The presence of two ovulatory follicles at timed artificial insemination affects ovulatory response to GnRH in high producing dairy cows

Running head: Two ovulatory follicles and ovulation

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Abstract

This study sought to examine the impact of the presence of two co-dominant (ovulatory) follicles at the time of artificial insemination (AI) on the ovulatory response to GnRH given in a fixed-time AI protocol. The study population comprised 622 lactating dairy cows: 306 (49.2%) with a single follicle, 198 (31.8%) with two bilateral follicles (one follicle per ovary) and 118 (19%) with two unilateral follicles (same ovary). Based on odds ratios, cows with two bilateral or unilateral follicles were less likely (by factors of 0.09 and 0.11, respectively) to undergo ovulation failure compared with cows with one follicle ($P = 0.01$ and $P = 0.02$, respectively); the likelihood of ovulation failure decreased 0.75 times with every one-mm increase in follicle diameter for cows with a single follicle, whereas individual follicle diameter was not related to ovulation failure in cows with two bilateral follicles ($P = 0.001$). The likelihood of double ovulation decreased 0.7 times with every one-mm diameter difference between the larger and smaller follicle for all cows with two follicles ($P = 0.001$), whereas cows with two unilateral follicles showed a higher ($P < 0.05$) double ovulation rate than cows with two bilateral follicles. In 116 (58.6%) of the cows with two bilateral follicles, only the larger follicle ovulated in 59.5% cows, whereas only the smaller one ovulated in the remaining 40.5% cows. In these cows, a one-mm size difference between the larger and the smaller follicle gave rise to a 1.12-fold increase in the ovulation failure rate for the larger follicles ($P = 0.0001$). Cows with two bilateral follicles were more likely (by a factor of 1.5) to conceive than cows with one follicle ($P = 0.001$). Significant right-left differences were not found in cows with two bilateral follicles, whereas the right ovary was more active than the left in the remaining cows. Our results indicate that cows with two co-dominant follicles at AI show different ovulation patterns to those with one dominant follicle. A higher rate of ovulation failure was observed among cows with one follicle than cows with two follicles, whereas the conception rate was higher for cows with two bilateral follicles than for the remaining cows. In cows with two follicles, double ovulations along with ovulation of the smaller follicle were related to the least size difference between the larger and smaller follicle.

Keywords: Co-dominant follicles; Follicular diameter; Ovulation failure; Double ovulation; Bilateral asymmetry.

1. Introduction

Pregnancy loss during the late embryonic/early fetal period severely affects dairy herd economy [1,2], and twin pregnancy is the main non-infectious factor compromising pregnancy maintenance during this period [3,4]. In some herds, the twin pregnancy rate may be higher than 18% [5] and twin pregnancy losses of up to 50% have been recorded, especially under heat stress conditions [6,7]. Understandably, the twin pregnancy rate is closely related to the twinning rate, which has a dramatic negative impact on the survival of dairy cows giving birth to twins [8e10]. Moreover, the real economic impacts of twinning are probably on the rise since twinning rates have increased considerably over the past three decades and will arguably continue to do so along with milk production [4,11]. The most probably reason for this is an increased double ovulation rate related to high milk production [12]. Double ovulation can occur in as many as 25% of cows during their third or later lactation [13].

During the estrus cycle, there are two or three consecutive waves of follicular growth comprising the inter-ovulatory interval. In each wave a dominant follicle and several subordinate follicles develop during a process termed deviation [14,15]. The mean diameter of the dominant follicle at the time of deviation may be 1.6mm larger than the largest subordinate follicle [16], and each follicular wave usually ends with the development of a single nonovulatory or ovulatory dominant follicle [17]. Only the last of the two or three waves is ovulatory. During the ovulatory wave, the largest follicle that finally ovulates enlarges as estrus approaches, and is especially large on Day 18 of the cycle [18,19]. However, there is significant variation in the numbers of various sized antral follicles present during the estrus cycle of dairy cows [20]. In cows [21] and women [22] delivering twins, increased follicular recruitment and growth has been described. In essence, the selection of beef cattle for twin births may increase the twinning rate to an annual frequency higher than 50% [23]. Therefore, two or more ovulatory follicles are often present at the time of insemination. Natural double ovulations result from the simultaneous formation of two co-dominant (ovulatory) follicles in the same ovary or from one follicle in each ovary within a follicular wave [21,24].

