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

PhD dissertation

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1 Introduction

My work was done in the semiarid region of Brazil where I was working 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 centre close to the city Petrolina, state Pernambuco. The transportation stress caused only one loss of pregnancy.

The climate in Petrolina was semiarid. To reduce the heat stress, the stable was opened and covered with tailed roof. The heifers were gathered in 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 cultivating alfalfa hay, elephant grass and corn plant. The corn was planted in 18 days periods to provide the fresh plant for green mass. The forage was PURINA. The milking system was full automatic ALFA-LAVAL.

The change of meteorological conditions caused serious stress to the animals. First, there were only two seasons of year with continuous high temperature, and second the lighting hours were nearly the same throughout the year because of the short distance from the Equatorial line. The management and meteorological conditions are published in the article *Bényei Balázs, Gáspárdy András, Barros Celso Walter Costa, 2001. Changes in embryo production results and ovarian recrudescence during the acclimatisation to the semiarid tropics of embryo donor Holstein-Friesian cows raised in a temperate climate. Animal Reproduction Science 68:57-68.*

The animals were transported to Petrolina in 1995 and the same year they were calved. My work started in the local in February of 1996. The herd was in silencious stage in the viewpoint of reproduction, eg. there was no observed heating. This diagnosis based on the observations and confirmed by rectal palpation and finally its consequence was the extremely long calving-to-pregnant interval (112.1 days). The first publication about the acclimatization problems was the poster presentation in the 13th Scientific Meeting of the European Embryo Transfer Association (A.E.T.E., Association Europenne de Transfert Embryonnaire) Lyon, France and was published as abstract, *Bényei Balázs, 1997. Superovulation response of the Holstein-Friesian cattle - born and grown up in mid-Europe - in tropical environment. Abstract book of 13th Conference of the European Embryo Transfer Association (A.E.T.E.). Lyon, France. p. 130.*

The analysis of the above referred reproduction problems suggested that the animals suffered from acclimatization problems caused by the changed meteorological environment. The lack of the four seasons, relative constant lighting hours throughout the year, and mostly the continuous heat stress caused long problematic period for the animals. The acclimatization events are analysed in the article *Bényei Balázs, Gáspárdy András, Barros Celso Walter Costa, 2001. Changes in embryo production results and ovarian recrudescence during the acclimatisation to the semiarid tropics of embryo donor Holstein-Friesian cows raised in a temperate climate. Animal Reproduction Science 68:57-68.*

After the acclimatization period, the donors entered to the good embryo production stage and I started the intensive superovulation phase. About the results (highlighting the specialities, eg. effect of tropical climate and repeated superovulation on the cycle of cows) an abstract was published in Theriogenology *Bényei Balázs, Barros Celso Walter Costa, 2001. Nymphomania and irregular cycle are the limits to repeated bovine superovulation programs. Theriogenology 55:356.*

I also compared the dates of embryo production of milking cows (20) and heifers (19). I concluded that because of the intensive milk production, milking cows have reduced thermotolerancy. Heat stress damage more of the cows reproduction system than the heifers' which may cause reduction in embryo quality. The results are presented in *Bényei Balázs, Fári Miklós, Barros Celso Walter Costa, Solti László, 1999. Superovulatory response of continuously heat stressed heifers and cows in Brazil. Theriogenology 51: 260.*

In an other study, I analised the superovulation and embryo production results of 1st, 2nd, 3rd lactation phase and dry cows. As I concluded similarly, the lactation is against the accomodation for heat stress. The results are revealed in the *Bényei Balázs, Barros Celso Walter Costa, 2000. Embryo production of heat-stressed donor cows at different lactation stages. Theriogenology 53:492.*

My earlier studies allowed me to chose the most effective hormone in tropical climate (Pluset, Serono, Italy), and in an another trial I managed to reduce the recommended 1000 IU dose to 600 IU, maintining similar embryo production. With 40% reduced amount of the Pluset, I economized and less weighted the endocrin system, also. Projeting superovulation programs using this schedule I reduced the interval between the two embryo collection programs in those donors who produced highest number of embryos. The results are published in the paper *Bényei Balázs, Barros Celso Walter Costa, 2001. The effects of FSH dose and frequency of embryo collection on superovulatory response in lactating Holstein cows. Theriogenology 55:512.*

To have higher quality frozen-thawed embryos, I tested the EMCARE (ICP, New Zealand) products: embryo flushing solution, handling medium and deepfreezing medium with Ethylene Glycol. These new embryo production solutions are based on MOPS-base (Zwitterion) which was published to be more adequate for embryo surviving. In my experiment there was no difference between the traditional Dulbecco and Zwitterion flushing medium but for deepfreezing, the MOPS-based ethylene glycol medium gave better result. About this experiment, the abstract *Barros Celso Walter Costa, Bényei Balázs, 2000. Comparison between zwitterionic and phosphate buffer-based bovine embryo handling solutions for embryo storage and transfer in a tropical environment. Theriogenology 53:308* was published.

As I showed, the continuous heat stress caused high impose to the lactating herd, because the milk production is a hard work with accelerated anabolitic processes and with it, the body temperature is higher and can not reduce the high corporal temperature by convention and radiation. The clima in Petrolina was semiarid but in the rainy season, the temperature is reduced, however, the humidity of air was higher. In the period between 1997 December to 1998 Jun in the South hemisphere, appeared the meteorological phenomenon, called El Niño, that did not allow to reduce the high temperature and didn't let the rainy season arrive, higher temperatures were observed than normally, in the dry season. In the well adapted donors there was no difference in embryo quality between the rainy and dry seasons and, in the El Niño period, the heat stress was so high that not only the embryo quality but the superovulation results were reduced. The complete information is written in the title *Bényei Balázs, Gáspárdy András, Cseh Sándor, 2003. Effect of the El Niño phenomenon on the ovarian responsiveness and embryo production of donor cows. Acta Veterinaria Hungarica 51:209-218.*

The principal aim of the donor center was the embryo production in highest number as possible, thus I superovulated dam/daughter couples in high number. The standard management and meteorological conditions allowed to calculate the repeatability (R) and heritability (h^2) of number of corpora lutea (CL) and gained embryo of the donors. However, in the literature there are some data about the R value, the calculation of h^2 has not been published jet. The earlier published R values were based on the number of transferable/freezable embryos.

The number of collected embryos and mainly the quality of them depends on various factors, e.g. effects which change the milieu enterieur of the uterus, embryo flushing staff, etc. In my work, I calculated the R and h^2 values counting the number of CL on the ovary and all gained embryos. The whole material is written in *Bényei Balázs, Gáspárdy András, Komlósi István, Pécsi Anna, 2004. Repeatability and heritability of ovulation number and embryos in dam-daughters pairs in superovulated Holstein-Friesian cows. Reproduction in Domestic Animals 39:1–4.*

To follow the progesterone (P4) serum concentration of superovulated cows in tropical climate after the embryo recovery, I collected blood samples up to 70 days. The first samples were taken at the embryo collection and later blood collections happened two times/week. Last bleedings were done when cows completed two regular cycles or in those cows who suffered from nymphomania and did not returned to cycle in long silent phase, the oestrus was observed. Further sample collections were not taken because my contract had finished. I found that half of donor cows showed cycle irregularity, 25% had short silent phase and only 25% returned to normal cycle immediately after embryo collection. The results are accepted for publication in *Acta Veterinaria Hungarica: Progesterone profiles and estrus cycle changes following superovulation treatment on Holstein-Friesian dairy cows in tropical environment.*

In an other study, blood samples were collected to measure serum concentrations of metabolic hormones e.g. insulin, leptin, insulin-like growth factor-I (IGF-I), thyroxin (T4), tri-jodothyronine (T3) P4 and beta-hydroxy-butyrate (BHB) to study whether in the tropical environment the serum concentration altered and has effect on superovulation results. The sampling occurred at the embryo collection. The serum samples were taken to Hungary, to the Endocrine Laboratory of the Faculty of Veterinary Sciences. In this study, I analyzed the hormone interactions with number of CL, embryo production, lactation and body condition score. The detailed analysis is in the manuscript: *The influence of elevated temperature on serum insulin, leptin, IGF-I, T4, T3, BHB and progesterone concentrations and their effect on superovulation results in Holstein-Friesian cow.* The MS is prepared to be published in the Animal Reproduction Science.

2 Effect of heat stress and heat regulation on cattle reproduction

2.1 Introduction

Cattle, as other mammalians is a homeotherm creature that controls its temperature within a narrow range (38.0-39.3^{o}C). Constant body temperature is the result of heat produced by metabolic processes passing towards the environment in the form of conduction, currency and radiation. The quantity of the heat given down depends on the temperature and the humidity of the surrounding. The high temperature and humidity of the environment restricts the passing of the heat from the surface of the body that can lead to a retrograde heat flow. Intensive sun radiation is also capable of bringing tremendous amount of heat to the body of the cattle, hereby it may cause disturbance in the heat regulation.

Hyperthermia occurs when the capacity of reduce of the heat is smaller than the heat production. For the prevention and reduction of hyperthermia homeokinetic control systems come to work, that lead to several physiological changes for the maintenance of the body temperature. A high degree of hyperthermia destabilizing proteins and cell membranes may induce harmful processes that can be even deadly. An animal in hyperthermic condition to become homeotherm changes its behavior metabolism, blood flow respiration, appetite and other physiological processes as well reducing the heat production and increasing the passing of heat.

