EMBRYO SURVIVAL AT DAY 9, 21 AND 35 OF PREGNANCY IN INTACT AND UNILATERALLY OVIDUCT LIGATED MULTIPAROUS SOWS 5A-105

Report prepared for the Co-operative Research Centre for High Integrity Australian Pork

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Executive Summary

Embryo mortality in pigs ranges from 10 to 50%, and losses are high predominantly in multiparous sows. Because embryo losses are related to the available uterine space, it is often assumed that embryo survival at the end of the embryonic phase (d35-40 of gestation) is limited by uterine capacity. Most studies, however, have assessed the extent of embryo mortality at this stage and hence it is not clear at what time during early gestation the losses actually occur and to what extent the losses at various stages of the embryonic phase are space related. This study was undertaken to assess when embryo mortality occurs and how available uterine space affects it.

Multiparous sows were mated and sacrificed at day 9, day 21, or day 35 of gestation to measure the number of ovulations, embryo survival, and a number of placental and uterine characteristics. In a subsample of sows one oviduct was ligated to reduce the number of embryos entering the uterus by 50% on average, and hence double the available space for the embryos. These sows were sacrificed at day 21 or day 35.

Average ovulation rate was 20.9 in this study. At day 9, 92 % of the ovulations were represented by an embryo, showing that at this stage there has been virtually no embryo loss since unrecovered embryos were presumably accounted for by the efficiency of the recovery procedure.

At day 21, which is shortly after embryos have implanted, 24 % of embryos were lost and this was not affected at all by providing more space (in oviduct ligated sows). At day 35, available uterine space was limiting with implantations taking up almost all the available space in intact sows, and the area of individual implantations in intact sows being significantly smaller than in oviduct ligated sows. As a consequence, embryo loss increased by another 17% in intact sows by day 35, whereas in oviduct ligated sows there was no more embryo mortality after day 21. Number of embryos was strongly correlated to the number of ovulations, regardless the available uterine space. Each extra ovulation resulted in 0.6-0.8 extra embryos at day 35 of gestation.

In conclusion, ovulation is a strong limiter of embryo number and ultimately, litter size. This finding underlines the importance of management measures to increase ovulation rate in commercial operations and genetic strategies to increase the overall potential of ovulation rate in the breeding herd.

Further findings of this study are not immediately applicable at a commercial level, but provide strong direction for further research that aims to increase litter size. We have shown that total embryo loss averages 40 % in multiparous sows, and that two-thirds of embryo loss occurs before or at implantation. This loss is not affected by uterine capacity and is presumably related to intrinsic embryo quality, variation between embryos, and the interaction between embryos and between embryos and the uterine environment. Additionally, some embryo losses occur after implantation and these are related to uterine capacity.

Table of Contents

1. Introduction

Embryo survival in pigs ranges from 50 to 90 %, with uterine space relative to the initial number embryos having a positive effect on embryo survival. Hence gilts tend to have a higher embryo survival (70 to 90%) than older parity sows (50 to 70%), as ovulation rate in gilts (14 to 20 as opposed to 20 to 30 in sows) is probably more limiting than uterine space.

Because at higher ovulation rates embryo mortality increases, uterine space has been presumed to be a physical limitation to embryo survival, which has led to the term 'uterine capacity' to be introduced as the limitation to ultimate litter size. Uterine capacity is a physical limitation in the third part of gestation, when available uterine space clearly affects weight gain (and survival) of the foetuses. However, during the embryonic phase there is hardly competition for nutrients, and the term uterine space may reflect a different effect of space on embryo survival.

Most embryo mortality is assumed to occur in the later embryonic phase (day 28 to 35 of gestation), and studies have mainly focussed on this phase. Unilateral oviduct ligation models have been employed to study the effect of uterine space on survival of embryos at d30-35 of gestation. In a study by Town et al. (2004), for example, embryo survival at d30 of gestation was increased from 79% to 91% by unilateral oviduct ligation in third parity sows with 19.9 ovulations on average. Similarly, Pere et al. (1997) reported an increase in embryo survival from 69% to 76% in gilts with 17.4 ovulations on average. However, some data in multiparous sows (Gerritsen et al., 2008) suggest that by three weeks of gestation (d23) most embryo mortality has already occurred with 61% of embryos surviving. This would indicate that uterine capacity is not so much a physical limitation, as at this stage the physical size and nutrient demand of the embryos is not such that physical space would limit their survival. Rather, uterine capacity would reflect the number of embryos that can cohabit in a given space. This may be due to interaction between embryos competing for the given space whilst migrating, spacing and implanting. As embryos space before implantation, they may cross-talk through the secretion of factors like oestrogens, which communicates the availability of uterine surface for implantation. This may impact generically on all embryos in a given uterine space, but additionally, this may also affect early pregnancy adaptations of the uterine endometrium in a way that disadvantages embryos that are less developed, a scenario that would have more impact when more embryos are cohabiting the same space.

