

—Technology Report—

Anti-Muellerian hormone levels in plasma of Holstein-Friesian heifers as a predictive parameter for ovum pick-up and embryo production outcomes

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Abstract. The aim of this study was to investigate whether plasma anti-Muellerian hormone (AMH) levels of Holstein-Friesian heifers could be used to predict ovum pick-up (OPU) and embryo production outcomes. Plasma samples and data were collected from 64 heifers, which underwent repeated OPU with subsequent *in vitro* embryo production followed by embryo flushing after superovulation. AMH levels were significantly positively correlated with the number of follicles aspirated per OPU session ($r = 0.45$), recovered oocytes per OPU ($r = 0.43$) and *in vitro* produced embryos per OPU ($r = 0.28$). No significant correlations between AMH and *in vivo* produced embryos were ascertained. Our results suggest that correlations between AMH and outcomes of an OPU-IVF program are too low to use AMH as a precise predictive parameter for the success of a particular OPU procedure in Holstein-Friesian heifers. However, AMH can help to identify groups of very good or very poor oocyte donors.

Key words: Anti-Muellerian hormone (AMH), Cattle, IVF, Ovum pick-up (OPU), Superovulation

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Anti-Muellerian hormone (AMH) is a dimeric glycoprotein of the inhibitory TGF-beta superfamily that is expressed only in the gonads. Secretion of AMH from Sertoli cells is essential for embryonic male sex differentiation. In females, it is produced only from granulosa cells. Mainly granulosa cells from growing secondary follicles produce AMH during follicle recruitment. It acts as an inhibiting growth factor on the pool of resting follicles [1, 2]. As shown in mice, humans and cattle, the concentration of AMH in plasma reflects the total number of oocytes in the ovary and the number of follicles, which are involved in preantral and antral follicular activity [3–5]. Therefore, AMH is used as an endocrine marker for this pool of small gonadotropin-responsive follicles, which are also termed the ovarian follicular reserve [6]. In human medicine, AMH has developed into a factor with a wide array of clinical applications, mainly based on its ability to represent the number of antral and preantral follicles. Since recognition of the linear relationship of AMH, the antral follicle count and the oocyte yield in women [7, 8], plasma AMH levels have become an important predictive parameter for the success of assisted reproductive technologies in humans, which include oocyte recovery and *in-vitro* embryo production [6]. Plasma AMH level is also used for the diagnosis of hyperstimulation

syndrome, menopause, granulosa cell tumor and the polycystic ovary syndrome (PCOS) in women [5]. However, in veterinary reproductive medicine, this parameter is not used very often. During the last few years, strong linear regressions between plasma AMH levels and the number of antral follicles have also been described in different breeds of cattle [9, 10]. From puberty on, the AMH levels of individual heifers stay at a constant level [9, 11]. Through the estrus cycle, the plasma AMH levels show only slight dynamics with a decrease one week after ovulation [12]. But that decrease of the AMH level is not big enough, that animals with high AMH levels will reach the plasma AMH levels of animals with low plasma AMH levels [11]. Therefore, AMH can also be considered as an independent marker of the ovarian follicle reserve in cattle. Recently, it was shown that AMH can be used as a predictive marker for *in vivo* embryo production in cattle [13, 14]. However, despite a notable increase in commercial *in vitro* embryo production of cattle worldwide [15], reports about the usefulness of the parameter AMH for *in vitro* embryo production after ovum pick-up (OPU) in cattle are very rare to date. One recent publication reports about high correlations between plasma AMH levels and outcomes of an OPU-IVF program in heifers, when blood samples were taken immediately before the OPU session [16]. However, to prove if AMH can be used as a prognostic diagnostic tool for outcomes of an OPU-IVF program like in human assisted reproductive medicine, this study aimed to find out whether the AMH levels of Holstein-Friesian heifers, determined only in one plasma sample, obtained prior to the use of reproductive biotechnologies, could be a predictive marker for further *in vitro* and *in vivo* embryo production under field conditions.