The increased temperature affects the reproductive functions in two main pathways. On the one hand the main physiological control mechanisms are occupied by heat control processes, even those that would be responsible for the reproduction. The heat stress disturbing these systems stops or ruins the breeding functions. For instance, in case of a low weight embryo attributed to heat stress the backward development is due to the decreased amount of blood flow through the placenta (Reynolds et al., 1985). On the other hand, the heat production damages several tissues involved in the reproduction (Malayer et al., 1990; Malayer and Hansen, 1990; Malayer et al., 1988). The preimplantation embryo reacts particularly sensitively to the heat due to the mother's elevated temperature (Putney et al., 1988b; Ealy et al., 1993; Malayer et al., 1992).

The above mentioned factors altogether deteriorate the fertility index, as stated by numerous authors (Monty and Wolff, 1974; Rosenberg et al., 1982; Badinga et al., 1985; Cavestany et al., 1985; King et al., 1988; Ryan et al., 1993). To prove that the decline of the reproductive functions can be ascribed to the heat stress and to exclude the possible influence of the environmental factors researches were carried out in climatic chamber (Putney et al., 1988a; Putney et al., 1989b; Ealy et al., 1993), and also in stable conditions where animals were cooled by water spraying and ventilation (Roman-Ponce et al., 1977; Wolfenson et al., 1988).

2.2 Effect of heat stress previous to conception

Few effect of heat stress can be attributed to the increased production of ACTH (Roman-Ponce et al., 1981b; Wise et al., 1988a; Elvinger et al., 1992) and it was published, that it inhibits the sexual behavior induced by estradiol (Hein and Alldrich, 1992). In some experiment the increase of the ACTH concentration in the blood is not proven (West et al., 1991; Wise et al., 1988b) other authors reported raise in the cortisol level (Christinson and Johnson, 1972; Miller and Alliston, 1974; Elvinger et al., 1992) others found evidence on the decline of the same hormone level (Abilay and Johnson, 1973). In result of the heat stress the period of the oestrus is shortened (Hall et al., 1959; Gwazdauskas et al., 1981) brought about not only the apathic behavior of the animals but the fall in the estrogen level in the circulating

blood (Gwazdauskas et al., 1989) and the heat stress rise the occurrence of the silence estrous as well (Thatcher and Collier, 1986; Nebel et al., 1997).

The pregnancy index of the artificial insemination programs may be deteriorated due to these altered forms of behavior, since the screening of the individuals in estrus is more complicated.

The period prior to the ovulation can also be critical to the reproduction. In superovulated cows, heat is harmful for the embryo development and vitality if donors are exposed to high ambient temperature from the first sign of the oestrus until the 15-20th hours following the artificial insemination (Putney et al., 1989a). A possible reason of the phenomenon is, that on higher temperature (41^{o}C) under in vitro circumstances lower percentage of the developing embryos reach methaphase II compared to those developed in neutral heat condition (Lenz et al., 1983). According to another possible theory the increased body temperature has negative effect on the fertility of the semen. Sperm kept on high temperature significantly looses its motility and decreased acrosomal reaction can be observed (Lenz et al., 1983).

Heat stress damages the quality of the follicle (Badinga et al., 1993) besides the fact that the degree of steroid synthesis decreases (Howell et al., 1994; Roman-Ponce et al., 1977; Rosenberg et al., 1977; Wilson et al., 1988a; Faust et al., 1993) as well. During summer, the development of the dominant follicle is less manifested, more middle size follicles grow (Wolfenson et al., 1995; Roth, 1996; Wilson et al., 1988a) and ovulate increasing the risk of twin pregnancy (Ryan and Boland, 1991).

The higher temperature effect has also impact on the production of spermatozoa. It is known, that the normal spermatogenesis requires lower temperature than the body temperature. According to recent findings it is proven, that the ova production is heat sensitive, as well. Therefore heat stress may end up in insufficient quality of ova production.

2.3 Changes occurring around the time of nidation

The developing embryo is highly sensitive to the increased body temperature of the mother. By the progress of the pregnancy the sensitivity decreases. In a basic experiment it was found, that while among of the control ewe groups the lamb-producing ratio was 85%, this ratio fell to 10% if at the time of covering or day after they were treated with 32^{o}C. Heat stress three days after mounting gave 35% result while treating those 5 days after the covering resulted in 40% concerning the lamb-producing ratio (Dutt, 1963). Concluding from the evidence in sheep, throughout the process of pregnancy thermotolerancy is acquired.

High environmental temperature damages embryos in preimplanting stage though this effect declines by the development of the embryo (Ray et al., 1993). Researches on the heat (stress) tolerance of the embryo came to the result, that cells of the embryo produce Heat Shock Protein (HSP) that protects it from the harmful effect of the heat (Ealy et al., 1993; Baumgartner and Chrisman, 1987; Biggers et al., 1987; Ealy et al., 1995; Edwards et al., 2001; Pelham, 1988; Putney et al., 1988b). Resistance of mouse embryos against heat stress starts from the 8-cell stage (Burel et al., 1992), which is in synchrony with the activation of the whole mouse genome (Prather and First, 1988). In case of cattle embryo development the three day stage is equal to the 8-cell embryo therefore according to the previous statement it can be assumed that cattle genome similarly activates in the 8-16-cell stage (Barnes and Eyestone, 1990) that corresponds with the beginning of the production of the HSP molecules.

Heat stress harms the endometrium as well partly in a direct way by disturbing the protein synthesis and secretion, partly in an indirect way through the changes of the hormones

circulating in the blood flow. Protein synthesis of the In Vitro cultured tissue of the uterine tube (collected ipsylateral to the CL) reduced slightly kept on 43°C compared to the 39°C control temperature. At the same time the quantity of the protein in the tissue sample gained from the contra lateral side grew somewhat (Malayer and Hansen, 1990). While culturing endometrium tissue of the uterus taken at estrus increased protein synthesis was observable due to the higher (43°C) temperature. The same phenomenon was noticed whether taking the sample on the 2nd, 5th, 7th or on the 17th day of pregnancy (Malayer and Hansen, 1988).

Moreover malfunction of the blood supply in the uterus was observed. In attribution to heat stress blood spread from the inner organs over the periphery, resulting disturbances in the nutrition and hormonal supply of them. In addition the temperature of the uterus increases (Roman-Ponce et al., 1978; Gwazdauskas et al., 1975). The above listed changes lower the efficiency of fertilization.

2.4 Effect of heat stress on the function of the hypophysis-hypothalamo-ovary axis

Since the main regulators of the ovary are GnRH produced in the hypothalamus and LH, FSH are produced in the frontal lobe of the hypophysis, intensive experiments were carried out testing the effect of increased temperature on these hormones.

The effect of heat stress on LH production is unclear, since the published data are contradictory. Some authors did not find change (Gwazdauskas et al., 1981; Gilad et al., 1993) others stated increase (Roman-Ponce et al., 1981a) or decrease (Madam and Johnson, 1973; Wise et al., 1988a; Gilad et al., 1993; Lee, 1993) in the blood concentration. The frequency of LH surges remains stable under higher environmental temperature (Gilad et al., 1993). There is a controversy about the effect of heat stress on the LH peak prior to ovulation, as well. Lower (Madam and Johnson, 1973) and altered (Gwazdauskas et al., 1981; Rosenberg et al., 1977; Gauthie, 1986) LH peeks were reported.

Summarizing the published data, most study states that the LH level decreases due to the heat stress. Therefore, in the summer the dominant follicle develops under lower LH level in the blood depressing the reproductivity index. Data on the effect of heat stress influencing the FSH level in the blood not sufficient yet.

FSH concentration prior to ovulation shows higher value in the summer, which appears with lower inhibin level (Roth, 1998).

Decrease of the estradiol concentration in the blood occurs due to heat stress exposure (Roth, 1998; Wolfenson et al., 1995; Wolfenson et al., 1997; Wilson et al., 1998a). Reduction of the blood supply in the uterus can be traced back to this phenomenon (Roman-Ponce et al., 1978).

Concerning the progesterone (P4) concentration there is no standard point of view. Increased (Wilson et al., 1998a; Wilson et al., 1998b) and decreased (Younas et al., 1993; Howell et al., 1994; Jonsson et al., 1997) hormone levels were published either.

Environmental heat stress is more likely to have lowering effect on the P4 concentration in the plasma influencing significantly the fertility. It is well known, that the low P4 concentration before the conception during the phase of the CL injures the follicle development resulting in damaged ova. Lower P4 level during conception causes disturbance in embryo implantation or may even lead to absorbance of the embryo. The effect of P4 level in the conception on embryogenesis likely to have connection with the necessary synchrony of the development of embryo and CL and the faster or lower embryo growth leads to reduced conception index. The effect of exogenic P4 therapy following conception or embryo

implantation aiming the improvement of the results is not clear. Upturn (Robinson et al., 1989) and ineffectiveness were (Breuel et al., 1990) detected as well. Based on my personal experience on several thousand embryo implantation, great number of factors influence the pregnancy however the hormonal treatment whether they are CIDR or CRESTAR ear implants or other progestagen products (data under evaluation) is at least unlikely to have impact on the adherence of the embryo.

Based on the Roman-Ponce experiments, the increased corticosteroid concentration may lead to the rise of the GnRH and gonadotropin production (Roman-Ponce et al., 1977; 1981a). Gilad (1993) found, that in case of heat stress the gonadotropin production was moderate in cows with low estradiol concentration and it was higher when the estradiol concentration was high. This study shows, that increased estradiol concentration can alleviate the harmful effect of heat stress to a certain extend. Under low estradiol concentration the gonadotropin secretion regulated by the neuroendocrin system is more sensitive for the high temperature. Any heat stress included change in the hormone production of the ovary might be the reason of the fall in fertility in the summer. Heat stress has direct influence on the ovary reducing its sensitivity on gonadotropin hormones.

