Synchronization and Superovulation of Boer Goats with PGF$_{2\alpha}$ and GnRH or hCG and Parentage Analysis using Microsatellite Markers

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Dedication

To

My beloved mother and adored father

My wife and sons Ali, Bilal and Husam

Table of Contents

Chapter IV

Chapter V

Chapter VI

Chapter VII

List of Tables

List of Figures

List of Abbreviations

°C	Celsius
ADME	Absorption, distribution, metabolism and elimination of hCG
AFLP	Amplified fragment-length polymorphism
AI	Artificial insemination
AUC	Area under the curve
bp	Base pair
CIDR	Controlled internal drug release
CL	Corpus luteum
cm	Centimeter
CNS	Central nervous system
Cp1	hCG concentrations time t_1
Cp2	hCG concentrations time t_2
C_{peak}	Peak concentration
d	Day
DNA	Deoxyribonucleic acid
e.g.	Abbreviation of Latin *exempli gratiā* (for example)
eCG	Equine chorionic gonadotropin
ECLIA	Electro- Chemi Luminescence Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
E_{PP}	Probability of excluding two putative parents
ET	Embryo transfer
FAO	Food and Agriculture Organization
FGA	Flurogestone acetate
F_{IS}	Inbreeding coefficient
FSH	Follicle stimulating hormone
GH	Growth hormone
GnRH	Gonadotropin releasing hormone
h	Hour
HAP	horse anterior gonadotropin extracts
hCG	Human chorionic gonadotropin
H_E	Expected heterozygosity
hMG	human menopausal gonadotrophin
H_o	Observed heterozygosity
HWE	Hardy-Weinberg Equilibrium
i.e.	abbreviation for Latin *id est*, meaning "that is; in other words"
ICM	Inner cell mass
IGFBP	Insulin-like growth factor-binding protein system
IGF-I	Insulin-like growth factor-I
ISAG	International Society for Animal Genetics
IU	International unit
K_a	Absorption rate constant
kDa	Kilo Dalton
K_e	Elimination rate constant
LH	Luteinizing hormone
LLCs	Large luteal cells
MAP	Methyl acetoxyprogesterone
mg	Milligram
MHz	Megahertz
ml	Milliliter

mRNA	Messenger ribonucleic acid
NaCl	Natrium chloride
N_e	Effective number of alleles
ng	Nanogram
OPS	Open pulled straw
Ovsynch	Ovulation synchronisation
P	Probability
PBS	Phosphate buffered saline
PCR	Polymerase Chain reaction
PGE2	prostaglandin E2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGI2	prostaglandin I2
P_I	Probability of identity
PIC	Polymorphic information content
PKC	Protein kinase C
PLC	Phospholipase C
PMSG	Pregnant mare serum gonadotropin
PVP	Polyvinylpyrrolidone
RAPD	Randomly amplified polymorphic DNA
SEM	Standard error mean
SLCs	Small luteal cells
SSR	Simple sequence repeats
STR	Short tandem repeat
T_0	Lag time
$T_{0.5Abs}$	Absorption half life
$T_{0.5Elm}$	Elimination half life
TPA	Tripropylamine
T_{peak}	Time of peak concentration
TSH	Thyroid stimulating hormone
Vd	Volume of distribution
VNTRs	Variant number of tandem repeats
vs.	Versus

Chapter I

State of the art of embryo transfer in goats

The critical step in embryo transfer program (ET) is superovulation, which is responsible for the number of ovulations and the number of transferable embryos. One of the drawback aspects of superovulation is the variable response of donors to superovulatory protocols and premature regression of corpus luteum (CL). The extreme variability in ovulatory response to similar gonadotropin remains an unsolved problem particularly in goats and may related to estrus synchronisation ptotocols. None of the presently available means of controlling the estrous cycle in goats will bring about tight synchronization of preovulatory LH surge and ovulation. According to Baril and Vallet (1990) this is partly responsible for unsatisfactory pregnancy rates with timed insemination of estrus-synchronized does. The 'ovsynch' protocol, comprising induced ovulation, is commonly applied in the cattle industry for accomplishing fixed-time insemination and has recently been shown to be suitable for goats as well (Holtz et al., 2008). In the context of ovsynch, GnRH or hC may be used.

The effectiveness of hCG in the context of estrus control and superovulation is controversial. In cows encouraging results were achieved with the synchronization of ovulation and timed AI (Schmitt et al., 1996); in goats hCG in the context of superovulatory treatment appears to be less effective (Saleh et al., in preparation). The rationale with the deployment of hCG for induction of ovulation in the context of estrus control is the appallingly high incidence of premature luteal regression encountered with the application of GnRH (Taponen et al., 2003; Holtz et al., 2008; Saleh et al., 2009). Whereas the pharmacodynamic effect of hCG in farm animals is well documented, its pharmacokinetics has received less attention. In order to design an effective protocol for ovulation induction it appears useful to study the pharmacokinetics of injected hCG.

Intensive application of AI and embryo transfer, selection for fecundity traits such as litter size and misidentification of parentage are potential contributors to inbreeding which may negatively affect the viability and reproductive fitness of the breeding stock (Pariacote et al., 1997; Frankham et al., 2004). Drawbacks of traditional methods of paternity control i.e. progeny testing (Baron et al., 2002) have justified the application of more accurate methods. Recently, DNA technologies were introduced for molecular characterization of breeds and paternity testing in farm animals. Microsatellites have received the highest attention and have extensively been used for genetic profiling of individuals due to advantages over other DNA markers as they combine high genetic variability with nuclear co-dominance inheritance (Jarne and Lagoda 1996; Heyen et al., 1997).

1.1 Physiology and endocrinology of the estrous cycle

In regions other than the tropics, the goats are seasonally poly-estrus. Cycling begins under the influence of decreasing photoperiod. The term *'estrus cycle'* refers to the rhythmic phenomenon observed in the female involving regular period of sexual receptivity i.e. estrus. Estrus is the behavioural manifestation of sexual receptivity in the cow or doe and is characterized by the female being willing for others, male or female, to mount her (Phillips, 2002). The length of the caprine estrus cycle averages 21.5 days (Camp et al., 1983). The estrus cycle is divided into four phases: proestrus, estrus, metestrus and diestrus. More commonly, the estrus cycle is divided into two phases; the follicular and luteal phase. The estrus cycle is regulated by hormones of the hypothalamic-pituitary- ovarian axis (Hafez and Hafez, 2000; Senger, 2005). The most important endocrine events during the estrus cycle are as follows: just before the onset of estrus there is a rise in estrogen concentration. Estrogen is secreted by the growing follicles and peak values occur at the beginning of the estrus with a subsequent decline to basal values at the time of ovulation. This rise in estrogen stimulates the

hypothalamus to secrete GnRH that causes the release of FSH and the LH surge (Senger, 2005). Both gonadotrophins play an important role to induce final maturation of the preovulatory follicle resulting in increased secretion of estradiol which acts in the presence of basal levels of progesterone on specific receptors in the brain to induce sexual behaviour and releases the LH surge which in turn triggers the ovulation. The duration of behavioural estrus is quit variable among goat breeds and averages 37 hours in Boer goats (Greyling and van Niekerk, 1990a). The mean interval from onset of estrus to the preovulatory LH surge is 10.5 to 13.1 hours and the ovulation occurs approximately 24.7 hours later (Cameron et al., 1988; Greyling and van Niekerk, 1990a).

1.2 Follicular waves

The process of follicular development from primary follicle stage to ovulation or atresia is known as follicular dynamics. Sequential ultrasonic inspections of ovaries, in cattle, have revealed that the follicular development during the estrus cycle occurs in a wave-like pattern; usually two and three waves are predominant but also one and four waves of follicular waves were reported (Aerts and Bols, 2008). Comparable follicular waves were also observed in goats with 1 to 4 follicular waves (Medan et al., 2003a), 2 to 4 follicular waves (de Castro et al., 1999) and 2 to 5 follicluar waves and the four follicular waves are the predominant pattern in goats (Rubianes and Menchaca, 2003). Studies have shown that 95% of bovine estrous cycles contain two or three waves (Lucy, 2007). Each follicular wave involves the following phases: recruitment, selection, dominance and finally ovulation or atresia (Fortune et al., 2001; Aerts and Bols, 2008). Follicular recruitment is usually preceded by a transient rise in peripheral FSH concentrations. A single follicle from the pool of recruited follicles is selected and becomes the single large dominant follicle, which in turn, continues to grow and suppresses the growth of other smaller follicles (Savio et al., 1993; Fortune et al., 2001). In

polytocous species such as pigs, sheep and goats multiple follicles are selected. Therefore, follicular dominance in these species is less apparent than in cattle, and is more common during waves 1 and 4 than during waves 2 and 3 (Ginther and Kot, 1994). Dominance is a mechanism in which a single follicle (the dominant follicle) or several follicles undergo a rapid development in an environment where the growth and development of other follicles is suppressed (Fortune et al., 2001; Lucy, 2007). Follicular growth to diameters greater than 3 to 4 mm is dependent upon FSH, but large antral follicles (about 7 to 9 mm diameter) transfer their gonadotrophic requirements to LH (Webb et al., 2004).

1.3 Estrus synchronisation

The goats are seasonal breeders and exhibit estrus only during few months of the year. Methods of induction and synchronisation of estrus can be divided into 2 main groups: hormonal and non-hormonal methods. The non-hormonal methods include manipulation of photoperiod and the male effect. The onset of ovarian cyclicity in the goats is dependent upon changes in the hours of daylight. The doe is stimulated to ovarian cyclicity by the effect of decreasing of photoperiod. Manipulation of photoperiod may increase the reproductive performance in goats (Robin et al., 1994). Exposure to males after a period of isolation can be used for induction and synchronization of estrus during the breeding and non-breeding season without additional treatments in goats (Veliz et al., 2002; Whitley and Jackson, 2004). The physiological basis for this response is due in part to smell and sight and the bucks don't need to be in contact with the does to exhibit this effect (Senger, 2005). However, a direct contact with the buck increases the response (Chemineau, 1987). The presence of a buck can exert a positive effect upon the ovarian activity during the transition from non-breeding to breeding season (Romano, 1998). The hormonal methods are intended to shorten the life span of an existing corpus luteum by administering of an exogenous luteolysin, or to simulate the corpus

luteum function by administering progestogens for many 9 to 19 days. Alternatively, esrtus synchronisation may be achieved by manipulation of both follicular and luteal phase through GnRH in combination with $PGF_{2\alpha}$.

1.4 Principles and methods of estrus/ovulation synchronisation

1.4.1 Induction of corpus luteum regression

Secretion of $PGF_{2\alpha}$ by the endometrium of nonpregnant doe terminates the luteal phase by causing regression of the corpus luteum (CL) and initiates a new estrus cycle. Prostaglandin $F_{2\alpha}$ or one of its analogs can be effectively used to synchronize estrus in cycling goats during the breeding season (Wildeus, 2000; Goel and Agrawal, 2005). Early studies have revealed that an injection of $PGF_{2\alpha}$, or one of its analogs, during the mid-luteal phase of the estrus cycle can induce a premature CL regression and does, therefore, can be expected to exhibit estrus symptoms approximately 50 hours later (Bretzlaff et al., 1980; Bretzlaff et al., 1983). In cattle, this treatment is effective only between days 5 to 16 after estrus to regress the CL (Wiltbank et al., 1995) and in goats between days 4 to 16 of the cycle (Holtz, 2005). This is followed by an augment in secretion of estradiol-17 β and gonadotropins culminating in the preovulatory surge of LH and finally ovulation. The drop in progesterone concentrations occurs rapidly, consistently reaching basal levels within 30 hours after injection. Occurrence of ovulation after the $PGF_{2\alpha}$ injection can be quite variable. To achieve high synchronization rate, $PGF_{2\alpha}$ has been used to control the estrus in several different methods, such as:

- Following the identification of an active corpus luteum (by progesterone measurement, rectal examination and following estrus detection).

- The two $PGF_{2\alpha}$ injections protocol, 10 to 11 days apart, was reported in many studies with no adverse effects on fertility (El-Amrawi et al., 1993; Kumar and Thomas 1994; Holtz, 2005; Khanum et al., 2006). This was designed to synchronize groups of

animals cycling at random without prior knowledge of their accurate ovarian status. Artificial insemination is performed either 2 times, three and four days after the second injection of $PGF_{2\alpha}$, or animals may be bred at observed estrus. At the time of the first injection a portion of animals will have active CLs and be responsive to $PGF_{2\alpha}$, i.e. between days 5 and 16 of the cycle. These will experience a premature regression of CL in response to the first injection of $PGF_{2\alpha}$. Therefore, they will show estrus and ovulate after four days or later. At the time of the second injection of $PGF_{2\alpha}$ (11 days later) all animals will have active CLs and will response to the second $PGF_{2\alpha}$ (i.e. between day 5 to 8 of the cycle). The cows or does that did not response to the first $PGF_{2\alpha}$, i.e. those between days 18 to 4 of the cycle, would be between days 8 to 15 at the time of the second injection. Therefore, in both cases, all animals will be in the responsive mid-luteal phase at the time of the second $PGF_{2\alpha}$ and may be inseminated either at a fixed time or at detected estrus. To reduce cost and to improve the pregnancy rates, a modified two-$PGF_{2\alpha}$ protocol is used. In this protocol, all animals are injected with $PGF_{2\alpha}$ on the same day and observed for estrus during the following days, all females exhibit estrus are inseminated and those not exhibit estrus receive the second injection of $PGF_{2\alpha}$ and inseminated as well.

Administration of $PGF_{2\alpha}$ is restricted to cyclic fenales during the breeding season. During the non breeding season, progesterone or one of its synthetic analogs is preferred (Holtz, 2005).

1.4.2 Simulation of a functional corpus luteum by exogenous progestogens

In this method, the function of the corpus luteum is simulated by application of progesterone or one of its analogous compounds. The release of gonadotropins is inhibited by progesterone, and, hence, the ovulation is also inhibited until progesterone is removed. If progesterone is applied for a group of females and withdrawn simultaneously, this will synchronize the estrus

and ovulation in this group. Progesterone was initially delivered for a period equal to the length of the natural luteal phase (i.e. 18 to 21 days). This period is long enough for corpora lutea to undergo timely regression in all animals no matter what stage of the cycle the animals were at the outset (Holtz, 2005). Long-term progesterone treatments (18 to 21 days) resulted in poor fertility rates. This is partially due to the ovulation of persistent follicles which contains oocytes of reduced quality. The poor fertility may also be due to adverse effects of progestagens in the intra-uterine environment, which affect sperm transport and survival (Leboeuf et al., 2003). Short-term progesterone treatments (7 to 12 days), generally, resulted in more acceptable fertility rates, but unfortunately the synchronization rate is reduced, because in such cases, the natural corpus luteum may outlive beyond the progesterone removal. Therefore, it is crucial to include a luteolytic agent in combination with short-term progesterone treatments in order to get rid of any natural corpus luteum. This technique is applicable for cycling and acycling does during the breeding and non-breeding season, but in this case, ovulation induction is required e.g. administration of 500 to 700 IU eCG (Corteel et al., 1988; Wildeus, 2000; Pierson et al., 2001). Equine CG is administered either upon progestagen removal or 48h before (Ritar et al., 1989; Greyling and van Niekrik, 1990b). Progestagens can be delivered through:

- Intravaginal sponges impregnated with progesterone (i.e. flurogestone acetate FGA or methyl acetoxyprogesterone MAP). These sponges contain 30, 45 and 60 mg FGA or 60 mg MAP. They are inserted over a period of 8 days (Greyling and Van Niekerk, 1991), 11d (Freitas et al., 1996; Freitas et al., 1997; Pintado et al., 1998), 16 days (Battye et al., 1988) and 21 d (Cairoli et al., 1987). Ninety eight percent of treated does exhibited estrus between 24 to 72 hours after sponge removal (Baril et al., 1993). In other study, about 91% of does ovulated by 54 hours after sponge removal during the breeding season (Pierson et al., 2001). Vaginal sponges are not preferred, since

they may adhere to the vaginal wall, causing discomfort and other problems for the female at removal or they may be lost before the end of treatment (Holtz, 2005).

- Controlled internal drug release (CIDR) devices: This device is made of progesterone-impregnated medical silicone elastomers and used for 16 to 20 d (Ritar et al., 1989).

- Implants impregnated with norgestomet, a potent synthetic progestagen, are inserted under the skin of upper side of the ear for 9 d (East and Rowe, 1989) and 11 d (Bretzlaff and Madrid, 1989; Freitas et al., 1997).

Currently, from a practical point of view, CIDR and ear implants are more preferable than the vaginal sponges, especially for small or nulliparous does (Holtz, 2005). Comparison between vaginal pessaries and ear implant as estrus synchronizing agents have revealed no significant differences in terms of prevalence of estrus, time of onset of estrus or pregnancy rates (Bretzlaff and Madrid, 1989; Pendleton et al., 1992a). In a comprehensive study, no significant differences were recorded among $PGF_{2\alpha}$-based and progestagen-based treatments for estrus synchronisation in goats (Kusina et al., 2000). Fixed-time AI in progestagen-based synchronisation treatments is performed, relative to the time of progestagen removal, either once 43 hours or twice at 30 and 50 hours (Corteel et al., 1988; Baril et al., 1993), 40 to 48 h after progestagen removal (Ritar et al., 1989). In both estrus synchronisation strategies, the emphasis is placed on controlling or mimicking luteal function to control the time of estrus, and thus the ovulation. These two principles to cycle control are the basis for commercially available products that effectively synchronize estrus.

1.4.3 GnRH in combination with prostaglandin $F_{2\alpha}$

This strategy, is called Ovsynch protocol, was designed to reduce the variability in the time of ovulation permitting AI to be performed at a fixed-time (Pursley et al., 1995). In this protocol either gonadotropin releasing hormone (GnRH) or human chorionic gonadotropin (hCG) is

commonly used. GnRH and hCG have been shown to be effective for the synchronisation of LH surge and ovulation. GnRH is injected at a random stage of the estrus cycle (day 0), followed by an injection of $PGF_{2\alpha}$ on day 7 and a further GnRH injection 48 hours later. Fixed-time AI is performed 16 hours later. The first GnRH injection is designed to either:

- Manipulate ovarian follicular development by ovulating and/or luteinizing the existing dominant follicle and initiating the emergence of a new follicular wave with a new dominant follicle, or

- It would be injected during a period of time in the estrus cycle when a new follicular wave, with the presence of active corpus luteum, was forming spontaneously so that it is still responsive to prostaglandin 7 days later.

