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A field study to compare OPU/IVP results of a once in two weeks DFR and GnRH protocol in FSH stimulated dairy heifers

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The objective of the present study was to compare two super stimulation protocols, which differ in dominant follicle removal (DFR) and gonadotropin-releasing hormone (GnRH), prior to ovum pick (OPU) in 45 Holstein-Friesian heifers in a field study which lasted eight weeks. Both protocols consist of a 14-day routine with one difference: DFR on the 10th day and GnRH treatment on the 9th day.
Results were evaluated for five groups (DFR/GnRH, synchronization (sync.)/once weekly OPU (non-sync.), young/old, # of the OPU session) on per sessions and week basis in antral follicle-, oocyte- and blastocysts count. Furthermore, the effect of high (≥2 ng/ml) /low (<2mg/ml) blood progesterone concentration (P_4) at the beginning of the FSH (follicle stimulating hormone, FSH) stimulation was evaluated.
The results show that the GnRH protocol in comparison with the DFR scheme resulted in a, non-significant, improvement in all parameters (26,6 ± 16,6 – 24,4 ± 12,6 antral follicles (AF), 16,47 ± 19,2 – 11,7 ± 6,9 lab counted oocytes (oocytes) and 4,5 ± 4,2 – 3,4 ± 3,8 blastocysts, P>0,05). The sync. group showed, compared with the non-sync. group, an elevation of the same parameters (26,6 ± 15,6 – 20,6 ± 11,6 AF, 14,9 ± 15,3 – 10,2 ± 6,3 oocytes and 4,1 ± 4,1 – 3,2 ± 3,9 blastocysts, P > 0,05). Younger heifers showed the same elevation compared with older heifers (31,6 ± 19,7 – 21,7 ± 10,0 (AF), 18,3 ± 21,2 – 11,3 ± 6,0 oocytes and 4,5 ± 5,0 – 3,5 ± 3,3 blastocysts, P>0,05). No distinct time trend (OPU sessions) was discovered in any parameter during the experiment. High P_4 levels, at the beginning of the FSH treatment, resulted in a non-significant elevation of the AF (27,9 ± 19,8 – 26,2 ± 11,5) and oocytes (17,0 ± 20,9 – 13,8 ± 10,1). Low P_4 levels did result in a non-significant elevation of the blastocysts (4,2 ± 4,0 – 3,8 ± 4,1).
Concluding, this treatment did not harm the heifers in such way that the fertility was negatively influenced during this study. Likewise, this study shows that DFR is not superior to GnRH treatment in OPU/IVP efficiency. Moreover, a not significant detrimental effect was observed regarding the DFR treatment in every parameter. Besides, the P_4 level at the beginning of the FSH treatment did not have any effect on all parameters.

Contents list

Introduction

During the last decades, new methods for artificial reproduction were investigated. In vitro production (IVP) of cumulus oocyte complex (COC) aspirated from slaughter house-obtained (stimulated with follicle stimulation hormone, FSH) ovaries were first used to produce calves (1). When puncturing of follicles with the help of transvaginal ultrasonography was introduced, which was first used in human medicine (2), it became possible aspirating large numbers of COCs in vivo (ovum pick-up, OPU) from donor cows to significantly raise the number of calves per cow (3). The combining of OPU and IVP with the selection of the pheno- and genotypically best cows, lead subsequently to the reduction of the generation interval and a gain in genetic improvement. There are two advantages of the OPU compared with standard embryo transfer. First, OPU may produce more embryos and second, different bulls can be used for all individual oocytes.

Many factors contribute to the efficiency of OPU and IVP. The factors can be divided into biological and technical factors. The technical factors consist of two aspects: needle type (length and width), aspiration vacuum and the technician(s) handling the materials. For example: a wrong adjusted vacuum could potentially result in an impaired recovery rate (RR). The biological factors consist of timing of OPU itself and OPU in relation to the stimulation prior (substance(s), dosage, administration and timing), animal factors (age, BCS, nutrition, breed and stage in cycle (5)) and the micro-environment of the ovarian and follicles itself (4). These factors are potentially able to either in- or decrease the number and quality of aspirated follicles. For example: BCS within normal limits, use of super stimulation hormones (FSH) and OPU at day 14 - 16 of the oestrus cycle could improve these factors.

In addition, a dominant follicle (diameter >5 mm) produces hormones (e.g. inhibine and oestradiol) that impair the growth of other (subordinate) follicles. Disabling that function by removal of the DF in combination with super stimulating (with follicle stimulation hormone, FSH) of the ovaria will result in more follicles per OPU session. A study of *Hendriksen et al.* showed that puncturing the biggest follicles (dominant follicle removal, DFR) results in an elevation in the number of oocytes which reach the blastocyst stadium. This is often used as a developing competence parameter (10). In that study, all follicles of 5 mm or bigger were punctured at day 5-7 of the cycle (a P_4 synchronised cycle).

Gonadotropin releasing hormone (GnRH) treatment will cause the dominant follicle to undergo atresia or form a corpus luteum (11). In a normal situation, three to five days after this injection a new dominant follicle will develop.

FSH is used because *Chaubal et al.* has shown that schedules with FSH produces more and bigger follicles than without FSH (6). In addition, *Adams et al.* showed, while using bFSH, that FSH administration can delay the process of follicles to turn into dominant and subordinate follicles. However, the use of pFSH did not always consistently resulted in more punctured COCs, because of a reduction of the RR (8). The reason for this is still not exactly known. Fortunately, since the introduction of a coasting period (time between last FSH injection and moment of OPU), results of OPU improved. Especially when a coasting period of 44 - 66 hours are used (9).

In this study two different OPU schedules within a population of Holstein-Friesian breed heifers (all older than 12 months of age) were compared. Both schedules aimed to 'remove' the dominant follicle (DF). This is attempted by applying DF removal (DFR) of all follicles bigger than 5mm in diameter or by applying GnRH. Those treatments have the intention to stimulate the new follicular wave two (GnRH) and one (DFR) days after this treatment. This way, the stimulation of the wave is synchronised with FSH treatments (11). Thereafter, four OPU sessions were performed and the total number of COC and total number of oocytes that reaches the blastocyst stadium were counted. Those schemes were used to evaluate the FSH treatments under, expected, high P_4-levels. This was done to study if the quality and number of the follicles and blastocysts improved.

Research hypothesis

Below, the research hypothesis of this study are described. The goals of this study are split up in three different questions.

1. Is there a significant difference in the antral follicle count (AFC), collected numbers of cumulus oocyte complex's (COCs) and number of blastocysts and their respective quality (COCs and blastocysts) using FSH and ovum pick-up (OPU) between a two-week schedule with GnRH removal of the dominant follicle (DF) or a two-week schedule with DFR?

H_0= # AF-, COCs- and blastocysts count DFR = # AF-, COCs- and blastocysts count GnRH.

H_1= # AF-, COCs- and blastocysts count DFR ≠ # AF-, COCs- and blastocysts count GnRH.

2. Is there a significant difference between the groups (origin, # OPU session and age) in the antral follicle count (AFC), number of COCs and blastocysts and their respective quality?

H_0= # AF-, COCs- and blastocysts count = # AF-, COCs- and blastocysts count, between groups.

H_1= # AF-, COCs- and blastocysts count ≠ # AF-, COCs- and blastocysts count, between groups.

3. Does a high P_4 (>2 ng/ml) blood concentration (at the start of FSH treatement) result in a higher AF-, COCs- or blastocysts count compared with a low (<2ng/ml) concentration P_4 during FSH treatment?

H_0= # AF-, COCs- and blastocysts count high [P_4] = # AF-, COCs- and blastocysts count low [P_4].

H_1= # AF-, COCs- and blastocysts count high [P_4] ≠ # AF-, COCs- and blastocysts count low [P_4].

Material and Methods

The experiment has taken place at the CRV nucleus: CRV ET-station, Bûtewei 1, 8407 EA Terwispel. All the heifers were healthy before and during the experiment, Holstein-Friesian, born in The Netherlands, Belgium or Germany, older than 10 months, cyclic and were fed grass silage with brewer's grain. No additional concentrates were fed. Water was provided ad libitum. The body condition score of the heifers ranged from approximately 2.75 till 3,5. The heifers were kept in a free walking stable and were housed in groups of 10-15 heifers according to Dutch (welfare) standards.

The heifers were submitted to a synchronisation (sync., 35 heifers) program or were already in a once weekly OPU program (10 heifers). Nine heifers, which underwent the sync. program, were newly recruited heifers and 26 had already been subjected to other CRV OPU programs. Those programs are briefly discussed in the discussion. The synchronisation program included a device which was placed intra-vaginally. This device releases progesterone and is called a controlled internal drug release (CIDR). This CIDR source was placed on day zero, for seven days. Six days after the placement a PGF2α injection* (5cc Dinoprost©) was given I.M. On day eight the OPU (day zero of the OPU experiment) program (DFR or GnRH) started.

The heifers were subjected to a total of four OPU sessions. Furthermore, one heifer was subjected to six OPU sessions. In the section 'results', the quantity of the OPU sessions is mentioned per group.

In the other groups, 17 heifers were considered young heifers (approximately 10 months) and 28 as older heifers (older than one and a half year).

To answer the main question, 27 heifers were subjected to the DFR treatment and 18 heifers to the GnRH/transova treatment. Selection and assignment to that treatment of the heifers was done 'as randomly as possible'. Due to this an unknown number of heifers, which responded very well to another CRV trail/treatment, were not selected for this experiment. It is also possible that the initial treatment was kept unchanged. Finally, the exact number of heifers, which were selected and not divided randomly, is not known by the author.

* All injection, in this experiment, were given intramuscular in the trapezius muscle or gluteal muscle.

OPU protocols

Since OPU was performed once every two weeks, both protocols consist of a fourteen-day cycle. The experimental procedures were repeated for 4 times. So, 8 weeks in total. Treatments and experimental design are explained below and summarized in figure 1.

DFR Protocol: At day nine of the protocol, DFR was performed. Approximately 24 hours after that treatment, four FSH injection were given S.C. The interval between those injection was approximately 12 hours. Approximately 36 hours later OPU was performed.

GnRH Protocol: At day ten of the protocol, a GnRH injection was given I.M. Approximately 24 hours after that treatment, four FSH injection were given S.C. The interval between those injection was approximately 12 hours. Approximately 36 hours later OPU was performed.

Consequently, FSH stimulation was planned one day after DFR and two days after GnRH treatment. Folltropin-V© (87,5 IE / 20mg/ml) was used for this purpose.