2.5 Effect of heat stress on pregnancy keeping ability

Heat stress causes the greatest damage on the first day of the pregnancy. If the heat affects the pregnant animal later on the 8-16 day either lower weight of the fetus or fall in the fertility index occur (Geisert et al., 1988). In this period there is a fast embryo growth and the embryo produces the so-called embryonic signal (bovine trophoblast protein-1, bTP-1) that inhibits the decline of the CL and promotes its transformation into CL pregnancy by preventing the endometrial $PGF_{2\alpha}$ production (Hansen, 1991), which was proved by in vitro essays. Embryonic bTP-1 production kept on 43°C cultures in vitro reduced by 74% (Putney et al., 1988b). Besides it was also observed that in endometrial culture the $PGF_{2\alpha}$ production increased (Putney et al., 1988b; Putney et al., 1989c; Malayer et al., 1990) by the heat reducing the survival of CL. According to another experiment the embryonic bTP-1 production In Vitro culturing on higher temperature is unchanged if pretreated with heat In Vivo (Geisert et al., 1988). It turned out, that the $PGF_{2\alpha}$ production increases in case of non-pregnant uterus in contrast with the pregnant one (Putney et al., 1989c) which suggests that the pregnancy is capable of making the uterus endometrium resistant to heat induced $PGF_{2\alpha}$ production.

2.6 Effect of heat stress on placenta formation

Heat stress influences negatively the size and hormone production of the placenta after its formation. According to publication in Florida it can be stated that the net weight of the placenta and the weight of the fetus in the summer is lower than those in the winter (Head et al., 1981). This seasonal alteration is due to the raised body temperature of the mother. Cows in third phase of the pregnancy exposed to sun radiation prior to calving showed reduced oestron sulphate production, lower weight of the fetus and lower milk production (Collier et al., 1982).

2.7 Possibilities of overcoming the low fertility index

As analyzed above, the embryo after conception is more capable of excluding the harmful effects of the heat; therefore, it is essential to overcome the sensitive period of the conception. Two possible method exits to achieve it. One of them is the effective cooling of cattle body temperature below the critical level around the time of insemination (Armstrong, 1984; Ealy et al., 1994; Flamenbaum et al., 1986; Her et al., 1988). The other is the transplantation of heat resistant 7-day-old embryos to heat stressed recipients (Ray et al., 1993; Ealy et al., 1993), though, with it we will face the synchronization and the estrus detection problem of the recipient animals.

2.8 Final conclusion

Fertility index of cattle kept in warm environment is significantly reduced because of the changes in the inner environment of the uterus, change of the follicle and spermatozoa, hormonal changes and disturbed development of the placenta. HSP-1 production of the embryo partly balances offering possibility to exclude the harmful effect of the heat on the fertility.

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3 Reproduction occurrences of donor cows during the adaptation period for semiarid climate and El Niño phenomenon

3.1 Changes in embryo production results and ovarian recrudescence during the acclimatisation to the semiarid tropics of embryo donor Holstein-Friesian cows raised in a temperate climate

3.2 Effect of the El Niño phenomenon on the ovarian responsiveness and embryo production of donor cows

4 Intensification of embryo production

4.1 Superovulation response of the Holstein-Friesian cattle - born and grown up in Mid-Europe - in tropical environment

The objective of this study was to test the ability of HF cattle, transported from Hungary to Brazil, to get acclimatized to new environment and respond to superovulation treatment. Donor animals were born in Hungary in 1994 and were transported to Brazil in 1995 to the savanna area of the state Pernambuco as 3 months pregnant heifers.

The following major differences between the climate of Middle-Europe and the savanna area of Pernambuco could potentially influence the reproductive function. Temperature is stable through the difference seasons (the daily maximum is 38-43°C during the hot season: while it is 30-35 °C in the moderate season). The temperature does not drop below 25°C at night. The average quantity of rain is 650 mm/year (D: 100-1500 mm), humidity is 35-38% the dry season and 50-55% during the rainy season, which lasts 3-4 month. Day light is stable (12 h/day ± 30 min), because this state is 9° South.

The animals were fed with cut whole plant, cut elephant grass, alfalfa hay and fodder mixture was added depending on milk production. Donors were housed in pens (10 animals /group) and a roof was built over each pen in order to protect the animals against direct sunlight. In addition, animals received a shower by a micro-aspersion system for 10 min, every hour if the temperature went above 38°C.

Results

No signs of ovarian activity were observed during the first 3 moths after calving. The standard superovulation treatment (AUSA-Superov) started 6 months after calving.

	1996									1997		
	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar
N° donors	1	7	11	-	16	6	6	6	16	7	10	8
Embryo*	2	4	18	-	2	9	17	1	21	10	59	44
Average	2	0.57	1.60	-	0.12	1.50	2.83	0.16	1.31	1.42	5.90	5.50

*Embryo freezable

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.

It is concluded that it took around one year for those HF cows to adapt to the new environmental conditions.

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4.2 Nymphomania and irregular cycle are the limits to repeated bovine superovulation programs

In MOET programs donors are superovulated and flushed repeatedly. In this study we investigated the effect of consecutive ovarian stimulations on the oestrus cycle of the donors (nymphomania and/or irregular cycle). The study was carried out in semiarid climate. Holstein-Friesian donor cows were treated with FSHp (Pluset, Serono, Italy) starting on Day 8-12 of cycle. A total dose of 650 IU (8 injections IM. over 4 days) was administered and PGF2alpha (Veteglan, Serono, Italy) was injected on Day 3 of the treatment. AI was performed twice, 48 (Day 0) and 60 hours later. Non-surgical embryo collection was carried out on Day 7. Number of obtained ova/embryos was registered and analysed by t test. Donors were grouped on the basis of the number of oocytes harvested. Records refer to calving interval. Proportional data were compared by chi-square analysis. Data of 75 donors and 364 superovulation programs were included in this study. 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 the semiarid climate the embryo production is affected by the high environmental temperature (Bényei, B. et al., Theriogenology, 53:492, 2000). In this study, donors of Group 3 to 6 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. Further research is needed to establish whether cows produce anti-FSHp antibody, as was found in primates (Cseh, S. et al, Theriogenology, 51: 282, 1999) resulting in failure of ovarian stimulation.

4.3 Superovulatory response of continuously heat stressed heifers and cows in Brazil

The superovulatory response of Holstein-Friesian cattle was evaluated under a dry and hot environment in the semi-arid area of Brazil; the daily maximum temperature was 33.5°C and the relative humidity was 35%. Cycling Holstein-Friesian heifers (n = 19; >12 months old and >370 kg body weight) and lactating Holstein-Friesian cows (n = 20) were superstimulated starting on Days 8 to 11 of the cycle with a total dose of 75 mg (2 x 12.5 mg/day for 3 d) porcine FSH (Superov, Ausa International Inc. USA). 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 IETS criteria. Quantitative data were compared by t-test and proportional data were compared by chi-square analysis.

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 (CL) detected by rectal palpation or in the numbers of ova/embryos recovered by the nonsurgical collection technique (Table 1). However, there were significantly more unfertilized ova and degenerate embryos collected from cows and more freezable (IETS Code 1) embryos collected from heifers ($P < 0.05$; Table 1).

Table 1. Superovulatory response (mean ± SE and %) in heat-stressed dairy cattle in Brazil

		Ova/embryos						
Donors		CL	Total	Unfert. (%)	Degen. (%)	Freezeable (%)	Morulae	Blast.
Heifers	(n=19)	9.1	6.00	0.2 (4) [a]	0.4 (7) [a]	5.4 (89) [a]	72%	28%
		±1.6	±1.1	±0.5	±0.8	±1.0		
Cows	(n=20)	6.55	5.80	1.40 (24) [b]	1.20 (21) [b]	3.20 (51) [b]	77%	23%
		±7.22	±7.22	±2.16	±2.40	±4.48		

[ab] Percentages with different superscripts are significantly different ($P < 0.05$)

Overall, data suggest that 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.

4.4 Embryo production of heat-stressed donor cows at different lactation stages

A MOET program has been running in the semi-arid tropical region of Brazil in order to increase milk production. The objective of this study was to determine the effect of different lactating stages to the embryo production of heat stressed Holstein-Friesian 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 two year study, all donors were superovulated with FSHp (Pluset, Serono, Rome, Italy) according to standard protocols. Non-surgical embryo collection was carried out 7 d after the first artificial insemination. Quantitative data were compared by t-test and proportional data were compared by chi-square analysis. As Table 1 shows, 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.

Table 1. Mean±SD values for number of corpus lutea and embryo production of dry donors and those at three different stages of lactation

	1st stage	2nd stage	3rd stage	Dry
No. of CL	7.4±6.7 [a]	8.9±6.7 [a]	9.8±8.0 [a]	13.6±7.0 [b]
No. of embryos[1]	10.0±13.0 [a]	8.0±5.7 [a]	7.8±6.0 [a]	10.3±7.0 [a]
No. of good quality embryos	2.0±2.9 [a]	3.0±4.7 [a]	3.8±5.1 [a]	5.0±4.8 [a(b)]
Overall % of good quality embryos	20.0 [a]	37.9 [b]	47.5 [c]	49.8 [c]

[1] Cows from which no ova or embryos were recovered were excluded.
[aa] Dates within rows with the same superscripts do not differ $P > 0.05$.
[a,b,c] Dates within rows with different superscript differ $P < 0.05$.
[a(b)] Comparing the 1st and 2nd stages, significant $P < 0.05$.

Overall, these 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.