Alternatively, the intrinsic quality of the embryos or the variation between embryos may be at the basis of increased embryo mortality in sows with increased ovulation rates.

The current study was performed to assess the extent of embryo mortality during the early embryonic phase (before and around implantation). We hypothesised that by three weeks of gestation most embryonic mortality has already occurred, in contrast to the assumption that it occurs during the late embryonic phase. To test the hypothesis of limiting space during this early period, unilateral oviduct ligation was employed.

2. Methodology

Multiparous sows (sows with 2 litters or more, up to 11) were weaned and then either left intact (CTR, n = 42) or subjected to unilateral oviduct ligation (LIG, n = 23) after the first postweaning oestrus. The sows that were left intact were either fed at 0.9 M (negative control, n = 31) and mated at first post weaning oestrus or fed 12.5 MJ DE per day above Maintenance requirements and treated with altrenogest for one week to postpone the first post weaning oestrus, and were subsequently mated at the oestrus following the end of altrenogest treatment (n = 11).

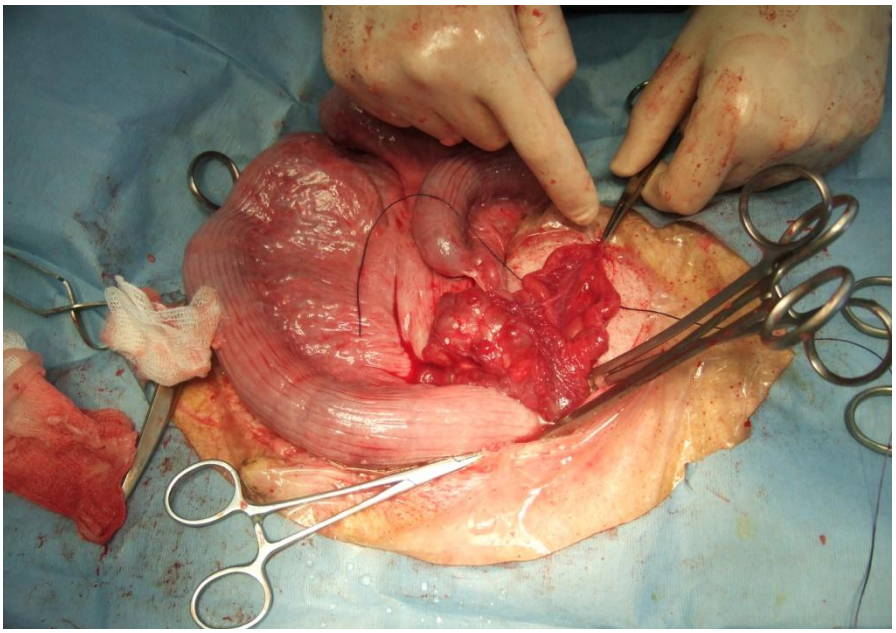
Altrenogest was supplemented from the night before weaning and then for 7 days. Altrenogest was supplemented to provide two times 20 mg per day as a top dress on the feed. The reason for providing a "double dose" was because body weight varied between 200 and 350 kg, being more than twice the average for gilts, for which altrenogest is generally prescribed. Also, use of a single dose of altrenogest in primiparous sows postweaning has been reported to allow some follicle growth up to a size that is typical for the mid-follicular phase, and this was (negatively) related to embryo survival. We assumed that the use of a double dose would be needed to inhibit follicle growth sufficiently.