Synchronization protocols for fixed-time artificial insemination (FTAI) have become routine practice in the reproductive management of lactating dairy cows [25]. However, such protocols or hormone treatments following AI can increase the risk of twin pregnancy [5,26]. A number of studies have sought to prevent the negative impact of twinning through the use of hormone synchronization protocols [27,28]. Even so, to our knowledge, no prior report has correlated the presence of two ovulatory follicles at AI with ovulation patterns in lactating dairy cows subjected to ovulation synchronization protocols for FTAI. The purpose of our study was to obtain information on the effects of the presence of two ovulatory follicles at AI on the ovulatory response to GnRH in a five-day progesterone-based FTAI protocol. Such information is important to determine the effects of synchronization protocols for FTAI on twin pregnancies. A further point of interest should be possible reduction of the risk of twin pregnancies. Puncture and drainage of the subordinate follicles at timed artificial insemination may prevent the risk of twin pregnancy in dairy cows [29]. Cows with one ovulatory follicle in each ovary are a good model to assess the fate of each follicle. Accordingly, we hypothesized that the relationship between ovulatory follicle size and the probability of ovulation would be similar for the larger follicle in cows with two bilateral follicles and for the single follicle in cows with only one follicle.

2. Materials and Methods

2.1. Cattle and herd management

This study was performed on a commercial Holstein-Friesian dairy herd in northeastern Spain. During the study period (May 2015 to September 2017), the mean number of lactating cows in the herd was 285 and mean annual milk production was 10,950 kg per cow. The mean annual culling rate was 31%. Cows were grouped according to age (primiparous plus secundiparous versus multiparous), milked three times daily and fed complete rations. All cows were artificially inseminated and the herd was subjected to a weekly reproductive health program, as described elsewhere [30, 31]. Only healthy cows inseminated following a FTAI protocol, free of detectable reproductive disorders and free of clinical diseases during the study period (Days -7 to 28 of insemination) were included. Exclusion criteria were the following disorders: mastitis, lameness, digestive disorders and pathological abnormalities of the reproductive tract detectable by ultrasonography. Spontaneous estrus was detected by visual observation several times throughout the day.

2.2. Fixed-time AI protocol, insemination and pregnancy diagnosis

During the weekly reproductive visit, open cows more than 60 days in milk with no estrous signs for at least 21 days were treated with a controlled intravaginal progesterone-releasing device (CIDR) (CIDR, containing 1.38 g of progesterone; Zoetis Spain SL, Alcobendas, Madrid, Spain) plus GnRH (100 µg im; Cystoreline, CEVA Salud Animal, Barcelona, Spain) upon CIDR insertion. The CIDR was left in place for

5 d, and these animals were also given PGF2α (25 mg dinoprost im; Enzaprost, CEVA Salud Animal, Barcelona, Spain) on CIDR removal. Twenty-four h and 36 h later, the cows received a second PGF2α dose and a second GnRH dose, respectively, and were inseminated 50-56 h after CIDR removal.

All cows were inseminated by two technicians with frozen-thawed semen from 12 bulls 14-20 h after the second GnRH dose. If a cow returned to estrus, its status was confirmed by transrectal palpation, and the animal was inseminated at this time and recorded as non-pregnant. In the remaining cows, pregnancy diagnosis was performed by ultrasound 28 d post-AI. Cows diagnosed as not pregnant received a further treatment, but the subsequent data were not included in this experiment. This meant that a cow was included only once per lactation in the study. All gynecological exams and pregnancy diagnoses were performed by the first author.