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4.5 The effects of FSH dose and frequency of embryo collection on superovulatory response in lactating Holstein cows

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 Holstein-Friesian cows in the semiarid area of Brazil. 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 (recommended dose of Pluset; n = 30, Group 1) or 600 IU (reduced dose; n = 26, Group 2) was administered. In Experiment 2, embryo collections were repeated at 30-day intervals (n = 15, Group A) or 50-day intervals (n = 18, Group B) using a total of 600 IU of Pluset. On Day 3 of superstimulation (Day 1 = first day of FSH treatment), PGF (3 ml of Veteglan, Serono, Italy) was administered to induce luteolysis and all animals were inseminated twice with a single straw of semen 48 and 60 hours later. Ovarian responses were determined by rectal palpation and ova/embryos were recovered non-surgically 7 days after the first insemination. Embryos were evaluated according to the IETS Manual. Quantitative data were compared by t-test and proportional data by chi square analysis. Data obtained from three consecutive superovulations are shown as means. In Experiment 1, no significant differences were found between Group 1 and 2 concerning the number of ovulations (12.3±6.8 vs. 13.4±6.0), good quality embryos (4.8±2.3 vs. 5.3±2.1) and eggs (unfertilized oocytes and degenerated embryos; 3.7±1.9 vs. 3.9±2.0). However, there was a significant difference between the two Groups in the proportion of donors having large unovulated follicles (20.2% vs. 3.6%, for Group 1 and 2, respectively; $P < 0.05$). In Experiment 2, no significant differences were found between Group A and Group B in the number of corpora lutea (11.7±6.8 vs. 14.5±7.2), good quality embryos (4.7±3.6 vs. 5.9±2.7) and number of eggs (3.4±1.7 vs. 2.0±1.6).

Our results show that the superovulation response and embryo production of the donor cows is similar regardless the dose of the FSHp applied (recommended or reduced). However, significant difference was found in the occurrence of large unovulated follicles. Donor cows return to estrus at about 12.2±3.2 d 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 unovulated follicles and the cost of ovarian stimulation can be reduced.

4.6 Comparison between zwitterion and phosphate buffer-based bovine embryo handling solutions for embryo storage and transfer in a tropical environment

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 IVP-produced bovine embryos and the transfer of fresh sheep embryos. This study was designed to evaluate the differences between ZBS and PBS on embryo recovery, handling, storage and pregnancy rate of bovine embryos following transfer in elevated ambient temperatures.

In an embryo transfer center located in the semi-arid region of Brazil, Holstein-Friesian donor cows were superovulated with FSHp (Pluset, Serono, Rome, Italy) and inseminated with a single straw of thawed semen on D 5 and D 6 (D 0 was the first FSH treatment). 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 (ZBS, EMCARE™, ICP, Auckland, New Zealand or PBS, NUTRICELL, São Paulo, Brazil) 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 practitioners can use either phosphate buffer-based or zwitterion buffer-based solutions for collecting and transferring fresh embryos in a tropical environment; however, only zwitterion buffer-based solutions should be used for frozen-thawed embryos.

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5 Repeatability and heritability of ovulation number and embryos in dam-daughters pairs in superovulated Holstein-Friesian cows

6 Endocrinological investigations

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

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Abstract

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 of FSHp 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. 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.

Key words: superovulation, heat stress, P4 concentration, Holstein-Friesian cows, cycle changes

Introduction

Earlier studies have shown that apart from the inherent unpredictability and variability in superovulatory response, there are also several factors that can influence the outcome of FSH treatment (Hasler et al., 1983; Lerner et al., 1986).

Reproductive efficiency is depressed when cattle are maintained under environmental conditions of high ambient temperatures and relative humidity. During heat stress, cattle are unable to dissipate environmental heat and are often hyperthermic, with elevated body-core temperatures.

Following superovulatory treatment, the final developing phase of the first wave dominant follicle may have three ways to further development (Beam and Butler, 1997; 1999; Rajamahendran and Taylor, 1990; Savio et al., 1990; Wiltbank et al., 2002): i. ovulation of the matured ova, ii. atresia, which can be followed by a development of a new dominant follicle from the next follicular wave or iii. development of anovulatory cyst.

Cyclic disorders, e.g. ovarian cystic degeneration (follicular or luteal cysts) is a common ovarian disorder among dairy cattle in tropical regions, 18.9% was reported in a Tanzanian study (Mujuni et al., 1993) and short luteal phases were observed in an Ethiopian work (Tegegne et al., 1993). These findings indicate that heat stressed cows may frequently express irregular cycles. Though the Holstein-Friesian cow appears to be the most susceptible to the deleterious effects of heat stress, nevertheless, embryo producing may improve under tropical conditions. Information about cycle and progesterone (P4) profiles changes for a longer time after the superovulation in tropical environment is not available. Therefore, we collected blood samples for up to 70 days after embryo recovery to track P4 profiles with the specific

objectives: 1) to evaluate changes in P4 concentration and number of corpora lutea (CL) on the superovulated ovary and 2) to study the serum P4 profiles and estrus cycle changes of the donor cows following the FSH treatment in semiarid Brazil.

Materials and Methods

Climatic and management conditions

The experiment was done in semiarid Brazil (Petrolina-PE, 09 °09'S latitude, 40 °22'W longitude and 465.5 m altitude) in the donor farm of the Multiply Ovulation and Embryo Transfer (MOET) project of the Company for Development of Rio Santo Francis Valley (CODEVASF). The sampling was initiated in the mid January 2000 and finished at the end of April 2000.

At the project site in Brazil, there is little variation in the ambient temperature between the different seasons. The average temperature was 24.8 ± 1.4 °C and ranged between 31.5 ± 1.6 °C and 20.0 ± 1.4 °C. Maximum daily temperature corresponds to the Temperature Humidity Index (THI) of 75.14 ± 1.35 where the animals suffer from mild heat stress (Armstrong, 1994). Meteorological data were collected at 40-km distance from the project.

The donor animals were bred in the MOET center, housed in open barns, in pens (capacity 10 cows/pen with 15 m^2/animal) with sprinkling system and fed with freshly chopped whole corn plant, elephant grass, alfalfa hay and concentrate, according to the milk production. They were milked twice a day in a milking parlor with Alfa-Laval system. The embedding system was hydrolyzed bagasse of sugar cane.

Animals and superovulatory treatment

For the study, randomly chosen, 20 Holstein-Friesian cows were used as donor animals. Most animals at the experiment were scored mean Body Condition Score (BCS) 3, which is considered the best from the reproduction point of view (Niekerk, 1982).

Cows with observed estrous were synchronized between day 5 and 15 of the natural cycle with PGF2α, (Veteglan, Serono, Rome, Italy, 47 µg/animal). For superovulation, 600 IU FSHp (Pluset, Serono, Rome, Italy), was administered as 2x150, 2x75, 2x50 and 2x25 IU per day (Bényei et al., 2001a), starting on days 8 to 11 of the synchronized cycle (day 0 was the estrus). On day 3 of the treatment 3 mL PGF2α was administered i. m. (225 µg D-Cloprostenol, Veteglan, Serono, Rome, Italy) and 48 and 60 h later the donors were inseminated artificially (AI) twice. Seven days after the first AI, ovarian structures were palpated and the number of CL was counted. Nonsurgical embryo collection was performed using Dulbecco's Phosphate Buffer Solution (DPBS, Nutricell, São Paulo, Brazil) supplemented with 25 mg/1000 mL kanamycin sulphate and 0.2% bovine serum albumin. Day 7 embryos were morphologically evaluated and assigned a quality grade from 1 to 4 using criteria issued by the International Embryo Transfer Society (Stringfellow and Seidel, 1998). Immediately after embryo recovery, the donors were treated with 3 mL PGF2α.

Sampling and P4 assay

Blood samples were collected from the coccygeal vein into heparinized tubes following the next schedule:

- Day of the first FSH treatment;

- At the PGF2α treatment;
- Day of the AI;
- Two and four days later;
- Day of the embryo recovery;
- Further samples were collected three times a week on Mondays, Thursdays and Fridays.
- End of sampling in each case was 70 days after the embryo recovery or in case of animals having irregular cycle when signs of regular sexual activity appeared.

P4 levels were determined by locally developed direct ^{3}H-RIA method used originally for human (Csernus, 1982) and equine (Nagy et al., 1998) samples, respectively, and validated for bovine plasma (Kulcsár et al., 1996) without any modification (sensitivity: 0.27 and 0.20 nmol/L, intra- and interassay CVs: ≤8.7 and ≤10.3 %, as well as ≤4.5 and ≤8.9% in P4 determination, respectively).

Statistical analysis

In the first examination we evaluated the data of 20 cows. The relationship between the serum P4 level at embryo recovery (P4C0) and the number of CL was determined by correlation coefficient using a linear regression model, where the number of CL was the dependent and the P4 concentration was the independent variable. Before the calculation, Kolgomorov-Smirnov one-sample test (K-S) for normality was used to prove the distribution of number of CL. The D statistic gave non significant result (K-S d = 0.127, p>0.20) and the Gaussian distribution of CL should not be rejected.

After embryo recovery, the whole cow population was allocated into 4 groups. In the first one, we grouped 5 donors with immediate cycle normalisation (normal cows), another 5 cows were put in the second group with a delayed cycle normalisation (cows with a short atresia), whilst, 4 animals in which the cycle recovered after a prolonged atresia (cows with long atresia) were put in the third group. The remaining 6 cows (30%, group 4) were diagnosed as cystic animals, therefore excluded from this evaluation. Kruskal-Wallis nonparametric alternative test was applied to differentiate the frequency of cows with cysts from the others. According to this, in the latter group, significant difference was found ($P < 0.0003$) from the other cow groups, therefore, the degree of occurrence of the cysts was remarkable.

The following time and P4 parameters were calculated:
a, length of baseline P4 level between the embryo recovery and first P4 wave (L0),
b, length of the first P4 phase (L1),
c, length of the second P4 phase (L2), as well as the
d, maximum concentration of P4 in the first P4 phase (P4C1),
e, maximum concentration of P4 in the second P4 phase (P4C2).
The evaluated parameters were processed by one-way analysis of variance, where the group of cows was the fix effect.