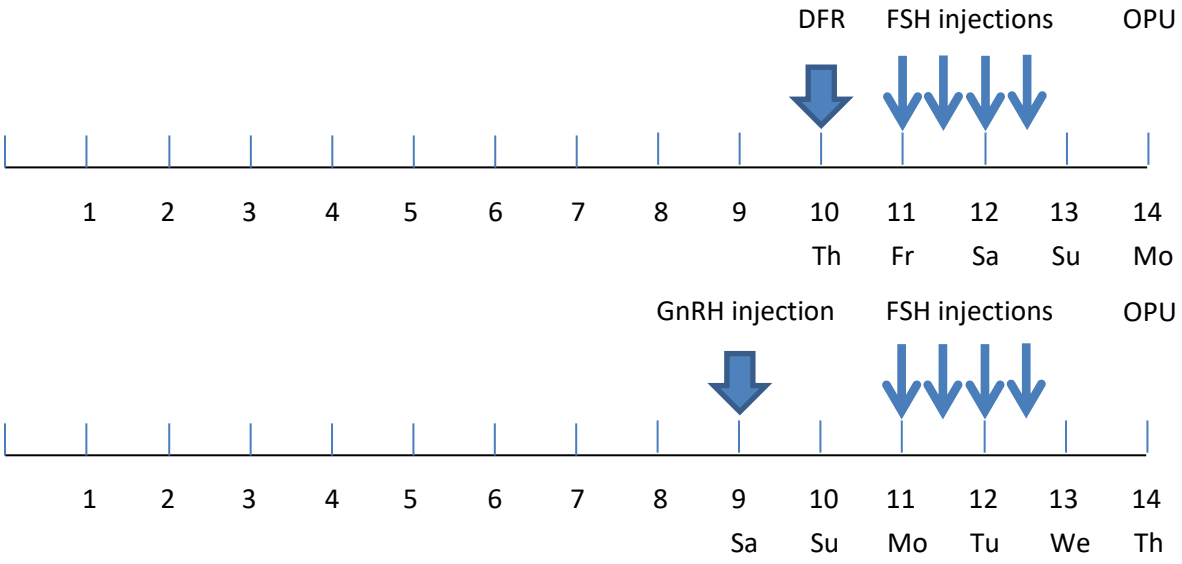


Figure 1 (CRV)

For practicality reasons the scheme below is added to exactly clarify which procedures were undertaken at the different time frames. These are separated for the two treatments, because DFR was always done on a Thursday and the following Monday the OPU session. In contrast, the GnRH group was always subjected to the GnRH treatment on a Saturday and the OPU session on the following Thursday.

OPU protocol group DFR (biweekly program)

Week 1:		
t= 128 – 131 h	day 4	Dominant follicle-removal, AFC and CL detection
t= 126 – 128 h	day 5	Blood collection (P_4)
t= 126 – 128 h	day 5	3,0 ml Folltropin-V©
t= 138 – 140 h	day 5	2,5 ml Folltropin-V©
t= 150 – 152 h	day 6	1,5 ml Folltropin-V©
t= 172 – 174 h	day 6	1,0 ml Folltropin-V©
Week 2:		
t= 7 – 12 h	day 1	OPU – IVP

OPU protocol group GnRH (biweekly program)

Week 1:		
t= 152 – 154 h	day 6	6-8 cc of GnRH
Week 2:		
t= 6 – 8 h	day 1	Blood collection (P_4)
t= 0 - 1	day 1	3,0ml Folltropin-V©
t= 10 – 12h	day 1	2,5ml Folltropin-V©
t= 23 – 25h	day 2	1,5ml Folltropin-V©
t= 34 – 36h	day 2	1,0ml Folltropin-V©
t= 74 – 78h	day 4	OPU - IVP

Ovum pick-up sessions

Before the OPU/DFR session all heifers received a spinal anaesthesia (epidural) with 3-5 cc of procaine (procamidor© 20 mg/ml AST FARMA) depending on the weight of the heifer. Thereafter, the faeces from the distal colon descendens and rectum was removed and the perineum and labia majora/minora were cleaned with soap and alcohol (70%).
For the OPU and DFR procedures an 18-g needle (Aluminium 47,3 cm in length and 9 mm diameter) was used. In order to acquire the required negative pressure, a labotech aspirator 5© was used which produced a 38-52 mm Hg pressure. By rectally holding the ovaria against the wall of the vagina combined with the help of an ultrasound scanner (Mylab 5©, 7,5 MHz), the OPU/DFR session could be performed. This way AFC, CL or ovarian cysts('s) detection, OPU and DFR were performed. All follicles bigger than 5 mm in diameter were punctured at the DF removal(DFR) session. In the GnRH group small and young heifers were given 6 cc of GnRH (Receptal©: $4,2x10^{-3}$ mg/ml) and older heifers were given 8 cc of GnRH.
The OPU and DFR sessions were performed by CRV OPU specialists. Rotation of these specialist was limited to the minimum. During OPU the specialist counted the follicles, determined the size of the follicles and checked the appearance of a CL or a cyst (follicular or luteal) on each ovarium. Time of the OPU, name of the specialist, the number of the heifer/cow and other comments were recorded on the checklist.

IVP

All COCs were gathered, counted and scored. The COCs were being scaled on a one to four scale with one being the best. In vitro production of the selected COCs was conducted in the CRV lab. The quality of the blastocysts was determined by CRV lab specialists on a one to three scale, with one being the best. The criteria for the selection of the COCs/blastocysts is not known by the author because of secrecy reasons. Moreover, the blastocysts, which obtained a class three grade, were not processed.

Blood P_4 determination

Blood samples from the tailvene were taken at the time of the super stimulation and on the OPU day. The blood was collected in heparin tubes and centrifuged the same day at 4000 RPM for 10 minutes. Thereafter, the supernatant was poured into a (smaller) tube and was kept in the fridge at -20 °C until analysis. After the experiment, the P_4-concentration of every heifer at the beginning of the FSH treatments was measured. heifers with a concentration of <2 ng/ml are considered to be in a non-luteal (follicular) phase of the cycle and heifers ≥ 2 ng/ml are considered to be in the luteal phase.

Statistical analysis

All gathered data in this study was analysed statistically to test the three hypotheses (see p. 3) and to obtain a well substantiated answer for the research questions. Statistics were performed in the 24th version of SPSS provided by the University of Utrecht. To obtain the results in this program a 'linear regression analysis' was performed using the 'mixed models' procedure. All the variables* were tested (see appendix) for statistical significance (P<0,05) and correlation for the different outcomes**. All variables except for the P_4 concentration were handled as a fixed effect. The P_4 variable was considered a random effect. The AFC and lab counted follicles were used as a covariate for the blastocysts statistics. A backward procedure for model reduction was used based on the F-values. These procedures were performed with the help of Dr. J. van den Broek, a theoretic epidemiologist at the UU.

* (Co)variables include: age, origin before synchronisation (sync.): once a week OPU or sync. week, measurement number (1-6), P_4-concentration (<2 ng/ml) and treatment(DFR/GnRH) for the AFC statistics.
AFC and small, medium and big follicles and lab counted COCs were included as (co)variables for the blastocysts statistics.
** Number of antral follicles, lab counted follicles and blastocysts. The respective qualities of those groups were also included.

Results

In this experiment, a comparison within five groups was performed to test the hypothesis mentioned in the introduction. The groups consisted of two treatments (DFR and GnRH), two age groups (young and old), two different origins (sync. / non-sync.), four OPU sessions, and P_4-level. These groups were evaluated based on size of the AF during OPU, the AFC during OPU sessions, the total lab follicle count, the COC quality's (1 and 2 combined, 1, 2, 3 alone and 4 combined and broken ones) and the blastocyst count (total count and class 1 and 2). The diameter/size of the antral follicle was measured and thereafter divided in three groups: small (<4 mm) medium (4-8 mm) and big (>8 mm).
In the charts (which are in the appendix), the mean, the standard deviation, the standard error of the mean, the 95% confidence interval and the P-value of all parameters are recorded. A P-value smaller than (<) 0,05 is considered statistical significant.

Number of measurements

Since, not all heifers were subjected to four OPU sessions and the groups were not divided 50-50%, the number of measurements per groups differs. This resulted in a total of 91 DFR measurements and 72 GnRH measurements, a total of 59 'young heifer' measurements and 104 'old heifer' measurements, 36 measurements originated from the once weekly OPU heifers and 127 measurements from synchronized heifers. 45 measurements were collected during the first OPU session, 44 during the second, 41 during the third, 31 during the fourth, one during the fifth and sixth session. 60 measurements of the P_4 level showed a concentration below 2 ng/ml and 52 measurement a concentration above 2 ng/ml.

Small, medium and big sized follicles (<4, 4-8 and >8 mm)

There were no statistical differences in any of the groups when all small or medium sized follicles were used as a dependable variable. The average amount of small respectively medium aspirated antral follicle during OPU was 12,45 (± 8,52) and 9,09 (± 7,45) for the dominant follicle removal group and 14,51 (± 15,4) and 8,25 (± 5,70) for the GnRH group ($P \geq 0,05$).
The big sized A.F. had an average of 2,81 (± 3,3) in the DFR group and 3,67 (± 3,2) at the GnRH group ($P \geq 0,05$). Nevertheless, the P_4 level at the time of the beginning of the FSH treatement had a significant effect on that number (P=0,022). The remaining's of the groups did not differ significantly.

Total AFC and lab counted follicles

The sum of the AFs (small, medium and big) is the antral follicle count (AFC). The age factor was marginal significant (P=0,050) on the AFC during OPU sessions: 31,6 ± 19,7 AFC for the young heifers versus 21,7 ± 10,0 AF for the older heifers. The groups, which originated from heifers of the non-synchronized/once OPU a week (1x7days), had an average of 20,6 (±11,7) follicles compared to 26,6 (±15,6) follicles in the sync. The other groups did not affect the outcome. The average AFC in the DFR group was 24,3 (± 12,6) and in the GnRH group 26,6 (±16,6), this difference was not significant.
After the recovery of the follicles during the OPU, in the proces phase, the total number of cummulus oocyte complex's (COC's) were being counted. These numbers are also recorded for all the groups. Unfortunatly, there was no significant difference among these groups. The average number of lab counted follicles in the DFR group was 11,7 (± 7,0) and in the GnRH group 16,5 (± 19,2). Overall, a total number of 4119 AFC were punctered during all the OPU sessions which resulted in a retrieval of 2254 follicles. This corresponds with a RR of 54,7%.

