AN ABSTRACT OF THE THESIS OF

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A series of studies was performed to more clearly evaluate gonadal function in llamas. Luteinizing hormone (LH) and progesterone (P_4) concentrations were compared following natural or hormone-induced ovulation. Luteal regression was evaluated following administration of the prostaglandin $F_{2\alpha}$ analog cloprostenol. The interaction of age, season and castration on testosterone (T) and LH concentrations in the male llama were examined in peripubertal and adult animals.

Study I. Progesterone increased ($P < 0.05$) following treatment with 1 or 5 μg GnRH/kg ; 1.25, 6.25 or 31.25 IU hCG/kg; vasectomized or fertile mating. Peak P_4 concentrations were found on d 8 (4.4 ± 0.4 ng/ml, mean ± SEM) post-treatment with return to basal concentrations (< 0.5 ng/ml) by d 12 after all treatments except fertile matings. No treatment differences in time course of rise and decline, magnitude released or peak concentrations of P_4 were noted. Release of LH was examined following treatment with 1 μg GnRH/kg and breeding to a vasectomized or a fertile male. While the magnitude and duration of the LH peak (4.3 ± 0.6 ng/ml) was similar for all 3 treatments, GnRH caused LH to peak approximately 1 h sooner than mating.

Study II. Six llamas treated with cloprostenol aborted and underwent a 2 phase decline in P_4: 1) an initial rapid phase to half of pretreatment levels within 1 h of treatment and 2) a slower phase to basal levels (< 0.5 ng/ml) over the succeeding 48 h.

Study III. In 5 peripubertal males studied at 2 mo intervals from 8 to 20 mo of age, 24 h mean T concentrations increased with age, except for a decline at 14 mo. Basal LH and response to GnRH did not change as a function of age while response of T to GnRH increased with age. Although all animals were producing sperm at 20 mo of age, 3 of 5 had preputial-penile adhesions. In 6 adult male llamas, season had no effect on basal T or response to GnRH or on testicular dimensions.

Study IV. Although 24 h basal LH concentrations rose (0.7 ± 0.1 to 1.2 ± 0.1 ng/ml) 1 mo following castration in 4 male llamas, response to GnRH did not differ pre- or post-castration. While mean T in hourly samples for 12 h following 0.1, 1.0 or 5.0 μg GnRH/kg BW in 4 male llamas increased relative to the 12 h control period, there was no difference in T concentrations between dosages of GnRH. After castration, T in male llamas decreased sharply by 15 min and was undetectable by 3 h, exhibiting a 2-phase clearance pattern.

Llamas appear to have similar responses within the hypothalamo-hyophyseal-gonadal axis to other species, but with a lower "setpoint". Basal LH is low in the llama and small increases of LH are capable of causing ovulation and stimulating T production.

Studies on Luteinizing Hormone and Gonadal Steroids in Male and Female Llamas (*Lama glama*)

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Pamela J. Reed, Author

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CHAPTER I. INTRODUCTION

The llama is a member of the family Camelidae and is one of four camelid species native to South America. The llama (*Lama glama*) and the alpaca (*Lama pacos*) have been domesticated for centuries, while wild members of the genus include the guanaco (*Lama guanicoe*) and the vicuña (*Lama vicugna*, sometimes classified as *Vicugna vicugna*). All members of the family generally live in the altiplano region of the Andes mountains, although the guanaco is also found down to sea level in Southern Chile and all are capable of tolerating climatic extremes, including limited periods of drought (Jessup and Lance, 1982). The South American camelids (SAC) do not, however, have the same level of drought tolerance observed in the phylogenetically closely related Old World camelids (OWC), the two-humped Bactrian and one-humped Arabian (dromedary) camels.

During the past 20 years there has been a dramatic increase in interest in the llama, and to a lesser extent, the alpaca. This interest has been reflected in the steady increase in numbers of llamas (> 70,000) and alpacas (> 7,000) in North America (Smith, 1993). While the alpacas are primarily used for fiber production, the llamas are used as pack animals, as a source of moderate to high quality fiber, and as backyard companion animals. Associated with the shift from the extensive management styles employed in South America to the intensive management schemes employed in North

America, has been a concurrent shift in the composition and concern about the medical problems seen in these species. Of particular concern to North American producers has been the relatively high incidence of reproductive problems in both the llama and alpaca. As a result, reproductive problems have routinely been identified by producer groups (International Llama Association, Lama Association of North America) as one of the highest priority health issues facing the industry.