Similarly in two cases, the relation of P4C0 and CL to the L1 were evaluated by linear regression model and expressed by correlation coefficient, where the P4C0 and the CL were the independent variables and L1 was the dependent one.

The relation between the serum P4 level and number of CL was calculated with a simple linear regression schema, following the next form:
No of CL = 2.435 + 0.277 x P4 concentration.

Results

Days of the cycles and intervals between them and P4 levels at the embryo recovery, first and second CL phases are demonstrated in the four groups of donor cows (Table 1). Normal ovarian function was assumed for cows, which showed elevated P4 concentrations above 1 nmol/L for approximately 2 weeks, interspaced with concentrations at or near the baseline for about 1 week.

Table 1. P4 concentrations at the embryo recovery and at subsequent luteal phases, number of days of the cycles and intervals between them in four groups

Group of the experimental animals**	1	2	3	4	
	Mean	Mean	Mean	Mean	p
P4 levels at the recovery (nmol/L)	30.7	46.6	52.2	30.8	0.536
±SD	14.7	23.5	34.0	33.8	
No of CL at the recovery	13.6	13.4	17.5	10.0	0.714
±SD	12.1	8.3	11.0	8.49	
No of days between the recovery and new cycle (L0)*	2.2	11.0	28.8	-	<0.001
±SD	0.8	1.9	2.2		
P4 levels at the peak of the first luteal phase (P4C1, nmol/L)	14.7	12.3	8.4	-	0.317
±SD	6.0	7.0	3.4		
No of days of the first cycle (L1)	17.6	15.6	18.8	-	0.212
±SD	2.9	2.5	2.1		
No of days between the two luteal phase*	1.6	3.6	-	-	0.008
±SD	0.6	1.1			
P4 levels at the peak of the second luteal phase (P4C2, nmol/L)	10.0	11.4	-	-	0.543
±SD	2.5	4.5			
No of days of the second cycle (L2)	18.2	17.4	-	-	0.587
±SD	1.64	2.7			

* baseline P4 level

** Groups: 1. cows returned to cycle immediately after the embryo recovery; 2. animals with short silencious phase; 3. cows with long atretic phase; 4. donors with cystic degeneration

Superovulatory treatment resulted in high concentrations of P4 in circulation (between 30.7 ± 14.7 and 52.2 ± 34.0 nmol/L) in all groups, which is non-significant ($P > 0.05$).

There were no significant differences in the number of CL-s at the embryo recovery in all groups (between 10.0 ± 8.49 and 17.5 ± 11.0 nmol/L, $P > 0.05$).

At the embryo recovery, PGF2α treatment was applied in order to luteolyse the high number of CL and thereafter, serum P4 concentration returned to baseline. It must be mentioned that 30% (6/20) cows were diagnosed as cystic and they did not return to estrus within

70 days. For these reasons, data from these cows were not included in the further analysis. After the PGF2α injection, short (11.0 ± 1.9 days in 5 animals) and long (28.8 ± 2.2 days in 4 cows) and no atretic phase (2.2 ± 0.8 in 5 donors) were observed until the first luteal phase, which is significant ($P < 0.001$).

In the group 1 and 2, the first and second luteal phases and in the group 3, the unique one, mean plasma P4 peak concentrations were between 10.0 ± 2.5 and 14.7 ± 6.0 nmol/L, ($P > 0.05$).

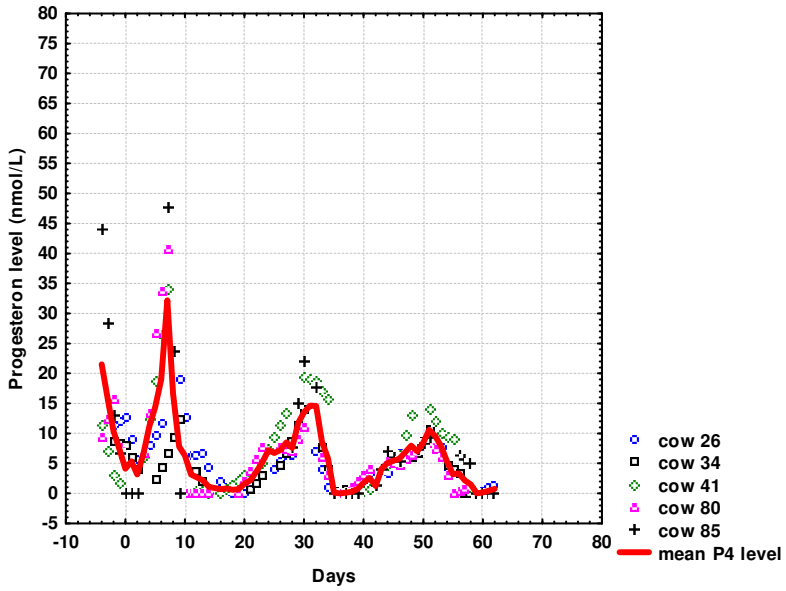
There were no significant differences between the number of days of the first and second cycles in the groups 1 and 2.

Days between the first and second luteal phase tended to be higher but non-significant.

There was a close relationship between the number of corpora lutea and serum P4 concentration ($r = 0.792$, $P < 0.0001$).

i. Serum P4 level regression curve of donor of group 1 (normal cows, Figure 1)

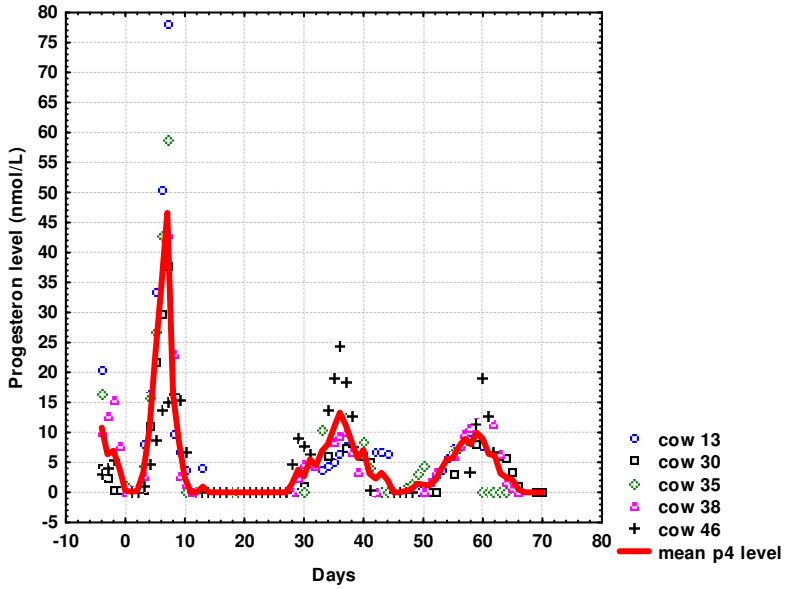
Figure 1. Average P4 concentrations in donor cows that returned to normal cycle immediately after the embryo collection



At the embryo collection, we found 13.6 ± 14.7 CL on the ovaries. The P4 level at the embryo recovery was 30.7 ± 14.7 nmol/L and in P4C1 and P4C2 cycles were 14.7 ± 6.0 and 10.0 ± 2.5 nmol/L. Number of days of L0 wave is 2.2 ± 0.8, the L1 was 17.6 ± 2.9 days long and L2 was 18.2 ± 1.64 days and the interval between the two cycle was 1.6 ± 0.6 days.

ii. Animals returned to normal cycle a short atretic phase after the embryo recovering (Figure 2)

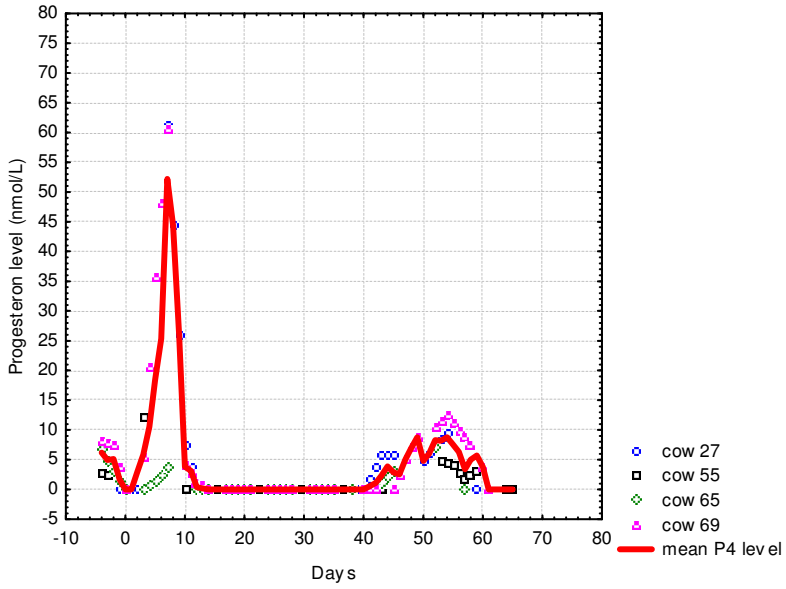
Figure 2. P4 concentrations in the cows having short regression phase



In average, 13.4 ± 8.3 CL-s were counted on the ovaries at the embryo recovery. There was a short atretic phase after the embryo collection (11.0 ± 1.9 days) which was followed by the first (15.6 ± 2.5 days) and the second (17.4 ± 2.7 days long) luteal phases. Between the two cycles 3.6 ± 1.1 days long interval was found. P4 concentration in the serum was 46.6 ± 23.5 nmol/L in the first (12.3 ± 7.0), and the second cycle was 11.4 ± 2.5 nmol/L.

iii. Group of animals returned to normal cycle with a long atretic phase after the embryo recovery (Figure 3)

Figure 3. Regression curve for the cows having long acyclic period



For these cows, we counted mean 17.5 ± 11.0 CL-s on the ovaries when the P4 concentration was 52.2 ± 34.0 nmol/L followed a 28.8 ± 2.2 days L0. The L1 was 18.8 ± 2.1 days long and the P4C1 was 8.4 ± 2.1 nmol/L.

iv. Group of animals with cystic ovarian formations and no cycle observed

Cystic ovarian formations were rectally palpated, blood samples evaluated and found that in cows 25, 66, 76 and 86 the P4 levels were in baseline and in cows 50 varied between 6 and 12 nmol/L and 71 altered between 5 and 10 nmol/L.