Follicle quality

The quality (Q) of the follicle is determined in the lab on a one to four scale. None of the groups had an significant effect on the total of Q 1 and 2 combined. However, both the origin (P=0,042) as the number of the OPU sessions (all P-value's were <0,50) had a significant effect on the number of Q2 follicles alone. The non-synchronized group had an average of 7,6 (± 5,4) follicles versus 10,9 (± 11,1)

in the synchronized group. The number of Q2 follicles declined significantly (see appendix) from 11,7 (± 14,6) till 8,8 (±7,2) till the third OPU session. Nevertheless, the fourth OPU session was sigfinicantly higher compared to the intercept (10,13 ± 9,2).
Among the Q3, 4 or broken follicles, there were no significant differences (see appendix) between the groups.

Blastocysts

During the IVP of the embryo's, the quality and number of blastocysts were determined. The quality was classed on a one to three system. Consequently, class one and two are used in the CRV program and class three is not used.
Although some differences are obvious, none of the them were statically significant. The average total- respectively without class three blastocysts for the DFR was: 3,37 (± 3,85) and 2,01 ± (2,84). In contrast, the GnRH group showed an average of 4,49 ± 4,182 for the total blastocysts variabele and 2,38 (± 2,21) for the same variabele minus class 3. None of those numbers had an P-value smaller than 0,50; see appendix.
The blastocysts developments rate ($\frac{Total\ blastocysts}{Lab\ counted\ COCs + Total\ blastocysts} * 100\%$) for the DFR is calculated to be 22,3 % and for the GnRH group 23,7%.

	Small AFs	**Middle AFs**	**Big AFs**	**Total AFs**
DFR	*12,45 ± 8,5*	*9,09 ± 7,4*	*2,81 ± 3,3*	*24,4 ± 12,6*
GnRH	*14,5 ± 15,4*	*8,25 ± 6,7*	*3,67 ± 3,2*	*26,6 16,6*
	Q_{1+2} COCs count	**Q_{3+4} COCs count**	**Broken COCs count**	**Total lab counted COCs**
DFR	11,47 ± 6,2	*0,96 ± 1,3*	*11,7 ± 7,0*	*11,7 ± 7,0*
GnRH	16,61 ± 20,0	*1,63 ± 2,5*	*16,5 ± 19,2*	*16,5 ± 19,2*
	Total blastocysts count	**Blastocysts count class one and two**		
DFR	*3,37 ± 3,9*	*2,01 ± 2,9*		
GnRH	*4,49 ± 4,2*	*2,38 ± 2,2*		

Table 1, All parameters for the treatment groups.
Q: Quality of the COC's on a one to four base.
Mean and standard deviation were mentioned.

Progesterone

The P_4 had had no significant effect on any of the parameters. The heifers, in which a blood P_4-level below 2 ng/ml was measured, showed an average of 26,2 ± 11,5 total antral follicles during the OPU sessions, an average of 13,8 ± 10,1 lab counted COC's and an average of 4,15 ± 4,0 – 2,32 ± 2,6 blastocyst (total – class one and two), see table 2. The other heifers (P_4-level ≥ 2ng/ml) showed an average of 27,9 ± 19,9 total antral follicles, 17,0 ± 20,9 lab counted COC's, 3,79 ± 4,1 blastocysts (total) and 1,94 ± 2,2 (class one and two blastocysts).

[P_4]	**Small AFs**	**Middle AFs**	**Big AFs**	**Total AFs**
<2 ng/ml	*14,2 ± 8,3*	*8,87 ± 6,9*	*3,12 ± 3,5*	*26,2 ± 11,5*
≥2 ng/ml	*14,1 ± 17,5*	*10,1 ± 6,0*	*3,75 ± 3,4*	*27,9 ± 19,9*
[P_4]	**Q_{1+2} COCs count**	**Q_{3+4} COCs count**	**Broken COCs count**	**Total lab counted COCs**
<2 ng/ml	11,47 ± 6,2	*1,12 ± 1,4*	*1,56 ± 2,5*	*13,8 ± 10,1*
≥2 ng/ml	16,61 ± 20,0	*1,43 ± 2,3*	*1,41 ± 3,3*	*17,0 ± 20,9*
[P_4]	**Total blastocysts count**	**Blastocysts count class one and two**		
<2 ng/ml	*4,15 ± 4,0*	*2,32 ± 2,6*		
≥2 ng/ml	*3,79 ± 4,1*	*1,94 ± 2,2*		

Table 2, All parameters for the P_4 – level groups.
Q: Quality of the COC's on a one to four base.
Mean and standard deviation are mentioned.

Excluding non-synchronized heifers

Due to the fact that the heifers, which were not synchronized before the OPU sessions, were included in the first analysis, the statistical procedures was repeated for a different dataset. For this purpose, see the discussion.

A dataset was used in which those heifers were excluded in order to obtain the results below. In the table 3 a summary for the treatment group is provided (main question). No statistical differences (data not shown) were found in the groups for all the parameters (same procudure as above). Overall, a total number of 3379 AFC were punctered during all the OPU session which resulted in a retrieval of 1887 follicles. This corresponds with a RR of 55,8%.

	Small AFs	**Middle AFs**	**Big AFs**	**Total AFs**
DFR	*12,3 ± 8,0*	*10,4 ± 7,8*	*3,18 ± 3,6*	*25,9 ± 12,5*
GnRH	*15,6 ± 12,5*	*8,18 ± 5,2*	*3,77 ± 3,4*	*27,6 ± 18,9*
	Q_{1+2} COCs count	**Q_{3+4} COCs count**	**Broken COCs count**	**Total lab counted COCs**
DFR	11,47 ± 6,2	*0,80 ± 1,1*	*0,97 ± 1,8*	*12,3 ± 7,4*
GnRH	16,61 ± 20,0	*1,86 ± 2,7*	*1,77 ± 3,5*	*18,2 ± 21,2*
	Total blastocysts count	**Blastocysts count class one and two**		
DFR	*3,65 ± 4,1*	*2,27 ± 3,1*		
GnRH	*4,57 ± 4,0*	*2,48 ± 2,2*		

Table 3, All parameters for the treatment groups without old OPU heifers.
Q: Quality of the COC's on a one to four base.
Mean and standard deviation are mentioned.

Discussion

This paper presents a big study on the effect of GnRH or DFR treatment in once per two-week OPU scheme. No statistical differences were found between the two treatments, due to the large standard deviation between heifers in one group. Although, it was possible to discover a non-statistical difference between the AFC, lab COCs count, blastocysts count (class one and two and total count) in the treatment group. The GnRH treated heifers showed an average increase of + 2,2 counted AFs during the OPU session (26,6 ± 16,6 v.s. 24,4 ± 12,6), + 4,8 counted COCs in the lab (16,5 ± 19,2 v.s. 11,7 ± 7,0), + 1,12 total blastocysts (4,49 ± 4,2 v.s. 3,37 ± 3,9) and + 0,37 (2,38 ± 2,2 v.s. 2,01 vs 2,9) in used blastocysts (class one and two).

The GnRH treatment could result in more follicles and subsequently more blastocysts. This is due to the bivalent effect of GnRH: an LH-effect and GnRH mimics the effect of FSH in smaller follicles with sufficient FSH-receptors. In the heifers, in which no dominant follicle was developed, this could cause those follicles to grow more compared to the DFR treated heifers. In the heifers which have a DF, GnRH will result in the ovulation of the bigger follicles (> ± 10mm, 12). DF ablation will result in the same effect in those heifers.

On the other hand, it is shown that a DFR session causes an endogenous FSH surge, approximately one till one and halve days after the session (25,26). This could have been a contributing factor for the new follicular wave.

The calculated RR in this study was 54,7 %. This is rather low compared to other studies: 60-73% (14). On the other hand, Petyim et al. calculated a RR of 45,6 and 58,5 % in different once weekly schemes (27). Boni et al. reviewed some articles which studied different ways to increase the RR (17). It was concluded that using long-bevelled disposable needles, with a thickness corresponding with an 18-g needle, are the most efficient to prevent damage to the follicles during aspiration and subsequently raise the RR (21). Naturally, it is also important to replace the disposable needle after every OPU session or even sooner if the OPU sessions takes long due to a high number of AF. Sassamoto showed that it is possible to raise the RR (+ 30,6 % in his experiment) and quality of the aspirated follicles even more if the needle is twisted inside the follicle during OPU (22). This way the COC is probably better detached from the follicular wall (17). The CRV specialist followed almost the same procedures during this study except for pressure. This was set on 38-52 mm Hg, which is lower than the optimum pressure found in the study of Bols in 1997 (21) and Sassamoto (22). Bols et al. studied pressures ranging from 50 – 130, and found an optimum of 70 mm Hg for the RR (70%). Sassamoto studied pressures ranging from 50 – 150 mm Hg and found 125 mmHg to be the optimal pressure (RR of ±75%). Another reason for the low RR could be that in this study a higher number of small sized follicles were punctured. Pieterse et al. (3) has concluded that a higher number of smaller AF results in a decline of the RR.

Scheme	Number of Sessions	Number of COCs / session	Number of COCs / week	Number of embryos / session	Number of embryos / week
Once weekly (NSFH)	*102*	*7,30*	*7,30*	*0,98*	*0,98*
Once weekly (FSH)	*104*	*8,45*	*8,45*	*1,54*	*1,54*
OETW (FSH)	*217*	*9,6*	*4,8*	*1,8*	*0,9*
Twice weekly (NFSH)	*135*	*6,78*	*13,56*	*0,81*	*1,62*
DFR (OETW, FSH)	*91*	*11,7*	*5,85*	*2,01*	*1,005*
GnRH (OETW, FSH)	*72*	*16,5*	*8,25*	*2,38*	*1,19*

Table 4, Total COCs and blastocysts (embryos) for six different CRV OPU schemes (CRV trail 2016).
(N)FSH: (no) FSH used.
OETW: once every two weeks OPU.

The number of COCs and embryos in the two groups observed in this study, differ from the number observed in older once weekly OPU schemes (table 4, CRV). The twice weekly (with FSH) and once weekly (without FSH) have a lower number of COCs and embryos on a weekly basis compared to two in this study. Furthermore, the once every two weeks protocol with FSH and twice weekly without FSH protocol show a much higher result in embryos (± 0,5 more) and COCs on weekly basis (table 4). Naturally, it is unknown if this difference is statically different.

