Corpus luteum development and its morphology after aspiration of a preovulatory follicle is related to size and steroid content of the follicle in dairy cows

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ABSTRACT: Secretion of adequate levels of progesterone from a proper *corpus luteum* (CL), which develops out of the cells of a healthy preovulatory follicle, is a key-factor for establishment of a pregnancy. The aim of this study was to investigate the relationship between morphological and secretory characteristics of preovulatory follicles and their corresponding *corpus luteum* with regard to the post-partum period in high-yielding dairy cows. Therefore, ultrasound-guided aspirations of preovulatory follicles were performed repeatedly, using 20 first lactating cows between 26 and 121 days after parturition. Heat was induced with a PGF analogon followed by administration of a GnRH analogon. The dominant follicle was aspirated 21 h after administration of the GnRH analogon. The diameters of the follicles were estimated at aspiration and the morphology of the resulting luteal tissue was examined on day 14 after follicle aspiration using ultrasonographic examinations. Concentrations of progesterone (P_4) and 17-beta-oestradiol (E_2) were determined in the follicular fluids (FF) and P_4 concentration was estimated at the time of CL examination in plasma. A CL development occurred in 82% after dominant follicle aspiration. The interval of time between parturition and follicle aspiration did not affect the investigated variables. The diameter of the aspirated preovulatory follicle was positively correlated to the cross-section area of the developed luteal tissue ($R = 0.60$; $P < 0.01$) as well as to the plasma P_4 concentration on day 14 after follicle aspiration ($R = 0.47$; $P < 0.05$). Also, E_2 concentrations in FF were positively correlated to cross-section area of the luteal tissue ($R = 0.54$; $P < 0.05$). Comparing the FF of the follicles that gave rise to a CL after aspiration to follicles that did not, both types had comparable P_4, but the former type harboured higher E_2 concentrations. In conclusion, preovulatory follicle diameter as well as steroid concentrations in the follicular fluid could be used prospectively to identify cows which will have well-developed CLs and high plasma P_4 levels later. On the other hand, CL development after follicle aspiration can be used as a retrospective quality parameter of dominant follicles. These results will help to identify suitable animals for breeding or recipients for embryo transfer.

Keywords: post-partum period; ultrasound; fertility; progesterone; oestradiol; follicle fluid

List of abbreviations

CL = *corpus luteum*, **DM** = dry matter, **E_2** = 17-beta-oestradiol, **FF** = follicle fluid, **GnRH** = gonadotropin realising hormone, **P_4** = progesterone, **PGF** = prostaglandin F2 alfa, **TMR** = total mixed ration

Ovulation of a healthy follicle and the consecutive development of an actively secreting *corpus luteum* (CL) from the follicle cells are basic processes for the further establishment of a pregnancy. In high-yielding dairy cows both processes seem to take place in the post-partum period (Lopez-Gatius et al. 2002; Wiltbank et al. 2006), particularly in the early post-partum period, a period which is characterised by a negative energy balance (Leroy et al. 2008). Ovulation failure due to inadequate follicle quality leads to an increasing occurrence of follicle-teka-cysts in dairy herds (Lopez-Gatius et

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al. 2002). Additionally, lower progesterone levels in the plasma during dioestrus are described in cows compared to heifers (Sartori et al. 2004). Both could be a cause for the reduced fertility in the early post-partum period, which is observed in high-yielding dairy herds (Lopez-Gatius et al. 2002; Morris and Diskin 2008; Clemente et al. 2009; Walsh et al. 2011). In recent years there have been incredible advances in our knowledge regarding the molecular regulation of the ovulation process and regulation of growth or regression of the Cl. However, most of these studies were performed in heifers or with material of unknown origin, collected at the slaughter house. As usual, the developmental stage of follicles recovered at slaughter houses are defined retrospectively by their hormone content in the follicular fluids (Nishimoto et al. 2009), but of course their real further development cannot be ascertained. By using the ultrasound technique we have the opportunity to observe the follicle and CL development without any influence on their physiology (Roelofs et al. 2004). Moreover, the ultrasound technique offers additionally the possibility to collect target-oriented samples from follicles by ultrasound-guided follicle aspiration (Cech et al. 2013) or from the CL by an ultrasound-guided biopsy (Kot et al. 1999). In heifers, as well as in cows, it is described that following sample collection of follicle fluid by follicle aspiration from a preovulatory follicle (after the luteinising hormone surge), development of a CL does occur (Hayashi et al. 2006; O'Hara et al. 2012). However, there is nothing known regarding the impact of lactation or time interval to parturition on these processes and also no information about the steroid metabolism of the aspirated follicles are available.