- The second GnRH injection is designed to synchronize ovulation further by synchronizing the LH surge (Pursley et al., 1995). Peters and Pursley. (2003) found that 'Ovsynch', with the second GnRH being given on day 9.5, was effective and suggested that the major role of the first injection appeared to be the extension of the cycle in late luteal phase cows and that the second GnRH injection was the most critical in determining the synchrony of ovulation.

1.5 Superovulation

Superovulation is the hormonal manipulation of ovaries to increase the development rate of subordinate follicles that would become naturally atretic by overcoming the effects of the dominant follicle (Mapletoft et al., 2002). More ovulations than normal rate may be achieved through either an exogenous gonadotrophic stimulation at a particular stage of follicular development to overcome the natural mechanism that would normally allow only one follicle to become dominant followed by control of luteolysis or a removal of the original dominant follicle (Bergfelt et al., 1997; Mapletoft et al., 2002). Gonadotropins are administered either

toward the end of estrus cycle or around the end of estrus synchronisation treatment (Ishwar and Memon, 1996). The majority of donors show the best superovulatory response when superovulatory treatment is initiated between days 8 and 12 of the cycle during the presence of full active CL (Bergfelt et al., 1997). Ultrasonic inspections of the ovaries had shown that at around this time the second or third follicular wave emerged (Bo et al., 2002; Aerts and Bols, 2008). Therefore, synchronisation of estrus cycle or more precisely the follicular waves increases the ovulation rate and reduces the variability in ovulatory response (Menchaca et al., 2010). Superovulation is still not a well controlled technique. Variable response was observed among donors; about 20% of donors have yielded no transferable embryos, another 20% have yielded 1 to 3 transferable embryos. The best response was obtained from about one third of the donors and yielded 5 to 12 transferable embryos (Seidel and Seidel, 1991; Greve et al., 1995). The number of transferable embryos averages 6 (Armstrong, 1993). Superovulation is traditionally induced using equine chorionic gonadotropin (eCG) (previously called PMSG: pregnant mare's serum gonadotrophin) and follicle stimulation hormone (FSH) extracted from domestic animal pituitaries, particularly from the pig's pituitary. Other hormones were used, with less intensive, to induce superovulation such as (hMG: human menopausal gonadotropin) extracted from women post-menopausal urine (McGowan et al., 1985), and HAP: horse anterior gonadotropin extracts (Staigmiller et al., 1992). Either multiple or single injection of HAP can also be used to induce a satisfactory superovulatory response comparable to that induced with FSH in cattle (Staigmiller et al., 1992). Alternatively, superovulation may be induced by means of immunization against endogenous inhibin.

1.5.1 Equine chorionic gonadotropin (eCG)

Equine CG is a glycoprotein, secreted by specialized trophoblast cells which invade the maternal endometrium between days 40 to 130 of gestation in mares, consists of 2 chemically

dissimilar α- and β-subunit. The β-subunit consists of 149 amino acids and is identical with the β-subunit of equine LH (Pineda et al., 2003). It has, among other gonadotropins, a unique property that possesses both FSH and LH biological activities (Mapletoft et al., 2002). The β-subunit is responsible for this dual action. Furthermore, eCG has a very long half-life which extends to 5 days due to its high content of carbohydrate side chains and sialic acid which influences liver degradation (Betterigde and Rieger, 1993). The conventional protocol of superovulation in goats involves administering a single dose of 750 IU (Pendelton et al., 1992b) to 1200 IU of eCG during the mid-luteal phase of the estrous cycle (Kumar et al., 1992; Espinosa-Marquez et al., 2004). In cattle, the dose of eCG averages between 2000 to 3000 IU (Hahn, 1992). A luteolytic dose of $PGF_{2\alpha}$ or an analog is administered simultaneously or 2 to 3 days later to artificially induce the regression of CL. The donor is expected to show estrous symptoms 2 days after prostaglandin injection. The advantages of using eCG are:

- its availability in large quantities for a low cost.
- a single dose of eCG induces successfully superovulation compared with the multiple injections of FSH (Alfuraiji et al., 1993) due to its half-life being longer than FSH (Ishwar and Memon, 1996).

Whereas, the disadvantages are:

- Due to its prolonged half-life, it causes a continuous growth of a second postovulatory follicular wave, which secretes high levels of estrogen for a long time after estrus (Monniaux et al., 1984; Ishwar and Memon, 1996; Mapletoft et al., 2002). This may have a deleterious effect on early embryonic development and reduce the quality of recovered embryos.

- eCG induces the formation of eCG-antibodies, which in turn, reduces or inhibits ovarian response to further superovulatory treatment with eCG (Roy et al., 1999; Herve at al., 2004).

- Increases the number of large follicles that fail to ovulate (Ishwar and Memon, 1996; Mapletoft et al., 2002). This may be due to higher levels of estrogen which persisted longer in eCG- treated than FSH- treated does.

- Increase the incidence of premature regression of the induced corpus luteum, resulting in short estrus cycles (Amoah and Gelaye, 1990).

To alleviate the negative effects of eCG as superovulatory agent on the superovulatory response and embryo quality, four strategies were proposed:

- Administration of eCG antibodies to neutralize eCG molecules. To get the best results, anti-eCG should be administered 6 to 8h after the preovulatory LH surge to suppress the continuous growth of follicles after eCG-administration (Alfuraiji et al., 1993; Dieleman et al., 1993; Vos et al., 1994; Mapletoft et al., 2002). This combination of eCG and anti-eCG increased ovulation rate, decreased number of unruptured follicles and decreased number of cysts. The beneficial effect of giving eCG- antiserum could be variable, because of the variability of timing the LH-surge (Callesen et al., 1992).

- Administration of progestins after mating (Cervantes et al., 2007).

- Administration of luteotropic hormones (hCG or GnRH) 84h after the onset of estrus (Saharrea et al., 1998).

- Administration of compounds that inhibit prostaglandin synthetase such as the aspirin-like drugs or meclofenamic acide (Flower, 1974; Cooke and Homeide, 1983).

1.5.2 Follicle stimulating hormone (FSH)

Follicle stimulating hormone is a glycoprotein extracted from the domestic animal pituitaries. To induce superovulation, multiple consecutive injections are required due to its short half-life, which is approximately 5 hours and disappears within 10 to 12 hours (Demoustier et al., 1988; Mapletoft et al., 2002). The 2 most acceptable protocols comprise 6 or 8 injections of FSH twice daily in descending doses over 3 or 4 days. Administration of decreasing doses of FSH has proved to give better response than the administration of equal doses (Torres et al., 1987). Prostaglandin $F_{2\alpha}$ or one of its analogs is administered simultaneously to the last 2 injection of FSH to induce the lysis of the corpora lutea to allow for precise timing of the onset of estrus and ovulation (Mapletoft et al., 2002). Purity degree of FSH preparations is of crucial importance since all FSH preparations are pituitary extracts and contain variable amounts of LH (LH:FSH ratio) (Kanitz et al., 2002). The LH:FSH ratio affects the superovulatory response; the high LH content in gonadotropin preparation reduces the superovulatory response and affects the fertilization rates and embryo quality (Kelly et al., 1997; Kanitz et al., 2002). However, LH plays an important role in follicular development. Early studies indicated that LH should be delivered in FSH preparations used for superovulation with a FHS:LH ratio of approximately 5:1 (Murphy et al., 1984). Higher ratio in goats (40% LH) proved to be close to the optimum range (Nowshari et al., 1995).

A series of comparisons between eCG and FSH as superovulatory agents have been performed and revealed evidence that FSH is superior (Tsunoda and Sugie, 1989; Pendleton et al., 1992b; Ishwar and Memon, 1996). Although the majority of superovulations were carried out using of multiple injections protocol, others attempted to reduce the number of injections of FSH. A single subcutaneous injection of FSH dissolved in polyvinylpyrrolidone for superovulation has been reported with encouraging results. This method is capable of achieving a similar profile to that obtained with well established multiple-injection procedure

(Yamamoto et al., 1993; Takedomi et al, 1995; Satoh et al., 1996). An alternative approach to reduce the dose and number of FSH-injections to induce superovulation is the administration of a single dose of FSH combined with a low dose of eCG (Watanbe et al., 1998). In goats, a single injection of FSH combined with eCG resulted in responses comparable to those of multiple injections of FSH (Batt et al., 1993). However, others didn't find benefit in a combined gonadotropin treatment (Peebles and Kidd, 1994). There is an evidence supporting that a higher and more consistent response can be obtained with FSH preparations than with eCG in terms of ovulatory response and embryos yield (Tsunoda and Sugie, 1989; Pendelton et al., 1992b). Up to date, not a single superovulatory protocol fulfils all desired expectations from the treatment. The large variability in the number of ovulations and viable embryos remains a major drawback (Holtz, 2005).

1.5.3 Immunization against inhibin

This technique was established based on the observation that the increase in maturing follicles and rate of ovulation is preceded by an overall decrease in ovarian output of inhibin and elevation in secretion of FSH. Suppression of the endogenous inhibin occurs after either passive or active immunization against inhibin.

- **Passive immunization** can be achieved by administration of inhibin antiserum i.e. anti-inhibin antibodies (Medan et al., 2003b). In this protocol estrus was synchronized by 2 injections of prostaglandin $F_{2\alpha}$ 11 days apart. On day 10 inhibin antiserum was injected and 48 hours later another $PGF_{2\alpha}$ injection was administered to induce estrus and ovulation.

- **Active immunization** can be achieved by vaccination against inhibin (Padilla et al., 2008). This protocol, as described by Padilla et al. (2008), comprises an initial injection of inhibin followed 4 weeks later by a booster injection and another 51 weeks

a second booster injection. Formation of antibodies considerably increased 2 weeks after administration.

Though ovulations and ovarian activity enhanced by immunization against inhibin (Wrathall et al., 1992; Glencross et al., 1994; Morris et al., 1993; Tannetta et al., 1998; D'Alessandro et al., 1999; Medan et al., 2003b; Padilla et al., 2008), it led to formation of inhibin antibodies accompanied by a large proportion of retained follicles and a high incidence of short estrous cycles (Padilla et al., 2008).

1.6 Ovulation induction

Ovulation induction is an effective means of helping females which do not ovulate or ovulate irregularly and involves stimulating the ovary to produce one or more oocytes. It may be accomplished using a number of different treatment regimens such as GnRH or hCG.

1.6.1 Gonadotropin releasing hormone (GnRH)

Gonadotropin releasing hormone is a decapeptide of small molecular weight synthesized and released in pulsatile manner by specific neurons in the hypothalamus and causes the release of FSH and LH from the pituitary (King and Millar, 1995; D'Occhio et al., 2000; Parhar, 2002; Senger, 2005). Binding of GnRH to its receptors causes internalization of these receptors and induces a transient insensitivity to GnRH until new receptors are synthesised and returned to the surface of the gonadotropic cells (D'Occhio et al., 2000). The GnRH receptor is a member of the large family of G-protein-coupled receptors which have seven transmembrane domains (Flanagan et al., 1997). GnRH exerts effects in other peripheral tissues including the brain, gonads and placenta (King and Millar, 1995). Its release is controlled by the positive and negative feedback mechanisms (Senger, 2005). Since it triggers the release of pituitary gonadotropins in mammals, this has justified the use of 'GnRH' or one of its analogs to

manage the animal reproduction. The physiological response of the pituitary is caused by specific pathways including phosphatidylinositol-specific phospholipase C (PLC), the release of calcium (Ca^{2+}) from intracellular stores and Protein kinase C (PKC) (Parhar, 2002). GnRH analogs can be divided into 2 categories according to the action mode:

1. GnRH antagonist competitively binds to the pituitary GnRH receptors and causes a prolonged inhibition of gonadotropin release (Heber et al., 1982).

2. GnRH agonist, which also can be divided into 2 sub-groups:

   - Decapeptide agonist is an oligopeptide containing ten amino acid residues such as Gonadorelin.

   - Nonapeptide agonist is an oligopeptide containing nine amino acid residues such as Buserelin and Fertirelin.

GnRH agonist is characterized by a higher affinity for GnRH-receptors and protects against enzyme degradation, thus, increasing half life in the circulation from 8 min to 5 hours (Thatcher et al., 1993; D'Occhio et al., 2000; Ghumman, 2006). It exerts a biological action through *'flare effect'*, followed by downregulation. Due to alteration in the chemical structure, GnRH agonists stimulate the pituitary with different potencies (Thatcher et al., 1993). According to Ghumman. (2006) a single dose of GnRH agonist increases both the release of high levels of gonadotropins from the pituitary and the number of their receptors (5 fold in FSH- receptors and 10 fold in LH- receptors). In cattle, the GnRH agonist Buserelin is 10 to 20 times more potent than Fertirelin and this (Fertirelin) is 2.2 to 10 times more potent than Gonadorelin (Chenault et al., 1990). On the other hand, continuous administration of GnRH agonist causes the opposite effect. It down-regulates or desensitizes the pituitary to endogenous GnRH preventing the release of LH surge and inhibiting the ovulation (Heber et al., 1982; D'Occhio et al., 2000; Parhar, 2002; Ghumman, 2006).

1.6.2 Human chorionic gonadotropin (hCG)

Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein of 57 kDa consisting of a noncovalently bound α- (92 amino acids) and a distinctive β-subunit (134 amino acids). The β-hCG has an 80% homology to the 121 amino acid subunit of the LH β-subunit (Norman and Litwack, 1997). Some glycoproteins hormones contain 2 side-by-side polypeptide chains (α- and β-subunits). The glycoprotein hormones hCG, LH, FSH and TSH have the same α-subunits but different β-subunits which gives a specific function of the hormone (Gupta and Dighe, 2000; Senger, 2005; De Rensis et al., 2010). Due to the similar structure of hCG and LH (Lei and Rao, 1994; Birken et al., 1996), the β-subunit of hCG is believed to interact specifically with LH receptors to stimulate the production of progesterone by the corpus luteum during the first stages of embryo development until the placenta becomes able to produce adequate levels of progesterone in the pregnant woman (usually at 6 to 8 weeks) (Norman and Litwack, 1997). Both hCG and LH can directly regulate GnRH gene expression in the hypothalamus (Lei and Rao, 1994). Receptors for LH/hCG are located in follicular theca, granulosa cells and the corpus luteum with similar binding characteristics (Henderson, 1984). hCG specifically binds to the theca interna and slowly dissociates in a biphasic process (Henderson, 1984; De Rensis et al., 2010). Binding of hCG to its receptors is a time- and temperature- dependent process (Henderson, 1984). At 37°C, specific binding to theca interna rapidly increased for the first 4 h, but thereafter proceeded more slowly to reach a maximum by 20 hours. The theca interna LH/hCG receptor shows a greater affinity for hCG than for LH (Henderson, 1984). hCG stimulates the synthesis of progesterone and maintains the CL beyond its normal life span in the cow (Litch and Condon, 1988) and has a biological half-life 12 times longer than LH (24 vs. 2 h) due to greater glycosylation rates (Birken et al., 1996; Speroff and Fritz, 2005; De Rensis et al., 2010). Early reports have indicated that hCG may be commercially used as substitute for LH to induce ovulation and CL formation in infertile

cows. hCG can initiate a normal luteinisation even in the absence of an endogenous LH surge as deduced from the normal luteal function in treated cows and ewes (Dobson, 1975; Bolt, 1979; Kamomae et al., 1989). Furthermore, it induces ovulation and formation of accessory corpora lutea when large follicles are present (De Rensis et al., 2010). The estimated time for hCG to induce the ovulation is about 36 hours after treatment in ovarian quiescent cattle (Kamomae et al., 1989). hCG may add a valuable contribution to estrus synchronization protocols by reducing the luteolytic effect of $PGF_{2\alpha}$, or even, when it is available in sufficient quantity, would prevent $PGF_{2\alpha}$-induced luteal regression (Bolt, 1979; Litch and Condon, 1988; Fonseca and Torres, 2005).

Exogenous GnRH and hCG had been used in livestock to synchronize the ovulation and improve the reproductive performance (Rajamahendran and Sianangama, 1992; Pursley et al., 1995; Schmitt et al., 1996a; Pursley et al., 1997; Santos et al., 2001; Thatcher et al., 2001; Fischer-Tenhagen et al., 2008). Though GnRH and hCG proved to be potent agents to induce ovulation (Rajamahendran and Sianangama, 1992; Schmitt et al., 1996a), hCG was superior in terms of better luteotropic effect on the original CL, formation of accessory CLs (Schmitt et al., 1996b) and progesterone production (Schmitt et al., 1996bc).

1.7 Embryo collection

Embryos are usually collected from donors 7 days after breeding or AI at this time; embryos are freely suspended in a small amount of fluid in the uterus horns. Methods of embryo recovery are divided into a surgical (Amoah and Gelaye, 1991), Laparoscopic (Mckelvey, 1986) and non- surgical procedure (Suyadi et al., 2000).

1.8 Satellite markers

Tandemly repeated DNA sequences can be categorized according to the size of the repeat-unit, into three groups: satellites, minisatellites and microsatellites (Charlesworth et al., 1994;

Ellegren, 2004). The term *satellite* came up after DNA extraction in equilibrium density gradient centrifugation when two minor satellite bands were observed (Geldermann, 2005).

- Satellite DNA, the largest repeat units ranging in size between 100 to more than 1000 bp is predominantly found surrounding the centromeric regions (Charlesworth et al., 1994; Schueler et al., 2001).

- The medium repeat-units, with a size of approximately 10 to more than 100 bp in length, are defined as **minisatellites** or as variant number of tandem repeats (**VNTR**s). In humans, minisatellites are a class of highly polymorphic GC-rich tandem repeats that are mainly located in the subtelomeric chromosomal regions (Valdes et al., 1993; Bois 2003).

- The shortest repeat units with a size of approximately 1-6 bp in length are referred to **microsatellites**, simple sequence repeats (SSR) or short tandem repeats (STR).