Blood samples and data were collected from 64 young Holstein-Friesian heifers, which underwent repeated OPU with subsequent

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in vitro embryo production and repeated embryo flushing after superovulation on a commercial embryo transfer station of a breeding association. Overall, it was estimated that there was an average of 0.368 ± 0.028 ng/ml AMH in the plasma of the 64 studied heifers (range 0.091–1.391 ng/ml). Furthermore, 9.1 ± 0.5 follicles were aspirated in 130 investigated OPU sessions for 54 heifers (range 1–35), and 7.2 ± 0.3 oocytes were recovered per OPU session in 202 studied OPU sessions for 64 heifers with known AMH levels (range 0–20; recovery rate $78.4 \pm 2.1\%$). After IVF, 1.3 ± 0.1 blastocysts were produced per OPU session from the 64 heifers and their 202 OPU sessions (blastocyst rate $17.6 \pm 1.4\%$). After subsequent superovulation of 52 of the investigated heifers, an average of 4.5 ± 0.6 embryos were flushed in 90 flushes, of which 3.3 ± 0.4 were transferable. AMH levels were positively correlated with the average number of follicles, which were aspirated in repeated OPU sessions of those animals ($r = 0.45$, $P < 0.001$), and linear regression between these parameters could be shown (Fig. 1). The numbers of aspirated follicles and recovered oocytes per OPU session were highly correlated ($r = 0.88$, $P < 0.001$, Fig. 2). As shown in Fig. 3, a correlation between AMH and the mean number of recovered oocytes per OPU session was apparent ($r = 0.43$, $P < 0.001$). While correlations between *in vitro* produced embryos per OPU session and aspirated follicles or recovered oocytes per OPU session reached correlation coefficients as high as 0.54 and 0.46 ($P < 0.001$), respectively, the correlation between plasma AMH levels and the mean number of *in vitro* produced embryos per OPU session was low ($r = 0.28$, $P = 0.02$). However, no significant correlations between AMH and *in vivo* produced embryos were determined. Dividing the heifers into groups (quartiles) according to their AMH plasma levels, it became apparent that the animals in the lower quartile (heifers with AMH levels of 0.236 ng/ml or lower) had significantly fewer follicles in OPU and a significantly lower number of oocytes was recovered compared with animals of the upper quartile (heifers with AMH levels of 0.410 ng/ml or higher; Table 1). However, the number of *in vitro* produced embryos per OPU session and the number of total or transferable embryos flushed after subsequent superovulation increased only slightly in the heifers of the lower quartile compared with to heifers of the upper quartile.

The measured plasma AMH levels are comparable to previously described levels of Holstein-Friesian heifers [9, 10] and slightly higher than described for dairy cattle [5, 9, 14]. High AMH levels in the plasma of Holstein-Friesian heifers of about 1 ng/ml or more, like we found in a few animals, have been discussed as a sign of granulosa-theca cell tumor [17]. However, we did not notice any clinically apparent pathology in the ovaries of the investigated heifers, and other investigators have also reported about AMH levels over 1 ng/ml in healthy heifers [10, 16]. The estimated number of follicles per OPU session in our study was higher than those of others, who described about 6 to 7 follicles aspirated in postpuberal, non-stimulated Holstein-Friesian heifers [18, 19]. Furthermore, in those studies, the numbers of recovered oocytes were about 3 to 7 oocytes per OPU session, and recovery rates of 40 to 60% were realized; thus, they were both lower than our results. Our OPU results are comparable to those of other studies that also used non-stimulated Holstein-Friesian heifers, even if they used different OPU schedules with time intervals from a few days up to two weeks between OPU sessions

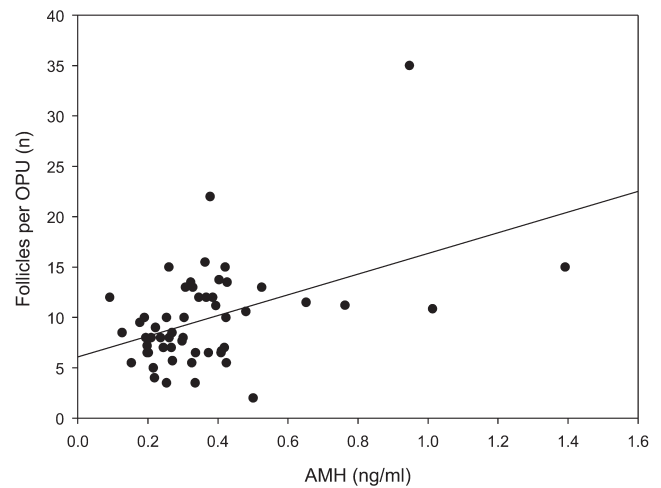


Fig. 1. Relationship between the mean number of aspirated follicles in repeated OPU sessions and anti-Müllerian hormone (AMH) plasma levels of Holstein-Friesian heifers ($n = 54$, $r = 0.45$, $f(x) = 6.08 + (10.28 * AMH \text{ ng/ml})$).