Heat detection and ultrasound

All sows received boar contact twice a day (morning and afternoon) from 5d before expected oestrus, in a detection-mating-area, until oestrus had ceased. Sows were mated with 3 billion sperm cells from pooled semen at first standing and then every 24 h until ovulation was detected. Ovulation was assessed by using transrectal ultrasound (Aquila Vet, Pie Medical, The Netherlands, 3.5 MHz sector probe). Ultrasound was also used to monitor follicle development in intact sows, on the day of weaning or the first day after last altrenogest, and 4 days later, to assess the effect of altrenogest on follicle status at the onset of the follicular phase, and to relate follicle development to ovulation time, ovulation rate, and embryo characteristics. Follicle status was measured as the average diameter of the five largest follicles on either ovary.

Oviduct ligated sows

Oviduct ligation was performed in LIG sows within 10 days following the first oestrus and sows were mated and ovulation was assessed as described above, at the second oestrus. Oviduct ligation was performed by mid-ventral laparotomy.
Sows were fasted for 24 h before surgery. The animals were anaesthetised by thiopentone sodium at a dose rate of 10 mg kg-1 of bodyweight administered by



Oviduct ligation procedure. Black arrow indicates uterine horn; white arrow indicates section of oviduct to be ligated.

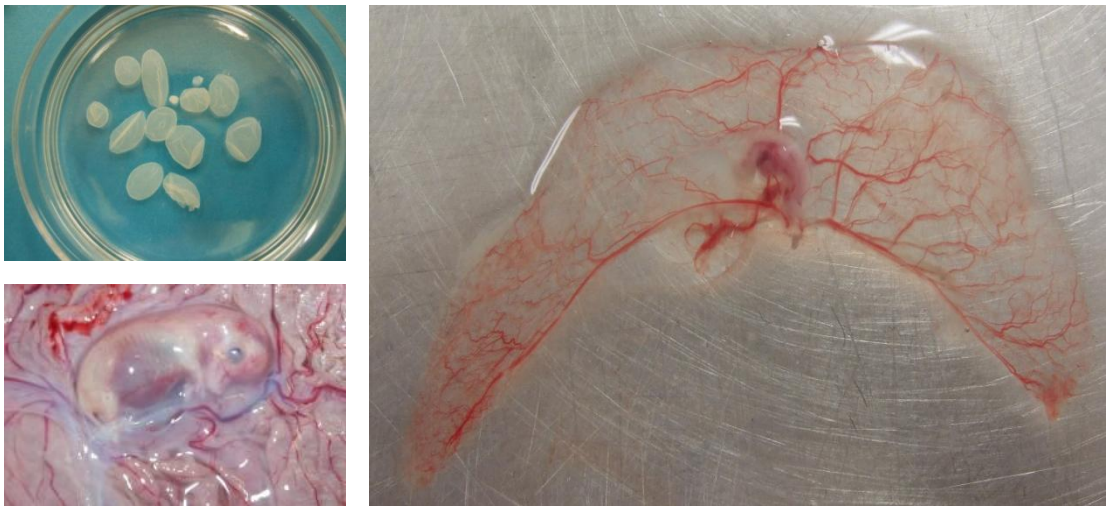
injection via an ear vein. Anaesthesia was maintained using a combination of isoflurane and oxygen. Unilateral oviduct ligation was performed by mid-ventral laparotomy. Randomly, either the right or the left oviduct was looped and then tied off at the base of the loop with one absorbable suture. The strictured part of the loop was then excised. The operation wound was closed using vicryl absorbable sutures. Animals were given 250 mg IM (intramuscular) of Flunixil (Flunixin-Meglumine, Norbrook Laboratories, N. Ireland) as an analgesic and 1050 mg IM of Moxylan (amoxicillin; Jurox, Rutherford, NSW, Australia) as an antibiotic. Sows then received 1050 mg of IM per day of Moxylan for 2 days post-surgery. The rationale for the unilateral oviduct ligation was to reduce the number of embryos entering the uterus after fertilisation by 50% on average, and as a result doubling the available uterine space per embryo, compared to intact sows.

Reproductive tracts

Intact sows were slaughtered at day 9, day 21, or day 35 of gestation (day 0 is day of ovulation). Reproductive tracts were collected to assess ovulation rate, weight of individual corpora lutea after excision, number of embryos, and gross morphological characteristics of uterine horns, placentae, and implantations (day 21 and day 35). Length of implantations was measured and embryos were weighed. Foetal part of the placentae were removed from the implantation sites and then spread on waxed paper and traced. After drying the size of the traced area was calculated by relating its weight to a standard size piece of waxed paper. The 10% embryos lowest in weight were considered as unviable.
For the day 9 gestational stage, uterine horns were flushed using phosphate buffered saline, and subsequently embryos were counted and dimensions were measured under a dissecting microscope. Presence of unfertilised oocytes was also recorded.