Not least, a better understanding of the relationships between follicle and CL characteristics is of interest for veterinarians *in praxis*. Parameters, which are suitable for predicting the individual fertility of a healthy animal in an individual cycle and which can be measured easily in clinical examinations like ultrasound, are very rare. Especially in order to find recipients for an embryo transfer, it is important to identify prospectively individual animals with the best chance of getting pregnant (Spell et al. 2001; Chagas e Silva et al. 2002; Chebel et al. 2008; Yoshida et al. 2012).

Therefore, the first aim of the current study was to investigate the impact of the post-partum period on preovulatory follicle and CL quality by collecting data and samples minimal-invasively using ultrasound techniques in dairy cows during this period. Secondly, we wanted to find out whether CL development after follicle aspiration could be helpful in defining retrospectively the quality of a preovulatory follicle more precisely. Thirdly we aimed to determine, whether clinical determinable parameters of preovulatory follicles can be used prospectively in order to find animals which will go on to develop a healthy, actively secreting CL.

MATERIAL AND METHODS

Animals and experimental design

Twenty first-lactating German Holstein cows were housed at the Dummerstorf dairy cattle research farm in a stall run during the experiments (from 10 to 120 days post-partum). All animals were offered a total mixed ration (TMR) with an energy content of 6.9 NEL/kg *ad libitum*, based on grass (22% of DM) and corn silage (42% of DM), concentrates (17.5% of DM, MLF2000, Vollkraft, Germany), hay (6.5% of DM) extracted rapeseed and soy (4.5% of DM, respectively), dried beet pulp (2% of DM) and a mineral mix (0.7% of DM, Rinderstolz 9522, Silvana,

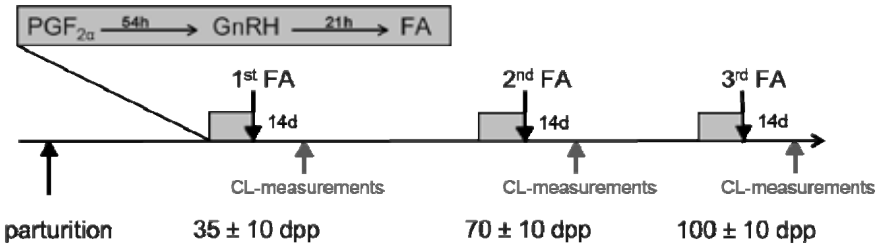

Figure 1. Experimental design: Three aspirations of pre-ovulatory follicles in dairy cows after hormonal heat and ovulation induction during the post-partum period, followed by an examination of the *corpus luteum* development 14 day thereafter (PGF2alfa = prostaglandine F2alfa, GnRH = gonadotropine releasing hormone, FA = follicle aspiration, CL = *corpus luteum*, dpp = days post-patrum)