2.3. Data collection and statistical analysis

As in dairy cows the dominant follicle acquires the capacity to ovulate when it reaches a diameter of about 10mm [32], the presence of all follicles of 10 mm or more was recorded. Ovarian follicular structures and the absence or presence of one or more corpus luteum (CL) at least 10mm in diameter were assessed by ultrasonography immediately before AI, and seven days after AI. Depending on the number of ovulatory follicles detected at AI, cows were grouped as having a single follicle, two bilateral follicles (one follicle in each ovary) or unilateral follicles (two follicles in the same ovary). Corpus luteum size was taken as the mean of two measurements approximating the greatest length and width. In cows returning to estrus, the physical properties of the largest ovulatory follicle, uterus and vaginal fluid were used as reference to confirm estrus at AI [33,34]. A cow was classified as ready for service when the CL was either less than 10mm or non-detectable, the diameter of the largest follicle was equal to or greater than 10 mm and the uterus was highly turgid and contractile to the touch. Conception ratewas defined as the percentage of cows that became pregnant at FTAI out of the total number of cows in the corresponding group. The maximum temperature-humidity index (THI) on the day of AI was used to evaluate the effects of heat stress (THI values higher than 72 [35]) on subsequent reproductive performance. It should be noted that in our geographical region, a strong negative effect of heat stress on the reproductive performance of lactating dairy cows has been well established [28].

The following data were recorded for each animal: parturition and treatment dates; parity (primiparous versus multiparous); maximum THI at AI (≤72 versus > 72); milk production at AI (low producers < 40 kg versus high producers ≥ 40 kg); days in milk at AI (DIM; < 90 days postpartum versus ≥ 90 days postpartum); follicular size at AI (diameter of the follicles ≥ 10 mm); ovulation failure (absence of a CL ≥ 10mm seven days after AI); double ovulation (presence of two CL seven days after AI); ovary in which follicular or luteal structures were recorded (right versus left ovary); sire; pregnancy after FTAI; and presence of twins after FTAI. Possible significant ($P < 0.05$) effects of number of ovulatory follicles on categorical variables were explored by a Tukey-Kramer multiple comparison test using the logistic procedure of PASW Statistics for Windows version 18.0 (SPSS Inc., Chicago, IL, USA). Differences in diameters between larger and smaller follicles in cows with two bilateral follicles were analyzed by the Student's t-test.

Three binary logistic regression analyses were performed using ovulation failure, double ovulation, and conception at FTAI as the dependent variables. The factors entered in the model as independent dichotomous variables (where 1 denotes presence and 0 denotes absence) were parity (multiparous) and heat stress at AI (THI > 72). Number of follicles, days in milk and milk production at AI and sire (class variables), and follicular diameter of the larger follicle in cows with two follicles (continuous variables) were considered factors in the analyses. In the case of the dependent variable conception at FTAI, double ovulation was added as a factor. Possible interactions between number of follicles and the dichotomous variables heat stress and parity were also analyzed.

Regression analyses were conducted according to the method of Hosmer and Lemeshow [35] using the logistic procedure of PASW Statistics for Windows Version 18.0 (SPSS Inc., Chicago, IL, USA). Basically, this method consists of five steps as follows: preliminary screening of all variables for univariate associations; construction of a full model using all the significant variables arising from the univariate analysis; stepwise removal of non-significant variables from the full model and comparison of the reduced model with the previous model for model fit and confounding; evaluation of plausible interactions among variables; and assessment of model fit using Hosmer-Lemeshow statistics. Variables with univariate associations showing P values < 0.25 were included in the initial model. Model reduction continued until only significant terms according to the Wald statistic remained in the model at P < 0.05.

3. Results

Twelve cows with three or more follicles equal to or larger than 10 mm, and eight cows with one single follicle at AI but with two corpora lutea seven days later were withdrawn from the study. The final study population was comprised of 622 cows: 306 (49.2%) with a single follicle, 198 (31.8%) with two bilateral follicles (one follicle in each ovary) and 118 (19%) with two unilateral follicles (two follicles in the right or left ovary). Double ovulation was recorded in 113 cows amounting to 35.8% of the 316 cows with two follicles and 18.2% of all 622 inseminated cows.

Mean milk production and days in milk at AI and number of lactations were 40.2 ± 9.8 kg, 132.4 ± 48.2 days, and 2.7 ± 1.7 lactations, respectively (mean ± SD). All cows had at least a follicular structure equal to or larger than 10 mm, the absence of CL or a CL smaller than 10mm was considered ready for insemination. The independent variables recorded for each of the three ovulatory follicle states at AI and effects of the different states on each dependent variable are shown in Table 1. According to Tukey-Kramer multiple comparison tests, parity, milk production, days in milk and maximum THI at AI had no effects on follicular patterns. The ovulation failure rate was higher for cows with one follicle (16%) than for cows with two follicles (10.4%). Cows with two unilateral follicles showed a higher rate of double ovulation (48.6%) than cows with two bilateral follicles (34.8%). Mean follicle diameter, considering the largest follicle for cows with two follicles, did not differ among groups. One hundred and seventy eight cows (28.6%) of the 622 cows enrolled became pregnant following FTAI. Twin pregnancy was recorded in 17 cows (9.6%) of these 178 pregnant cows. The conception rate at AI was higher for cows with two bilateral follicles (34.8%) than for the remaining cows (25.7%).

