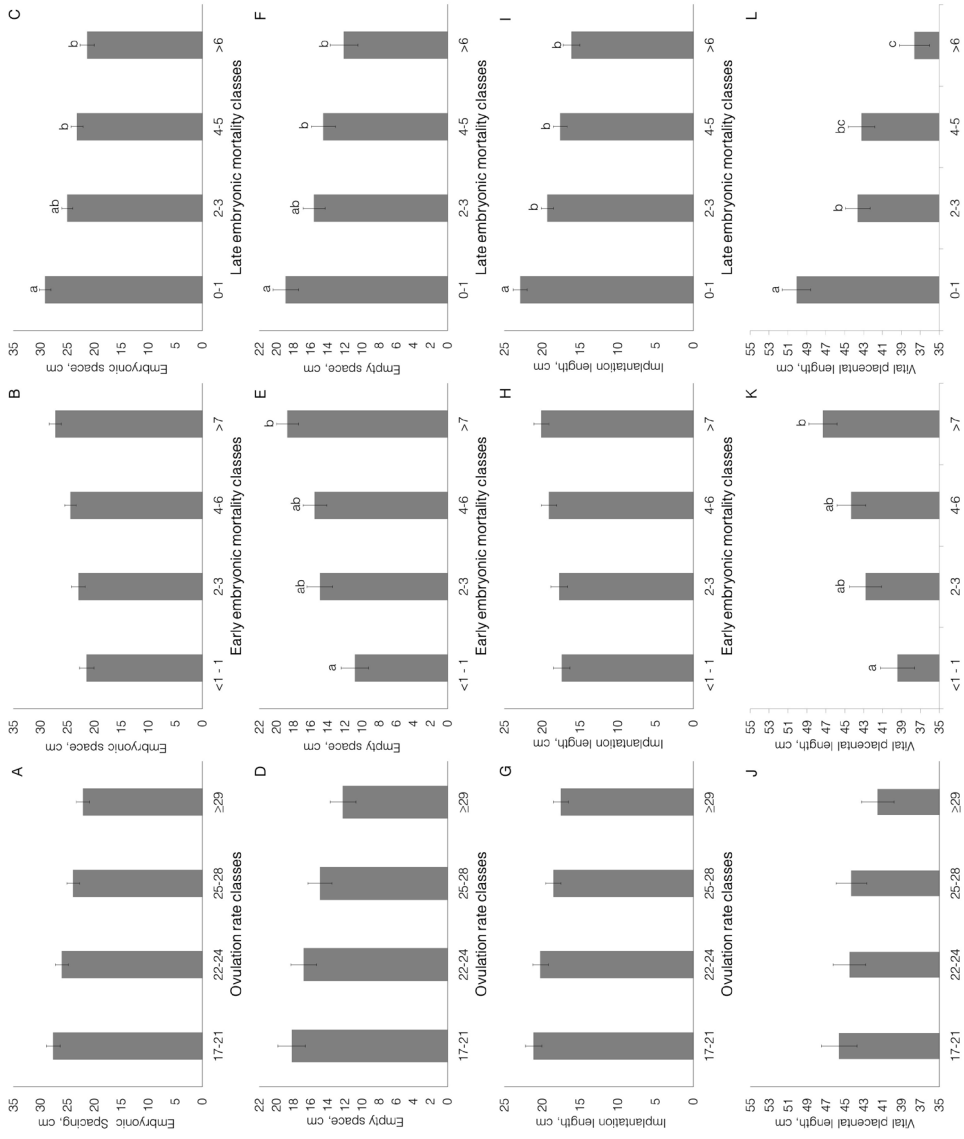

Figure 3.2 Estimated least square means for the effect of ovulation rate (OR) classes (number of corpora lutea; panels A, D, G and J), early embryonic mortality (EM) classes (corpora lutea that do not account for an embryo, panels B, E, H and K) and late embryonic mortality (LM) classes (evidence of implantation, combined with placental or embryonic remnants or both; panels C, F, I and L) on embryonic spacing (OR, $P = 0.003$; EM, $P = 0.12$; LM, $P < 0.0001$) and empty space around embryonic-placental units (OR, $P = 0.06$; EM, $P = 0.003$; LM, $P = 0.02$), on implantation length (OR, $P = 0.12$; EM, $P = 0.08$; LM, $P < 0.0001$) and on vital placental length (OR, $P = 0.43$; EM, $P = 0.01$; LM, $P < 0.0001$) at day 35 of pregnancy for the vital embryos. Significant differences between classes are indicated by letters above the columns and the error bars indicate a single SE of the estimates.

also shows that a higher incidence of late embryonic mortality was related with a decrease in these four parameters (Figures 3.2C, 3.2F, 3.2I and 3.2L).

Discussion

This study investigated relationships between ovulation rate (OR) and embryonic and placental characteristics at 35 days of pregnancy, aiming to clarify consequences at early pregnancy for litter characteristics at term. The results show that a higher OR in sows is related with a considerable increase in early and late embryonic mortality and only a moderate increase in number of vital embryos at day 35. Increased OR also result in a compromised placental development in the vital embryos at day 35. The latter may cause reduced further growth and increased foetal mortality in later stages of pregnancy.

In the multiparous sows in this study, with OR varying between 17 and 38, each extra ovulation represented an increase in the incidence of early embryonic mortality of 0.49. Many factors can explain this. First, one should consider that it might be related with fertilization failure. Fertilization rates are normally considered to be approximately 95% (Geisert and Schmitt 2002), however, sows with a higher OR might have an earlier and higher rise in progesterone, which would affect sperm transport to the site of fertilisation and thereby reduce fertilization rate, and/or would induce early embryo mortality (Day and Polge 1968; Mao and Foxcroft 1998; Soede et al. 2012). The higher OR might also result in a higher early embryonic mortality related with a higher embryo diversity. This higher diversity might arise from an increase in follicular and oocyte diversity (Pope et al. 1990) or from a prolonged variation in ovulation time between oocytes, that might not only affect fertilisation rate, but also the time of fertilisation, contributing to embryo diversity (Soede and Kemp 1993). Embryo diversity results in peri-implantation losses as the oestrogen production of the more developed embryos stimulate uterine secretions to their own benefit but creates an hostile environment for the less developed embryos (Pope 1992; Geisert and Schmitt 2002), compromising their survival chances. Therefore, considering the relation between OR and early embryonic mortality, increased follicular heterogeneity and/or embryo diversity might account for part of the observed increase in the early embryonic mortality.

Besides an increase in early embryonic mortality, the current study also showed an increase in late embryonic mortality with an increase in OR of 0.24 non-vital embryos per extra ovulation at day 35 of pregnancy. The category of non-vital embryos included the sites in the uterus with evidence of implantation but without a vital embryo, so also sites with only placental remnants. It thereby gives an estimate of post-implantation mortality up to day 35 of pregnancy. This mortality presumably is related with competition for uterine space by the embryos. Around days 10 and 14 of pregnancy, embryos elongate and start to attach to the uterine wall. Further developed embryos will elongate quicker and get a larger implantation site (Bazer et al. 2009), which will provide an increased placental surface area (Stroband and van der Lende 1990). Foxcroft et al. (2000) showed that substantial embryonic losses occur between day 26 and 44 of pregnancy. Although placental attachment occurs around

day 13 to 17, the placenta is not functionally complete until day 35 of pregnancy (van der Lende et al. 2000; Geisert and Schmitt 2002). In the course of pregnancy, embryos become more dependent of their placenta for further growth, and placental functionality can therefore limit embryonic development and survival (Vallet et al. 2014). Thus, embryos that, due to the competition for space, have acquired a smaller implantation site and associated smaller placenta, might die during pregnancy in case of insufficient placental supply of nutrients. This process already takes place before day 35 of pregnancy, not only in animals with a very high OR [(van der Waaij et al. 2010); superovulation, 45 ovulations], but also in animals with a normal OR [(Langendijk et al. 2012); 20 ovulations]. In addition, Vonnahme et al. (2002) found a correlation between OR (average 26.6) and number of viable conceptuses at day 25 of pregnancy ($r = 0.50$; $P < 0.0001$), but not at day 36 of pregnancy ($r = 0.02$; $P = 0.98$) due to a further loss of embryos in sows with a high OR. Further, van der Waaij et al. (2010) also found a smaller implantation site and lighter and a shorter placenta in non-vital foetuses, which indeed suggests that limited uterine space was the cause of this mortality. In this study, the number of vital embryos at day 35 of pregnancy linearly increased by 0.26 with each extra ovulation, meaning that to achieve one more vital embryo at this stage at least four ovulations are needed. Further analyses –dividing sows in four classes of OR- showed hardly any increase in the number of vital embryos in sows in the OR classes above 21. Therefore, at this stage of pregnancy, the number of vital embryos seems to reach a plateau at about 17 embryos. At 40 days of pregnancy, van der Waaij et al. (2010) also found a plateau of about 17 vital foetuses in gilts with OR varying between 20 and 50. Therefore, a further increase in OR does not result in higher embryo numbers at this early foetal stage of pregnancy. However, the increase in OR affected the development of the surviving embryonic-placental units at day 35 of pregnancy. The increase in OR was not related with a reduction in embryonic weight, but was related with a reduction in placental length and also with a reduction in embryonic spacing, in implantation length and in the empty spaces around each vital embryonic-placental unit. These results suggest that the development and survival of these vital embryos might become compromised in the remainder of pregnancy due to insufficient uterine space and reduced placental development (van der Lende et al. 2000).

The observed vital embryonic and placental development at 35 days of pregnancy was found to be related to the incidence of early and of late embryonic mortality. We observed that sows with a high level of early embryonic mortality had a longer vital placental length and larger empty spaces around each embryonic-placental unit. This could indicate that sows that have a high OR followed by a high level of early embryonic mortality, provides the surviving embryos with more space and therefore a better opportunity to grow, as has also been shown by van der Lende (1989). We also observed that sows with a high level of late mortality had less foetal spacing and smaller empty spaces around each embryonic-placental unit, they had less implantation length and a shorter vital placenta length. This suggests that the increased late embryonic mortality is already a consequence of uterine crowding, in which the lack of space also compromises placental development of the –as yet- vital embryos. Such a

decreased placental weight due to intra-uterine overcrowding was observed by Town et al. (2004) already at 30 days of pregnancy. Further, as the recently died embryos still occupy space that cannot or can hardly be used by the surviving embryos (Vallet et al. 2011), late embryonic mortality can also be seen as a cause, and not only a consequence, of the crowding conditions faced by the surviving embryos. Like in our study, van der Waaij et al. (2010) observed that embryonic-placental development and implantation length of vital embryos at day 40 of pregnancy was more related to late embryonic mortality than to early embryonic mortality. In contrast to our study, van der Waaij et al. (2010) also found a lower embryonic weight in gilts with a high level of late embryonic mortality. This difference may be related with the very high OR and associated levels of uterine crowding in the study of van der Waaij et al. (2010) (45 vs 25 CL in our study and 11.2. non-vital embryos vs 3.7 in our study). It could also be related with the somewhat later evaluation time (day 40 vs day 35 in our study). The present study did not show relationships between OR and embryonic uniformity of the vital embryos at 35 days of pregnancy. However, as discussed before, a higher embryonic diversity in early pregnancy might be related with a higher mortality at earlier stages of pregnancy, reducing the impact of diversity on the surviving embryos. However, in sows with a high ovulation rate and low levels of early embryonic mortality, the compromised placental development in surviving embryos might increase the variation in foetal weight at a later stage of pregnancy, resulting in lower uniformity in large litters (Quesnel et al. 2008). In conclusion, higher OR especially increased early embryonic mortality and to a lesser extent late embryonic mortality. The resulting number of vital embryos at day 35 of pregnancy showed an increase with a higher OR, however, this increase seems to reach a plateau of ~ 17 embryos at an OR of 22. This seems to confirm the findings of Johnson et al. (1999), who found low correlated responses between an increase in ovulation rate and subsequent litter size. Further, at high OR, vital embryos had a reduced uterine space and placental development, which might cause growth retardation and increased mortality in later stages of pregnancy and thereby affect piglet birth weight. Research is needed in later stages of pregnancy to confirm such relationships between OR and foetal development and survival. On the other hand, ovarian ultrasound during pregnancy may become a reliable technique to assess ovulation rate (Gonzalez-Añover et al. 2009) and relate the findings to litter characteristics at term.

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CHAPTER 4

Relationship between ovulation rate and embryonic characteristics in gilts at 35 days of pregnancy

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Abstract

This study investigated the relationship between ovulation rate (OR) and embryonic characteristics in gilts. Landrace (n = 86) and Yorkshire x Landrace (n = 212) gilts were inseminated with semen stored for 3 to 5 days (SS1, n = 59), 6 to7 days (SS2, n = 133), or 8 to 10 days (SS3, n = 106), and slaughtered at 35 days of pregnancy. Ovulation rate was assessed by dissection of the corpora lutea on both ovaries. Embryos were classified as vital (VE) by visual appearance and individually weighed (VEg), and the SD of the weight calculated (SDVEg). Early embryonic mortality (EM) was estimated as the difference between OR and the number of vital plus nonvital embryos. Embryonic characteristics were analysed with a model that included linear and quadratic terms of OR, and fixed class effects of semen storage duration (SS) and genetic line (GL). Landrace gilts had a higher OR than Yorkshire x Landrace gilts (22.1 ± 0.4 vs 20.3 ± 0.2, $P \leq 0.05$) and also a higher EM (6.1 ± 0.4 vs 3.5 ± 0.3, $P \leq 0.05$). EM was also higher in gilts inseminated with semen stored for more than 8 d. Also, Yorkshire x Landrace gilts had a higher number of VE (16.9 ± 0.7) than the Landrace gilts (13.3 ± 0.8) when inseminations were done with semen stored for up to 5 d. Yorkshire x Landrace gilts had the highest VEg when inseminated with semen stored for 3 to 5 days (SS1: 4.9 ± 0.2 g, SS2: 4.1 ± 0.1 g and SS3: 4.0 ± 0.2 g; $P \leq 0.05$). VE and VEg did not differ within Landrace gilts between different SS classes. A quadratic relationship of OR ($P \leq 0.05$) was found with VE: a maximum of 16.8 VE was observed at 26 ovulations [(2.5 (± 0.6)*OR - 0.05 (± 0.01)*OR^2]. A quadratic relationship of OR ($P \leq 0.05$) was also found for EM: a minimum of 3.33 EM was observed at 15 ovulations [(-1.1 (± 0.6)*OR -0.03 (± 0.01)*OR^2]. VEg was not related with OR, but SDVEg had a positive linear relationship with OR [0.01 (± 0.003)*OR, $P \leq 0.05$]. Results show that Yorkshire x Landrace gilts perform better than Landrace when inseminated with fresh semen, but not with semen stored for longer time. Also, the VE increases with an increase in OR up to 26, but at a lower level at higher OR, which is likely related with the increase in EM. The higher EM at higher OR might arise from a higher variation in follicular/oocyte quality leading to a higher variation in embryonic quality and development, increasing mortality before uterine implantation and the variation in embryonic weight already at 35 days of pregnancy.

Key words: embryonic mortality, embryonic weight, gilts, ovulation rate.

Introduction

A high ovulation rate (OR) and high litter size are characteristics of modern sows (Town et al. 2004). However, OR reaches higher values than litter size, with averages above 25 described in literature (Patterson et al. 2008; Wientjes et al. 2013). This discrepancy between OR and litter size in multiparous sows is mainly due to an increase in early embryonic mortality, i.e. mortality before uterine implantation at 13 days of pregnancy (Da Silva et al. 2016). The increase in early embryonic mortality with the increase in OR is probably due to an increase in heterogeneity in embryonic development originated from an increase in heterogeneity in the pool of ovulatory follicles and oocytes in sows with more ovulations (Geisert et al. 1982; Pope et al, 1990), leading to a higher number of embryos with suboptimal development capacity. An increase in OR was also related with a decrease in implantation length and placental length of the vital embryos in multiparous sows at 35 days of pregnancy (Da Silva et al. 2016), indicating uterine crowding already at this stage of pregnancy. This might lead to a higher fetal mortality, but might also compromise fetal growth, leading to lower piglet birth weight and birth weight uniformity, both associated with a higher piglet mortality after birth (Milligan et al. 2002). Gilts, however, have a lower average OR than multiparous sows (Belstra 2003), which could imply different relationships between OR and embryonic characteristics during pregnancy compared to sows. Thus, gilts from two different genetic lines and inseminated with semen stored for different duration were slaughtered at 35 days of pregnancy and we investigated the effects of genetic line and semen storage duration on ovulation rate and embryonic characteristics, and the relationship between OR and embryonic mortality and development in early pregnancy, aiming to understand the mechanisms that lead to litter characteristics at birth.

Material and methods

The experiment and all measurements were approved by the Animal Welfare Committee of Wageningen University and Research in compliance with the Dutch Law on Animal Experimentation. The experiment was conducted between May and August 2016 at Wageningen University and Research (Wageningen, The Netherlands).

Animals and housing

The study included a total of 298 pregnant gilts, from 1 farm, being 212 crossbred **(C)** gilts (Yorkshire x Landrace; Topigs Norsvin, Vught, The Netherlands) and 86 purebred **(P)** Landrace gilts (Topigs Norsvin, Vught, The Netherlands), which were used in 14 batches, 1 batch per week.

The gilts were group housed (6 animals per 8 m²), with individual feeding stations and received liquid feeding. From weaning at day 25 till day 49 of age the gilts were fed a starter diet (9.68 NE MJ/kg, 9.13 g/kg of ileal digestible lysine), from day 50 up to day 105 gilts were fed a rearing diet (9.42 NE MJ/kg, 8.03 g/kg of ileal digestible lysine), and from

day 106 until the first insemination at 255 ± 12 days (mean ± SD, ranging from 231 to 292 days) the gilts were fed a second rearing diet (9.24 NE MJ/kg, 7.35 g/kg of ileal digestible lysine). During the first 70 days the gilts were fed three times a day and from 71 days onwards the gilts were fed twice a day. Gilts had free access to water at all times.

Gilts were inseminated at 255.2 ± 11.7 days (ranging from 231 to 292), 1 or 2 times with semen stored for 3 to 5 days (SS1, n = 73), 6 to 7 days (SS2, n = 156) or 8 to 10 days (SS3, n = 143). The semen was collected from 9 boars from the Tempo breeding line (Topigs Norsvin, Vught, The Netherlands). The Tempo boar is bred from a Topigs Norsvin E-line (Large White type). Semen was processed at 1 Specific Pathogen Free (SPF) artificial insemination station and homospermic (i.e. not pooled) insemination doses of 1.2 billion cells per 80 mL were produced (Varkens KI Nederland, Vught, the Netherlands). The boars used in the inseminations did not affect the embryonic characteristics of the gilts measured at 35 days of pregnancy ($P > 0.05$) and therefore were not included in the statistical models. Semen was stored and transported to the farm at 17°C ± 2°C.

The pregnant gilts were slaughtered at 34.9 ± 0.9 days of pregnancy. Pregnancy rate did not differ between semen storage duration classes (80.8 %, 85.3 % and 74.1 %, for SS1, SS2 and SS3, respectively). The weight at first insemination for the P and C gilts was 168.2 ± 2.1 kg vs 159.2 ± 1.6 kg, respectively ($P \leq 0.05$), and average back fat thickness, measured by ultrasound at 6 to 8 mm away from body midline at the last rib level, was 14 ± 2 mm for P and C gilts. Gilts were slaughtered with an average weight of 184 ± 14 kg.

Ovarian, embryonic and uterine measurements

After slaughter, uterus and ovaries of the gilts were collected. Ovulation rate (OR) was assessed by dissection of each individual corpus luteum (CL) present on left and right ovaries. After dissection, individual corpus luteum were cleaned of remaining connective tissue and individually weighed to assess average and standard deviation of individual corpus luteum weight (g). Total luteal mass was calculated as the sum of all corpora lutea weights.

Both uterine horns were separated from the mesometrium and opened at the anti-mesometrial side. After opening the uterus, embryos were classified as vital, non-vital or degenerated according to their visual appearance and were considered as nonvital when there was haemolysed amniotic fluid, and degenerated when there were resorbed embryonic membranes or evidence of implantation, combined or not with placental or embryonic remnants (van der Waaij et al. 2010). After classification, embryos were separated from their placentas and counted. The total number of embryos was calculated as the sum of the vital embryos, nonvital embryos and of the degenerated embryos. The difference between OR and the total number of embryos was considered as early embryonic mortality, and the nonvital plus the degenerated embryos were considered as late embryonic mortality.

The embryonic-placental units were separated from the uterine wall. After removal of the embryonic-placental units, implantation sites were recognized by the reddening of the endometrium, compared to the whiter area (unoccupied/empty uterine space) in between. The length and width of each implantation site on the uterine wall containing a vital embryo was

measured and vital implantation area was calculated as the product of implantation length and implantation width. The average length of the whiter areas of the uterine wall on each side of a vital embryo implantation site was defined as vital empty space. The length of the left and right uterine horns were measured in wet surface, from the utero-tubal junction to the uterine body. Total uterine length (cm) was measured as the sum of the left and right uterine horn length. In 161 of 212 gilts (batches 7 to 14), all vital embryos were individually weighed for assessment of average and standard deviation of vital embryonic weight (g).

Statistical analyses

Analyses on ovulation rate (OR) and luteal, embryonic and uterine characteristics were performed using PROC MIXED in SAS 9.3 (SAS Inst. Inc. Cary, NC). In all models, batch was included as a random class effect to account for possible environmental variation. Fixed class effects consisted of genetic line [crossbred Yorkshire x Landrace gilts (C, n = 212) and purebred Landrace gilts (P, n = 86)] and semen storage duration (SS) divided into 3 categories: 3 to 5 days (SS class 1, n = 59); 6 to 7 days (SS class 2, n = 132) and 8 to 10 days (SS class 3, n = 106). First (model 1), the fixed class effects of genetic line and of semen storage duration were assessed on OR and on luteal, embryonic and uterine characteristics.

Further analyses focused on the relationships between OR and luteal, embryonic and uterine characteristics. To investigate the relationship between OR and luteal, embryonic and uterine characteristics, model 1 was extended with linear and nonlinear relationships as fixed regressions. Two types of nonlinear relationships were considered: quadratic relationships (model 2a) were checked for all traits and an inverse relationships (1/OR, model 2b) for number of embryos (total and vital) only. If the quadratic relationship is a mountain parabola, i.e. the regression coefficient on the quadratic term is negative, it would indicate the maximum value of a trait in relation with OR. Conversely, if the regression coefficient on the quadratic term is positive, it would indicate the minimum value in relation with OR. For model 2b, if the relationship follows an inverse relationship and the regression coefficient on 1/OR is negative, it would indicate the existence of a biological maximum for total number of embryos and number of vital embryos. In model 2a, linear and quadratic effects of OR were fitted as a fixed regression. When the quadratic term was not significant ($P > 0.05$), it was omitted, leaving only the linear term of OR in the model. For traits that had a significant linear (but not quadratic) relationship with OR, OR was subsequently divided in 3 categories (OR class 1 [8 to 18 ovulations, n = 71]; OR class 2 [19 to 22 ovulations, n = 143] and OR class 3 [23 to 34 ovulations, n = 84]) and replaced the linear effect as a fixed class effect in the model to calculate least square means-estimates for the OR classes.

All models included the fixed class effect of genetic line and semen storage duration, and the interaction between genetic line and semen storage class plus its interactions with OR. The fixed class effects and interactions were excluded from the models when not significant. The fixed class effects of genetic line and semen storage duration were adjusted using Bonferroni. Residuals from all models approximated normality.

Results were considered significant at $P \leq 0.05$ and are presented as regression coefficients (β) with their SE for continuous fixed effects, as the intercept and the inverse coefficient for the inverse function and as LS means for fixed class effects.

Results

The observed averages and standard deviations (SD) of the luteal, embryonic and uterine characteristics are presented in Table 4.1. Average OR was 20.9 ± 3.2, ranging from 14 up to 34; the average corpora lutea (CL) weight was 0.45 ± 0.1 g. The early embryonic mortality was on average 4.3 ± 4.2, and the late mortality 1.5 ± 1.6. The number of vital embryos at 35 days of pregnancy was 15.1 ± 4.1, with an average implantation length of 21.6 ± 4.2 cm.

Effect of genetic line and semen storage duration on embryonic characteristics

Effects of genetic line and of semen storage duration classes on luteal, embryonic and uterine characteristics are presented in Table 4.2. Purebred Landrace gilts had a higher OR than the crossbred Yorkshire x Landrace gilts (22.1 ± 0.4 vs 20.3 ± 0.2, respectively, $P < 0.0001$), and also a higher incidence of early embryonic mortality (6.1 ± 0.4 vs 3.5 ± 0.3, $P < 0.0001$). Also, the vital embryos of the purebred Landrace gilts had a higher length and area of uterine implantation (23.0 ± 0.5 cm. vs 21.2 ± 0.3 cm, $P = 0.001$ and 214.5 ± 4.8 cm^2 vs 187.1 ± 3.2 cm^2, $P < 0.0001$, respectively). These effects were independent of the semen

Table 4.1 Summary statistics of ovarian, embryonic and uterine characteristics of gilts at slaughter at 35 days of pregnancy.

Variables	n	Mean	SD	Min	Max
Averages					
Ovulation rate (OR)	298	20.9	3.2	14	34
Corpus luteum (CL) weight, g	298	0.45	0.1	0.3	0.8
Total luteal mass, g	298	9.4	1.6	5.4	17.5
Uterine length, cm	298	502	64	346	675
Number of embryos	298	16.6	4.3	3	27
Number of vital embryos	298	15.1	4.1	3	24
Early embryonic mortality [1]	298	4.3	4.2	-2	21
Late embryonic mortality [2]	298	1.5	1.6	0	10
Vital embryonic weight, g	161	4.2	0.8	2.5	6.8
Vital empty space [3], cm	298	21.2	17.1	5.7	126.7
Vital implantation length, cm	298	21.6	4.2	11.3	38.4
Vital implantation area, cm^2	298	193	46	77	398
Standard deviations					
Corpus luteum weight, g	298	0.1	0.1	0.01	1.7
Vital embryo weight, g	161	0.4	0.1	0.2	0.9
Vital empty space, cm	298	10.7	9	2.1	69.8
Vital implantation length, cm	298	5.5	1.9	2.4	29.2

[1] Difference between the number of corpora lutea (ovulation rate) and the total number of embryos.

[2] Number of non-vital embryos counted at 35 days of pregnancy.

[3] Total empty uterine space around each vital embryo-placental unit.

storage duration.

The duration of semen storage affected the incidence of early embryonic mortality irrespective of genetic line. Gilts in SS3 (semen stored for 8 to 10 d) had a higher level of early mortality (6.1 ± 0.4) than gilts in SS2 (4.2 ± 0.4; semen stored for 6 to 7 d) and SS1 (4.2 ± 0.5; semen stored for 3 to 5 d). This shows that gilts inseminated with semen stored for longer time have a higher estimation of early embryonic mortality. Semen storage duration also affected the area of implantation of the vital embryos. Gilts inseminated with semen stored for 3 to 5 d (SS1) had a higher area of uterine implantation for the vital embryos (208.7 ± 5.7 cm^2) than gilts inseminated with semen stored for 6 to 7 d (SS2, 191.3 ± 4.0 cm^2).

There was a significant interaction between the genetic lines and the classes of semen storage duration (SS) for total number of embryos, number of vital embryos, weight of the vital embryos, and average and standard deviation of empty uterine space around the vital embryos. Crossbred gilts had the highest number of total and vital embryos when inseminated with semen stored for 3 to 5 d (SS1), resulting in a significant difference in embryo numbers for crossbred gilts in SS1 compared to crossbred gilts inseminated with semen stored for 8 to 10 d (total: 18.2 ± 07 vs 15.7 ± 0.5; vital: 16.9 ± 0.7 vs 14.3 ± 0.4). Also, there was a significant difference in embryo numbers between purebred and crossbred gilts in SS1 (total: 14.3 ± 0.9 vs 10.2 ± 0.7; vital: 13.3 ± 0.8 vs 16.9 ± 0.7, $P \leq 0.05$). On the other hand, the number of total and vital embryos did not significantly differ between purebred gilts in different semen storage classes. This indicates that there is a higher reduction in the number of vital embryos in crossbred gilts inseminated with semen stored for longer time than in purebreds.

Both purebred and crossbred gilts had the highest vital embryonic weight when inseminated with semen stored for up to 5 d (SS1; 4.6 ± 0.2 g and 4.9 ± 0.2 g, respectively), but only significantly so compared to crossbred gilts inseminated with semen stored for longer (4.1 ± 0.1 g and 4.0 ± 0.2 g for SS2 and SS3, respectively, $P = 0.04$). This indicates that insemination with semen stored for more than 6 d is related with a reduction in the weight of vital embryos. Thus, crossbreds gilts perform better than purebred gilts when inseminated with semen stored for a shorter time (up to 5 d), but not when semen stored for a longer time is used.

The average and standard deviation of the empty uterine space around the vital embryos was higher in purebred gilts than for crossbred gilts (28.0 ± 3.5 vs 15.3 ± 2.8 cm and 14.4 ± 1.9 vs 7.5 ± 1.5 cm, respectively) when the inseminations were done with semen stored for 3 to 5 d (SS1). This indicates that inseminations using semen stored for shorter time in purebred gilts resulted in less embryos and therefore more empty uterine space between embryos.

Relationships between OR and luteal, embryonic and uterine characteristics

The regression equations for the relationship between ovulation rate (OR) and luteal, embryonic and uterine characteristics are presented in Table 4.3. Significant quadratic relationships are shown in Figure 4.1, the fitted inverse function for total number of embryos

Table 4.2 Least square means and SEM for ovarian and embryonic characteristics of gilts at 35 days of pregnancy for two different genetic lines (GL; Purebred Landrace and Crossbred Yorkshire x Landrace, P and C respectively) and three different semen storage duration classes (SS; 3 to 5 days, 6 to 7 days and 8 to 10 days).

Variables[1]	Genetic line		Semen storage classes, days			P values	
	P	C	3 to 5	6 to 7	8 to10	GL	SS
n=	86	212	59	133	106		
Averages							
Ovulation Rate (OR)	22.1±0.4[a]	20.3±0.2[b]	21.0±0.4	21.2±0.3	21.5±0.3	<0.001	ns
Corpus luteum weight, g	0.44±0.01	0.46±0.01	0.45±0.01	0.46±0.01	0.44±0.01	ns	ns
Total luteal mass, g	9.7±0.2	9.3±0.2	9.4±0.3	9.6±0.2	9.5±0.2	ns	ns
Uterine length, cm	494.3±7.5	506.2±5.0	506.4±8.7	502.5±6.1	491.9±6.9	ns	ns
Number of embryos[2]	15.7±0.5	17.0±0.3	16.3±0.6	17.2±0.4	15.7±0.5	0.03	ns
Number of vital embryos[3]	14.5±0.4	15.6±0.3	15.1±0.5	15.5±0.4	14.4±0.5	0.04	ns
Early embryonic mortality	6.1±0.4[a]	3.5±0.3[b]	4.2±0.5[a]	4.2±0.4[b]	6.1±0.4[c]	<0.001	0.001
Late embryonic mortality	1.3±0.2	1.5±0.1	1.2±0.2	1.7±0.1	1.3±0.2	ns	ns
Embryo weight[4], g	4.5±0.2	4.3±0.2	4.8±0.2	4.3±0.2	4.2±0.2	ns	<0.001
Empty space[5], cm	23.4±1.9	19.7±1.3	21.6±2.2	19.1±1.6	24.0±2.0	ns	ns
Implantation length, cm	23.0±0.5[a]	21.2±0.3[b]	22.2±0.5	21.6±0.4	22.4±0.4	0.001	ns
Implantation area, cm^2	214.5±4.8[a]	187.1±3.2[b]	208.7±5.7[a]	191.3±4.0[b]	202.3±4.6[ab]	<0.001	0.02
Standard deviations							
Corpus luteum weight, g	0.1±0.01	0.1±0.01	0.04±0.01	0.1±0.01	0.1±0.01	ns	ns
Embryo weight, g	0.4±0.03	0.4±0.03	0.4±0.03	0.4±0.02	0.4±0.03	ns	ns
Empty space[6], cm	11.6±1.0	10.0±0.7	10.9±1.2	9.7±0.8	11.7±1.1	ns	ns
Implantation length, cm	5.7±0.2	5.5±0.2	6.0±0.3	5.4±0.2	5.4±0.2	ns	ns

[1] Batch was included in the models as a random fixed effect. Measurements of embryonic weight, length and area of implantation, and empty uterine space were collected only for vital embryos. ns Not significant ($P > 0.05$).

[2] Least square means estimates for the interaction GL and SS ($P = 0.02$): Purebreds * SS1: 14.5 ± 0.9 a; Purebreds * SS2: 17.1 ± 0.7 ab; Purebreds * SS3: 15.6 ± 0.9 ab; Crossbreds * SS1: 18.2 ± 0.7 b; Crossbreds * SS2: 17.2 ± 0.4 ab; Crossbreds * SS3: 15.7 ± 0.5 a.

[3] Least square means estimates for the interaction GL and SS ($P = 0.01$): Purebreds * SS1: 13.3 ± 0.8 a; Purebreds * SS2: 15.6 ± 0.7 ab; Purebreds * SS3: 14.4 ± 0.9 ab; Crossbreds * SS1: 16.9 ± 0.7 b; Crossbreds * SS2: 15.3 ± 0.4 ab; Crossbreds * SS3: 14.3 ± 0.4 a.

[4] Least square means estimates for the interaction GL and SS ($P = 0.04$): Purebreds * SS1: 4.6 ± 0.2 a; Purebreds * SS2: 4.4 ± 0.2 ab; Purebreds * SS3: 4.4 ± 0.2 ab; Crossbreds * SS1: 4.9 ± 0.2 a; Crossbreds * SS2: 4.1 ± 0.2 b; Crossbreds * SS3: 4.0 ± 0.2 b.

and vital embryos is shown in Figure 4.2; and significant linear relationships are shown in Figure 4.3.

There was a quadratic relationship between OR and average CL weight (Figure 4.1A); which shows a minimum CL weight of 0.42 g at an OR of 28. This indicates that individual CL weight decreases with the increase in OR.

Ovulation rate had a positive linear relationship with total luteal mass, and each extra ovulation was related with an 0.32 g increase in total luteal mass. When OR was considered as class effect, gilts with OR from 23 up to 34 had the highest total luteal mass (Figure 4.3A).

An increase in OR was also related with a linear increase in uterine length, with each extra ovulation leading to an increase of 10.6 ± 2.1 cm of uterine length. However, this relationship was different between gilts inseminated with semen with different storage duration, and the increase in uterine length with the increase in OR was smaller for gilts inseminated with semen stored for 6 to 7 d (SS2) than for gilts inseminated with semen stored for 3 to 5 d (SS1) and for 8 to 10 d (SS3). Thus, a high OR is related with a higher uterine length in gilts at 35 days of pregnancy, but this increase in length seems to be lower in gilts inseminated with semen stored for 6 to 7 d.

Ovulation rate had significant quadratic relationships also with the total number of embryos and the number of vital embryos (Figure 4.1B). The equation predicted a maximum number of embryos of 19.6 at an OR of 30 and for the number of vital embryos a maximum of 16.8 at an OR of 26. The decrease in number of embryos in gilts after a certain OR indicates that the response in number of embryos becomes less in gilts with the increase in OR.

The inverse model estimated the total number of embryos to be 19.9 at 30 ovulations and the number of vital embryos to be 16.6 at 26 ovulations. Also, the regression coefficient predicts a maximum of 28.7 total number of embryos and of 24.6 number of vital embryos (Figure 4.2). This matches the estimations of the quadratic model and suggests that with an unlimited number of ovulations, the maximum uterine capacity would be to carry 24.6 vital embryos at 35 days of pregnancy.