Germany). The cows were milked twice daily and reached an average milk yield of 26.8 ± 0.6 litres per cow per day during the experimental period. Continuous heat observation was carried out twice daily by an experienced person and additionally ovaries were examined by ultrasound weekly. After observation of the first heat and CL formation after parturition the cows were integrated into the following experimental design of heat and ovulation synchronisation (Figure 1): Between day 9 and day 13 after the last heat CL were regressed by injection of an analogue of prostaglandin F2 alfa (0.5 mg Cloprostenol/animal, PGF Veyx forte®). A gonadotropin realising hormone (GnRH) analogon (0.1 mg Gonadorelin, Gonavet Veyx®) was administrated 54 h later to induce ovulation. The dominant follicle was aspirated 21 h after administration of the GnRH analogon as described below (according to Pursley et al. (1995), ovulation can be expected between 24 to 32 h after GnRH administration). The procedures of animal synchronisation and follicle aspiration were scheduled repeatedly over three periods post-partum: 35 ± 10, 70 ± 10 and 100 ± 10 days post-partum (Figure 1). Fourteen days after each follicle aspiration the ovaries were examined by ultrasound to detect and measure CL development and morphology as described below. Also a blood sample was taken on day 14 after follicle aspiration from the coccygeal vessels. All procedures involving animal handling and treatment were approved by the Agricultural Ministerial Department of Mecklenburg-Vorpommern, Germany.

Follicle aspiration

The samples of follicle fluid from the preovulatory follicles were obtained using transvaginal ultrasound-guided follicle aspiration. Prior to follicle aspiration, from prostaglandin administration to follicle aspiration, follicle development was monitored using transrectal ultrasound examination with a 10^{-5} MHz linear ultrasound probe (L52, MicroMaxx®, SonoSite Inc., USA). Follicles were considered as pre-ovulatory and chosen for aspiration if they showed a minimum average growth in their diameter of one millimetre daily, a minimum diameter of 12 mm at aspiration and a clear visible vascularisation of the follicle wall in the Color-Doppler mode (Acosta 2007). If more than one follicle fulfilled these criteria, all possible preovulatory follicles were aspirated. For the aspiration, the animals were fixed and peridural anaesthesia with 6 ml of an 2% Procainhydrochlorid solution (Isocain ad. us. vet.®, Selectavet Dr. Otto Fischer GmbH, Germany) was induced. The perineal area was cleaned and a carrier of a 6.5 MHz sector finger-tip-probe (EUP-F331, Hitachi Medical Corporation, Japan) was placed in the cranial virgina. The ovary was moved via the rectum in front of the probe and was visualised using a Picker CS 9000 ultrasound system (EUP-405, Hitachi Medical Corporation, Japan). Under the visual control in the ultrasound picture, the pre-ovulatory follicle was aspirated through a canal in the probe carrier with a 55 cm, single lumen 17 G Ovum Pick-Up aspiration needle (V-OPAA-1755, Cook Australia Pty Ltd, Australia) and a direct appointed 5 ml syringe. With this short procedure the follicle fluids were aspirated completely and 1.5 to 2.5 ml were available for the further measurements.

Ultrasound examination of follicles and corpora lutea

All examinations were carried out using a linear ultrasound probe (L52 transducer, SonoSite Inc., USA) linked to a MicroMaxx ultrasound system (SonoSite Inc., USA). For the measurements the 2D-B-mode and a frequency of 7.5 MHz was used. Just before follicle aspiration, the diameters of the follicles were estimated by the distance measurement function (Caliper key) at two frozen images of the apparent maximal areas of each follicle and the calipers were positioned at the inner follicle wall. Measurements of the corpa lutea (diameter, perimeter, cross-section area of the luteal tissue) 14 days after aspiration were performed using the ellipse-function at two frozen images of the apparent maximal areas of each CL, according to Luttgenau et al. (2011a) and Rizos et al. (2012). The cross-section areas of solid CLs were considered as the cross-section area of the luteal tissue (31 cases). If the *corpus luteum* had a cave, the cross-section area of the cave was also measured and subtracted from the cross-section area of the whole *corpus luteum* to calculate the cross-section area of the luteal tissue (14 cases). If more than one CL developed after the aspiration of more than one follicle in one animal, the cross-section area of the corpora lutea were totalled to calculate the cross-section area of the luteal tissue for that animal (four cases).