Compared with other studies the number of embryos on a weekly basis appeared to be rather low (AFC: 12,6 and COCs: 6,9 on weekly basis). Chaubal reported, using once weekly OPU and multiple FSH

injections, a retrieval of 13-14 AFs, 9 - 9,5 COCs and 1,9 - 2,2 blastocysts (14). Chaubal's group reported in another experiment that even more beneficial results could be achieved over a 10-week period using a scheme in which DFR was performed on day 0, FSH treatment (120 mg SC and 80 mg IM) 36 hours later and the OPU session 48 hours after the prior FSH treatment (22). This schedule provided 16 AF, 10,6 COCs and 2,4 blastocysts. However, Peytim observed lower numbers of AF (6,0 – 6,6) during OPU and in the lab: 2,6 – 3,7 COCs. No IVP-procedure was performed on those COCs. Numerous reasons could be named to explain these differences.

It is stated that a follicle develops into a dominant follicle at the moment it reaches a diameter of at least one cm (13 & 15). This is the same size as a dominant follicle and therefor becomes sensitive to ex-/endogenous GnRH/LH (12). In this study and Chaubal (14), follicles bigger than 5 mm were considered a DF and consequently punctured during the DFR sessions. This difference could result in higher number of punctured DF and subsequently in a lower number of remaining follicles on the ovaria. This has both advantages as some disadvantages. An advantage is that less oestradiol and inhibin is produced, which reduce the growth and development of the remaining small and medium sized follicles (12). This is supported in an experiment of Rouillier (15). His group performed an experiment in which was investigated, with the help of slaughterhouse ovaria, if FSH treatment was effective with the presence of a DF (>10mm). It was concluded that FSH treatment provides less atretic follicles in the absence of a DF. Another positive effect may be that deviation of another subordinate follicle is largely prevented (see figure 2). The subordinate follicle could become the DF, after ablation of the first DF. This is called deviation and occurs occasionally (16). DFR could mimic this if only the biggest (>1 cm) DF would be removed. Consequently, the odds that a day after these DFR treatments a DF is present, is very small in any heifer in that group. This is supported by figure 2, a future DF, of that size, grows approximately 2 mm a day, so all follicles <5 mm would take a couple of days (2,8) to become the DF (deviation time). In contrast, in the GnRH protocol, the heifers are subjected to GnRH injection two days before the FSH stimulation. If a follicle has deviated, which occurs at a mean diameter of DF ± 8-9 mm (16) and after approximately 2,8 days (blue arrow in figure 2 (16) before the FSH treatment, could this subsequently lead to a situation that at the day of the treatment with GnRH no DF, which harbours sufficient LH receptors to ovulate, is present. Nevertheless, it is also possible that a specific follicle develops to a certain level in those two days. If that occurs, that DF could harbour all the negative consequences, as mentioned above.

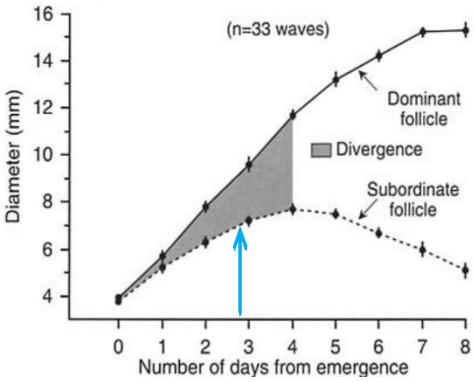


Figure 2 Ginther et al, 1996 (16). Follicular dominance in relation to size.

A negative effect of the DF puncture is the fact that the ovaria are exposed to an extra 'injection' of mechanical damage induction, which could result in ovarian inflammation/adhesions and consequently less harvested AFC, blastocysts and quickened removal of the heifer. Another disadvantage could be presented in a heifer which has a couple of 'almost DF' (≥ five till ± 10-12 mm). These follicles were to be thrown away. It is unlikely that the new (competent) follicular wave (even in cows/heifers (20%, 12) which own a three wave of follicles) is presented the day after this procedure and thus will result in low OPU efficiencies.

In this study the FSH treatment starts approximately 24 hours after the DFR procedure. This way the remaining follicles are subjected to an extra day to develop and restart their race for dominance. It might have been better if the FSH treatment is started right after the DFR treatment. This statement is supported by the fact that the FSH peak normally occurs when the future DF has a diameter of 4 mm. This occurs before the day of emergence. So, the follicles which remain in the ovaria are possibly FSH restricted for a couple of days. If FSH were to be given immediately, the small and almost atretic follicles could possibly have had the opportunity to develop and collected at the OPU session three days later (16).

In addition, Chaubal (14) showed significant differences (P<0,05), in a once weekly OPU scheme, between the groups which received three days FSH in comparison to two one-time FSH treated groups,

in follicular response and total oocytes recovered. The timing of OPU relative to stimulation is comparable with the schemes in this study.
Since deviation occurs approximately 2,8 days after the emergence of the DF, the timing relative to the DFR/GnRH treatment is logical in respect to the prevention of the production of E_2, inhibin, inhibition of FSH secretion and other negative effects on the follicles in the ovaria. The GnRH treatment was planned a day before the DFR treatment while 24-32 hours after the LH surge a DF is completely ovulated. Naturally, the DF must hold sufficient LH-receptors to ovulate (>10mm in size, (12). Here to, all the heifers in both protocols should, no longer, not hold a DF on their respective ovaria. Nevertheless, it is possible to start the FSH treatment the day before.
The protocols in this study enable to plan the FSH stimulation during the luteal phase. Nevertheless, combined with the expectation that this will not be achieved for all the OPU sessions (see below), the results in this study show that high P_4 during FSH stimulation do not correlate with better result in AFC, COCs and blastocysts. However, Domínguez (5) describes that the AFC (quality and number) did not differ among heifers (n=449) with or without a CL at slaughter. This is also described in Chaubal (14). In that experiment, it is shown that OPU in angus cows, in absence of a CIDR and IV LH injection six hours before a OPU session, resulted in more blastocysts per session (once weekly scheme) compared to the same group except for the fact that a CIDR was present in those heifers during FSH treatments (3,44 vs 2,22 - 1,56). The same trend is seen in the grade wise distribution of the high(est) quality COCs (33,7 % vs 21,8 %, P<0,05). Remarkable is fact that the number of follicles aspirated (18,6 vs 11,7) and retrieved (11,5 vs 6,9) were also significantly higher in the LH treated groups compared the groups which did not received that treatment. Noteworthy is the fact that all heifers in the groups were subjected to DFR on day 0 to warrant that the CIDR would be the only source of P_4 during the FSH treatment(s).
Injection of LH or LH-mimicking glycoproteins could (potentially) boost the theca cells inside follicles which have become (partly) LH-dependant for further development and improved number of blastocysts (14 & 16). This will not exert the intended effect if the heifer has relatively many smaller sized follicles (<8 mm), because with the increasing diameter of the AF the more aromatase enzyme is present in the follicle and the more LH-dependent the follicle will be (12 & 16). Unfortunately, this did not result in the excepted blastocyst development rate in the Chaubal' study (17,2 – 21,7%). This number is comparable with the number found in this study (22,3 – 23,7%). In a study of Blondin (20) this rate was calculated to be 80,9 % (± 9,4) in that experiment. So, a LH treatment (IM or IV) does not always result in a higher blastocyst development rate due to higher E_2 and possibly P_4 (depending of the cycle), which can block the action of LH on the follicles (19). The halve life of FSH and LH varies from approximately 30 – 40 minutes (18). So, it seems logical that a I.V. treatment could provide a better result than I.M. injection. Furthermore, the SC injection (14) could result in a prolonged period in which the threshold inside the follicles is exceeded.
One of the major reasons why the differences were found to be statically indifferent in this study could largely be due to the study design. For what reasons and how many heifers were selected for a certain group (treatment) is not known by the author. In addition, Lopes (13) showed that in a herd the variation between heifers can be very big. So, due to selection of heifers in a certain group could result in a better result (higher yield of COCs and blastocysts). The opposite could also be true, shifting heifers which do not respond well to another treatment (GnRH or once weekly OPU) could decline the overall yield in that group.
Another reason why some heifers failed to respond well, among all the groups, could be that their fertility was impaired because the BSC of those heifers was too high (5). A couple of heifers showed an BSC of ≥ 3,5 during the experiment. Unfortunately, no data was recorded for the heifers during this study.
Another factor, which varied during the experiment, was the technician who operated the OPU machinery and ovaria. In the past it is proven that this is contributing factor to the AF and COCs yield.
In this experiment, no detrimental effect of multiple OPU session was seen on any of the parameters (AFC, COCs and blastocysts). The AFC even increased (P>0,05) during the eight weeks from 24,5 till 27,5 AF, the total lab counted number declined (P>0,05) from 14,6 till 13,5 and the total number of

blastocysts varied from 3,5 till 4,4 and the number of blastocysts class one and two varied between 1,8 and 2,6. The last two parameters showed no distinct trend in time. The same result is seen in other once weekly OPU scheme studies (23-24 & 27).
Before the experiment, all young and older heifers were (supposed to be, see below and figure 4 and 5) synchronized. This was done with a seven days synchronization program with progesterone supplementation with a CIDR placed in the vagina. After the six days the heifers received an injection with PGF2α and after seven days the CIDR was removed. This way all heifers (see figure 4 in the appendix) which were on day 1 to 20 of the oestrus* cycle at the insertion of the CIDR were to be synchronised. heifers, which were at day -1 (is day 20) or (most likely) ** day 0 of their respective oestrus cycle (figure 4 in the appendix) at the time of the placement are most likely not synchronised because the CL in those heifers are too young to respond to the PG injection (only a CL beteen six days and 17 days old is responsive to PG (12), see figure 4 and 5 in the appendix.
In the study nine heifers (five DFR and four GnRH treatment) were included which were never subjected to the sync. protocol. Due to this, it is very unlikely that those heifers would show the same P_4 levels during the experiments as the sync. heifers. The effect of this 'mismatching' is tested in the last paragraph of the result section. No statically differences were found between any of the groups in any of the parameters.
A reason, why no difference was found, could be that the CIDR did not always stayed in the heifers during the whole sync. week. There is no data how frequent and how long some CIDR were 'out', but this could be a factor which interfered with all the results.
Another reason why not all heifers show the same P_4 course could be due to the ablation of the follicles. Boni states in his review that OPU does not interfere with the normal cycle of the cow, if OPU is performed on day 3-4, 9-10, 15-16 of the normal cycle (17). This is true unless a DF is removed during OPU or a DFR session and that DF would be the new CL. This way the cow enters a para-physiological state with a prolonged cycle. Before the cow enters a new cycle, a new DF must ovulate. This usually happens within six days after the last OPU session (17). It can be imagined that in some cows the ablation of the DF happens twice: The DFR sessions, in a cow in the follicular phase, removes the DF (and all other follicles) because all follicles ≥ 5 mm were being removed. This follicle would have become a CL. It is possible that the new DF from the new wave hasn't ovulated yet on the OPU day (four days later). This way the cycle could potentially be prolonged with four till 10 days, messing up the scheme (figure 3).
If the goal of CRV is to study the main goal of this study, and not to produce as much embryo's on an annual basis, a potential more beneficial manner could be developed. This protocol should be controlled in a way that every cow is in the same phase in the cycle during the study. This could be achieved, if after every OPU session a new sync. week was incorporated (figure 6). All heifers should be synchronized the same way as intended as the first sync. week.
Another method in order to achieve the same results, could be acquired if a PGF2α injection is incorporated in the scheme at the time same of the OPU session. Thereafter, every heifer will enter the same cycle unless a new CL is active or an old one was active. The presence of this new CL is unlikely; because that CL must have been created between the DFR and the OPU session or in the coasting period. Those options are very unlikely. In addition, The GnRH protocol produces most likely a young CL, because the intention of GnRH was to produce a new CL (age = five days at the time of OPU). In conclusion, this treatment will most likely will not be a success in this GnRH scheme.
The author did not exclude any heifers/measurements. Even though some heifers did not produce any blastocysts. This could prove to be a contributor to the fact that the differences between the means in the treatment groups were not significant (see results). Since, not all the heifers were divided randomly across the treatment groups, as mentioned above.