Since the Spanish conquest of Peru, Chile, and Bolivia in the 16th century, the South American camelid industry has been in decline. Historical records suggest that Inca management of the llama and alpaca was excellent prior to the introduction of sheep by the Spaniards. Recent archaeologic discoveries of desiccated sacrificial camelid remains in Peru have indicated that the Incas were raising animals with very high quality fiber more than 5000 years ago (Wheeler et al., 1995). Associated with the introduction of sheep, cattle, and pigs to the region was a concerted effort by the Spaniards to eliminate the llama and alpaca as major livestock species in the region (Von Hagen, 1961). These efforts were largely successful and in more modern times the reproductive rate in the llama and alpaca in South America has been reported to be very low, with one report noting as few as 5 to 20% of females bearing young each year (Foote et al., 1968). An additional report indicated a somewhat higher birth rate, but still estimated that no more than half of female alpaca of reproductive age bear young each year. It has been suggested, however, that with good management this rate may be increased to approximately 80% (Fernandez-Baca, 1975). Other reports indicate that neonatal mortality rates of 30 to 40% are not unusual. The low overall production rate observed in South America appears to be the result of a relatively low fertility and a

high rate of neonatal loss, particularly due to neonatal infections with *Clostridium perfringens* Type A (Fernandez-Baca, 1975).

Another significant change associated with the introduction of European livestock species to this region was a displacement of the llama and alpaca from lower altitude regions with higher quality forage. As the numbers of cattle, sheep, and pigs increased, the llama and alpaca were displaced into the higher, more nutritionally marginal regions of the altiplano. This displacement has continued to the present day with few llamas and alpacas found outside of the altiplano region.

Since most llamas and alpacas in South America are owned by campesinos with very limited financial resources, a low to moderate fertility rate and a high neonatal loss rate has been acceptable. In North America, however, the value of the animals is markedly higher and the fertility and neonatal loss rates experienced in South America have not been acceptable to producers. As a result, there has been a major emphasis in the industry for enhanced diagnostic and treatment protocols. Developments in this area have, however, been hampered by a poor understanding of significant aspects of normal reproduction in these species. Accordingly, the overall focus of this thesis is to address specific aspects of normal reproduction in the llama.

Specific Issues: Llamas and all camelids have long been known to be induced ovulators (Novoa, 1970). Although llamas normally ovulate only following breeding, ovulation can also be hormonally induced with human chorionic gonadotropin (hCG) (England et al., 1969) or gonadotropin releasing hormone (GnRH) (Bravo et al., 1992). Recommended dosages and routes of administration to reliably induce ovulation vary widely and are often extrapolated from other species, specifically cattle and sheep. The

third chapter in this thesis contains results of a study designed to investigate ovulatory treatments in female llamas, including comparisons of different treatments with hCG and GnRH. The fourth chapter contains results from a study investigating the termination of early pregnancy in llamas by the prostaglandin $F_{2\alpha}$ analog cloprostenol.

Less is known about reproduction in the male llama than in the female, probably as a result of several factors. One factor is the relative overabundance of males in both South and North America, which has made the focus of reproductive research remain primarily on the female since a male which fails to perform to desired specifications can often be more easily replaced than evaluated. While the value of average quality males in North America is relatively low, breeding quality males frequently command very high prices. In this population of animals, questions of infertility have become economically important. There is also the recurring question of the proper age to use a young male for breeding. Resolution of these issues requires a better understanding of normal reproduction in the male llama.

While the OWC have distinct rutting seasons during which physiological and behavioral changes occur in the male, and the female undergoes a corresponding period of cyclic receptivity, similar changes are not observed in the SAC (Novoa, 1970). There are few reports in the literature on the interaction of season and reproduction, normal age at puberty, normal luteinizing hormone and testosterone concentrations, hormonal response to gonadotropin releasing hormone and effect of castration on reproductive hormones in the llama. The studies performed for the fifth and sixth chapters of this thesis partially address these issues.