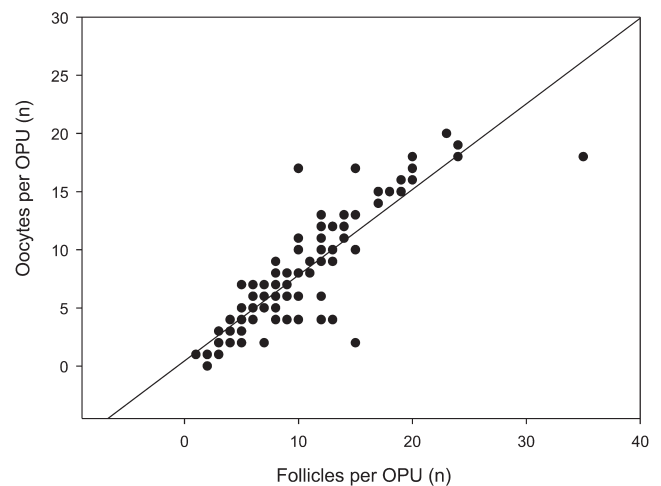


Fig. 2. Relationship between recovered oocytes and aspirated follicles per OPU session of Holstein-Friesian heifers (results of 130 OPU sessions of 54 heifers, every dot represents the result of one OPU session (dots overlapping partly), $r = 0.88$, $f(x) = 0.45 + (0.74 * n \text{ follicles})$).

[18–21], as further studies demonstrated that the interval between OPU sessions has no impact on the number of follicles or oocyte yielded in non-stimulated heifers [20, 21]. We found correlations between AMH and the number of aspirated follicles that were much lower, as described previously for the correlations between AMH and the antral follicle count (follicles larger than three millimeters, measured by ultrasound examinations) in Friesian heifers [9, 10], where correlation coefficients of about 0.70 to 0.80 were estimated. Moreover, recent studies have reported a correlation coefficient of 0.61 for the correlation between the plasma AMH levels and the

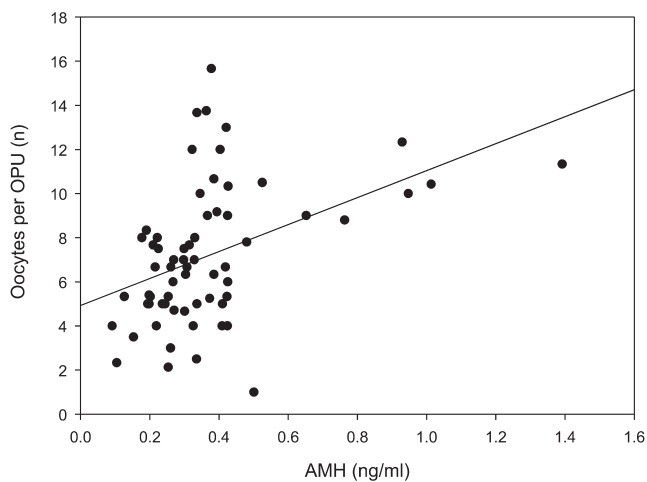


Fig. 3. Relationship between mean recovered oocytes per ovum pick-up session and anti-Muellerian hormone (AMH) plasma levels of Holstein-Friesian heifers (results of 202 OPU sessions of 64 heifers, $r = 0.43$, $F(x) = 4.93 + (6.11 \cdot \text{AMH ng/ml})$).

number of aspirated follicles during OPU in Holstein heifers when blood samples were collected immediately before the OPU session started [16]. It has to be acknowledged that the numbers of aspirated follicles per OPU session in our and other studies [18–21] were only about half of the antral follicle counts of the Holstein-Friesian heifers in other studies [9, 10, 16], which compared the number of antral follicles to markers of the follicular reserve like AMH. Since it is known that the correlation between AMH and small antral follicles is stronger than between AMH and large antral follicles in cows [9, 11], the differences between our study and the other studies in terms of the total number of counted follicles and the correlations between AMH and follicle number might be partly explained by the preference to aspirate middle and large follicles during OPU. Without doubt, there was an effect of the different practitioners who performed the OPU on the total number of aspirated follicles or the number of recovered oocytes in our study, too. As we could not see a significant difference in the correlations between AMH and the investigated parameters between the OPU practitioners, we pooled the data to get a general result for field conditions. However, the