Follicular patterns related to the presence of one follicle on the right or on the left ovary are shown in Table 2. The right ovary was more active than the left one in cows with a single follicle and in cows with two unilateral follicles. Significant right-left differences were not found in cows with two bilateral follicles.

3.1. Ovulation failure

For all cows (n = 622), number of follicles at AI was the only variable related to ovulation failure. Based on the odds ratios (Table 3), cows with two bilateral or unilateral follicles were less likely (by a factor of 0.09 and 0.11, respectively) to suffer ovulation failure compared to cows with one follicle (P = 0.01 and P = 0.02, respectively). No significant effects of the remaining variables or interactions were found.

In cows with one follicle (n = 306), the likelihood of ovulation failure decreased 0.75 times with a one-mm increase the follicle diameter at AI (odds ratio: 0.75; 95% confidence interval: 0.60-0.9; P = 0.001). No significant effects of the remaining variables examined were found. Fig. 1 shows ovulation failure values for different follicle diameters. Lower ovulation failure rates were observed for follicles measuring 16mm or more in diameter.

Among the cows with two bilateral follicles at AI (n =198), a single ovulation was recorded in 116 (58.6%). In these 116 single ovulating cows, the larger follicle ovulated in 69 cows (59.5%), whereas the smaller one ovulated in the remaining 47 cows (40.5%). The mean diameter (±SD) of the larger follicles that failed to ovulate (17.5 ±

3.26 mm)was greater (P = 0.003; Student's t-test) than that of the larger ovulating follicles (15.5 ± 2.97 mm). A one-mm larger diameter of the larger versus smaller follicle was found to give rise to a 1.12-fold increase in the ovulation failure rate for the larger follicles (odds ratio: 1.12; 95% confidence interval: 1.05e1.23; P = 0.0001). This meant that the chances of the smaller follicle ovulating increased greatly with increasing diameter differences between larger and smaller follicles. No significant effects of the remaining variables examined were found. Ovulation failure rates were not related to individual follicle diameters (Fig. 1). In Fig. 1, values for five smaller follicles larger than 19mm were grouped with the values for follicles measuring 16e18 mm. Mean diameter differences between larger and smaller follicles were 3.7 ± 2.4mm and 5.7 ± 2.9mm for single ovulating cows in which the larger follicle and smaller follicle ovulated, respectively.

3.2. Double ovulation

For all cows with two follicles (n = 316), the likelihood of double ovulation decreased 0.7 times with every one-mm increase in the difference in diameter between the larger and smaller follicle (odds ratio: 0.7; 95% confidence interval: 0.55-0.87; P = 0.001). No significant effects of the remaining variables examined were found. Mean diameter differences between larger and smaller follicles were 3.1 ± 2.7mm and 3.8 ± 3.3mm for double ovulating cows and remaining cows, respectively.

3.3. Conception and twin pregnancy rates

Odds ratios for the conception rate variables recorded in all cows are provided in Table 4. Cows with two bilateral follicles were more likely (by a factor of 1.5) to conceive than cows with one follicle (P = 0.001). Conception rates were similar for cows with one and with two unilateral pre-ovulatory follicles (25.5 and 26.3%, respectively). No significant effects of the remaining variables or interaction terms were detected. None of the factors examined had an impact on the twin pregnancy rate.