Sows subjected to oviduct ligation were slaughtered at day 21 or day 35 of gestation, and reproductive tracts were treated as described above for the other

two treatments, with the exception that embryo survival was calculated as the percentage of corpora lutea on the intact ovary represented by embryos.



Clockwise from top left: Day 9 embryos in a petri dish; Day 21 embryo enclosed in the chorio-allantoic membrane; Day 35 embryo.

Outcomes

Ovulation rate was 22 ± 1.5 for sows with delayed heats (Regumate), 21.6 ± 0.9 for sows mated at first oestrus after weaning, and 11.1 ± 0.6 for ligated sows (ovary contralateral to the ligated oviduct). Embryo and uterus characteristics were not evaluated between these three groups because of the number of observations and only compared for ligated and intact sows.

Follicle diameter at weaning was 4.0 ± 0.1 mm for all sows. After one week of Regumate treatment follicle diameter decreased to 3.7 ± 0.2 mm (P = 0.08). At 4 days after weaning or after last Regumate treatment, follicle diameter was 6.4 ± 0.1 mm for sows mated at first post weaning oestrus and 5.8 ± 0.3 mm for Regumate sows (P = 0.05). At 2 days after weaning or last regumate, oestradiol in systemic circulation was 13.7 ± 1.6 pg/ml in sows mated at first oestrus after weaning and 9.4 ± 2.5 pg/ml in Regumate sows (P = 0.15).

At day 9 of pregnancy almost every (92%) ovulation was represented by an embryo. The increased number of available embryos in relation to the available space in intact animals therefore had a direct impact on space related uterine characteristics. Even though at d35 (not at d21) the length of the uterine horn increased with the number of ovulations (r = 0.47), the available space per ovulation was doubled by d35 due to the ligation procedure, and available space was strongly correlated to the number of ovulations (r = -0.85). At day 21 this did not have much effect on the area of the placentas (67 ± 9 cm2 vs 84 ± 22 cm2, n.s.), but at d35 placentas in ligated animals spanned an 18% greater area (600 ± 54 vs 709 ± 23 cm2). The crowding effect is also illustrated by the length of the implantation sites in intact animals (15.5 ± 1.3 cm) being almost similar to the available space (16.1 ± 0.8 cm), whereas in ligated sows the space taken up by implantation sites (19 ± 1 cm) was longer than for intact sows but far smaller than

the available space (30 ± 2.9 cm). Embryo weight at d35 tended to be lower in intact animals (Table 1; Fig 1).

By day 21 most embryo mortality (~24%) had already occurred and was not influenced by available space. In ligated animals there was no more embryo mortality after day 21. In intact animals, however, another 8 to 14 % mortality occurred, depending on whether outliers were included, bringing the viable embryo survival by d35 down to 59% in intact animals as opposed to 75% in ligated sows. Therefore providing more space per embryo by unilateral oviduct ligation, does not increase embryo survival at d21, but does at d35. Although the reduced available space in intact animals clearly reduced the placenta area, there was no clear relationship between mean placenta area and embryo survival rate. Nevertheless, it may be assumed that with a reduction in average available space the frequency of inadequate sized implantations would have increased, probably compromising the survival of some embryos. There was no clear relationship between placenta area and embryo weight (Fig 1), however, in the ligated group the extra available space tended to increase embryo weight.

The increased crowding in intact animals obviously increased embryo mortality after d21. Nevertheless, the number of surviving embryos increased with the number of ovulations, with every extra ovulation resulting in an extra 0.64 embryos in intact animals and in 0.80 embryos in ligated animals (Fig 2).

In this study ovulation rate seemed to increase only after the second litter. Ovulation rate was 18.7 ± 0.7 (n=18) for second litter sows, 20.5 ± 0.9 (n=12) for third litter sows, 19.5 ± 2 (n=6 for fourth litter sows, and 22.8 ± 0.8 (n=28) for older sows. Overall, ovulation rate 20.9 ± 0.5 was lower than reported in studies from Northern Europe and North America.