correlations we found between AMH and the number of aspirated follicles during OPU are comparable to analogous studies in humans and horses [22, 23] but lower than in reports from sheep or bos indicus [16, 24]. Since a strong correlation between the numbers of follicles and recovered oocytes was evident, linear regressions between AMH and the number of follicles or the number of recovered oocytes, respectively, were similar, and therefore, they verify each other indirectly. The correlations between AMH and recovered oocytes are comparable to the results from studies in woman [7, 8]. However, the results in women revealed a high variability, and correlation coefficients between 0.3 and 0.7 were reported. Since the OPU results of an individual are variable and we wished to minimize the effects of the estrus cycle on the OPU results [25], we analyzed at minimum the outcome of three oocyte recoveries and IVF procedures. High numbers of blastocysts per OPU were realized due to the high number of recovered oocytes but not due to high blastocyst rates in our study. However, the correlations between AMH and oocyte yield as well as the number of *in vitro* produced embryos, as very important parameters for *in vitro* embryo production success, remained at a low level. Studies in women have also reported low correlations ($r = 0.24$) between AMH in plasma and *in vitro* produced embryos [22]. In a recent study in Holstein-Friesian heifers, the correlation coefficients between plasma AMH and the oocyte yield or number of produced blastocysts per OPU session were estimated to be about 0.70 and 0.36, respectively [16]. In that study the researchers took the blood sample immediately before the OPU session, but for the use of AMH as a prospective diagnostic parameter for the outcome of assistant reproductive biotechnologies, it is necessary to take the sample a few weeks before the use of reproductive biotechnologies, as is done in human medicine. Maybe the lower correlations in our study are due to the time span between blood sampling and the onset of OPU-IVF or superovulation, but on the other hand, it has been stated that from puberty onward, AMH levels remain at a constant level and do not show substantial fluctuation throughout the estrous cycle [11, 17]. This shows clearly the difficulties in predicting the *in vitro* embryo production outcomes for a particular OPU-IVF cycle or a few OPU-IVF cycles for an individual with only one parameter like AMH. The same can be stated for *in vivo* embryo production, where we were unable to replicate high correlations (with correlation coefficients of about 0.5) between plasma AMH levels and the results of embryo flushing after superovulation in

Table 1. Average results of repeated ovum pick-up followed by *in vitro* embryo production and embryo flushing after superovulation of Holstein-Friesian heifers ($n = 64$), divided into groups (quartiles) according to their anti-Muellerian hormone (AMH) plasma levels

		Lower quartile AMH $\leq 25\%$	Middle quartiles 25% > AMH < 75%	Upper quartile AMH $\geq 75\%$
AMH	(ng/ml)	0.185 \pm 0.011 ^a	0.321 \pm 0.001 ^c	0.634 \pm 0.073 ^b
Follicles per OPU	(n)	7.7 \pm 0.6 ^a	9.9 \pm 0.8	11.9 \pm 2.0 ^b
Oocytes per OPU	(n)	5.7 \pm 0.5 ^a	7.4 \pm 0.6	8.2 \pm 0.8 ^b
IVF blastocysts per OPU	(n)	1.1 \pm 0.3	1.3 \pm 0.2	1.4 \pm 0.2
Flushed embryos	(n)	3.9 \pm 1.1	4.3 \pm 0.8	5.4 \pm 1.3
Transferable embryos	(n)	3.3 \pm 1.1	3.0 \pm 0.5	3.7 \pm 0.8

Values are presented as means \pm SE. Values with different superscripts in rows differ significantly ($P < 0.05$).

cattle [13, 14]. In contrast to our study, others have correlated the AMH levels to average results of *in vivo* embryo production, which were recorded over several embryo production campaigns over the course of four years for each cow. Additionally, it should be noted that our results of *in vivo* embryo production in the investigated young heifers were lower, as described for cows or Holstein-Friesian heifers [13, 14, 26], and may not be indicative of their full potential for *in vivo* embryo production. As shown in our study, it is difficult to predict the success of a single or a few embryo flushings based on the AMH level. However, it was possible to divide the heifers into groups of very good or very poor oocyte donors according to their plasma AMH levels (Table 1); but due to high variability of oocyte yields between animals with low AMH levels (Fig. 3), we would not recommend a boundary value for AMH under which a group of animals would not be suitable for oocyte recovery, although boundary values have been recommended for superovulation and embryo transfer programs in cattle [13, 14].