Discussion

In heat stressed environment, cystic ovarian formation occurs in low incidence in Nelore zebu (Resende et al., 1972) or other heat stress resistant tropical cattle (Basile and Megale, 1974) but in European breeds they can be found in 30% (Youngquist, 1986) or 18.9% (Mujuni et al., 1993). These results are comparable with our study, our donor cows showed cystic degeneration in 30% case after superovulation. This phenomenon may occur due to the excessive dose of FSH treatment applied in heat-stressed condition.

We compared plasma P4 concentration with superovulation result in day 7 and found significant ($P < 0.0001$) elevation of serum P4 level, in accordance with the number of CL on ovaries which was in agreement with the finding of Yadav et al. (1986). For prediction of embryo production, there was no significant correlation between the P4 blood concentration and embryo collection results (Solti et al., 1978).

This day, mean P4 level was 39.0 ± 27.1 nmol/L, which is similar to the previously reported 39.4 nmol/L (Kanuya et al., 1997). Observing the P4 concentration at the embryo collection, there was no effect on the return rate to the normal cycle, animals having 30.7 ± 14.7 nmol/L showed regular cycle immediately after embryo recovery and others having 30.8 ± 33.8 nmol/L developed cysts.

Most of the available data indicate that the duration of the estrus cycle is about 21 days, even in tropical environment (Jaiswal et al., 1979, Sharma et al., 1984). Other authors in a Nigerian study (Zakari et al., 1981) and in a Tanzanian case (Kanuya et al., 1997) published that length of the estrus cycle was longer in hot environment. They results are not confirmed with those presented in this paper. We found that after the embryo recovery, the P4 phase was 17.2 ± 2.7 days with 2.6 ± 1.4 days P4 zero level in the first cycle and 17.8 ± 2.2 days in the second cycle. Our results suggest a shorter estrous cycle than previously estimated. The discrepancy in these findings could be due to difference in response to elevated temperature in different breeds.

In an earlier paper, we observed that nymphomania and irregular cycles may limits to repeated bovine superovulation programs in tropical climate (Bényei et al., 2001b).

With completed results in this experiment, we found that after embryo collection, the donor cows returned to cycle: i. immediately, when the first luteal phase was commenced (5 cows, 2.2 ± 0.8 days after); ii. with a short (5 cows, 11.0 ± 1.9 days delay); iii. after a long silencious phase, 28.8 ± 2.2 days later (4 cows) and iv. cystic formations appeared on the ovary and no cycle was observed during the study (6 cows, 60 days). As their P4 level suggests, two of them suffered lutein cyst and in 4 cases follicular theca cysts were observed. We found significant differences among the four groups, $P < 0.001$. Kanuya et al. (1997) pub-

lished in a Tanzanian study that 72% of the superovulated Jersey and Ayrshire cows returned to cycle after superovulation and only 10% showed cystic formations. These findings can be explained with the high sensitivity of the Holstein-Friesian breed and individual differences among them, which influence the hormonal balance even when they were in the same management system.

There are discrepancies in the literature regarding the P4 secretion under conditions of elevated ambient temperature. Several studies have found P4 concentration to be lower (Ronchi et al., 2001; Howell et al., 1994) in heat-stressed cows, while others have observed higher concentrations of P4 (Roman-Ponce et al., 1981; Wilson et al., 1998a; Wilson et al., 1998b; Abilay et al., 1975). In contrast, Wolfenson et al. (1995) and Younas et al. (1993) found no differences in P4 concentrations between heat-stressed and cooled cows. Another authors published P4 serum concentration changes in different seasons of the year (Badinga et al., 1994, Trout et al., 1997, Wise et al., 1988a, and 1988b). The disparity in results in these studies is probably due to several differences, including comparison of P4 concentrations in summer and winter months and difference in degree and duration on heat stress exposure. In our work, the P4 level varied between 8.4 ± 3.4 nmol/L and 14.7 ± 6.0 nmol/L which is in agreement with the study of Kanuya et al. (1997) where 14.4 ± 0.8 nmol/L was reported.

In this study we followed P4 and cycle changes up to 70 days after embryo recovery and concluded that although the P4 level of the investigated 20 cows was similar during the embryo recovery, the superovulation treatment causes cycle irregularity (long atretic phase and cystic ovaries) in 50% in Holstein-Friesian donor cows in tropical environment. Furthermore, the commencement of the new cycle was immediate in 25% of the donors; a short silent phase was detected in another 25% of the cows. Number of days of the first and second luteal phases seemed to be shorter than published previously. Our work indicates the high sensitivity of this breed to elevated thermal condition.

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

The manuscript is prepared to be published in the *Reproduction in Domestic Animals*.

Summary

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-]) 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.

Introduction

Early reports on nutritional influence on reproductive performance in domestic ruminants have documented numerous observations showing the close relationship between fertility, body condition and nutritional status of females (Hammond, 1927; Robinson, 1996).

Nutrition has long been known to have profound influence on reproductive performance of female cattle, but the underlying mechanism remains poorly understood. Whilst early investigations focused on the modulation of nutrition on hypothalamic–pituitary axis, more recent studies have tested the hypothesis that metabolic hormones as nutritional signals exert direct effect at the ovarian level. (Rhodes et al., 1996; Gutierrez et al., 1997).

The superovulation technique has played an important part in the genetic improvement of cattle and the production of viable embryos for various applications in biotechnology. However, the efficiency and wider use of this technique are severely limited by the fact that there are always a proportion of animals from a group respond poorly to superovulatory treatment (Armstrong, 1993). Several factors, including purity of gonadotrophin preparations, treatment regimes and ovarian status, have been proposed to be responsible for this problem. (Adams, 1994).

It has been well established that in cattle, as in most other mammalian species, ovarian function is controlled primarily by an integrated GnRH–gonadotrophin–ovarian axis (Ginther et al., 1996; Webb et al., 1999), several papers have shown that factors classically thought to be mainly involved in the regulation of metabolic processes, such as GH, insulin, and IGF, play an important part in the control of follicular development in cattle (Spicer and Echternkamp, 1995; Webb et al., 1999; Lucy et al., 1999). Metabolic hormones can act either directly to control gonadotrophin-independent stages of follicle development (Gong et al., 1996), or in synergy with gonadotrophins to modulate follicular recruitment and final development and

maturation of preovulatory follicles (Armstrong et al., 2001; 2002; Spicer and Echternkamp, 1995).

Materials and methods

Animals and superovulatory treatment

First and second lactation (at the end phase of the lactation, n = 9) and dried off (n = 14) Holstein-Friesian donor cows in the semi-arid region of Brazil were superovulated with FSHp[1]. Ovarian stimulations were started on Days 9 - 11 of the estrous cycle (estrous = Day 0). The FSH was injected twice daily over 4 days as 150, 150; 75, 75; 50, 50 and 25, 25 IU/day, respectively. On the third day of FSH treatment, 3 mL of PGF2α (225 µg D-cloprostenol[2]) was administered i.m. once in the afternoon and animals were inseminated with a single straw of frozen-thawed semen 48 and 60 h later. Embryos were recovered non-surgically on Day 7 after estrous. Donors were rectally palpated and the number of CL were counted by ultrasonograph and recorded.

The experiment was done in semiarid Brazil (Petrolina-PE, 09 °09'S latitude, 40 °22'W longitude and 465.5 m altitude) in the donor farm of the MOET project of the Company for Development of Rio Santo Francis Valley (CODEVASF). The sampling was initiated 15th of January 2000 and finished at the end of April 2000.

The donors were kept in the MOET center, housed in open stable, in pens (capacity 10 cows/pen with 15 m^2/animal) with sprinkling system and fed with freshly chopped whole corn plant, elephant grass, alfalfa hay and concentrate, according to the milk production. The cows were milked twice a day in a milking parlor with Alfa-Laval system. The embedding system was hydrolyzed bagasse of sugar cane. (Bényei et al., 2001).

At the project site in Brazil, there is little variation in the ambient temperature between the different seasons. The average temperature was 24.8 ± 1.4 °C and ranged between 31.5 ± 1.6 °C and 20.0 ± 1.4 °C. Maximum daily temperature corresponds to the Temperature Humidity Index (THI) of 75.14 ± 1.35 where the animals suffer from mild heat stress. Meteorological data was collected at 40-km distance from the project.

Sampling and hormone assays

Blood samples were collected simultaneously with embryo collection from the coccygeal vein to heparinized tubes, cooled immediately, and centrifuged (1000 g / 10 min.) within 1 hour. The plasma was stored at –20°C.

Plasma leptin concentration was quantified by a local version of the ruminant-specific, homologue, double-antibody, non-equilibrium ^{125}I-RIA of Delavaud et al. (2000, 2002). Under routine use the sensitivity of this assay (defined as the lower leptin quantity able to generate a diminution of 5% of the B/B0 ratio) was 0.49 ng/mL. The calculated inter- and intraassay coefficients of variation (CV) were 12.01, 5.50 and 6.01%, as well as 9.99, 4.51 and 5.21% in ranges of the regularly used quality control samples with "low", "medium" and "high" leptin content, respectively.