3. Application of Research

There is no immediate commercial application of the results from this study. The results from this study rather provide directions for future research (see recommendations). However, a major outcome of this study is that ovulation rate is a strong limitation for litter size, even if average ovulation rates of over 20 may seem sufficient. Management approaches that are known to increase ovulation rate therefore should be encouraged whenever litter size is below average on a commercial operation.

4. Conclusion

Embryo survival in this study is in the range reported by other papers, although this range is fairly large. The majority of embryo loss (~24%) occurred between d9 and d21, so presumably before and around the period of implantation. Assuming fertilisation rate is 100%, there was no embryo loss before day 9 and recovery rates lower than 100% were presumably due to efficiency of the flushing procedure. Since implantation rate was 82%, of the potential number of embryos, 18% did not even implant. Embryo loss before d21 was not influenced by the available uterine space as it was not altered by oviduct ligation, implying that the loss of embryos before d21 is driven by other factors. In sows with oviduct

ligation, there was no mortality after d21. In the intact sows, mortality after d21 was 8% to 14%, depending on inclusion of outliers. It is debatable whether the outliers should be included in the analysis of mortality. On one hand, they may be a consequence of crowding as they mainly occurred in the intact animals. On the other hand, the outliers were animals with below average ovulation rate. Either way, the majority of embryo mortality occurred before d21 and embryo mortality after d21 was only observed in intact animals with limited space per embryo, and not in the ligated sows. Similarly, studies by Town et al. (2004) and Pere et al. (1997) show that doubling the uterine space per embryo by ligation does increase embryo survival at d30-35. However, those authors did not assess embryo mortality at earlier stages.

The current study shows that the majority of embryo mortality (~24%) occurs early, around the period of implantation, and is not a result of physical crowding, since providing more space per embryo did not increase embryo survival.

This raises the question of what causes embryo mortality around implantation. From d10-d13, embryos migrate through the uterine horns and will even cross the bifurcation between the left and right horn. Around d12, embryos start to secrete oestrogens, and at the same time, they start the process of spacing, in which they position themselves through the uterine horns and aligning themselves prior to implantation around d15. The process of spacing seems to be a co-ordinated mechanism, during which the available uterine space is distributed amongst the embryos. Studies with inert, oestrogen-soaked beads have shown that this process may be coordinated by mild contractile activity of the uterus, induced by oestrogens originating from the embryos, with presence of embryos in localised areas of the uterus inducing more or less uterine contractility (Pope et al., 1986). As a matter of fact, pig embryos are generally neatly aligned during the implantation process. This suggests that there is a mechanism that co-ordinates the spacing process, whether it is some form of communication between the embryos, or between the embryos and the uterus or both. In this process, developmental stage of the embryos and variation between embryos may play a role in determining which embryos acquire sufficient space to implant. In that sense less developed embryos may acquire insufficient space to implant, providing an explanation of early embryo mortality. Therefore variation in embryo development has been proposed to be a cause for embryo mortality (Geisert et al., 2006).

Even before spacing and implantation, variation in embryo development may be a cause for negative impacts of uterine environment on embryo development. Geisert et al. (2006) showed that treatment of sows with oestrogens around before d12 had a negative impact on embryo survival, suggesting that there is a window in which oestrogens are detrimental to embryo development through their effect on the uterine environment relative to the developmental stage of the embryos. Treatment with oestrogens at a later stage, when embryos start to secrete oestrogens themselves, is not harmful to embryo survival. Advanced embryos may start to secrete oestrogens at a stage when retarded embryos are compromised by the same oestrogens, and this may be another mechanism through which variation in embryo development causes embryo mortality.

A third mechanism may be the intrinsic quality of embryos, that results in some embryos not surviving. It is interesting to note that in multiparous sows, with higher ovulation rates, embryo mortality seems to be higher. Whether this is a reflection of the fact that with higher ovulation rates more embryos of less quality are generated, or whether this results in more variation, is not clear.