1.9 Definition, nature and polymorphism of microsatellites

Microsatellites are flanked by conserved sequences and generally found in non-coding regions of the genome but are relatively rare in protein-coding regions (Li et al., 2002; Ellegren, 2004). They are extremely plentiful in eukaryotic genomes, but also in prokaryotes and eubacteria at lower frequencies. Moreover, they dispersed frequently over a genome with at least one STR every 10kb of DNA sequence in eukaryotes (Tautz, 1989), whereas, they are less frequently in the human genome with at least one STR every 300 to 500 kb (Edwards et al., 1992). Regardless, microsatellites account for approximately 3% of the total human genome, where dinucleotide repeats dominate, followed by mono- and tetranucleotide repeats, whereas trinucleotide repeats are least dominant (Ellegren, 2004). The nature of polymorphism is due to high mutation rate which was estimated to be about 2×10^{-3} per meiosis in humans to 6×10^{-6} - 9×10^{-6} in *Drosophila melanogaster* (Ellegren, 2000).

1.10 Microsatellite evolution

There are two different mechanisms causing microsatellite loci to be hypervariable: strand slippage and unequal crossing-over:

1.10.1 Strand slippage occurs during DNA replication (Schlötterer and Tautz, 1992; Ellegren, 2004). A replicating DNA strand can slip one or more repeat units within a repeat resulting in formation of 'transiently unpaired bulges' or single stranded regions. If DNA replication continues on this molecule, the non-paired bases will be added or deleted resulting in altered strands. In vivo, most of these errors can be repaired, because of exonucleolytic proofreading and mismatch DNA repair enzymes, but some of them can escape repairs and become mutations (Li et al., 2002). These *'repair enzymes'* can reduce the mutation rate of microsatellites 100-1000 fold (Schlötterer, 2000). There is evidence that the addition or deletion (increase or decrease of allele size caused by mutations) usually involves a single repeat unit of a up to 4 bp sequence stretch (Valdes et al., 1993; Garza et al., 1995; Holm et al., 2001). The most widespread mutations are modifications of a single repeat unit, which allow microsatellite mutations to be interpreted as a very good approximation of a stepwise mutation process (Schug et al., 1997). The rate of slippage depends on the size of the repeat unit (highest for dinucleotides such as AAT/ATT) and on its sequence composition (lowest for GC-rich repeats such as GCC/GGC) (Schlötterer and Tautz, 1992; Valdes et al., 1993). Moreover, the mutation rate may be correlate with the repeat length. In the *Drosophila melanogaster* genome, the short length of microsatellite repeats is most likely the factor that contributes to relatively lower mutation rates compared to the numbers known for vertebrates (Schug et al., 1997; Ellegren, 2004).

1.10.2 Unequal crossing-over (gene conversion) occurs during recombination at meiosis (Valdes et al., 1993). Recombination could potentially alter the lengths of microsatellites by unequal crossing-over (Li et al., 2002). Gene conversion results in alteration of motif numbers when crossing-over occurs between misaligned microsatellites on sister chromatids of homologous chromosomes (Wierdl et al., 1997). Misalignment between sister chromosome strands occurs more easily for longer tandemly repeated sequences. Although, the strand slippage appears to be the most predominant mechanism generating microsatellite variability (Schlötterer, 2000; Valdes et al., 1993), other studies reported that in some cases a combination of strand slippage and crossing over effects/causes microsatellite stability (Li et al., 2002).

1.11 Putative roles of microsatellites

Although a large fraction of microsatellites is considered as non-functional or neutral (Charlesworth et al., 1994), they play in some cases important roles in chromatin organization, regulation of DNA metabolic processes and gene function (Schlötterer, 2000; Li et al., 2002; Ellegren. 2004). Furthermore, microsatellites may also be linked to genes that contribute to some diseases such as the STR locus TGLA116 which closely linked to the Weaver disease gene in cattle (Georges et al., 1993a). In humans, microsatellites associated with diseases are mainly located in the coding region (Ellegren, 2004).

1.12 Multiplex PCR

One of the key factors to analyze populations on a large scale is the investigation of multiple loci in one reaction tube to reduce the time and the amount of pipetting work as well as to spare DNA. The multiplex PCR techniques, is the simultaneous amplification of several microsatellite loci in a single PCR tube (Luikart et al., 1999; Tettlin et al., 1999). Prior to that

microsatellites should be selected carefully, i.e. strong clean signals and the ability to be combined with further primers. Furthermore, the multiplex PCR primers must be chosen to amplify STR with different size ranges across different chromosomes in order to avoid linkage among loci on the same chromosome and overlapping of alleles from different loci (Maudet et al., 2001). The primers are provided with different fluorescent dyes to distinguish among overlapping allele sizes. Although microsatellite multiplexing is advantageous, some factors could limit its usage such as allelic drop out and production of null alleles (Luikart et al., 1999).

1.13 Applications of microsatellites

Microsatellite loci have a wide range of applications:

- Studies of gene mapping (Barendse et al., 1993; Vaiman et al., 1996) and disease diagnostics (Georges et al., 1993a).

- Parentage and kinship testing (Luikart et al., 1999; Wenk, 2004).

- Population structure, genetic diversity and linkage studies (Vaiman et al., 1996; Saitbekova et al., 1999; Aggrawal et al., 2007).

- They are also used as markers linked to economic trait loci, such as the ovine Booroola fecundity gene, gene controlling ovulation rate in sheep (Montgomry et al., 1993; 2001; Davis, 2004) and 'horn development' in *Bos taurus* (Georges et al., 1993b). Horn development is under control of the autosomal polled locus characterized by 2 alleles: 'P' dominant over 'p' and causing the polled or hornless phenotype. There is a genetic linkage between polled locus and 2 STR markers: GMPOLL-1 and GMPOLL-2.

1.14 References
See chapter V

Chapter II

Superovulation of ovsynch-synchronized Boer goats

induced to ovulate with GnRH or hCG

Abstract

The aim of the present investigation was to devise an efficient protocol for getting a satisfactory embryo yield accomplished by combining the recently established ovsynch protocol with pFSH superovulatory treatment. Furthermore, the intention was to minimize the incidence of premature luteal regression frequently encountered in superovulated goats by substituting hCG for GnRH. A total of 51 pluriparous Boer goat does, 2-6 years of age, was subjected to superovulatory treatment and, thereafter, randomly allocated to one of three treatment groups. The does of Group 1 were subjected to an im injection of 0.004 mg of the GnRH analog Buserelin (Receptal[®]) 18 hours after the superovulatory treatment; does of Group 2 received 500 IU hCG (Chorulon[®]) and does of Group 3, 1 mL sterile physiological saline solution. Blood samples, collected every 2 h from 1 h before until 42 h after treatment, were analyzed for plasma LH concentration. With the intention to exactly determine ovulation time, in a subsample of each group ovaries were scanned ultrasonically at 2 hour intervals from 18 hours after the ovulation inducting treatment until after ovulation had taken place. Estrous does were mated and embryos were flushed non-surgically 6 or 8 d after the last mating, depending on whether morulae or blastocysts were to be collected. In GnRH-treated does the LH surge was tightly synchronized; it commenced 1.0 (SEM 0.03) h after treatment. In the hCG- and saline treated groups it commenced 11.8 (SEM 0.5) hours and 14.9 (SEM 1.2) hours after treatment, respectively ($P<0.05$). The duration of the LH surge was 7.2 (SEM 0.6) hours for the GnRH group, which was significantly shorter than the 11.2 (SEM 0.8) hours for the hCG- and 12.1 (SEM 0.6) hours for the NaCl group ($P<0.05$). Ovulation was synchronized most effectively with GnRH. With regard to number of transferable embryos (3.2 (SEM 1.2) for the GnRH-, 1.9 (SEM 1.0) for the hCG- and 4.6 (SEM 1.3) for the NaCl group) there were no significant differences. After GnRH and hCG treatment the incidence of

corpus luteum insufficiency amounted to 100% and 88%, respectively; after saline treatment it was 56%, which is significantly less (P<0.01), though still substantial. Application of ear implants compensating premature luteal regression resulted in a substantial increased in number of transferable embryos (P<0.05). This study indicates that both GnRH and hCG are suited for synchronizing ovulation in does superovulated in connection with an ovsynch protocol, permitting fixed-time insemination. GnRH, however, was more effective in synchronizing groups of does. The expected reduction in the incidence of luteal insufficiency when substituting hCG for GnRH did not materialize. Only when luteal insufficiency was compensated by providing the does with norgestomet-releasing ear implants, a modest yield of transferable embryos was accomplished.

Key Words: Superovulation, GnRH, hCG, goats

2.1 Introduction

Estrus control is a management tool helping to reduce time and effort involved with estrus detection by clustering individual estrous periods and, if combined with ovulation induction, permit timed AI. Traditionally in goats estrus is controlled by the use of progestogen-impregnated vaginal pessaries (polyurethane sponges or CIDR) combined with an injection of eCG just before or at the time of withdrawal (Corteel et al., 1988; Leboeuf et al., 1998; Freitas et al., 1997; Holtz et al., 2005). Occasionally a luteolytic dose of prostaglandin $F_{2\alpha}$ is administered toward the end of progestogen treatment (Ritar et al., 1989; Freitas et al., 1997; Fonseca et al., 2005). Especially in nulliparous does intravaginal pessaries tend to cause discomfort (Leboeuf et al., 2003; Holtz, 2005). Alternatively, prostaglandin $F_{2\alpha}$ or one of its analogs may be administered, either as a single injection or as two injections 10 to 14 days

apart (Goel and Agrawal, 2005). Prostaglandins have the disadvantage that they are only effective in the presence of functional corpora lutea, hence during the breeding season. None of the presently available means of controlling the estrous cycle in goats will bring about tight synchronization of ovulation. This is, according to Baril and Vallet (1990), partly responsible for unsatisfactory pregnancy rates with timed insemination of estrus-synchronized does. The 'ovsynch' protocol, comprising induced ovulation, is commonly applied in the cattle industry for accomplishing fixed-time insemination and has recently been shown to be suitable for goats (Holtz et al., 2008).

In goats, FSH has repeatedly been shown to be more effective than eCG for eliciting a superovulatory response; yet, FSH application is more laborious and time consuming (Tsunoda and Sugie, 1989; Nowshari et al., 1992, 1995; Yuswiati and Holtz, 1996). None of the superovulation protocols commonly in use will deliver fully satisfactory results in terms of a predictable and reliable superovulatory response (Holtz, 2005). Neither alterations of traditional protocols, such as temporary LH-suppression by progestogens or a GnRH antagonist, followed by LH or hCG administration (Krisher et al., 1994; Baril et al., 1996); hCG administration at the onset of estrus (Tsunoda and Sugie, 1989; Goel and Agrawal, 2005) nor active or passive immunization against endogenous inhibin (Dietrich et al., 1995; Medan et al., 2003; Padilla et al., 2008), delivered satisfactory solutions.

The objective of the present study was to devise an efficient protocol for getting a satisfactory embryo yield accomplished. The recently established ovsynch protocol (Holtz et al., 2008) was combined with pFSH administration, an approach that ought to, eventually, permit fixed-time insemination. Furthermore, the intention was to minimize the incidence of premature luteal regression frequently encountered in superovulated goats (Pintado et al., 1998; Saharrea

et al., 1998; Espinosa-Marquez et al., 2004; Cervantes et al., 2007) by substituting hCG for GnRH.

2.2 Materials and Methods

The experiment was conducted on 2 to 6 year old pluriparous Boer goat does from our own breeding stock in Goettingen, Germany (9º 41' E, 51º 46' N) during the breeding season (October to January). The does were group-housed in open barns with straw-bedding and outdoor concrete runs, fed a daily ration of 600 g concentrate consisting of equal parts of a pelleted diet for breeding ewes (16% crude protein, 10.2 MJ ME/kg, supplemented with 43 mg/kg Se, 12 mg/kg I and 5000 mg/kg Zn), oats and dried sugar beet pulp, and had free access to wheat or barley straw, salt lick and water. Once daily the complete flock was routinely tested for estrus with an aproned male.

Donor does were tested for plasma progesterone concentration from day 5 of the cycle onward. Does with a progesterone level exceeding 5 ng/mL were subjected to im treatment with 5.0 mg of the prostaglandin $F_{2\alpha}$ preparation Dinoprost (1mL Dinolytic[®], Pfizer, Germany) followed, seven days later, by an im injection of 0.004 mg of the GnRH analog Buserelin (1mL Receptal[®] Intervet, Unterschleissheim, Germany). Five days later does were treated with 4, 4, 2, 2, 2 and 2 armour units (AU) pFSH, supplemented with 40% pLH (Nowshari et al., 1995) administered at 12 h intervals. Along with the last two FSH injections, 5 mg Dinoprost was administered im. Thereafter, the does were randomly allocated to three groups of 17. One group received, 18 hours after the last Dinoprost injection, an im injection of 0.004 mg buserelin. Another group was, instead, treated with 500 IU hCG (Chorulon[®], Intervet, Unterschleissheim, Germany) and the third group with 1 mL of sterile physiological

saline solution. From that time onward until 6 hours after the end of estrus does were tested for estrus with an aproned adult buck every six hours (6 am, 12 am, 6 pm, 12 pm). Estrous does were hand mated twice daily as long as they would permit a male to mount. When it was noticed that a substantial portion of the does returned to estrus prematurely, of each group 11 does were provided with subcutaneous progestogen-containing ear implants (Crestar®, 3.3mg Norgestomet, Intervet, Beaucouze, France) 12 hours after the last mating. Twenty hours before embryo collection the implants were removed simultaneously with an im administration of 5.0 mg Dinoprost.

Blood samples of 5 mL were drawn via jugular venipuncture at 2 day-intervals from the first prostaglandin injection until the onset of the FSH treatment. Thereafter, sampling frequency was increased to once daily until embryo collection. Ten randomly selected does of the GnRH group, 9 does of the hCG group and 11 does of the control group were provided with permanently indwelling jugular catheters and subjected to sampling at 20 minute intervals from one hour before until four hours after the ovulation inducing treatment. Thereafter sampling frequency was reduced to once hourly for three hours and to once every two hours for the next 32 hours. Collection tubes contained three drops of Na-citrate to prevent clotting. Within less than four hours after collection the blood was centrifuged for 10 minutes at 1000 g and plasma was stored at -20°C until being assayed for progesterone content by ELISA according to Van de Wiel and Koops (1986), modified by Moeller (1991) and for LH content by ELISA according to Moeller (1991). The onset of the LH surge was defined as the time when the plasma LH concentration exceeds basal levels by two standard deviations.

From 6 hours before until 42 hours after the ovulation inducing treatment ovarian structures were monitored at 12 hour intervals with the aid of a real-time ultrasound scanner (ALOKA

SSD 500, Japan) equipped with a 7.5 MHz rectal linear array transducer. Antral follicles more than 3 mm in diameter were counted and measured with electronic calipers. The number of ovulations was deduced from the reduction in number of large follicles between two consecutive observations. With this approach ovulation rate can be closely approximated as shown by data correlating laparoscopic and ultrasonic measurement on 50 does ($r = 0.82$, $P < 0.01$) by Suyadi and Holtz (unpublished). On 4 does of the GnRH group, 3 does of the hCG group and 5 does of the control group, ovaries were inspected at 2 hour intervals to precisely pinpoint the time when ovulation occurs.

Non-surgical embryo collection was conducted as described in Suyadi et al. (2000) and Holtz et al. (2000). For obtaining morulae, does were flushed 6 days after mating, for obtaining blastocysts, 8 days after mating. Collection was preceded by an im luteolytic dose of 5.0 mg Dinoprost, administered 20 hours in advance. After ten lavages with 20 mL Dulbecco's phosphate buffered saline supplemented with bovine serum albumin per uterine horn, embryos were recovered from the flushings and inspected under a stereo microscope at X 20-50.

Data were statistically analyzed using the program SAS$^{®}$ 9.2 according to the model fitted by the GLIMMIX procedure: $Y_i = X_i\beta + Z_i\gamma + \varepsilon_i$ where, Y_i is the response variable for the ith observation (LH parameters, time of ovulation and area under the LH curve). The quantity X_i is a column vector of explanatory variables for observation i that is known from the experimental setting and is considered to be fixed (the treatment of ovulation induction). The vector of unknown coefficients β is estimated by a least squares fit to the data Y. The ε is assumed to be independent, normal random variables with zero mean and constant variance, and γ is an unknown vector of random-effects parameters (age, animal) with known design

matrix Z, and ε_i is an unknown random error vector whose elements are no longer required to be independent and homogenous. Differences between the mean values were tested for significance by *t-test* with the predicted difference PDIFF adjusted to Tukey (SAS 9.2; 2008). The area under the LH curve was calculated by the linear trapezoidal approximation as described by Jambhekar and Breen, (2009).

2.3 Results

Of 51 does treated (17 per treatment group) two, belonging to the saline control group, had to be excluded from the statistical analysis; one, because it came into estrus before being treated, the other because it responded with atypical delay (Fig. 2).

As shown in Table 1, all does responded. The time from ovulation inducing treatment to onset of estrus was 6.3 hours (SEM 1.0, range 3-17) for the GnRH group, 5.9 hours (SEM 0.9, range 3-15) for the hCG group and 6.6 hours (SEM 1.3, range 3-15) for the saline controls. The corresponding data for estrus duration were 34.2 hours (SEM 2.2, range 18-54), 40.6 hours (SEM 3.2, range 18-66) and 37.4 hours (SEM 3.4, range 14-66), respectively. None of these differences were statistically significant (P>0.05). The onset of tail flagging and immobility reflex did always coincide. At the end of estrus tail flagging outlasted the immobility reflex in 22% of the does by, on average, 7.2 hours. A significant difference (P<0.05) was detected for the proportion of does exhibiting short estrous cycles. It comprised 100% of GnRH treated does, 88% of hCG-treated does and 56% of saline treated control does.