[1] Pluset, Serono, Rome, Italy
[2] Veteglan, Serono, Rome, Italy

P4 level was determined by locally developed direct ^{3}H-RIA methods used originally for human (Csernus, 1982) and equine (Nagy et al., 1998) samples, respectively, which could be validated for assaying bovine plasma (Kulcsár et al., 1996) without any modification (sensitivity: 0.27 and 0.20 Nmol/L, intra- and interassay CVs: ≤8.7 and ≤10.3%, as well as ≤4.5 and ≤8.9% in cortisol and P4 determinations, respectively). Neutralised acid-ethanol extracts of plasma were assayed for IGF-I using a heterologue ^{125}I-RIA system modified for bovine samples (Nikolic et al., 2001) (sensitivity: 0.20 Nmol/L, intra- and interassay CVs: ≤6.2 and ≤12.0%). The other laboratory procedures were based on the use of commercially available test kits of T4: ^{25}I-T4-Spec RIA coated tube kit (Institute of Isotopes Co., Ltd. Budapest, Hungary), T3: ^{125}I-T3 RIA coated tube kit (Institute of Isotopes Co., Ltd. Budapest, Hungary); BHB: D-3-Hydroxybutyrate kit, Kat. # RB 1007, Randox Laboratories Ltd, Ardmore, UK. In the entire endocrine assay systems employed in this study the binding pattern of serially diluted bovine samples was parallel to that of the standard curves, and the recovery of added known quantity of hormones varied between 95 and 106%.

Statistical methods

Three groups were formed: i. donors with unsuccessful superovulation result (number of CL 0-2); ii. animals with fear superovulation result (number of CL 3-11) and; iii. good superovulation result (No. CL 12-).

To evaluate the parameters we used one-way analysis of variance (sums of square estimated by Type VI [unique], sigma-restricted parameterisation), where the dependent variables were the investigated parameters and the categorical factor was the group of cows according to the number of CL categories.

For significant differences calculation the Duncan's test was applied.

For the presentation of the relationship among the parameters we used a linear correlation coefficient (StatSoft, Inc. (2003). STATISTICA (data analysis software system, version 6.).

Results

I. Overall leptin, insulin, thyroid hormones, BHB, P4 hormones and body weight, BCS values and number of CL, live, degenerated and total embryos as means, highest and lowest values and correlations are presented in the Table 1.

Table 1. Overall leptin, insulin, IGF-I, thyroid hormones, BHB, P4, BCS concentrations, milk kg, number of CL, live, degenerated and total embryos as means (± SD), minimum and maximum values and correlations

	*Leptin	**Insulin	***IGF-I	***T4	***T3	+BHB	***P4	BCS	Milk	CL	Live emb.	Degen. emb.	Total emb.
x	6.20	12.51	10.68	73.61	1.89	0.35	37.04	2.79	14.6	13.5	2.4	8.4	10.8
sd	1.69	2.46	3.18	8.76	0.24	0.10	26.29	0.41	4.9	9.2	3.2	7.5	8.2
n	23	23	23	23	23	23	20	23	8	23	19	19	19
min	4.12	8.71	5.51	59.66	1.51	0.23	3.74	2.00	9.4	0.0	0.0	0.0	1.0
max	9.67	16.14	14.97	87.44	2.31	0.55	87.36	3.50	24.6	34.0	11.0	28.0	28.0
Correlations													
Leptin		0.5077	0.404					0.5407					
Insulin						0.3215	0.2688						
IGF-I				0.4443	0.3727	0.3162	0.5871			0.5778			
T4					0.8763	0.3115			0.3361				
T3						0.3553							
P4										0.6282			0.3585
CL												0.3662	0.5125
Emb. deg.													0.8504

* values presented in ng/mL

** values presented in µIU/mL

*** values presented in Nmol/L

+values presented in Mmol/L

Low correlations are not seen. The results indicate correlations between leptin and insulin, IGF-I and BCS, between insulin and BHB and P4. IGF-I was correlated with highest number of other hormones, e.g. T4, T3, P4, BHB and number of CL. High correlation was between P4 level and number of CL.

II. Univariate and multivariate test of significance was done for leptin, insulin, IGF-I, T4, T3, BHB, P4 and number of live embryos on three groups, according the number of CL. Body weight, BCS, milk production, and number of degenerated embryos were also considered. We found significant effect of groups on IGF-I, P4 and live embryos (Table 2.).

Table 2. Least squares means (LSM ± SD), univariate and multivariate tests of significance for number of CL, concentrations of IGF-I, P4 and number of live embryos on non-responding (No CL = 0-2) responding (No CL = 3-11) and well responding (No CL = 12-) donors

Groups of cows, according to the CL number	Number of donors	Number of CL	IGF-I, Nmol/l	P4, Nmol/l	Number of live embryos
1 (0-2)	4	1.25 ± 0.96	6.25 ± 0.84	5.03 ± 1.32	-
2 (3-11)	6	7.00 ± 2.19	9.78 ± 3.05	23.57 ± 14.42	0.00 ± 0.00
3 (12-)	13	20.31 ± 5.66	12.46 ± 2.03	54.81 ± 19.54	3.46 ± 3.33
All groups	23	13.52 ± 9.21	10.68 ± 3.18	37.04 ± 26.29	2.37 ± 3.18
*P-value		<0.001	<0.001	<0.001	0.023
**1-2		0.198	0.056	0.279	-
**1-3		<0.001	<0.001	0.002	-
**2-3		<0.001	0.059	0.022	0.023
***1	4	1.05 ± 2.50	6.65 ± 1.16	7.06 ± 8.40	-
***2	6	6.88 ± 2.11	9.26 ± 0.98	19.04 ± 7.53	0.60 ± 1.28
***3	13	20.28 ± 1.39	12.16 ± 0.64	52.75 ± 4.92	3.11 ± 0.84

*Effect of CL group
**Approximate probabilities
***Effects in multivariate test (values in LSM ± SEM)

Leptin, insulin, T4, T3 and BHB concentrations have no effect on superovulation result, however, as the multivariate analysis shows, the CL group has significant effect on T4, T3, BHB and P4 concentrations in 1-2, 1-3 and 2-3 groups. These results are due to the consequence that age group has significant effect on T4, T3, BHB serum concentrations. BCS and proportion of degenerated and total number of CL did not alter according to the three groups. Body weight also does not effect on the number of CL, however, the multivariate test shows significant influence on the superovulation result (P = 0.006).

III. Univariate and multivariate tests of significance were executed according to the milking categories (dried up or milking donors) and we found that insulin, T4, T3 and BHB serum concentrations changed significantly (Table 3.).

Table 3. Least squares means (LSM ± SD), univariate and multivariate tests of significance for insulin, IGF-I, T4, T3, BHB and P4 concentrations on milking and dried-up donors

Groups of cows according to milk categories:	Number of donors	Insulin µIU/mL	T4 Nmol/l	T3 Nmol/l	BHB Mmol/l
(1) Milking	8	11.01 ± 2.32	64.74 ± 3.30	1.67 ± 0.12	0.43 ± 0.12
(2) Dried-up	15	13.31 ± 2.20	78.35 ± 6.79	1.99 ± 0.21	0.32 ± 0.07
*P-value		0.029	<0.001	<0.001	0.011
**1	8		63.67 ± 2.12	1.63 ± 0.07	0.44 ± 0.04
**2	15		78.37 ± 1.78	2.02 ± 0.06	0.33 ± 0.03

*Effect of group Milk
** Effects in multivariate test (values in LSM ± SEM)

In association with IGF-I and P4 blood level, there were no significant changes in the milk group 1 and 2, but, overall groups show significant differences (IGF-I; $p = 0.51$ and P4; $p = 0.107$). Leptin serum concentrations, body weight, number of CL and embryo gain results did not differ in the dried off and lactating groups.

IV. We analyzed the above parameters in the Age group and found that there were no significant differences between the Group 1 and Group 2. Only body weight changed significantly in the two groups (Table 4.).

Table 4: Least squares means (± SD), univariate and multivariate tests of significance for body weight on heifers and cows

Groups of cows according to age categories:	Number of donors	Body weight Kg
(1) Primiparous	14	530 ± 47
(2) Cows with 2 or 3 calves	9	615 ± 84
*P-value		0.005
**1	4	535 ± 19
**2	6	624 ± 25

*Effect of group Age
**Effects in multivariate test (values in LSM ± SEM)

However, overall Age group has significant effect on IGF-I and P4 concentrations, separately; differences in both, Group 1 and 2 were not observed.

Discussion

The objective of this study was to evaluate the effect of heat stress on serum concentration of metabolic hormones and P4 and evaluate their effect on the superovulation result in Holstein-Friesian cows under tropical environment.

In cows, IGF-I serum concentration at the calving as published by Watches et al. (2003) was 140 ng/mL in primiparous and 80 ng/mL in multiparous cows, which decreases to 70 ng/mL and 40 ng/mL soon after the delivery that is maintained throughout the lactation. Spicer et al. (1990) published that serum IGF-I was 60 ng/mL at delivery which augments to 110 ng/mL 5 weeks later and did not decrease below 110 ng/mL. In our experiment, we found the overall 10.68 ± 3.18 Nmol/l that corresponds to 81.17 ng/mL.

Circulating insulin profile was reported to be 0.45 ng/mL (12.15 µIU/mL, Taylor et al., 2003), 0.46 ± 0.01 ng/mL (12.42 µIU/mL), in low and 0.34 ng/mL (9.18 µIU/mL) in high genetic

merit cows (Gong et al., 2002), 10.9 μIU/mL (Chilliard et al., 1998), 9.4 μIU/mL (Rukkwamsuk et al., 1999). Itoh et al. (1998) reported that insulin serum concentration is higher in elevated (28°C) than thermo neutral (18°C) environmental temperature (18.3 μIU/mL vs 13.2 μIU/mL) which is in contrast to our study, in our Holstein cows we found 12.51 ± 2.46 μIU/mL which is similar to that the same author was found in thermo neutral environment.