After day 21 available space clearly becomes a limiting factor, as manifested by the smaller implantation are in the intact sows and by the fact that virtually all the available space was taken up by placentations. As a result embryo weight tended to be lower in the intact sows, and even more obvious, intact sows lost another 8-14% of embryos between d21 and d35, whereas ligated sows maintained all the embryos present at d21. There was no clear relationship between implantation area and embryo weight at d35. This suggests that at this stage of pregnancy the available placenta tissue can still sustain embryo growth, although some embryos with inadequate implantations will not survive. However, later in gestation the smaller placenta area may become limiting, as shown by brain:body size ratio in a study by Town et al. (2004).

With ovulation rate sin modern sows exceeding 20, it is often assumed that ovulation rates are not a limiting factor for litter size. This study, however, clearly shows that the number of embryos at d35 increases with ovulation rate also in intact sows, with every ovulation resulting in 0.6 extra embryo. Furthermore, with an average ovulation over 20, a considerable proportion of sows still have ovulation rates lower than 20.

In conclusion, around 24 % of embryos are lost before and around implantation, and this loss is not related to uterine space, but probably due to intrinsic embryo quality or variation, interaction between embryos and/or the uterine environment, or a combination of these factors. After implantation uterine space becomes a limiting factor for the survival of embryos to d35. In this period uterine space available for implantation is reduced with increasing ovulation rate, and although at this stage does not yet seem to compromise embryo development as such, it may later in gestation.

5. Limitations/Risks

There are no risks identified for the application of the results in this study.

6. Recommendations

As a result of the outcomes in this study the following recommendations have been made:

Ovulation rate is strongly related to the number of embryos and therefore sets a limit to litter size, even in older parity sows with relatively high ovulation rates. In general, but specifically in lower parity sows, it is beneficial to increase ovulation rates where possible. In lower parity sows (gilts and first litter sows) this can be achieved through management procedures during gilt rearing, during lactation, and after weaning. In older sows, management interventions will hardly affect

ovulation rate. For the breeding herd in general, genetic gain can be achieved for ovulation rate by stronger and BLUP assisted selection for litter size.

Considering the large loss of embryos in early gestation, before uterine capacity becomes limiting, it is worth investigating whether intrinsic embryo quality, or the variation in embryo quality, can be improved.

It is also worth investigating whether the interaction between embryos, and the interaction between the uterine environment and the developmental stage of embryos can be modified to benefit survival of embryos.

Lastly, it is worth considering research that improves the maternal environment for survival and implantation of embryos. Strategies that can improve the uterine receptivity to embryos, such as skip-a-heat or delayed matings, but also such as immunological priming of the uterus, may improve survival of embryos also in multiparous sows that have considerable embryo loss (up to 40%) by 3 wks of lactation. An altrenogest strategy (delayed mating) was applied in this study but the number of observations was small to compare with control sows. It has to be stressed that the large part of embryo loss (two-thirds) that occurs before or around implantation is not likely to be improved by manipulations that increase size or efficiency of placentation, nevertheless, such interventions may benefit embryo or foetal development at later stages.

7. References

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8. Appendix of Table and Figures

Table 1 - Embryo survival and characteristics of placentation and embryo development at d9, d21, and d35 of gestation.

	Day 9	Day 21	Day 21 oviduct ligated	Day 35	Day 35 oviduct ligated
N	10	15	11	17	12
Ovulations	24.2 ± 1.5	20.9 ± 1.5[a]	11.6 ± 0.8[b]	20.3 ± 0.9[a]	10.7 ± 0.9[b]
Embryos, n	22.0 ± 1.0	15.7 ± 0.9[a]	9.0 ± 0.6[b]	13.0 ± 1.0[a]	8.5 ± 0.8[b]
Implantation rate, %		82 ± 5	83 ± 5	69 ± 4[a]	84 ± 4[b]
Embryo survival, %	92 ± 3	78 ± 4	79 ± 5	64 ± 4*[a] **70 ± 3*[a]**	79 ± 3[b]
(range)	(77-106)	(50-100)	(57-100)	(33-83)	(60-100)
Viable embryo survival, %		76 ± 5	75 ± 5	59 ± 4	77 ± 3
Space, cm per ovulation		14.3 ± 1.3[a]	22.7 1.5[b]	16.1 0.8[a]	30.0 ± 2.9[b]
Length of implantations, cm		9.9 ± 1.1	11.4 1.2	15.5 ± 1.3[x]	19.0 ± 1.2[y]
Placenta attachment area, cm2		66.8 ± 9.3	88.6 ± 23.0	600 ± 54[x]	709 ± 23[y]
Embryo weight, g		0.22 ± 0.03	0.25 ± 0.05	4.3 ± 0.3[x]	4.9 ± 0.2[y]
Efficiency, g/10 cm		0.30 ± 0.06	0.25 ± 0.05	3.2 ± 0.4	2.8 ± 0.3
Efficiency, g/100 cm2		0.42 ± 0.07	0.37 ± 0.04	0.87 ± 0.08	0.71 ± 0.06
Uterine weight full, g		1459 ± 72	1330 ± 150	3980 ± 279[x]	3272 ± 302[y]
Uterine weight empty, g		1133 ± 59	1110 ± 70	1920 ± 141	1830 ± 103
Uterine length, cm		274 ± 16	255 ± 14	334 ± 15[x]	296 ± 16[y]