Steroid analyses

Native follicle fluid of the aspirated pre-ovulatory follicles were stored on ice immediately after recovery, the cells were spun off and aliquots of the supernatant were stored (–80 °C) for analysis. Plasma, taken on day 14 after follicle aspiration, was prepared from 6mM K-EDTA blood samples from the coccygeal vessels using centrifugation (at 4 °C, 4000 × g, 10 min) and stored (–80 °C) for analysis. Progesterone concentrations in the plasma as well as progesterone and oestradiol concentrations in the follicle fluid were measured as described previously (Schneider et al. 2002). Briefly, steroid levels were estimated using a direct single purified rabbit antibody H^3RIA, after ethylether extraction. The sensitivities of the assays were 0.4 ng/ml for progesterone and 3 pg/ml for oestradiol. The intra- and inter-assay coefficients of variation for progesterone were 9% and 15% and for estradiol 7% and 10%.

Statistical analysis

Calculation of means and their standard errors, analyses of differences in mean values between groups, calculations of correlations and linear regressions as well as the generation of graphs was carried using the software package SigmaPlot 11.0. Differences between parameters with a normal distribution were tested by multiple pairwise comparison using the *t*-test. If the test of normality failed, a Mann-Whitney Rank Sum test was used. Differences of $P < 0.05$ were considered significant. Correlations between parameters of the follicle and the CL were calculated using a Spearman rank order correlation, in which only datasets of single follicle aspiration followed by a single CL development were considered. Relations between the cross-section area of the Cl and the plasma progesterone concentration were computed with the Spearman correlation and linear regression calculation function of SigmaPlot.

RESULTS

As a number of cows lacked ovarian activity in the post-partum period, the induction of heat and consecutive pre-ovulatory follicle aspiration could not be carried in all of the 20 cows at the three scheduled times (Table 1). With increasing time after parturition more animals could be included in the study. However, no impact of the time interval between parturition and follicle aspiration was seen on the occurrence of development of a CL after aspiration of a pre-ovulatory follicle. Also, significant differences were neither noted between

Table 1. Repeated ultrasound-guided aspirations of pre-ovulatory follicles during the post-partum period in a herd of 20 dairy cows: Characteristics of cows, aspirated pre-ovulatory follicles and subsequently developing corpora lutea (14 days after follicle aspiration) at three aspiration times (means ± SE)

		Follicle aspiration		
		1st	2nd	3rd
Animal	days post-partum (mean)	37 ± 1.9	70 ± 2.2	108 ± 1.8
	days post-partum (range)	26–48	57–84	96–121
	milk yield (kg/day)	27.1 ± 1.2	27.8 ± 1.2	26.0 ± 1.1
	number of realized aspirations (*n* of 20)	12	14	18
	development of CL after aspiration n (%)	10 (83)	13 (93)	14 (77)
Follicle	diameter of follicle (mm)	16.2 ± 0.96	15.7 ± 1.01	16.5 ± 0.97
	oestradiol concentration in FF (ng/ml)	226.3 ± 34.9	172.0 ± 40.1	213.8 ± 29.7
	progesterone concentration in FF (ng/ml)	489.0 ± 36.5	637.4 ± 121.5	549.7 ± 95.9
Corpus luteum	diameter of CL (mm)	22.0 ± 0.2	24.5 ± 0.2	23.0 ± 0.1
	perimeter of CL (mm)	61.4 ± 0.6	69.0 ± 0.5	63.0 ± 0.4
	cross-section area of luteal tissue (cm^2)	4.02 ± 0.66	3.76 ± 0.53	4.01 ± 0.54
	progesterone concentration in plasma (ng/ml)	1.84 ± 0.32	1.87 ± 0.21	1.78 ± 0.19