Ovulation rate had a quadratic relationship with the incidence of early embryonic mortality, with a minimum value of early mortality of 3.3 achieved at 15 ovulations, increasing thereafter at higher OR (Figure 4.1C). The incidence of late mortality also showed a quadratic relationship with OR, with the lowest value of 1.2 at 18 ovulations increasing thereafter with the increase in OR (Figure 4.1C). Results shows that, regardless of the effects of semen storage duration and genetic line, gilts with a higher OR

[5] Least square means estimates for the interaction GL and SS ($P = 0.03$): Purebreds * SS1: 28.0 ± 3.55 a; Purebreds * SS2: 18.3 ± 2.6 ab; Purebreds * SS3: 24.0 ± 3.6 ab; Crossbreds * SS1: 15.3 ± 2.8 b; Crossbreds * SS2: 20.0 ± 1.8 ab; Crossbreds * SS3: 24.0 ± 1.8 ab.

[6] Least square means estimates for the interaction GL and SS ($P = 0.01$): Purebreds * SS1: 14.4 ± 1.9 a; Purebreds * SS2: 9.0 ± 1.4 ab; Purebreds * SS3: 11.4 ± 1.9 ab; Crossbreds * SS1: 7.5 ± 1.5 b; Crossbreds * SS2: 10.4 ± 0.9 ab; Crossbreds * SS3: 12.0 ± 1.0 ab.

[abc] Least square means estimates differ between genetic lines or semen storage duration classes and their interactions ($P \leq 0.05$).

Table 4.3 Regression equations (ß) for the relationship between ovulation rate (OR) and luteal, embryonic and uterine characteristics for gilts at 35 days of pregnancy.

Variables[1]	Linear/Quadratic function		P values			
	β OR	β OR²	OR	OR²	SS	GL
Averages						
Corpus luteum weight, g	-0.03±0.01	0.001±0.0002	0.03	<0.001	ns[2]	ns
Total luteal mass, g	0.32±0.02	-	<0.001	-	ns	ns
Uterine length[3], cm	10.6±2.1	-	<0.001	-	0.02	0.01
Number of embryos	2.0±0.6	-0.03±0.01	0.001	0.02	0.001	0.001
Number of vital embryos[4]	2.5±0.6	-0.05±0.01	<0.001	0.001	0.04	0.003
Early embryonic mortality	-1.1±0.6	0.03±0.01	0.11	0.02	0.001	0.001
Late embryonic mortality	-0.6±0.3	0.02±0.01	0.02	0.004	ns	0.02
Embryo weight, g	0.06±0.1	-0.001±0.002	0.56	0.60	0.001	ns
Empty space, cm	-10.3±2.7	0.2±0.1	<0.001	0.001	0.02	ns
Implantation length, cm	-0.7±0.7	0.01±0.02	0.33	0.44	ns	0.001
Implantation area, cm²	3.2±7.3	-0.09±0.2	0.66	0.59	0.03	0.001
Standard deviations						
Corpus luteum weight, g	0.01±0.02	-0.0002±0.0004	0.68	0.66	ns	ns
Embryo weight, g	0.01±0.003	-	<0.001	-	ns	ns
Empty space, cm	-3.9±1.4	0.1±0.03	0.01	0.01	ns	ns
Implantation length, cm	0.08±0.03	-	0.02	-	ns	ns

[1] Statistical models included genetic line (GL, Purebred Landrace and Crossbred Yorkshire x Landrace) and semen storage duration classes (SS, 3 to 5 days, 6 to 7 days and 8 to 10 days) as fixed class effects and batch as a random fixed effect. Fixed effects and interactions were excluded from the models when not significant. Measurements of embryonic weight, length and area of implantation, and empty uterine space were collected only for vital embryos.

[2] Equation for the interaction between OR and SS (P = 0.03): SS 3 to 5 days (317.7 ± 113.5 + 9.6 ± 5.4 * OR); SS 6 to 7 days (422.7 ± 96.8 + 4.3 ± 4.6 * OR); SS 8 to 10 days (278.3 ± 43.4 + 10.6 ± 2.1 * OR).

[3] Least square means estimates for the interaction GL and SS (P = 0.03): Purebreds * SS1= 13.4 ± 0.8 a; Purebreds * SS2= 15.3 ± 0.6 ab; Purebreds * SS3 = 13.4 ± 0.8 a; Crossbreds * SS1 = 16.9 ± 0.6 b; Crossbreds * SS2 = 15.8 ± 0.4 ab; Crossbreds * SS3= 14.3 ± 0.4 a. Difference between estimates (P ≤ 0.05) are represented by different letters (abc).

[4] Least squares means estimates for the interaction of GL and SS (P = 0.03): Purebreds * SS1 = 13.4 ± 0.8 a; Purebreds * SS2 = 15.3 ± 0.6 ab; Purebreds * SS3 = 13.4 ± 0.8 a; Crossbreds * SS1 = 16.9 ± 0.6 b; Crossbreds * SS2 = 15.8 ± 0.4 ab; Crossbreds * SS3 = 14.3 ± 0.4 a. Difference between estimates (P ≤ 0.05) are represented by different letters (abc).

ns Not significant (P > 0.05).

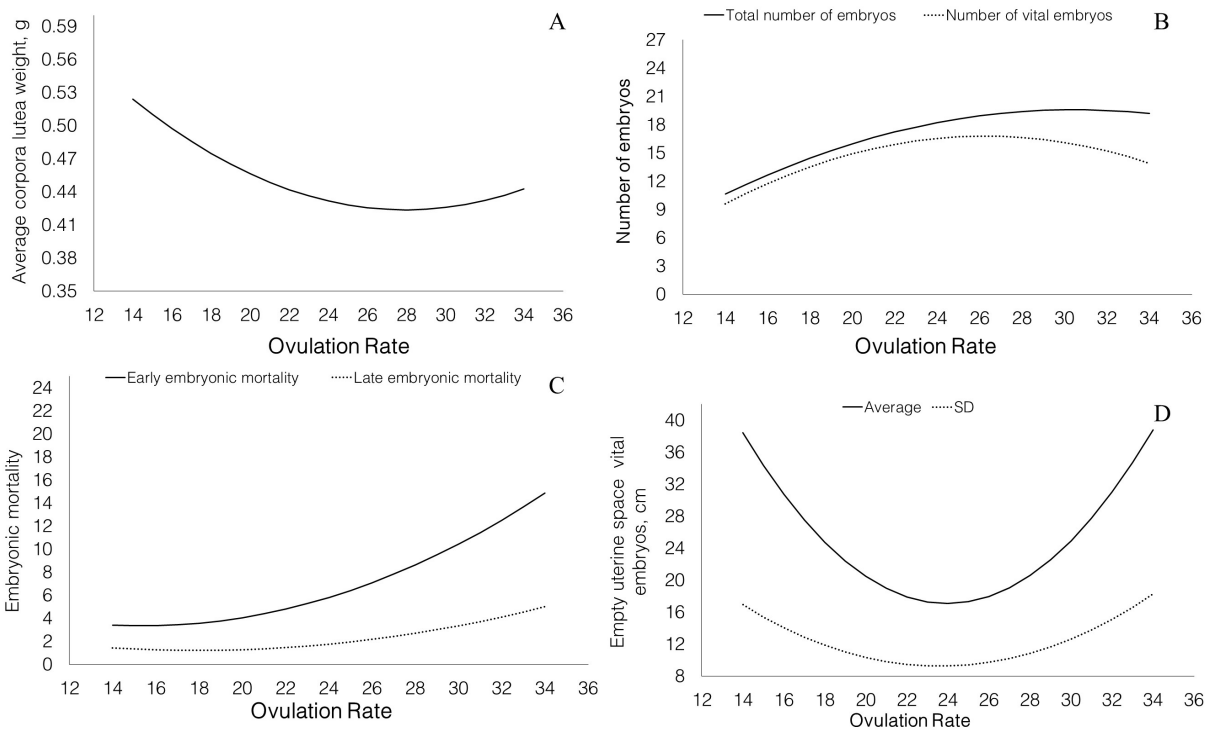

Figure 4.1 Predicted quadratic relationships between ovulation rate and embryonic characteristics in gilts at 35 days of pregnancy, corrected for fixed class effects of gilts genetic line (GL; purebred Landrace, n = 86 and crossbred Yorkshire x Landrace, n = 216) and semen storage duration classes (SS; 3 to 5 days, n = 59; 6 to 7 days, n = 133; 8 to 10 days, n = 106). Panel A, average corpus luteum weight (-0.03 ± 0.01 * OR + 0.001 ± 0.0002 * OR2, $P \leq 0.05$); panel B, total number of embryos (solid line, 2.0 ± 0.6 * OR -0.03 ± 0.01 * OR2 , $P \leq 0.05$; SS P = 0.001; GL P = 0.001) and number of vital embryos (dotted line, 2.5 ± 0.6 * OR -0.05 ± 0.01*OR2 , $P \leq 0.05$; SS P = 0.04; GL P = 0.003); panel C, early embryonic mortality (solid line, -1.1 ± 0.6 * OR + 0.03 ± 0.01 * OR2, $P \leq 0.05$; SS P = 0.001; GL P = 0.001) and late embryonic mortality (dotted line, -0.6 ± 0.3*OR + 0.02 ± 0.01 * OR2 , $P \leq 0.05$; GL P = 0.02), and panel D, average (solid line, -10.3 ± 2.7 * OR + 0.2 ± 0.1 * OR2; $P \leq 0.05$, SS P = 0.02) and SD of the length of empty uterine space around vital embryos (dotted line, -3.9 ± 1.4 * OR +0.1 ± 0.03 * OR2, $P \leq 0.05$).

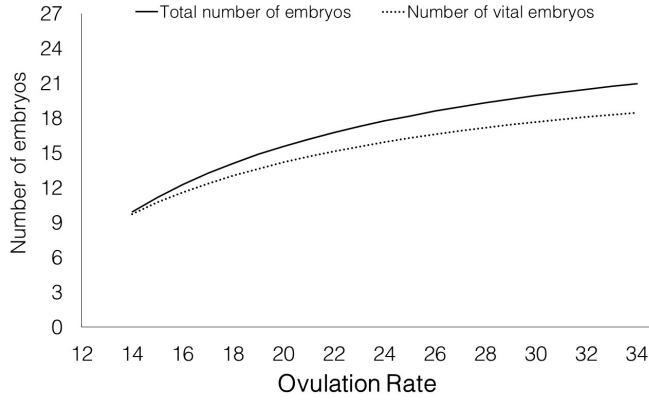

Figure 4.2 Inverse regression function of ovulation rate in relation with the total number of embryos and number of vital embryo, corrected for fixed class effects of gilts genetic line (GL; purebred Landrace, n = 86 and crossbred Yorkshire x Landrace, n = 212), and semen storage duration classes (SS, 3 to 5 days, n = 59; 6 to 7 days, n = 133; 8 to 10 days, n = 106). The solid line represents the predicted values for total number of embryos [28.7 ± 1.5 -262.5 ± 30.3/OR, ($P < 0.0001$)] and the dotted line represents the predicted values for number of vital embryos [24.6 ± 1.5 - 207.9 ± 29.9 /OR, ($P < 0.0001$)].

had a specifically higher incidence of early embryonic mortality and to a lesser extend of late embryonic mortality.

Ovulation rate had a quadratic relationship with the average and SD of empty uterine space around the vital embryos, with a minimum average of 17.1 cm and a minimum SD of 9.2 cm at an OR of 24 (Figure 4.1D). This shows that both gilts with a low and a high OR have a larger and less uniform empty uterine space surrounding the vital embryos than gilts with OR around 24.

Ovulation rate had linear relationships with the SD of vital embryo weight and with the SD of the vital embryos implantation length. An increase in OR was related to an increase in the SD of the vital embryos weight, with each extra ovulation related to an increase of 0.01 g in variation in embryo weight. When considered as a class effect, gilts with 23 to 34 ovulations had a higher variation in embryo weight than gilts with 8 to 18 ovulations (Figure 4.3B). An increase in OR was related to an increase in the SD of the implantation length of the vital embryos. Each extra ovulation was related to an increase in variation in implantation length of 0.08 cm. When OR was considered as a class effect, there was no significant difference in implantation length between gilts in different OR classes, but it was numerically higher in gilts with OR from 19 up to 34 (Figure 4.3C). This shows that gilts with a high OR have a higher variation in both the vital embryonic weight and in their length of uterine implantation.

Discussion

This study investigated the relationship between OR and embryonic characteristics in Landrace and Yorkshire x Landrace gilts inseminated with semen stored for 3 up to 10 d and slaughtered at 35 days of pregnancy. The objectives were first to investigate the effects of

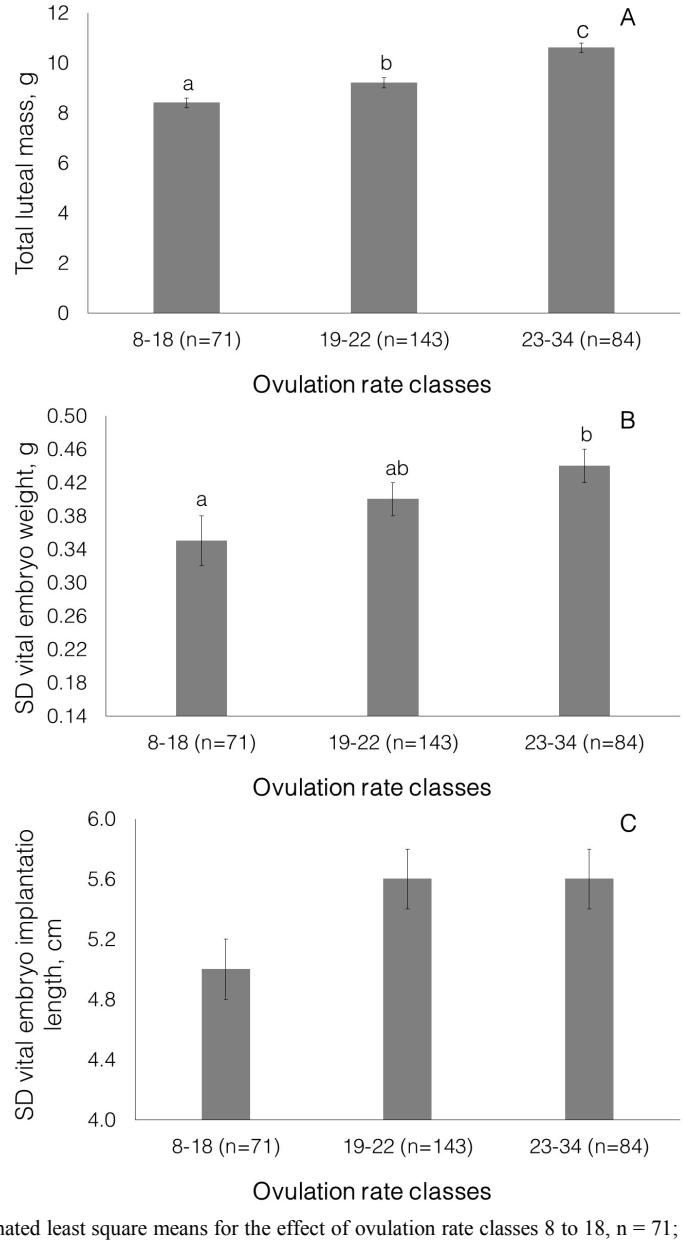

Figure 4.3 Estimated least square means for the effect of ovulation rate classes 8 to 18, n = 71; 19 to 22, n = 143; and 23 to 34, n = 84, respectively on total luteal mass (panel A, $P < 0.0001$); standard deviation of vital embryo weight (panel B, $P = 0.002$) and on vital embryos length of uterine implantation (panel C, $P > 0.05$) in gilts at 35 days of pregnancy. The models included the fixed class effect of gilts genetic line (GL, purebred, n = 86 and crossbred Yorkshire x Landrace, n = 216) and semen storage duration classes (SS, 3 to 5 days, n = 59; 6 to 7 days, n = 133; 8 to 10 days, n = 106) and interactions, which were excluded when not significant ($P > 0.05$). Significant differences between classes are indicated by letters above the columns and the error bars indicate a single SE of the estimates.

genetic line and duration of semen storage on the reproductive traits and second to investigate how OR influences early embryonic mortality and development, aiming to better understand the mechanisms that lead to variation in litter characteristics at birth.

Gilts of 2 different genetic lines were used. Comparison between genetic lines showed that purebred Landrace gilts had a higher OR than the crossbred Yorkshire x Landrace gilts (22.1 vs 20.3, respectively). This was not expected since previous studies found OR in purebred gilts to be lower or not significantly different from OR in crossbred gilts (Johnson et al. 1978, Irgang et al. 1993). However, in this study the purebred gilts were heavier at insemination than the crossbreds, despite being on average at the same age, and an increase in weight at first insemination was positively related with OR ($\beta = 0.09 \pm 0.01$ ovulations/kg, $P < .0001$, *results not shown*). Therefore, the higher OR in purebred gilts in our study seems to be a reflection of the higher body weight at insemination. Purebred Landrace gilts also had a higher incidence of early embryonic mortality than the crossbreds, which might be related with their higher OR, since at a higher OR embryonic mortality increases (Da Silva et al. 2016). However, lower embryonic mortality observed in crossbred gilts can be also related with maternal heterosis (Johnson and Omtvedt 1973), since crossbreeding studies have shown that maternal heterosis for prenatal survival is one of the major reasons for the value of crossbreeding in pigs (reviewed by Blasco et al. 1993). So, the higher early embryonic mortality in purebred compared to crossbred gilts can be related with both the higher OR and maternal heterosis.

The incidence of early embryonic mortality was also affected by semen storage duration, being higher in gilts inseminated with semen stored for more than 5 d. Despite the wide range of extenders that have been developed for optimal *in vitro* boar semen preservation, most inseminations are still performed within 3 d after semen collection, since the fertilization rate decreases with semen stored for more than 4 d (Waterhouse et al. 2004) due to structural and functional changes in the spermatozoa mitochondria, flagellum, acrosome and plasma membrane (reviewed by Johnson et al. 2000). So, a lower fertilization rate due to longer semen storage might have led to a decrease in number of embryos formed and therefore increased the difference between OR and the total number of embryos, leading to an overestimation of the early embryonic mortality. Also, the increase in semen storage duration leads to a decrease in the number of competent spermatozoa reaching the fertilization site after insemination, increasing time to fertilization and reducing the competition among spermatozoa, thereby allowing fertilization by less competent spermatozoa, which might lead to formation of embryos with lower quality (Saacke et al. 1994). This might also explain the decrease in vital embryonic weight we observed in crossbred gilts inseminated with semen stored for more than 6 d. Soede et al. (1995) observed that a high fertilization rate, i.e. more than 90% of normal embryos relative to all embryos and oocytes recovered, was significantly associated with better embryonic development at d 5 of pregnancy. Thus, long semen storage may increase embryo mortality by increasing the percentage of unfertilized oocytes and might also influence embryonic quality.

The main objective of this study was to investigate the relationship between OR and embryonic mortality and development at early pregnancy. Despite the effects of both genetic line and semen storage duration in the gilts reproductive characteristics at d 35 of pregnancy, there were only limited effects of these factors on the relationship between OR and the reproductive characteristics. Results show that at 35 d of pregnancy a maximum of on average 16.8 vital embryos was achieved at 26 ovulations. Although the quadratic function predicts a decrease in the number of vital embryos in gilts with OR above 26, this should be interpreted carefully due to the low number of animals with OR above 30. On the other hand, a quadratic relationship between OR and number of vital embryos at 24 to 34 days of pregnancy was also reported in Norwegian Landrace gilts, with OR ranging from 7 to 23, and a maximum number of 12.2 vital embryos was achieved at 18 ovulations (Blichfeld and Almlid 1982). A linear increase in the number of embryos with an increase in OR up to 18 (β = 0.76 live foetuses/ovulation, $P \leq 0.05$) was also described for multiparous sows slaughtered between 3 and 15 weeks of pregnancy, in which a maximum of 14 live foetuses was achieved. However, there was no further significant increase in the number of live foetuses in sows when OR went from 19 up to 25 (Wu et al. 1987). A *plateau* in the number of vital embryos was also observed in multiparous sows slaughtered at 35 days of pregnancy, when no more than 17 vital embryos were observed in sows with OR from 22 up to 38 (Da Silva et al. 2016). Therefore, a further increase in the number of ovulations does not result in an further increase in the number of vital embryos at 35 d of pregnancy. Indeed, further results from this study based on the inverse model estimated a similar number of vital embryos (16.6) at 26 ovulations as the quadratic model, and estimated that the number of vital embryos could have reached 24.6 if there would be no limitations related with OR. Altogether, this suggests that there is a limit for the correlated increase in number of vital embryos in relation with the increase in OR, and also that the maximum number of embryos achieved at 26 ovulations does not represent the maximum uterine capacity in gilts at 35 d of pregnancy.

The limited increase in the number of vital embryos at d 35 of pregnancy, achieved with the increase in OR, is related with the incidence of embryonic mortality. Gilts in this study had an average embryonic mortality of 5.8, which was classified as early or late embryonic mortality. Early embryonic mortality is estimated as the difference between OR and the total number of embryos and corresponds to the embryonic losses occurring before uterine implantation, i.e. before 13 days of pregnancy. Gilts in this study had an average early embryonic mortality of 4.3 and, in relation with OR, early embryonic mortality had the lowest value at 15 ovulations and increased progressively from 16 up to 34 ovulations. Blichfeldt and Almlid (1982) observed the lowest embryonic mortality around 13 ovulations in gilts at 28 up to 34 days of pregnancy.

In super ovulated crossbred gilts (average OR 45.2, ranging from 22 to 76), van der Waaij et al. (2010) observed a linear positive relationship between OR and early embryonic mortality (β = 0.71/ovulation); and Da Silva et al. (2016) also found a positive relationship (β = 0.49 ± 0.11/ovulation) in noninduced multiparous sows (average OR 25.5, ranging from 17 to 38).

Independent of having a linear or a quadratic relationship with OR, it is clear that gilts and sows with a higher OR have a higher incidence of early embryonic mortality, which has multiple possible causes. First, it is possible that gilts with a higher OR have a higher and faster increase in progesterone after the beginning of the ovulation process. This premature progesterone rise might affect sperm transportation to the fertilization site, thus compromising fertilization, but might also induce early embryonic mortality by directly affecting the oocyte or by changing the environment in the oviduct (Day and Polge 1968). Higher OR might also result in higher early embryonic mortality due to higher embryonic diversity originating from a higher diversity in follicles and their oocytes at ovulation (Pope et al. 1990). The increase in early embryonic mortality in gilts with a high OR might also be related with a decrease in follicular and oocyte quality. In this study, gilts with a higher OR had a lower average CL weight (minimum CL weight of 0.42 g at 28 ovulations). This indicates smaller follicles at ovulation, as a positive relationship between pre-ovulatory follicle diameter and CL weight ($r = 0.28$, $P < 0.01$) was observed by Soede et al. (1998), and Wientjes et al. (2012) also found that each mm increase in follicle diameter at ovulation led to a 1.23 mm increase in CL diameter ($P = 0.03$) in multiparous sows at 10 days of gestation. So, the lower average CL weight in gilts with a higher OR in this study may indicate that these gilts had a lower follicle size at ovulation. In cattle, follicles with lower diameter at ovulation also have oocytes with lower diameter and with a reduced potential for development and survival (Gandolfi et al. 2005). Thus, smaller follicles at ovulation develop into smaller CL and release smaller oocytes of lower quality that lead to the development of embryos with reduced quality (Ding and Foxcroft 1994; Gandolfi et al. 2005) therefore increasing early embryonic mortality. This indicates that the higher early embryonic mortality in gilts with a higher OR might be related with a reduced follicular and oocyte quality in these gilts. Studies in humans have shown that the follicular and oocyte quality impacts not only the incidence of early embryonic mortality, but also late mortality and fetal development throughout pregnancy and may even affect adult disease (reviewed by Krisher 2004).

Late embryonic mortality is defined as the mortality occurring after uterine implantation and accounts for the number of degenerated and nonvital embryos (van der Waaij et al. 2010). In this study, the lowest values of late mortality were observed at 18 ovulations, progressively increasing from 19 up to 34 ovulations. Like early mortality, the incidence of late embryonic mortality can be related with variation in embryonic development in early pregnancy. At day 11 of pregnancy, pig conceptuses start to elongate and the length of embryonic elongation will determine the length of their uterine implantation site (Geisert et al. 1982). However, there is a high variation in development stages and length of elongation between conceptuses at this stage (Pusateri et al, 1990), and conceptuses with delayed development and therefore smaller elongation length, will attach to a smaller uterine area. A smaller uterine area for implantation will result in a smaller placenta (Stroband and van der Lende 1990), which in turn might lead to late embryonic mortality and compromised embryonic development in later pregnancy (Vonnahme et al. 2002a). Thus, the increase in late embryonic mortality in

gilts with high OR might be related with an increase in variation in length of elongation between conceptuses. Another possible explanation for the higher late embryonic mortality in gilts with a higher OR is uterine crowding, since gilts with a high OR, considering the incidence of early embryonic mortality, can have a higher number of embryos arriving in the uterus and will, therefore, have a higher competition for uterine space. Langendijk et al. (2016) observed that in sows with an average OR of 21.6 ± 0.9, the available uterine space per embryo at 35 d of pregnancy had a negative correlation with OR (r = -0.85, $P \leq 0.05$). Also, Da Silva et al. (2016) found that an increase in OR in multiparous sows was related to a decrease in the empty uterine space around the vital embryos (β = -0.40 ± 0.2 cm/ovulation) and also to a decrease in their length of implantation (β = 0.35 ± 0.1 cm/ovulation,) and length of the placenta (β = -0.39 ± 0.3 cm/ovulation) at 35 days of pregnancy, indicating uterine crowding. In the current study, however, we observed an increase in empty uterine space around vital embryos in gilts with higher OR (above 24) and no relationship with the average uterine implantation length of the vital embryos. The extent of uterine crowding is critically dependent on the interaction between OR and early embryonic mortality (Town et al. 2005); thus, the higher empty space around vital embryos in gilts with high OR, and the lack of effect on the implantation length, might be due to the higher early embryonic mortality in these gilts. Thus, the higher late embryonic mortality in gilts with a higher OR seems not to be caused by uterine crowding, as it was suggested for multiparous sows, since in the gilts there were no decrease in implantation length and in empty uterine space with the increase in OR.

The increase in OR affected not only the survival but also the development of the vital embryos at 35 d of pregnancy. An increase in OR was related with a linear increase in variation in the vital embryo weight and in the variation in vital embryos uterine implantation length at 35 d of pregnancy. Interestingly, the variation in vital embryo weight was correlated with the variation of the uterine implantation length (r = 0.23, $P = 0.003$*, results not shown*). Because uterine implantation length relates with length of elongation of the embryos, as discussed above (Geisert et al. 1982), this increase in variation in the uterine implantation length and in embryonic weight might reflect a higher embryonic diversity in gilts with higher OR. A higher variation in embryo weight in early pregnancy might lead to a higher foetal mortality, since the lighter embryos might die during late pregnancy due to the acquired smaller placenta (reviewed by Vallet et al. 2014). The higher variation in embryo weight might also lead to a higher variation in piglet birth weight, since vital embryos have limited ability to benefit from the demise of an adjacent conceptus if mortality occurs after 35 d of pregnancy (Vallet et al. 2011). Thus, an increase in OR leads to a higher variation in weight of the vital embryos at 35 d of pregnancy which might be related with the variation in implantation length of these embryos. This high variation in embryo weight at 35 d of pregnancy might lead to a higher foetal mortality and to a higher variation in piglet birth weight.

Knowing what might influence embryonic development in gilts and sows may help in clarifying the origin of the differences in litter characteristics between gilts and sows. It is

known that gilts have a lower OR (Belstra 2003; Town et al. 2005), lower litter size (Zindove et al. 2014) and a lower average piglet birth weight (Redmer et al. 2004) than multiparous sows. Data available from approximately 1,300 sows and 276 gilts shows that sows had an average number of live born piglets of 15.2, with an average piglet birth weight of 1,346 grams, while gilts had an average live born piglets of 13.9 with an average piglet birth weight of 1,290g (Topigs Norsvin, Vught, The Netherlands). In this study, the OR of gilts was lower compared to studies with multiparous sows (20.9 vs 23.6 and 25.5, in the studies of Town et al. 2005 and Da Silva et al. 2016). Interestingly, the number of vital embryos observed in the gilts of the current study (15.1 ± 4.1) at 35 d of pregnancy was similar to the number observed for multiparous sows at similar stages of pregnancy (14.9 ± 0.5, Town et al. 2005 and 16.4 ± 3.9, Da Silva et al. 2016).

In both gilts and sows an increase in OR is related with an increase in the number of vital embryos up to a certain maximum, which was reached at 26 ovulations in the gilts of this study and at 22 ovulations in the sows in the study of Da Silva et al. (2016). Interestingly, the maximum number of embryos achieved at d 35 of pregnancy is similar in these studies (respectively 16.4 and 17.0). In sows, different from that observed in the gilts in the current study, an increase in OR was also related with a decrease in implantation and placental length (Da Silva et al. 2016) and weight (Vonnahme et al. 2002b) and also to a decrease in empty uterine space around the vital embryos (Langendijk et al. 2016; Da Silva et al. 2016), which indicates an increase in uterine crowding in sows with an increase in OR. On the other hand, in the gilts of the current study, no relationship between OR and average implantation length was found, and the empty uterine space around the vital embryos actually increased with the increase in OR, which suggests that there is no increase in uterine crowding with the increase in OR. There was, however, an increase in the variation in implantation length and in embryo weight with the increase in OR in the gilts, which was not observed in previous studies with multiparous sows at early pregnancy. Thus, the relationship between OR and embryonic development seems to be different between gilts and sows. High OR in sows lead to smaller placental and implantation lengths that may potentially affect birth weights. In gilts high OR lead to more variation in implantation length and embryo weight which may potentially increase variation in birth weight. However, this speculation is built on a comparison between different experiments and an experiment including both gilts and sows is needed.

In conclusion, crossbred Yorkshire x Landrace gilts had a lower OR and a lower embryonic mortality than the purebred Landrace gilts. The lower early embryonic mortality in crossbred gilts could be an effect of the lower OR but also of maternal heterosis. Also, early embryonic mortality was higher in gilts inseminated with semen stored for more than 5 d, and this is most likely due to a decrease in fertilization rates and therefore the inclusion of nonfertilized oocytes as early embryonic mortality. Crossbred gilts performed better than purebred gilts when inseminations were done with semen stored for up to 5 d, but when old semen was used crossbred gilts had a decrease in the number of vital embryos and in vital embryonic weight, suggesting purebreds to perform better when semen stored for more than 5 d is used. Despite the effects of genetic line and semen storage duration in some of the reproductive traits, the

relationship between OR and luteal and embryonic characteristics was hardly ever affected. The number of vital embryos increased with the increase in OR from 14 up to 26, where it reached the highest value, decreasing its response to OR in gilts with OR above 26. This suggests that there is a limit in the number of vital embryos to be achieved at 35 d of pregnancy with the increase in OR, which is probably imposed by the increase in early embryonic mortality that follows the increase in OR. Also, since the highest number of vital embryos was yet below the number predicted as the maximum uterine capacity of the gilts, it seems like it would still be possible to increase litter size by increasing OR and by decreasing early embryonic mortality. However, an increase in OR was also related with an increase in variation in vital embryonic weight, which might indicate increased variation in embryonic development at earlier stages of pregnancy and might result in a higher foetal mortality and a higher variation in piglet birth weight. So, since it appears that an increase in OR negatively affects follicular and oocyte quality, therefore decreasing embryonic quality and influencing the available uterine space per vital embryo due to its effect on embryonic mortality; to gain knowledge about how OR might affect litter characteristics at birth would help clarify the mechanisms that lead to piglet birth weight and birth weight variation.

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CHAPTER 5

Validation of transrectal ultrasonography for assessment of corpora lutea characteristics in pregnant sows and its relationship with litter characteristics at birth

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Abstract

In experiment 1 we investigated the accuracy of transrectal ultrasonography (TUS) to assess the number (OR) and diameter of corpora lutea (CL) in 45 and 25 sows, respectively, at 23.4 ± 2.9 d of pregnancy. The diameter was calculated as the average diameter of 10 biggest CL. Sows were subsequently slaughtered and OR was assessed by dissection of CL from both ovaries (n = 45) and average diameter of the 10 biggest CL was also calculated after measurement of CL with the calliper rule (n = 25). There was a weak relationship between OR counted after dissection of the ovaries and OR counted with TUS ($\beta = 0.28 \pm 0.01$ CL/CL, $P = 0.01$), but there was a strong relationship between the average CL diameter measured with the calliper rule after dissection and the average CL diameter based on TUS ($\beta = 1.0 \pm 0.1$ mm/mm, $P < 0.0001$). This shows that TUS is not a valid method to assess OR in pregnant sows but it is a valid method to assess average CL diameter. In experiment 2, we investigated the relationship between the average CL diameter assessed by TUS (n = 100) at 23.8 ± 2.4 d of pregnancy and average piglet birth weight (BW) and observed an increase of 37.6 ± 17.8 g in piglet BW per mm increase in average CL diameter measured by TUS ($P = 0.04$). This relationship is probably because larger CL develop from bigger follicles at ovulation, which might have ovulated oocytes of higher quality that developed into embryos with higher growth potential and thus higher birth weight.

Keywords: corpora lutea, piglet birth weight, pregnancy, sows, transrectal ultrasonography.

Introduction

Transrectal ultrasonography (TUS) is an established technique for assessing ovulation rate (OR) by counting the number of pre-ovulatory follicles in sows during oestrus (Soede et al. 1992; Soede et al. 1998; Lucy et al. 2001; Hazeleger et al. 2005; Madej et al. 2005), and it was used by Gonzalez-Añover et al. (2009) to assess OR by counting the number of corpora lutea (CL) in Iberian sows at 9 to 11 d after estrus with an accuracy of 86.7%. However, TUS has not yet been used to assess OR in Western commercial sows during pregnancy. So far, most assessments of OR in pregnant sows to study its relationship with embryonic characteristics have been based on post-mortem findings (Vonnahme et al. 2002; Town et al. 2005; Da Silva et al. 2016; Da Silva et al. 2017). It was observed that an increase in OR is related with vital embryos at 35 days of pregnancy with lower placental length in sows (Da Silva et al. 2016) and with a higher variation in weight in gilts (Da Silva et al. 2017). This might decrease foetal survival and consequently litter size, but might also lead to a decrease in piglet birth weight and birth weight uniformity. Additionally, average CL weight decreased with the increase in OR in gilts (Da Silva et al. 2017), indicating a compromised follicular growth, with oocytes and possibly embryos of lower quality (Ding and Foxcroft 1994), compromising embryonic and foetal survival and development. Thus, we hypothesised that the number and size of CL in pregnant sows might be related with litter characteristics at birth, and the objectives of this study were first to investigate the accuracy of TUS to assess the number and average diameter of CL in modern crossbred sows in early pregnancy, and second to investigate the relationship between the CL characteristics evaluated by TUS and subsequent litter characteristics at birth.

Material and Methods

The experiments and all measurements were approved by the Animal Welfare Committee of Wageningen University and Research Centre in compliance with the Dutch Law on Animal Experimentation. Experiment 1 was conducted at Schothorst Feed Research B.V. (Lelystad, The Netherlands), and at a commercial farm (Nijmegen, The Netherlands). Experiment 2 was conducted at the Pig Innovation Centre, Sterksel (VIC, Sterksel, The Netherlands).

Animals and Housing

Experiment 1

The study included a total of 45 pregnant multiparous (parity 7.3 ± 3.2, ranging from 2 to 13) crossbred sows (Yorkshire x Landrace; Topigs Norsvin, Vught, The Netherlands), at 2 different farms (farm 1, n = 20 and farm 2, n = 25) in 3 batches each.

Experiment 2

The study included a total of 100 pregnant multiparous (parity 5.0 ± 1.9, ranging from 2 to 9) crossbred sows (Yorkshire x Landrace; Topigs Norsvin, Vught, The Netherlands) at 1 farm, which were used in 6 batches. At the first day after weaning, that took place at

d 27.0 ± 3.8 (mean ± SD) of lactation, the sows were group housed with individual feeding stalls, where they received a commercial lactation diet (NE = 9.50 MJ/kg; CP = 149 g/kg and ileal digestible lysine= 7.6 g/kg) *ad libitum*. From the second day post weaning to the day of first insemination, sows were housed in individual crates, and received 4.5 kg of the lactation diet per day. The light schedule consisted of 16 consecutive hours of light (100 lux) and 8 hours of darkness. After insemination, sows were moved to a gestation group housing system in groups of 11 to 45 sows, where they received a commercial diet (NE = 9.06 MJ; CP = 119 g/kg, ileal digestible lysine = 4.6 g/kg); at 2.8 kg/d from days 1 to 34 of pregnancy, 2.7 kg/d from days 35 to 76, and 3.3 kg/d from 76 d to farrowing. The light schedule during gestation consisted of 12 consecutive hours of light (100 lux) and 12 hours of darkness. Sows had free access to water at all times.

