Acclimatization to heat stressed environment and embryo production of donor cows transported from Hungary to semiarid region of Brazil.

Thesis of the PhD dissertation

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2004

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1 Introduction

My work was done in the semiarid region of Brazil where I worked as an expert in a multiply ovulation and embryo transfer (MOET) program. The project was done as part of a large-scale genetic program, a co-operation between AGROINVEST (Hungary) and the firm of the Brazilian govern, Compania de Desenvolvimento de Vale do Rio São Francisco (CODEVASF) between February of 1996 and July of 2000. The genetic work was based on the herd of high-pregnant Holstein-Friesian heifers transported from Hungary to Brazil. They were carried in 11 hours cargo jet flight and more 12 hours truck transportation to the final destination, the MOET center close to the city Petrolina, state Pernambuco. The transportation stress caused only one loss of pregnancy.

The climate in Petrolina was semiarid. To alleviate the heat stress, the stable was opened and covered with tailed roof. The heifers were accommodated is small groups, 10 animals/box allowing 15 m^2 for a cow. In the hottest hours in the afternoon, the animals were sprinkled with water. The floor was asphalt and covered by bagasse of sugar cane which was changed daily. The production of food was close to the farm in irrigated plough cultivating alfalfa hay, elephant grass and corn plant. The corn was planted in 18 days periods to provide the fresh plant for chopped green mass. The forage was PURINA. The milking system was full automatic ALFA-LAVAL. There was no food supply in the milking parlour.

At the project site in Brazil, there is little variation in the ambient temperature between the different seasons. Average daily temperature in 1996 and 1997 at the location of the experiment was 25.8 ± 1.4 °C, ranging from 34.4 ± 1.6 to 23.2 ± 1.3 °C. In the hottest months (from September to

January), the average temperature was 27.3 ± 1.1 °C and ranged from 35.6 ± 1.1 °C (THI = 80) to 22.5 ± 0.9 °C. In the cooler months (from February to August), the average temperature was 24.8 ± 1.4 °C and ranged between 31.5 ± 1.6 °C (THI = 75) and 20.0 ± 1.4 °C. These data do not take account of some extreme periods when the daily temperature exceeded 40 °C and did not drop below 33 °C at night. Annual precipitation was 650 mm per year (with extremes 100–900 mm) with humidities of 35–38% in the afternoon, 70–85% during the night and early morning. Daylight is relatively constant (12.0 ± 0.5 h per day) at 9° south of the equator. The environmental conditions during the experiment were consistent through the experiment, with little fluctuation in either temperature or humidity between superovulations.

The dissertation is based on four major topics:

- investigation of acclimatization problems,
- intensification of the embryo production,
- calculation of repeatability and heritability of ovulation numbers and embryo production of donor cows,
- endocrinological investigations.

2 Investigation of acclimatization problems

I found that after the calving, the animals were suffered from reproduction problems, which were revealed in cycle disorders, infertility, and low embryo production results.

In this study, imported animals were superovulated in 1996 (n = 63) and 1997 (n = 96), compared to 38 and 45 cows in the control herd. The variates recorded were: the interval post-partum to first oestrus; changes in ovarian size and activity; responses to superovulation; and, embryo quality.

The average daily milk yields of the imported cows were 20.0 and 23.3 L in 1996 and 1997, respectively compared to 22.1 L throughout the experiment for cows in the control herd.

The cycle disorders and infertility caused an extreme long post partum interval which was 112.1 ± 30.5 days long in 1996 in the imported cows compared to 55.0 ± 18.0, 48.2 ± 12.0 and 42.6 ± 10.7 days in 1997 for control cows.

The size and functionality of the ovaries was lowest for the imported animals in 1996 but did not differ between other group–year combinations.

Cows observed in oestrus were synchronized by intramuscular administration of 2 mL of prostaglandin (150 mg cloprostenol). Cows were superovulated by administration of FSHp twice daily over 4 days starting on Days 8–11 of the cycle (oestrus = Day 0). The FSH was administered as 150, 150; 75, 75; 50, 50 and 25, 25 IU per day. On the third day of FSH, 3 ml of prostaglandin (187.5 mg cloprostenol) was administered once in the afternoon and animals were inseminated with a single straw of semen 48 and 60 h later. Embryos were recovered non-surgically on Day 7 after oestrus. The standard superovulation treatment started 6 months after calving.