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CHAPTER II. REVIEW OF THE LITERATURE

II.1. The Camelidae Family

Depending upon the phylogenetic scheme used, the family Camelidae is divided into two or three genera: *Camelus*, comprised of two species, *Camelus dromedarius* (the dromedary or one-humped Arabian camel) and *Camelus bactrianus* (the two-humped Bactrian camel); and *Lama*, divided into four species, *Lama glama* (the llama), *Lama pacos* (the alpaca), *Lama guanicoe* (the guanaco) and *Lama vicugna* (the vicuña). The vicuña is sometimes classified as a separate genus, *Vicugna*, on the basis of open-rooted incisors (Franklin, 1981). Although the camelids originated in North America, forerunners of the family migrated approximately 3 million years ago to Asia by way of the Beringia land bridge and to South America by way of the Panamanian land bridge. The Asian branch of the family further evolved into members of the genus *Camelus*, represented by the one-humped camel of southwest Asian and northern African deserts and the two-humped Bactrian camel of southern Russia and Mongolia. The South American branch of the family became members of the *Lama* genus, now known as South American camelids (SAC). The llama and alpaca are domesticated species while the vicuña and guanaco are wild species (Franklin, 1981).

The camelids are uniquely adapted to their respective environments. All live in relatively arid areas with only periodic rainfall. The Old World camelids (OWC) are adapted in various ways for water conservation in areas of water scarcity and to travel on sand. In addition, Bactrian camels have shaggy hair which helps insulate them from

the cold temperatures found in their native lands (Dorman, 1986). The SAC are adapted to life at high altitudes and significant diurnal variations in temperature (Flores, 1979). All members of the Camelidae family are capable of efficient utilization of poor-quality forage by means of fermentation in a compartmentalized forestomach. Although their digestive system shares some characteristics with ruminants, specifically compartmentalized stomachs and fermentation, they are not closely phylogenetically related, having diverged from common ancestry approximately 35 million years ago (Franklin, 1989). Camelids are capable of greater utilization from poor quality forages than ruminants (Franklin, 1981). The SAC in particular have approximately 37% less digestible energy requirements than sheep and consume up to 26% less dry matter on range sites than sheep (Johnson, 1989, 1994; Carmean et al., 1992).

Camelids include species that are among those domesticated by man for the longest periods of time. Old World camelids have been used for transportation, fiber and food for centuries and were probably first domesticated 5000 to 4000 B.C. (Dorman, 1986). The SAC were hunted as sources of meat and fiber by preceramic societies of man as early as 10,000 years ago. Later societies domesticated the SAC and began selective breeding programs to develop the types known today as llamas and alpacas, probably 4,000 to 5,000 years ago (Franklin, 1981). It is believed by archeologists that the guanaco served as foundation stock for breeding programs that developed the llama as a beast of burden and the alpaca as a source of meat and fiber. The smaller, finely wooled vicuña may have contributed some to breeding programs as well, particularly in the case of alpacas. Recent studies of DNA in these species support the theory that the guanaco served as the base stock for the llama and the alpaca

although the role of the vicuña is less clear (Stanley et al., 1994; Vidal-Rioja et al., 1994; Wheeler, 1995). All four species of SAC are capable of interbreeding, producing fertile offspring, arguing against classification of the vicuña as a separate species. The two types of OWC can also interbreed with each other, although such matings may be difficult due to the smaller size and presence of the second hump in the Bactrian camel. Karyotypes of all camelids are similar with 37 pairs of chromosomes (Franklin, 1981).

While initial development of the llama and alpaca occurred in pre-Incan societies, the Inca empire refined the breeding types and herd management to a high degree (Von Hagen, 1961; Franklin, 1981). The SAC held an important place in Inca civilization, in daily life and as religious objects (Markham, 1969). With the destruction of the Inca empire by the conquering Spaniards, both domesticated and wild South American camelids were forced higher into the Andes mountains. The ranges of the guanaco and vicuña became even smaller through loss of habitat and competition for forage with domestic cattle and sheep (Franklin, 1981). The domesticated llama and alpaca became animals left to the poorest of Indians and were underutilized for centuries in the lands to which they were uniquely adapted. The wild SAC, particularly the vicuña, were hunted nearly to extinction and it was not until the early to mid twentieth century that protective measures were initiated and not until the mid to late twentieth century that these measures were sufficiently enforced to encourage an increase in numbers of vicuña. Some interest in reviving economic usefulness of domesticated SAC has also resurfaced in the twentieth century, along with intensification of research in normal anatomy and physiology. In spite of their long association with man, there has been relatively little research done in these species until recently. This interest was

heightened when the llama and to a lesser extent the alpaca became popular in North America, primarily as companion animals in the last 15 to 20 years. This literature review will focus on reproductive physiology of the camelids, with comparison to other induced ovulator species and other domestic species where relevant.

II.2. Reproduction in the Female Camelid

II.2.1. **Anatomy**.