Transrectal ultrasonography

A list of all abbreviations is provided in Appendix 1. In experiments 1 and 2 transrectal real time B-mode ultrasonography (TUS) of the ovaries was performed using an Aquila MyVet30 LAB with a convex transducer at 7.5 MHz (Pie Medical/Esaote, Maastricht, The Netherlands). Sows were scanned in early pregnancy (21.7 ± 1.1 days of pregnancy in experiment 1, and 23.8 ± 2.4 days in experiment 2). To perform the ultrasonography, sows were placed in individual crates. The scanning procedure involved manual cleaning of the rectum and rectal insertion of a transducer covered with a disposable glove containing scanning gel to prevent the presence of air bubbles in contact with the probe. During the entire procedure lubricated disposable transrectal examination gloves were used, to minimize animal discomfort. Ovulation rate (OR_{TUS}) was considered as the total number of corpora lutea (CL) counted on both ovaries with transrectal ultrasonography. In experiment 1, the CL counting was performed by 2 examiners separately in a subset of sows (E1 and E2, n = 29), to check for inter-examiner agreement in assessment of OR_{TUS}.

Also, a movie clip of the examination of each ovary was saved (25 sows in experiment 1 and 100 sows in experiment 2) and the diameter of the 5 biggest CL on each ovary (10 per sow) was later assessed, and the average CL diameter (mm) measured by TUS was calculated ($DIAM_{TUS}$). An example of ultrasound images of the ovaries can be seen in Figure 5.1, and a movie of ovarian examination has been provided as supplementary material.

Dissection of the ovaries: ex vivo examination of the ovaries

Sows from experiment 1 (n = 45) were slaughtered at a local abattoir at 29.8 ± 1.9 d of pregnancy and the uterus and ovaries were collected. Ovulation rate was assessed by dissection of each individual corpus luteum present on left and right ovaries (OR_{DIS}).

In the 25 sows in which CL diameter was measured with TUS (farm 2), each individual corpus luteum was cleaned of connective tissue, their individual diameter was measured using a caliper rule and the average and standard deviation (SD) of CL diameter were assessed. Further, each individual CL was weighed and the average and SD of CL weight per sow was calculated. Total luteal mass was calculated as the sum of all corpora lutea weights.

The diameter (mm) of the 5 biggest CL in each ovary (10 per sow) was used to estimate the average CL diameter measured after dissection ($DIAM_{DIS}$), and the weight of the 5 CL with the highest diameter in each ovary (10 per sow) was used to assess average CL weight (g) after dissection (WT_{DIS}). All CL measurements were done by the same person.

Litter characteristics

Sows from experiment 2 (n = 100) farrowed and the length of gestation, number of piglets born alive (live born), number of piglets born dead (stillborn), and number of mummified piglets were assessed. Total number of piglets born (litter size) was defined as the sum of the number of piglets born alive and dead. Piglets were weighed within 24 h after birth and from this, average piglet birth weight (total born and live born), within litter SD of piglet birth weight (total born and live born), and total litter birth weight were calculated.

Statistical analyses

All analyses were performed using PROC MIXED in SAS 9.3 (SAS Inst. Inc. Cary, NC).

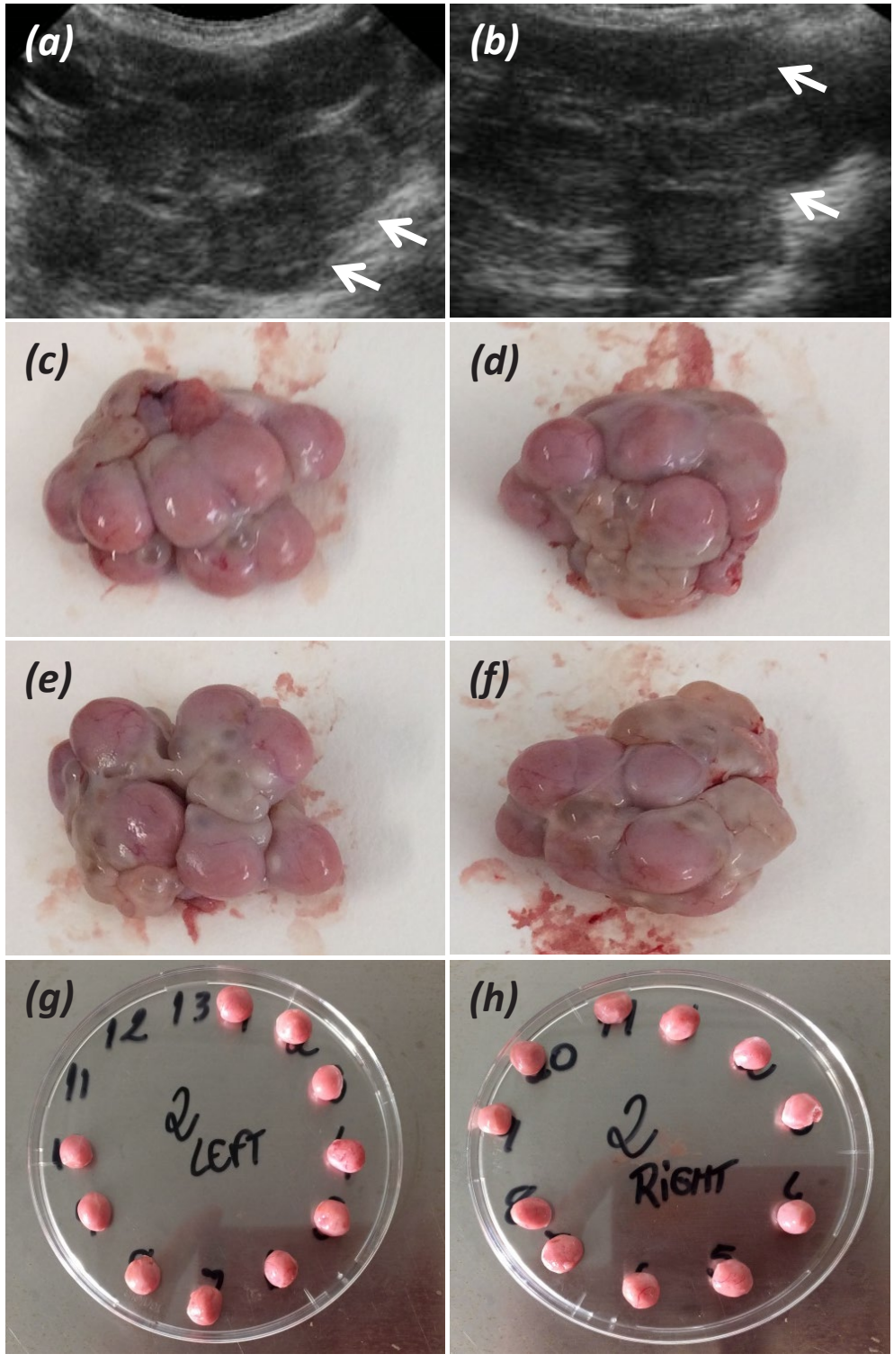
Experiment 1

Preliminary analyses showed that there was a farm difference in average OR assessed after dissection [least square means of OR_{DIS} for farm 1 was 27.5 ± 1.0 and for farm 2 was 21.4 ± 0.9, $P < 0.0001$]. Thus, all statistical models for accuracy of CL counting included the fixed class effect of farm, and the random class effect of batch to account for possible environmental variation. Statistical models for accuracy of CL diameter measurements did not have farm in the model since the measurements were done for a subset of sows coming from the same farm (n=25). There was no relationship between the parity of the sows and the CL measurements done by TUS and after ovarian dissection ($P > 0.05$), so parity was not included in the models. In all models, fixed class effects were corrected with Bonferroni and if non-significant were removed from the models. Interactions were never significant and were therefore removed from the models.

First, the relationship between the OR_{TUS} as assessed by the two examiners separately (E1 and E2) was investigated to check for inter-examiner agreement. For this, the continuous linear effect of OR_{TUS} counted by E2 was assessed on OR_{TUS} counted by E1.

Analyses on the accuracy of TUS were done using the data of E1 (n = 45). First, the difference between OR_{TUS} and OR_{DIS} in number of CL counted was calculated. Further, to check the accuracy of TUS in assessing OR (i.e. how does OR_{TUS} relates with OR_{DIS}) the continuous linear effect of OR_{DIS} was assessed on OR_{TUS}. To check if the accuracy of TUS was affected by OR_{DIS} the continuous fixed effect of OR_{DIS} was assessed on the difference between OR_{TUS} and OR_{DIS}.

Regarding CL diameter measurements, the difference between average CL diameter estimated based on TUS and after ovarian dissection were assessed (i.e. $DIAM_{TUS} - DIAM_{DIS}$). Further, to check the accuracy of TUS in measuring the diameter of the 10 biggest CL, the continuous fixed effect of the average diameter of the 10 biggest CL


(a)
(b)
(c)
(d)
(e)
(f)
(g)
12
13
11
2
LEFT
(h)
2
RIGHT

at dissection ($DIAM_{DIS}$) was assessed on the average diameter of the 10 biggest CL by TUS ($DIAM_{TUS}$). To check if the accuracy of TUS in measuring CL diameter was affected by OR_{DIS}, the continuous fixed effect of OR_{DIS} was assessed on the difference between $DIAM_{TUS}$ and $DIAM_{DIS}$.

Also, to investigate the relationship between OR and CL size, the continuous linear and quadratic effect of OR_{DIS} was assessed on $DIAM_{TUS}$, $DIAM_{DIS}$ and on average CL weight after dissection (WT_{DIS}).

Moreover, aiming to check the relationship between OR and characteristics of all CL dissected, the continuous linear and quadratic effect of OR_{DIS} was assessed on the average and standard deviation of CL diameter and weight of all CL, and on total luteal mass.

Residuals of all models approximated normality based on skewness and kurtosis. Results are presented as the regression coefficients (β) with their standard errors (SE) for the continuous linear and quadratic fixed effects and as least square means and their SE for fixed class effects. Results are considered significant at $P \leq 0.05$.

Experiment 2

To assess the effect of sow parity on CL, gestation and litter characteristics, parity was divided into 3 categories: class 1 (parities 2 and 3, n = 27), class 2 (parities 4 and 5, n = 35), and class 3 (parities 6 to 9, n = 38) and the fixed class effect of parity (parity class 1, 2 and 3) was included in the model together with the random effect of batch.

To assess relationships between the measurements of CL done by TUS and sow and litter characteristics at birth, the continuous fixed effect of $DIAM_{TUS}$, together with the fixed class effect of parity (1, 2 and 3) and the fixed class effect of litter size [class 1 (9 to 16 piglets born, n = 34); class 2 (17 to 19 piglets born, n = 36); and class 3 (20 to 26 piglets born, n = 30)] and their interaction, were assessed on litter characteristics. In all models, batch was included as a random class effect to account for possible environmental variation. If no significant, the interactions and the fixed class effects were removed from the models. Residuals from all models approximated normality based on skewness and kurtosis. Results are presented as the regression coefficients (β) with their SE for continuous fixed effects and as least squares means and their SE for fixed class effects. Results are considered significant at $P \leq 0.05$.

Results

Experiment 1

The averages and standard deviations (SD) of the corpora lutea (CL), litter and sows characteristics are presented in Table 5.1. In experiment 1, the total number of CL (mean ±

Figure 5.1 Example of ultrasound image of the ovaries (a and b, left and right ovary respectively), with the white arrows indicating individual corpus luteum. Panels c, d show the left ovary and e, f show the right ovary, before dissection. Panels g and h show the individual *corpus luteum* dissected from left and right ovary, respectively.

Table 5.1 Summary statistics of sows, corpus luteum and litter characteristics.

Variables	n	Mean	SD	Min	Max
Experiment 1					
Parity	45	7.3	3.2	2	13
TUS* pregnancy age, d	45	23.4	2.9	20	28
Slaughter pregnancy age, d	45	29.8	1.9	27	32
Ovulation rate by TUS	45	23.4	5.8	13	34
Ovulation rate after dissection	45	24.1	5.3	13	37
Average CL diameter TUS [1], mm	25	10.3	0.73	8.4	11.7
Average CL diameter DISS [2], mm	25	10.3	0.64	8.7	11.5
Average CL weight DISS [3],g	25	0.40	0.07	0.22	0.56
Total average CL diameter [4], mm	25	9.8	0.7	7.8	11.0
Total average CL weight [4], g	25	0.38	0.1	0.20	0.51
Total luteal mass [5], g	25	7.9	1.5	5.2	10.7
Experiment 2					
Parity	100	5.0	1.9	2	9
TUS pregnancy age, d	100	23.8	2.4	21	29
Gestational length, d	100	115	1.7	111	120
Average CL diameter TUS [1], mm	100	8.4	0.8	5.5	10.5
Litter size [6]	100	17.9	3.0	9	26
Number of live born	100	16.3	2.9	6	23
Number of stillborn	100	1.53	1.8	0	9
Number of mummies	100	0.42	0.8	0	5
Average piglet BW [7], g	100	1277	165	914	1618
SD BW [7], g	100	303	76	161	555
Average live born piglets BW [7], g	100	1299	167	933	1674
SD BW live born [7], g	100	292	76	115	492
Litter BW, kg	100	23	4.0	15	30

* TUS Transrectal ultrasonography.

[1] Average calculated based on the diameter of the 5 biggest corpora lutea in each ovary (10 per sow) measured by TUS.

[2] Average calculated based on the diameter of the 5 biggest corpora lutea in each ovary (10 per sow) measured with calliper rule after slaughter of the sows and ovarian dissection.

[3] Average weight of the 5 corpora lutea in each ovary (10 per sow) that had the highest diameter measured after ovarian dissection.

[4] Average diameter and weight calculated based on the measurement of all corpora lutea dissected from each ovary.

[5] Sum of the weight of all the corpora lutea dissected from the ovaries.

[6] Litter size is the sum of piglets born alive (live born) and stillborn piglets.

[7] Piglets were weighed within 24 hours after birth.

SD) assessed by TUS (OR_{TUS}) was 23.4 ± 5.8 (ranging from 13 to 34) and the number counted after dissection of the ovaries (OR_{DIS}) was 24.1 ± 5.3 (ranging from 13 to 37). The average diameter of the 10 biggest CL measured with TUS ($DIAM_{TUS}$) was 10.3 ± 0.7 mm and after dissection ($DIAM_{DIS}$) was 10.3 ± 0.6 mm. The average diameter of all CL dissected was 9.8 ± 0.7 mm with an SD of 0.8 ± 0.4 mm. The average weight of the 10 biggest CL (WT_{DIS}) was 0.40 ± 0.07 g, and the average weight of all CL dissected was 0.38 ± 0.07g. Average total luteal mass per sow was 7.9 ± 1.5g.

Relationship between CL characteristics after ovarian dissection and with TUS

Results show that there was a strong relationship between OR_{TUS} assessed by the 2 examiners, E2 and E1 ($\beta = 0.87 \pm 0.09$ CL E1/ CL E2, $P < 0.0001$, $R^2 = 0.93$, Figure 5.2A), showing that there was inter-observer agreement in the assessment of OR using TUS.

Differences in CL number measured with TUS and after ovarian dissection are presented in Figure 5.3. The average ± SD of the difference in OR assessed by TUS and OR assessed after ovarian dissection was 0.7 ± 4.7 CL (ranging from -8 up to +12 CL difference). Results show that in 24.4% of the sows OR_{TUS} differed from OR_{DIS} in 0 or 1 CL; in 28.9% of the sows the difference was 2 or 3, in 24.4% 4 or 5 and in 22.2% of the sows the difference was 6 or more. So, there was not a close relationship between OR_{DIS} and OR_{TUS} [$\beta = 0.28 \pm 0.01$ TUS/DISS, $P = 0.01$, farm 1 = 28.0 ± 1.3 and farm 2 = 20.5 ± 1.2; $P = 0.0002$, $R^2 = 0.79$; figure 5.2B]. Furthermore, there was a positive linear relationship between OR_{DIS} and the difference in OR assessed by TUS and after dissection ($\beta = 0.72 \pm 0.10$ difference/CL dissected, $P < 0.0001$, farm $P = 0.0002$, $R^2 = 0.68$). So, TUS is not an accurate method to estimate OR in sows at early pregnancy, and there is an increase in the inaccuracy of TUS with an increase in OR.

Differences in the average CL diameter measured by TUS and the average CL diameter measured after ovarian dissection ($DIAM_{TUS} - DIAM_{DIS}$) were assessed. The average ± SD of the difference between the $DIAM_{TUS}$ and $DIAM_{DIS}$ was 0.02 ± 0.20 mm (ranging from -0.36 up to 0.34 mm difference). There was a positive linear relationship between the $DIAM_{DIS}$ and $DIAM_{TUS}$ ($\beta = 1.00 \pm 0.07$ mm TUS/mm DIS; $P < 0.0001$, $R^2 = 0.96$; Figure 5.2C). Moreover, there was no relationship between OR_{DIS} and the difference between $DIAM_{TUS}$ and $DIAM_{DIS}$ ($\beta = 0.005 \pm 0.01$ mm difference/CL dissected, $R^2 = 0.44$; $P = 0.58$). So, TUS is an accurate method to estimate the average diameter of the 10 biggest CL in sows in early pregnancy and the accuracy of the measurements done by TUS is not related with an increase in OR.

Regarding the relationship between OR_{DIS} and the average CL diameter and weight of the 10 biggest CL, there was a quadratic relationship between OR_{DIS} and the $DIAM_{TUS}$ [$0.49 \pm 0.2 * OR_{DIS}$ ($P = 0.02$) – $0.01 \pm 0.0004 * OR_{DIS}^2$ ($P = 0.01$), $R^2 = 0.76$; Figure 5.2D] and $DIAM_{DIS}$ [$0.47 \pm 0.2 * OR_{DIS}$ ($P = 0.02$) – $0.01 \pm 0.0004 * OR_{DIS}^2$ ($P = 0.01$), $R^2 = 0.69$; Figure 5.2D], which shows a maximum average diameter of the 10 biggest CL of 10.5 mm at 19 ovulations for measurements done with TUS and also after dissection ($DIAM_{TUS}$ and $DIAM_{DIS}$, respectively). Also, there was a negative linear relationship between OR_{DIS} and the average weight of the 10 biggest CL ($\beta = - 0.01 \pm 0.003$ g/CL dissected, $R^2 = 0.51$; $P = 0.01$). So, the average diameter and weight of the 10 biggest CL is lower in sows with a higher OR. There was a quadratic relationship between OR_{DIS} and the average diameter of all CL measured after dissection [$0.43 \pm 0.2 * OR_{DIS}$ ($P = 0.05$) – $0.01 \pm 0.01 * OR_{DIS}$ ($P = 0.03$)], which shows a maximum average CL diameter of 10.1 mm at 18 ovulations. There was a negative linear relationship between OR_{DIS} and total average CL weight ($\beta = - 0.01 \pm 0.03$ g/CL dissected, $P = 0.002$). However, OR_{DIS} was not related with the SD in CL diameter ($P = 0.85$) and with the SD in CL weight ($P = 0.12$). Furthermore, there was a positive linear

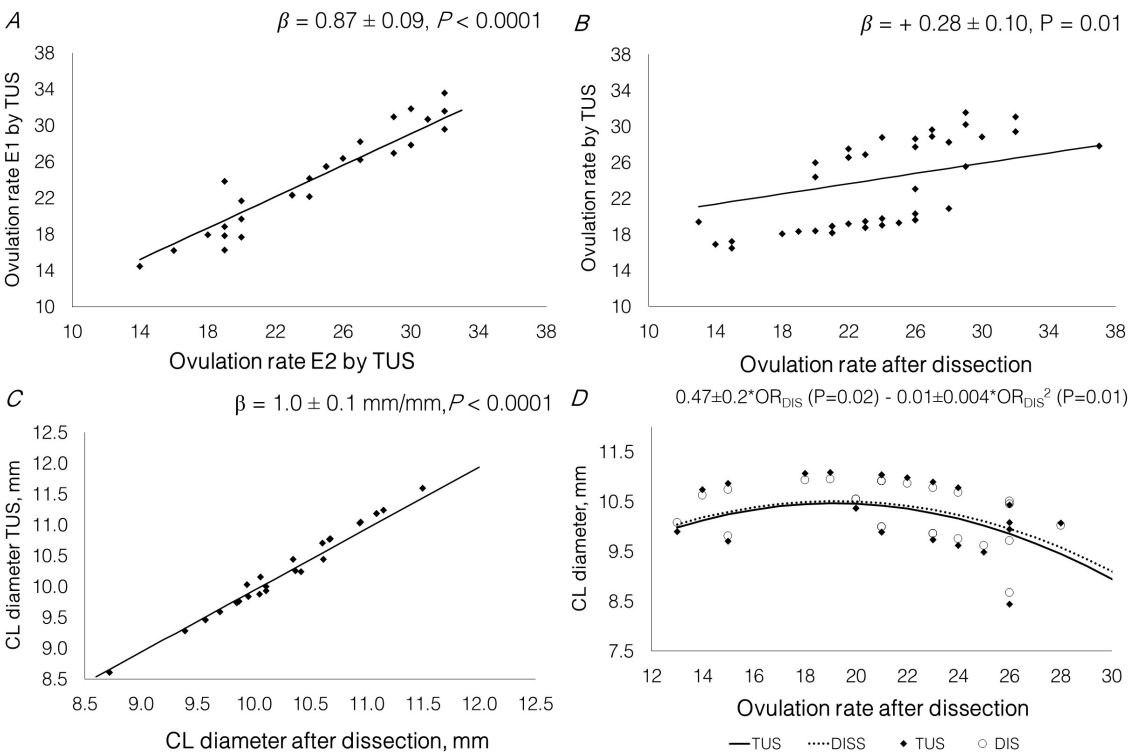


Figure 5.2 Panel A, predicted relationship between OR assessed by TUS performed by examiner 2 and the OR assessed by TUS performed by examiner 1 [(n = 29), β = 0.87 ± 0.09 CLTUS E1/CLTUS E2, P < 0.0001, farm P = 0.12]. Panel B, predicted relationship between the OR assessed by E1 by TUS and OR after dissection [β = 0.28 ± 0.01 TUS/DIS, P = 0.01, farm P = 0.0002, (n = 45)]. Panel C; predicted relationship between the average diameter of the 10 biggest CL assessed after ovarian dissection and the average diameter of the 10 biggest CL assessed by TUS [(n = 25), β = 1.0 ± 0.07 mm TUS/mm dissected, P < 0.0001]. Panel D, predicted relationship between the OR after dissection and the average CL diameter [TUS = 0.49 ± 0.19 (P = 0.02) – 0.01 ± 0.005 (P = 0.01) and DISS = 0.47 ± 0.18 (P = 0.02) – 0.01 ± 0.004 (P = 0.01)]. Statistical models included the fixed class effect of farm (n=2, for panels a and b), and the random effect of batch (n=6). Data points (♦) represent the predicted values estimated as the difference with the model residuals.

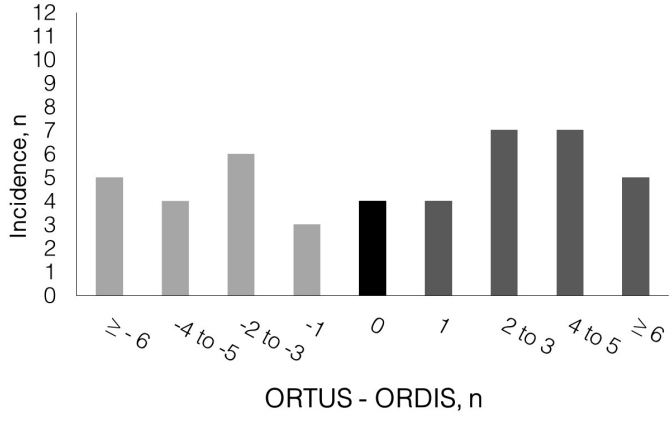


Figure 5.3 Summary of the differences between the number of corpora lutea (CL) counted with transrectal ultrasonography (OR_{TUS}) and the number of CL counted after slaughter and dissection of the ovaries (OR_{DIS}) in 45 multiparous sows at early pregnancy. Underestimations are shown in light grey and overestimations are shown in dark gray.

relationship between OR_{DIS} and total luteal mass ($\beta = + 0.19 \pm 0.1$ g/CL dissected, $P = 0.003$). So, the total average diameter and weight of the CL is lower in sows with a higher OR, but there is no increase in variation in CL size with the increase in OR. Also, the total luteal mass increases linearly with the increase in OR, despite the decrease in average CL weight.

Experiment 2

The averages and SD of sows, CL and litter characteristics are presented in Table 5.1. The average CL diameter measured with TUS was 8.4 ± 0.8 mm, ranging from 5.5 to 10.5 mm. The average total number of piglets born was 17.9 ± 3.0, and 16.3 ± 2.9 were born alive. The average birth weight (BW) of the total piglets born was 1,277 ± 165 g and the average within litter BW variation was 303 ± 76 g, the average BW of the piglets born alive was 1,299 ± 167 g and average within litter BW variation was 292 ± 76 g.

Effect of parity on gestation length, CL and litter characteristics

Parity did not affect the gestation length, the average diameter of the 10 biggest CL measured by TUS ($DIAM_{TUS}$), litter size, average and SD piglet birth weight or litter weight ($P > 0.05$). Sows in parity 6 up to 9 had a higher (2.13 ± 0.3, $P = 0.02$) number of stillborn piglets than sows in parity 2 up to 3 (0.86 ± 0.4) and 4 up to 5 (1.34 ± 0.3).

Relationship between CL diameter and litter characteristics

Relationships between $DIAM_{TUS}$ and litter characteristics are presented in Table 5.2. There was a positive linear relationship between $DIAM_{TUS}$ and average piglet birth weight [($\beta = + 37.6 \pm 17.8$ g/mm; $P = 0.04$, Figure 5.4A) + c value dependent on litter size ($P < 0.0001$): 9 to 16 piglets = 1,367 ± 25 g, 17 to 19 piglets= 1,280 ± 24 g, and 20 to 26

Table 5.2 Relationship between average corpora lutea diameter measured by transrectal ultrasonography ($DIAM_{TUS}$) and litter characteristics at birth.

Dependent Variables	$DIAM_{TUS}$ [1], mm		Litter size	Parity
	β (SEM)	P value	P value	P value
Gestational length, d	0.48 (0.26)	0.06	ns	ns
Litter size [2]	-0.098 (0.43)	0.82	-	ns
Number of live born	-0.084 (0.43)	0.84	-	ns
Number of stillborn [3]	-0.015 (0.22)	0.95	0.02	0.01
Number of mummies	-0.013 (0.10)	0.90	ns	ns
Average piglet BW [4], g	37.57 (17.84)	0.04	<.0001	ns
SD BW [4], g	24.25 (10.37)	0.02	ns	ns
Average live born piglets BW [4], g	32.98 (18.41)	0.08	<.0001	ns
SD BW live born [4], g	27.33 (10.52)	0.01	ns	ns
Litter BW, kg	0.81 (0.47)	0.09	<.0001	ns

[1] Average calculated based on the diameter of the 5 biggest corpora lutea in each ovary (10 per sow) measured by TUS.

[2] Litter size is the sum of piglets born alive (live born) and stillborn piglets.

[3] Interaction between parity (PC) and litter size (LS) classes ($P = 0.002$): PC1*LS1 (n = 9): 0.67 ± 0.55b; PC2*LS1 (n = 12): 0.48 ± 0.48b; PC3*LS1 (n = 6): 1.54 ± 0.47ab; PC2*LS2 (n = 12): 1.02 ± 0.49b; PC2*LS2 (n = 12): 2.01 ± 0.49b; PC3*LS2 (n = 11): 0.92 ± 0.49ab; PC1*LS3 (n = 13): 0.81 ± 0.66b; PC2*LS3 (n = 12): 1.58 ± 0.51ab; PC3*LS3 (n = 13): 3.83 ± 0.47a.

[4] Piglets were weighed within 24 hours after birth.

ns $P > 0.05$.

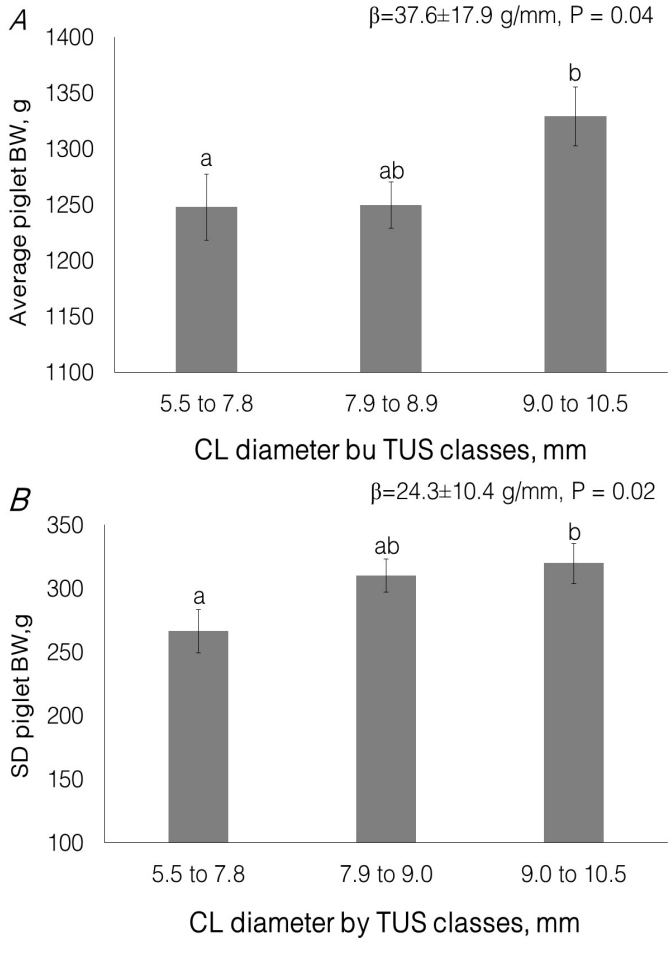


Figure 5.4 Panel A: estimated least square means for the effect of average CL diameter classes [5.5 to 7.8 mm (n = 23); 7.9 to 8.9 mm (n = 47); and 9.0 to 10.5 mm (n = 30)] on BW of total piglets born [$P = 0.04$; litter size class $P < 0.0001$]. Panel B:estimated least square means for the effect of CL diameter classes on standard deviation (SD) of BW of the total piglets born ($P = 0.02$). Statistical models included the fixed class effect of litter size [LS, 8 to 18 piglets born (n = 35); 17 to 19 piglets born (n = 36) and 20 to 26 piglets born (n = 30)] and its interactions, which were excluded from the models when not significant ($P > 0.05$). Significant differences between classes are indicated by letters above the columns and the error bars indicate the SE of the estimates.

piglets = 1,171 ± 26 g]. There was also a positive linear relationship between $DIAM_{TUS}$ and the SD in BW of the total born piglets (ß= 24.3 ± 10.4 g/mm; $P = 0.02$, Figure 5.4B) and the SD in BW of the piglets born alive (ß= 27.3 ± 10.5 g/mm, $P = 0.01$). There were no interactions between $DIAM_{TUS}$, litter size classes and parity classes. So, an increase in the average CL diameter in early pregnancy is related with an increase in average piglet birth weight and in within litter birth weight variation.

Discussion

This is, to our knowledge, the first study that investigated the accuracy of transrectal ultrasonography to assess CL number and diameter in sows in early pregnancy and investigated the relationship between CL diameter measured by transrectal ultrasonography and litter characteristics at birth.

Transrectal ultrasonography (TUS) did not provide an accurate estimation of the number of corpora lutea (OR) in sows in early pregnancy. The difference between the OR assessed after ovarian dissection and with TUS was on average 0.7 ± 4.7 CL, ranging from an underestimation of 8 CL up to an overestimation of 12 CL. Moreover, only in 24.4 % of the sows, the difference between OR assessed with TUS and after ovarian dissection was of only 1 CL, with 46.7% of the TUS estimations differing with more than 4 CL. This inaccuracy occurred almost equally due to under and over estimations of the number of CL assessed after dissection of the ovaries (33.3 and 42.2%, respectively). Underestimations are probably related with the difficulty in visualizing all individual CL. Ultrasound machines uses high frequency sound waves and their echo to produce images (Pierson et al. 1988) and different tissues have different abilities to reflect the sound waves (Pierson et al. 1988). Thus, characteristics of a tissue determine the proportion of the sound wave that will be reflected, which will then be represented on the ultrasound image display by dots of different shades of gray, varying from black to white. Liquids, like follicular fluid, do not reflect sound waves (non-echogenic) and therefore the image appears as black on the screen, in contrast with the surrounding ovarian tissue, that reflects part of the sound waves (echogenic) and can be seen as different shades of gray (Pierson et al. 1988). This contrast facilitates visualization of individual follicles and explains the high accuracy of transrectal ultrasonography in assessing the number of pre ovulatory follicles in sows. Soede et al. (1992) described a difference of only 0.4 ± 1.8 between the number of follicles counted by transrectal ultrasonography and the number of CL counted after slaughter of the sows (18.6 ± 3.5). Also Bolarin et al. (2009) observed a significant correlation between the number of pre ovulatory follicles (6 to 10 mm) counted per ovary with transrectal ultrasonography and with laparoscopy ($r = 0.98$, $P < 0.001$). Corpora lutea, however, are tissue filled glands and are echogenic, surrounded by the also echogenic tissue of the ovarian stroma, which makes visualization of each individual CL more difficult. Moreover, despite the fact that transrectal ultrasonography is believed to provide clearer images of the ovaries due to less interference of intestinal tissues (Kauffold and Althouse 2007), is still possible that the ovaries were only partially visible due to interference of the intestinal and uterine tissue of the pregnant sows.

Overestimations, on the other hand might be explained by counting CL from part of an ovary 2 times, which might happen due to the proximity of the 2 ovaries during the examination and the difficulty in distinguishing between the 2 separate ovaries. The lack of accuracy of transrectal ultrasonography in assessing the number of CL in pregnant sows might also be related with the high number of CL present in each ovary (high ovulation rate), where crowding of the ovaries makes it more difficult to differentiate the CL. Gonzalez-Añover et

al. (2009) investigated the accuracy of transrectal ultrasonography in accessing the number of CL in Iberian sows in the mid luteal phase (average ovulation rate of 6.0 ± 1.3), and achieved accuracy close to 100% in ovaries with 5 CL or less, which decreased with the increase in number of CL present per ovary. A decrease in accuracy with the increase in OR was also observed by Soede et al. (1992) and Bolarin et al. (2009) when counting the number of pre-ovulatory follicles. In the present study (average OR of 24.1 ± 5.3) there was also a decrease in accuracy of TUS (difference between OR counted by TUS and after slaughter) with the increase in OR after ovarian dissection. This could be related with the decrease in CL size with the increase in ovulation rate. Average CL diameter in sows with 13 up to 22 ovulations was predicted to be 10.3 mm, decreasing to 8.7 mm in sows with more than 23 ovulations. The same is true for the average CL weight, which can be 0.13 g smaller in sows with more than 23 ovulations in comparison with sows with 13 up to 22 ovulations. A decrease in average CL weight (and thus size, this study) with the increase in ovulation rate was also observed in gilts at 35 d of pregnancy (Da Silva et al. 2017). This indicates that with a higher OR, not only the ovaries are more crowded, but also individual CL are smaller, which might increase the difficulty in visualization of the individual CL, thus decreasing the accuracy of counting CL with TUS. The reason for the decrease in CL size with the increase in ovulation rate is not known. During follicular growth, recruited follicles respond to FSH by increasing the production of oestradiol-17β (Britt and Findlay 2002; Drummond 2006). The increase in oestradiol-17β (E2) production leads to an increase in granulosa cell number and therefore to further follicular development (Drummond and Findlay 1999). So, together with the increase in E2 production, follicles increase in size. E2 production increases until it reaches a certain systemic threshold concentration that triggers a pre-ovulatory GnRH/LH surge that subsequently triggers ovulation (Drummond and Findlay 1999; Drummond 2006). With more follicles recruited from the pool (i.e. higher OR), and therefore more follicles producing E2, the threshold of E2 necessary to trigger ovulation might occur when follicles are of smaller size than in sows with a lower OR. Indeed, Knox et al. (2003) observed that systemic E2 levels were the same at days -1 to +1 relative to the LH peak (day 0) in gilts selected for high OR (OR 18.8 ± 0.4, E2 37.9 ± 3.4 ng/mL) and in gilts of the control line (OR 14.3 ± 0.6, E2 44.7 ± 3.6 ng/mL), indicating that the threshold of E2 that precedes ovulation is independent of OR. Soede et al. (1998) observed that the average volume of pre-ovulatory follicles at ovulation per sow was significantly correlated with the average CL weight at 5 d of pregnancy ($r = 0.28$, $P < 0.01$), and Wientjes et al. (2012) observed that each mm increase in follicle diameter at ovulation was related with 1.23 mm increase in CL diameter ($P = 0.03$) in multiparous sows at 10 d of pregnancy. Thus, high OR sows may have smaller follicles at ovulation that develop into smaller CL.