4. Discussion

Our study sought to examine the effects of the presence of two ovulatory follicles (defined as follicles of diameter 10mm or more [32]) at AI on the ovulatory response to GnRH in a five-day progesterone- based FTAI protocol. The FTAI protocol proved valuable and all cows had at least one 10mm or larger follicular structure and were considered ready for insemination at FTAI, consistent with our recent findings [37]. The points to be highlighted in the present study are: 1) the ovulation failure rate was significantly higher for cows with one follicle than for cows with two follicles; 2) the likelihood of ovulation failure was 0.75 times lower for every one-mm increase in follicle diameter for cows with a single follicle, whereas individual follicle diameter was not related to ovulation failure in cows with two bilateral follicles; 3) the mean size of the larger follicle in cows with two follicles, and of the single follicle in cows with one follicle was similar; 4) the likelihood of double ovulation was 0.7 times lower for every one-mm increase in the size difference between follicles for all cows with two follicles, whereas cows with two unilateral follicles showed a higher double ovulation rate than cows with two bilateral follicles; 5) the conception rate at AIwas higher for cows with two bilateral follicles than for the remaining cows; and 6) a single ovulation was recorded in 58.6% of the cows with two bilateral follicles and only the smaller one ovulated in 40.5% of these cows. Our results thus suggest that the presence of two ovulatory follicles at FTAI affects the ovulatory response to GnRH in lactating dairy cows.

In cows with two bilateral follicles, every one-mm difference in diameter between the larger and the smaller follicle increased the ovulation failure rate 1.12 times for the larger follicle. Hence, our starting hypothesis that the larger follicle or single follicle in cows with two or a single follicle respectively would show a similar Likelihood of ovulation was not supported. This finding clearly explains differences in ovulation patterns between cows with one or two ovulatory follicles. In cows with one follicle, the likelihood of ovulation increased with increasing follicle diameter, reinforcing previous results [37,38], whereas individual follicle diameter was not related to the likelihood of ovulation in cows with two bilateral follicles. It is difficult to explain why it is the smaller follicle that ovulates in these cows whereas the larger one remains anovulatory. A switch in diameter rank between the dominant and largest subordinate follicle may occur just before or at deviation within a follicular wave [39]. Also, a second deviation could occur in cows with co-dominant follicles [40]. These processes could be related to ovulation of the smaller follicle. Thus, an original dominant follicle could maintain its likelihood of ovulation regardless of its loss of size rank with regards to the largest subordinate follicle. Studies of functional changes in developing follicles are needed to improve our understanding of the relationships between two co-dominant follicles. For instance, the altered expression of cytokines in ovarian follicular structures may contribute to anovulation in cattle [41].

If key determinants of a successful ovulation include the amount of circulating gonadotrophin sequestered by a maturing follicle and physical modifications to the ovarian surface, then the presence of two ovulatory follicles on one ovary being more likely both to ovulate than one follicle on each ovary is easier to interpret. Whether there is also a particular relevance of inter- and intraovarian gradients in temperature remains to be explored, but such putative gradients would impact on proteolytic enzyme activity [42]. Double ovulation and ovulation of the smaller follicle were related to the least size differences between the larger and smaller follicle. Thus, the development of two dominant follicles seems more related to a small difference in size rather to the individual diameter of each follicle. Turning to mechanisms underlying multiple ovulations, a transient increase of FSH and LH just before the process of follicular deviation stimulates growth of the subordinate follicles at a rate similar to the dominant follicle [24,43], especially in highproducing dairy cows [24]. This mechanism together with the increased follicular recruitment which occurs in cows delivering twins [21] may favor the presence of co-dominant follicles. Scaramuzzi et al. [44] proposed the “window theory”. In most cases, the development of one dominant follicle occurs when the FSH level reaches a required threshold before the deviation process. The time span of this threshold constitutes either a narrow window that allows a single follicle to ovulate, or a wider window allowing multiple ovulations [44]. An increased pool of growing follicles may increase the number of ovulatory follicles. Last but not least, relative distribution of capillary blood-flow and humoral factors therein could contribute to the selection and development of one or two dominant follicles, whether or not a regressing corpus luteum is present [45].

Half of the cows had two follicles at AI, and the subsequent percentage of double ovulations for all cows was 18.2%. The prevalence of double ovulating cows in this study is within the ranges of 12e28% referred to the total AIs reported in several studies following FTAI [13,14,31,37], or of 14.5e22.5% for cows showing spontaneous estrus not given hormone treatment [13,46]. This means that the incidence of double ovulation is not a true reflection of the rate of follicular co-dominance at AI. Probably, the mean prevalence of cows developing two co-dominant follicles is close to 50%. As an increased twinning rate has been linked to increased milk production in the past few decades [4,11], the incidence of follicular co-dominance at AI and therefore the double ovulation rate will doubtless continue to increase over the years to come. Among other factors, improvements in nutrition and management practices related to high milk production are likely to favor follicular co-dominance [47].