[a,b]Different superscripts in the same row indicate significant differences (P<0.05).
[x,y]Different superscripts in one row indicate a trend (P<0.10). *When including "outliers" embryo survival was 64% and when excluding these outliers (n=3) embryo survival was 70%. Outliers had embryo survival lower than 45% and these were only found at d35 in the control group.

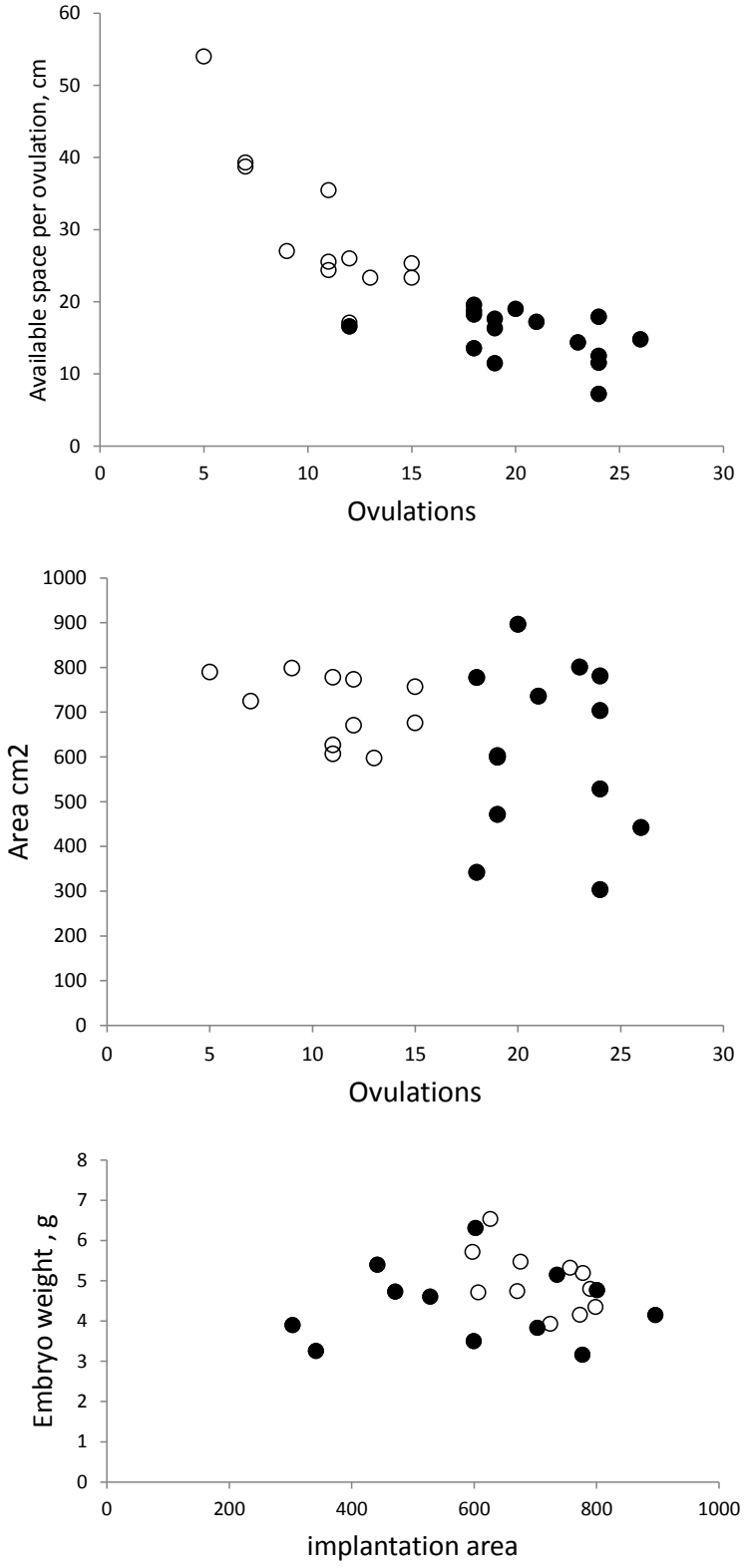

Figure 1 - Top two panels: Available length (cm) of uterine horn per ovulation (calculated) and actual mean area of placentation (cm^2), in relation to the number of available ovulations