We also investigated the validity of transrectal ultrasonography (TUS) to measure CL diameter in sows. Results show that TUS is an accurate method to assess CL diameter in sows at 3 to 4 weeks of pregnancy. There was a strong relationship between the CL diameter measured by transrectal ultrasonography and the CL diameter measured after dissection of the ovaries. Further, we investigated the relationship between average CL diameter assessed

by transrectal ultrasonography in early pregnancy and litter characteristics at birth and observed that there is an increase in average piglet birth weight with an increase in average diameter of the 10 biggest CL. This might indicate that sows with a higher average CL diameter ovulated oocytes of better quality, that developed into embryos with higher growth potential and consequently into piglets with higher birth weight. Indeed, in a recent study with 390 gilts slaughtered at 35 d of pregnancy we found that there is an increase of 2.3 g in the weight of the vital embryos per gram of increase in average CL weight [$P = 0.001$; C.L.A. Da Silva, *unpublished results*]. Heavier embryos at d 35 of pregnancy might develop into heavier piglets at birth.

Larger/heavier CL develop from larger follicles at ovulation, as discussed above. Larger follicles at ovulation are known to release oocytes with superior quality due to a more advanced maturational status (Hunter 2000; Gandolfi et al. 2005), which might lead to the development of embryos with higher growth potential (Krisher 2004). At d 11 and 12 of pregnancy, pig conceptuses transition from spherical to tubular and filamentous blastocysts in a process called elongation (Geisert et al. 1982a; Geisert et al. 1982b). Timing of rapid conceptus elongation is established by the conceptus (Geisert et al. 2014) and more advanced embryos [derived from the more developed oocytes, Pope et al. (1990)] elongate earlier and will have an increased uterine implantation length (Geisert et al. 1982b). The uterine implantation length is related with the placental length of vital embryos at 35 d of pregnancy in sows [$\beta = 0.98 \pm 0.14$ cm / cm, $P < 0.0001$. C.L.A. Da Silva, *unpublished results*], which will possibly favour further foetal development and consequently piglet birth weight.

Corpora lutea produce progesterone, which is of primary importance for maintenance of the pregnancy in the pig (Spencer et al. 2004). So, it could be hypothesized that higher CL size in pregnant sows is related with higher progesterone production favouring embryonic growth and piglet birth weight. However, systemic progesterone levels in 238 gilts at 35 d of pregnancy (C.L.A Da Silva, *unpublished results*), were not related with average CL weight ($P = 0.69$). It could also be possible that average CL weight and piglet birth weight have a common origin in early pregnancy. Corpora lutea regression occurs on d 15 to 16 of the oestrus cycle due to the increase in pulsatile endometrial secretion of prostaglandin F2-α (PGF-2α,) (Moeljono et al. 1976). Thus, pregnancy recognition requires the development and maintenance of CL beyond the luteal phase, and is a result of oestrogens (mainly oestradiol-17β, E2) secretion by the conceptuses on d 11 and 12, followed by a second peak between days 15 and 25 through 30 of pregnancy (Geisert et al. 1990). Conceptuses E2 increases luteal LH receptor concentration, and together with Il-β1, favours the production of luteoprotective PGE2, which stimulates the expression of Vascular Endothelial Growth Factor (VEGF) in luteal cells, increasing luteal permeability and delivery of cholesterol to the luteal cells (Ziecik et al. 2011), which might favour CL growth. So, conceptus development at the time of elongation and maternal recognition of pregnancy may influence luteal vascularization (throughout the effects of PGE2 and VEGF), and thereby increase CL size. However, further investigations are needed to understand the mechanisms underlying the relationship between CL size, embryonic growth and piglets birth weight.

An increase in average CL diameter was not only related with average piglet birth weight as discussed above, but also with an increase in within litter variation in piglet birth weight. Within litter piglet birth weight variation increases with the increase in litter size (Milligan et al. 2002; Quiniou et al. 2002; Wolf et al. 2008), and is seen as a consequence of uterine crowding on placental development in the early post implantation period (Foxcroft et al. 2006). However, in the current study, we did not observe a higher variation in piglet birth weight in bigger litters. Before uterine implantation, an increase in variation in the length of implantation in the uterus will contribute to variation in timing and capacity to establish an adequate surface area for placentation, thus increasing variation in foetal growth and in birth weight (Yuan et al. 2015). But it is also possible that this increase in variation is caused by a limitation in uterine capacity imposed by the increased competition during foetal growth, i.e. an increase in competition for blood flow and nutrient uptake between littermates, which might also lead to variation in foetal growth and in piglet birth weight (Ford et al. 2002).

In conclusion, transrectal ultrasonography is not a valid method to assess OR in sows in early pregnancy, but it is a valid method to assess CL diameter. Also, a higher average CL diameter measured by transrectal ultrasonography in sows around 25 d of pregnancy was related with a higher average piglet birth weight and with a higher within litter variation in piglet birth weight. This might be related with the influence of follicular/oocyte quality in embryonic development. However, the mechanisms leading to increase in birth weight and in birth weight variation needs further investigation.

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APPENDIX 1 LIST OF ABBREVIATIONS

Abbreviations	Description
TUS	Transrectal ultrasonography
OR	Ovulation rate, i.e. the total number of CL present in both ovaries
CL	Corpora lutea
OR_{TUS}	Total number of corpora lutea counted on both ovaries with transrectal ultrasonography
E1	Transrectal ultrasonography examiner one
E2	Transrectal ultrasonography examiner two
$DIAM_{TUS}$	The average diameter of the 10 biggest corpora lutea measured by transrectal ultrasonography in a sow
OR_{DIS}	Total number of corpora lutea counted after slaughter and dissection of the ovaries for individual corpora lutea
$DIAM_{DIS}$	The average diameter of the 10 biggest corpora lutea measured with a calliper rule after slaughter and dissection of the ovaries for individual corpora lutea
WT_{DIS}	The average weight of the 10 biggest corpora lutea measured after slaughter and dissection of the ovaries for individual corpora lutea
LS	Litter size, i.e. the sum of the number of piglets born alive and dead
BW	Piglet birth weight

CHAPTER 6

Consequences of genetic selection for litter traits at birth on ovarian and embryonic traits in gilts and their genetic background

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Abstract

We investigated how genetic selection based on estimated breeding values (EBV) for total number of piglets born (TNB), average piglet birth weight (BW), and for within litter piglet birth weight standard deviation (BWSD) influences ovulation rate (OR), average corpora lutea (CL) weight and embryonic survival and development at 35 days of pregnancy, and estimated the additive genetic variance of these underlying traits using pedigree information. Landrace (n = 86) and Yorkshire x Landrace (n = 304) gilts were inseminated with semen stored for 3 to 5 days (n =109), 6 to 7 days (n = 159) or 8 to 10 days (n = 122) and slaughtered at 35 days of pregnancy. Ovulation rate (OR) was assessed by dissection of the corpora lutea (CL) on both ovaries. Individual CL were weighed and the average CL weight calculated. The number of embryos (total and vital) were counted and the vital embryos were individually weighed for calculation of within litter average and SD of the embryo weight. Length of the uterine implantation site of the vital embryos was measured and the average per gilt calculated. Results show that an increase in EBV for TNB is related with a proportional increase in OR and number of embryos, while decreasing the average CL weight. On the contrary, an increase in EBV for BW and for BWSD is related with an increase in average CL weight. There was no relationship between the EBVs for BW and for BWSD and vital embryonic weight at 35 days of pregnancy. Ovulation rate, average CL weight, number of embryos, average weight and implantation length of the vital embryos had all moderate to high heritabilities, ranging from 0.36 to 0.70. Thus, results indicate that there is ample genetic variation in ovulation rate, average CL weight and embryonic development traits. This knowledge could be used to optimize the balance between selection for litter size, average piglets birth weight and within litter birth weight uniformity.

Keywords: precision phenotyping, ovulation rate, corpora lutea weight, embryo, gilts.

Introduction

Genetic selection for total number of piglets born (TNB) has been successful and more than 30 piglets weaned per sow per year can be achieved nowadays. However, genetic selection for sows ability to farrow a high number of piglets has led to a decrease in piglet mean birth weight (Quiniou et al. 2002), and an increase in within litter birth weight variation (Quiniou et al. 2002; Wolf et al. 2008). These negative associations between litter traits are partly genetic (Damgaard et al. 2003; Wolf et al. 2008), which makes it difficult to improve all traits simultaneously. It has been suggested that genetic selection for TNB has altered the balance between other litter component traits, specifically ovulation rate (OR) and uterine capacity, resulting in uterine crowding and compromised embryonic and foetal development (Foxcroft et al. 2006; Da Silva et al. 2016; Da Silva et al. 2017a). Ovulation rate (OR), the major genetic component of TNB (Schneider et al. 2014), has increased disproportionally due to genetic selection for TNB (Blasco et al. 1993), reaching averages of 25 up to 30 (Patterson et al. 2008; Da Silva et al. 2016). In sows, an increase in OR is related with a decrease in placental length of the vital embryos at 35 days of pregnancy (Da Silva et al. 2016), and in gilts an increase in OR is related with a higher variation in vital embryonic weight at 35 days of pregnancy (Da Silva et al. 2017a). This might compromise further foetal development leading to foetal mortality, but it might also lead to a lower average piglet BW and higher within litter BW variation. Therefore, knowledge about the underlying genetics of ovarian, uterine and embryonic development characteristics might help understanding the mechanisms leading to litter characteristics at birth and the physiological consequences of genetic selection for litter traits at birth. Moreover, OR, embryonic survival and development traits are new phenotypic traits that could be used to improve litter characteristics at birth. Thus, the objectives of this study were 1) to investigate the relationship between the estimated breeding values for litter traits at birth and ovulation rate, average corpora lutea weight, uterine length and embryonic survival and development traits in gilts at 35 days of pregnancy, 2) to estimate the genetic variation of OR, average corpora lutea weight, uterine length and embryonic survival and development traits at 35 days of pregnancy and 3) to estimate the genetic correlations between these traits.

Material and methods

Ethics and approval statement

The experiment and all measurements were approved by the Animal Welfare Committee of Wageningen University and Research in compliance with the Dutch Law on Animal Experimentation. The experiment was conducted between May and December 2016 at Wageningen University and Research (Wageningen, The Netherlands).

Animals and housing

The study included a total of 390 pregnant gilts, from one farm, being 304 crossbred (C) gilts (Yorkshire x Landrace; Topigs Norsvin, Vught, The Netherlands) and 86 purebred (P) Landrace gilts (Topigs Norsvin, Vught, The Netherlands), which were used in 18 batches, one batch per week.

The gilts were group housed (6 animals per 8 m^2), with individual feeding stations and received liquid feeding. From weaning at day 25 till day 49 of age, gilts were fed a starter diet (9.68 NE MJ/kg, 9.13 g/kg of ileal digestible lysine), from day 50 up to day 105 gilts were fed a rearing diet (9.42 NE MJ/kg, 8.03 g/Kg of ileal digestible lysine), and from day 106 until first insemination gilts were fed a second rearing diet (9.24 NE MJ/Kg, 7.35 g/kg of ileal digestible lysine). During the first 70 days the gilts were fed three times a day and from 71 days onwards the gilts were fed twice a day. Gilts had free access to water at all times.

Gilts were inseminated at 248.4 ± 16.6 days (ranging from 212 to 292), 1 or 2 times with semen stored for 6.5 ± 1.6 days (ranging from 3 to 10 days). The semen was collected from 17 boars from the Tempo breeding line (Topigs Norsvin, Vught, The Netherlands). The Tempo boar is bred from a Topigs Norsvin E-line (Large White type). Semen was processed at one Specific Pathogen Free (SPF) artificial insemination station (Varkens KI Nederland, Vught, the Netherlands) and insemination doses of 1.2 billion cells per 80 ml were produced. Semen was stored and transported to the farm at 17°C ± 2°C.

The weight at first insemination for the P and C gilts was 165.6 ± 2.9 Kg vs 154.5 ± 2.3 Kg, respectively ($P \leq 0.05$), with an average back fat thickness of 14.0 ± 0.3 mm for P and 13.3 ± 0.2 mm for C gilts. Gilts were slaughtered at 34.7 ± 0.9 days of pregnancy (32 up to 37 days) with an average weight of 180.0 ± 15.5 Kg.

Ovarian, embryonic and uterine measurements

After slaughter, uterus and ovaries of the gilts were collected. Ovulation rate (OR) was assessed by dissection of each individual corpus luteum present on left and right ovaries. After dissection, individual *corpus luteum* were cleaned of remaining connective tissue and individually weighed to assess average and standard deviation of corpora lutea (CL) weight (g). Total luteal mass was calculated as the sum of all corpora lutea weights.

Both uterine horns were separated from the mesometrium and opened at the anti-mesometrial side. After opening the uterus, embryos were classified as vital, non-vital or degenerated according to their visual appearance and were considered as non-vital when there was haemolysed amniotic fluid, and degenerated when there were resorbed embryonic membranes or evidence of implantation, combined or not with placental or embryonic remnants (van der Waaij et al., 2010). After classification, embryos were separated from their placentas and counted. The total number of embryos was calculated as the sum of the vital embryos, non-vital embryos and of the degenerated embryos. The difference between OR and the total number of embryos was considered as early embryonic mortality, and the non-vital plus degenerated embryos were considered as late embryonic mortality.

The embryonic-placental units were separated from the uterine wall. After removal of the embryonic-placental units, implantation sites were identified by reddening of the endometrium, compared to the whiter area (unoccupied/empty uterine space) in between. The length and width of each implantation site on the uterine wall containing a vital embryo was measured and vital implantation area was calculated as the product of implantation length and implantation width. The length of the left and right uterine horns were measured on a wet surface, from the utero-tubal junction to the uterine body. Total uterine length (cm) was measured as the sum of the left and right uterine horn length. In 254 of 390 gilts (batches 7 to 18), all vital embryos were individually weighed for assessment of average and standard deviation of vital embryonic weight (g).

Relationship between estimated breeding values and the phenotypic traits

Analyses on the relationship between the estimated breeding values (EBV) for gilts litter characteristics at birth and gilts ovarian, uterine and embryonic characteristics at 35 days of pregnancy were performed using PROC MIXED in SAS 9.3 (SAS Inst. Inc., Cary, NC). Estimated Breeding Values (EBV) are best estimates of genetic merit. They come from the daily routine of Topigs Norsvin Research Center, where 17 reproductive traits are analyzed simultaneously in a multi-trait single step genomic BLUP evaluation (this includes genomic information for genotyped animals); data of 3 million sows and 10 million litters of a multitude of lines and crosses are used and corrected for known fixed effects as herd, year, season and parity.

For clarification, an increase of one EBV for TNB indicates an increase of one genetic piglet, an increase of one EBV for BW indicates an increase of one genetic Kg in piglet birth weight, and an increase of one EBV for BWSD indicates an increase of one genetic gram in within litter piglet birth weight standard deviation. Therefore, higher EBV for TNB and for BW indicates a higher genetic potential for higher litter size and higher average piglet birth weight, while higher EBV for BWSD indicates a higher variation and a lower genetic potential for within litter piglet birth weight uniformity.

Estimated breeding values for total number of piglets born (EBV_TNB), average piglet birth weight (EBV_BW) and within litter standard deviation of piglet birth weight (EBV_BWSD) were provided by Topigs Norsvin based on their routine genetic evaluation. The effects of EBV_TNB, EBV_BW and EBV_BWSD were fitted as linear regressions.

In all models, batch was included as a random effect to account for possible environmental variation. All models included the fixed class effects of gilts genetic line (GL) and of semen storage duration classes (SS), and the interactions between GL and SS, plus the interactions between GL, SS with the EBVs. The fixed class effects and interactions were excluded from the models when not significant. Fixed class effects of GL and SS were adjusted using Bonferroni. Residuals of all models were approximately normality distributed. Results were considered different at $P \leq 0.05$ and are presented as regression coefficients (β) with their SE.

Genetic parameters

The following linear animal model was used for estimating variance components for the reproductive traits:

$$Y_{ijx} = \mu + GL_i + SS_j + a_x + e_{ijx}$$

where Y_{ijx} are the reproductive traits [ovulation rate, average and standard deviation (SD) of CL weight, total lutal mass, uterine length, number of embryos, early and late embryonic mortality, average and SD of vital embryonic weight, of empty uterine space around the vital embryos, of vital embryos uterine implantation length, and area], μ is the overall mean, GL_i is the fixed class effect of genetic line (86 Landrace and 304 Yorkshire x Landrace), SS_j is the fixed class effect of semen storage duration classes (SS1= 3-5 days, n=109; SS2= 6-7 days, n= 159 and SS3= 8-10 days, n= 122), a_x is the random additive genetic effect of the xth animal assumed to be ~N(0, $\mathbf{A}\sigma_a^2$), and e_{ijx} is the residual term assumed to be ~N(0, $\mathbf{I}\sigma_e^2$). Assumed (co)variance structures of the random model terms are $\mathbf{A}\sigma_a^2$, and $\mathbf{I}\sigma_e^2$, in which **A** is the additive genetic relationship matrix, σ_a^2 is the additive genetic variance, **I** is an identity matrix, and σ_e^2 is the residual variance. The pedigree used to construct **A** consisted of 5,082 individuals, and was based on 7 generations of ancestors.

Heritabilities were calculated as:

$$h^2 = \frac{\sigma_a^2}{\sigma_p^2}$$

and phenotypic variance was $\sigma_p^2 = \sigma_a^2 + \sigma_e^2$, where σ_a^2 is the additive genetic variance, and σ_e^2 is the residual variance. Genetic correlations between traits were estimated using bivariate versions of the model given estimating both genetic correlations and residual correlations between traits. Phenotypic correlations were calculated by summing the genetic and residual covariance divided by the phenotypic standard deviations of both traits. Fixed effects were tested for significance by an incremental Wald F statistics analysis ($P \leq 0.05$). Analyses were performed using by ASReml 4.0.

Results

Descriptive statistics are shown in Table 6.1. In total 390 pregnant gilts, of which 86 purebred Landrace and 304 crossbred Yorkshire x Landrace were phenotyped at 35 days of pregnancy for ovarian, uterine and embryonic characteristics. Average ovulation rate (OR) was 20.7 ± 3.0, ranging from 14 up to 34; average corpora lutea (CL) weight was 0.44 ± 0.1 g, ranging from 0.27 to 0.61 g. Early embryonic mortality was on average 4.5 ± 4.3 and late embryonic mortality 1.2 ± 1.5. The number of vital embryos was 15.0 ± 4.1, with an average weight of 4.2 ± 0.8 g and an average uterine implantation length of 21.7 ± 4.3 cm. Within litter variation in vital embryo weight was 0.4 ± 0.1 g, ranging from 0.2 to 1.0 g and in uterine implantation length 5.4 ± 1.8, ranging from 2.1 to 29.2 cm.

Estimated breeding values of the gilts are based on pedigree information. The average estimated breeding value for total number of piglets born was -0.09 ± 0.61, ranging from -1.80 to 2.10; for average piglet birth weight it was 31.0 ± 76.7 g; ranging from

Table 6.1 Summary statistics of phenotypic traits (ovarian, uterine and embryonic characteristics) and of the estimated breeding values (EBV) of litter traits of gilts at 35 days of pregnancy.

Variables	n	Mean	SD	Min	Max
Averages					
Ovulation rate, OR	390	20.69	3.04	14	34
Average corpus luteum weight, g	390	0.44	0.06	0.27	0.61
Total luteal mass, g	390	9.10	1.56	5.31	16.43
Uterine length, cm	390	502.03	63.30	346	675
Number of embryos, n	390	16.19	4.32	3	26
Number of vital Embryos, n	390	14.97	4.05	3	24
Early embryonic mortality, n	390	4.50	4.32	-2	21
Late embryonic mortality, n	390	1.22	1.45	0	9
Vital embryonic weight, g	254	4.17	0.77	2.51	6.79
Vital empty space, cm	390	21.9	19.5	5.7	231.3
Vital implantation length, cm	390	21.66	4.31	11.27	38.4
Vital implantation area, cm^2	390	192.66	44.83	77.37	397.78
Standard Deviations					
Corpus luteum weight, g	390	0.05	0.02	0.02	0.13
Vital embryo weight, g	254	0.39	0.14	0.15	0.97
Vital empty space, cm	390	11.2	10.3	2.1	103.7
Vital implantation length, cm	390	5.40	1.81	2.08	29.16
Estimated Breeding Values					
Total number of piglets born, n	390	-0.09	0.61	-1.80	2.10
Average piglet birth weight, Kg	390	0.031	0.077	-0.246	0.282
Within litter birth weight variation, g	390	-0.04	15.7	-51.2	40.3

-245.6 g to up to 282.3 g; and for within litter birth weight variation it was -0.04 ± 15.7 g, ranging from -51.2 g to up to 40.3 g.

Effect of genetic line and semen storage duration on ovarian, uterine and embryonic characteristics

Effects of genetic line and semen storage duration classes on ovarian, embryonic and uterine characteristics are presented in Table 6.2. Purebred Landrace gilts had a higher ovulation rate (OR) than the crossbred Yorkshire x Landrace gilts (22.1 ± 0.4 vs 20.3 ± 0.2, $P < 0.0001$) and also a higher incidence of early embryonic mortality (6.2 ± 0.5 vs 4.0 ± 0.2, $P < 0.0001$). Also the vital embryos of the purebred Landrace gilts had a higher weight (4.4 ± 0.2 vs 4.2 ± 0.1 g, $P = 0.05$), and a higher length and area of uterine implantation (22.9 ± 0.5 vs 21.3 ± 0.2 cm, $P = 0.003$ and 213.2 ± 4.8 vs 187.6 ± 2.5 cm^2, $P < 0.0001$, respectively). These effects were independent of the semen storage duration.

The duration of semen storage affected the incidence of early embryonic mortality irrespective of genetic line. Gilts inseminated with semen stored for 8 to 10 days had higher early embryonic mortality (6.2 ± 0.4) than gilts inseminated with semen stored for 6 to 7 days (4.8 ± 0.4) and with semen stored for 3 to 5 days (4.3 ± 0.4). This shows that gilts inseminated with semen stored for longer time have a higher estimation of early embryonic

Table 6.2 Least square means and SEM for ovarian and embryonic characteristics of gilts at 35 days of pregnancy for two different genetic lines (GL, Purebred Landrace and Crossbred Yorkshire x Landrace, respectively) and three different semen storage duration classes (SS; 3-5, 6-7 and 8-10 days, respectively). Batch (n=18) was included in the models as a random effect.

Variables	Genetic Line (GL)		Semen Storage Classes (SS, d)			P Values		
	P	C	3 -5	6-7	8-10	GL	SS	GL*SS
n=	86	304	109	159	122			
Averages								
Ovulation Rate, OR	22.10±0.35	20.31±0.19	21.06±0.32	21.13±0.27	21.42±0.31	**<.0001**	0.62	NS
Corpus luteum weight, g	0.44±0.01	0.44±0.01	0.44±0.01	0.45±0.01	0.44±0.01	0.68	0.26	NS
Total luteal mass, g	9.68±0.3	8.97±0.17	9.26±0.22	9.41±0.20	9.30±0.21	**0.006**	0.74	NS
Uterine length, cm	493.40±7.6	504.25±4.2	504.71±6.9	503.13±5.8	488.63±6.6	0.20	0.09	NS
Number of embryos [1]	15.61±0.5	16.33±0.3	15.93±0.5	16.56±0.4	15.42±0.5	0.18	0.18	**0.008**
Number of vital embryos [2]	14.46±0.5	15.13±0.2	14.88±0.5	15.20±0.4	14.32±0.5	0.18	0.34	**0.004**
Early embryonic mortality	6.18±0.5	4.03±0.2	4.30±0.4a	4.84±0.4a	6.17±0.4b	**<.0001**	**0.002**	NS
Late embryonic mortality	1.19±0.2	1.20±0.1	1.16±0.2	1.36±0.1	1.07±0.2	0.98	0.25	NS
Embryo weight, g	4.39±0.2	4.16±0.1	4.44±0.2a	4.31±0.1a	4.06±0.2b	**0.05**	**0.002**	NS
Empty space [3], cm	23.44±2.3	21.46±1.2	22.58±2.3	20.95±1.8	23.81±2.3	0.44	0.60	**0.03**
Implantation length, cm	22.86±0.5	21.30±0.2	21.85±0.4	22.18±0.4	22.21±0.4	**0.003**	0.77	NS
Implantation area, cm^2	213.17±4.8	187.56±2.5	204.2±4.5	196.7±3.7	200.2±4.3	**<.0001**	0.39	NS
Standard Deviations								
Corpus luteum weight, g	0.05±0.003	0.05±0.002	0.05±0.002	0.05±0.002	0.05±0.002	0.47	0.08	NS
Embryo weight, g	0.42±0.02	0.39±0.02	0.40±0.02	0.39±0.02	0.42±0.02	0.21	0.46	NS
Empty space [4], cm	11.58±1.1	11.05±0.6	11.51±1.2	10.71±0.9	14.40±2.1	0.68	0.76	**0.009**
Implantation length [5], cm	5.78±0.2	5.31±0.1	5.94±0.2	5.30±0.2	5.41±0.2	**0.05**	0.06	**0.03**

[1] Least square means estimates for the interactions genetic line*semen storage duration (P=0.008): Purebreds*SS1=14.4±0.9a; Purebreds*SS2=17.0±0.7ab; Purebreds*SS3=15.5±0.9ab; Crossbreds*SS1=17.5±0.5b; Crossbreds*SS2=16.2±0.4ab; Crossbreds*SS3=15.3±0.4a.

[2] Least square means estimates for the interactions genetic line*semen storage duration (P=0.008): Purebreds*SS1=13.4±0.8a; Purebreds*SS2=15.6±0.6ab; Purebreds*SS3=14.4±0.9ab; crossbreds*SS1=16.4±0.4b; Crossbreds*SS2=14.5±0.4ab; Crossbreds*SS3=14.2±0.4a.

[3] Least square means estimates for the interactions genetic line*semen storage duration (P=0.03): Purebreds*SS1=27.9±4.1ab; Purebreds*SS2=18.4±3.1ab; Purebreds*SS3=24.0±4.2ab; Crossbreds*SS1=17.3±2.2a; Crossbreds*SS2=23.5±1.8b; Crossbreds*SS3=23.6±1.9b.

[4] Least square means estimates for the interactions genetic line*semen storage duration (P=0.01): Purebreds*SS1=14.4±2.1a; Purebreds*SS2=8.98±1.6b; Purebreds*SS3=11.4±2.2ab; Crossbreds*SS1=8.6±1.1b; Crossbreds*SS2=12.4±0.9a; Crossbreds*SS3=12.1±1.0a.

mortality. Gilts inseminated with semen stored for 8 to 10 days had a lower average weight of the vital embryos (4.1 ± 0.2 g) than gilts inseminated with semen stored 3 to 5 days or 6 to 7 days (4.4 ± 0.2 g and 4.3 ± 0.1 g, respectively).

There was a significant interaction between gilts genetic line and the classes of semen storage for total number of embryos, number of vital embryos, average and standard deviation of empty uterine space around the vital embryos and standard deviation of uterine implantation length of the vital embryos. Crossbred gilts had the highest number of total and vital embryos when inseminated with semen stored for 3 to 5 days, resulting in a significant difference in embryo numbers for crossbred gilts at the same semen storage class (3 to 5 days) compared to crossbred gilts inseminated with semen stored for 8 to 10 days (total: 17.5 ± 0.5 vs 15.3 ± 0.4; vital: 16.4 ± 0.4 vs 14.2 ± 0.4). Also there was a significant difference in number of embryos between purebred and crossbred gilts inseminated with semen stored for 3 to 5 days (total: 14.4 ± 0.9 vs 17.5 ± 0.5; vital: 13.4 ± 0.8 vs 16.4 ± 0.4). On the other hand, the number of embryos (total and vital) did not significantly differ between purebred gilts in different semen storage classes. This indicates that there is a higher reduction in the number of vital embryos in crossbred gilts inseminated with semen stored for more than 5 days than in purebreds.

The average and standard deviation of the empty uterine space around the vital embryos were higher in purebred than in crossbred gilts (27.9 ± 4.1 vs 17.3 ± 2.2 cm and 14.4 ± 2.1 vs 8.6 ± 1.1 cm, respectively) when the inseminations were done with semen stored for 3 to 5 days. This indicates that inseminations using semen stored for a shorter time in purebred gilts resulted in less embryos and therefore more empty uterine space around the vital embryos.

The standard deviation of the uterine implantation length was higher in purebred gilts inseminated with semen stored for 3 to 5 days than in crossbred gilts inseminated with semen stored for 8 to 10 days. This indicates that inseminations using semen stored for a shorter time in purebred gilts results more variation in embryonic development.

Relationship between estimated breeding values for litter traits and ovarian, uterine and embryonic characteristics

The regression equations for the relationship between Estimated Breeding Values (EBV) for total number of piglets born (EBV_TNB), average piglet birth weight (EBV_BW) and within litter birth weight standard deviation (EBV_BWSD) and ovarian, uterine and embryonic characteristics of gilts at 35 days of pregnancy are presented in Table 6.3.

There were hardly any interactions between the EBVs and gilts genetic line and semen storage duration classes. An increase in EBV_BW was related with the variation in the vital embryos implantation length. However, this relationship was

[5] Least square means estimates for the interactions genetic line*semen storage duration (P=0.03): Purebreds*SS1=6.59±0.4a; Purebreds*SS2=5.26±0.3ab; Purebreds*SS3=5.50±0.4ab; Crossbreds*SS1=5.28±0.2ab; Crossbreds*SS2=5.35±0.2ab; Crossbreds*SS3=5.31±0.2b.

Table 6.3 Relationship between the gilts estimated breeding values for total number of piglets born (EBV TNB), average piglet birth weight (EBV BW) and within litter piglet birth weight standard deviation (EBV BWSD) and their ovarian, uterine and embryonic survival and development characteristics at 35 days of pregnancy. Results come from three different models (one for each EBV).

Variables	EBV TNB, n				EBV BW, Kg				EBV BWSD, g			
	β ± SE	P value	GL	SS	β ± SE	P value	GL	SS	β ± SE	P value	GL	SS
Averages												
Ovulation rate, OR	1.12±0.2	**<.0001**	**	-	3.18±2.9	0.08	**	-	0.05±0.01	0.62	**	-
Corpus luteum weight, g	-0.011±0.004	**0.02**	ns	-	0.14±0.04	**0.0006**	ns	-	0.0006±0.0002	**0.003**	ns	-
Total luteal mass, g	0.27±0.12	**0.02**	**	-	4.35±1.48	0.11	**	-	0.024±0.01	**<.0001**[B]	*	-
Uterine length, cm	14.1±5.3	**0.001**	ns	ns	17.2±42.2	0.68	ns	ns	-0.20±0.2	0.34	ns	ns
Number of embryos	1.22±0.4	**0.001**	ns	ns	6.37±4.2	0.60	ns	ns	0.06±0.02	0.25	ns	**
Number of vital embryos	1.12±0.3	**0.002**	ns	ns	5.72±3.9	0.69	ns	ns	0.05±0.03	0.76	ns	ns
Early embryonic mortality	-0.13±0.4	0.70	**	**	-0.17±2.9	0.95	**	**	0.01±0.01	0.33	**	**
Late embryonic mortality	0.10±0.1	0.42	ns	ns	-0.59±0.97	0.55	ns	ns	-0.004±0.01	0.44	ns	ns
Embryo weight, g	-0.05±0.1	0.41	*	**	-0.065±0.49	0.90	ns	**	0.001±0.003	0.68	ns	**
Empty space, cm	-3.3±1.6	**0.04**	ns	ns	-3.63±13.02	0.78	ns	ns	-0.25±0.1	0.52	ns	ns
Implantation length, cm	1.1±0.7	0.76	**	ns	3.67±2.9	0.21	*	ns	0.05±0.03	0.36	ns	ns
Implantation area, cm^2	12.3±7.8	0.22	**	ns	53.06±29.9	0.08	**	ns	0.31±0.14	**0.03**	**	ns
Standard Deviations												
Corpus luteum weight, g	-0.003±0.002	0.07	ns	ns	0.0097±0.002	0.51	ns	ns	-0.00001±0.0001	0.94	ns	ns
Embryo weight, g	0.02±0.01	0.09	ns	ns	-1.62±0.46	0.58	ns	ns	-0.003±0.002	0.13	ns	ns
Empty space	-1.9 ± 0.9	**0.03**	ns	ns	2.34±7.02	0.74	ns	ns	-0.035±0.04	0.34	ns	ns
Implantation length, cm	0.21±0.3	0.52	ns	ns	0.75±2.2	**0.03**[A]	ns	ns	0.02±0.1	0.62	*	*

[A] Interaction between semen storage duration classes and EBV_BW (P = 0.01): SS1 = 5.3 ± 0.1 + 7.5 ± 5.2*EBV_BW; SS2 = 5.3 ± 0.1 – 0.081 ± 0.49*EBV_BW; SS3 = 5.3 ± 0.1 + 0.75 ± 2.21*EBV_BW.

[B] Interaction between genetic line and EBV_BWSD (P = 0.01): Purebreds: 9.7 ± 0.4 + 0.005 ± 0.004*EBV_BWSD; Crossbreds: 9.0 ± 0.2 + 0.03 ± 0.01*EBV_BWSD.

dependent on semen storage duration classes (P = 0.01). In gilts inseminated with semen stored for 6 up to 7 days, an increase in EBV for BW was related with a decrease in variation in vital embryos implantation length [β = - 0.08 ± 0.49 cm / Kg of EBV]. Thus, in gilts inseminated with semen stored for 6 to 7 days, differently from gilts inseminated with semen stored for 3 to 5 or 8 to 10 days, there is no increase in variation in vital embryos implantation length with the increase in EBV for BW. Also, an increase in EBV_BWSD was related with the total luteal mass. However, this relationship was different in Landrace and Yorkshire x Landrace gilts (P = 0.01).

Although in both genetic lines an increase in EBV for BWSD was related with an increase in total luteal mass, in Landrace gilts an increase in EBV for BWSD was related with an increase in total luteal mass of + 0.005 ± 0.004 g / g of EBV; and in Yorkshire x Landrace gilts an increase in EBV for BWSD was related with an increase in total luteal mass of + 0.03 ± 0.01 g / g of EBV. Thus, gilts with a higher genetic potential for within litter birth weight variation (i.e. lower uniformity) have a higher total luteal mass at 35 days of pregnancy.

An increase in the EBV_TNB was related with an increase in OR (β= 1.1 ± 0.2 CL / EBV, P < 0.0001) and with a decrease in average CL weight (β = -0.01 ± 0.01 g / EBV, P = 0.02). Thus, a higher genetic potential for total number of piglets born is related with a higher OR but with a lower average CL weight.

An increase in the EBV_TNB was related with an increase in the total number of embryos (β = 1.2 ± 0.4 embryos / EBV, P = 0.001) and in the number of vital embryos (β = 1.1 ± 0.3 embryos / EBV, P = 0.002) in gilts at 35 days of pregnancy, but not with early and late embryonic mortality (P > 0.05). Thus, gilts with a higher genetic potential for total number of piglets born have a higher number of vital embryos at 35 days of pregnancy.