* Note that a 20-day cycle of a young heifer is neglected.
** The heifers, which receive PGF2α on this date, were on the cut off time. If luteolysis will occur depends e.g. on the timing of the day and other men- and cow factors.

Conclusion

Concluding, in this study differences are reported among the two treatment groups and in the controlled groups (age, number of the OPU session, origin and P_4-level). These differences are calculated to be statically indifferent. If a better answer is needed whether the DFR protocol or GnRH protocol is more beneficial, changes should be incorporated.

Appendix

Progesterone sheets

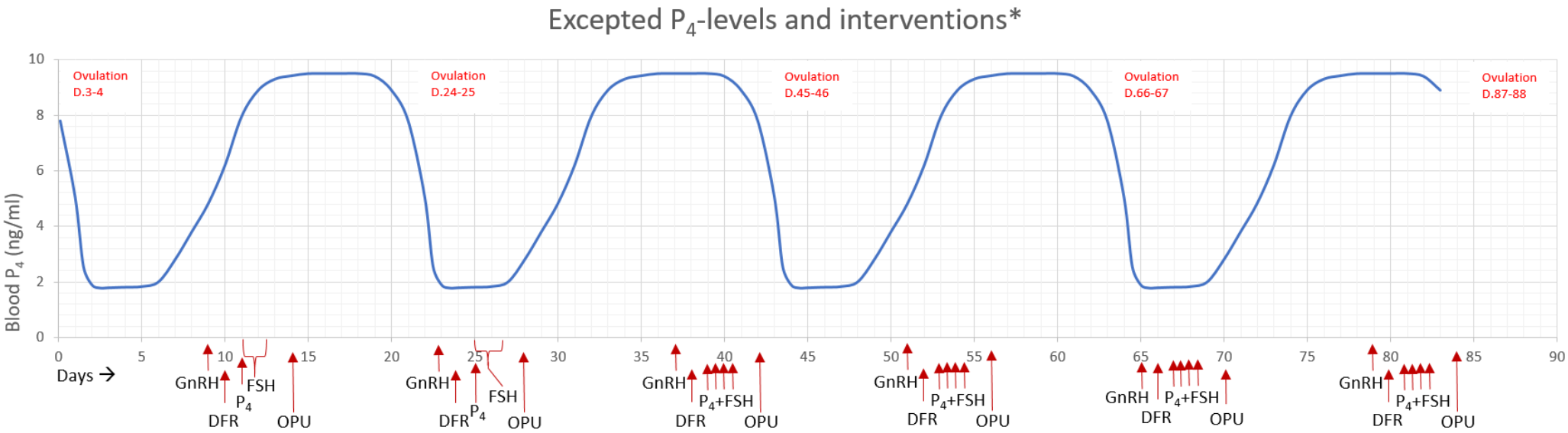


Figure 3, Excepted progesterone levels during the experiment after synchronization week.
*(DFR/GnRH and P_4 measure).

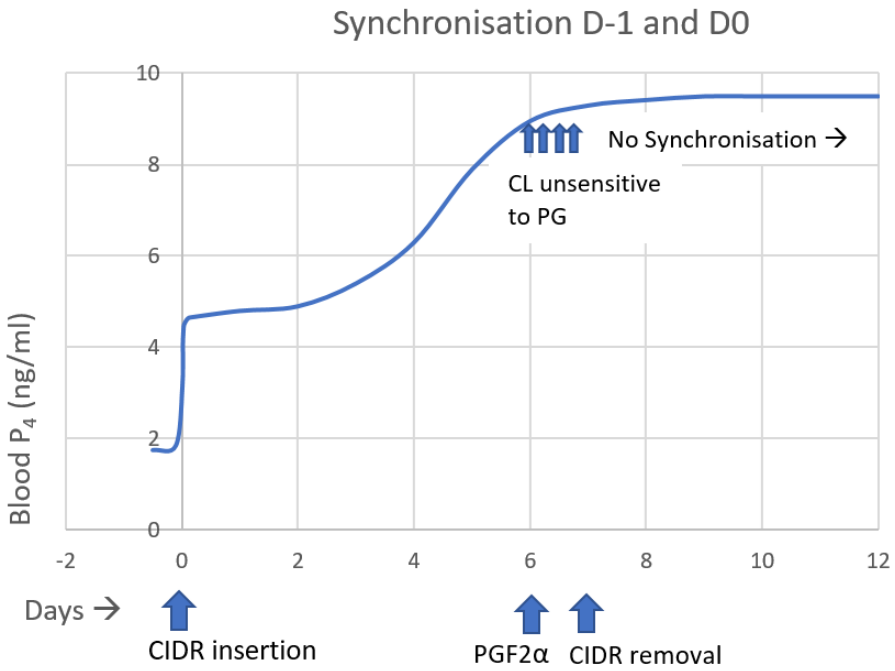


***Figure 4**, Excepted progesterone levels for heifers at day -1 and day 0 of the oestrus cycle in relation with the synchronization procedures.*

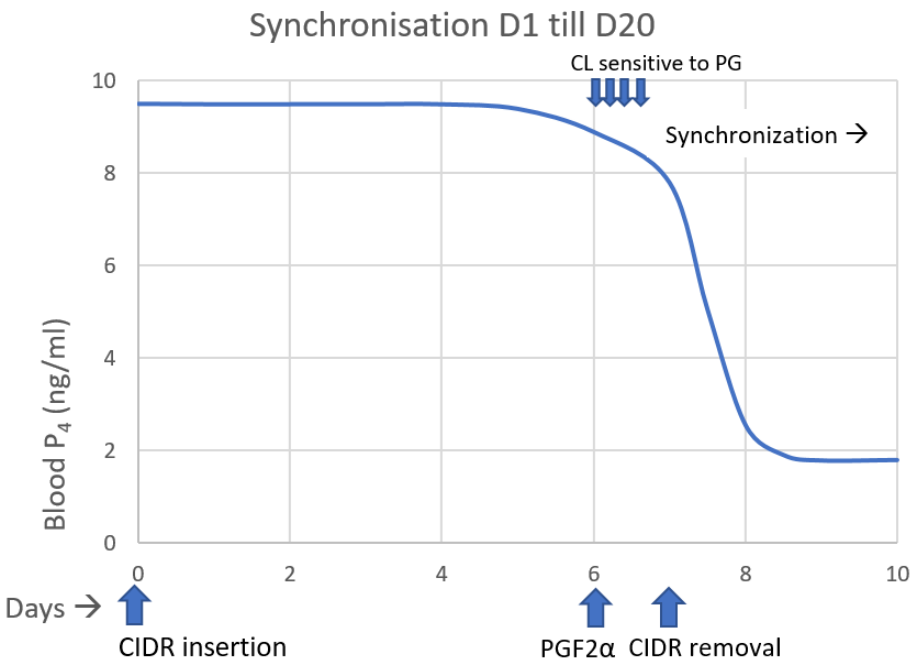


***Figure 5**, Excepted progesterone levels for heifers at day 1 till day 20 the oestrus cycle in relation with the synchronization procedures.*

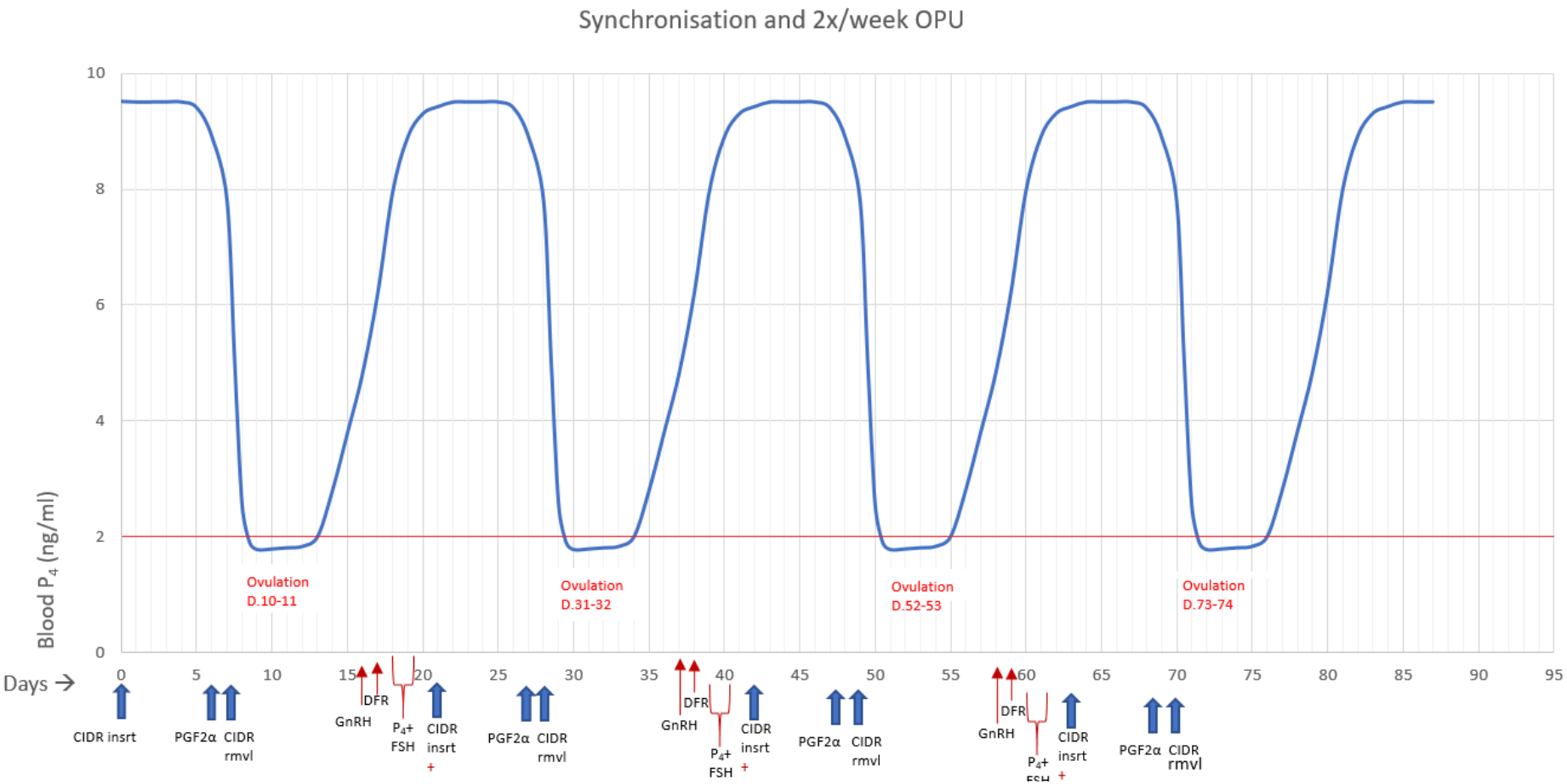


Figure 6, *New study proposal (first).*

Appendix (results extensively)