The imported animals had a lower superovulatory response in 1996 than control cows in terms of the number of ovulations (6.4 ± 4.3 versus 13.6 ± 5.9, $P < 0.05$) and good quality embryos (1.2 ± 0.9 versus 4.4 ± 2.1, $P < 0.05$). The two groups of cows did not differ in respect of these characters in the second year of the study.

These results show that during the first year of presence, donors did not respond well to the superovulation treatment. After that time, embryo production started to increase to reach an average number of freezable embryos per donor higher than 5. During that time animals were not ready to be included in the superovulation program, however, the herd returned to normal cyclic earlier.

In conclusion, I found that Holstein-Friesian donors were changed in embryo production results and ovarian recrudescence during the acclimatisation to the semiarid tropics and a period of approximately 1.5 years is required for full adaptation.

During my work, the El Niño, a meteorological phenomenon caused an extreme hot period. The effects of different Temperature Humidity Index values in cold, hot and El Niño climates on superovulation and embryo production were analysed on donor cows. There were significant differences in the THI among the three climates. The average temperature in the El Niño period was 6 °C higher than in the summer period of the previous 30 years. The number of corpora lutea and embryos were log- and back-transformed, Kolmogorov-Smirnoff test was used for normality and Lilliefors test was applied for significance. In the cold season Temperature Humidity Index was 70.74 ± 1.35 and the average number of CL was 9.84 ± 4.37. In the hot season the Temperature Humidity Index was 73.99 ± 0.72 and the average number of CL was 9.70 ± 4.49. When the Temperature Humidity Index, in the El Niño period, increased up to 79.74 ± 4.01, the superovulation response was

significantly ($P < 0.01$) reduced (average number of CL = 5.22 ± 2.53). The embryo production result showed a similar tendency. In the hot period the average number of embryos obtained was 5.87 ± 2.98. However, in the El Niño period it decreased to 4.21 ± 2.05. Higher temperature reduced embryo quality. The proportion of live embryos was 59.2 ± 37.4% in the cold and 38.2 ± 38.5% in the El Niño periods of the year ($P < 0.01$). However, ovarian sensitiveness showed adaptation to summer environment while the heat stress, which was more severe in the El Niño period, negatively affected the superovulation response and embryo production.

3 Studies for intensification of embryo production

My researches were executed to reach the maximum embryo production affectivity, using minimum number of donors, with reduced costs.

In the first phase, I superovulated all of available donors to allocate the herd for groups of different superovulation responsiveness and choose the most effective hormone preparation, which was available. In the second phase, I superovulated the herd and made three researches:

i. Cycling Holstein-Friesian heifers (n = 19; >12 months old and >370 kg body weight) and lactating Holstein-Friesian cows (n = 20) were stimulated starting on Days 8 to 11 of the cycle with pFSH. On Day 3 of treatment (Day 1 = day of first FSH treatment), PGF was administered to induce luteolysis and animals were inseminated with a single straw of semen 48 and 60 h later. Seven days later, ova/embryos were harvested by a nonsurgical collection technique and evaluated according to International Embryo Transfer Society criterias. Although the numbers of animals responding with more than one ovulation did not differ between heifers (16/19) and cows (14/20), the percentage of heifers yielding embryos (14/19, 74%) was higher than that in cows (11/20, 55%; $P < 0.05$). Based on CL counts, ova/embryo collection efficiency was 66% in heifers and 89% in cows ($P < 0.05$). There was no significant difference between heifers and cows in numbers of ovulations detected by rectal palpation or in the numbers of ova/embryos recovered. However, there were significantly more unfertilized ova and degenerate embryos collected from cows and more freezable embryos collected from heifers (P <

0.05). Under high environmental temperatures Holstein-Friesian heifers were superior in terms of embryo production when compared to lactating Holstein-Friesian cows. Although other factors were not ruled out, data are consistent with a hypothesis that high environmental temperatures are detrimental to ova/embryo quality in superovulated lactating dairy cattle. The effect may be due to an inability to maintain normal body temperature under heat stress conditions, possibly because of anabolic demands associated with lactation.