An increase in the EBV_TNB was related with an increase in uterine length (β = 14.1 ± 5.3 cm / EBV, P = 0.001), and with a decrease in average empty uterine space (β = -3.3 ± 1.6 cm / EBV, P = 0.04) and in variation in the empty uterine space (β = -1.9 ± 0.9 cm / EBV, P = 0.03) around the vital embryos at 35 days of pregnancy. Thus, in gilts with a higher genetic potential for total number of piglets born there is less empty uterine space around the vital embryos, despite the higher uterine length at 35 days of pregnancy.

An increase in EBV_BW was related with an increase in the average CL weight (β = 0.14 ± 0.04 g / Kg of EBV, P = 0.0006). Thus, gilts with a higher genetic potential for average piglet birth weight have a higher average corpora lutea weight at 35 days of pregnancy. There was no relationship between the EBV_BW and the average and SD vital embryonic weight at 35 days of pregnancy (P = 0.90 and 0.58, respectively). EBV_BW was also not related (P > 0.05) with the vital embryos average implantation length and area and empty uterine space. Thus, there is no relationship between gilts genetic potential for piglets birth weight and the weight of the vital embryos in early pregnancy.

An increase in EBV_BWSD was related with an increase in the average CL weight (β = 0.001 ± 0.0002 g / EBV, P = 0.003). Thus, gilts with a higher genetic potential for within litter birth weight variation (i.e. lower uniformity) have a higher average corpora

lutea weight at 35 days of pregnancy. There was no relationship between the EBV_BWSD and average and SD of the vital embryonic weight at 35 days of pregnancy (P = 0.68 and 0.13, respectively). EBV_BWSD was also not related (P > 0.05) with the vital embryos average and SD in implantation length and average and SD empty uterine space. There was, however, a positive relationship between EBV_BWSD and the vital embryos implantation area ($\beta = 0.31 \pm 0.14$ cm^2 / EBV, P = 0.03). Thus, a higher genetic potential for within litter piglet birth weight variation does not represent an increase in average or variation in weight of vital embryos at 35 days of pregnancy, but it seems to increase their implantation area at 35 days of pregnancy.

Heritabilities, genetic and phenotypic correlations

Heritabilities and additive genetic variation of ovarian, uterine and embryonic traits are shown in Table 6.4. Genetic and phenotypic correlations between ovarian, uterine and embryonic traits are presented in Table 6.5. Heritabilities and genetic correlations were considered as low when ≤ |0.19|; moderate when |0.20| to |0.40| and high when ≥ |0.41|.

Heritability estimates were high for OR, average CL weight, total luteal mass, uterine length, number of embryos (vital and total) and vital implantation length and area; moderate for average vital embryonic weight at 35 days of pregnancy, and average and standard deviation of vital empty space around the vital embryos; and low for early and late embryonic mortality, and the SD of CL weight, SD of vital embryo weight, and the SD of vital empty space around the vital embryos. The majority of the genetic correlations between the ovarian uterine and embryonic number and development traits were high. For the traits with low heritabilities (≤0.19), genetic and phenotypic correlations were not estimated, because of convergence issues with ASReml or extremely high standard errors.

High positive genetic correlations included the correlations between OR and total luteal mass, uterine length and the number of embryos (total and vital), between average CL weight and total luteal mass, between uterine length and number of embryos (total and vital) and the average implantation length. In other words, all traits related to numbers or total mass or length are highly positively correlated indicating that these traits are genetically similar.

High negative genetic correlations included the correlations between OR and average CL weight, average and SD of empty uterine space around the vital embryos, between the average CL weight and the number of embryos (total and vital), the average and SD of vital empty space around the vital embryos, and the average implantation length and area. In other words, higher OR or higher number of vital embryos are genetically associated with lower CL weight, average and SD of empty uterine space and average implantation length and area.

Moderate positive genetic correlations included the correlations between total luteal mass and the average implantation length and area, and the SD of empty uterine space around the vital embryos; between uterine length and the average weight of the vital embryos and implantation area, and between the average weight of the vital embryos and the

Table 6.4 Estimated variance components and heritabilities of ovarian, uterine and embryonic characteristics in gilts at 35 days of pregnancy (n=390).

Traits	Variance component [1]			h^2
	σ_p^2	σ_a^2	σ_e^2	
Averages				
Ovulation rate, OR	9.3 ± 0.87	5.1 ± 1.73	4.1 ± 1.18	0.55 ± 0.15
Corpus luteum weight, g	0.004 ± 0.0004	0.0025 ± 10^{-3}	0.001 ± 10^{-3}	0.70 ± 0.17
Total luteal mass, g	1.7 ± 0.15	0.80 ± 0.29	0.91 ± 0.21	0.47 ± 0.15
Uterine length, cm	4558.7 ± 461.22	3098.9 ± 962.60	1459.8 ± 628.50	0.68 ± 0.16
Number of embryos	19.4 ± 1.72	8.1 ± 3.25	11.2 ± 2.39	0.42 ± 0.14
Number of vital embryos	17.0 ± 1.50	7.0 ± 2.86	10.0 ± 2.11	0.41 ± 0.15
Early embryonic mortality, n	17.9 ± 1.36	1.3 ± 1.79	16.6 ± 1.88	0.07 ± 0.09
Late embryonic mortality, n	2.1 ± 0.15	0.1 ± 0.17	2.0 ± 0.21	0.03 ± 0.08
Embryo weight, g	0.36 ± 0.04	0.13 ± 0.07	0.23 ± 0.06	0.36 ± 0.18
Empty space, cm	383.3 ± 30.37	73.0 ± 46.24	310.3 ± 41.55	0.19 ± 0.11
Implantation length, cm	19.6 ± 1.77	8.7 ± 3.45	10.9 ± 2.50	0.44 ± 0.15
Implantation area, cm^2	2101.1 ± 201.46	1162.0 ± 411.92	939.5 ± 282.74	0.55 ± 0.16
Standard Deviations				
Corpus luteum weight, g	0.001 ± 0.00003	0.00001 ± 0.00002	0.001 ± 0.00004	0.01 ± 0.06
Embryo weight, g	0.02 ± 0.002	0.0 ± 0.002	0.02 ± 0.002	0.002± 0.09
Empty space, cm	108.8 ± 9.21	29.9 ± 17.12	78.8 ± 13.72	0.28 ± 0.14
Implantation length, cm	3.2 ± 0.24	0.22 ± 0.29	3.02 ± 0.32	0.07 ± 0.09

[1] σ_p^2 permanent environment variance; σ_a^2 additive genetic variance; σ_e^2 residual variance.

Table 6.5 Estimated genetic correlations (*in italic below the diagonal*) and phenotypic correlations (above the diagonal) of ovarian, uterine and embryonic characteristics in gilts at 35 days of pregnancy (n=390).

Traits[1]	OR	CL	LM	UL	E	VE	VEW	VES	VIL	VIA	SDVES
OR	-	-0.47±0.05	0.58±0.05	0.30±0.06	0.40±0.05	0.33±0.06	0.04±0.08	-0.17±0.06	-0.13±0.06	-0.11±0.07	-0.12±0.06
CL	*-0.58±0.16*	-	0.48±0.05	-0.08±0.07	-0.24±0.06	-0.23±0.06	0.17±0.08	0.11±0.06	0.24±0.06	0.23±0.07	0.15±0.07
LM	*0.53±0.18*	*0.42±0.20*	-	0.20±0.07	0.20±0.06	0.14±0.06	0.16±0.08	-0.14±0.06	0.02±0.06	0.05±0.07	-0.05±0.06
UL	*0.45±0.20*	*-0.37±0.21*	*0.12±0.24*	-	0.33±0.06	0.38±0.06	0.22±0.07	-0.11±0.06	0.35±0.06	0.31±0.06	-0.08±0.06
E	*0.93±0.12*	*-0.88±0.12*	*0.03±0.27*	*0.49±0.21*	-	0.95±0.01	-0.05±0.07	-0.76±0.02	-0.59±0.04	-0.40±0.05	-0.66±0.03
VE	*0.85±0.15*	*-0.87±0.11*	*-0.03±0.27*	*0.58±0.19*	*0.99±0.01*	-	-0.06±0.07	-0.74±0.03	-0.51±0.04	-0.31±0.06	-0.64±0.03
VEW	*-0.03±0.32*	*-0.01±0.34*	*-0.05±0.36*	*0.23±0.30*	*-0.08±0.35*	*0.01±0.35*	-	0.05±0.07	0.25±0.07	0.20±0.07	0.09±0.07
VES	*-0.71±0.29*	*0.86±0.17*	*0.11±0.33*	*-0.48±0.30*	*-0.84±0.13*	*-0.86±0.13*	*0.14±0.46*	-	0.43±0.05	0.23±0.1	0.72±0.03
VIL	*-0.29±0.24*	*0.64±0.19*	*0.27±0.27*	*0.56±0.19*	*-0.31±0.24*	*-0.22±0.26*	*0.19±0.33*	*0.13±0.36*	-	0.83±0.02	0.40±0.05
VIA	*-0.34±0.22*	*0.58±0.18*	*0.22±0.26*	*0.27±0.22*	*-0.42±0.22*	*-0.29±0.25*	*0.31±0.29*	*0.12±0.35*	*0.88±0.07*	-	0.28±0.06
SDVES	*-0.59±0.27*	*0.93±0.11*	0.38±0.28	*-0.37±0.30*	*-0.92±0.12*	*-0.98±0.14*	*0.11±0.42*	*0.80±0.17*	*0.41±0.28*	*0.49±0.25*	-

[1]OR: ovulation rate; CL: average corpora lutea weight (g), LM: luteal mass (sum of all corpora lutea weight, g); UL: total uterine length (cm); E: total number of embryos; VE: number of vital embryos; VEW: average individual vital embryo weight (g); VES: average empty uterine space around the vital embryos, VIL: average length of vital embryos uterine implantation (cm); VIA: average area of vital embryos uterine implantation (length x width of the implantation, cm^2); SDVES: within litter variation in empty uterine space around the vital embryos.

implantation area.
Moderate negative genetic correlations included the correlations between OR and the average implantation length and area, between the average CL weight and uterine length; between uterine length and the SD of empty space.

Discussion

We investigated how genetic selection based on the estimated breeding values for litter traits at birth influences ovarian, uterine and embryonic traits in gilts at 35 days of pregnancy and estimated the additive genetic variation of these underlying traits. To our knowledge this is the first study to estimate the consequences of genetic selection for litter traits at birth on early prenatal piglet development and also the additive genetic variation of corpora lutea weight and vital embryonic survival and development traits estimated.
Litter traits at birth are genetically and phenotypically negatively correlated, and genetic selection for a higher total number of piglets born seems to have simultaneously compromised average piglet birth weight and within litter birth weight uniformity (Milligan et al. 2002; Damgaard et al. 2003; Wolf et al. 2008). Litter traits are composite traits, being dependent on the interactions between several underlying traits, such as ovulation rate (OR), embryonic survival and development and uterine capacity. It is important to know how these underlying traits are influenced by genetic improvement up to now and if these traits are heritable, as this information could be used in genetic selection programs for litter traits at birth.

Relationship between EBVs and traits at 35 days of pregnancy
Ovulation rate has a high genetic correlation with the total number of piglets born [0.98; (Haley and Lee 1993)], and is considered as the main component trait of litter size (Schneider et al. 2014). In this study, an increase in the estimated breeding values (EBV) for total number of piglets born (TNB) was related with a proportional increase in OR: a genetic increase of one piglet leads to one more ovulation. This proportional increase in OR is surprising, as the much higher phenotypic values of OR in relation with the number of piglets born suggested that genetic selection programs aiming to increase litter size led to an disproportional increase in OR. This might have been the case in the past – when genetic selection programs mainly focused on increase the TNB. However, the proportional relationship between EBV for TNB and OR found in this study, might have been the results of modern balanced genetic selection programs, that select also for higher piglets birth weight and birth weight uniformity for example. Despite the high relationship between OR and the EBV for TNB, the phenotypic correlated response in piglets born with the increase in OR is much smaller (Johnson et al. 1984), due to the increase in embryonic mortality that follows the increase in OR (Da Silva et al. 2016; Da Silva et al. 2017a). However, an increase in the EBV for TNB was not related with an increase in embryonic mortality and it was related with an proportional increase also in the number of vital embryo at 35 days of

pregnancy, despite the increase in OR. Thus, these results indicate that limitations in the phenotypic response in the total number of piglets born with the increase in OR are probably established after 35 days of pregnancy, and that the increase in embryonic mortality that follows the increase in OR is not genetically determined.

Genetic selection for a higher TNB has been associated with an increase in uterine crowding (Bérard et al. 2010), which leads to an increase in competition between littermates for an adequate uterine space for placental development and has negative consequences for embryonic survival and development. However, an increase in the EBV for TNB was related with an increase in uterine length, and that despite the decrease in average empty uterine space around the vital embryos, gilts with higher EBV for TNB seems to do not have a compromised development of the vital embryos, as shown by the absence of relationship between EBV for TNB and the uterine implantation length and area, both important traits for placental development (Stroband and van der Lende 1990). Also, an increase in the EBV for TNB did not influence the weight of the vital embryos at 35 days of pregnancy. Thus, an higher genetic potential for TNB is not related with an compromised development of the vital embryos at 35 days of pregnancy.

Although gilts with a higher EBV for TNB did not have compromised early embryonic development, they had a smaller average corpora lutea (CL) weight at 35 days of pregnancy, which indicates smaller follicles at ovulation (Wientjes et al. 2012). The ovulation of smaller follicles indicates the release of oocytes with lower quality, leading to the development of embryos with lower quality and compromised growth potential (Ding and Foxcroft 1994; Gandolfi et al. 2005). Thus, in gilts with a higher genetic potential for TNB, the lower average CL weight suggests a compromised follicular and oocyte quality, that might lead to compromised foetal development (i.e. after day 35 of pregnancy).

Another indication of the importance of CL size for embryonic and foetal development, is the surprising absence of association between the EBV for BW and uterine length, empty uterine space and also not with most measurements of development of the vital embryos at 35 days of pregnancy (average implantation length and area, and weight of the vital embryos), and its significant positive relationship with the average CL weight. At the phenotypic level an increase of 1 g in the average weight of the CL is related with an increase of 2.3 g in the average weight of the vital embryos at 35 days of pregnancy for the gilts in these study ($P = 0.001$, *results not shown*). In multiparous sows at ~ 3 weeks of pregnancy with an average CL diameter ranging from 9.0 to 10.5 mm, the average piglet birth weight was 1,338.3 ± 27.3 g while in sows with an average CL diameter ranging from 5.5 to 7.8 mm the average piglet birth weight was 1,270.5 ± 30.9 g [$P < 0.05$; Da Silva et al. (2017b)].

There are 3 possible explanations for the relationship between CL size and piglet birth weight. First, as discussed above, CL size is related with the follicular size at ovulation (Wientjes et al. 2012), and larger follicles at ovulation might release oocytes with higher development potential (Marchal et al. 2002), which might favour early embryonic development. At days 10 to 11 of pregnancy, pig conceptuses start to elongate (Geisert et

al. 1982) and timing of rapid elongation is established by the conceptuses (Geisert et al. 2014). Conceptuses with higher development potential might elongate earlier and quicker, taking up more uterine space (i.e. uterine implantation length) (Vallet et al. 2009). The length of the implantation site determines the length of the placenta (Stroband and van der Lende 1990). Thus, larger CL might be associated with embryos with higher quality, that acquire a larger placenta and are heavier at 35 days of pregnancy. Heavier embryos at 35 days of pregnancy are likely to develop into heavier piglets at birth, since most of the eventual weight of the embryos and its placentas are established up to 35 days of pregnancy (Vallet et al. 2009). Second, since CL produce progesterone, heavier CL could produce higher amounts of progesterone, favoring embryonic growth and piglet birth weight. However, systemic progesterone levels analyzed for a subset of 238 gilts at 35 days of pregnancy were not related ($P = 0.69$) with average CL weight [*results not shown*]. Third, CL and embryonic development may share a common origin. During elongation conceptuses produce 17β- oestradiol (E2), which increases the expression of luteinizing hormone (LH) receptor in the luteal cells (Ziecik et al. 2011). Luteinizing hormone, together with interleukin β1, favors the production of prostaglandin E2 by the CL, a potent luteoprotective that increases luteal permeability and delivery of cholesterol to the luteal cells by stimulating the expression of Vascular Endothelial Growth Factor (Ziecik et al. 2011), which might increase CL growth. Thus, the relationship between EBV for BW and average CL weight and not with any trait regarding vital embryonic development at 35 days of pregnancy, indicates that genetic improvement in piglet birth weight might occur through improvements in follicular and oocyte quality.

Similarly to what was observed for EBV for BW, an increase in the EBV for BWSD was not related with uterine length, empty uterine space around the vital embryos and also not with most of the measurements of development of the vital embryos at 35 days of pregnancy. An increase in EBV for BWSD was related with an increase in average CL weight and total luteal mass. As a higher EBV for BWSD indicates gilts with a higher within litter piglet birth weight variation, genetic improvement in piglet birth weight uniformity occurs through a decrease in EBV for BWSD. Within litter variation (SD) in piglet birth weight is genetically positively correlated with average piglet birth weight (Damgaard et al. 2003; Sell-Kubiak et al. 2015), and consequently the EBVs for BW and for BWSD are correlated [$r = 0.69$, $P < 0.0001$, *this study, results not shown*]. This correlation can also explain the higher average CL weight in gilts with higher EBV for BWSD. Phenotypically, multiparous sows at ~ 3 weeks of pregnancy with an average CL diameter ranging from 9.0 to 10.5 mm had not only a higher average piglet BW but also a higher within litter piglet birth weight variation (318.6 ± 17.0 g) than sows with an average CL diameter ranging from 5.5 to 7.8 mm [252.2 ± 17.9 g, $P < 0.05$; Da Silva et al. (2017b)]. Thus, the increase in average CL weight in gilts with a higher genetic potential for within litter piglet birth weight variation might be a consequence of the genetic association between BW and BWSD.

The genetic association between BW and BWSD might also explain the higher variation in implantation length observed in gilts at 35 days of pregnancy with a higher EBV for BW. The length of uterine implantation is a consequence of the length of embryonic elongation at earlier stages of pregnancy (Geisert et al. 1982). So, a higher variation in implantation length indicates a higher variation in embryonic elongation length before implantation. A higher variation in implantation length will lead to a higher variation in placental length (Stroband and van der Lende 1990), which might lead to a higher variation in foetal growth and in piglet birth weight. Thus, gilts with a higher EBV for BW might have a higher piglet birth weight variation due to an higher variation in uterine implantation length and consequently in placenta growth at early pregnancy.

Heritabilities and genetic correlations

Ovarian, uterine and embryonic characteristics in gilts at 35 days of pregnancy are underlying traits influencing litter characteristics at birth. Although the study population was small, resulting in high standard errors, results showed that these traits can be highly heritable and could be used to improve accuracy of genetic selection programs for litter characteristics at birth. Ovulation rate is known to be heritable, and in this study 55% of its phenotypic variation is explained by genetic variation. Previous studies on the heritability of OR reported values of 0.27 in gilts at 27 to 30 days of pregnancy (Bidanel et al. 1996), 0.24 in gilts at 50 days of pregnancy (Johnson et al. 1999) and, more recently, 0.32 in a genome wide association study (Schneider et al. 2014). Despite the higher heritability in this study in comparison with literature, the additive genetic variation of OR in the present study was 5.1, similar to the genetic variation observed by Johnson et al. (1999) (6.6) and Schneider et al. (2014) (4.9). Thus, the higher heritability of OR observed in this study could be related with a lower residual or environmental variation, since it was based on a very homogeneous dataset (i.e. only gilts from a single farm), and estimations were done by dissection of ovaries and counting individual CL, which is the most precise method for estimations of OR (i.e. precise phenotyping). This can also explain the high heritabilities described for other traits in this study.

Average CL weight and total luteal mass are also highly heritable. Phenotypically, an increase in OR is related with a decrease in average CL weight and an increase in total luteal mass in gilts (Da Silva et al., 2017a). These relationships are at least partly genetic, as OR had a negative genetic correlation with average CL weight, and a positive genetic correlation with total luteal mass. This indicates that genetic selection to increase OR might simultaneously select for a decrease in average CL weight, which could compromise embryonic weight at 35 days of pregnancy and piglet BW.

Heritability for the number of embryos (total and vital) at 35 days of pregnancy were also higher (0.42 and 0.41, respectively) than previously reported in literature: a heritability estimate of 0.14 for number of embryos at 30 days of pregnancy (Bidanel et al. 1996) and a heritability estimate of 0.18 for number of foetuses at day 50 of pregnancy (Johnson et al. 1999), but are still within the range of heritability described for total number of piglets born

(up to 0.76) and number of piglets born alive (up to 0.66) (Bidanel 2011). This indicates that it is possible to improve the number of vital embryos at 35 days of pregnancy, although it is not clear from this data if this increase would represent an improvement in the number of piglets born alive.

Early and late embryonic mortality have low heritability (0.07 and 0.03, respectively) similarly to that previously reported for embryonic survival up to 30 days of pregnancy (Bidanel et al. 1996). Although early embryonic mortality could still be decreased through genetic selection, the low additive genetic variation shows the importance of environmental factors. For instance, early embryonic mortality is estimated as the difference between OR and the total number of embryos in the uterus at 35 days of pregnancy, and assumes an optimal fertilization rate (~100%), which is normally achieved with the use of fresh semen (< 24 hours of storage) (De Ambrogi et al. 2006). However, in this study, gilts were inseminated with semen stored for 3 up to 10 days, and a decrease in fertilization rate might have led to an overestimation in early embryonic mortality. Regarding late embryonic mortality, genetic selection to improve it might not be possible, considering that this trait has an additive genetic variation close to zero. Thus, early and late embryonic mortality are mainly controlled by environment factors.

Average implantation length and area of the vital embryos at 35 days of pregnancy had high heritabilities. As the length and area of uterine implantation determines the size of the placenta (Stroband and van der Lende 1990), improvement of these traits likely benefits embryonic and foetal growth and consequently piglet birth weight. Also, vital embryonic weight at 35 days of pregnancy has a heritability similar to what has been described for piglet birth weight by Knol (2001) (0.30) and by Damgaard et al. (2003) (0.39). Moreover, vital embryonic development can be compromised by uterine crowding, when there is competition between littermates for uterine space, leading to compromised placental development (Père et al. 1997; Foxcroft et al. 2006). Uterine crowding at 35 days of pregnancy could be alleviated by genetic selection, as both uterine length and the average empty uterine space around the vital embryos in gilts at 35 days of pregnancy were heritable traits. Although uterine length at 35 days of pregnancy is influenced by the number of embryos (Wu et al. 1987), it had a higher heritability than the uterine length in prepuberal gilts [0.50; (Young et al. 1996)]. Thus, both vital embryonic development and the uterine space for such development up to 35 days of pregnancy could be improved through genetic selection, and could lead to an increase in piglet birth weight.

To use ovarian, uterine and embryonic survival and development traits at 35 days of pregnancy in genetic selection programs, it is important to understand how selecting for one trait might influence other traits genetically (i.e. correlated responses). Phenotypically, an increase in OR is related with an increase in uterine length at 35 days of pregnancy (Da Silva et al. 2016; Da Silva et al. 2017a) and with an increase in the number of embryos at early pregnancy (Vonnahme et al. 2002; van der Waaij et al. 2010; Da Silva et al. 2016; Da Silva et al. 2017a). Moreover, an increase in OR is related with a decrease in the average implantation length (Da Silva et al. 2016; Da Silva et al. 2017a), in placental length and in

average empty uterine space around the vital embryos at 35 days of pregnancy (Da Silva et al. 2016). These relationships are at least partly genetic, as the genetic correlations between these traits were similar to the phenotypic correlations above described. This indicates that, genetic selection for a higher OR might simultaneously compromise vital embryonic development at 35 days of pregnancy, and, since most of the eventual weight of the embryonic-placental unit is established up to 35 days of pregnancy (Vallet et al. 2009), this might lead to a decrease in the average piglet birth weight.

Moreover, average CL weight has a positive phenotypic correlation with the average weight of the vital embryos at 35 days of pregnancy [β = +2.3 g embryo weight / g of CL weight, *P = 0.01, results not shown*], and although the genetic correlation between these two traits was close to zero, average CL weight had strong positive genetic correlations with vital embryonic implantation length and area at 35 days of pregnancy. This indicates that genetic improvement in CL weight at early pregnancy, might select gilts with embryos with a better development potential, leading to piglets with a higher average birth weight. However, it is important to consider that average CL weight has strong negative genetic correlations with, OR (-0.58), uterine length (-0.37) and number of embryos (-0.88) at 35 days of pregnancy. Thus, genetic improvement of these traits simultaneously might be difficult, and development of a genetic selection index balancing these traits might be necessary.

Piglets born in large litters have a lower average piglet birth weight and within litter birth weight uniformity (Père et al. 1997; Milligan et al. 2002), which can be related to compromised placental development at early pregnancy (Père et al. 1997; Town et al. 2005). The negative associations between litter traits at birth are partly genetic (Damgaard et al. 2003; Wolf et al. 2008). Although at 35 days of pregnancy, the genetic correlations between the number of embryos (total and vital) and the average vital embryonic weight was close to zero, it was negatively genetically correlated with the average uterine implantation length and area. This indicates that, the negative genetic associations between litter size and birth weight are not yet present at early pregnancy in this population, but it might originate in later pregnancy, as foetal development might be compromised in gilts with higher number of embryos.

Thus, genetic improvement in total number of piglets born, average piglet birth weight and within litter piglet birth weight variation is mainly related with ovulation rate and average corpora lutea weight in gilts at 35 days of pregnancy. This study also provides genetic parameters estimates of component traits of litter characteristics at birth, although the study population is small. It confirms the existence of additive genetic variation for OR, and also indicates the existence of additive genetic variation for average CL weight, a trait that has been phenotypically related with vital embryonic weight at 35 days of pregnancy. Also uterine and embryonic development traits at 35 days of pregnancy are also heritable. This gives opportunity to include precise phenotyping in genetic selection programs, in order to minimize the undesired associations between litter traits at birth. Moreover, future genome wide association studies might help unravelling genetic variation of the underlying traits influencing litter characteristics at birth.

Conflict of interest statement

This study was in kind funded by Topigs Norsvin. In addition, EK and MB are employed by Topigs Norsvin Research Centre B.V. MB was involved in the data collection, and both EK and MB contributed to the interpretation of the results. However, the possible conflict of interest did not interfere with the outcome of this paper.

Author contributions

CS performed the research, analyzed and interpreted the data, and wrote the manuscript. HM helped in the data analyses and in the interpretation of the data. MB helped in the data collection and in the interpretation of the data. BK and NS helped in the data interpretation. EK designed the research and interpreted the data. All authors critically revised the manuscript.

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CHAPTER 7

General discussion

Introduction

Litter characteristics at birth (total number of piglets born, number of piglets born alive, average piglet birth weight and within litter piglet birth weight variation) are dependent on interactions between many underlying component traits, such as ovulation rate (OR), embryonic survival and development, and uterine capacity affecting foetal survival and development (Spötter and Distl 2006). Ovulation rate is the main component trait influencing the total number of piglets born (Bidanel et al. 1996; Schneider et al. 2014) and these two traits have a high positive genetic correlation [0.85 to 0.98, (Blasco et al. 1993)]. Genetic correlations, generally are a result of pleiotropy, which means that alleles that influence one trait on average also have an influence on a second trait; or that these same traits share the same underlying biology, and therefore the genetic variants that influence one trait also influence the other trait (Paaby and Rockman 2013). Due to its high genetic correlation with total number of piglets born, OR increased sharply in the last decades, following the genetic selection towards higher total number of piglets born. An overview of published data on OR from 1980 up to 2017 shows an increase of 0.2 ovulations per year in both gilts and sows (Figure 7.1), and averages OR of 25 up to 30 are relatively common nowadays (Patterson et al. 2008; Wientjes et al. 2013).

This increase in OR might have changed the relationship between the underlying traits influencing litter characteristics at birth (Foxcroft et al. 2006), specially embryonic survival and uterine capacity, consequently affecting embryonic and foetal survival and development. An increase in OR leads to an higher number of embryos surviving to the post-implantation period (> 13 days of pregnancy), thus leading to a higher competition between littermates for adequate uterine space (i.e. an increase in uterine crowding) (Foxcroft et al. 2006). This increase in uterine crowding due to the increase in OR and in the number of embryos and foetuses competing in the uterus leads to a decrease in piglet birth weight and to an increase

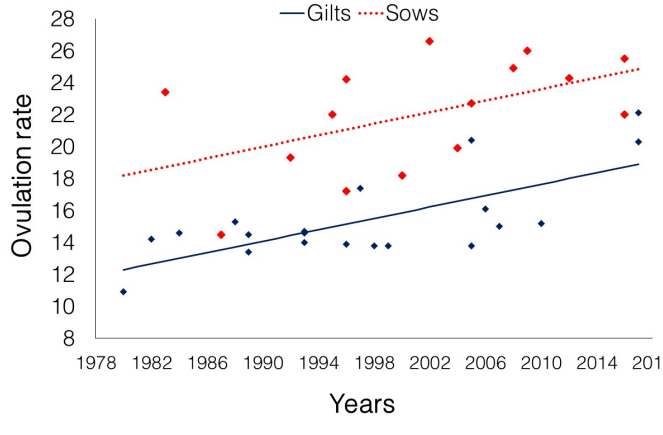

Figure 7.1 Predicted values for ovulation rate in relation with year of study showing an increase of 0.2 ovulations per year from 1980 up to 2017 ($P \leq 0.05$), in gilts and in sows. Adapted from Table 1 [*Chapter 1*].

in within litter piglet birth weight variation in large litters (Quiniou et al. 2002; Quesnel et al. 2008). Moreover, sharp increases in OR might negatively influence follicular and oocyte quality, compromising embryonic development and piglet birth weight.
Thus, the main hypothesis of this thesis is that the increase in ovulation rate, i.e. the number of oocytes a sow sheds in each oestrus cycle, is related with both the increase in total number of piglets born in commercial dam line sows, and also with the decrease in average piglet birth weight and the increase in within litter piglet birth weight variation. More specifically, it is hypothesised that an increase in ovulation rate (OR) compromises embryonic survival and embryonic and placental development at 35 days of pregnancy.

Therefore, the aims of this thesis are:
(1) to understand the physiological consequences of increased number of corpora lutea (OR) for embryonic survival and development in multiparous sows (high average OR) and in gilts (lower average OR) at 35 days of pregnancy;
(2) to investigate relationships between corpora lutea number and size in pregnant sows, and their litter characteristics at birth;
(3) to study differences in underlying physiological components of litter characteristic between gilts with low and high genetic potential for total number of piglets born, average piglet birth weight and within litter piglet birth weight variation (based on their estimated breeding value); and
(4) to investigate the genetic variation in underlying litter characteristics traits like, OR, corpora lutea characteristics and embryonic survival and development in gilts in early pregnancy.

In the following paragraphs the results of this thesis will be discussed. In paragraph 7.1 the relationship between OR and embryonic survival and development up to 35 days of pregnancy is discussed, including its differences between gilts and sows. In paragraph 7.2 the importance of corpora lutea size during pregnancy is discussed for embryonic survival and development and for piglet birth weight. In paragraph 7.3 consequences of genetic selection for total number of piglets born, average piglet birth weight and for within litter piglet birth weight uniformity on ovarian and embryonic characteristics at 35 days of pregnancy is discussed. In paragraph 7.4 the possibilities to use these underlying reproductive traits for genetic improvement of litter traits at birth is discussed. Finally, general conclusions and recommendations for future research and for the industry are given in paragraph 7.5.

7.1. Relationship between ovulation rate and embryonic survival and development

Ovulation rate (OR) has continued to increase in the last decades (Figure 7.1). We hypothesised that this increase in OR might have disrupted the balance between embryonic survival and development and uterine capacity, thus influencing foetal survival and development and consequently the litter characteristics at birth (total number of piglets born, the number of piglets born alive, average piglet birth weight and within litter birth weight variation). Therefore, we investigated the relationship between OR and embryonic survival and development in gilts and sows at 35 days of pregnancy. The major findings of the relationship between OR and embryonic survival and development at 35 days of pregnancy [*Chapter 3 (sows) and Chapter 4 (gilts)*] have been summarized in figures 7.2, 7.3 and 7.4 and are discussed below.

An increase in OR was related with an increase in the number of vital embryos at 35 days of pregnancy. However, the increase in number of embryos with the increase in OR is smaller at higher OR [*Figure 7.3*]. In sows, at least 4 ovulations are needed to achieve one more vital embryo [β = + 0.26 embryos/ovulation, P = 0.01]. Further analyses, with OR analysed in classes, showed that the number of vital embryos reaches a *plateau* of 17 vital embryos at 22 ovulations, with no further significant increase in sows with more ovulations [*Figure 3.1a*]. Similarly, in gilts a maximum of 16.8 vital embryos was observed at 26 ovulations, with no further increase at higher OR [*Figure 4.1*]. This limitation in the increase in number of embryos with an increase in OR was previously found by Blichfeldt and Almlid (1982) in gilts at 30 days of pregnancy, by Wu et al. (1987) in gilts at 3 to 15 weeks of pregnancy, and by Blasco et al. (1996) in sows at birth. Thus, the increase in the number of embryos with the increase in OR decreases at higher OR.

This limited increase in the number of vital embryos (and in piglets born) with an increase in OR is related with an increase in pre-natal mortality (Haley and Lee 1993), both an increase in early embryonic mortality, in late embryonic mortality (and in foetal mortality after 35 days of pregnancy). Early embryonic mortality is the mortality occurring before uterine implantation at ~13 days of pregnancy, and is defined as the number of potential embryos (i.e. ovulation rate) not accounted for by embryos or embryonic remnants at 35 days of pregnancy. Late embryonic mortality, is defined as the mortality occurring after uterine implantation at ~13 days up to 35 days of pregnancy, resulting in remnants of embryonic/placental tissue or in embryos with haemolysed amniotic fluid indicating death.

In this thesis, there was an increase in both early and late embryonic mortality with the increase in OR in both sows and gilts [*Figure 7.3*]. **Early embryonic mortality** accounted for 59.3 % and 74.1 % of the total mortality in sows and gilts, respectively. In sows, each extra ovulation from 17 up to 38 led to an increase of 0.49 in early embryonic mortality [*P <0.0001; Figure 3.1c*].