As depicted in Fig. 1, the plasma progesterone concentration, averaging 12.5 (SEM 1.0) ng/mL at the outset of the experiment, dropped to 1.0 (SEM 0.1) ng/mL within two days after prostaglandin treatment. By the time of the first FSH injection the progesterone level had recovered to 16.7 (SEM 1.0) ng/mL. At the time of prostaglandin $F_{2\alpha}$ injection at the end of FSH treatment, within a single day a decline from 20.1 (SEM 0.8) ng/mL to 1.7 (SEM 0.1) ng/mL took effect. One day later a basal level of 1.3 (SEM 0.1) ng/mL had been reached and, since estrous symptoms occurred, it was considered day 1 of the estrous cycle. Four days after prostaglandin treatment (day 3 of the cycle) plasma progesterone had increased to between 2.8 ng/mL (saline group) and 6.8 ng/mL (hCG group), which was substantially higher than the concentrations from 0.9 ng/mL (saline group) to 1.2 ng/mL (GnRH group) recorded at the comparable stage after the previous prostaglandin treatment (day -12). On day 6 of the estrous cycle, in 8 of 17 (44%) saline-treated control does progesterone levels had further increased to 24.4 (SEM 0.7) ng/mL, whereas, in the remaining nine control does, as well as all does of the GnRH- and 15 of 17 (88%) does of the hCG-group, progesterone had dropped to basal level (Fig. 1).

In 10, 9 and 11 randomly chosen animals in the GnRH-, hCG- and saline group, respectively that had been provided with indwelling jugular catheters, LH profiles were established. These are presented in Fig. 2; the corresponding data are provided in Table 1. The does treated with GnRH exhibited a tightly synchronized LH surge commencing, on average, 1.0 hours after injection and reaching a peak of 88.9 ng/mL 2.5 hours after treatment to return to basal level 7.2 hours after commencement. In a single goat a secondary LH peak of almost similar magnitude than the initial peak made its appearance 11.7 hours later. In hCG treated does LH surges emerged, on average, 11.8 hours after injection and peaked 15.1 hours after treatment. The average duration of the LH surge was 11.2 hours. The LH surges were much less

synchronous as evidenced by a SEM of 0.50 vs. 0.03 for the GnRH group. The differences between the two groups were significantly different (P<0.05). In saline treated does onset and peak of the LH surge were 14.9 and 17.6 hours after treatment; the surge lasted 12.1 hours. The peaks were even less synchronous than after hCG treatment (SEM for commencement: 1.2), but the differences were not significant. One doe trailed the mean of the others by 18 hours (Fig. 2) but was excluded from the statistics.

Average pattern and amplitude of the LH surges recorded after GnRH, hCG and saline treatment are summarized in Fig. 3. The general appearance was quite uniform. After GnRH administration the incline was somewhat steeper, the amplitude higher (P<0.05) and the duration until return to basal non-significantly less than in the saline controls yet, due to the higher amplitude, the area under the curve was similar (P>0.05). The LH surge recorded after hCG treatment appeared to be slightly, though non-significantly, lower in amplitude than those of the other groups. Kinetic parameters for the LH surge in the respective treatment groups are presented in Table 1. The time from LH peak to half the peak concentration ($t_{1/2}$) was 1.9 hours for GnRH-, 2.6 hours for hCG- and 1.8 hours saline treated animals, respectively. The clearance rate was as high as 1.6, 2.4 and 1.8 L/hour for the GnRH-, hCG- and saline group, respectively, with no significant difference among treatment groups.

Five of nine does of the saline control group in which LH was recorded featured normal luteal function. In these the LH surge commenced more than 44 hours after $PGF_{2\alpha}$ administration and in four of these the amplitude of the LH surge exceeded 70 ng/mL.

Number and size of ovarian follicles recorded ultrasonically at 12 hour intervals from 6 hours before until 42 hours after the ovulation inducing treatment are presented in Table 2.

Throughout the inspection period the number of large follicles (>7mm in diameter) did not differ among treatment groups (P<0.05). According to the limited data available on does inspected at two hour intervals (Table 3), does of the GnRH group ovulated, on average, 24.5 hours after GnRH treatment, accordingly 23.5 hours after the onset of the LH surge, with a range of six hours (21 to 27 hours). The average interval from the peak of the surge to ovulation was 22.0 hours. In the hCG-treated does ovulation came to pass, on average, 34.3 (range 31 to 41) hours after hCG injection, respectively 21.7 (range 17 to 31) hours after the onset and 19.0 hours after the peak of the LH surge. In the saline controls, time from injection to ovulation amounted to 39.3 (range 32 to 44) hours and from onset and peak of the LH surge to ovulation 26.8 (range 22 to 32) and 24.3 hours, respectively. Ovulations in the GnRH treatment does occurred, on average, 15.0 (range 13 to 19) hours after the onset of estrus, as compared to 27.3 (range 22 to 38) hours and 34.8 (range 28 to 41) hours in the hCG- and saline treated does, respectively.

In does of the GnRH-, hCG- and saline treated group not provided with progestogen releasing ear implants between mating and flushing merely 0, 1 and 1 transferable embryo, respectively, was yielded. When luteal insufficiency was compensated, from the same groups, on average, 3.2, 1.9 and 4.6 transferable embryos were obtained, additionally from three does of the GnRH-, two does of the hCG- and one doe of the saline group another 3, 6 and 2 unfertilized ova, respectively. The differences among groups were not statistically significant.

Table 1. Characterization of the LH surge in superovulated does induced to ovulate with GnRH (n=10) and hCG (n=9) as compared to saline controls (n=10).

Parameter	GnRH	hCG	Saline [d]
Ovulation induction to commencement of LH surge (h)			
Mean	1.0 [a]	11.8 [b]	14.9 [b]
SEM	0.03	0.5	1.2
Range	0.7-1	10-14	10-20
Ovulation induction to peak of LH surge (h)			
Mean	2.5 [a]	15.1 [b]	17.6 [b]
SEM	0.2	0.8	1.4
Range	1.7-4	12-20	12-24
Commencement of LH surge to LH peak (h)			
Mean	1.6 [a]	3.7 [b]	2.7 [b]
SEM	0.2	0.8	0.3
Range	1-3	12-20	2-4
Duration of LH surge (h)			
Mean	7.2 [a]	11.2 [b]	12.1 [b]
SEM	0.6	0.8	0.6
Range	3-10	8-15	10-16
Amplitude of LH surge (ng/mL)			
Mean	88.9 [a]	50.4 [b]	69.7 [b]
SEM	3.0	6.1	8.6
Range	68-99	24-74	31-103
Area under LH surge (h ng/mL)			
Mean	372.5 [a]	277.2 [a]	320.7 [a]
SEM	41.6	33.7	30.4
Range	161-559	156-480	185-492
Clearance rate of peripheral LH (L/h)			
Mean	1.6 [a]	2.4 [a]	1.8 [a]
SEM	0.2	0.3	0.2
Range	1-3	1-4	1-3
Onset of estrus to commencement of LH surge (h)			
Mean	-7.6 [a]	5.9 [b]	8.5 [b]
SEM	1.2	1.1	1.4
Range	-16.2--2	-1-9	3-17
Onset of estrus to LH peak (h)			
Mean	-6.1 [a]	9.2 [b]	11.2 [b]
SEM	1.5	1.0	1.4
Range	-15.3-0.3	5-13	7-19

[abc] Within rows values with different superscripts differ (p<0.05, t-test)
[d] Excluding one doe that came into estrus before treatment and one that responded with atypical delay (see Fig 1)

Table 2. Number of small (3 to 7 mm in diameter) and large follicles (> 7 mm) from 6 hours before until 42 hours after ovulation induction with GnRH and hCG as compared to saline controls (17 does per treatment group).

Time after treatment	Parameter	GnRH		hCG		Saline	
		Small	Large	Small	Large	Small	Large
-6 hours							
	Mean	12.0 [a]	2.2 [a]	12.5 [a]	2.6 [a]	14.8 [a]	1.3 [a]
	SEM	1.5	0.5	1.2	0.6	1.2	0.4
	Range	0-21	0-5	2-19	0-8	9-22	0-3
+6 hours							
	Mean	15.8 [ab]	4.2 [a]	18.6 [b]	3.9 [a]	19.8 [b]	3.7 [ab]
	SEM	2.4	0.9	1.4	0.7	2.1	0.8
	Range	0-27	0-9	11-33	0-8	10-38	0-9
+18 hours							
	Mean	18.8 [b]	3.8 [a]	17.3 [b]	5.4 [ab]	17.1 [b]	4.9 [b]
	SEM	2.2	0.7	1.2	0.7	2.0	1.0
	Range	3-28	0-7	9-27	0-9	8-30	0-10
+30 hours							
	Mean	14.6 [ab]	2.2 [a]	17.7 [b]	3.6 [a]	19.8 [b]	4.9 [b]
	SEM	1.8	0.6	0.9	0.5	2.5	1.2
	Range	3-23	0-6	12-26	1-7	10-38	0-13
+42 hours							
	Mean	15.5 [ab]	2.1 [a]	16.1 [ab]	2.6 [ac]	15.8 [ab]	3.0 [ab]
	SEM	1.9	0.5	1.4	0.3	1.5	0.6
	Range	7-27	0-6	9-24	1-4	6-26	1-9

[abc] Within columns values with different superscripts differ ($p<0.05$, t-test)
Within rows there were no significant differences ($p>0.05$, t-test)

Table 3. Time relationships assessed on a subsample of does subjected to 2-hourly monitoring of ovarian structures.

Parameter	GnRH n=4	hCG n=3	Saline n=4	Overall n=11
Ovulation induction to onset of estrus (h)				
Mean	9.0[a]	7.0[a]	4.5[a]	6.8
SEM	0	2.0	1.5	0.9
Range	9	3-9	3-9	3-9
Ovulation induction to commencement of LH surge (h)				
Mean	1.0	12.7	12.5	8.4
SEM	0.	1.3	1.0	1.8
Range	1	10-14	10-14	1.-14
Ovulation induction to LH peak (h)				
Mean	2.5	14.7	15.0	10.4
SEM	0.5	0.7	1.3	1.9
Range	2-4	14-16	12-18	2-18
Ovulation induction to ovulation (h)				
Mean	24.5[a]	34.3[bc]	39.3[c]	32.5
SEM	1.5	3.3	2.9	2.4
Range	22-28	31-41	32-44	22-44
Duration of estrus (h)				
Mean	26.0[a]	32.7[a]	38.3[a]	32.3
SEM	2.8	1.8	4.1	2.4
Range	18-30	30-36	30-48	18-48
Onset of estrus to commencement of LH surge (h)				
Mean	-8.0	5.7	8.0	1.5
SEM	0	0.7	1.3	2.3
Range	-8.0	5-7	5-11	-8-11
Onset of estrus to LH peak (h)				
Mean	-6.5	7.7	10.5	3.5
SEM	0.5	1.8	1.7	2.5
Range	-7--5	5-11	7-15	-7-15
Onset of estrus to ovulation (h)				
Mean	15.0[a]	27.3[ab]	34.8[b]	25.7
SEM	1.5	5.3	3.6	3.2
Range	13-19	22-38	28-41	13-41
Ovulation to end of estrus (h)				
Mean	10.5[a]	5.3[a]	3.5[a]	6.5
SEM	3.9	5.9	1.3	2.2
Range	-1-17	-6-14	1-7	-6-17
Onset of LH surge to ovulation (h)				
Mean	23.5[a]	21.7[a]	26.8[a]	24.2
SEM	1.5	4.7	2.5	1.6
Range	21-27	17-31	22-32	17-32
LH peak to ovulation (h)				
Mean	22.0[a]	19.0[a]	24.3[a]	22.0
SEM	1.4	4.0	2.3	1.5
Range	20-26	14-27	20-30	14-30

[abc] Within rows values with different superscript differ (p<0.05, t-test)

37
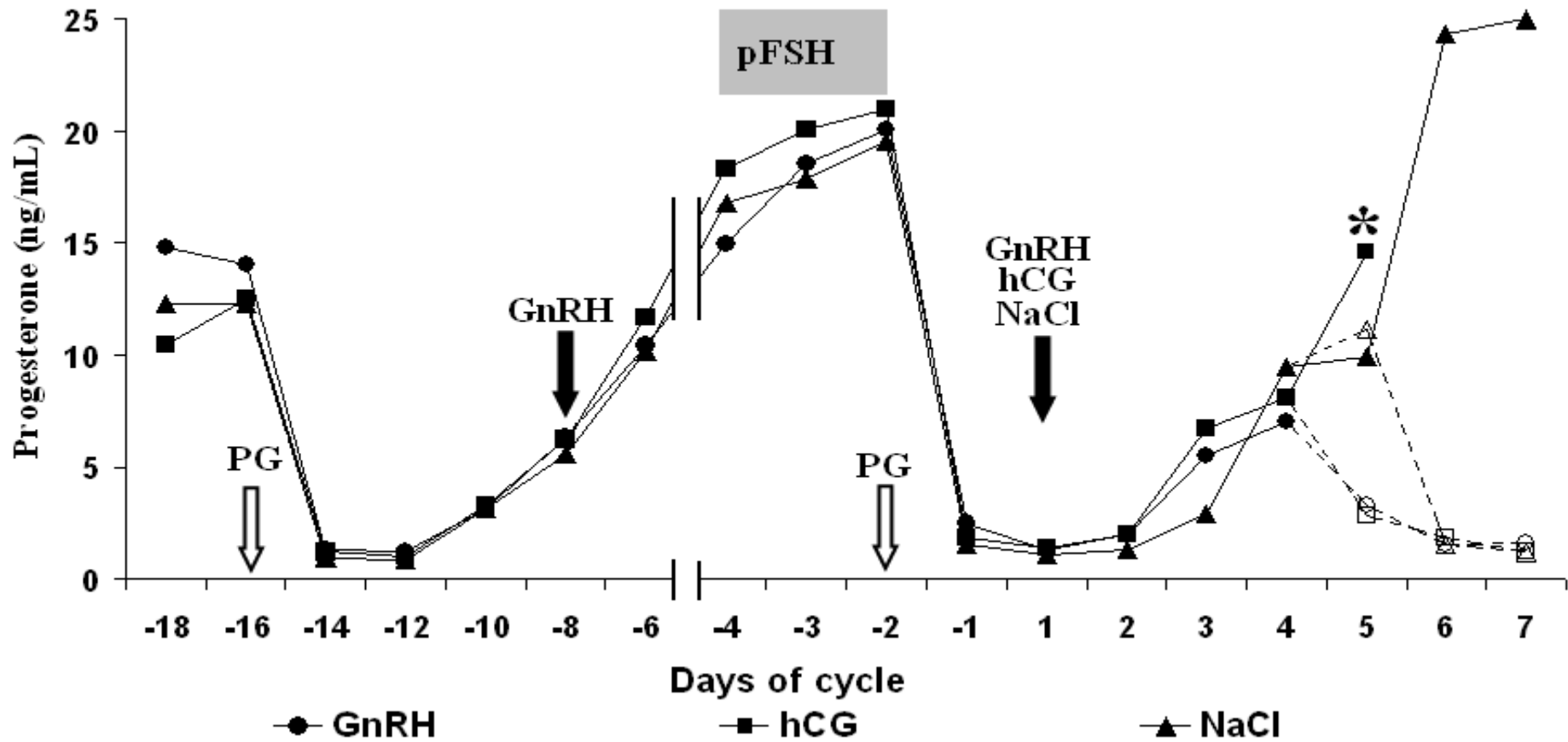
Fig 1: Mean plasma progesterone concentrations in three groups of does treated with $PGF_{2\alpha}$ (PG) during the luteal phase, followed by an Ovsynch regimen in combination with superovulatory pFSH treatment and ovulation induction with GnRH or hCG as compared to physiological saline (NaCl) controls. Dotted lines and open symbols indicate premature corpus luteum regression.