for intact sows (closed circles) and sows with unilateral oviduct ligation (open circles). Bottom panel: Embryo weight in relation to area of placentation.

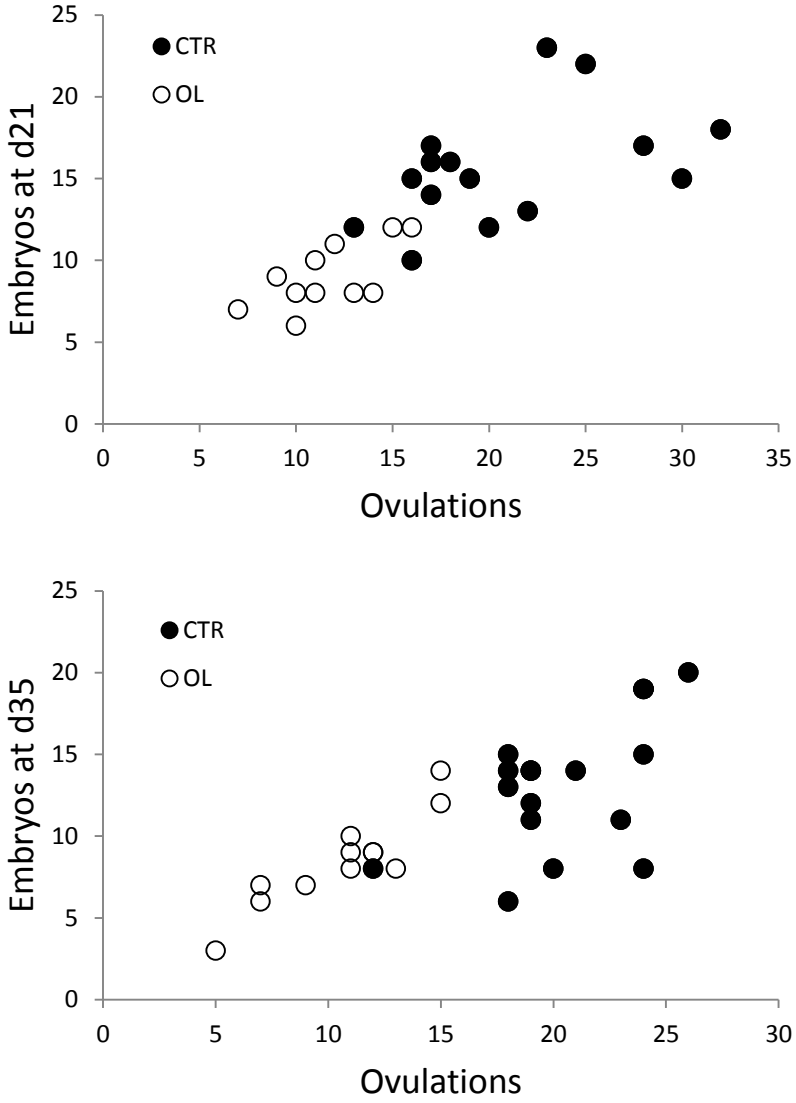

Figure 2 - Number of embryos surviving at d21 (top panel) and at d35 (bottom panel) of gestation in relation to ovulation rate for intact sows (closed circles) and oviduct ligated sows (open circles). At d21, each extra ovulation resulted in 0.73 extra embryo (R^2 = 0.68). At d35, each extra ovulation resulted in 0.8 extra embryo in ligated sows, and 0.6 extra embryo in intact sows (R^2 = 0.52).