Small sized follicles (<4 mm)

Group		Mean and Sd	SEM	95 % CI	P-value
Treatment	*DFR*	12,45 ± 8,524	0,894	10,68 – 14,23	0,874
	GnRH	14,51 ± 15,439	1,820	10,89 – 18,14	
Age	*Young*	17,63 ± 17,119	2,229	13,17 – 22,09	0,278
	Old	10,94 ± 6,935	0,680	9,59 – 12,29	
Origin from scheme	*1x/7days*	12,06 ± 8,744	1,457	9,10 – 15,01	0,787
	Synchronizised	13,73 ± 12,873	1,142	11,47 – 15,99	
Number of the OPU session	*First OPU*	11,31 ± 9,613	1,433	8,42 – 14,20	0,819
	Second OPU	12,25 ± 7,637	1,151	9,93 – 14,57	0,632
	Third OPU	15,17 ± 13,468	2,103	10,92 – 19,42	0,496
	Fourth OPU	15,90 ± 17,432	3,131	9,51 – 22,30	0,526
[P_4]	< 2 ng/ml	14,22 ± 8,338	1,076	12,06 – 16,37	0,077
	≥ 2ng/ml	14,10 ± 17,502	2,427	9,22 – 18,97	

Medium sized follicles (4-8 mm)

Group		Mean and Sd	SEM	95 % CI	P-value
Treatment	*DFR*	9,09 ± 7,446	0,781	7,54 – 10,64	0,981
	GnRH	8,25 ± 5,698	0,672	6,91 – 9,59	
Age	*Young*	10,44 ± 7,278	0,948	8,54 – 12,34	0,096
	Old	7,74 ± 6,213	0,609	6,53 – 8,95	
Origin from scheme	*1x/7days*	6,19 ± 5,731	0,955	4,26 – 8,13	0,077
	Synchronizised	9,43 ± 6,831	0,606	8,23 – 10,63	
Number of the OPU session	*First OPU*	9,44 ± 7,847	1,170	7,09 – 11,80	0,964
	Second OPU	8,48 ± 6,472	0,976	6,51 – 10,44	0,966
	Third OPU	8,51 ± 6,345	0,991	6,51 – 10,51	0,955
	Fourth OPU	8,71 ± 6,007	1,079	6,51 – 10,91	0,984
[P_4]	< 2 ng/ml	8,87 ± 6,870	0,887	7,09 – 10,64	0,022
	≥ 2ng/ml	10,06 ± 6,005	0,833	8,39 – 11,73	

Big sized follicles (>8 mm)

Group		Mean and Sd	SEM	95 % CI	P-value
Treatment	*DFR*	2,81 ± 3,300	0,346	2,13 – 3,50	0,050
	GnRH	3,67 ± 3,162	0,373	2,92 – 4,41	
Age	*Young*	3,49 ± 4,053	0,528	2,44 – 4,55	0,649
	Old	3,02 ± 2,713	0,266	2,49 – 3,55	
Origin from scheme	*1x/7days*	2,31 ± 2,026	0,338	1,62 – 2,99	0,369
	Synchronizised	3,44 ± 3,495	0,310	2,83 – 4,05	
Number of the OPU session	*First OPU*	3,76 ± 3,517	0,524	2,70 – 4,81	0,853
	Second OPU	3,57 ± 3,938	0,594	2,37 – 4,77	0,763
	Third OPU	2,49 ± 2,541	0,397	1,69 – 3,29	0,597
	Fourth OPU	2,87 ± 2,553	0,458	1,93 – 3,81	0,620
[P_4]	< 2 ng/ml	3,12 ± 3,523	0,455	2,21 – 4,03	0,209
	≥ 2ng/ml	3,75 ± 3,446	0,478	2,79 – 4,71	

AFC during OPU sessions

Group		Mean and Sd	SEM	95 % CI	P value
Treatment	*DFR*	24,35 ± 12,57	1,317	21,73 – 26,97	0,664
	GnRH	26,63 ± 16,64	2,078	22,29 – 30,57	
Age	*Young*	31,56 ± 19,73	0,568	26,42 – 36,70	0,050
	Old	21,70 ± 10,00	0,997	19,76 – 23,64	
Origin from scheme	*1x/7days*	20,56 ± 11,65	1,941	16,61 – 24,50	0,130
	Synchronizised	26,61 ± 15,61	1,385	23,87 – 29,35	
Number of the OPU session	*First OPU*	24,51 ± 13,98	2,084	20,31 – 28,71	0,950
	Second OPU	24,30 ± 11,40	1,719	20,83 – 27,76	0,895
	Third OPU	26,17 ± 15,58	2,433	21,25 – 31,09	0,802
	Fourth OPU	27,48 ± 19,92	3,577	20,81 – 34,79	0,934
[P_4]	< 2 ng/ml	26,20 ± 11,52	1,487	23,23 – 29,17	0,761
	≥ 2ng/ml	27,90 ± 19,87	2,755	22,37 – 33,44	

Lab follicle count

Group		Mean and Sd	SEM	95 % CI	P-value
Treatment	*DFR*	11,74 ± 6,962	0,7297	10,29 – 13,19	0,218
	GnRH	16,47 ± 19,24	2,267	11,95 – 20,99	
Age	*Young*	18,29 ± 21,17	2,756	12,77 – 23,80	0,329
	Old	11,30 ± 6,008	0,589	10,13 – 12,47	
Origin from scheme	*1x/7 days*	10,19 ± 6,315	1,052	8,06 – 12,33	0,242
	Synchronizised	14,86 ± 15,31	1,359	12,17 – 17,55	
Number of the OPU session	*First OPU*	14,62 ± 12,63	1,882	10.83 – 18,41	0,452
	Second OPU	13,34 ± 11,02	1,661	9,99 – 16,69	0,431
	Third OPU	13,98 ± 19,41	3,031	7,85 – 20,10	0,426
	Fourth OPU	13,45 ± 11,78	2,115	9,13 – 17,77	0,406
[P_4]	< 2 ng/ml	13,77 ± 10,12	1,307	11,15 – 16,38	0,562
	≥ 2ng/ml	17,02 ± 20,94	2,904	11,19 – 22,85	

Follicle quality in lab 1 and 2

Group		Mean and Sd	SEM	95 % CI	P value
Treatment	*DFR*	11,47 ± 6,182	0,866	9,73 – 13,21	0,416
	GnRH	16,61 ± 19,82	2,581	11,44 – 21,78	
Age	*Young*	16,95 ± 19,99	2,603	11,74 – 22,16	0,192
	Old	10,23 ± 5,805	0,575	9,09 – 11,37	
Origin from scheme	*1x/7 days*	9,03 ± 6,088	1,104	6,91 – 11,15	0,148
	Synchronizised	13,67 ± 14,50	1,286	11,12 – 16,21	
Number of the OPU session	*First OPU*	14,84 ± 19,10	2,879	9,04 – 20,65	0,150
	Second OPU	12,63 ± 10,18	1,552	9,50 – 15,76	0,138
	Third OPU	11,54 ± 10,62	1,659	8,18 – 14,89	0,170
	Fourth OPU	11,77 ± 10,50	1,886	7.92 – 15,63	0,215
[P_4]	< 2 ng/ml	12,88 ± 9,388	1,222	10,43 – 15,33	0,591
	≥ 2ng/ml	15,78 ± 20,04	2,806	10,15 – 21,42	

Follicle quality in lab 1

Group		Mean and Sd	SEM	95 % CI	P value
Treatment	*DFR*	1,84 ± 2,256	0,239	1,37 – 2,32	0,099
	GnRH	3,18 ± 5,059	0,597	1,99 – 4,37	
Age	*Young*	2,95 ± 5,664	0,737	1,47 – 4,43	0,881
	Old	2,15 ± 2,131	0,211	1,73 – 2,53	
Origin from scheme	*1x/7 days*	1,38 ± 1,706	0,293	0,79 – 1,98	0,085
	Synchronizised	2,72 ± 4,178	0,371	1,99 – 3,46	
Number of the OPU session	*First OPU*	3,11 ± 4,895	0,738	1,63 – 4,60	0,420
	Second OPU	2,19 ± 2,986	0,455	1,27 – 3,11	0,528
	Third OPU	2,71 ± 4,343	0,678	1,34 – 4,08	0,509
	Fourth OPU	1,65 ± 2,058	0,370	0,89 – 2,40	0,772
[P_4]	< 2 ng/ml	2,22 ± 2,901	0,378	1,46 – 2,98	0,329
	≥ 2ng/ml	3,33 ± 5,552	0,777	1,77 – 4,89	