In conclusion, our results support the assumption that the correlations between AMH and outcomes of an OPU-IVF program or a few embryo flushings after superovulation are too low to use AMH as a precise predictive parameter for the success of a particular OPU-IVF or superovulation procedure in Holstein-Friesian heifers. However, plasma AMH levels can help to identify groups of very good or very poor oocyte and embryo donors.

Methods

Animals

Blood samples and data from *in vitro* and *in vivo* embryo production were collected from 64 young Holstein-Friesian heifers (9–14 months old at the beginning), which were housed in a commercial embryo transfer station of a breeding association (Masterind, Verden, Germany). All heifers were included in a breeding program, which is based on repeated *in vitro* and subsequent *in vivo* embryo production and transfer. Heifers were held in stable cubical and fed *ad libitum* a mixed ration based on grass and corn silage, supplemented with minerals and vitamins. Estrus was monitored by automatic heat detection (SCR Heitime® HR Sytem SCR Europe, Podenzano, Italy) and/or proved visually. All procedures involving animal handling and blood sampling were approved by the State Office of Agriculture, Food Safety and Fisheries Mecklenburg-Vorpommern, Germany.

Blood sampling and anti-Muellerian hormone determination

To avoid possible impacts of the ovarian cycle on AMH plasma levels and to be sure that the heifers had passed puberty, samples were only taken at a date when the animals were in heat or one day thereafter. The heifers were 11.7 ± 0.2 months old at blood sampling. Blood samples were taken 25 ± 3 days apart from the next OPU session of the repeated OPU-IVF procedure and a mean of 94 ± 9 days before the subsequent repeated embryo flushing after superovulation. Blood was recovered by puncture of the coccygeal vessels and collected in blood collection tubes for plasma preparation containing 1.6 mg EDTA-K/ml blood (S-Monovette® EDTA 9 ml, Sarstedt, Nuembrecht, Germany). Samples were centrifuged directly after collection, and 2 ml of plasma was stored for a maximum of three months at -20°C until analysis. To estimate AMH concentrations,

we used an ELISA kit (DSL-10-144400, Beckman Coulter, Brea, CA, USA) for human AMH, which was previously validated for bovine blood samples [5]. Samples were analyzed all together with ELISA kits in one batch with duplicate measurements according to the manufacturer's instructions. As modifications to the manufacturer protocol, we diluted 30 μl of the plasma in 150 μl of the assay buffer and added 180 μl of the assay buffer in a second step to the solution. For the analysis, we used 120 μl of the final dilution in the ELISA. According to the manufacturer, the sensitivity of the assay is 6 pg/ml . The intra- and interassay coefficients of variation were 1.5% and 7.6%, respectively.