* Does were flushed for obtaining morulae.
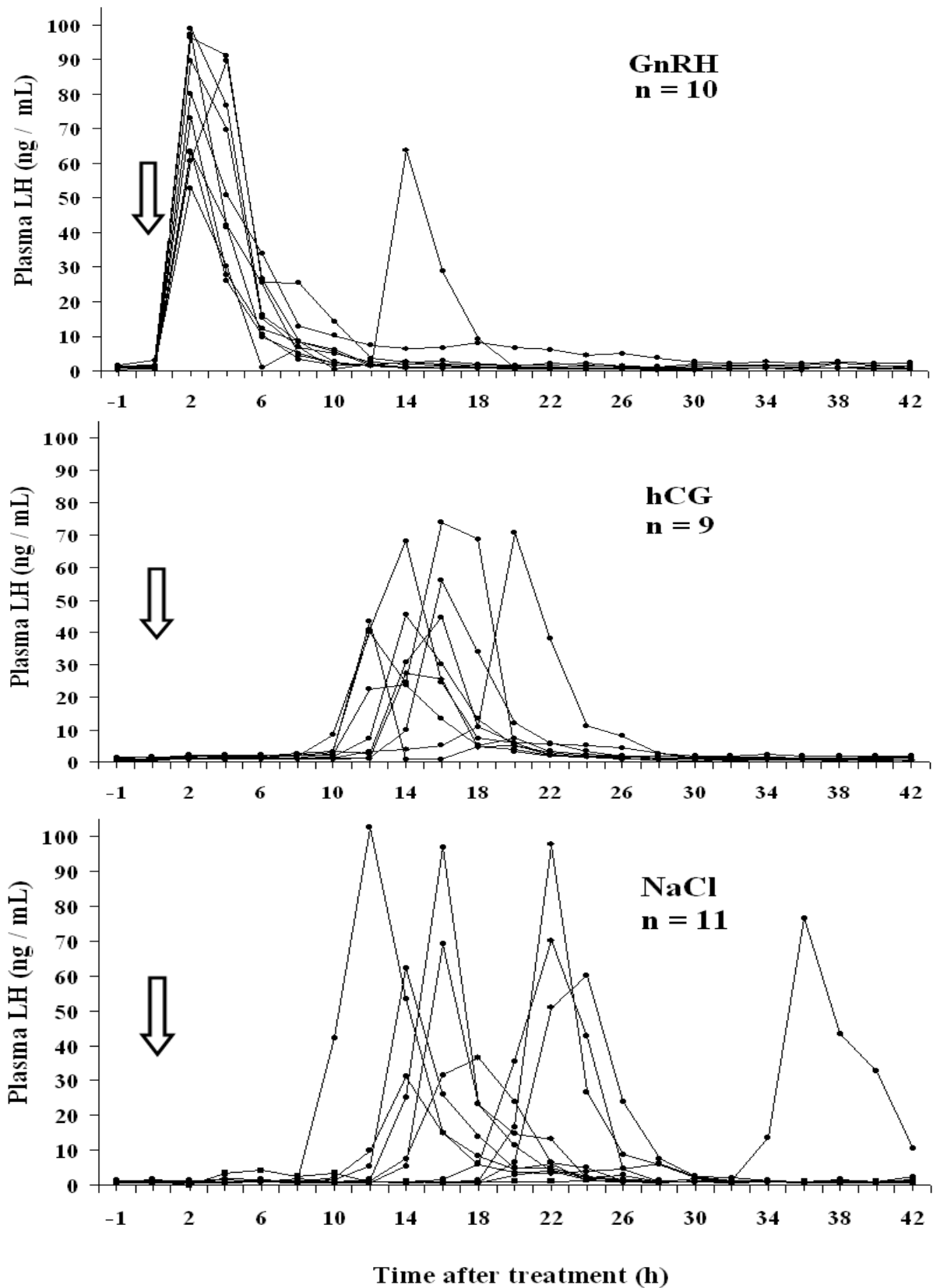
Fig 2: Plasma LH in individual does after ovulation induction with GnRH or hCG as compared to physiological saline (arrows). The single spike trailing the others in the GnRH-treated group is second to a previous one.
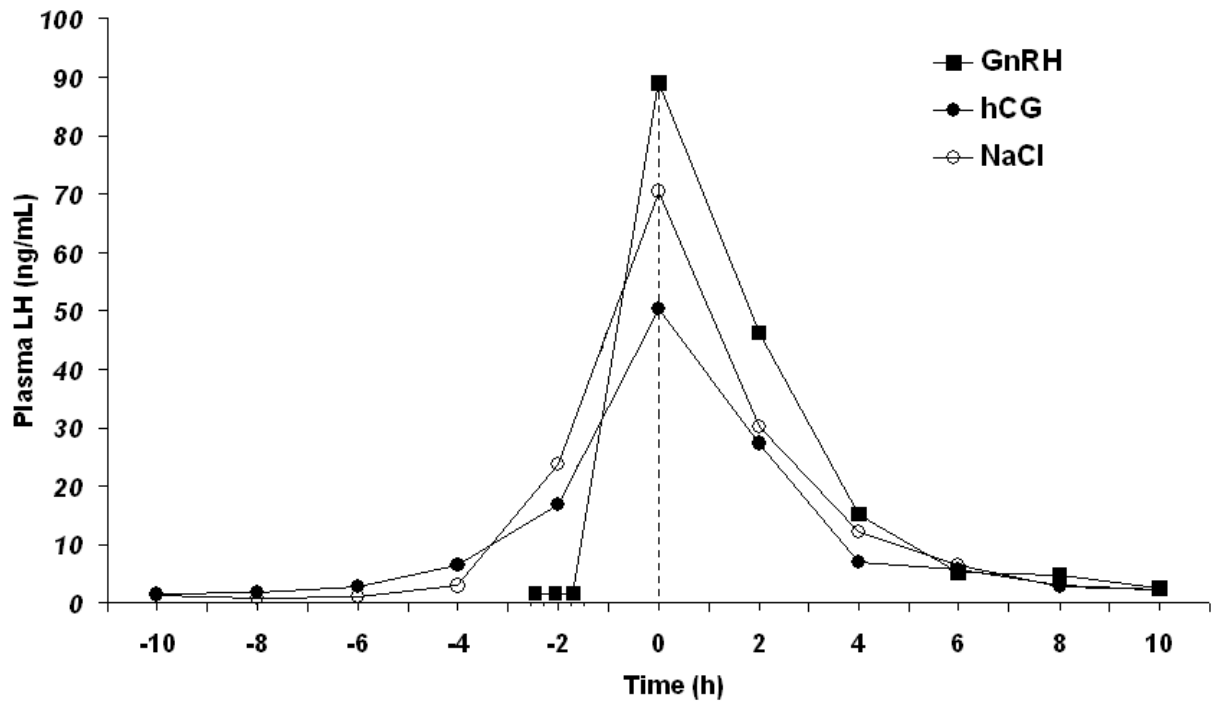
Fig 3: LH surges after treatment with GnRH, hCG and physiological saline (NaCl), respectively. Data were arranged around the LH peak (dotted line).

2.4 Discussion

Estrous symptoms were typical for the Departmental flock with no apparent effect of treatment. This is an indication that estrogen secretion, playing a pivotal role in the exhibition of estrous symptoms (Jeong and Kaiser, 2006; Etgen and Garcia-Segura, 2009) was normal regardless of treatment. The observation that immobility reflex and tail flagging at the onset of estrus coincided agrees with earlier findings on the same population of goats (Holtz et al., 2008). This observation is of practical relevance in that tail flagging may be considered a reliable sign of estrus and it is not necessary to go to the trouble of having a teaser mount a doe before being able to decide whether she is ready to be inseminated.

The prompt luteolytic response recorded in all does after the two prostaglandin treatments is proof of the high susceptibility of cyclic corpora lutea in goats to prostaglandin $F_{2\alpha}$. The double injection of prostaglandin $F_{2\alpha}$ conducted at 12 hour interval in association with the administration of FSH, therefore, might have been redundant. In cows two injections are considered necessary to assure complete corpus luteum regression (Drost et al., 1986). On the second day after prostaglandin administration estrous symptoms were recorded in all does (Fig. 1).

The plasma progesterone pattern between first and second prostaglandin treatment was typical for goats (Boscos et al., 2003; Gonzalez et al., 2004; Fonseca and Torres, 2005; Khanum et al., 2006). After the superovulatory treatment of the does, in those not subject to premature corpus luteum regression progesterone reached a higher level (Fig. 1). This indicates the presence of supernumerary corpora lutea brought about by FSH treatment. Similar observations have been reported before (Quirke et al., 1979; Jarrell and Dziuk, 1981); in fact,

a direct linear relationship with a correlation coefficient of r = 0.9 between number of corpora lutea and serum progesterone level in superovulated goats was observed by Appavu and Holtz (1992).

The high mid-luteal level of progesterone, as compared to several other reports, appears to be assay-related, as pointed out elsewhere (Holtz et al., 2008).

The finding that in almost all does subject to induced ovulation - no matter whether with GnRH or hCG - and more than half of the saline treated controls corpora lutea failed to function beyond day 4 was most disconcerting. Short cycles are not uncommon in goats early and late in the season (Armstrong et al., 1983; Chemineau et al., 1986; Rivera et al., 2003), after superovulatory treatment (Espanosa-Marquez et al., 2004; Cervantes et al., 2007) especially when eCG is involved (Pendleton et al., 1992b) or when applying the ovsynch regimen (Holtz et al., 2008). According to Horton and Britt (1990), Saharrea et al. (1998) and Taponen et al. (2003) a reason for luteal malfunction might be untimely prostaglandin $F_{2\alpha}$ secretion, presumably caused by elevated plasma estrogen level (Filicori et al., 2005; Clifton and Steiner, 2009; Kaiser et al., 2011). Others suspect an inadequate LH surge (Armstrong et al., 1983; Taponen et al., 2003), insufficient progesterone priming (Rivera et al., 2003) or the lack of responsiveness to LH of corpus luteum cells derived after prostaglandin $F_{2\alpha}$-induced estrus (Hansen et al., 1987; Skarzynski, et al. 2009). We do not have a plausible explanation for the extent of the phenomenon in the present study.

The pattern of the LH surge recorded in the three treatment groups was quite uniform. The rapid clearance, responsible for the short half-life, could be a consequence of a lack of sugar moieties which are responsible for the protracted clearance rate of hCG (0.2 L/h; Saleh et al., in preparation). The prompt and closely synchronized response to buserelin is indicative of

immediate binding of the GnRH agonist to hypophyseal GnRH receptors as has been shown to be the case by Catt et al. (1985). A single injection of buserelin was found to be just as effective as the endogenous GnRH release which occurs in a pulsatile fashion (Schuiling et al., 1984). Studies in cattle (Nawito et al., 1977) and sheep (Schilling and Minar, 1971) have shown that the gonadotropin releasing effect of the nonapeptide buserelin is 50 to 70 times as intense as that of a decapeptide with the amino acid sequence of endogenous GnRH.

From the data of the present investigation it appears unlikely that there is a direct effect of hCG on the release of LH. Time and degree of synchronization of the LH surge following hCG administration closely resembled that in the saline group. Whether hCG was responsible for the induction of ovulation is not entirely clear either. It may be deduced from Tables 2 and 3 that the hCG treated does ovulated slightly sooner than the saline controls. Yet, the interval between LH surge and ovulation being not significantly different from that of the other treatment groups, it is more likely that ovulation was a sequel to the endogenous LH release. If this were the case, it would not sensible to use hCG as a means of inducing ovulation in goats.

The ovsynch protocol is designed to permit fixed-time insemination. Nevertheless, in the present experiment the does were mated when displaying estrous symptoms, the reason being that the prime intention was to produce viable embryos and at the time the most suitable time for insemination was not known. With the majority of the goats being affected by corpus luteum insufficiency, a prerequisite for embryo collection was administration of norgestomet releasing subcutaneous implants from mating to embryo collection. Even then embryo yield was not up to expectations. Under comparable conditions with regard to breed and husbandry

43

of goats, FSH preparation and treatment regimen, with the only difference being the means of synchronization which was accomplished by intravaginal progestogen sponges, a yield of 9.1 (SEM 1.5) transferable embryos was achieved (Nowshari et al., 1995) as compared to, on average, 3.2, 1.9 and 4.6 transferable embryos in GnRH-, hCG- and saline treated does in the present experiment.

By way of conclusion, it was possible to induce superovulation by combining the ovsynch protocol with the administration of FSH. The response to prostaglandin $F_{2\alpha}$ was prompt and all does showed estrous symptoms. The objective to attain timed ovulation appears to have been accomplished by treatment with GnRH 30 hours after prostaglandin $F_{2\alpha}$ as it caused a prompt and full-fledged LH release. On the average the LH surge made its appearance almost 20 hours in advance of the saline controls. The role of hCG as an ovulation inducing agent was found to be questionable. The kinetics of the LH release emerging after GnRH-, hCG- and saline treatment was quite uniform, the GnRH-induced discharge being slightly steeper, higher and longer-acting. Ovulation occurred, on the average, 24.2 hours after the onset and 22.0 hours after the peak of the LH surge with no significant difference among treatment groups. A dominant feature of the present study was the disconcertingly high incidence of corpus luteum insufficiency, affecting factually all animals subjected to ovulation inducing treatment and more than half of the saline treated goats. As a consequence, in order to yield transferable embryos, does have to be provided with progestogen releasing implants from mating to embryo collection. From a quantitative point of view embryo yield was not up to expectations. More research will be needed to solve the pending problems of premature luteal regression and unfavorable embryo yield.

2.5 References

See chapter V

Chapter III

Pharmacokinetics of human chorionic gonadotropin

(hCG) in superovulated goats

Abstract

The present investigation addresses the pharmacokinetics of human chorionic gonadotropin (hCG) administered as a single im injection as ovulation inducing agent in the context of superovulation treatment. Nine pluriparous Boer goat does, 2 to 6 years of age, received a single im injection of 500 IU hCG (Chorulon®) 18 h after the end of superovulatory FSH-treatment. Blood samples were drawn two-hourly until 22 hours after hCG administration, thereafter at 26, 32, 38, 42, 66, 90 and 114 h. Plasma hCG concentration was assessed by electro-chemiluminescence immunoassay. Lag time (0.4, SEM 0.1) h, absorption rate constant (0.34, SEM 0.002) h and absorption half life (2.7, SEM 0.5) h, elimination rate constant (0.02, SEM 0.002) h, biological half life (39.4, SEM 5.1) h and apparent volume of distribution (16.9, SEM 4.3) L were calculated as pharmacokinetical parameters of hCG. The hCG curve was characterized by an absorption phase of 11.6 h (SEM 1.8) h and an elimination phase of 70.0 h (SEM 9.8). Considerable individual variation was found in both bioavailability and pharmacokinetical parameters. Biological half life was correlated with peak concentration ($r=-0.76$), absorption rate constant ($r=-0.78$) and elimination rate constant ($r=-0.87$). These results indicate that, following intramuscular administration, hCG is rapidly absorbed, whereas clearance occurs rather gradually, with considerable individual variation in bioavailability and pharmacokinetical parameters.

Key Words: human Chorionic Gonadotropin, hCG, Superovulation, Goat, Pharmacokinetics

3.1 Introduction

Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein of 57 kDa consisting of a noncovalently bound α- (92 amino acids) and a distinctive β-subunit (134 amino acids). The α-subunit is common among other glycoprotein hormones such as LH, FSH and TSH whereas, the β-subunit is unique for each glycoprotein and responsible for a specific function of the hormone (Gupta and Dighe, 2000; Senger, 2005; De Rensis et al., 2010). The β-hCG has an 80% homology to the 121 amino acid subunit of the LH β-subunit (Norman and Litwack, 1997). hCG is primarily produced by trophoblast cells of human embryo and after implantation by villous syncytiotrophoblast cells of the placenta (Cole, 2010).

This hormone contributes to the maternal recognition of the developing embryo (Senger, 2005; Cole, 201), initiates angiogenesis caused by growth factors to prepare the uterus for implantation (Zygmunt et al., 2002; Filicori et al., 2005; Cole, 2010), and stimulates the production of progesterone by the corpus luteum during the first stages of embryo development until the placenta becomes able to produce adequate levels of progesterone in the pregnant woman (usually at 6 to 8 weeks) (Norman and Litwack, 1997).

Due to the similar structure with LH (Lei and Rao, 1994; Birken et al., 1996), hCG exerts its effects through binding to LH receptors. This action is characterized by a prolonged duration owing to high glycolysation rate (Cole, 2010) which is also responsible for the low clearance rate from the circulation. More than 75% of the hCG molecules are cleared from the circulation by the lever and the remainder by the kidney (Cole, 2010). The pregnant woman is, therefore, used to extract hCG for commercial administration (Farrag et al., 2008). Alternatively, hCG can be derived by genetic modification i.e. recombinant hCG (Farrag et al., 2008).

In human fertility, hCG is extensively used as an ovulation inducing agent in lieu of LH (Nader and Derkowitz, 1990), and after introduction of IVF and ICSI, in the final maturation of oocyte in women (Farrag et al., 2008). In the man, it is used to stimulate the Lydig cells to synthesize testosterone production which is necessary for spermatogenesis (Heller and Leach, 1971). In domestic animals, hCG has a wide range of application. It was used in the context of estrus synchronisation in cattle and horse (Schmitt et al., 1996, Ginther et al., 2009), ovulation induction in fish (Kahkesh et al., 2010), superovulation in sheep and goat (Wani et al., 1997; Saleh et al, in preparation), to overcome the negative effect of premature CL regression after superovulatory treatment in goat (Saharea et al., 1997) and to improve pregnancy rates in cattle and goat (Rajamahendran and Sianangama, 1992; Fonseca and Torres, 2005). The effectiveness of hCG in the context of superovulatory treatment was unsatisfactory (Saleh et al., in preparation). To our knowledge, except for cat, no information is available about pharmacokinetics of hCG in the circulation of domestic animals. The objective of the present study was, therefore, to characterize the pharmacokinetics of hCG after im injection in superovulated goats.

3.2 Materials and Methods

The experiment was conducted at Goettingen, Germany (9º 41' E, 51º 46' N) during the breeding season (October to January) on pluriparous Boer goat does, 2 to 6 years of age, from our own breeding flock. The does were group-housed in open barns with straw-bedding and outdoor concrete runs. They were fed a daily ration of 600 g concentrate, consisting of equal parts of a pelleted diet for breeding ewes (16% crude protein, 10.2 MJ ME/kg, supplemented with 43 mg/kg Se, 12 mg/kg I and 5000 mg/kg Zn), oats and dried sugar beet pulp, and had

free access to wheat or barley straw, salt lick and water. Once daily the complete flock was routinely tested for estrus with an aproned male.

Donor does in the luteal phase with plasma progesterone concentration in excess of 5 ng/ml - assessed by ELISA as described by Van de Wiel and Koops (1986), modified by Moeller (1991) - received an im injection of 5.0 mg Dinoprost (1mL Dinolytic®, Pfizer, Karlsruhe, Germany), followed, seven days later, by an im injection of a GnRH analog (1mL Receptal® = 0.004mg Buserelin, Intervet, Unterschleissheim, Germany). Five days later superovulation was induced by 6 sc injections of 4, 4, 2, 2, 2 and 2 armour units of pFSH supplemented with 40% pLH (Nowshari et al., 1995) at 12 hour intervals. Along with the last 2 pFSH-injections the does received im injections of 1 mL Dinolytic®. Ovulation was induced 18 hours after the last Dinoprost injection by im injection of 500 IU hCG (Chorulon®, Intervet, Unterschleissheim, Germany). To counteract occasionally occurring premature corpus luteum regression, of 9 does treated, 6 were provided with progesterone-containing ear implants (Crestar®, 3.3 mg Norgestomet, Intervet, Beaucouze, France) 12 hours after the end of standing estrus. Twenty hours before embryo collection the implants were removed together with an im injection of 1mL Dinolytic®. Does were monitored for estrus symptoms by confronting them with an aproned buck at 6 hour intervals from 6 hours before treatment until 6 hours after the end of estrus. Estrous does were handmated twice daily as long as they would allow a male to mount.

Daily blood samples of 5 mL were drawn via jugular venipuncture from the initial prostaglandin treatment until 7 days after the end of the experiment. Sampling frequency was increased to once every two hours for 24 hours following hCG administration and once every six hours for the subsequent 18 hours. Collection tubes contained three drops of Na-citrate to prevent clotting and were centrifuged at $1000 \times g$ for 10 min. Plasma was stored at -20°C until

being analyzed for hCG concentration by ECLIA (Electro - Chemi Luminescence Immuno Assay; ELECSYS®, Roche) as described by Forest et al. (1998). The assay is based on an electro-chemiluminescent label (Ruthenium (II) tris (bipyridyl)$_3^{2+}$; Ru(bpy)$_3^{2+}$) which can undergo multiple oxidation-reduction cycles when immobilized at the surface of an electrode in the presence of a co-reactant included in the assay buffer. The co-reactant, tripropylamine (TPA), when oxidized at the electrode, produces a radical cation which acts as reducing agent. After the release of a proton, a TPA radical is formed that reacts with the oxidized form of the label to generate Ru(bpy)$_3^{2+}$ in an excited state. After emission of a photon, the label can undergo another cycle and, therefore, generate multiple photons for each labeled molecule. Immobilization of the labeled complex is achieved by using magnetic microparticles coated with streptavidin which bind to biotin, linked, covalently, to reaction antibodies. A magnet immobilizes the bound fraction at the electrode to allow elimination of the unbound label before proceeding to the electrochemiluminescent detection.