ii. The objective of another study was to determine the effect of different lactating stages to the embryo production of heat stressed cows. Animals were allotted by dry (n = 121), first (n = 20, daily milk production = 22.5 kg), second (n = 40, daily milk production = 28.3 kg) and third (n = 25, daily milk production = 20.2 kg) lactation stages. During the study, all donors were superovulated with FSHp (Pluset, Serono, Rome, Italy) according to standard protocol. The number of CL was significantly higher in the dry cows compared to the lactating donors. The number of good quality embryos also tended to be higher in the dry cows, but there were no significant differences by stage of lactation. However, the proportion of good quality embryos in cows being on the first and second stages of lactation was significantly lower than in those ones being in the third lactation phase and in the dry cows. Overall, data indicate that under high ambient temperature conditions the ovarian responsiveness and embryo production of Holstein-Friesian donor cows vary between dry and different lactation periods. The best results were achieved in dry donors suggesting that lactation affects the superovulation response of heat stressed cows.

iii. Two experiments were designed to compare the effects of dose of FSHp, Pluset, Serono, Italy and the frequency of embryo collection on superovulatory response and embryo production in lactating cows. Ovarian stimulations

were started between Days 9 and 11 of the estrous cycle (estrus = Day 0) with FSHp in a twice daily schedule over 4 days. In Experiment 1, a total of either 1000 IU or 600 IU was administered. In Experiment 2, embryo collections were repeated at 30-day or 50-day intervals using a total of 600 IU of Pluset. On Day 3 of stimulation (Day 1 = first day of FSH treatment), PGF was administered to induce luteolysis and all animals were inseminated 48 and 60 hours later. Ova/embryos were recovered non-surgically 7 days after the first insemination. Our results show that the superovulation response and embryo production of the donor cows is similar regardless the dose of the FSHp applied. However, significant difference was found in the occurrence of large anovulated follicles. Donor cows return to estrus at about 12.2 ± 3.2 days after embryo collection. Generally, a new superovulation treatment is commenced following one or two estrous cycles. In this study ovarian stimulations were initiated in the estrous cycle following the first estrous after embryo recovery and results indicate that the embryo production was similar to the programs started after the second estrus. Our data indicate that in the semiarid climate the donor cows can be successfully stimulated with reduced dose of Pluset and the interval between the embryo collections can be shortened. Using lower dose of FSHp occurrence of large anovulated follicles and the cost of ovarian stimulation can be reduced.

Based on my experiments, I continued superovulation programs adding heifers to donor herd; inseminating the non-responsive and bad embryo producing donors; drying the good and best embryo producing ones; reducing the amount of the used hormone quantity and reducing the days between the embryo collection and forthcoming superovulation treatment. With these measures, the embryo production became as intensive as possible. Unfortunately, the frequently superovulated programs have the next disadvantage.

In this study we investigated the effect of consecutive ovarian stimulations on the oestrus cycle of the donors. The cows were treated with FSHp (Pluset, Serono, Italy) starting on Day 8-12 of cycle. A total dose of 650 IU was administered by the above mentioned protocol. Cows with no embryo (Group 1; 21.8%) or poor embryo production (Group 2; 23.1%) were superovulated 2.1 ± 0.9 and 3.5 ± 1.6 (Mean±SD) times. The average number of stimulations of animals producing 3 to 5 embryos (Group 3; 28.2%) was 5.5 ± 3.0; cows with 6 to 9 embryos (Group 4; 12.8%) was 5.8 ± 3.0; and excellent donor animals with >10 embryos harvested (Group 5; 14,1%) was 7.8 ± 4.6, respectively. Donor cows in group 3, 4 and 5 were dried on Day 180 ± 10 of lactation. Animals of group 3 and 4 were withdrawn after 6 superovulations or earlier, when 32.5% and 38.0% of the donors showed signs of irregular oestrous cycle and 18.2% and 20% of the cows had symptoms of nymphomania. Donors having good embryo production (Group 5) were superovulated as many times as possible until they showed irregular oestrous cycle (60% of the animals) or nymphomania (40% of the donors) and the program was suspended. No significant difference was found in the occurrence of the irregular cycle and/or nymphomania between group 3 and 4. Significantly more animals having nymphomania and irregular cycle were found in group 4 and 5 ($P < 0.05$). In this study, donors of Group 3 to 5 were dried and frequently superovulated in order to produce the maximum number of embryos. We found that donors superovulated more than 5 times become nymphomaniac and/or loose their regular cycle. It was also observed that this frequently happen to donors with excellent embryo production. Although other factors were not ruled out, our results suggest that the endpoints of the consecutive superovulations are nymphomania and irregular cycle.