In vitro embryo production

In vitro embryo production was performed on all of the investigated 64 heifers after puberty, which was ascertained by detection of heat. Oocytes were obtained by repeated transvaginal ultrasound guided ovum pick-up (OPU) on a biweekly schedule, independent of cycle stage. Prior to OPU, heifers were fixed in position and prepared by a peridural anesthesia with 4 ml of a 2% procaine solution (Procasel®, Selectavet, Weyarn-Holzolling, Germany). Ovaries were visualized by a 6.5 MHz fingertip ultrasound probe (EUP-F-331) and a Hitachi ultrasound machine (EUB 405, Hitachi Medical Systems, Norderstedt, Germany). All follicles of or larger than 3 mm were aspirated via a 550 mm 17G single lumen puncture needle (14851, Labotect, Goettingen, Germany) with a vacuum of 65 mmHg provided by an aspiration pump (V-MAR 5100, Cook Veterinary Products, Spencer, IN, USA). Aspirates were collected in 50-ml tubes, containing 20 ml warmed Phosphate Buffered Saline (PBS) solution with Ca^{2+} and Mg^{2+} (L1815, Biochrom, Berlin, Germany) supplemented with 0.3% BSA (A9674, Sigma-Aldrich, Munich, Germany) and 2.5 IU/ml heparin (Heparin-Natrium-Ratiopharm®, Ratiopharm, Ulm, Germany) and stored in a 37 C water bath while the OPU session was ongoing. For this study, in the OPU sessions of 54 heifers, all follicles that have seen to be aspirated, were counted directly during the OPU session. Immediately after the OPU procedure was completed, the aspirated fluid was filtered through a 50- μm filter (Analysensieb ISO 33101, Retsch, Haan, Germany). The collected cells were flushed from the filter into a Petri dish using the PBS solution with Ca^{2+} and Mg^{2+} supplemented with 0.3% BSA and 2.5 IU/ml heparin. Using a stereomicroscope at 15–63 \times magnification, the cumulus-oocyte complexes (COCs) were identified. All recovered oocytes were used for *in vitro* embryo production despite their quality. For *in vitro* maturation, COCs were transferred to modified TCM 199 media (N2520, Sigma-Aldrich, Munich, Germany) supplemented with 50 $\mu\text{g/ml}$ gentamicin, 22 $\mu\text{g/ml}$ sodium pyruvate, 2.2 mg/ml carbonate, 0.5 $\mu\text{g/ml}$ FSH, 0.03 IU/ml HCG and 1 $\mu\text{g/ml}$ estradiol and cultured for 22 to 24 h in an atmosphere of 5% CO_2 in humid air at 38.9 C. All oocytes were then transferred into a 100–120 μl drop *in vitro* fertilization media together with 1×10^6 spermatozoa/ml, prepared by the swim-up method, for 18–21 h. The used spermatozoa were from frozen-thawed semen from several very young Holstein-Friesian breeding bulls with unknown fertility that had been chosen by analysts and a computerized breeding program of the breeding association according to their genomic breeding values. Fertilized oocytes were set in SOF-stock media (19990/0040, Minitube International, Tiefenbach, Germany) supplemented with BSA, amino acids and

bovine fetal serum as suggested by the culture medium manufacturer and cultured in an atmosphere of 5% CO₂ in humid air at 38.9 C for seven days before counting developed blastocysts.

In vivo embryo production

Superovulation was induced by intramuscular injections of a solution containing 50 IU/ml follitropin (FSHp) and 50 IU/ml lutropin (LHp) (PLUSET[®], Laboratorios Calier, Barcelona, Spain). Treatment started at mid diestrus and in total, 8 ml of solution, split into seven parts, was injected over 3.5 days in a descending doses, starting with 1.5 ml on the evening of the first day and ending with 0.5 ml on the fourth day. A luteolytic dose of cloprostenol (2.0 ml, Estrumate 250 µg/ml[®], Intervet Deutschland, Unterschleissheim, Germany) was given together with the last injection of FSHp/LHp. Artificial insemination was performed 36 h and 48 h after the last hormone injection with a dose of 20 million spermatozoa from frozen-thawed semen from several Holstein-Friesian breeding bulls with proven fertility, that had been chosen by analysts and a computerized breeding program of the breeding association. Seven days after the first artificial insemination, embryos were flushed with a transcervical catheter (Embryo-Transfer-Kathether “Neustadt/Aisch” Ch. 15, RMP Med Produkte, Ansbach, Germany) by flushing repeatedly every uterine horn separately with warmed PBS solution with Ca²⁺ and Mg²⁺ (L1815, Biochrom, Berlin, Germany). Directly after flushing, embryos were picked up and classified according to the IETS (International Embryo Transfer Society) standard under a stereomicroscope.

Data presentation and statistical analysis

As the base data for the analysis in this study, the number of aspirated follicles per OPU was counted in 130 OPU sessions of 54 heifers, and oocyte yields from 202 OPU sessions of 64 heifers with known AMH levels were available; data from a minimum of three OPU sessions per animal were used, and data for 202 IVF cycles after OPU were available and compared to the AMH levels of 64 heifers. *In vivo* embryo production was performed with 52 heifers, and data from 90 embryo flushings after superovulation were available; 17 heifers were flushed only once, 32 heifers were flushed twice, and three heifers were flushed three times. Values are presented as means ± SE. Differences in mean values between groups, correlations and linear regression, as well as the presentations of the graphs were computed with the software package SigmaPlot 11.0. As the Shapiro-Wilk test of normality was failed, differences in mean values between groups (Table 1) were determined by the Mann-Whitney Rank Sum test. Differences of P < 0.05 were considered significant. Correlations between the parameters of OPU, IVF or *in vitro* embryo production and AMH were calculated by a Spearman Rank Order correlation. For the linear regressions, shown in the graphs, functions are given in the legend, as they were calculated by linear regression function in SigmaPlot.

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