To characterize the pharmacokinetic parameters of hCG; lag time (T_0; onset after treatment) absorption rate constant (K_a), absorption half life ($T_{0.5Abs}$), elimination rate constant (K_e), elimination half life ($T_{0.5Elm}$) and the apparent volume of distribution (Vd) were assessed (Jambhekar and Breen, 2009). The absorption rate- and elimination rate constants were calculated as $K_a = 0.693/T_{0.5Abs}$ and $K_e = 0.693/T_{0.5Elm}$, respectively. To assess the absorption half-life $T_{0.5Abs}$ and the biological half life $T_{0.5Elm}$, plasma hCG concentrations of individual does were plotted against time. Thereafter, the absorption half-life was determined as the time required for half the hCG to be absorbed from the administration site and the biological half life was assigned as the time necessary for the maximum concentration of hCG in plasma to decrease by half. The apparent volume of distribution, the volume to which a given dose of

hCG would have to be diluted in order to have a concentration equal to the concentration detected in blood, was calculated as Vd= Dose / hCG concentration.

To characterize bioavailability parameters for hCG, observed and calculated peak concentration (C_{peak}), observed and calculated time of its occurrence (T_{peak}) and the area under the hCG curve (AUC_{0-114}) were determined. The observed values were calculated based on individual plasma concentration, whereas the calculated values for the peak concentration and the time of its occurrence (T_{peak}) were determined using $T_{peak} = \frac{\ln(Ka/Ke)}{Ka-Ke}$ and

$C_{peak} = I \times [e^{-KeT_{peak}} - e^{-KaT_{peak}}]$, respectively, where I stands for intercept. The intercept was assigned after subjection of individual elimination curves to linear regression analysis. The area under the hCG curve (AUC_{0-114}) was calculated by means of the linear trapezoidal approximation using $(AUC)_{t1}^{t2} = \frac{(Cp1+Cp2)}{2} \times (t_2-t_1)$, where Cp1 and Cp2 are hCG concentrations at the corresponding times t_1 and t_2, respectively. Pearson correlation coefficients were calculated to identify relationships between the pharmacokinetics parameters using the procedure CORR (SAS 9.2). Means and standard errors were calculated using the MEAN procedure (SAS 9.2).

3.3 Results and Discussion

The present study addresses the pharmacokinetics of hCG in goats. The pharmacodynamics were discussed in a different context elsewhere (Saleh et al., in preparation).

Figure 1 depicts mean, standard deviation as well as minimum and maximum plasma concentration over time in relation to im administration of 500 IU hCG. Fitting the hCG curve to the most appropriate model revealed that hCG follows a two-compartment pharmacokinetic model with absorption and elimination phases (Fig. 2). The same pattern was also observed in human (Weissman et al., 1996; Chan et al., 2003) and domestic cats (Swanson et al., 1997). Bioavailability and kinetic parameters for hCG are presented in Tables 1 and 2. In the absorption phase hCG concentration increased from pretreatment level in a linear fashion (Fig. 2). Bio-availability of plasma hCG became evident as early as 0.4 hours (T_0) after administration, indicating rapid absorption from the administration site with an absorption rate constant (K_a) of 0.34, to reach a maximum (C_{peak}) of 64.6 mIU/mL (SEM 4.9) after 11.6 hours. Peak hCG levels showed considerable individual variation (range 40 to 85 mIU/mL, Table 1) which may be due to individual differences in the hypothetical volumes of distribution (range 12 to 25 L, Table 2). The more the hCG molecules bind to target tissues, the lower will be the plasma concentration which is, therefore, an indication of the hypothetical volume into which hCG is distributed.

The absorption rate constant showed a negative relationship with elimination half life (*r=-0.78*; p=0.01) indicating that, when hCG is rapidly absorbed, it is also rapidly cleared from the circulation. Pearson's correlation coefficients showed that, in does with low peak concentration, half-lives were longer (*r=-0.76*; p=0.02); elimination rate constants were lower

(*r=0.67*; p=0.05); apparent volumes of distribution were higher (*r=-0.97*; P=0.0001), whereas the clearance rate was not affected (*r=0.06*; p=0.90).

hCG was eliminated from the circulation in a manner resembling that described by a two-compartment model by Jambhekar and Breen (2009). The elimination phase (Fig. 2) reflects the time the hCG molecules reside in the circulation. It is characterized by a gradual decrease in hCG concentration over a relatively long duration of, on average, 70 hours (SEM 9.8, range 30 to 106). This phase was approximately 7 times as long as the absorption phase, indicating that hCG was cleared from the circulation rather slowly. hCG remained in the system for 114 hours owing to sialic acid residuals on the β–subunit (Kalyant et al., 1982; Nisula et al., 1989; Kobata, 2010). This is responsible for the low clearance rate of 0.2 L/h which is very slow in comparison with LH (1.9 L/h; Saleh et al., in preparation). Compared to data reported for woman (Weissman et al., 1996), cattle (Schmitt et al., 1996) and domestic cats (Swanson et al., 1997) duration of hCG in the circulation in this study fell within an intermediate range. The sialic acid residuals are enzymatically dissociated from the hCG molecule which is, thereafter, degraded after binding to hepatic receptors. This takes care of more than three quarters of the biological activity; the remainder is cleared via the kidney (Kalyant et al., 1982; Apparailly and Combarnous, 1994, Cole, 2010). Approximately 21% is excreted into urine as heterodimeric hCG, nicked heterodimeric hCG, free subunits (some nicked), and, predominantly, as the hCG *β* core fragment. Elimination rates by liver and the kidney might differ according to physiological state (Rao, 1985), interactions with other hormones or substances (Liu et al., 1995), administration route (Saal et al., 1991; Wikland et al., 1995; Stelling et al., 2003), body mass index (Chan et al., 2003; Detti et al., 2007), and the presence of large ovarian follicles (Detti et al., 2007). The biological half life was, on average, 39.4 hours (SEM 5.07, range 25 to 66). This value is almost comparable to that in human (36 h: Cole, 2010) but longer than that in domestic cats (Swanson et al., 1997). According to Liu et

al. (1995) the term *biological half life* is not a constant value. It represents the theoretical volume of blood which is completely cleared of hCG per unit time and does not indicate how much hCG is being removed.

From the present study it may be concluded that hCG, injected intramuscularly, will rapidly appear in the circulation, whereas clearance from the blood occurs rather gradually and may last more than 80 hours. By means of ultrasonography it was determined that ovulation occurred approximately 34 hours after treatment. Taking this into consideration a prolonged bioavailability of hCG for approximately 47 hours after ovulation may be supportive of the function ability of newly formed corpora lutea and may thus reduce the incidence of premature luteal failure. This aspect of hCG administration will be addressed in a separate study.

Table 1. Characterization of plasma hCG profile (observed and calculated time of maximum concentration: T_{peak}; observed and calculated peak plasma concentration: C_{peak}; duration and the area under the curve: AUC) after im administration of 500 IU hCG in superovulated Boer goat does.

Parameter	Mean	SEM	Range
T_{peak} (h)			
Observed	11.6	1.8	4-20
Calculated	11.0	1.9	5-20
C_{peak} (mIU/mL)			
Observed	64.6	4.9	40-85
Calculated	70.9	4.6	50-99
Duration (h)			
Absorption phase (h)	11.6	1.8	4-20
Elimination phase (h)	70	9.8	30-106
AUC (h mIU/mL)	2427	177	1632-3118

Table 2. Pharmacokinetic parameters; lag time (T_o), absorption rate constant (K_a), absorption half life ($T_{0,5abs}$), elimination rate constant (K_e), elimination (biological) half-life ($T_{0,5elm}$), apparent volume of distribution (Vd) and clearance rate after intramuscular administration of 500 IU hCG in nine adult Boer goat does.

Parameter	Mean	SEM	Range
T_o (h)	0.40	0.06	0.33-0.66
K_a (per h)	0.34	0.06	0.13-0.62
$T_{0,5abs}$ (h)	2.70	0.54	1.1-5.3
K_e (per h)	0.02	0.002	0.01-0.03
$T_{0,5elm}$ (h)	39.4	5.1	25-66
Vd (L)	16.9	4.3	12-25
Clearance rate (L/h)	0.2	0.02	0.2-0.3
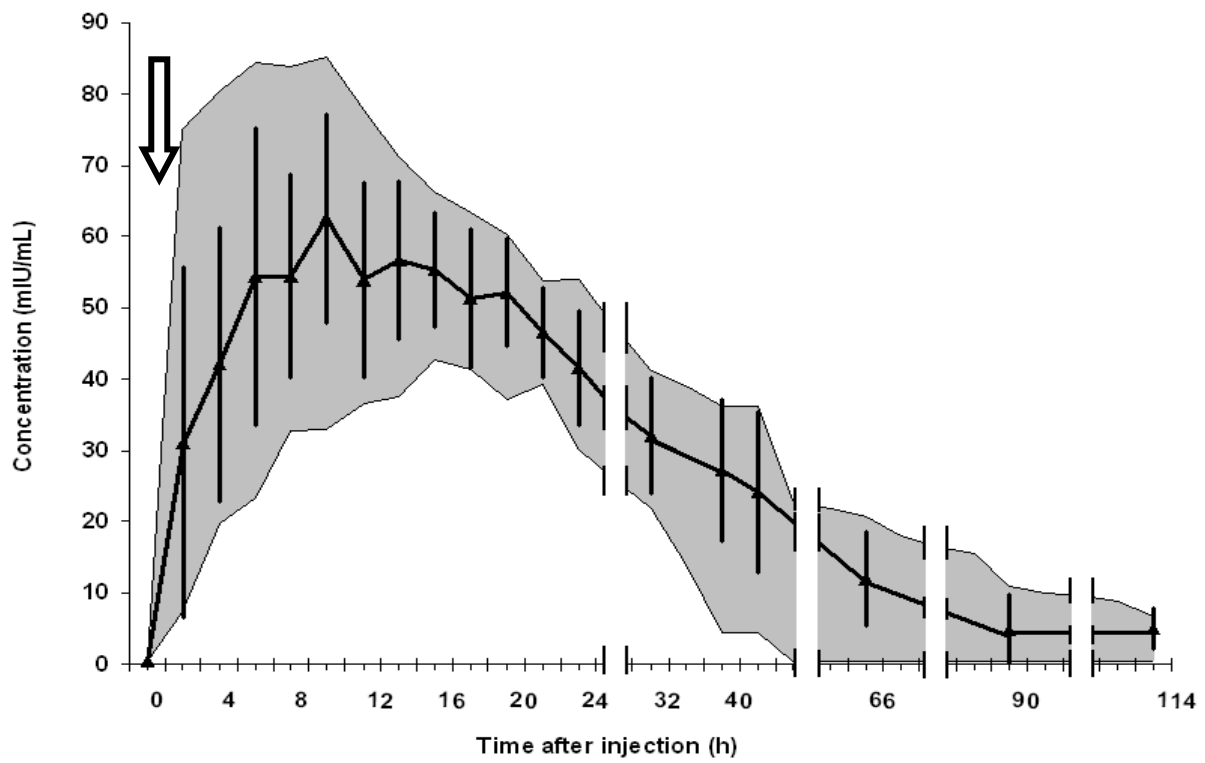
Fig 1. Change in hCG level (mean, standard deviation, minimum and maximum values) in

nine adult Boer does after a single im injection of 500 IU hCG (arrow).

$$y = -0.011x^4 + 1.0594x^3 - 13.034x^2 + 60.839x - 48.08$$
$$R^2 = 0.9927$$

$$y = 0.0109x^4 - 0.3111x^3 + 2.5722x^2 - 9.6336x + 68.572$$
$$R^2 = 0.9894$$

Fig 2. Absorption (top) and elimination phase (bottom) of the plasma hCG profile in the wake of a single im injection of 500 IU in nine adult Boer goat does, fitted with polynomial trend lines.

3.4 References

See chapter V

Chapter IV

Parentage analysis of Boer goats using

microsatellite markers

Abstract

Parentage testing of 13 ET kids using 13 microsatellite markers originally isolated from sheep and goats was done. In addition, means for expected and observed heterozygosity, number of alleles per locus, effective number of alleles, inbreeding coefficient, polymorphic information content and exclusion probabilities were calculated for 80 unrelated individuals. Four loci (*McM527, OarFCB20, BM1258* and *INRA0132*) deviated significantly (p<0.01) from the Hardy-Weinberg equilibrium; 69% of the loci were highly polymorphic with an average number of 7.46 alleles per locus. The inbreeding coefficient was 0.178, the average expected heterozygosity 0.632 and the mean polymorphic information content 0.591. Considering all l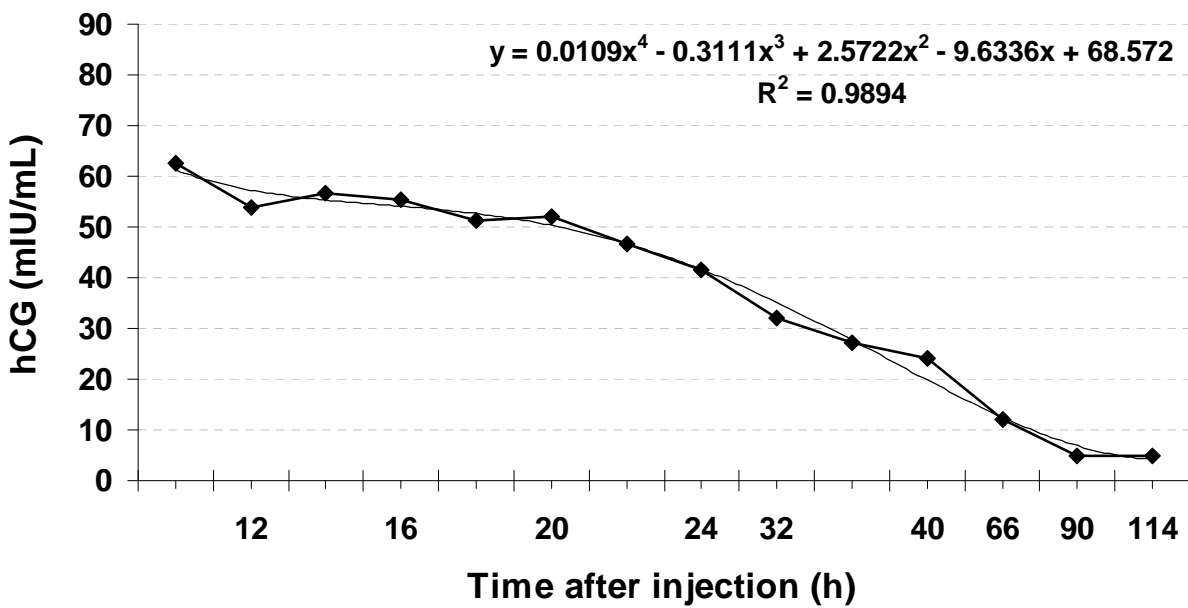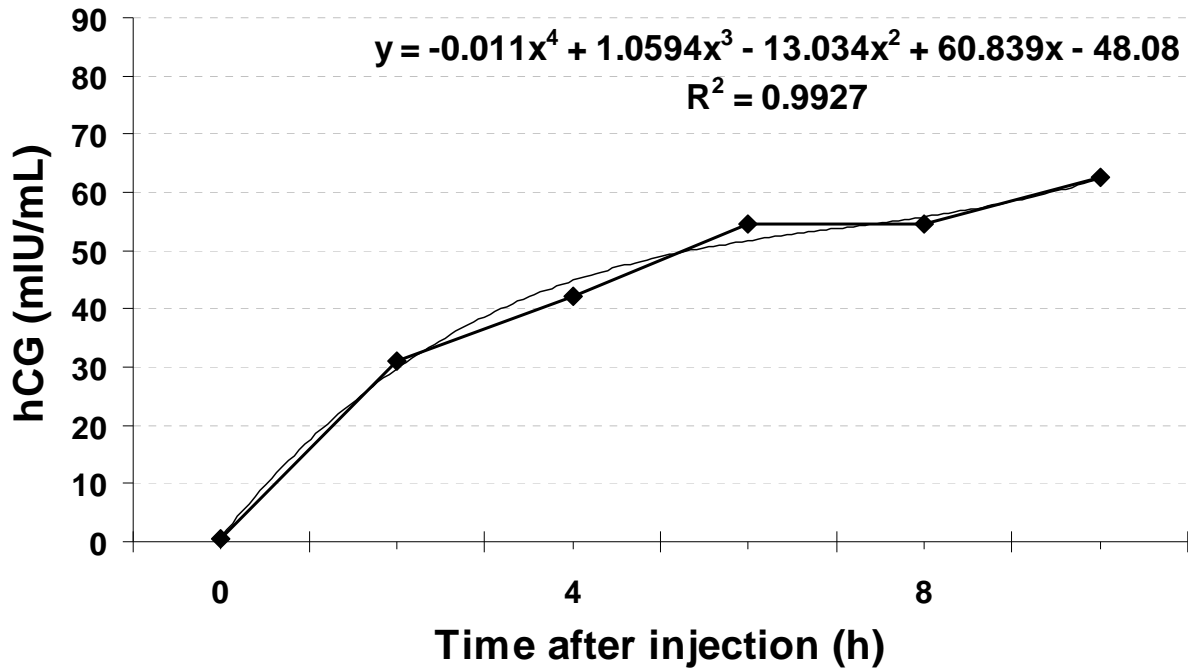oci, the probability of excluding two putative parents was 99.99974% and the probability of identity was instead 4.9×10^{-11}. Paternities of 13 kids were resolved with an estimated pedigree error rate of 15.4%. Mismatching of alleles in at least 4 microsatellite loci led to the exclusion of paternity.