The embryos were deepfreezed and stored in liquid Nitrogen. I made experiments with a new embryo holding and deepfreezing solution containing zwitterion to have better pregnancy results. Initial experiments have shown that zwitterion buffer-based solutions (ZBS) are superior to phosphate buffer-based solutions (PBS) in terms of the handling and storage of IVF-produced bovine embryos and the transfer of fresh sheep embryos. Cows were submitted for non-surgical embryo recovery with ZBS (n = 20) or PBS (n = 19) on D 7 (D 0 was the first AI). In each case, the buffer-based solution used for embryo collection was applied for subsequent treatment. Non-surgical embryo transfer was carried out 7 days after estrus. Quantitative data were compared by t-test and proportional data by chi square analysis.

No significant differences in the proportion of cows with a minimum of one ovum collected relative to the number of flushed donor cows (embryo collection efficiency; 85.3% vs. 82.9%), number of collected ova (mean ± SD: 8.4 ± 6.8 vs. 7.8 ± 6.3) or percentage of embryos plus ova recovered relative to the number of palpated corpora lutea (embryo collection rate; 73.0% vs. 76.4%) were observed between the use of ZBS and PBS, $P>0.05$. Moreover, no difference was found in the number of good quality embryos harvested (mean ± SD: 4.7 ± 2.8 vs. 4.2 ± 3.4, $P>0.05$). Embryos of IETS code 1 (n = 20 and 18) and 2 (n = 23 and 21) were freshly transferred or deep-frozen (number of deep-frozen code 1 embryos = 23 and 28 and code 2 embryos = 19 and 24) using either ZBS or PBS containing 1.5 M ethylene-glycol. No significant differences were observed in the implantation rate of freshly transferred IETS code 1 (60.0% vs. 55.6%) and IETS code 2 (34.8% vs. 28.6%) embryos. Significant differences, however, were found in the pregnancy results of thawed IETS code 1 (56.5% vs. 39.2%) and IETS code 2 (26.3% vs. 16.7%) embryos, $P < 0.05$.

The results of this study suggest that either phosphate buffer-based or zwitterion buffer-based solutions can be used for collecting and transferring fresh embryos in a tropical environment; however, only zwitterion buffer-based solutions should be used for frozen-thawed embryos.

4 Repeatability and heritability of ovulation number and embryos in dam-daughters pairs in superovulated Holstein-Friesian cow

Holstein-Friesian dams (n = 28) and daughters (n = 28) were superovulated (total number of observations was 235) to determine the repeatability and heritability of ovulation number and embryo collection result for FSH treatment. The donor cows were superovulated with FSHp (Pluset, Serono, Italy), artificial insemination was done and embryo collection was carried out seven days after. For the analysis the raw corpdata of number of corpora lutea (CL), the number of collected embryos (EM) and their log-transformed values were used (LogCL, LogEM). The genetic parameters were calculated by using the VCE4 software. For calculating heritability, the number of embryo collection was used as a random effect, for calculating repeatability, the permanent environment was fitted. The additive genetic variance of CL was 8.91, and that of the EM was 9.23. The additive genetic variance for the LogCL and LogEM were 0.457 and 0.340, respectively. The estimated heritability for CL and EM were 0.234 and 0.159, the repeatability were 0.386 and 0.301, respectively. Higher heritabilities but lower as the previous repeatabilities were observed for the logtransformed data, 0.266, 0.194 and 0.294, 0.208 for LogCL and for LogEM, respectively.