FF = follicle fluid, CL = *corpus luteum*

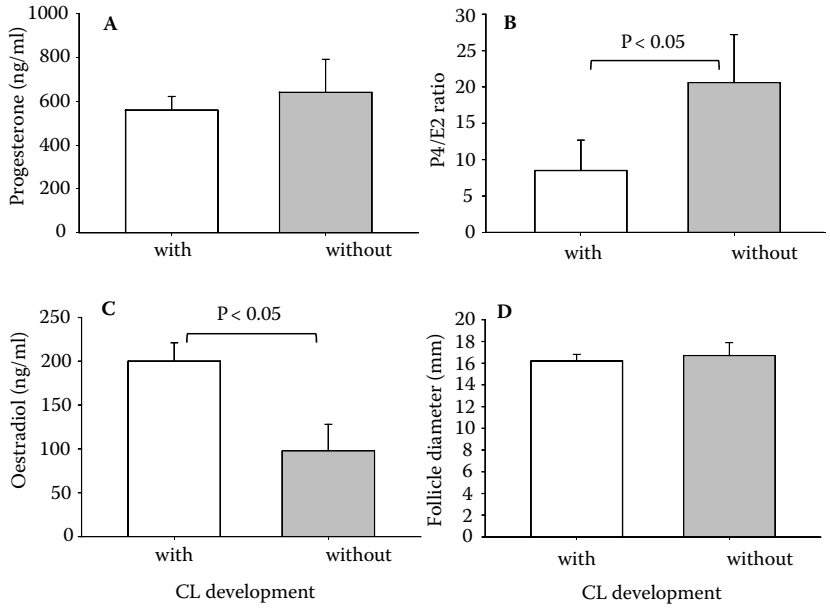

Figure 2. Oestradiol and progesterone concentrations in the follicle fluid of dominant follicles as well as follicle diameter from dairy cows, divided into groups of follicles, with (white bars) or without (grey bars) a subsequent development of a *corpus luteum* after aspiration of those follicles (means ± SE). A = progesterone concentration in follicular fluid, B = progesterone and oestradiol ratio in follicular fluid, C = oestradiol concentration in follicular fluid, D = follicle diameter

the three follicle aspiration times post-partum with respect to the size and steroid concentrations of the preovulatory follicles, nor between the size of the developed CLs or progesterone plasma levels on day 14 (Table 1). Pre-ovulatory follicles, with or without subsequent development of a CL after aspiration, showed significantly different patterns of steroid expression (Figure 2). The development of a CL is particularly connected to high oestradiol concentrations in a preovulatory follicle, while the progesterone levels in the FF or follicle diameter were not predictive of a follicle, which will later give rise to a CL. Taking into account all investigated CLs it became evident that the cross-section area of the luteal tissue is a valid marker for predicting progesterone concentrations in plasma (Figure 3), and also that the perimeter of a single CL in a cow had a positive correlation to the progesterone concentrations in plasma ($R = 0.58$, $P < 0.01$). Our results showed clearly that the properties of a pre-ovulatory follicle are significantly related to the qual-

Table 2. Summary of correlations between diameter and pattern of steroid levels of those pre-ovulatory follicles of dairy cows, which developed into a *corpus luteum*, and the morphology and secretory activity of their corresponding *corpus luteum* on day 14 after follicle

Follicle	*Corpus luteum*		
	cross-section area of luteal tissue	Perimeter	Progesterone in plasma
Diameter	$R = 0.60$ $P =$ **0.005**	$R = 0.53$ $P =$ **0.017**	$R = 0.47$ $P =$ **0.035**
E_2 in FF	$R = 0.54$ $P =$ **0.013**	$R = 0.38$ $P = 0.099$	$R = 0.30$ $P = 0.191$
P_4 in FF	$R = 0.32$ $P = 0.169$	$R = 0.40$ $P = 0.079$	$R = 0.45$ $P =$ **0.044**
P_4/E_2 quotient in FF	$R = -0.13$ $P = 0.581$	$R = -0.02$ $P = 0.927$	$R = 0.10$ $P = 0.658$

E_2 = oestradiol, P_4 = progesterone, FF = follicle fluid, R = correlation coefficient, P = P-value)