In gilts, a minimum incidence in early embryonic mortality of 3.3 was observed at 15 ovulations, increasing thereafter with the increase in OR up to 34 [*P = 0.02, Figure 4.1C*]. Also van der Waaij et al. (2010), working with super-ovulated gilts (OR ranging from

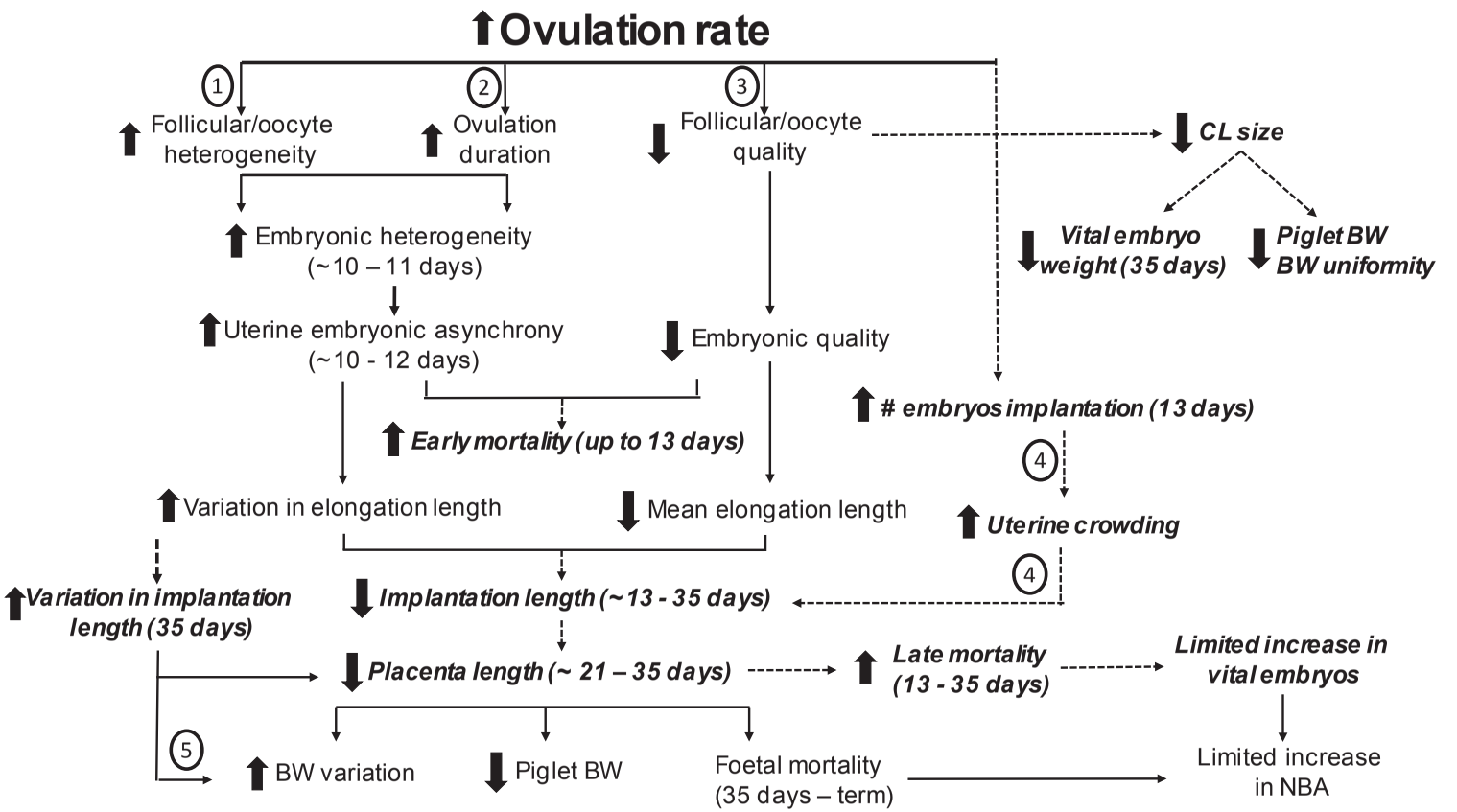

Figure 7.2 Schematic illustration of the relationship between ovulation rate and embryonic survival and development at 35 days of pregnancy and its consequences for litter characteristics at birth. Dashed arrows with italic and bold text indicate results of this thesis. Solid arrows with regular text indicate hypothetical physiological background of the relationships and their possible consequences.

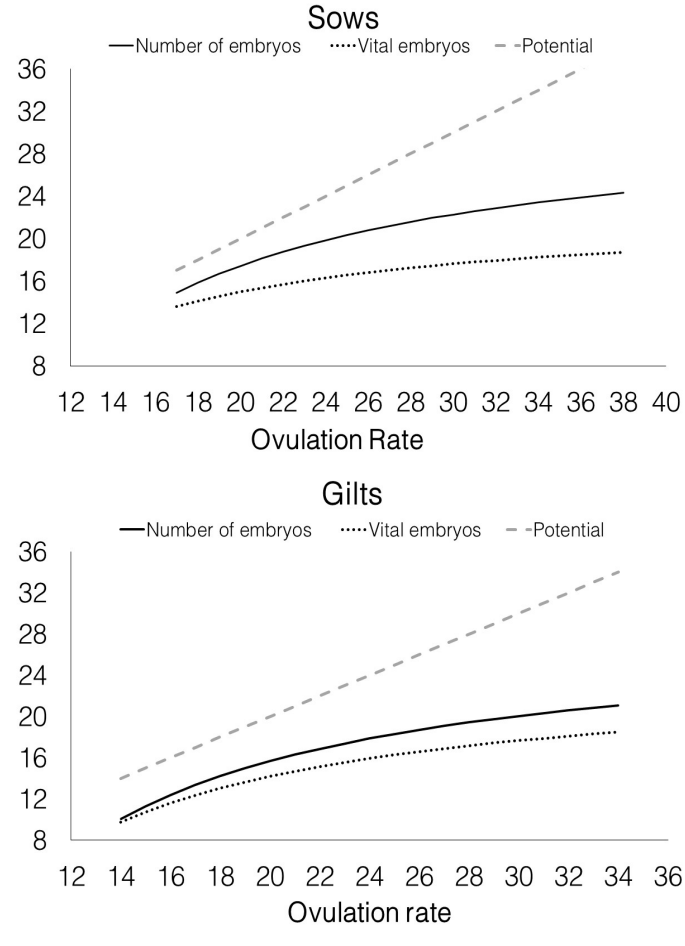

Figure 7.3 Inverse regression function of ovulation rate in relation with the total number of embryos and the number of vital embryos for sows and gilts. The grey dashed line represents the potential number of embryos (i.e. ovulation rate), the black solid line represents the predicted values for total number of embryos and the black dotted line the predicted values for number of vital embryos for sows. SOWS: Total number of embryos [31.9 ± 2.5 – 290.6 ± 60.5/OR (P < 0.0001)]; number of vital embryos [22.9 ± 3 – 176.8 ± 56.4/OR (P = 0.002), the model included the fixed class effect of parity: 2 to 3, n = 25; 4 to 10; n = 47; and 11 to 17, n = 19]. GILTS: Total number of embryos [28.7 ± 1.5 – 262.5 ± 30.3/OR (P < 0.0001), the model included the fixed class effect of gilts genetic line (GL): purebred Landrace, n= 86 and crossbred Yorkshire x Landrace, n = 212 (P = 0.0003) and semen storage duration (SS): 3 to 5 d, n = 59; 6 to 7 d, n = 133; 8 to 10, n = 106 (P = 0.001)], number of vital embryos [24.6 ± 1.5 – 207.9 ± 29.9/OR (P < 0.0001), GL and SS P > 0.05].

22 up to 76) at 40 days of pregnancy, observed an increase of 0.71 (P < 0.0001) in early embryonic mortality with each extra ovulation. Such increase in early embryonic mortality with an increase in OR might be related with fertilization failure. Fertilization rates can be close to 100% in pigs, however Day and Polge (1968) proposed that sows with a higher OR might have an earlier and higher rise of progesterone during and shortly after ovulation, compromising sperm transport to the fertilization site and decreasing fertilization rate, thus

leading to an overestimation in early embryonic mortality. However, comparison between gilts with high and low OR (18.8 ± 0.4 vs 14.3 ± 0.6) revealed no differences in progesterone rise up to 3 days after the LH peak in high and low OR gilts [3.2 ± 0.2 vs 3.4 ± 1.0 ng/mL, P > 0.05, Knox et al. (2003)]. Thus, there is an increase in early embryonic mortality with the increase in OR which, in normal circumstances, seems to do not be related with an decrease in fertilization rate. The reasons for the increase in early embryonic mortality with the increase in OR are discussed below.

Late embryonic mortality accounted for 41.8 % and 25.9 % of total mortality in sows and gilts, respectively. In sows, each extra ovulation from 17 up to 38 led to an increase of 0.24 in late embryonic mortality [*Figure 3.1b*]. In gilts, a minimum incidence of late mortality of 1.2 was observed at 18 ovulations, increasing thereafter with the increase in OR up to 34 [*Figure 4.1C*]. Vonnahme et al. (2002b) found that OR was positively correlated with the number of vital embryos at day 25 of pregnancy (r = 0.50, P < 0.0001), but not at days 36 (r = 0.02, P = 0.98) and 44 (r = 0.10, P = 0.41) of pregnancy, due to the increase in the incidence of late embryonic mortality. An increase in late embryonic mortality with an increase in OR was also observed in super-ovulated gilts with OR ranging from 22 up to 76, where each extra ovulation led to an increase of 0.36 in late embryonic mortality (P = 0.0003) (van der Waaij et al. 2010). Thus, although to a lesser extent then early embryonic mortality, there is also an increase in late embryonic mortality with the increase in OR. The reasons for the increase in late embryonic mortality with the increase in OR are discussed below.

Both the increase in early and late embryonic mortality in pigs seems to be associated with an increase in **heterogeneity in embryonic development** at early stages of pregnancy (Pope et al. 1990; Xie et al. 1990). Pig blastocysts undergo rapid changes in morphology before initial attachment to the uterine epithelium at ~ 13 days of pregnancy (Geisert et al. 1982), and between days 10 and 12 of pregnancy, pig conceptuses change from spherical to long tubular shape conceptuses, and start elongation, changing from tubular to filamentous forms (Anderson 1978; Geisert et al. 1982). However, not all embryos start development at the same time and at the same speed and at 11 days of pregnancy there is considerable within litter heterogeneity in embryonic development, with spherical, tubular and filamentous conceptuses being present in one litter (Stroband et al. 1984; Pusateri et al. 1990; Stroband and van der Lende 1990). During elongation, the most developed embryos start producing estrogens (Bazer and Thatcher 1977), that changes the uterine environment to their advantage, but that creates a hostile environment for the embryos that are still spherical or tubular (i.e. less developed embryos), leading to mortality of the less developed embryos due to uterine-embryonic asynchrony (Pope et al. 1990; Wilson and Ford 2001). Moreover, not all the elongating embryos are at the same stage of development, and some had started to elongate later than others, being not so delayed in development that it dies due to uterine-embryonic asynchrony, but being still smaller in length of elongation at the time of uterine implantation at 13 days of pregnancy. Such embryos with smaller elongation length attach to a smaller implantation site in the uterus (Geisert et al. 1982), and develop a smaller placenta (Stroband and van der Lende 1990), which might lead to late embryonic mortality

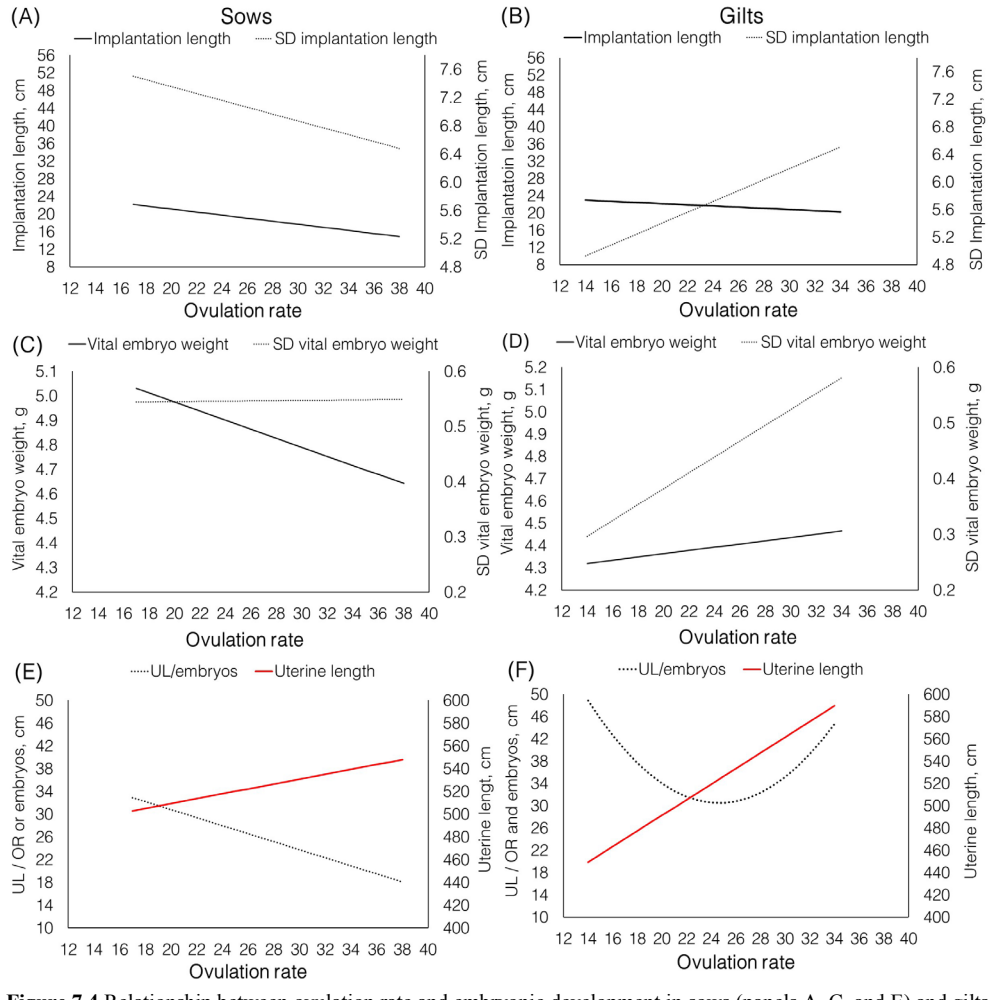

Figure 7.4 Relationship between ovulation rate and embryonic development in sows (panels A, C, and E) and gilts (panels B, D, and F) at 35 days of pregnancy. For SOWS, quadratic term of OR was never significant, so the predicted values were estimated with the linear term of ovulation rate (OR) and corrected for the fixed class effect of parity [PC: 2 to 3, n = 25; 4 to 10; n = 47; and 11 to 17, n = 19] and of genetic background [GB: Sire line cross, n = 20; Purebred Landrace, n = 10; crossbred Yorkshire x Landrace, n = 17]. (A) Average implantation length [(solid line), -0.35 ± 0.1 * OR, P = 0.003, PC = 0.92], within litter standard deviation of implantation length [(dotted line), – 0.05 ± 0.05 * OR, P = 0.34; PC = 0.21]. (C) Average vital embryonic weight [(solid line), -0.02 ± 0.02 * OR, P = 0.42, PC = 0.14], within litter standard deviation in vital embryonic weight [(dotted line) + 0.0002 ± 0.005 * OR, P = 0.96, PC = 0.73], (E) Uterine length available per total number of embryo (UL / embryo, cm) [(black dotted line), - 0.71 ± 0.2 * OR, P < 0.0001, GB x PC = 0.04]; uterine length [(red solid line), + 2.1 ± 2.1 * OR, P = 0.03, PC = 0.05]. For GILTS, predicted values were estimated for the quadratic and linear terms of ovulation rate (OR) and corrected for the fixed class effect of genetic line [GL, Purebred landrace, n = 86; crossbred Yorkshire x Landrace, n = 212], and of semen storage duration [SS, 3 to 5 days, n = 59; 6 to 7 days, n = 133; 8 to 10 days, n = 106]. The quadratic term of OR was excluded from the model when not significant (P > 0.05), and only the linear term was used for the estimations. (B) Average implantation length [(solid line), -0.14 ± 0.1 * OR, P = 0.08, GL= 0.0003], within litter standard deviation (SD) of implantation length [(dotted line), + 0.08 ± 0.03 * OR, P = 0.02]. (D) Average vital embryonic weight [(solid line), + 0.01 ± 0.01 * OR, P = 0.57, SS = 0.001],

or foetal mortality due to insufficient supply of nutrients (Vallet et al. 2014). Thus, heterogeneity in embryonic development leads to mortality of the less developed embryos before 13 days of pregnancy (i.e. early embryonic mortality) due to uterine-embryonic asynchrony; after 13 days up to 35 days of pregnancy (i.e. late embryonic mortality) and after 35 days of pregnancy (foetal mortality) due to the smaller uterine implantation and placental length.

An increase in OR might increase within litter embryonic heterogeneity in development due to an increase in **heterogeneity in follicular and oocyte development** [*see 1 in scheme, Figure 7.2*]. A higher follicular and oocyte heterogeneity at ovulation is related with a higher heterogeneity in early embryonic development (Pope et al. 1990; Xie et al. 1990). This higher follicular and oocyte heterogeneity in development in sows with a higher OR might be related with their extended follicular recruitment phase and longer follicular phase than sows with lower OR (Kirkpatrick et al. 1967; Kelly et al. 1988; Vatzias et al. 1991; Driancourt and Terqui 1996). An extended recruitment of follicles will result in selection and ovulation of follicles of different maturational status, as all follicles that escape atresia at the time of selection are mature enough to respond to the pre-ovulatory LH surge (Hunter and Wiesak 1990). Indeed, considerable heterogeneity in size (7 to 11 mm of diameter), morphology and hormonal status of selected follicles has been reported within gilts (Hunter et al. 1989). Even during estrus (1 to 2 days before ovulation) the group of ovulatory follicles still has considerable heterogeneity, which suggests that not all follicles are at the same stage of development at the time of selection and at ovulation (Knox 2005). A proof of the relationship between follicular heterogeneity and embryonic heterogeneity came from the fact that the elimination of the smallest follicles in gilts 34 hours after the onset of estrus by electrocautery, thus leaving only follicles of 6 to 8 mm, also eliminated the spherical embryos of 1 to 2 mm and reduced the number of 3 to 5 mm spherical embryos, resulting in higher embryonic uniformity at 11 days of pregnancy while maintaining the same mean number of embryos in cauterized and in control gilts (12.7 vs 12.6, respectively) (Pope et al. 1988). In this thesis, the variation (SD) in weight of the 10 biggest corpora lutea (CL) in sows reached its lowest value of 0.037 g at 21 ovulations and increased to up to 0.25 g at 37 ovulations at ~ 24 days of pregnancy [$n = 25$; *SD CL weight* $= 0.42 \pm 0.11 - 0.036 \pm 0.01 * OR (P = 0.01) + 0.001 \pm 0.0003 * OR^2 (P = 0.01)$; *results not shown*]. This suggests an increase in variation in follicular size at ovulation, as corpora lutea size reflects pre-ovulatory follicular size (Soede et al. 1998; Wientjes et al. 2012a). Thus, an increase in OR can increase follicular and oocyte heterogeneity, thus leading to a higher within litter embryonic heterogeneity in early pregnancy, thereby increasing embryonic mortality.

An increase in OR might also increase within litter embryonic heterogeneity due to a

within litter standard deviation (SD) in vital embryonic weight [(black dotted line), + 0.01 ± 0.003 * OR, P < 0.0001]. (F) Uterine length available per total number of embryo (UL / embryo, cm) [(black dotted line), - 8.0 ± 2.3 * OR (P = 0.001) + 0.16 ± 0.1 * OR2 (P = 0.002), GL = 0.02, SS = 0.02]; uterine length [(red solid line), + 10.6 ± 2.1 * OR, P < 0.0001, GL = 0.01, SS = 0.02].

prolonged **ovulatory process** [*see 2 in scheme, Figure 7.2*]. Ovulation in the pig occurs in follicles of 6 up to 8 mm in diameter (Soede et al. 1992) and the period between the rupture of the first and last follicle can vary between sows from 1 to up to 7 hours, although normally is estimated to last no longer than 2 to 3 hours (Soede et al. 1998). This period might be prolonged in sows with a higher OR, which might increase embryonic heterogeneity. Xie et al. (1990) compared the development of embryos originating from oocytes aspired from the 4 to 6 biggest pre-ovulatory follicles and from oocytes aspired from the remaining ovulatory follicles (3 hours after the pre-ovulatory follicles), and observed that the later ovulating follicles were associated with lesser developed embryos. Thus, embryonic heterogeneity can also arise from the difference in time of ovulation between follicles and consequently in the time of beginning of embryonic development. Soede et al. (1992) investigated the relationship between the duration of ovulation (using ultrasonography) and embryonic heterogeneity at day 5 after ovulation in sows that had an average OR of 19.3 ± 3.3 (ranging from 14 up to 25). The authors reported an average duration of ovulation of 1.8 ± 0.6 h (ranging from 0.75 to 3.3 h) and no relationship between the duration of ovulation and embryonic heterogeneity was found, nor between the duration of ovulation and OR. However, it is important to consider that those studies were done more than 20 years ago in animals with lower average OR, and that both the average and the variation in duration of ovulation might have changed in modern sows with higher average OR. Thus, an increase in OR might be related with a longer duration of ovulation that leads to a higher within litter embryonic heterogeneity in early pregnancy, increasing embryonic mortality.

Another possible explanation for the increase in embryonic mortality related with the increase in OR is a **decrease in the follicular and oocyte quality, and consequently in embryonic quality** [*see 3 in scheme, Figure 7.2*]. An indication of follicular and oocyte quality is the follicular size; as pre-ovulatory follicular diameter in pigs was strongly positively correlated with the number of granulosa cells per follicle, an indication of follicular maturation and an important factor for oocyte quality (Ding and Foxcroft 1992). In this thesis, although follicular, oocyte and early embryonic quality were not investigated, an increase in OR was related with a decrease in average corpora lutea (CL) weight and diameter [*Chapters 4 and 6*]. Smaller CL might be an indication of smaller follicles at ovulation, as CL size is related with pre-ovulatory follicular size (Soede et al. 1998; Wientjes et al. 2012a). It can be speculated that in sows with higher OR and therefore a higher number of pre-ovulatory follicles producing oestradiol - 17β (E2), the threshold of E2 necessary to trigger the pre-ovulatory LH peak might be reached when follicles have a smaller size. Indeed, systemic levels of E2 during the ovulatory process are similar ($P > 0.05$) in sows with high and low OR (37.9 ± 3.4 ng/mL at 18.8 ovulations vs 44.7 ± 3.6 ng/mL at 14.3 ovulations), showing that the threshold of E2 to trigger ovulation is independent of OR (Knox et al. 2003). Moreover, Driancourt and Terqui (1996) observed that sows with higher OR (23.1 vs 18.8 pre-ovulatory follicles) had smaller follicles at day 3 (5.6 vs 5.9 mm) and day 5 (6.8 vs 7.5 mm) of the follicular phase, but also had higher steroidogenic ability (i.e. higher ability to convert testosterone in E2) and therefore a higher concentration of E2 in the

follicular fluid at day 3 (12.2 ± 1.2 vs 9.9 ± 0.7 ng/mL) and day 5 (33.4 ± 0.6 vs 22.1 ± 0.7 ng/mL) of the follicular phase, which indicates that in sows with higher OR the threshold of E2 is achieved earlier due to both the higher number of pre-ovulatory follicles and also due to their higher steroidogenic ability. Although the increase in steroidogenic ability is a sign of higher follicular quality, high concentrations of E2 during the final maturation induce oocyte nuclear aberrations (Beker et al. 2002), which might compromise embryonic quality. Thus, sows with a high OR and consequently smaller follicles at ovulation, that develop into smaller CL, might release oocytes of lower quality that might lead to the development of embryos with lower quality and development potential (Ding and Foxcroft 1992; Ding and Foxcroft 1994). As previously explained, embryos with lower development potential are more likely to be delayed in development in relation with their littermates, consequently have a higher chance of dying before uterine implantation due to uterine-embryonic asynchrony (early embryonic mortality). This embryos are also more likely to acquire a smaller implantation length and die after uterine implantation due to the smaller placental length and consequently compromised nutrient uptake (late embryonic mortality). Thus, an increase in OR might be associated with a decrease in follicular and oocyte quality, leading to the development of embryos with lower quality that are more prone to die at early pregnancy.

An increase in OR might also increase late embryonic mortality by increasing **uterine crowding** [*see 4 in scheme, Figure 7.2*], which will also lead to the acquisition of a smaller uterine implantation length and smaller placental length. An increase in uterine crowding with an increase in OR occurs due to an increase in the number of embryos surviving to the post implantation period (Foxcroft et al. 2006), thus increasing the within litter competition for uterine space at early pregnancy to an extent that might exceed uterine capacity (Foxcroft et al. 2007). In sows with an average OR of 21.6 ± 0.9, OR was negatively correlated with the available uterine space per embryos [r = - 0.85, P ≤ 0.05, Langendijk et al. (2016)]. Additionally, van der Waaij et al. (2010) using super-ovulated gilts (45.2 ± 13.2 ovulations) to investigate the effects of uterine crowding, observed that an increase in OR was related with an increase in late embryonic mortality [+ 0.36 not-vital embryos/ovulation, P = 0.0003] at 40 days of pregnancy, and that non-vital embryos had a smaller implantation length [4.4 cm vs 11.6 cm] than the vital embryos. This indicates that the increase in uterine crowding that follows the increase in OR leads to an increase in late embryonic mortality due to a smaller implantation length. Thus, an increase in OR leads to an increase in the number of embryos competing for a uterine implantation site, which leads to a compromised placental development and consequently to embryonic mortality after implantation (late embryonic mortality) or to foetal mortality.

An increase in OR influences not only the survival but also the **development of the vital embryos** at 35 days of pregnancy. In sows, an increase in OR from 17 up to 38 was related with a decrease 0.35 cm/ovulation in the average implantation length of the vital embryos [P = 0.003, *Figure 7.4A*]. As a smaller implantation length is associated with reduced placental development (Stroband and van der Lende 1990), sows with OR ≥ 29 had smaller placental length than sows with OR ranging from 17 up to 21 [41 cm vs 46 cm, *Figure 3.2j*].

Wientjes (2013) also reported a negative correlation between OR and placental length ($r = -0.37$, $P = 0.01$) and placenta dry weight ($r = -0.37$, $P = 0.01$) at 42 days of pregnancy in sows. Although the average weight of the vital embryos at 35 days of pregnancy was not influenced by the increase in OR [*$P > 0.05$; Figure 7.4C and D*], a reduced development of the placenta at early pregnancy (between days 20 and 30) might affect subsequent growth and survival of the embryos in later pregnancy (Vonnahme et al. 2002a; Vallet et al. 2014). In gilts, an increase in OR was related with an increase in the within litter variation in implantation length of vital embryos [*+ 0.08 ± 0.03 cm/ovulation, $P = 0.02$; figure 7.4B*] and in the variation in vital embryonic weight at 35 days of pregnancy [*+ 0.01 ± 0.003 cm/ovulation, figure 7.4D*]. Since the length of elongation of the vital embryos at day 13 of pregnancy will determine their length of uterine implantation (Geisert et al. 1982), a higher within litter variation in implantation length indicates a higher within litter embryonic heterogeneity in elongation length (~ 10 - 12 days of pregnancy), which can explain the higher within litter variation in vital embryonic weight at 35 days of pregnancy [*variation in vital embryo weight was positively correlated with the variation in implantation length ($r = + 0.23$, $P = 0.003$)*].

Thus, an increase in OR leads to a smaller uterine implantation length and placental length of vital embryos in sows at 35 days of pregnancy, and to a higher variation in implantation length and probably in placental length of vital embryos in gilts at 35 days of pregnancy. This might lead to an increase in foetal mortality and to an decrease in average piglet birth weight due to a compromised placental development and nutrient uptake by the vital embryos in sows and gilts, as it occurs in cases of uterine crowding (Vallet et al. 2014). It might also lead to a higher within litter variation in piglet birth weight, as the compromised placental development of the vital embryos at 35 days of pregnancy might increase the variation in foetal weight at a later stage of pregnancy (Quesnel et al. 2008), or their higher within litter variation in embryonic weight, might persist during the foetal stage up to term.

7.1.1. Differences between gilts and sows in the relationship between OR and embryonic survival and development at 35 days of pregnancy

Gilts and sows have different litter characteristics at birth. Milligan et al. (2002) observed that the average piglet birth weight [1.32 Kg vs 1.41 Kg, $P \leq 0.05$] and the number of piglets born alive [9.0 ± 0.3 vs 10.8 ± 0.2, $P \leq 0.05$] was lower in gilts than in sows of parities 3 to 5. Wientjes et al. (2012b) observed an average total number of piglets born of 12.5 ± 0.2 in first parity gilts and an average of 14.3 ± 0.2 in sows from 3 up to ≥ 5 ($P \leq 0.05$). Recent data from ~ 1,300 sows and 276 gilts confirms these results: the average number of piglets born alive was 15.2 for sows and 13.9 for gilts, while average piglet birth weight as 1,346 g for sows and 1,290 g for gilts (Topigs Norsvin, Vught, The Netherlands). These differences in litter characteristics at birth might arise from differences in underlying litter traits such as OR, embryonic survival and development at early pregnancy, and uterine capacity and their interaction.

Box 1. Twins

Estimations of the incidence of early embryonic mortality were done by assessing the difference between the total number of corpora lutea (CL) dissected from the ovaries, and the total number of embryos at 35 days of pregnancy. However, in some animals, the number of embryos exceeded the number of CL. The explanation for a higher number of embryos than CL could be the presence of monozygotic twins. Monozygotic twins develop from one zygote that split and develop into two embryos (Bjerre et al. 2009). These embryos might share the same placenta (monochorionic twins) or not (Vejlsted et al. 2006). If embryonic splitting occurs during the cleavage or the blastocyst stages before 13 days of pregnancy, a separate set of extra-embryonic membranes is formed (Vejlsted et al. 2006). Moreover, in case of dizygotic twins (embryos that develop from different eggs, as most embryos in polytocous species like the pig within the same gestation), each embryo develops independently within its own set of membranes. However, in some cases the extra-embryonic membranes might fuse, generating monochorionic twins. In fact, while studying monochorionic twins in pigs, only one monozygotic twin pair, out of 72 monochorionic embryos, was identified by Bjerre et al. (2009). Thus, not all monochorionic embryos are monozygotic twins.

In pigs, identification of monochorionic twinning is often interpreted as proof of monozygotic twins (Bjerre et al. 2009). In our studies, 6.4% of the 390 gilts slaughtered at 35 days of pregnancy, presented at least one pair of monochorionic twins, and one gilt presented two pairs of monochorionic twins. Only in two gilts both monochorionic twins were alive at 35 days of pregnancy (Figure A). In the majority of the gilts both monochorionic twins were non-vital at 35 days (Figure B). Although the occurrence of monozygotic twins is still a possible explanation for the higher number of embryos than of CL, they can only be identified through genotyping of all embryos.

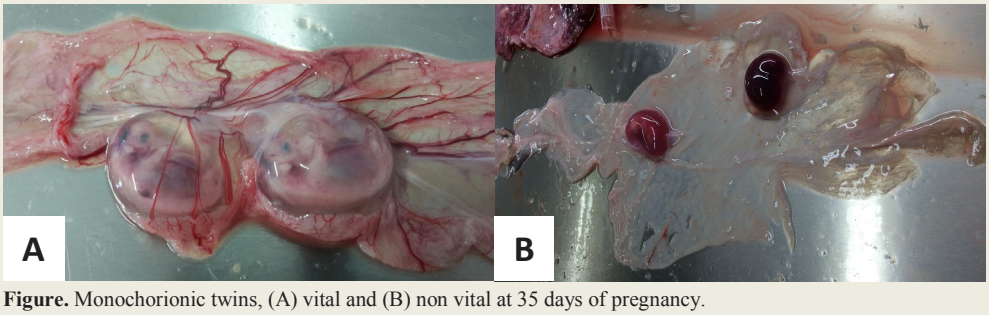

Figure. Monochorionic twins, (A) vital and (B) non vital at 35 days of pregnancy.

Box 2. "Uterine constrictions and non-attachment sites"

Constrictions in the uterine horns were observed in gilts at 35 days of pregnancy (n = 93). These were parts of the uterine horn where the uterus did not distend to accommodate the overlying placenta and placental fluids (Figure A) and at which sites the uterine wall itself was distinctively thicker and less flexible in comparison with the distended sites of the uterine horns. The constrictions were associated with lighter-coloured places within the implantation sites (Figure B), which seems to indicate that, at that place, the placentae did not attach to the uterus. Analyses in a subset of gilts (n = 25) showed that the number of non-attachment sites within an implantation site can range from 0 to 6, ranging from 11 up to 44 per gilt. The presence of the constrictions/non-attachment sites do not seem to influence the vitality of the embryos at 35 days of pregnancy. It is not clear yet if the incidence of constrictions/ non-attachment sites is related with ovulation rate, number of embryos or uterine length and if the non-attachment sites compromise the development of the placenta or embryo after 35 days of pregnancy. As far as we know, these constrictions/non-attachment sites have not been described before.

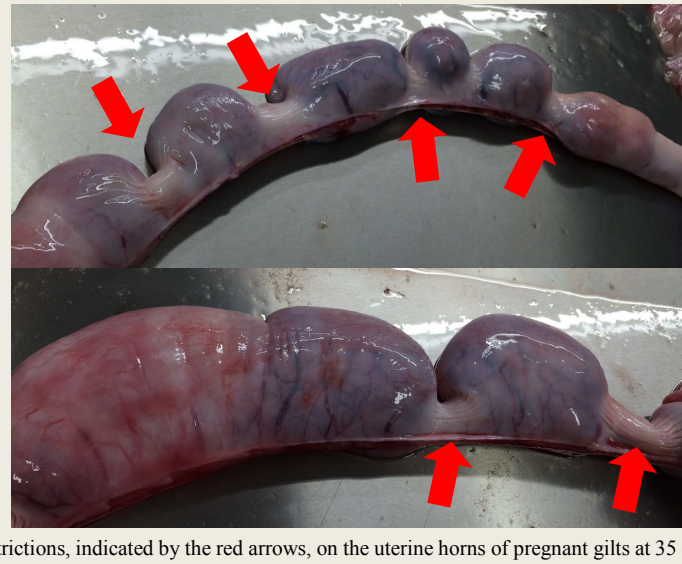

Figure A. Constrictions, indicated by the red arrows, on the uterine horns of pregnant gilts at 35 days of pregnancy.

Box 2. (*continued*)

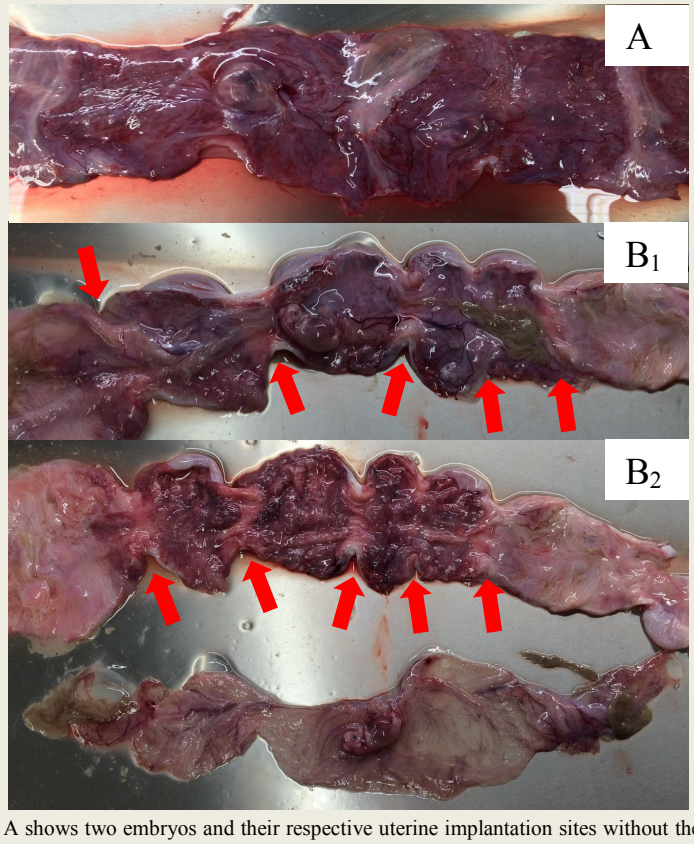

Figure B. Panel A shows two embryos and their respective uterine implantation sites without the non-attachment sites. These embryos were retrieved from sows at 35 days of pregnancy. Panels B1 (with embryo) and B2 (without the embryo) shows the non-attachment sites (red arrows), observed at the uterine constriction places, after opening the uterine horns from a gilt at 35 days of pregnancy. At this implantation site, 5 non-attachment sites (5 red arrows) were observed. The embryo was vital.

During pregnancy, myometrial growth occurs in two distinct phases: 1) myocyte hyperplasia, which is the growth due to an increase in reproduction rate of the smooth muscle cells ('proliferative' phase); and 2) myocyte hypertrophy associated with the increase in smooth muscle cell size ('synthetic' phase). Myocyte hyperplasia is responsible for early pregnancy uterine growth, and depends on oestrogen (Shynlova et al. 2010). Oestrogen stimulates uterine growth by binding to cytoplasmic receptors, and a decrease in the number of receptors would reduce uterine tissue sensitivity to oestrogen (Hsueh et al. 1975). So, it is possible that the constrictions are related with lower levels of oestrogen or with uterine insensitivity to oestrogen. However, the physiological background of the uterine constrictions and of the non-attachment sites still needs to be investigated.