Follicle quality in lab 2

Group		Mean and Sd	SEM	95 % CI	P value
Treatment	*DFR*	9,02 ± 5,442	0,577	7,88 – 10,17	0,538
	GnRH	11,76 ± 14,00	1,647	8,48 – 15,05	
Age	*Young*	14,00 ± 15,09	1,964	10,07 – 17,93	0,060
	Old	8,08 ± 4,732	0,469	7,15 – 9,01	
Origin from scheme	*1x/7 days*	7,65 ± 5,370	0,921	5,77 – 9,52	0,042
	Synchronizised	10,94 ± 11,10	0,985	9,00 – 12,89	
Number of the OPU session	*First OPU*	11,73 ± 14,62	2,204	7,28 – 16,17	0,042
	Second OPU	10,44 ± 8,084	1,233	7,95 – 12,93	0,049
	Third OPU	8,83 ± 7,190	1,123	6,56 – 11,10	0,038
	Fourth OPU	10,13 ± 9,157	1,645	6,77 – 13,49	0,031
[P_4]	< 2 ng/ml	10,66 ± 7,291	0,949	8,76 – 12,56	0,495
	≥ 2ng/ml	12,45 ± 15,26	2,137	8,16 – 16,74	

Follicle quality in lab 3 and 4

Group		Mean and Sd	SEM	95 % CI	P value
Treatment	*DFR*	0,96 ± 1,296	0,137	0,68 – 1,23	0,243
	GnRH	1,63 ± 2,492	0,294	1,04 – 2,21	
Age	*Young*	1,37 ± 2,228	0,290	0,79 – 1,95	0,793
	Old	1,19 ± 1,773	0,176	0,84 – 1,53	
Origin from scheme	*1x/7days*	1,21 ± 1,533	0,263	0,67 – 1,74	0,747
	Synchronizised	1,27 ± 2,049	0,182	0,91 – 1,63	
Number of the OPU session	*First OPU*	1,36 ± 1,740	0,262	0,83 – 1,89	0,281
	Second OPU	1,40 ± 2,269	0,346	0,70 – 2,09	0,303
	Third OPU	1,10 ± 2,035	0,318	0,46 – 1,74	0,413
	Fourth OPU	1,19 ± 1,721	0,309	0,56 – 1,82	0,356
[P_4]	< 2 ng/ml	1,12 ± 1,403	0,183	0,75 – 1,48	0,977
	≥ 2ng/ml	1,43 ± 2,343	0,328	0,77 – 2,09	

Broken follicles

Group		Mean and Sd	SEM	95 % CI	P value
Treatment	*DFR*	0,85 ± 1,682	0,178	0,50 – 1,21	0,102
	GnRH	1,67 ± 3,149	0,371	0,93 – 2,41	
Age	*Young*	1,86 ± 3,431	0,447	0,97 – 2,76	0,100
	Old	0,84 ±1,597	0,158	0,53 – 1,16	
Origin from scheme	*1x/7days*	0,82 ± 1,336	0,229	0,36 – 1,29	0,542
	Synchronizised	0,85 ± 2,693	0,239	0,85 – 1,80	
Number of the OPU session	*First OPU*	1,39 ± 3,350	0,505	0,37 – 2,40	0,460
	Second OPU	1,28 ± 2,423	0,370	0,53 – 2,02	0,424
	Third OPU	1,17 ± 1,801	0,281	0,60 – 1,74	0,406
	Fourth OPU	1,03 ± 1,958	0,352	0,31 – 1,75	0,566
[P_4]	< 2 ng/ml	1,56 ± 2,521	0,328	0,90 – 2,22	0,143
	≥ 2ng/ml	1,41 ± 3,251	0,455	0,50 – 2,33	

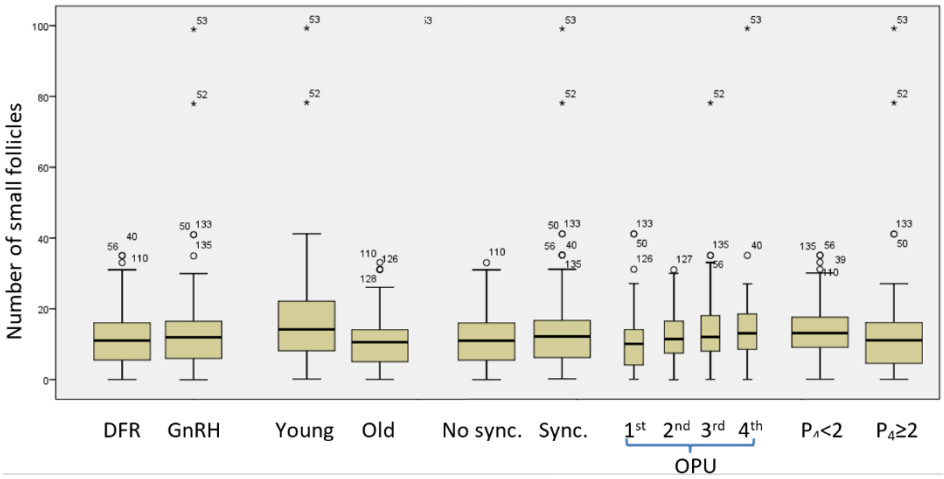
Total blastocyst count

Group		Mean and Sd	SEM	95 % CI	P value
Treatment	*DFR*	3,37 ± 3,849	0,403	2,57 – 4,18	0,130
	GnRH	4,49 ± 4,182	0,493	3,50 – 5,47	
Age	*Young*	4,53 ± 5,008	0,652	3,22 – 5,83	0,511
	Old	3,49 ± 3,312	0,325	2,85 – 4,13	
Origin from scheme	*1x/7 days*	3,19 ± 3,853	0,642	1,89 – 4,50	0,481
	Synchronizised	4,06 ± 4,068	0,361	3,34 – 4,77	
Number of the OPU session	*First OPU*	4,42 ± 4,934	0,735	2,94 – 5,90	0,524
	Second OPU	3,45 ± 3,217	0,485	2,48 – 4,43	0,497
	Third OPU	4,02 ± 3,765	0,588	2,84 – 5,21	0,614
	Fourth OPU	3,48 ± 4,130	0,742	1,97 – 5,00	0,425
[P_4]	< 2 ng/ml	4,15 ± 3,965	0,512	3,13 – 5,17	0,234
	≥ 2ng/ml	3,79 ± 4,137	0,574	2,64 – 5,17	

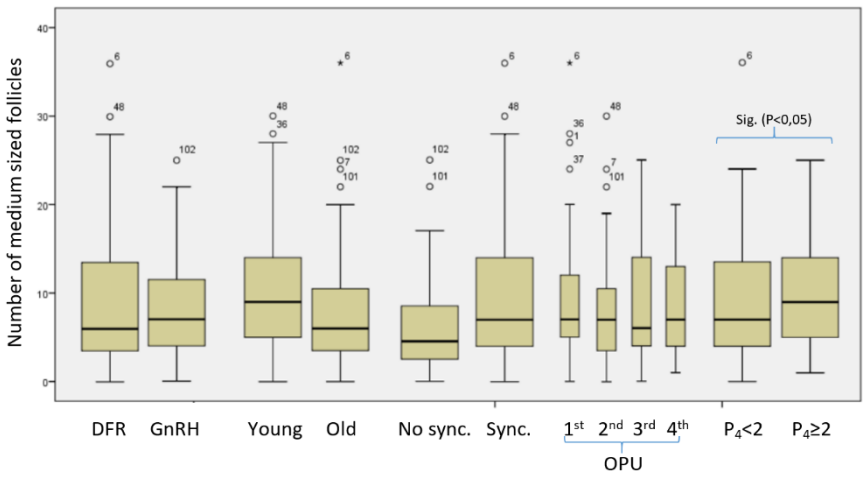
Blastocyst count without class 3 blastocyst*

Group		Mean and Sd	SEM	95 % CI	P value
Treatment	*DFR*	2,01 ± 2,846	0,298	1,42 – 2,60	0,293
	GnRH	2,38 ± 2,217	0,261	1,85 – 2,90	
Age	*Young*	2,73 ± 3,183	0,414	1,90 – 3,56	0,558
	Old	1,86 ± 2,129	0,209	1,44 – 2,27	
Origin from scheme	*1x/7 days*	1,50 ± 1,813	0,302	0,89 – 2,11	0,546
	Synchronizised	2,36 ± 2,742	0,243	1,88 – 2,84	
Number of the OPU session	*First OPU*	2,62 ± 3,406	0,508	1,60 – 3,65	0,707
	Second OPU	1,77 ± 2,022	0,305	1,16 – 2,39	0,534
	Third OPU	2,24 ± 2,538	0,396	1,44 – 3,04	0,670
	Fourth OPU	2,06 ± 1,982	0,356	1,34 – 2,79	0,685
[P_4]	< 2 ng/ml	2,32 ± 2,568	0,331	1,65 – 2,98	0,160
	≥ 2ng/ml	1,94 ± 2,200	0,305	1,33 – 2,55	