In this context, attempts to reduce the risk of twin pregnancies should be encouraged. In a recent study [29], the draining by ovum pickup procedures of all follicles 10mm except the largest follicle at AI eliminated the risk of twin pregnancies. Furthermore, all drained follicles developed as a corpus luteum seven days later, although probably sub-functional. Variable proportions of granulosa cells remaining in the follicle were a source for the subsequent development of luteal tissue which remained as an additional corpus luteum in cows that became pregnant [29]. Although these observations are preliminary, puncture and drainage of the smaller follicles at insemination may abolish the risk of twin pregnancies and reduce the risk of subsequent pregnancy loss by increasing the incidence of additional corpora lutea. In pregnancies in which the number of corpora lutea is higher than the number of embryos, this additional corpus luteum is a strong factor favoring pregnancy maintenance [11,28]. However, this idea of reducing the smaller follicle warrants caution. As our results show, only the smaller follicle ovulated in 23.7% (47/198) of cows with bilateral follicles determining that follicular drainage could reduce the fertility of cows with bilateral follicles. More work is needed to find ways of promoting ovulation of the largest follicle following follicular drainage; for example, by giving a further GnRH dose at drainage. In this situation, additional GnRH could favor both ovulation of the remaining largest follicle and luteal activity of the drained follicle. If we consider the cows with unilateral follicles, in which double ovulation occurred in practically half of them and twin pregnancies affected a third of the subsequent pregnant cows, the benefits should outweigh the possible negative effects of this strategy.

Double ovulation has been linked to higher fertility [12,48]. Since double ovulation occurs in older cows [12,13], increasing the ovulation rate might be a strategy to increase opportunities of pregnancy, both for cows and other low ovulating mammalian species [47]. Irrespective of the double ovulation rate, the conception rate at AI was higher for cows with two bilateral follicles than for cows with two unilateral or a single follicle. The inclusion here of two follicles as an independent factor probably masked the possible positive effects of double ovulation on the conception rate. However, in a further analysis excluding the number of follicles (data not shown), no factors could be related to the conception rate.

Well-documented evidence exists of the functional asymmetry of the mammalian body. In the cow, many studies have shown that the right ovary and uterine horn are larger and more active than the left one [49e52]. The right ovary was more active in cows with a single follicle and in cows with two unilateral follicles, but right-left differences were not highlighted in cows with two bilateral follicles.
Our observation of one co-dominant follicle in each ovary suggests no detectable asymmetry in ovarian function in these animals. Moreover, ovulation failure and conception rates were practically the same for the right and left sides. These results indicate that asymmetry in the reproductive system of the cow is

determined by the activity of the right versus left ovary rather than by physiological differences in the uterine horns. In other words, asymmetries in genital tract morphology and physiology would seem to be secondary to gonadal asymmetry.

In our geographical zone, heat stress conditions have been strongly linked to low fertility [53,54] and to a high incidence of ovulation failure [13,55]. However, heat stress, defined as an environmental THI >72 [35], had no effect on conception or ovulation failure. The progesterone-based protocol for FTAI used here probably offset the negative effect of heat stress, consistent with previous studies [30,31].

As an overall conclusion, cows with two co-dominant follicles at AI, whether unilateral or bilateral, showed different ovulation patterns to those observed in cows with only one dominant follicle. A higher ovulation failure rate was recorded in cows with one follicle than in those with two follicles, whereas the conception rate was higher for cows with two bilateral follicles than for cows with one follicle or two unilateral follicles. In cows with two follicles, double ovulation along with ovulation of the smaller follicle were related to the smallest size difference between the larger and smaller follicle.

Acknowledgments

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Table 1. Independent variables recorded at AI for each class concerning number of ovulatory follicles and effects of the different classes on each dependent variable (n = 622).