Box 3. Sow at 35 days of pregnancy with foetus in the abdominal cavity

One sow slaughtered at 35 days of pregnancy had an aperture in the left uterine horn which was associated with an extra-uterine foetus. The foetus was fully grown, which suggests that it crossed the uterine wall at the previous farrowing, approximately 65 days earlier. In the right uterine horn, the sow had 8 embryos, of which 6 were vital. Total ovulation rate was 14; no corpora lutea (CL) on the left ovary, 14 on the right ovary.

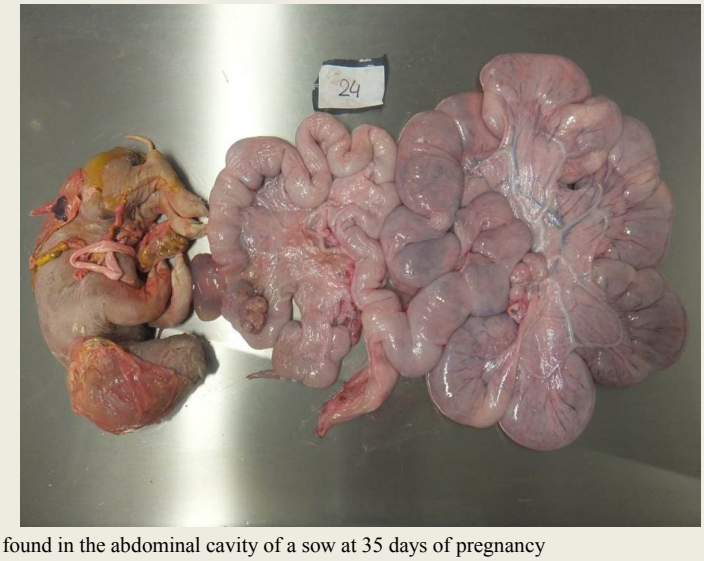

Figure. Foetus found in the abdominal cavity of a sow at 35 days of pregnancy

Spontaneous uterine rupture mainly occurs due to extreme myometrial contractions during parturition and due to other factors like, dystocia, failure of cervical dilatation and uterine torsion. Obstructions of the birth canal, simultaneously presentation of two foetuses and oversized foetuses are some of the predisposing factors for dystocia in swine (Schuh and Kurth 2005). Despite that, follicle development and ovulation may have been unaffected. However, uterine rupture probably prevented fertilisation of the oocytes from the left ovary. Normally during pregnancy, 17β – oestradiol is produced by the embryos, which ensures that the luteolytic PGF2α that is produced by the endometrium, does not reach the ovary and thus the CL are rescued from luteolysis and remain producing progesterone (Niswender et al. 2000). In this sow, the left horn did not contain embryos and did not have CL at the ovary. This suggests, that although there may have been trans-uterine migration of some of the embryos from the right uterine horn to the left uterine horn at day 8 to 11 of pregnancy, there were too few embryos present to prevent luteolysis of the CL on the left ovary. Interestingly, however, CL on the right ovary did not suffer luteolysis. Finally, the continued progesterone production on the right ovary prevented the growth of large follicles on the left ovary.

Gilts had a lower average OR than multiparous sows [*20.9 (14 up to 34)* vs *25.5 (17 up to 38)*], as it was described by Town et al. (2005) [gilts OR 20.2 ± 0.5 vs multiparous sows (parities 2 to > 4) OR 23.6 ± 0.4, P ≤ 0.05]. In both gilts [*Chapter 4*; Blichfeldt and Almlid (1982)] and sows [*Chapter 3*; Wu et al. (1987)] an increase in OR leads to an increase in the number of vital embryos up to a maximum. These results shows that in gilts and sows with higher OR there is a decrease in efficiency in the increase in number of embryos with the increase in OR (i.e. a weaker slope of the line). This limitation in the increase in the number of embryos is related with the increase in early and late embryonic mortality with the increase in OR in both gilts and sows [*Figure 7.3*].

Gilts had a lower total embryonic mortality up to 35 days of pregnancy and a higher proportion of early embryonic mortality than the multiparous sows [*74.1 % vs 59.3 %*]. Due to the higher proportion of early embryonic mortality, an increase in OR in gilts is associated with an increase in empty uterine space around the vital embryos [F*igure 4.1D*]. Moreover, the uterine space available per embryo reaches a minimum of 30.5 cm at 25 ovulations and increases to > 35 cm in gilts with OR ≥ 30 [*Figure 7.4F*]. This might indicate a decrease in uterine crowding in gilts at 35 days of pregnancy with an increase in OR. On the other hand, in sows an increase in OR leads to a decrease in the empty uterine space around the vital embryos [F*igure 3.2d*], and the uterine space available per embryo decreases with the increase in OR [*Figure 7.4E*], which might indicate an increase in uterine crowding in sows at 35 days of pregnancy with an increase in OR. However, also in sows, high levels of early embryonic mortality alleviates uterine crowding, as sows with a high (> 7) incidence of early embryonic mortality had longer average empty uterine space around the vital embryos compared to sows with a low (± 1) incidence of early embryonic mortality [*19 cm vs 11 cm, Figure 3.2e*]. Thus, in gilts it seems like there was no increase in uterine crowding with the increase in OR, while in sows, in general, an increase in OR led to a higher uterine crowding.

The higher proportion of early embryonic mortality in gilts might be related with lower fertilization rates as a consequence of the use of semen stored for 3 to up to 10 days at 17°C before the inseminations. Early embryonic mortality is estimated as the difference between OR and the total number of embryos in the uterus at 35 days of pregnancy, and assumes an optimal fertilization rate (~100%), which is normally achieved with the use of fresh semen (< 24 hours of storage) (De Ambrogi et al. 2006). The use of artificial insemination doses stored for more than 12 to 24 hours following extension of the semen may lead to a decrease in fertilization rate (De Ambrogi et al. 2006). Lower fertilization rate with the use of stored semen is related with changes in sperm morphology, as for example in the plasma membrane, mitochondria or in the nuclear deoxyribonucleic acid (DNA) (Peris et al. 2004). Analyses of the relationship between OR and early embryonic mortality per semen storage duration classes [*Figure 7.5*] shows that, although the increase in early embryonic mortality with the increase in OR occurs independently of semen storage (i.e. there is no interaction between OR and semen storage duration), there is a higher proportion of early embryonic mortality in gilts inseminated with semen stored for 8 to 10 days (P ≤ 0.05). Thus, the incidence of early

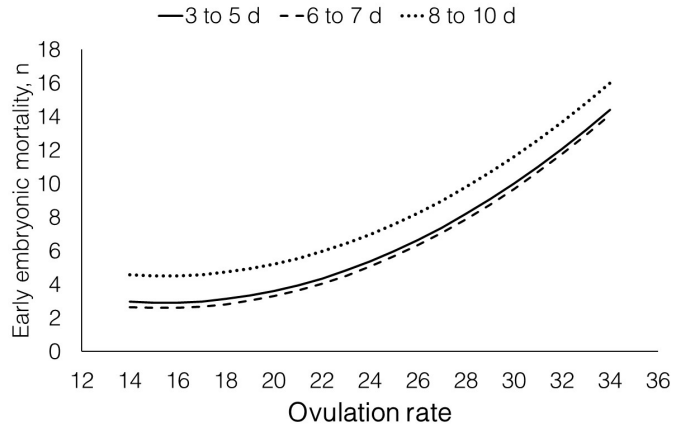

Figure 7.5 Relationship between ovulation rate (OR) and early embryonic mortality in gilts at 35 days of pregnancy (n = 298) within semen storage duration classes [3 to 5 days, n = 59; 6 to 7 days, n = 133; and 8 to 10 days, n = 106]. Model included the linear and quadratic term of OR, the fixed class effect of semen storage duration classes, and of gilts genetic line (GL, purebred Landrace, n = 6; crossbred Landrace x Yorkshire, n = 212). Semen storage duration (SS) 3 to 5 days = 9.9 ± 6.2 – 1.02 ± 0.6 * OR + 0.03 ± 0.01 * OR2; SS 6 to 7 days = 9.5 ± 6.3 – 1.02 ± 0.6 * OR + 0.03 ± 0.01 * OR2; SS 8 to 10 days = 11.5 ± 6.8 – 1.02 ± 0.6 * OR + 0.03 ± 0.01 * OR2; OR P = 0.11; OR2 P = 0.02; SS P= 0.001; GL P = 0.001.

embryonic mortality in this population of gilts might be higher than in sows due to an overestimation explained by a decrease in fertilization rate as a consequence of the use of old semen.

An increase in OR influences also the development of vital embryos at 35 days of pregnancy through different mechanisms, for example **(1)** by increasing within litter heterogeneity in embryonic development at early stages of pregnancy; or **(2)** by increasing uterine crowding and compromising placental development [*see in scheme, Figure 7.2*]. Although these mechanisms may occur simultaneously, it is also possible that some of the mechanisms become more or less important in different situations. For example, in gilts the use of semen stored for longer time might have increased the proportion of early embryonic mortality, and consequently, an increase in OR did not decreased the empty uterine space around the vital embryos or the uterine length available per embryo at 35 days of pregnancy, indicating that there is no increase in uterine crowding with the increase in OR. Consequently, an increase in OR did not influence the average implantation length and the average weight of the vital embryos at 35 days of pregnancy [*Figure 7.4 B and D*]. Since there is no indication of an increase in uterine crowding with the increase in OR in gilts, the higher within litter variation in implantation length and in weight of the vital embryos at higher OR might originate from a higher embryonic heterogeneity in development at earlier stages of pregnancy, as the length of uterine implantation is determined by the length of embryonic elongation at days 10 to 12 of pregnancy (Geisert et al. 1982). On the other hand, in sows an increase in OR was related with a decrease in empty uterine space around the vital embryos and in the uterine length available per embryo, indicating an increase in uterine crowding. An increase in uterine

crowding with the increase in OR in sows is also shown by the decrease in average implantation length and in average placental length of the vital embryos, but this did not influence the average and variation in weight of the vital embryos [*Figure 7.4A and C*]. This is not surprising, as embryonic growth rate is less sensitive to uterine crowding than placental growth rate (Vallet et al. 2003). Thus, results of this thesis indicate gilts with a higher OR had a higher within litter heterogeneity in pre-implantation embryonic development, which persisted to the post-implantation period even though they did not experience uterine crowding; while sows with a higher OR did have higher uterine crowding, which compromised the development of the placenta of the vital embryos.

Thus, in this thesis the relationship between OR and embryonic survival and development up to 35 days of pregnancy was different between gilts and sows; which might lead to different consequences for foetal development in later stages of pregnancy and consequently different litter characteristics at birth. In gilts, the higher variation in vital embryonic implantation length and weight at 35 days of pregnancy with higher OR might lead to **(1)** a decrease in the number of piglets born alive due to higher foetal mortality, **(2)** to a decrease in average piglet birth weight, as in case of mortality after 35 days of pregnancy, vital foetuses can hardly benefit from the uterine space occupied by the dead foetuses causing uterine crowding in later pregnancy (Vonnahme et al. 2002a; Vallet et al. 2011), and **(3)** to a higher number of piglets with lower birth weight thus increasing the within litter variation in piglet birth weight, in case the smaller embryos of the litter survive to term. In sows, the compromised placental development at 35 days of pregnancy with higher OR might also lead to **(1)** lower number of piglets born alive due to higher foetal mortality, **(2)** a decrease in average piglet birth weight due to the compromised placental development; and **(3)** to an increase in within litter variation in piglet birth weight due to the higher competition for uterine space and nutrients between embryos at the beginning of pregnancy (Père et al. 1997). Although these conclusions are based in results of only one population of gilts and sows, they indicate the importance of the interaction between the underlying traits for litter characteristics at birth.

7.2 Corpora lutea size and embryonic development at 35 days of pregnancy and litter characteristics at birth

Ovulation rate is related with embryonic survival and development up to 35 days of pregnancy, and it is, therefore, expected to influence litter characteristics at birth. Assessment of OR in gilts and sows has been mainly done by slaughter during pregnancy and dissection of the ovaries (Blichfeldt and Almlid 1982; Irgang et al. 1993; Vonnahme et al. 2002a; Vonnahme et al. 2002b; Town et al. 2004; Town et al. 2005; Hoving et al. 2012; Langendijk et al. 2012; Wientjes et al. 2012a). However, to check the relationship between OR and litter characteristics at birth, it would be necessary to assess OR in live animals. This could be done by laparoscopy (King and Williams 1984) or by the use of transrectal ultrasonography (Soede et al. 1992).

Transrectal ultrasonography has been used to assess ovulation rate (OR) by counting the number of pre-ovulatory follicles in sows (Soede et al. 1992; Soede et al. 1998; Bolarin et al. 2009), but it was only in 2009 that Gonzalez-Añover et al. investigated the use of transrectal ultrasonography to assess OR by counting the number of corpora lutea (CL) in Iberian sows, reporting an accuracy of ~ 100 %. However, the average OR of the Iberian sows used was only 6.0 ± 1.3, much lower than the average OR described for commercial crossbred sows (> 25); (Vonnahme et al. 2002b; Town et al. 2005; Da Silva et al. 2016).

The accuracy of transrectal ultrasonography (TUS) to assess the OR by counting the number of CL in pregnant modern crossbred sows with expected higher OR was investigated [*Chapter 5*]. Transrectal ultrasonography did not provide an accurate assessment of OR by counting the number of CL in pregnant modern crossbred sows with an average OR of 24.1 ± 5.3. The relationship between OR assessed by TUS and after slaughter and dissection of the ovaries was low [*β = + 0.28 ± 0.01 CL TUS/CL after dissection;* F*igure 5.2B*] and the difference in CL number counted with TUS and after ovarian dissection was > 1 CL in as many as 75.6% of the sows examined. The differences ranged from an underestimation of 8 CL up to an overestimation of 12 CL in the total number of CL counted per sow [F*igure 5.3*]. This lack of accuracy is probably related to the high number of CL present per ovary, which is aggravated by the decrease in average CL size [*Figure 5.2D*]. Thus, it is not possible to investigate the relationship between the number of CL (OR) and litter characteristics at birth.

The accuracy of transrectal ultrasonography to assess the average CL diameter in pregnant modern crossbred sows was also investigated [*Chapter 5*]. Transrectal ultrasonography (TUS) is an accurate method to assess the average CL diameter in pregnant sows, as shown by the strong relationship between the CL diameter measured by transrectal ultrasonography and after dissection of the ovaries [*β = 1.0 ± 0.1 mm TUS/mm CL after dissection,* R^2 *= 0.96, figure 5.2C*]. The average CL diameter observed for sows with TUS and after dissection was 10.3 mm, which is similar to the average CL diameter observed by Wientjes et al. (2012a), of 10.0 ± 0.3 mm in sows with similar OR [24.3 ± 1.20].

Results of this thesis shows that average CL diameter in sows at 3 to 4 weeks of pregnancy is related with piglet birth weight [*Chapter 5*]. Sows with higher average CL diameter (9.0 to 10.5 mm), as measured by TUS had a higher average piglet birth weight than sows with lower average CL diameter (5.5 to 7.8 mm) [*1,338.3 ± 27.3 g vs 1,270.5 ± 30.9 g, P = 0.04, Figure 5.4*]. Further analyses showed that CL size was also related with early embryonic development, as gilts at 35 days of pregnancy with higher average CL weight (0.46 to 0.61 g) also had a higher average weight of the vital embryos and a higher average length of uterine implantation site than gilts with lower average CL weight (0.27 to 0.40g) [*Figure 7.6*].

The physiological mechanisms that link corpora lutea size with piglet birth weight and with embryonic development are unknown. Corpora lutea are responsible for progesterone production during pregnancy, a hormone of primary importance for maintenance of pregnancy and early embryonic development (Spencer and Bazer 2002; Spencer and Bazer

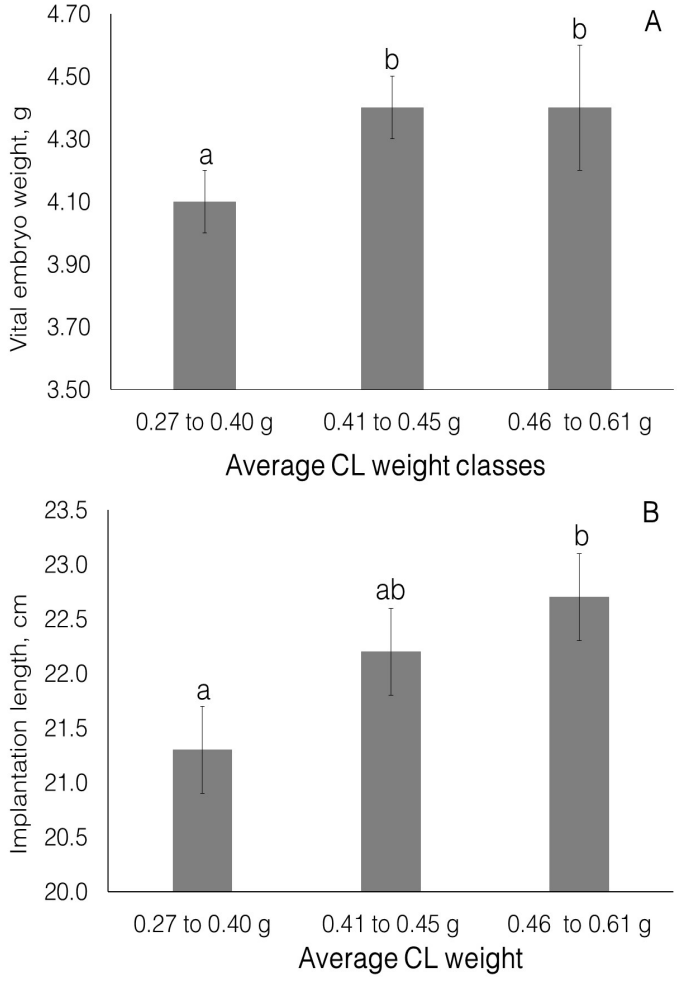

Figure 7.6 Estimated least square means for the effect of average corpora lutea (CL) weight classes on the average vital embryo weight and on the average implantation length of the vital embryos in gilts (n = 390) at 35 days of pregnancy. **(A)** The model included the fixed classes effect of average CL weight [0.27 to 0.40 g, n= 118; 0.41 to 0.45 g, n= 134; and 0.46 to 0.61 g, n = 138; P = 0.003]; of semen storage duration [SS, 3 to 5 days, n = 109; 6 to 7 days, n = 159; 8 to 10 days, n = 122, P = 0.004] and of gilts genetic line [Purebred Landrace, n = 86; crossbred Yorkshire x Landrace, n = 304]. **(B)** The model included the fixed class effect of average CL weight (P = 0.03) and of the gilts genetic line (P = 0.004). Letters above the columns indicate significant differences between the average CL weight classes ($P \leq 0.05$) and the error bars indicate the SE of the estimates.

Linear relationships between average corpora lutea weight and average weight of the vital embryos in gilts at 35 days of pregnancy (n = 390): β =+ 2.27 ± 0.7 g/ g of CL weight (P = 0.001) + intercept dependent on semen storage duration classes (P = 0.01), 3 to 5 days (n = 109): 3.42 ± 0.43; 6 to 7 days (n = 159): 3.29 ± 0.43 and 8 to 10 days (n = 122): 3.07 ± 0.33; and average corpora lutea weight and average length of uterine implantation site in gilts at 35 days of pregnancy (n = 390): *β =+ 10.3 ± 3.5 cm/ g of CL weight (P = 0.004) + intercept dependent of the gilts genetic line (P = 0.01), purebred Landrace (n = 86): 18.3 ± 2.2 and crossbred Yorkshire x Landrace gilts (n = 304): 16.7 ± 1.6.*

2002; Spencer and Bazer 2004). So it could be hypothesised that a higher CL size in pregnant sows is related with a higher progesterone production and higher plasmatic levels, thus favouring embryonic growth and piglet birth weight. However, systemic progesterone levels did not increase with the increase in average CL weight in gilts at 35 days of pregnancy [$P > 0.05$].

Another hypothesis is that there is an association between CL size and embryonic quality. Larger corpora lutea develop from larger follicles at ovulation (Soede et al. 1998; Wientjes et al. 2012a). Larger follicles might release oocytes with better development potential (Hunter 2000). For example, higher success in *in vitro* production of embryos in ruminants (i.e. higher percentage of embryos developing to the blastocyst stage) is achieved when oocytes are collected from large follicles. This indicates that some important events take place in the oocyte during late follicular growth, leading to its full development competence (Marchal et al. 2002). Oocytes become able to complete meiosis up to metaphase I and proceed to metaphase II in growing antral follicles ≥ 5 mm in diameter (Hunter 2000). However, beyond the acquisition of meiotic competence, oocytes have to acquire the ability to support cytoplasmic maturation and to be successfully fertilized to develop into a viable embryo. This terminal differentiation of meiotically competent oocytes (capacitation) occurs at the end of folliculogenesis (Mermillod et al. 1999), however, oocytes continue to store mRNA and proteins during further follicular growth that may be involved in the regulation of the fertilization process or necessary during the first steps of embryonic development (Marchal et al. 2002). Thus, larger follicles have oocytes with a higher development potential that also generates embryos of higher growth potential (Marchal et al. 2002). These embryos with a higher growth potential start elongation earlier and have a longer elongation length at implantation, and consequently acquire a longer implantation length (Geisert et al. 1982), and therefore an longer placenta length (Stroband and van der Lende 1990). The acquisition of a longer placental length would explain the higher weight of the vital embryos at 35 days of pregnancy in gilts with a higher average CL weight. It is also possible that CL weight and vital embryonic weight are not causally related but share the same origin. Oestradiol - 17β released by the embryos during elongation increases intra-ovarian production of prostaglandin E2 (PGE2), a luteoprotective that stimulates the production of VEGF (vascular endothelia growth factor) in luteal cells, which might increase CL blood flow (Ziecik et al. 2011) and thereby favour CL growth.

Sows with higher average CL diameter (9.0 to 10.5 mm), as measured by TUS, had a higher variation in piglet birth weight than sows with lower average CL diameter (5.5 to 7.8 mm) [*318.6 ± 17.0 g vs and 252.2 ± 17.9 g, P = 0.02, Figure 5.4*]. This is probably related with uterine crowding. A higher embryonic weight and a higher placental length at 35 days of pregnancy will favour foetal development in further pregnancy, thus leading to a higher average piglet birth weight. However, as pregnancy progresses, the negative effects of limited uterine space on foetal development increases (Vonnahme et al. 2002a), and an increase in competition for nutrient uptake between littermates during foetal growth, might increase the within litter variation in foetal growth (Ford et al. 2002), which could explain the increase in

within litter variation in piglet birth weight in sows with a higher average CL weight. Thus, an increase in CL weight and diameter favours embryonic growth and piglet birth weight, but it will also lead to a higher variation in piglet birth weight if there is limited uterine space.
Thus, results have shown that an increase in OR leads to a decrease in CL size in gilts and sows at early pregnancy [*Chapters 4 and 5*]. Considering the relationship between average CL diameter and piglet birth weight, as discussed above, this provides further indication that an increase in OR leads to a compromised development of the vital embryos which may culminate in a compromised piglet birth weight.

7.3. Consequences of genetic selection for litter characteristics at birth on ovulation rate, corpora lutea weight and embryonic survival and development at 35 days of pregnancy

Genetic selection for litter traits at birth is based on estimated breeding values (EBVs) to select sows with high genetic merit for *e.g.* total number of piglets born, average piglet birth weight and within litter piglet birth weight uniformity[1]. Estimated breeding values are expressed in units of measurements for each particular trait and are shown as positive or negative differences between a sows estimated genetic potential and the genetic average to which the sow is being compared (set to zero). Thus, for example, sows with an EBV for total number of piglets born (TNB) of + 1 are estimated to have a genetic merit of 1 piglet per litter above the breed average, while sows with an EBV for TNB of -1 are estimated to have a genetic merit of 1 piglet per litter below the breed average.
Ovulation rate (OR), average corpora lutea (CL) weight, uterine and embryonic survival and development at 35 days of pregnancy might influence litter traits at birth [*Chapters 3, 4 and 5*]. Therefore, understanding how these traits respond to genetic selection for litter traits at birth [*total number of piglets born (TNB), average piglet birth weight (BW) and within litter piglet birth weight standard deviation (BWSD)*] might help clarify the mechanisms leading to the compromised piglet birth weight often described in large litters. Therefore, the relationships between EBVs for TNB, BW and BWSD and ovarian, uterine and embryonic traits in gilts at 35 days of pregnancy were investigated [*Chapter 6*].
Results of the relationships between the EBVs for TNB, BW and BWSD and ovarian, uterine and embryonic traits at 35 days of pregnancy are summarized in Table 7.1. Interestingly, EBVs for litter traits at birth were mainly related with ovarian traits and not with embryonic mortality or development (weight, implantation length and area traits) at 35 days of pregnancy. Thus, at 35 days of pregnancy, ovarian traits seem more responsive to genetic selection for litter characteristics at birth than traits related with embryonic development.

[1] Selection for a higher within litter piglet birth weight uniformity occurs by selection of animals with the lowest estimated breeding values (EBV) for within litter piglet birth weight standard deviation (BWSD). So a higher genetic merit for litter uniformity means a lower EBV for BWSD.

Table 7.1 Relationship between the EBV for total number of piglets born (TNB), average piglet birth weight (BW) and within litter piglet birth weight standard deviation (BWSD) and the ovarian, uterine and embryonic traits in gilts at 35 days of pregnancy [adapted from Table 6.3].

Variables	EBV TNB	EBV BW	EBV BWSD
	P value	P value	P value
Averages			
Ovulation rate, OR	**	ns	ns
Corpus luteum weight, g	*	**	**
Total luteal mass, g	*	ns	**
Uterine length, cm	**	ns	ns
Number of embryos	**	ns	ns
Number of vital embryos	**	ns	ns
Early embryonic mortality	ns	ns	ns
Late embryonic mortality	ns	ns	ns
Embryo weight, g	ns	ns	ns
Empty space, cm	*	ns	ns
Implantation length, cm	ns	ns	ns
Implantation area, cm^2	ns	ns	*
Standard Deviations			
Corpus luteum weight, g	ns	ns	ns
Embryo weight, g	ns	ns	ns
Empty space	*	ns	ns
Implantation length, cm	ns	*	ns

ns Not Significant (P > 0.05); *P < 0.05; **P < 0.01.

An increase in the EBV for TNB was related with a proportional increase in OR and in number of vital embryos at 35 days of pregnancy, without influencing the incidence of early and late embryonic mortality [*Figure 7.7*]. Previous results from studies on direct selection for TNB also showed a proportional increase in OR and no improvements in prenatal survival (Driancourt et al. 1992; Bidanel 2011). However, genetic selection for TNB leads to a higher variance in TNB and probably also in OR, due to the higher genetic correlation between the two traits. In other words, with selection on TNB there is an increased probability that OR will reach extreme values i.e. decanalization (Gottlieb 2001). Thus, this suggests that genetic selection for higher TNB does not lead to an exponential increase in OR but instead increases the variance of OR leading to the phenotypic expression of extreme values in OR.

Interestingly, an increase in EBV for TNB did not decrease average uterine implantation length and implantation area of the vital embryos, which are important traits for placental development (Stroband and van der Lende 1990). Moreover, an increase in EBV for TNB did not influence the average weight of the vital embryos at 35 days of pregnancy, despite the higher OR and number of vital embryos, and the lower average empty uterine space around the vital embryos (which indicates a higher uterine occupation). Thus, genetic selection for higher TNB does not directly compromise embryonic development at 35 days of pregnancy.

Results suggests that uterine crowding is not aggravated in gilts with higher genetic merit for TNB at 35 days of pregnancy. However, this should be interpreted with careful as uterine

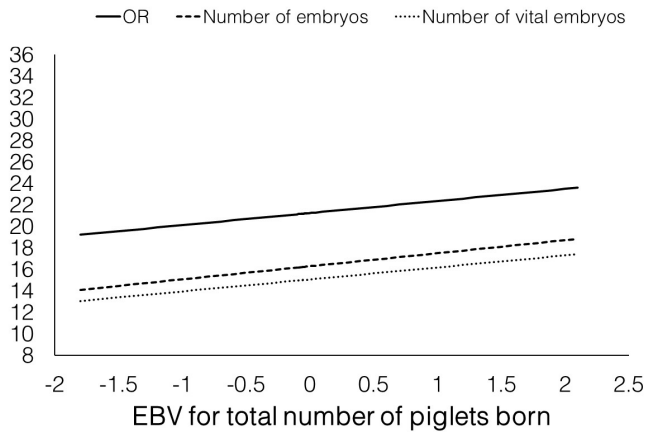

Figure 7.7 Relationship between the estimated breeding value (EBV) for total number of piglets born (TNB) in gilts ovarian and embryonic traits in gilts at 35 days of pregnancy. Ovulation rate (OR) [(black solid line); β = + 1.12 ± 0.2 ovulations / EBV (P < 0.0001); GL P < 0.0001]; total number of embryos [(black dashed line); β = 1.22 ± 0.4 embryos / EBV (P = 0.001)]; and number of vital embryos [(black dotted line); β = 1.12 ± 0.3 ovulations / EBV (P = 0.002)].

Statistical models included the linear and quadratic term of the EBV for TNB, the fixed class effect of gilts genetic line [(GL), purebred Landrace, n = 86; crossbred Yorkshire x Landrace, n = 304]; and of semen storage duration classes [(SS), 3 to 5 days, n = 105; 6 to 7 days, n = 159; 8 to 10 days, n = 122] and their interactions. Fixed class effects, interactions and the quadratic term of the EBVs were excluded from the models when not significant (P > 0.05). P values were corrected for Bonferroni.

length increases with the increase in OR [*Chapters 3 and 4*] and in number of embryos, which makes it difficult to estimate uterine crowding. Thus, although there were no significant negative effects on the development of the vital embryos at 35 days of pregnancy, it is still possible that uterine crowding compromises further foetal growth and survival (later in pregnancy) in gilts with higher EBV for TNB.

To understand if relationships between OR and embryonic survival and embryonic development are influenced by genetic selection for TNB, these relationships were investigated in gilts divided in classes of low, intermediate and high genetic merit for TNB [*Figure 7.8*].

Results show that in gilts with low and intermediate genetic merit for TNB there is an increase in the number of vital embryos with the increase in OR up to a maximum [*Figure 7.8A*], after which more ovulations do not result in more vital embryos at 35 days of pregnancy. This limited response in number of vital embryos with an increase in OR was also described for gilts and sows in previous chapters [*Chapters 3 and 4*]. However, gilts with high genetic merit for TNB kept a linear increase in number of vital embryos with the increase in OR [*Figure 7.8A*]. Thus, modern dam line gilts selected for higher TNB have a more efficient response in number of vital embryos at 35 days of pregnancy with the increase in OR.

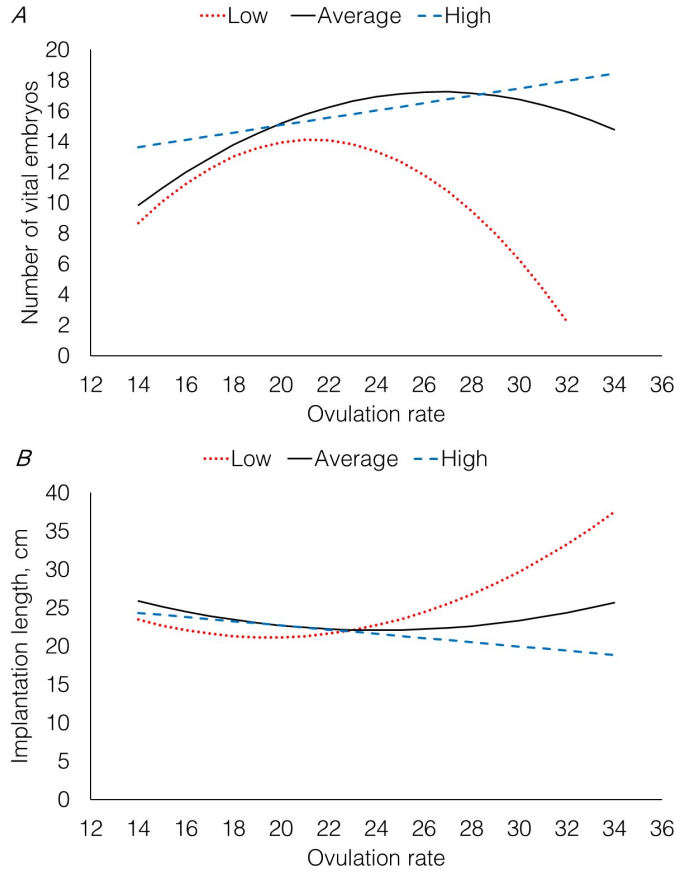

Figure 7.8 Relationship between OR and (A) the number of vital embryos and (B) their length of uterine implantation at 35 days of pregnancy in gilts with low [-1.8 to -0.39, n = 122], average [-0.38 to 0.09, n = 128] and high [0.10 to 2.1, n = 140] estimated breeding value (EBV) for total number of piglets born (TNB). (A) Low [(red solid line) + 4.4 ± 1.5 * OR (P = 0.01) - 0.10 ± 0.04 * OR2 (P = 0.01), GL = 0.01; GL * SS P = 0.02]; Average [(black solid line) + 2.5 ± 1.0 * OR (P = 0.01) - 0.04 ± 0.02 * OR2 (P = 0.04)]; High [(blue solid line) + 0.24 ± 0.11 * OR (P = 0.03), GL *SS P = 0.01]. (B) Low [(red solid line) -3.02 ± 1.7 * OR (P = 0.07) + 0.08 ± 0.04 * OR2 (P = 0.06)]; Average [(black solid line) -1.78 ± 1.11 * OR (P = 0.11) + 0.04 ± 0.03 * OR2 (P = 0.16), GL P = 0.003]; High [(blue solid line) -0.27 ± 0.13 * OR (P = 0.03), GL * SS P = 0.04.

Statistical models included the linear and quadratic term of OR and the fixed class effect of gilts genetic line [(GL), purebred Landrace, n = 86; crossbred Yorkshire x Landrace, n = 304]; and of semen storage duration classes [(SS), 3 to 5 days, n = 105; 6 to 7 days, n = 159; 8 to 10 days, n = 122] and their interactions. The quadratic term of OR was excluded from the models when not significant. Fixed class effects and interactions were excluded from the models when not significant (P > 0.05).

However, in gilts with high genetic merit for TNB, an increase in OR is related with a decrease in uterine implantation length of the vital embryos at 35 days of pregnancy [*Figure 7.8B*]. Smaller uterine implantation length results in smaller placental length (Stroband and van der Lende 1990), which compromises foetal growth due to insufficient nutrient uptake, leading to lower piglet BW. Thus, the decrease in piglet birth weight that follows genetic selection for higher TNB might be a consequence of compromised placental development of vital embryos that follows the increase in OR (see box 4 for more information).

An increase in EBV for TNB was related with a decrease in average CL weight at 35 days of pregnancy. Phenotypically, a lower average CL weight was related with smaller average uterine implantation length and lower average weight of the vital embryos at 35 days of pregnancy in gilts [*Chapter 4*]. Also, a lower average CL diameter at ~24 days of pregnancy was related with lower average piglet BW [*Chapter 5*]. Thus, the lower average CL weight in gilts with higher genetic merit for TNB might be part of the mechanism that leads to a lower BW in piglets born in large litters (see box 5 for more information).

Average CL weight was not only related with genetic selection for higher TNB, but also with genetic selection for piglet BW and BWSD. As highlighted in *Table 7.1*, average CL weight was the sole trait related with the EBVs for piglet BW and BWSD, being also the only trait influenced by the three EBVs investigated here. This indicates that CL weight in early pregnancy is an important trait influencing litter traits at birth.

One possible explanation for the fact that average CL weight is influenced by all EBVs for litter traits is that TNB, average piglet BW and within litter BWSD are genetically correlated

Box 4.

The decrease in uterine implantation length and placental length with the increase in OR might be related with: 1) an increase in within litter embryonic heterogeneity in development, which causes embryos with delayed development to have a smaller elongation length at the moment of implantation, consequently acquiring a smaller uterine implantation site (Geisert et al. 1982) and developing a smaller placental length (Stroband and van der Lende 1990); and 2) an increase in uterine crowding, when an increase in number of embryos surviving to the post-implantation period (and therefore competing for an implantation site in the uterus) exceeds the uterine capacity (Foxcroft et al. 2006).

Box 5.