5 Endocrinological investigations

5.1 Progesterone profiles and estrus cycle changes following superovulatory treatment on Holstein-Friesian dairy cows in tropical environment

Changes of progesterone (P4) profiles and estrous cycle were investigated up to 70 days in 20 superovulated Holstein-Friesian cows in semiarid environment. The donors were maintained in pens (10 animals/group, 15 m^2/donor) in open stables and fed with freshly chopped corn plant, elephant grass and concentrate. For the superovulation, 600 IU total amount was administered as 150, 150; 75, 75; 50, 50 and 25, 25 IU per day was applied and embryo collection was done at day 7 after the AI. At the same day, 225 µg prostaglandin injection was given. Samplings were done at the embryo collection and two times weekly. Superovulated cows showed no significant differences in relation to P4 level at the embryo recovery (39,0 ± 27,1 nmol/L, P = 0,536), first and second (12,0 ± 6,0 and 10,7 ± 2,2 nmol/L, P = 0,543) cycle. There was close correlation between the serum P4 concentration and CL number (13,3 ± 9,5) at the embryo recovery ($P < 0.0001$). After the embryo recovery, cows returned to cycle in different way: i. group of donors returning to cycle after 2,2 ± 0.8 days later, ii. group with 11,0 ± 1,9 days delay and iii. animals having a long (28,8 ± 2,2 days) atretic period, which is significant ($P < 0.001$). The remaining animals (30%) showed cystic ovarian malformations. P4 level at the embryo recovery

does not influence the estrus cycle change. Data suggest that Holstein-Friesian donor cows may suffer from cystic ovarian degeneration and long atretic phase after the superovulation treatment in tropical climate.

5.2 Influence of elevated temperature on metabolic hormones, beta-hydroxy-butyrat and progesterone concentrations and their effect on superovulation results in Holstein-Friesian cow

Metabolic hormones [insulin, leptin, insulin-like growth factor-I (IGF-I), thyroxin and tri-jodothyronine], progesterone and beta-hydroxy-butyrate (BHB) serum concentrations were evaluated and their effect was studied on the superovulation results of donor cows in tropical environment in this work. Body weight, body condition score (BCS) and lactation stage were also included in the analysis. Holstein-Friesian cows were superovulated (23) with 600 IU FSH following the routine procedure and flushed in day 7 in a MOET center in Brazil. The number of CL was counted, registered and blood samples were collected for assays. For group evaluation, one-way analysis of variance, for calculation of significant differences the Duncan's test was used and presentation of the relationship was by linear correlation coefficient. All of the investigated hormones and BHB serum concentrations were within physiological values in heat stressed conditions indicating that serum concentration did not changed in high temperature. Confirming the superovulation result, three groups were formed (unsuccessful [CL = 0-2], successful [CL = 3-11] and good [CL = 12-] embryo producing animals) and hormone levels evaluated for superovulation effectiveness. Insulin-like growth factor-I seems to have direct effect on the number of CL and change of

P4 concentrations highly correlated with the number of CL. However, leptin level increased with the number of CL and relatively well correlated with the BCS, less correlated with insulin and IGF-I level. Thus, we concluded that the role of leptin in superovulation success is unlikely and the role of other metabolic hormones on the number of CL also was not proved.

6 Resume of the dissertation

To alleviate the lack of food for human population in the developing countries, governs set up genetic programs to grow milk production. For this reason high genetic value cows – mainly Holstein-Friesian or Jersey - are transported from different meteorological conditions and bred separately from the local, weak milk-producing cows.

I was working as an expert in a MOET program; to improve cattle genetic in the semiarid region settled by the Brazilian govern. Therefore, pregnant heifers were imported from Hungary and put in a newly constructed donor center in Petrolina, PE.

At the initial stage of my work, I found that

- The donor cows were suffered from acclimatization problems due to the drastic climatic change causing serious problems in reproduction which was revealed in long acyclic period and 1.5 years long non-responding stage for superovulatory treatment.

As passed the acclimatization period, I established the embryo producing performance of the donor center. First,

- I superovulated all available cows to set up the good embryo producing donor nucleus inseminating the weak embryo-producing animals;
- Then, based on my experiments, I chose the most effective hormone preparations;
- Dried off the best embryo producing donors;
- Reduced to 60% of the amount of used hormone and shortened the interval between the embryo recoveries;
- Later, I showed that the limit of the repeated superovulatory programs is the appearing of the cycle

irregularities, e.g. nymphomania and long interval between the estrous.