Number of cows with	One single ovulatory follicle (n = 306)	Two bilateral ovulatory follicles (n = 198)	Two unilateral ovulatory follicles (n = 118)
Independent variables			
Parity (multiparous)	169 (55.2%)	124 (62.6%)	68 (57.6%)
Milk production (≥ 40 kg)	128 (41.8%)	82 (41.4%)	48 (40.7%)
Days in milk (>90 d)	183 (59.8%)	112 (56.6)	70 (59.3%)
Heat stress (max THI > 72)	188 (61.4%)	111 (56.1%)	72 (61%)
Dependent variable[a]			
Ovulation failure[b]	49 (16%)[a]	20 (10.1%)[b]	13 (11%)[b]
Double ovulation[c]	0 (0%)[a]	62 (34.8%)[b]	51 (48.6%)[c]
Follicle diameter (mm)[d]	16.1 ± 3.96 (range 10-30)	16.0 ± 3.35 (range 11-25)	16.1 ± 4.32 (range 10-32)
Conception rate at AI	78 (25.5%)[a]	69 (34.8%)[b]	31 (26.3%)[a]
[c]Twin pregnancy rate[e]	0 (0%)	7 (10.1%)	10 (32.3%)

[a]Values with different superscript differ within rows according Tukey-Kramer Tests ($P < 0.05$).
[b]For both follicles in cows with two ovulatory follicles.
[c]Percentages on ovulating cows.
[d]For the larger follicle in cows with two ovulatory follicles.
[f]Percentages on pregnant cows.

Table 2. Follicular patterns related to the presence of one or two ovulatory follicles in the right or left ovary.

Number of cows with	One single ovulatory follicle (n = 306)	Two bilateral ovulatory follicles (n = 198)	Two unilateral ovulatory follicles (n = 118)
Right ovary			
Number of cows	198 (64.7%)^{a}	198 (100%)	84 (71.2%)^{a}
Ovulation failure*	34 (17.2%)	76 (38.4%)	11 (13.1%)
Double ovulation			37 (44%)
Conception rate**	51 (25.8%)	42 (21.2%)	20 (23.8%)
Left ovary			
Number of cows	108 (35.3%)^{b}	198 (100%)	34 (28.8%)^{b}
Ovulation failure*	15 (13.9%)	82 (41.4%)	2 (5.9%)
Double ovulation			14 (41.2%)
Conception rate**	27 (25%)	34 (17.2%)	11 (32.4%)

$^{a, b}$Values with different superscript differ within columns according Tukey-Kramer Tests ($P < 0.05$).

*For this ovary in cows with bilateral pre-ovulatory follicles, irrespective of which occurs in the contralateral ovary, and for both follicles in cows with unilateral pre-ovulatory follicles.

**In the ipsilateral uterine horn in cows with bilateral pre-ovulatory follicles.

Table 3. Odds ratios of the ovulation failure variables included in the final logistic regression model (n = 622).

Factor	Class	n	% ovulation failure[a]	Odds ratio	95% confidence interval	P
Number of	1	49/306	16.0	Reference		
ovulatory	2 bilateral	20/198	10.1	0.09	0.03-0.55	0.01
follicles	2 unilateral	13/118	11.0	0.11	0.05-0.2	0.02

Hosmer and Lemeshow Goodness-of-fit test = 28.6; 3 df, P = 0.92.

R^2 Nagelkerke = 0.12

[a]For both follicles in cows with two pre-ovulatory follicles.

Table 4. Odds ratios of the conception rate variables included in the final logistic regression model (n = 622).

Factor	Class	n	% conception rate	Odds ratio	95% confidence interval	P
Number of	1	78/306	25.5	Reference		
ovulatory	2 bilateral	69/198	34.8	1.5	1.11-2.38	0.001
follicles	2 unilateral	31/118	26.3	0.11	0.54-3.52	0.6

Hosmer and Lemeshow Goodness-of-fit test = 22.6; 3 df, P = 0.90.

R^2 Nagelkerke = 0.14.

Figure 1. Ovulation failure rates recorded in cows with one ovulatory follicle (n = 306 “one follicle”), cows with two bilateral ovulatory follicles (n = 198 “bilateral. larger”, or the larger of the two follicles and “bilateral smaller”, or the smaller of the two follicles). Values for five smaller follicles larger than 19mm were grouped with the values for follicles measuring 16-18 mm. bil = bilateral.