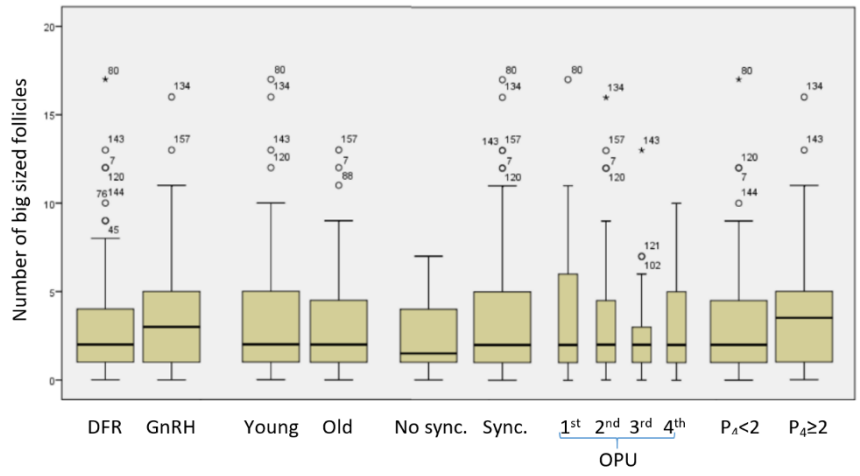
Small follicles during OPU



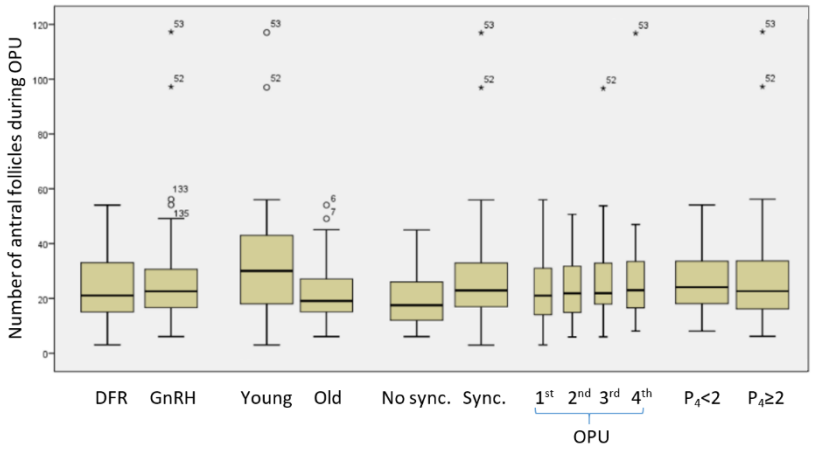
Medium sized follicles during OPU



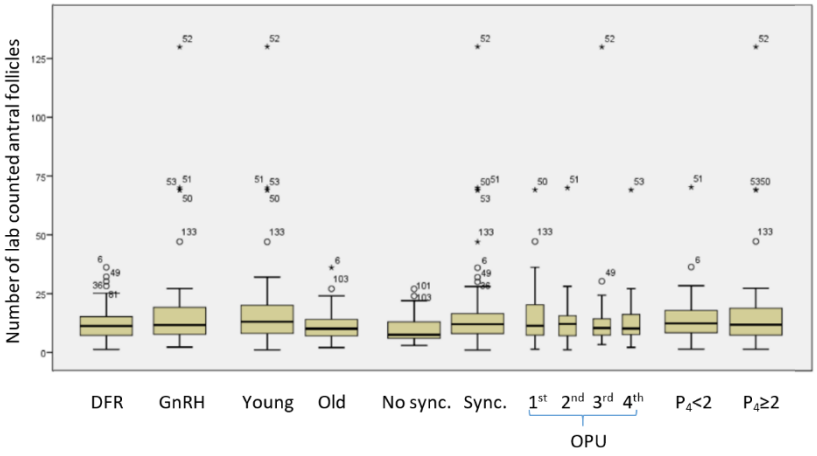
Big sized follicles during OPU



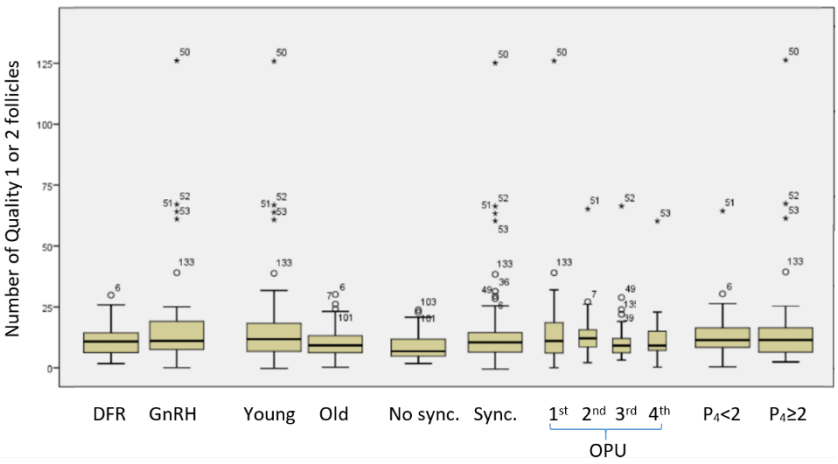
Total AFC count



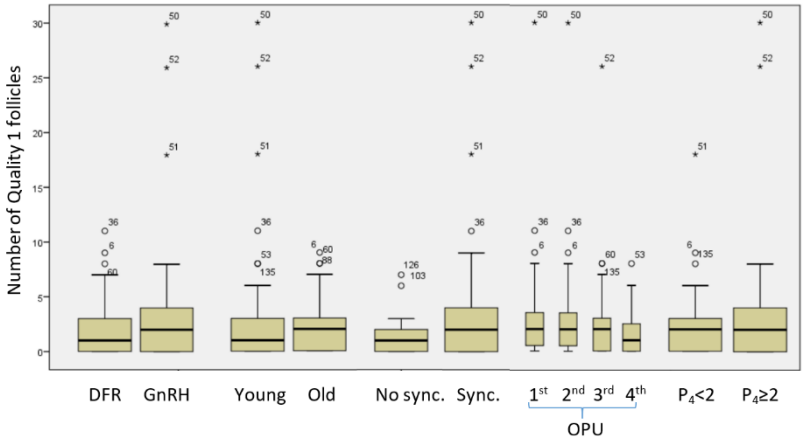
Total Lab counted follicles



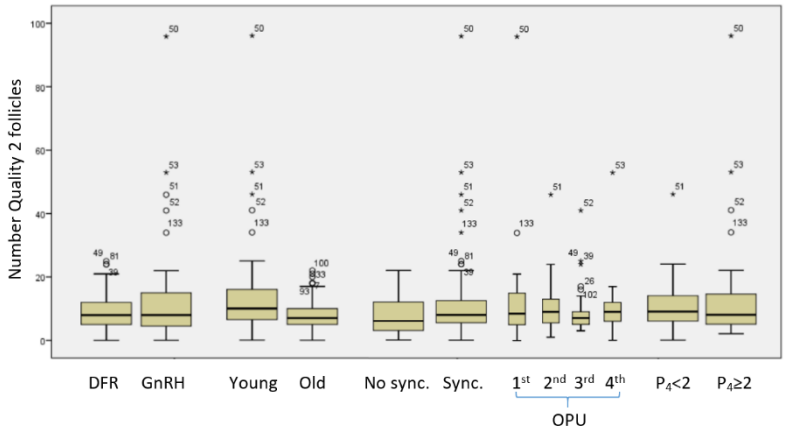
Quality 1 or 2 follicles



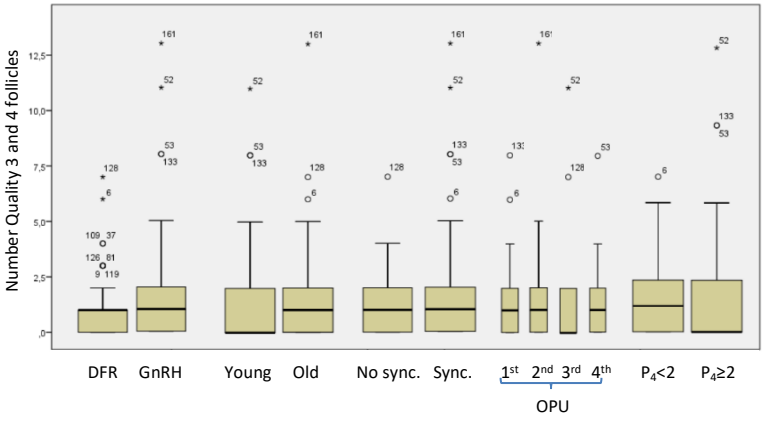
Quality 1 follicles



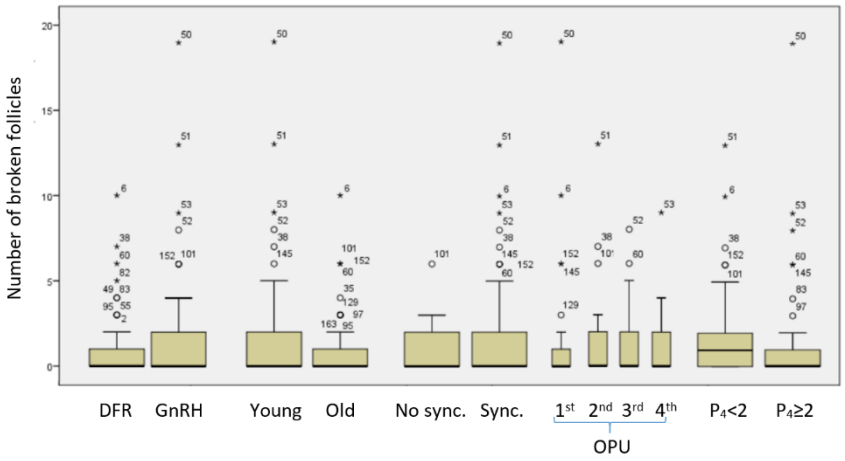
Quality 2 follicles



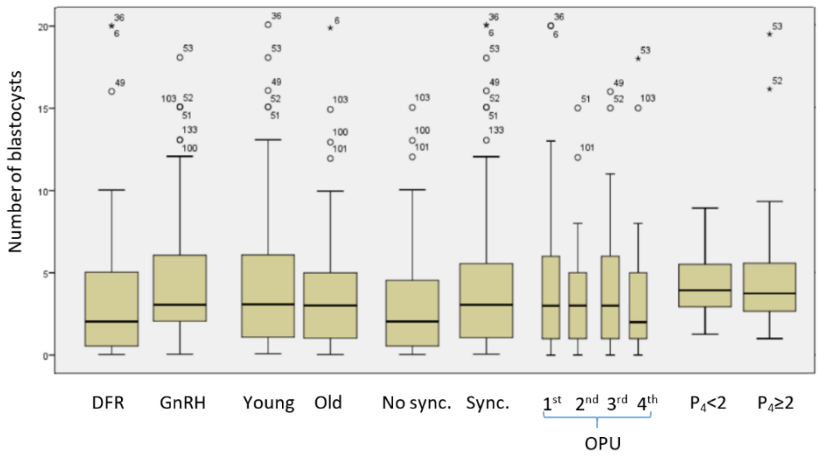
Quality 3 and 4 follicles



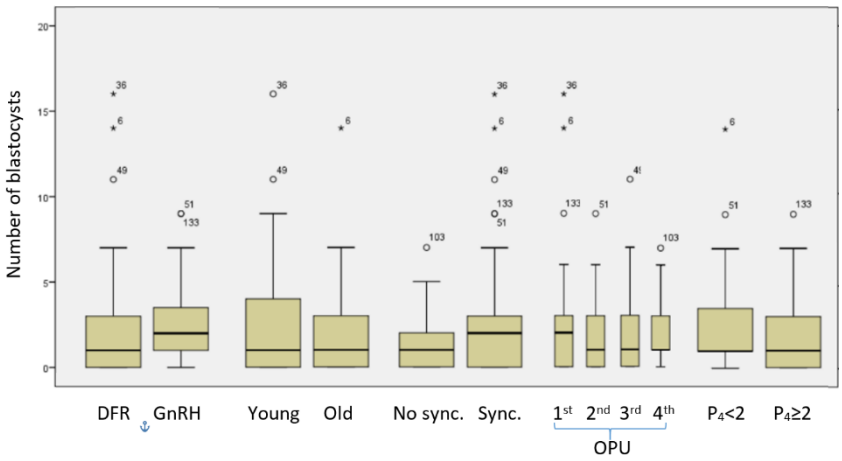
Broken follicles



Total blastocysts count



Blastocysts count (class 1 and 2)



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