I found that in tropical environment, the heifers and dried off cows are better in the point of view of embryo production than lactating animals. The anabolic and other heat producing processes of milk production (p.e. intensive heart work) produce high quantity of heat and to maintain the homeothermy of the body, it has to be dissipated to the environment. In the hot climate this excess amount of heat is difficult to dissipate due to the hot environmental temperature, therefore, the body temperature augments consequently the normal embryo development suffers damages.

In my studies I confirmed the statements of literature that donors kept in high ambient temperatures do not reduces the number of CL on ovary following superovulatory treatment. However, there's only a few publication whether donors exposed to extra high temperatures react for superovulatory treatment or not. In my work, cows during the El Nino phenomenon were superovulated and found that

- Not only the gained high number of eggs/degenerated embryos was higher in number; but
- The superovulation reaction was reduced, as well, which was not observable in the really hot summer periods.

The frequently carried out superovulations gave possibility to calculate the repeatability of number of CL and gained embryo for hormonal treatment. The available literature is not wide and the authors reported the analysis only little number of donors, in different management and meteorological conditions. In this dissertation using sufficient number of superovulation result in standard conditions, I added the literature with a complete number of repeatability.

During the work in Petrolina, I superovulated dam/daughter pairs in high number; thus, I collected enough dates to calculate the heritability of the superovulation

responsiveness and gained embryo number of donors. This kind of statistics has never been published.

In another trial I collected blood samples up to 70 days to follow the progesterone serum level. I found that

- No conclusion could be made about the later cycle disorder by the progesterone level at the embryo collection;
- At about 50% of the superovulated donors showed cycle irregularity (ovarian cystic formations and long anoestrus period); and
- Only half of them turned to normal estrous cycle immediately or after a short silence phase.

At the time of embryo collection, I collected blood samples to measure serum levels of metabolic hormones and BHB. The aim of this study was to investigate whether tropical environment has effect on these investigated parameters and what is the effect of certain hormones in the superovulation result. I concluded that

- In the tropical environment the metabolic hormone concentrations were not modified in the serum;
- The leptin and IGF-I levels were significantly higher in those donors where the superovulation responses were found. There were no significant differences in the blood concentration of some hormones (insulin, T3, T4) responsible for homeostatic regulation and similarly, there were no significant differences in the same point of view in the cows responding with 3-12 and 12- CL for superovulation treatment;
- The leptin concentration correlated well with BCS and fewer correlated with insulin and IGF-I. There was no correlation with the number of CL and P4 level at embryo recovery. This finding shows that significantly lower leptin level of non-responding cows refers to the lower BCS, thus, leptin has no direct effect on metabolic regulations on superovulation responsiveness;

- Relatively close correlation was observable between the level of IGF-I and number of CL and progesterone level at the embryo collection, which confirms the connection between the STH-IGF-I axis and superovulation responsiveness.

7 New scientific statements

- Determination of the acclimatization problems occurred due to the climatic change of Holstein-Friesian herd.

- Intensification of embryo production in the MOET donor center in the dry tropical climate.

- Determination of cycles disorders in the superovulated Holstein-Friesian donors in semiarid climate.

- Determination of embryo production and ovarian response of Holstein-Friesian donor cows in extreme high air temperature (El-Niño phenomenon).

- Determination of progesterone profiles and cycle changes up to 70 days after superovulatory treatment in tropical environment.

- Investigation of metabolic hormones in superovulated donor cows in hot climate and find effect of them in superovulation effectiveness.

- Calculation of repeatability and heritability of number of CL in superovulated ovaries and gained embryos of Holstein-Friesian donor cows.

8 Acknowledgements

I say thank for professional advices to my Supervisor
Prof. Dr. Cseh Sándor
and members of Advisory Committee
Prof. Dr. Solti László
Prof. Dr. Huszenicza Gyula

For professional assistance to my co workers

Celso Walter Costa Barros
Heraldo Marcos Cruviel
Dr. Gáspárdy András

To the leadership of the AGROINVEST

Dávid Imre
Varga Zsuzsanna
Balla Antal

And to my wife and children

9 Publications

9.1 Articles

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