Dropping of loci with (*PIC*) values less than 0.4 did not affect the effectiveness of other markers to distinguish individuals. From the results it may be concluded that the investigated set of 13 loci may indeed serve as a suitable tool for herd management, enabling confirmation of progeny records prior to selection of breeding animals.

Key Words: Microsatellites, Parentage, Boer goat

4.1 Introduction

Boer goats have been introduced into Germany since 1980. The nucleus herd has been maintained at the Department of Animal Science in Göttingen. To reduce inbreeding, frozen semen and embryos have been imported from South Africa. Intensive application of artificial insemination (AI), selection for fecundity traits such as litter size and misidentification of parentage are potential contributors to inbreeding which may negatively affect the viability and reproductive fitness of the breeding stock (Pariacote et al., 1997; Frankham et al., 2004). Drawbacks of traditional methods of paternity control i.e. progeny testing (Baron et al., 2002) have justified the application of more accurate methods. Recently, DNA technologies were introduced for molecular characterization of breeds and paternity testing in farm animals. Microsatellites have received the highest attention and have extensively been used for genetic profiling of individuals due to advantages over other DNA markers as they combine high genetic variability with nuclear co-dominance inheritance (Jarne and Lagoda 1996; Heyen et al., 1997). Moreover, microsatellite loci can be successfully amplified across related species (Pepin et al., 1995; Vaiman et al., 1996; Yang et al., 1999). For parentage analysis several polymorphic microsatellite markers have been recommended by the International Society for Animal Genetics (ISAG) and the Food and Agriculture Organization (FAO). A set of 10 microsatellites should be enough to exclude a parentage with a high confidence level (Tautz, 1989; Tracey, 2001). The objectives of this study were to utilize microsatellite markers to quantify the rate of pedigree errors in the local Boer goat population, to evaluate the degree of inbreeding and to investigate if less informative markers could be dropped from the panel with a negligible effect on the probability of exclusion. This study might, therefore, serve as a valuable mean for the genetic management of herds and to design rational strategies for optimum utilization and conservation of genetic diversity in a population.

4.2 Materials and methods

Blood samples were drawn by jugular venipuncture using EDTA-tubes from 13 embryo transfer program kids, 46 putative dams and 10 putative sires. Semen samples of further 5 putative sires were also included in the study. The does were either naturally or artificially inseminated. Genomic DNA was extracted from the blood samples by the SDS/Proteinase K/NaCl/Ethanol procedure and from frozen semen samples by the Phenol-Chloroform procedure according to Miller et al. (1988) and Sambrook et al. (1989).

4.2.1 Microsatellite Amplification

A panel of 13 polymorphic microsatellites was chosen according to the recommendation of the International Society for Animal Genetics (ISAG) (Table 1), due to their highly polymorphism and location on different chromosomes. Furthermore, they have been successfully amplified in different goat and sheep breeds worldwide. This permits a better comparison among breeds with different evolution histories. The primers used for the polymerase chain reaction (PCR) were fluorescent end-labelled with different fluorescent dyes.

Nine of the 13 microsatellite markers were originated from sheep and combined into two multiplexes (*CSRD247*, *HCS*, *INRA63*, *OarFCB11*) and (*INRA63*, *INRA23*, *MAF65*, *McM527*, *OarFCB20*). The remainder four markers were originated from goat and amplified separately. For the ovine multiplexes, PCR reactions were performed in a total volume of 14 μL containing 1 μL genomic DNA (20 ng), 1.4 μL primermix, 7 μL Multiplex-Mastermix and 4.6 μL double distilled water. The PCR amplification was performed in a thermo cycler (Biometra®) using an initial denaturation at 95°C for 15 min, 32 cycles of denaturation at 94

°C for 30s, primer annealing at (Table 1) for 90s, primer extension at 72°C for 1min followed by a final extension at 60°C for 30 min.

PCR reactions for the caprine markers (*BM1258, BM1329, BM1818 and INRA0132*) were performed in a total volume of 25 μL containing 1 μL (20 ng) genomic DNA, 2.5 μl 10X PCR buffer, 0.5 μl dNTP, 1 μL of each STR primer unit (forward and reverse), 0.3 μL *Taq*-polymerase. $MgCl_2$ was added to the reactions at 0.5, 0, 1.5 and 0.5 μL, respectively, and double distilled water was added to achieve final volume of 25 μL. The PCR reactions were performed on a Biometra T-Gradient Thermocycler (Biometra[®], Germany) using an initial denaturation at 94°C for 1.5 min, 34 cycles of denaturation at 94°C for 1 min, primer annealing at (see Table 1) for 1 min, primer extension at 72°C for 1 min followed by a final extension at 72°C for 5 min. To check fragment integrity PCR products were loaded on 1.5% agarose gels.

At the end of the PCR reaction, the total ovine- and caprine- PCR product was diluted with 100 μL and 40 μL double distilled water, respectively, and then 1.5 μL was loaded with 12.5 μL Hi-Di Formamide and 0.5 μL GenScan 500-ROX to put into in the ABI-3100 Genotyper (Applied Biosystems[®]) for microsatellite genotyping.

4.2.2 Genotyping

For genotyping of samples, the size separation was performed on an ABI PRISM[®] 3100 DNA analyzer (ABI, Weiterstadt, Germany), using GENSCAN-500ROX[TM] as internal size standard according to the manufacturers' specification. Evaluation of microsatellites and size determination of alleles were done with appropriate ABI-Software GENSCAN and GENOTYPER software (Applied Biosystems, Applera Europe B.V.), respectively.

4.2.3 Data analysis

The observed (N_A) and the effective number of alleles (N_e), the estimates for probability of excluding two putative parents (E_{PP}) and the probability of identity (P_I) were calculated using GenAlEX 6 software v.6.3 (Peakall and Smouse, 2006). Observed (H_o) and expected heterozygosity (H_E), polymorphic information content (PIC), tests for deviation from Hardy–Weinberg equilibrium and null alleles were calculated using CERVUS$^®$ v3.0.3 (Kalinowski et al., 2007). Inbreeding coefficient (F_{IS}) was estimated according to (Weir and Cockerham, 1984) using FSTAT$^®$ v2.9.3 (Goudet, 2001). To resolve suspected paternities, 15 sires and 46 does were considered as putative sires/dams. Mismatching of alleles at least 4 microsatellite loci led to the exclusion of paternity.

4.3 Results and Discussion

All selected markers were successfully amplified and generated multiple alleles at each locus (Table 3.1). The most important parameters for paternity testing are (H_E) and (*PIC*), because they take into account the number and frequency of alleles (Fig. 3-1) in a given population. Expected heterozygosity (H_E) ranged from 0.417 (*BM1818*) to 0.846 (*INRA5*) with an average of 0.632. The average number of alleles across all loci amplified was 7.46 ranging from 4 (*CSRD247*) to 12 alleles (*INRA5*) suggesting that all markers were suitable for parentage testing. Expected heterozygosity (H_E) was slightly higher than was previously reported for Boer goats in South Africa, which have been subjected to intensive artificial selection for improved production and application of A.I (H_E = 0.617; Visser et al., 2004; Pieters, 2007). The same microsatellites have generated variable number of alleles when they were amplified in different breeds or, even, in the same breed indigenous to different regions (Yang et al., 1999; Martinez at al., 2004; ISAG, 2005; Pieters, 2007), indicating events of different historical evolution, degree of genetic improvement, intensity of selection and population size. Three ovine microsatellite loci (*CSRD247, INRA23* and *McM527*) showed an expected heterozygosity (H_E) lower than Italian sheep breeds (ISAG, 2005). Deficiency of heterozygosity is a phenomenon occasionally observed after amplification of microsatellite markers in closely related species due to lack of conservation in flanking regions during evolution (Pepin et al., 1995; Jarne and Lagoda, 1996). Generally, segregation of null alleles, selection and inbreeding significantly decrease gene diversity (Dakin and Avise, 2004). In this study, deficiency of gene diversity is unlikely to result from segregation of null alleles and inbreeding (Tab 1). Low inbreeding level (F_{IS}=0.178) and high heterozygosity indicate that this population is maintained at an acceptable level of genetic variation probably due to an effective breeding system, exclusion of suspected individuals from the breeding stock and introduction of new genetic material (frozen semen and embryos).
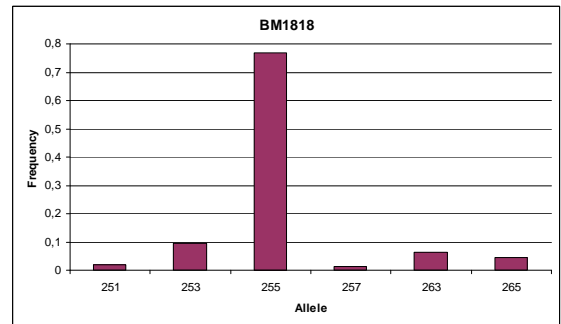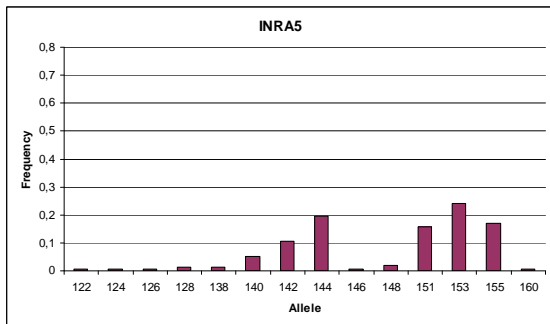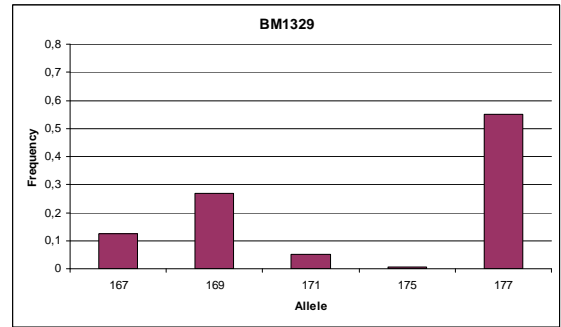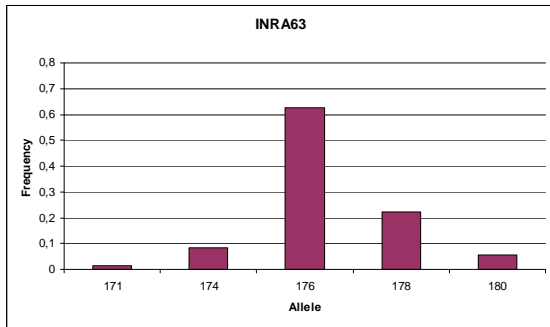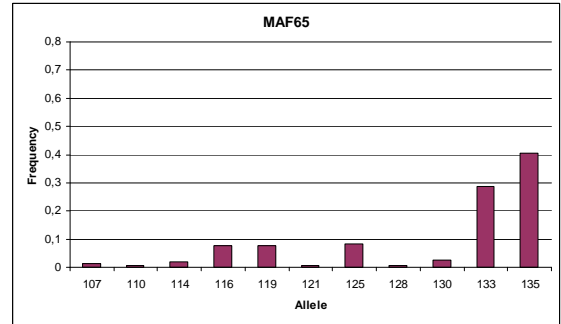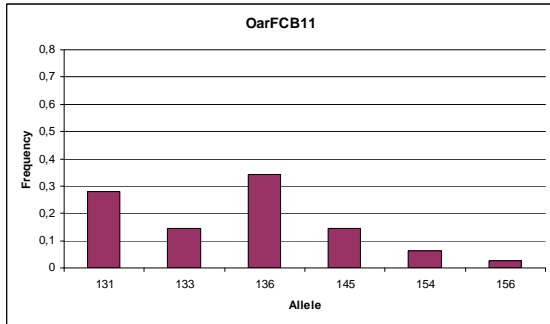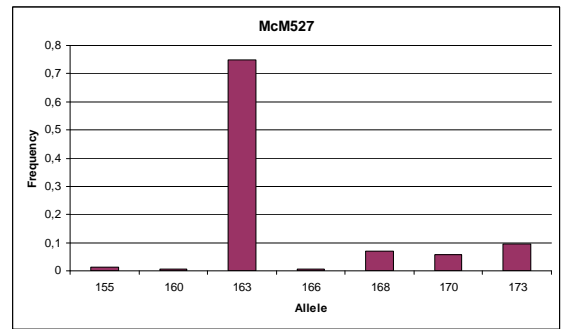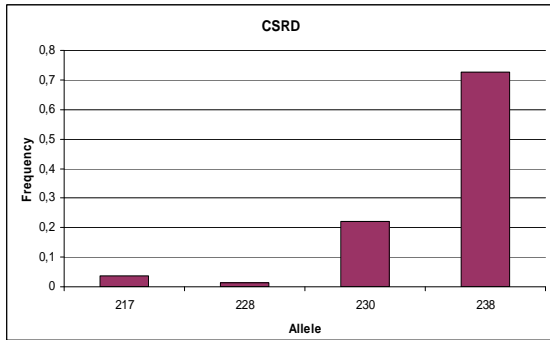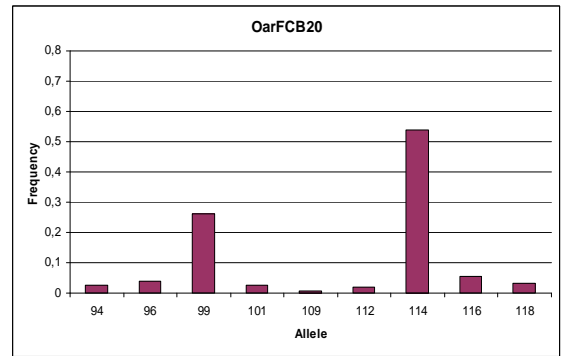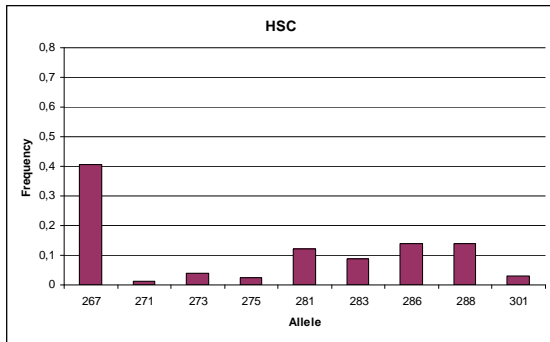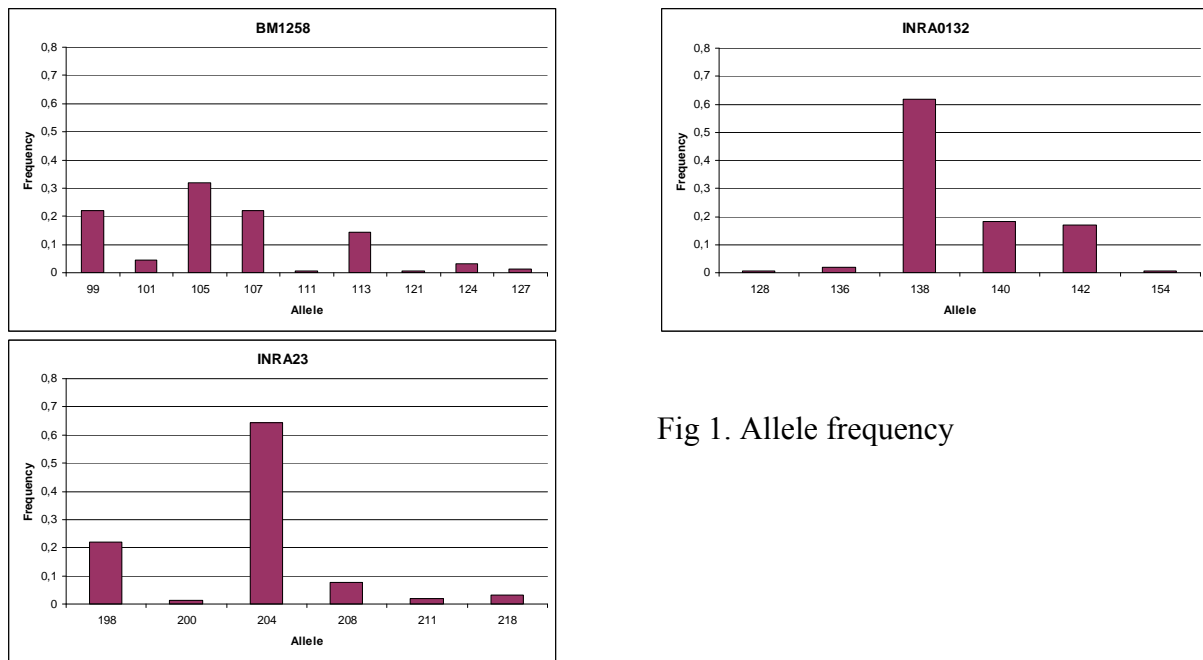
Fig 1. Allele frequency

1

2   After Bonferroni correction, genotype frequencies of 4 markers (*McM527*, *INRA0132*,

3   *BM1258* and *OarFCB20*) were not within the expected Hardy-Weinberg Equilibrium.

4   Disequilibrium of three loci (*McM527*, *INRA0132* and *BM1258*) could be demonstrated as a

5   result of potential segregation of null alleles and an high inbreeding index (Table 1; Dakin

6   and Avise, 2004). Otherwise, departure from *HWE* could be due to other factors such as

7   selection and insufficient sample size (Frankham et al., 2004). The equilibrium state of the

8   other 9 loci in this population provides a rational indicator for the present level of genetic

9   variability. In South Africa, *HWE* differs according to the region (Pieters, 2007). Similar

10   deviation from *HWE* was also reported in Indian goat breeds (Rout et al., 2008).

11   The overall mean of the PIC over all loci was 0.591. The most informative locus was (*INRA5*

12   with 0.822) while the least informative locus was (*BM1818 with* 0.338). Nine microsatellites

13   were highly polymorphic with a *PIC* value higher than 0.5, indicting that these loci are very

14   informative for parentage testing (Zhiguo et al., 2007).