In the literature, there is a disagreement about the P4 level, in tropical environment. Authors reported that with the augmented temperature, P4 level is higher (Roman-Ponce et al., 1981; Wilson et al., 1998a; Wilson et al., 1998b; Abilay et al., 1975), others found decreased P4 serum concentrations in the hot ambient (Ronchi et al., 2001; Howell et al., 1994). Wolfenson et al. (1995) and Younas et al. (1993) reported equal P4 serum concentrations in moderate and elevated ambient temperature. In our work, we found that the P4 level vary between 8.4 ± 3.4 Nmol/L and 14.7 ± 6.0 Nmol/L which is in agreement with the Tanzanian study (Kanuya et al., 1997) where 14.4 ± 0.8 Nmol/L was reported.

BHB was published to be 0.72 Mmol/l (Taylor et al., 2003), 600 μmol/l (Reist et al., 2000) and 0.47 Mmol/l (Rukkwamsuk et al., 1999). In our test we found 0.35 ± 0.10 Mmol/l, thus, we conclude that our experimental animals were not in negative energy balance. In an adequate feeding regime the BHB depends on the lactating and pregnancy status of the herd. In our milking and dried off cows we found significant differences, however, it is below the hyperketonaemic status (Andersson et al., 1991).

Thyroid status was reported to have an effect on ovarian response to exogenous FSH (Bernal et al., 1999), thus, hypothyroid cows have greater responses compared the control donors. In another study de Moraes et al. (1998) concluded that thyroid hormones (T3 and T4) do not seem to exert any direct effect on ovarian follicular dynamics or luteal function. In our study, we did not found differences in thyroid concentrations between the unsuccessfully and successfully superovulated and the superovulated and well-superovulated donors. In association with milk categories, there were significant differences in dried off and lactating groups. Our result is in accordance with other papers. In their review, Huszenicza et al. (2002) showed that higher thyroid hormone concentrations are measured in dry than lactating cows. The same hormone relations were reported in two Texan studies (Thrift et al., 1999a and Thrift et al., 1999b) and concluded that non-lactating Brahman heifers had higher thyroid concentrations than first lactation Brahman cows (T3 = 2.1 ± 0.2 vs. 1.6 ± 01 and T4 = 76.0 ± 4.1 vs. 50.8 ± 4.0 ng/mL). Spicer et al. (2001) reported a possible influence of the insulin on regulatory function of thyroid hormones on the ovary. In an in vitro study, in the presence of insulin, thyroid hormones augmented the P4 production in bovine theca cells. Comparing our results with this experiment, however, it should be expected, we did not found correlation between insulin and thyroid hormone concentrations. T3 was published to be stable throughout the cycle: 1.3 ng/mL, (Bernal et al., 1999), 1.6 ng/mL (De Moraes et al., 1997) and 2.1 ± 2 ng/mL (Thrift et al., 1999a), we found overall 1.89 ± 0.24 Nmol/l (1.28 ng/mL). Similar to T3, T4 serum concentration was reported also stable at 50-60 ng/mL (Bernal et al., 1999; De Moraes et al., 1997) and 76.0 ± 4.1 ng/mL (Thrift et al., 1999b). In this study, we found overall 73.61 ± 8.76 Nmol/l (57.19 ng/mL) values in the blood.

Insulin has an important part in the control of ovarian follicular development in cattle (Gong, 2002), however, the same author concluded (2002) that insulin is stable throughout the superovulatory process (effect of time on superovulatory treatment $P > 0.05$) indicating that this hormone does not have effect on superovulatory result. In our work we found no significant differences between hormone concentrations on non responding cows (CL = 0-2), responding cows (CL = 3-11) and well responding cows (CL 12-). According to the milking categories, significantly higher concentrations were found in dried-up group than the lactating one. Lomax et al. (1979) analyzed the insulin levels in lactating and non lactating cows and concluded that insulin secretion in the dairy cow, in response to an insulinotropic agent is diminished during lactation. A possible explanation of Vasilatos and Wangsness (1981) was that the level of insulin decreases with increased milk production is due to the adipose tissue mobilization. Another observation (McClary et al., 1988) was that

compared with that in the mature cows, the higher insulin concentration required by the first-lactation cows to utilize approximately the same glucose load suggested that first-lactation cows were insulin resistant.

IGF-I was found to have direct effect on increasing of small follicle number (Gong, 2002) and was implicated by Spicer et al. (1990) as a potential regulator of follicular growth and differentiation. In the present study, we found a correlation between the IGF-I secretion and ovarian activity during the superovulation. Our results demonstrate that the increase in the number of CL is significant with the change of IGF-I concentration.

Liefers et al. (2003) reported the leptin concentration 4.1 ng/mL and in another study, 7.23 ng/mL was published (Williams et al., 2002) in cows. In our trial we found similar, 6.20 ± 1.69 ng/mL overall serum level. In this work, serum leptin concentration was significantly higher in responding than non-responding cows suggesting that leptin has effect on superovulatory result. In a Japanese study, Tanizawa et al. (1997) published that leptin, in physiological concentration has direct stimulatory effect on insulin production in the pancreatic beta cells, in rats and Cases (2001) confirmed that there is an increase in plasma leptin that inhibits insulin secretion In Vivo in rats and Leury et al. (2003) published that in cows an insulin infusion causes a progressive rises of leptin serum concentration. This study is in agreement with our finding, leptin concentration correlated relatively well with insulin and IGF-I level (0.507 and 0.404), however, we found no correlation between leptin, number of CL (0.128) and P4 level (0.098). We found close correlation between leptin and BCS (0.547) which is not surprise because leptin was reported as a regulator the adipose mass (Chebab, 2000). These correlations indicate that the significantly lower leptin level of the non-responding animals may be due to the lower BCS. Thus, the direct role of leptin in the metabolic regulation of the superovulation responsibility is unlikely.

We found relatively close correlation between the IGF-I level and number of CL (0.682) and P4 (0.587) concentration at the embryo collection, which proves the connection of STH-IGF-I axis and superovulation sensitiveness of the cow. This conclusion confirms the previous result of Gong et al. (1993). This high correlation can be explained by the stimulatory effect of bST on the IGF-I production since IGF-I increases the number of growing follicles and stimulates the oocyte and embryo development (Armstrong, 1993).

During the superovulation process, two days after the first FSH treatment, a PGF2α injection is applied to eliminate the excess amount of CL-s on the ovary. As Saumande (1979) reported, serum P4 concentration increases rapidly 48 hours after this treatment and reaches the higher level at the embryo flushing and the author also found that the number of CL has significant effect on the P4 level which is in agreement to our study. As our univariate analysis shows, the change of P4 serum concentration was significant in the three CL groups, thus, high correlation between P4 level and total number of embryos was also expectable and however, this observation disagrees with the result of Solti et al. (1978) finding weak correlation between P4 blood concentration and embryo gain results.

Because of the complexity of interactions between the brain, pituitary, gonads and target organs in regulation of female reproduction system, it is vulnerable to disruption by a variety of noxious agents, including thermal stress. Surprisingly, our results did not show any changes in serum concentrations, the investigated hormones and metabolite levels were in accordance with the previous reports done in moderate climate, however, elsewhere, reduction in T4 (Collier et al., 1982a and 1982b), growth hormone (Collier et al., 1982a), and T3 (Nardone et al., 1997 and Collier et al., 1982b) and elevation in BHB (Nardone et al., 1997) serum concentrations were published in heat stressed cattle. The explanation may be that our imported Holstein-Friesian cows were well adapted for high THI values in the Brazilian semiarid region (Bényei et al., 2001).

The present study was undertaken to examine the possible relationship between the metabolite hormone serum concentrations and ovarian activity during the superovulation done in heat stressed climate. Our results demonstrate that IGF-I has a direct stimulatory effect on superovulation whereas leptin, has a direct effect on GnRH release and ovary, but no influence on superovula-

tion effectiveness. The effect of other metabolic hormones and BHB on the superovulation results not proved, however, they play an important role in the regulation of female reproduction.

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7 Resume of the dissertation

To reduce 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 introduce high genetic value cattle breed in the semiarid region settled by the Brazilian govern. Therefore, pregnant heifers were imported from Hungary and install in a newly constructed donor center in Petrolina, PE.

At the initial stage of my work, I found that

- The donor cows were disturbed 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 embryo production than lactating animals. The anabolic and other heat producing processes of milk production (pe. 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 gets higher and consequently the normal embryo development is damaged.

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 Niño phenomenon were superovulated and found that

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

The frequently done 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 a few number of donors, in different management and meteorological conditions. In my studies using sufficient number of superovulation result in standard conditions, I added the literature with a complete number of repeatability ($R = 0{,}386$).

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 ($h^2 = 0{,}234$). 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

- The later cycle disorder can not be estimated 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 showed 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

- The metabolic hormone concentrations were not modified in the serum by the tropical environment;
- 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.

8 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.

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10 List of abbreviations

AETE	Association Europenne de Transfert Embryonnaire
BCS	body condition score
bST	bovine somatotrop hormone
bTP-I	bovine trophoblast protein-I
CL	corpora lutea
FSHp	porcine folliculus stimulating hormone
IETS	International Embryo Transfer Association
IGF-I	insulin-like growth factor-I
IU	international unit
K-S	Kolgomorov-Smirnov one-sample test
LH	luteotrop hormone
MOET	multiply ovulation and embryo transfer
PGF2α	prostaglandin
P4	progesterone
SD	standard deviation
T3	tri-jodothyronine
T4	thyroxin
THI	temperature humidity index

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