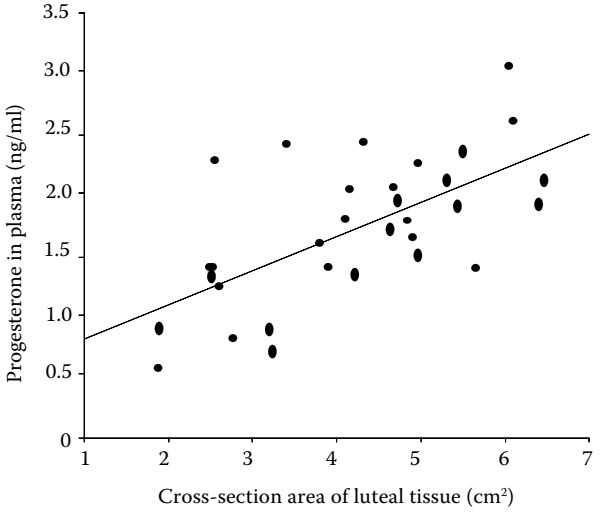

Figure 3. Progesterone concentrations in blood plasma of dairy cows with respect to the corresponding summarised cross-section area of luteal tissue in the ovaries, which developed until 14 days after aspiration of a preovulatory follicle (linear regression: progesterone in plasma (ng/ml) = 0.284 + (0.284 × cross-section area of luteal tissue (cm^2), $R = 0.65$, $P < 0.001$)

ity of the corresponding CL, which develops from that follicle (Table. 2). In partiuclar, the diameter of the aspirated follicle was significantly correlated to the cross-section area of the luteal tissue and perimeter of the consecutive developed CL as well as to the progesterone levels in the plasma 14 days after follicle aspiration. Oestradiol and progesterone concentrations in the follicle fluid can also be used to predict the cross-section area of the luteal tissue of the later CL or the later progesterone concentrations in plasma (Table 2). Interestingly, the oestradiol concentration in the follicle fluid was also strongly related to the diameter of the follicle ($R = 0.71$, $P < 0.001$).

DISCUSSION

For aspiration of the pre-ovulatory follicles three time points were chosen in order to allow investigation of different time periods post-partum, which are marked by different metabolic characteristics (Weber et al. 2013). First, a time period of deep negative energy balance, in which breeding of the animals is not reccomended was chosen for follicle aspiration (1st follicle aspiration, 37 ± 1.9 days post-partum). Secondly, a time period in which most of the high-yielding cows are in balanced energy metabolism and beginning of breeding is suggested was selected (2nd follicle aspiration, 70 ± 2.2 days post-partum), and thirdly, a time period in which dairy cows return to a positive energy balance and breeding success is known to reach its maximum was investigated (3rd follicle aspiration, 108 ± 1.8 days post-partum) (Inchaisri et al. 2011a,b; Weber et al. 2013). As expected, our results showed that a lack of ovarian function is a problem for fertility in dairy herds during the post-partum period within the time period of negative energy balance (Lopez-Gatius et al. 2002; Wiltbank et al. 2006; Walsh et al. 2011). However, if heat occurred, the quality of the pre-ovulatory follicle or CL quality was independent of the time point investigated. This supports the data of practitioners, who report about adequate breeding success in the early post-partum period, if they breed only cows which show a good heat in that usually non-recommended time (Beetz 2012). But these results are in contrast to other studies, which identified low follicle (Lopez et al. 2005; Mussard et al. 2007; Leroy et al. 2008) or CL quality (Lucy 2001; Wathes et al. 2003; Morris and Diskin, 2008; Clemente et al. 2009) in the early post-partum period as a reason for increased infertility in dairy herds. It is possible that infectious courses are more important than follicle or CL quality in determining the decrease in fertility in cyclic cows during the post-partum period (Azawi 2008).