Lower average CL weight in early pregnancy indicates smaller diameter of pre-ovulatory follicles (Soede et al. 1998; Wientjes et al. 2012a), that might also ovulate oocytes of lower quality (Ding and Foxcroft 1992; Ding and Foxcroft 1994). Lower oocyte quality leads to lower embryonic quality with a compromised potential to grow (Krisher 2004), possibly compromising foetal growth resulting in lower piglet BW.

traits (Damgaard et al. 2003; Wolf et al. 2008). This is the result of two possible mechanisms: 1) linkage disequilibrium, in which two genes are closely linked on the genome, influencing two unrelated traits; or 2) pleiotropy in which a gene influences two or more related traits (Rauw 2009). The latter is more likely (Rauw 2009), and therefore it can be hypothesised that this is also the case for CL weight and the litter traits studied here. However, considering the importance of CL weight for vital embryos uterine implantation length and weight of the vital embryos at 35 days of pregnancy and for piglet BW (at the phenotypic level), it is not surprising that this trait responds to an increase in EBV for BW. It is, however, intriguing that an increase in EBV for BW did not influence traits related with uterine crowding, such as uterine length, empty uterine space around the vital embryos, or the uterine space available per embryo (i.e. uterine length/number of embryos, an indication of uterine crowding) at 35 days of pregnancy [*Figure 7.9*]. Also measurements of placental development, such as average implantation length and area, or the average weight of the vital embryos at 35 days of pregnancy were not influenced by EBV for BW. Thus, at 35 days of pregnancy, genetic improvements in piglet BW seems to occur through increases in average CL weight (which indicates improvements in follicular and embryonic quality), and not through improvements in uterine and embryonic traits.

The EBV for BW does not influence uterine traits at 35 days of pregnancy that, if improved, could alleviate the negative effects of uterine crowding in further pregnancy. However, an

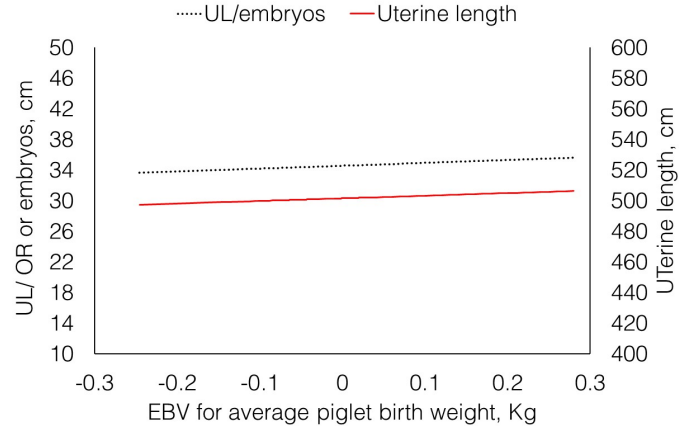

Figure 7.9 Relationship between the estimated breeding value (EBV) for average piglet birth weight (BW) in gilts and ovarian and uterine traits at 35 days of pregnancy. Uterine length available per total number of embryo (UL/embryo, cm) [(*black dotted line*); β = 3.74 ± 12.0 cm / Kg (P = 0.75)]; Uterine length (cm) [(*red solid line*); β = 17.2 ± 42.2 cm / Kg (P = 0.68)].

Statistical models included the linear and quadratic term of the EBV for BW or BWSD, the fixed class effect of gilts genetic line [(GL), purebred Landrace, n = 86; crossbred Yorkshire x Landrace, n = 304]; and of semen storage duration classes [(SS), 3 to 5 days, n = 105; 6 to 7 days, n = 159; 8 to 10 days, n = 122]; and their interactions. Fixed class effects, interactions and the quadratic term of the EBVs were excluded from the models when not significant (P > 0.05).

increase in EBV for BW seems to result in embryos with higher growth potential (heavier at 35 days of pregnancy and at birth). This combination of embryos with higher growth potential without improvements in available uterine space might lead to higher competition between littermates for uterine space and nutrient supply after 35 days of pregnancy. This might increase within litter BWSD. Thus, the absence of improvements in uterine traits at 35 days of pregnancy might explain the increase in BWSD seen in gilts with higher genetic merit for BW.

For breeding companies it is important to know that in modern gilts with high genetic merit for TNB, the increase in number of embryos with the increase in OR does not reach a point where this increase reduces. However although the efficiency of producing more vital embryos with the increase in OR does not seem affected at higher OR, the quality (implantation length) of these embryos seems to be compromised. Moreover, the decrease in average CL weight that follows genetic selection for higher TNB might also explain the lower average piglet BW in big litters. Lower CL weight is also related with lower genetic potential for piglet BW. Thus, genetic programs aiming to improve piglet BW in big litters must also take corpora lutea weight at early pregnancy (as an indication of follicular/oocyte and embryonic quality) into consideration, and not only improvements in uterine capacity.

7.4. Importance of ovarian, uterine and embryonic development traits for modern genetic selection programs

Estimations of heritability of ovarian, embryonic and uterine traits in gilts at 35 days of pregnancy were mainly high, ranging from 0.36 for vital embryonic weight to 0.70 for average corpora lutea (CL) weight [*Chapter 6*], which indicates that these traits can be genetically improved and therefore used in genetic selection programs aiming to improve litter traits at birth.

Constrains for the use of ovarian, uterine and embryonic traits in genetic selection programs are: the absence of information regarding their genetic correlation with production traits, the fact that these traits are not genetically independent from each other, and the necessity of slaughter to phenotype animals for uterine and embryonic traits.

However, differently from uterine traits and traits related with embryonic development (vital embryonic weight, implantation length and area), which can only be measured after slaughter, it is possible to assess ovulation rate (OR) and CL size in live sows. Although the use of transrectal ultrasonography to assess OR in pregnant sows by counting the number of CL was not proven successful [*Chapter 5*], assessment of OR by counting the number of pre-ovulatory follicles with transrectal ultrasonography can be an alternative (Soede et al. 1992; Soede et al. 1998), although its validation in modern crossbred animals with high OR is recommended. Moreover, transrectal ultrasonography provides accurate measurements of CL diameter in pregnant sows, which has a high correlation with CL weight [*Chapter 5*]. Thus, transrectal ultrasonography can be used to phenotype sows for OR and CL diameter, and can be used as a tool in genetic improvement programs.

The phenotypic relationships between OR and average CL weight, uterine length and embryonic survival and development in gilts and sows at 35 days of pregnancy [*Chapters 3 and 4*] are partly genetic, as shown by the moderate to high genetic correlations between these traits [*Table 6.5*]. This indicates that genetic selection for higher average CL weight (i.e. when selecting for higher piglet BW) might simultaneously select for lower OR [r_g = *-0.58*] and for lower number of vital embryos at 35 days of pregnancy [r_g = *-0.87*], which might result in lower TNB. Thus, balanced genetic selection is needed, which can be achieved by the use of a selection index.

Modern genetic selection programs counteract the necessity to improve piglet BW without decreasing TNB through the use of a **selection index,** in which different weights are given to different traits based on the final breeding goal. Similarly, to balance the negative association between average CL weight and OR, these traits could be included in the selection index. If the breeding goal (H) is to improve the survival of piglets born in large litters during lactation, phenotypes related with survival, such as average piglet BW and within litter BWSD, must be included in the selection index (I) with a higher relative weight than the total number of piglets born. In that sense, average CL weight should be also included as a phenotype related with piglet survival and given a higher weight than OR, included as a phenotype related with TNB. This would, ideally, improve the correlation between the breeding goal and the index selection (r_{IH}), balancing the correlated phenotypic response. Thus, despite the moderate negative genetic correlation between OR and average CL weight, the inclusion of these traits in the selection index, together with TNB, BW and BWSD, will likely balance selection for litter traits probably resulting in heavier litters without losses in TNB.

Moreover, the identification of specific genes influencing ovarian, uterine and embryonic development traits in early pregnancy would allow **marker-assisted selection** for litter traits or, alternatively, **genomic selection** could be used (Calus 2010). Genomic selection uses dense marker maps to predict the breeding value of animals with reported accuracies that are higher than those of pedigree indexes, without the need to phenotype the animals themselves (Goddard 2009). The key issue in genomic selection is estimation of effects of individual SNP alleles on the trait of interest in a reference population. This reference population preferably comprises a minimum of 1000 individuals with reliable phenotypic and genotypic information. By linking the phenotypic and genotypic information, estimates of the effect of each of the SNPs are obtained. By genotyping a juvenile population (that share their recent pedigree with the reference population) genomic estimated breeding values (GEBVs) are obtained by summing up all the relevant SNP effects for each phenotypic trait (Calus 2010). Although more accurate than the pedigree-based EBVs, the accuracy of the GEBVs strongly depends on the characteristics of the reference population, such as the number of phenotyped animals, but more important, it depends on the number of independent *loci* affecting the traits and of the heritability of the phenotypes (Goddard 2009). Based on this, a reference population with phenotypes and genotypes can be created for ovarian, uterine and embryonic development traits. Thus, since underlying traits influencing litter characteristics at birth have higher heritabilities than litter traits at birth itself (due to lower residual variation probably

related with the accuracy of the measurements collected from a very uniform population), they could be used to increase the accuracy of GEBVs and therefore the correlated response of litter traits at birth to genomic selection.

For example: the heritability for TNB is on average 0.11 (Bidanel 2011) and assuming the number of independent *loci* affecting this trait (M_e) as 500 in a reference population of 1,000 phenotyped sows (N), the estimated accuracy of GEBV for TNB using only traditional phenotype is 0.41. However, assuming the same number of independent *loci* in the same reference population, the accuracy of the GEBV for TNB is estimated to be 0.65 if GEBV would be estimated based on OR instead of TNB [*Figure 7.10A*], because the heritability of OR is higher [*0.55, Chapter 6*]. This increase in accuracy depends not only on a higher heritability, but also on the high genetic correlation between the underlying traits and litter traits at birth [for example, r_g *OR and TNB of 0.9,* Bidanel (2011)]. In case the genetic correlation is weak, as between average CL weight and BW [*0.19 (P = 0.0001), results not shown*] and between average CL weight and BWSD [*0.16 (P = 0.002), results not shown*] the estimated accuracy of GEBVs for BW and for BWSD based on traditional phenotypes is higher than the accuracy of GEBVs based on average CL weight [*Figure 7.10B and C*]. The genetic correlations between average CL weight and average piglet BW and BWSD were calculated as the correlation between the EBVs for these traits. However, it is important to consider that the genetic correlations between average CL weight and BW and BWSD are underestimated as the EBVs for BW and for BWSD were not estimated based on the gilts own phenotypes, but based on pedigree information (parent average), and the study population is small. Thus, underlying traits with a higher heritability and a high genetic correlation with litter traits at birth can be used to increase the accuracy of GEBVs and consequently increase the correlated phenotypic response to genomic selection.

Moreover, phenotyping sows for underlying traits influencing litter characteristics at birth, such as OR and average CL weight, will provide breeding companies with the opportunity to select animals based on a more accurate description of their reproduction capabilities: precision phenotyping (Cabrera-Bosquet et al. 2012). Two situations in which knowledge about OR and average CL weight might be helpful are presented in *Figure 7.11*. First, it might improve the accuracy of estimations of uterine capacity. Estimations of uterine capacity (UC) are done based on litter weight (LW), which is defined as the product of the TNB and average piglet BW. Thus, sows with the same TNB that have a higher LW are consider to have a higher uterine capacity [for example sows 1 vs 2]. However, part of the LW observed at birth might be a consequence of a better balance between OR (less competition for uterine space) and average CL weight (embryos with higher uterine implantation length and weight at 35 days of pregnancy) and not of a better uterine capacity [sow 1]. Second, it improves the possibilities to improve LW because it provides extra information on what is the limiting factor for each sow. For example, between two sows that have the same TNB and same average piglet BW, and consequently the same LW, in sow 4 the higher OR (more competition for uterine space) and lower average CL weight (embryos with lower uterine implantation length and weight at 35 days of pregnancy) might be limiting

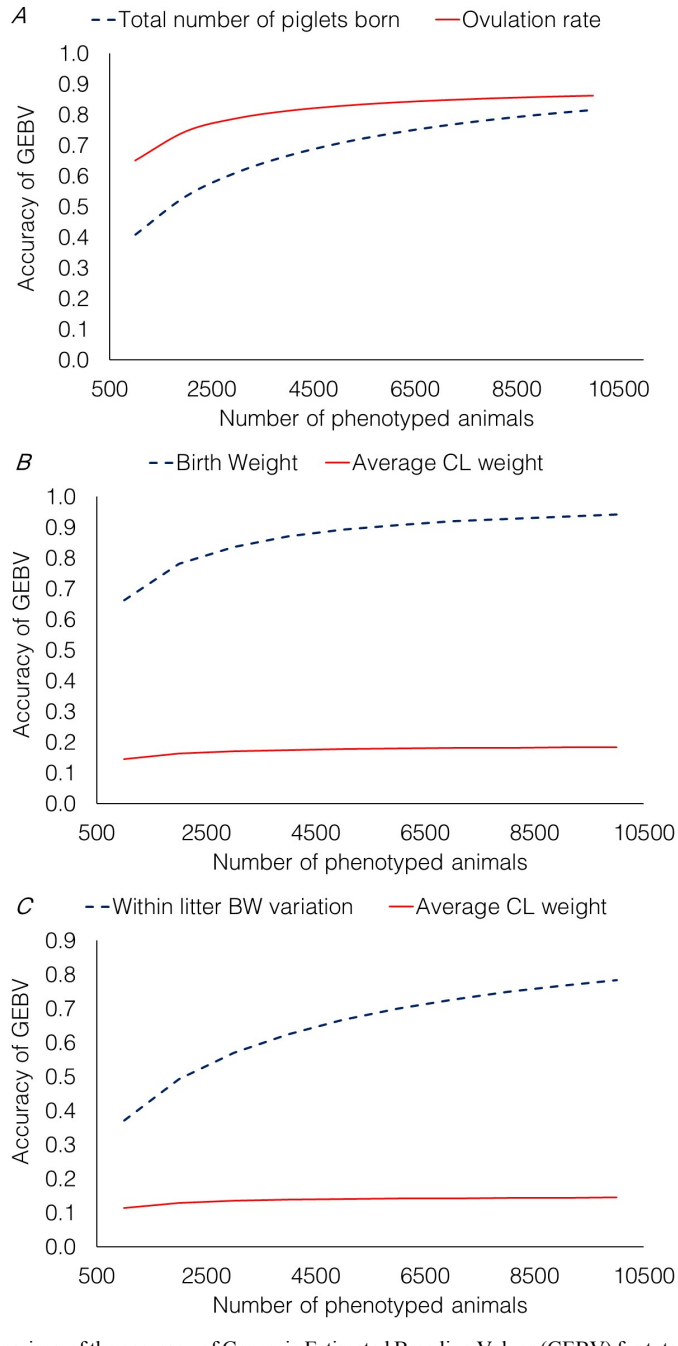

Figure 7.10 Comparison of the accuracy of Genomic Estimated Breeding Values (GEBV) for total number of piglets born [(TNB), panel **A**] for average piglet birth weight [(BW), panel **B**] and for within litter piglet birth weight variation [(BWV), panel **C**] using traditional phenotypes or using underlying phenotypes with higher heritabilities (OR and average corpora lutea (CL) weight), in relation with the number of phenotyped animals. Estimations of accuracy of GEBV where done considering the formula proposed by Daetwyler et al. (2008): $r_{ih} = \sqrt{\frac{Nh^2}{Nh^2 + M_e}} * r_g$;

LW instead of uterine capacity, while in sow 3 (lower OR and higher CL weight) uterine capacity might be the limiting factor. In other words, higher LW might not necessarily be a result of a higher uterine capacity, but might be also a result of a more optimal balance between OR and average CL weight that favours early embryonic development. Thus, estimations of uterine capacity are more accurate when OR and average CL weight are also known, which also provides breeding companies with a more accurate estimation of what is in fact leading to the achieved LW.

In conclusion, ovarian, uterine and embryonic traits in gilts at 35 days of pregnancy influence litter traits at birth, and because they are heritable, can be used in genetic selection programs. However, these traits are genetically correlated and, in order to improve piglet birth weight and consequently piglet survival (as part of the breeding goal), balanced genetic selection is needed. Moreover, these traits could increase the accuracy of genomic selection (GEBVs) for litter traits at birth, and provide breeding companies with a more accurate estimation the reproduction capabilities of their population.

where r_{ih} is the accuracy of GEBVs, N is the number of phenotyped animals in the reference population, h^2 is the heritability of the phenotype, and M_e is the number of independent loci affecting the trait of 500, and $\boldsymbol{r_g}$ is the genetic correlation between the underlying trait and litter traits at birth. Based on the equation proposed by Goddard (2009).

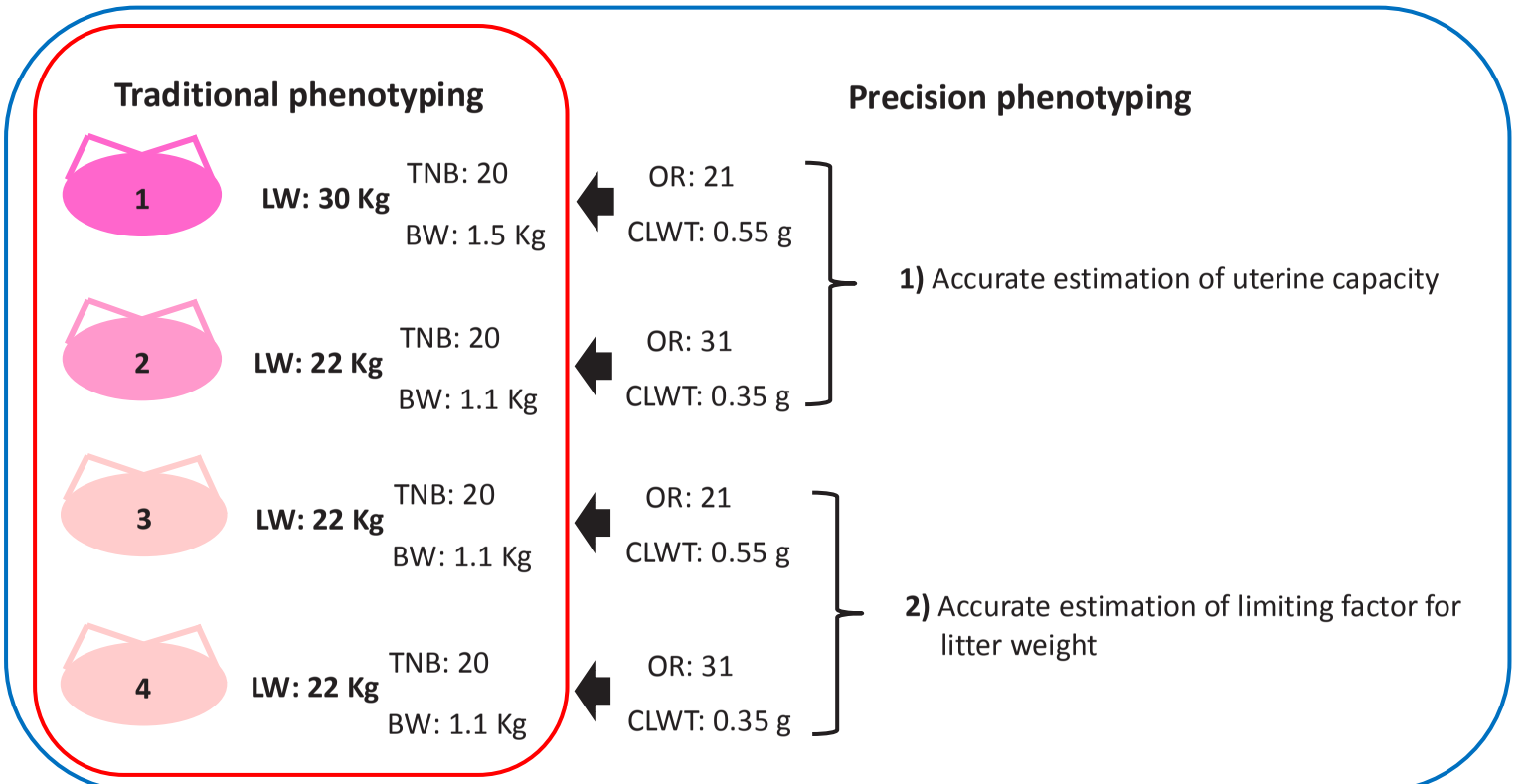

Figure 7.11 Schematic representation of how the phenotyping sows for ovulation rate (OR) and average corpora lutea weight (CLWT) might improve interpretation of uterine capacity. LW: litter weight, TNB: total number of piglets born, BW: average piglet birth weight, OR: ovulation rate, CLWT: average corpora lutea weight. 1) Estimations of uterine capacity that are done based on LW (TNB x BW, traditional phenotyping) might mistakenly consider a sow that was less challenged in the beginning of pregnancy (lower OR and higher average CL weight: less competition for uterine space and better embryos) as a sow with higher UC. 2) LW might be limited by uterine capacity or by a higher OR and lower average CL weight. Data on TNB and BW is based on sows used in Chapter 5, while the data on OR and CLWT is based on sows used in Chapter 3 and on gilts used in Chapter 4 (± 1 SD of mean).

7.5. General conclusions and recommendations

This thesis investigated how the number (OR) and size of corpora lutea (CL) in early pregnancy interacts with embryonic survival and development at 35 days of pregnancy aiming to unravel underlying mechanisms influencing litter characteristics at birth.

The main conclusions of this thesis are:

(1) an increase in OR led to a higher embryonic mortality, which limited the increase in number of vital embryos at 35 days of pregnancy with the increase in OR.
(2) an increase in OR compromised vital embryonic development at 35 days of pregnancy, as it was related with a lower placental length in sows, and with a higher within litter variation in embryonic implantation length and weight in gilts.
These results suggests that an increase in OR might be related with higher foetal mortality in further pregnancy and with lower piglet birth weight in large litters, and also with a higher within litter piglet birth weight variation.

(3) an increase in OR was related with a decrease in average CL weight at 35 days of pregnancy.
(4) lower CL weight was related with lower implantation length and weight of the vital embryos at 35 days of pregnancy and with piglets with lower weight at birth.
These results suggests that the compromised embryonic survival and development at 35 days of pregnancy that follows the increase in OR, and might compromise litter characteristics at birth, is at least partly related with a decrease in average CL weight.

The investigation of the consequences of genetic selection for litter traits at birth for ovarian, uterine and embryonic traits showed that:
(5) genetic improvements in total number of piglets born (TNB) occurs through an increase in OR and in the number of vital embryos, but decreases the average CL weight. More specifically, in gilts with a higher genetic merit for TNB an increase in OR results in an increase in the number of vital embryos and in a decrease in their uterine implantation length at 35 days of pregnancy.
(6) genetic improvements in average piglet birth weight (BW) occurs through an increase in average CL weight, but not in the estimations of uterine capacity (uterine length, empty uterine space around the vital embryos and uterine length per embryo) at 35 days of pregnancy.
These results suggests that the lower piglet BW in gilts selected for high TNB might be related with the lower average CL weight, which might be caused by the increase in OR. This fits with the fact that the average CL weight is lower in gilts with lower genetic merit for piglet BW. Altogether, this indicates that lower piglet BW might be related with a lower

quality of follicles and oocytes at ovulation, that generate embryos with lower growth potential (lower implantation length and weight) at early pregnancy.

(7) Ovulation rate and average CL weight were highly heritable but have a negative genetic correlation.
This suggests that genetic selection for higher average CL weight, aiming to improve average piglet BW (as part of the breeding goal to improve piglet survival after birth), has to be done through its inclusion in a selection index together with OR, to avoid a decrease in total number of piglets born. Since it is possible to phenotype a large number of animals for OR and average CL weight with the use of transrectal ultrasonography, these traits could be used to increase the relationship between the breeding goal and the selection index.

Based on these conclusions and their implications, some recommendations can be made:

Genetic selection programs aiming to improve piglet birth weight would probably benefit from more information regarding the relationship between OR, average CL weight and litter characteristics at birth. It is therefore recommended to start phenotyping sows of different parities and genetic lines for OR (by counting the number of pre-ovulatory follicles) and for CL size during pregnancy, and relate these findings with litter traits at birth. This would make it possible to stablish an optimal OR, based on the best results of total number of piglets born, birth weight and birth weight variation. Moreover, if these animals are also genotyped, genomic selection can be implemented.

Future genetic selection programs should take into consideration that genetic improvements in piglet birth weight and birth weight uniformity in large litters is dependent not only on improvements in uterine capacity, but also in CL weight, and possibly in follicular and oocyte quality, leading to higher embryonic quality during pregnancy. Therefore, it would be worthwhile to investigate the influence of OR in modern crossbred sows on follicular and oocyte quality at ovulation, and also on within litter embryonic development before uterine implantation (~ 10 to 12 days of pregnancy), to better understand the physiological background of litter traits at birth. Moreover, new information is also needed on the patterns of follicular growth and duration of ovulation in modern crossbred sows with high OR.

The negative consequences of the higher OR for average corpora lutea weight, and possibly for follicular and oocyte quality, might be counteracted or diminished by the use of specific nutritional strategies before insemination. It is known that gilts fed a high feeding level (3x maintenance) during the estrus cycle prior to insemination had higher oocyte maturation, and higher embryonic survival and quality at 12 days of pregnancy than gilts fed at maintenance level (Ashworth et al. 2000). For multiparous sows, the supplementation with dextrose plus lactose during lactation and during the weaning to estrus interval increased average piglet BW and reduced the within litter piglet birth weight standard deviation

(van den Brand et al. 2006). Both nutritional strategies influences on embryonic development and on piglet birth weight are believed to occur through an elevation in plasmatic levels of insulin, which has beneficial effects on insulin-like growth factor -1 (IGF-1), a growth factor directly related with follicular growth and maturation (Whitley et al. 1998). With that in mind, it would be worthwhile to provide gilts with high genetic merit for total number of piglets born, and/or with low genetic merit for average piglet birth weight, with high feeding levels or with other insulin stimulating diets before insemination and access the consequences for pre-ovulatory and post-ovulatory ovarian traits and also for litter traits at birth. Thus, precision farming, with special nutritional and/or management strategies implemented for specific sub-populations within the farm might optimize reproductive efficiency in modern herds.

Finally, an increase in ovulation rate reduced corpora lutea size during pregnancy, which indicates influences in follicular and oocyte quality, and negatively influenced embryonic survival and development at 35 days of pregnancy. Thus, the increase in ovulation rate influences not only the total number of piglets born but also piglet birth weight and within litter piglet birth weight standard deviation, as it is part of the mechanism compromising these two traits in large litters. These underlying traits and their relationships must, therefore, be taken into consideration in genetic selection programs aiming to improve piglet survival.

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Summary

This thesis focuses on how the number (ovulation rate) and the size of corpora lutea (CL) interact with embryonic survival and development at 35 days of pregnancy. The aim is to unravel the underlying mechanisms that lead to lower average birth weight and lower birth weight uniformity of piglets born in large litters. Lower piglet birth weight and lower within litter piglet birth weight uniformity in large litters increases piglet mortality during lactation, a major economic and welfare concern for modern pig production. The main hypothesis of this thesis is that an increase in ovulation rate (OR) compromises not only embryonic survival but also embryonic and placental development and thereby litter characteristics at birth.

First, a literature review was done on how OR might influence follicular and subsequent embryonic survival and development [*Chapter 2*]. Ovulation rate (OR) it is the result of factors influencing the growth and development of follicles during early antral follicle development. The pattern of growth of the ovulatory follicles seems different between animals with high and low ovulation rate, as the recruitment of ovulatory follicles is extended in sows with higher OR. This longer follicular phase may be used to recruit additional follicles for ovulation. Moreover, there appears to be considerable variation within and between animals in the size of the selected pre-ovulatory large follicles (7-11 mm), but also in morphology and steroidogenic activity. This may indicate that not all follicles are at the same stage of development at the time of selection; a variation that can be higher in animals with a higher OR. Thus, animals with a higher OR might have a higher variation in stage of follicular development at ovulation, which might indicate a higher variation in follicular quality. A higher variation in follicular quality may have also consequences for oocyte quality, but also on the corpora lutea development after ovulation and on embryonic development.

The physiological consequences of the increase in ovulation rate (OR) on embryonic survival and development in multiparous sows and in gilts at 35 days of pregnancy were investigated [*Chapters 3 and 4, respectively*].

An increase in OR resulted in a curvilinear increase in number of vital embryos at 35 days of pregnancy. This means that at a high OR ($\geq$ 22 for sows and $\geq$ 26 for gilts), more ovulations are needed to achieve one more vital embryo at 35 days of pregnancy (ideally each ovulation would be represented by one vital embryo in the uterus, *e.g.* 20 corpora lutea equals 20 embryos). This limited increase in number of vital embryos with the increase in OR was related with the increase in early (before uterine implantation at ~ 13 days of pregnancy) and in late embryonic mortality (from 13 up to 35 days of pregnancy) with the increase in OR. In sows, each extra ovulation from 17 up to 38 led to an increase of 0.49 in early and of 0.24 in late embryonic mortality. In gilts, a minimum incidence of 3.3 in early and of 1.2 in late embryonic mortality was observed at 15 ovulations and 18 ovulations, respectively, increasing thereafter with the increase in OR (up to 34). Thus, the number of ovulations that are represented by a vital embryo at 35 days of pregnancy was smaller in sows and gilts with high OR, because of higher embryonic mortality.

More importantly, also the development of the vital embryos is compromised with the increase in OR. In sows with high number of ovulations, vital embryos occupied a smaller

area in the uterus (implantation site) [- 0.35 cm/ovulation in implantation length of the vital embryos] and had a smaller placenta at 35 days of pregnancy [41 cm for OR ≥ 29 vs 46 cm for OR 17 to 21]. In gilts with a high number of ovulations there was more variation in the weight of vital embryos at 35 days of pregnancy [~ 0.44 g with OR ≥ 23 vs 0.35 g with OR 8 to 18]. Also, the average weight of the CL at 35 days of pregnancy was lower in gilts with higher OR [minimum of 0.42 g at 28 ovulations]. Moreover, gilts with lower average CL weight had vital embryos with lower weight at 35 days of pregnancy. Thus, an increase in OR compromised the development of the corpora lutea (CL) and also the survival and development of the vital embryos at 35 days of pregnancy.

The **hypothesis** behind these results is that in sows and gilts an increase in OR is associated with changes in follicular development, which causes ovulation to occur when part of the follicles are smaller and not completely developed and mature. These smaller follicles will liberate oocytes (eggs) of lower quality and will develop into smaller corpora lutea during pregnancy. The oocytes of lower quality will develop into embryos of lower quality. These lower quality embryos might have one of two fates: first, it is possible that the embryos die around the time of elongation (~ d12) of their more advanced littermates, which change the uterine environment making it hostile for the less developed (lower quality) embryo (early mortality); second, it is also possible that the less developed embryos reach elongation in time, but elongate less, therefore acquiring a smaller uterine implantation site and developing a smaller placenta. These embryos thus might, due to insufficient nutrient uptake, die after implantation (late embryo mortality and foetal mortality) or be born with lower birth weight. As the aim of this thesis was to unravel underlying mechanisms leading to litter characteristics at birth, the relationships between OR, corpora lutea (CL) size in pregnant sows and their litter characteristics at birth were investigated [*Chapter 5*].

First, the possibility to assess OR and average corpora lutea (CL) diameter using transrectal ultrasonography (TUS) in multiparous sows (n = 45) in early pregnancy (23.4 days) was investigated. Results showed that it is not possible to accurately count the number of CL with transrectal ultrasonography to assess OR in pregnant sows, as the relationship between OR assessed with TUS and after slaughter and dissection of the ovaries was of only 0.24 CL per CL. However, assessment of average CL diameter is reliable, as the relationship between the diameter measured with TUS and after slaughter and dissection of the ovaries was of 1.0 mm TUS / mm at dissection.

Consequently, the relationship between average CL diameter and litter characteristics at birth was investigated. Results showed that sows with a lower average CL diameter at ~24 days of pregnancy had lower average piglet birth weight (BW) and a lower within litter piglet birth weight standard deviation (BWSD), independent of the litter size they were born in. Thus, transrectal ultrasonography cannot be used to assess OR, but it can be used to assess average CL diameter in pregnant sows, which was related with piglet average piglet birth weight and with the within litter piglet birth weight standard deviation.

The consequences of genetic selection for total number of piglets born (TNB), average piglet birth weight (BW), and within litter piglet birth weight standard deviation (BWSD) were

investigated for ovarian (OR and CL weight), uterine (length, empty uterine space around the vital embryos) and embryonic survival (number of vital embryos, early and late embryonic mortality) and development (implantation length and area in the uterus, average and standard deviation in weight) traits in gilts at 35 days of pregnancy [*Chapter 6*].

The results revealed the importance of OR and average CL weight for litter characteristics at birth. Genetic selection for higher TNB increased OR and the number of vital embryos at 35 days of pregnancy, but did not influenced early and late embryonic mortality. Further analyses showed that in gilts with a higher genetic merit for TNB an increase in OR resulted in a linear increase in the number of vital embryos. This means that the number of ovulations needed to increase the number of vital embryos was the same in animals with low or high OR. Unfortunately, the increase in OR was related with vital embryos acquiring a smaller uterine implantation site. Thus, the increase in OR in gilts with higher genetic merit for TNB compromised vital embryonic development at 35 days of pregnancy.

Moreover, genetic selection for TNB decreased average CL weight at 35 days of pregnancy, which might be a consequence of higher OR. Phenotypically, gilts with lower CL weight had vital embryos with lower weight at 35 days of pregnancy and sows with lower average CL diameter farrowed piglets with lower birth weight. Thus, it seems that the lower average piglet BW in gilts with high genetic merit for TNB might be related with lower average CL weight at 35 days of pregnancy.

Genetic selection for higher average piglet BW did not influence vital embryonic development traits or uterine traits that can influence embryonic development (uterine length, empty uterine space around the vital embryos, and uterine length available per embryo) at 35 days of pregnancy. However, genetic selection for higher average piglet BW increased the average CL weight. Thus, at 35 days of pregnancy, average CL weight was the only trait influenced by genetic selection for higher BW. Interestingly, gilts with higher genetic merit for BWSD (i.e. lower genetic merit for within litter piglet birth weight uniformity) also had a higher average CL weight at 35 days of pregnancy. This might be related with the fact that these two traits are genetically correlated and might share gene(s) that are involved in CL weight.

The genetic variation of OR, average CL weight, and embryonic survival and development traits in gilts at 35 days of pregnancy was also investigated [*Chapter 6*]. Results showed that OR, average CL weight, embryonic and uterine traits at 35 days of pregnancy were all highly heritable and can be improved by genetic selection. However, most of these traits were also genetically correlated. This means that the phenotypic relationships between OR, average CL weight and embryonic development traits described previously are partly genetic.

Ovulation rate and average CL weight are important traits for embryonic development and piglet birth weight. Moreover, these traits can be measured in animals in a more practical approach with the use of transrectal ultrasonography. So, transrectal ultrasonography can be used as an breeding tool to phenotype animals for OR and average CL weight and use this information in breeding programs. For example, if the breeding goal is to improve piglet survival after birth by increasing the average piglet BW, average CL weight could be also

included in the genetic selection index (together with average piglet birth weight), but also OR to prevent losses in TNB. Thus, the inclusion of average CL weight and OR in the selection index, which includes also TNB, BW and BWSD, will likely balance selection for litter traits probably improving piglet birth weight without decreasing the total number of piglets born.

Thus, this thesis shows that, phenotypically, an increase in OR increases the number of embryos at 35 days of pregnancy, but up to a certain limit. Females with high OR have compromised placental and embryonic development at 35 days of pregnancy, which is likely to increase foetal mortality and to decrease piglet birth weight. Lower piglet birth weight in females with high OR might also be related with the decrease in average CL size at 35 days of pregnancy, and it might be a result from lower follicle development at ovulation. The fact that genetic improvement of piglet birth weight is associated with an increase in CL weight at 35 days of pregnancy might indicate the importance of follicular/oocyte/embryonic quality for piglet BW. Therefore, future genetic selection programs should take into consideration that genetic improvement in piglet birth weight and in within litter birth weight standard deviation (increased uniformity) in large litters is dependent not only of improvements in uterine capacity, but also in follicular quality at ovulation and corpora lutea size during pregnancy.

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