15

Table 1. Measures of polymorphism among the microsatellites in terms of number of alleles (N_A), effective allele number (N_e), observed (H_o) and expected (H_E) heterozygosity, polymorphic information content (PIC), inbreeding coefficient (F_{IS}), exclusion probability when both parent known ($PE1$), exclusion probability when one parent known ($PE2$), probability of excluding two putative parents ($PE3$), exclusion probability of identity (P_I), and null allele frequencies.

Locus	Chromo-some	T_m	Allele Size (bp)	N_A	N_e	PIC	H_E	H_O	F_{IS}	$PE1$	$PE2$	$PE3$	P_I	F(null)
HCS		62	267-301	9	4.237	0.737	0.769	0.738	0.041	0.571	0.389	0.767	0.083	+0.0377
INRA0132 ***		64	136-142	6	2.405	0.542	0.588	0.313	0.470	0.355	0.188	0.536	0.215	+0.3055
OarFCB11	2	62	131-231	6	4.040	0.710	0.757	0.438	0.424	0.521	0.343	0.700	0.103	+0.2608
OarFCB20 ***	2q23	53	94-118	9	3.354	0.661	0.706	0.813	-0.151	0.479	0.304	0.675	0.130	-0.0949
INRA23	3	53	198-208	8	2.470	0.557	0.599	0.613	-0.023	0.376	0.205	0.568	0.202	-0.0402
McM527 ***	5	53	155-170	7	1.723	0.399	0.422	0.213	0.498	0.247	0.096	0.412	0.357	+0.3368
BM1329	6q15	58	167-177	5	2.516	0.545	0.606	0.613	-0.010	0.349	0.194	0.518	0.215	-0.0102
INRA5	12	53	124-160	12	6.293	0.822	0.846	0.263	0.691	0.683	0.514	0.854	0.045	+0.5292
CSRD247	14	62	217-238	4	1.757	0.376	0.433	0.463	-0.068	0.211	0.094	0.338	0.379	-0.0377
MAF65	15	53	110-135	11	3.784	0.703	0.740	0.738	0.004	0.529	0.348	0.727	0.102	-0.0084
INRA63	18q22	62	171-180	5	2.152	0.482	0.539	0.550	-0.021	0.298	0.152	0.459	0.270	-0.0256
BM1818	23	58	251-265	6	1.707	0.338	0.417	0.463	-0.110	0.235	0.920	0.390	0.369	-0.0599
BM1258 ***	23	68	99-124	9	4.735	0.757	0.794	0.550	0.308	0.586	0.304	0.768	0.077	+0.1836
Multilocus				*7.46*	*3.157*	*0.591*	*0.632*	*0.521*	*0.178*	*0.9994*	*0.9828*	*0.9999*	*4.9×10^{-11}*	

Three asterisks as superscripts mean a significant deviation ($p<0.001$) from Hardy–Weinberg equilibrium. (T_m) *Annealing temperature*

The estimated probability of excluding two putative parents (E_{PP}) per locus varied from 0.338 (*CSRD247*) to 0.854 (*INRA5*) reflecting the relative informativeness of the markers.

The combined probability of excluding two putative parents over all loci was 99.99974% providing a high confidence level. These results are similar to exclusion probability of 22 microsatellites in Cashmer, Angora and Murciana-Granadina goats (Luikart et al., 1999). Excluding loci *BM1818, CSRD247* and *McM527* yielding PIC values less than 40%, the exclusion probability of putative parents dropped to 99.9989 %. Regarding the rate of erroneous paternities within the 13 kids resulted from ET program, the estimated value (15.4%) was slightly lower than reported previously in Murciano-Granadina dairy goats (16.2%; Jiménez-Gamero et al., 2006). Important factors contributing to misidentification are erroneous recordings of the semen source or the embryo(s) transferred to the recipients and when multiple does kidded in the same pen. Erroneous implications may also arise due to segregation of null alleles (Dakin and Avise, 2004), mutation and deviation from *HWE* (Luikart et al., 1999), which may cause a rejection of a correct parent. This reveals the importance of paternity testing using DNA markers for accurate assignment of sires/dams used in breeding programs.

From the results it may be concluded that this set of microsatellites was successfully amplified in the Boer goat and may indeed serve as a suitable tool for herd management, enabling confirmation of progeny records prior to selection of breeding animals.

4.4 References

See chapter V

Chapter V

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Chapter VI

Concluding Remarks

This dissertation includes three studies that address aspects associated with in-vivo embryo production in goats.

In Study 1, as a novel approach the Ovsych protocol (Holtz et al., 2008) was combined with a superovulatory dose of pFSH in an effort to achieve a satisfactory embryo yield. Furthermore, by substituting hCG for GnRH, it was attempted to minimize the incidence of premature luteal regression frequently encountered when superovulating goats (Pintado et al., 1998; Saharrea et al., 1998; Espinosa-Marquez et al., 2004; Cervantes et al., 2007). Estrous symptoms, ovarian functions, preovulatory LH surge and embryo yield were studied. The results obtained were unsatisfactory, both in terms of the maintenance of luteal function and embryo yield, in particular after ovulation induction with hCG. The findings from this experiment were as follows:

- Tail flagging and immobility reflex, as reflection of estrus, coincide and commence approximately 36 hours after prostaglandin-induced luteolysis. This observation is of practical relevance in that tail flagging may be considered a reliable sign of estrus when having to decide upon the best time for insemination.

- Injection of a GnRH analog 30 hours after prostaglandin treatment advanced ovulation by almost 20 hours in comparison to saline treated controls. Therefore, in these does AI should best be conducted 16 to 18 hours after GnRH treatment, respectively 46 to 48 hours after prostaglandin administration, especially when using cryopreserved semen.

- Five of nine does of the saline control group in which LH was recorded featured normal luteal function. In these the LH surge commenced more than 44 hours after $PGF_{2\alpha}$ administration and in four of these the amplitude of the LH surge exceeded 70 ng/mL.

- Ovulation induction with a GnRH agonist 30 hours after prostaglandin treatment, as executed in the present experiment, might not be appropriate. It is, therefore, recommended that the time of ovulation induction should not be less than 44 hours after prostaglandin treatment. This time will permit the follicles to reach the appropriate state of maturity when the preovulatory LH surge commences. In this respect GnRH is preferable due to its immediate and highly synchronized induction of the LH surge. This leads to an efficient control of ovulation and provides for a LH surge of sufficient amplitude.

In Study 2, characterization of the pharmacokinetics of intramuscularly injected hCG in female goats was addressed. It became apparent that peripheral hCG concentration increases rapidly after injection, whereas clearance from the blood occurs rather gradually, lasting up to more than 80 hours. Individual variability was substantial, which might be responsible for the variability in time of ovulation, as compared to GnRH-treated does. By way of ultrasonography it was determined that ovulation occurred approximately 34 hours after treatment. Taking this into consideration, it may be concluded that the ovulation, if not occurred with physiological LH surge, is triggered approximately 10 hours after hCG treatment. Consequently AI ought to be conducted 26 to 28 hours after hCG administration. The prolonged presence of hCG, lasting approximately 47 hours after ovulation, did not enhance the function of the corpora lutea and thus was ineffective in reducing the incidence of premature luteal failure as had been hoped for. Therefore, hCG is not recommended as ovulation inducing agent in the context of superovulatory treatment since it did not effectively synchronize the LH surge and, in comparison to saline controls, significantly increased the incidence of premature luteal regression. More research will be needed to solve the pending problems of premature luteal regression and unfavorable embryo yield.

Study 3 was complementary to an embryo transfer program and addressed the application of microsatellite markers, isolated from sheep and goats, for the confirmation of parentage in kids born to embryo transfer recipients. Furthermore, microsatellite markers were utilized to genetically characterize the population of Boer goats maintained at the Department of Animal Science. The departmental flock represents the nucleus of the national Boer goat population. Quantifying the inbreeding coefficient was considered an indicator of long-term breeding management. Eventually, it was attempted to reduce cost and labor of the use of microsatellite markers by reducing the number of markers required. Dropping the three less informative loci from the system did not diminish the effectiveness of the system. Resolving of the paternities of 13 kids revealed that two kids were assigned to the wrong parents. Since the set of markers used in this study corresponds with that used in sheep and goats worldwide, we were able to compare the genetic structure of the departmental Boer goat population with the Boer goat population of South Africa, where the breed originates from, and other breeds. The results of the present study indicate that the genetic structure of the population is comparable to its ancestors in South Africa, due to avoidance of inbreeding and introduction of semen and embryos imported from South Africa. It may be concluded, that this set of microsatellite markers may serve as a valuable means for the genetic management of breeding herds and the design of rational strategies for efficient utilization and conservation of genetic diversity in populations.

Chapter VII

German Abstract

Die vorliegende Arbeit besteht aus drei separaten Versuchen: Die erste Untersuchung beschäftigt sich mit der Frage der Ovulationsinduktion bei superovulierten Ziegen. Die zweite mit der Pharmakokinetik von injizierten hCG und die dritte mit der Elternschaftskontrolle bei Burenziegen mit Hilfe von Mikrosatelliten.

In dem ersten Versuch sollte untersucht werden, ob das Ovsynch-Verfahren in Kombination mit einer Superovulationsbehandlung bei der Burenziege eingesetzt werden kann. Die Untersuchung an 51 pluriparen Burenziegen erfolgte während der Paarungssaison von Oktober bis Januar. Tieren mit hohem Plasmaprogesteronwert (Gelbkörperphase) wurde 1 mL des Prostaglandinpräparats Dinolytic® verabreicht. Sieben Tage später erfolgte eine Receptal®-Gabe (GnRH-Analog; 1 mL = 0.004 mg Buserelin), und 5 Tage darauf eine Superovulationsbehandlung bestehend aus insgesamt 16 AU FSH/40%LH, verabreicht in absteigender Dosierung (4, 4, 2, 2, 2 und 2 AU) im Abstand von 12 Stunden. Gleichzeitig mit den letzten beiden FSH/LH-Gaben erfolgte eine weitere Dinolytic®-Behandlung. Nach weiteren 30 Stunden wurden die Tiere in drei Gruppen unterteilt, die unterschiedlichen Behandlungen unterworfen wurden.

- *Gruppe 1*: 0.004 mg Buserelin (1mL Receptal®)
- *Gruppe 2*: 500 IE hCG (Chorulon®)
- *Gruppe 3*: 1 mL 0.9% NaCl-Lösung (Kontrolle)

Um bei Ziegen häufig auftretenden Kurzzyklen entgegenzuwirken, wurde einem Teil der Tiere aus jeder Versuchsgruppe 12 Stunden nach Brunstende ein subkutanes gestagenhaltiges Ohrimplantat (3 mg Norgestomet®) gelegt. Um den LH-Verlauf zu bestimmen, wurden Blutproben wie folgt entnommen: Von einer Stunde vor bis vier Stunden nach der letzten Hormonbehandlung alle 20 Minuten, anschließend 3 Stunden stündlich und für weitere 32 Stunden zweistündig. Die Brunstbeobachtung mit einem Suchbock wurde alle 6 h, beginnend 6 h vor der Behandlung bis zum Brunstende, durchgeführt. Die Ovulation wurde festgestellt,

indem bei einigen Ziegen aus jeder Versuchsgruppe die Ovarien 18 h nach der Behandlung im Zweistundenintervall ultrasonographisch beurteilt wurden.

Bei den 17 Tieren der GnRH-Gruppe setzte durchschnittlich 57 min (20 bis 60 min) nach Receptal®, noch vor dem Brunsteintritt der LH-Anstieg ein. Dieser wies im Vergleich zum physiologischen Verlauf (9,6 h bei NaCl-Gruppe) eine verkürzte Dauer von 7,2 h auf, und verlief bei allen Tieren synchron. Bei einem Tier zeigte sich etwa 10 Stunden später ein zweiter Anstieg. Die Ovulationen erfolgten durchschnittlich 20,5 h (20 bis 22 h) nach dem LH- Gipfel. Zeitverzögert und asynchron erfolgte die LH-Ausschüttung bei den Tieren der hCG-Gruppe. Die Ovulationen fanden etwa 16 h nach dem LH-Gipfel bzw. 34,7 h nach der hCG-Gabe statt. Vermutlich war das verabreichte hCG nicht für die Ovulationauslösung verantwortlich. Bei dem physiologischen LH-Verlauf (NaCl-Gruppe) erfolgte der LH-Anstieg unsynchronisiert durchschnittlich 16.8 h (10 bis 32 h) nach der NaCl-Injektion und die Ovulationen 43,4 h (32 bis 58 h) danach. Wiewohl sich bei allen Tieren der GnRH-Gruppe Ovulationen einstellten, bildeten sich die Gelbkörper innerhalb von 4 bis 5 Tagen wieder zurück, so dass sämtliche Tiere Kurzzyklen aufwiesen. Der Anteil Kurzzyklen bei der hCG-Gruppe betrug 88,2 % und bei der NaCl-Gruppe 56,3%. Durch den Einsatz von gestagen-haltigen Ohrimplantaten ließ sich die Luteolyse zwar nicht verhindern, doch wurden die Spülergebnisse verbessert durchschnittlich 3.2, 2.0 und 4.6 transfertauglichen Embryonen gewonnen, gegenüber lediglich 0, 1 und 1 den Tieren ohne Ohrimplantat.

Schlussfolgernd ist zu sagen, dass eine synchronisierte Ovulationsauslösung bei superovulierten Ziegen mit Hilfe von GnRH möglich ist, während hCG bestenfalls eine geringe Wirkung ausübt. Bei einer terminorientierten Besamung ist der optimale Besamungstermin abhängig vom verwendeten Präparat. Aufgrund des vermehrten Auftretens von Kurzzyklen nach einer Ovulationsauslösung mit GnRH oder hCG sollten die superovulierten Tiere bis zur Embryonengewinnung einer Gestagenbehandlung unterzogen werden.

Ziel des zweiten Versuchs was, die Pharmakokinetik von hCG nach einer i.m. Verabreichung bei superovulierten Ziegen zu charakterisieren. Pharmakokinetik beschäftigt sich damit, den zeitlichen Verlauf von hCG im Organismus, d.h. in verschiedenen biologischen Flüssigkeiten und Geweben, zu beschreiben. Die Pharmakokinetik beinhaltet die Prozesse der Resorption, der Verteilung, der Metabolisierung und der Beseitigung.

Die Untersuchung erfolgte an 9 pluriparen Burenziegen, denen im Ausschluss an eine Superovulationsbehandlung hCG induziert wurde. Um den hCG-Verlauf zu bestimmen, wurden Blutproben wie folgt entnommen: Vom Anfang bis 22 Stunden nach der Hormonbehandlung alle 2 Stunden, dann nach 26, 32, 38, 42, 60, 90 und 114 Stunden.

Die hCG-Konzentration im Blut stieg 20-40 min nach der hCG- Verabreichung an, um nach etwa 11 Stunden ihren Höhepunkt zu erreichen. Absorptionskonstante (0.34, SEM 0.06), Absorptionshalbwertszeit (2.7, SEM 0.5 h), Eliminationskonstante (0.02, SEM 0.002 h), Halbwertzeit von hCG (39.4, SEM 5.1 h) und scheinbares Verteilungsvolumen (16.9, SEM 4.3 L) wurden als pharmakokinetische Parameters berechnet. Die individuelle Variabilität war hoch. Die Halbwertzeit von hCG korreliert mit der Gipfelkonzentration ($r=-0.76$), der Resorptionskonstante ($r=-0.78$) und der Eliminationskonstante ($r=-0.87$).

Schlussfolgernd kann festgestellt werden, dass hCG nach einer intramuskulären Verabreichung schnell im Blut nachgewiesen werden kann, während sich der konzentrationsabfall über einen langen Zeitraum erstreckt.

Ziel des dritten Versuchs war, die Effizienz der Elternschaftskontrolle bei Burenziegen mit Hilfe von caprinen und ovinen Mikrosatelliten zu beurteilen. Mit dem Set von 13 Mikrosatelliten wurde zum einen die genetische Vielfalt innerhalb der Göttinger Burenziegenpopulation, zum anderen die Elternschaft von 13 ET-Lämmern untersucht. Die statistische Auswertung des Ausschlusses bzw. der Bestätigung der korrekten Abstammung wurde mit Hilfe der Programme CERVUS[®] und GenAlEX[®] vorgenommen. Der

Inzuchtkoeffizient wurde mit Hilfe des Programms F-STAT berechnet. An 112 mit 13 Mikrosatellitenloci analysierten Tieren wurden insgesamt 97 unterschiedliche Allele ermittelt. Alle untersuchten Marker erwiesen sich als polymorph, wobei die Anzahl der Allele zwischen vier (*CSRD247*) und 12 (*INRA5*) lag. Die mittlere Anzahl an Allelen betrug 7,46. Die erwartete Heterozygosität erstreckte sich von 0,42 (*BM1818*) bis 0.85 (*INRA5*) und betrug im Durchschnitt 0,63. Sie lag somit höher als die der südafrikanischen Herdbuch-Burenziegen. Der PIC (Polymorphism Information Content), erstreckte sich von 0,34 (*BM1818*) bis 0,82 (*INRA5*) mit einem Mittelwert von 0,59. Die Allelfrequenzen der vier Loci *McM527, OarFCB20, BM1258* und *INRA0132* wichen signifikant (p<0,01) vom Hardy-Weinberg-Gleichgewicht ab. Diese Abweichungen können sowohl auf Inzucht als auch auf Nicht-Äquilibrium innerhalb der Population zurückzuführen sein. Bei drei dieser Marker (*McM527, BM1258* und *INRA0132*) gibt es Hinweise auf das Vorliegen von Nullallelen. Der Inzuchtkoeffizient war mit 0,18 sehr gering, was sowohl auf eine erfolgreiche Vermeidung von Inzucht als auch auf den Import von Tiefgefriersperma und -embryonen aus Südafrika zurückzuführen ist. Die mit Hilfe der 13 Mikrosatelliten erzielbare Ausschlusswahrscheinlichkeit betrug 99,99%. Bei Nicht-Berückschtigung von drei der loci (*CSRD247*, *McM527* und *BM1818*) verringerte sie sich nur unmassgeblich. Es gelang, die vermeintliche Elternschaft von zwei der 13 ET-Lämmer aufgrund der Nichtübereinstimmung an mindestens vier Loci auszuschließen. Der Stammbaumfehler betrug somit in diesem Fall 15.4 %.

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