Concentrations of progesterone and oestradiol as well as their ratio in the FF as well as follicle size are commonly used to define the developmental stage of a follicle (Nishimoto et al. 2009). We showed in the current study for the first time that assessing the rate of CL development after the taking of follicle fluid samples from a pre-ovulatory follicle, allows discrimination between follicles with different qualities, which might be taken as the same group if they were judged commonly on the basis of oestradiol and progesterone concentrations (Lohrke et al. 2005, Nishimoto et al. 2009) or morphology (Acosta 2007). Both groups of pre-ovulatory follicles (with or without subsequent development of a *corpus luteum*) showed expected, or even higher, progesterone concentrations in their FF (Lohrke et al. 2005; Nimz et al. 2009). However, our results indicate that the oestradiol concentration is more important for predicting the further physiological development of a pre-ovulatory follicle into luteal tissue and this might fit with recent *in vivo* findings regarding plasma oestradiol and pregnancy rates (Jinks et al. 2013). However, the follicle size alone is not sufficient for predicting the further development of a follicle. Rather, both follicle size and oestradiol concentration in the FF (which were positively correlated), seemed to be very important for the further development of a pre-ovulatory follicle into a healthy, actively secreting CL. From a clinical point of view, therefore, hormonal treatment protocols for time insemination should give the developing dominant follicle the maximum possible time to grow prior to the hormonal induction of ovulation, as suggested by Mussard et al. (2007).

One important factor for embryonic health and the establishment of pregnancy in cows is a suitable progesterone level in the plasma during the dioestrus, delivered by a proper CL. However, the importance of progesterone levels is a matter of controversy. Recent studies have shown that progesterone levels did (Forro et al. 2012), or did not (Larson et al. 2009) have a positive effect on pregnancy rates in cows. The current understanding is that an early rise in progesterone alters endometrial secretions that stimulate embryo development and

lead to a large conceptus that is better able to signal maternal recognition of pregnancy (Clemente et al. 2009; Lonergan 2011). In accordance with others this study showed that the cross-section area of the luteal tissue is a good parameter for estimating the progesterone concentration in the plasma of a cow in dioestrus (Luttgenau et al. 2011a; Rizos et al. 2012). It should be taken into account that for breeding it is necessary to have an earlier marker, which can predict which animal will have a well-developed CL, producing a high level of progesterone in the plasma during the time of embryo implantation. In particular, before transfer of a valuable embryo to a recipient animal on day 8 of its cycle, it must be decided which animal could provide the best conditions for the later establishment of a pregnancy. In the current study it became apparent that the diameter of a pre-ovulatory follicle, which can be measured easily by ultrasound, is a suitable parameter for predicting the morphology of a subsequently developing CL and the plasma progesterone concentration for the following dioestrus, as long as ovulation occurs. These results are in accordance with Jinks et al. (2013) Rizos et al. (2012) and Luttgenau et al. (2011b), but run counter to those of Luttgenau et al. (2011a). Relationships between an increased size of preovulatory follicles, increased CL and increased pregnancy rates after artificial insemination have been clearly shown (Mussard et al. 2007; Perry et al. 2010; Sa Filho et al. 2010). However, whether the maintenance of a pregnancy after an embryo transfer increases with an increasing pre-ovulatory follicle size of the recipient is still under discussion (Atkins et al. 2010; Jinks et al. 2013). Because CL morphology and activity could be used to find good recipients for an embryo transfer (Spell et al. 2001; Chagas e Silva et al. 2002; Chebel et al. 2008; Yoshida et al. 2012), we support the hypothesis that follicle size could be a suitable prospective marker for identifying suitable recipients very early.

In conclusion, as follicle size and concentrations of progesterone and oestradiol in the follicular fluid showed significant correlations with the later development of a *corpus luteum* as well as with the corpus luteum morphology and the progesterone concentrations in the plasma regardless of the time post-partum, ultrasound-measured parameters can be used retrospectively to determine follicle quality or prospectively to assess *corpus luteum* quality in dairy cows.

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