While there is relatively little published information concerning the reproductive anatomy of the female llama, there is a large body of information about OWC anatomy and some reports on alpaca anatomy. The reproductive tracts of all camelids are similar in gross appearance and microscopic structure, with differences in relative size relating to size of the animal. The llama (average adult weight about 150 kg; Smith et al., 1985) is much smaller than the camel (average adult weight about 450 kg; Yagil, 1982) but larger than the alpaca (average adult weight about 60 kg; Smith et al., 1985).

Ovaries in the camel are approximately 4 cm long by 2.5 cm wide and 0.5 cm deep (Arthur et al, 1985), while the ovaries in the llama are approximately 2 cm long by 1 cm wide by 0.5 cm deep (Johnson, 1989). These dimensions can double with follicular activity or when an active corpus luteum is present. The corpus luteum projects from the surface of the ovary in the camel but is not typically crowned as in the bovine (Arthur and Al-Rahim, 1982). There is no ovulation fossa and follicles of all stages are distributed throughout the ovary. The dynamics of primary and secondary

follicular development and subsequent atresia are similar to those observed in the cow and sheep (Bravo et al., 1990a). In contrast with the domestic ruminant species, ovulation in camelids occurs only following mating, although spontaneous ovulation is occasionally observed in the absence of mating (Novoa, 1970) .

The uterine tubes of camelids possess a well-developed ovarian bursa and are highly convoluted. The uterine tubes are approximately 17 to 28 cm long in the camel (Wilson, 1983) and 12 to 14 cm long in the llama (Smith et al., 1994). A unique feature of the uterine tubes of all camelids is the presence of a distinct papillae at the entrance to the uterine tubes. These papillae have sphincters which may prevent fluid from the uterus from entering the oviducts, making salpingitis rare (Smith et al., 1994).

The uterus of the camel and llama is bicornuate, somewhat similar in external appearance to that of the horse, with relatively short uterine horns and a distinct body. In llamas, however, the cut surface of the uterus reveals a velum separating the caudal portion of the uterine horns, making them longer than they appear on the surface. This velum is occasionally observed on ultrasound of the pregnant uterus and can be incorrectly interpreted as fetal membranes or abnormal structures. The velum is not complete to the cervix and a short uterine body (2.5 by 2.5 cm) is present. The uterine horns measure approximately 2 by 6 cm in the nulliparous female llama and are bilaterally symmetrical. In contrast, the left uterine horn will usually measure approximately 3 by 10 cm in a nonpregnant previously gravid female llama (Johnson, 1989). This asymmetry in the multiparous animal is due to > 95% of all pregnancies in camelids being carried in the left uterine horn.

The cervix of the llama has 2 to 3 cartilaginous folds. These folds are not, however, as distinct as those found in cattle. Some authors report that the folds in a llama cervix are actually a counterclockwise spiral of the cervix rather than distinct rings as observed in the bovine (Smith et al., 1994). It has been speculated that this would allow the clockwise spiral of the male penis to more easily enter the cervix and facilitate intrauterine deposition of semen during mating. The vagina in the llama is unremarkable, measuring approximately 20 to 25 cm in length, ending in a small vulva with a well-developed clitoris (Smith et al., 1994).

II.2.2. **Puberty and breeding practices.**

The female llama is reported to reach puberty at 1 to 2 years of age, although little research data are available in the literature to substantiate this observation. Pregnancy has been reported by owners of llamas to occur as young as 6 months of age in female animals pastured with males, indicating that puberty can occur at an earlier age. Common breeding practices in South America usually involve first breeding of female alpacas at 1 to 2 years of age (Novoa, 1970). Yearling alpacas may ovulate but have lower fertility than adults with up to 40% being nonovulatory (Sumar et al., 1993). In North America, it is customary to breed llamas of average frame size at a body weight of 200 pounds if they are over 12 to 16 months of age, whichever occurs latest.

In other species, it has been shown that puberty in both males and females occurs when a certain critical weight has been reached, usually about 50 to 60% of adult body weight. The relationship between body weight and puberty is usually more

important than age in cattle and sheep (Hafez, 1993). Consequently, animals on higher planes of nutrition tend to reach puberty at a younger age. There is also a relationship between breeds and age at puberty in cattle and sheep, often related to adult body weight and the length of time required to achieve mature body weight. Llamas do not have recognized breeds in the United States, although there are recognized body types in favor with breeders. In llamas, a practical recommendation has been to begin breeding when about 60% of expected adult body weight is achieved.

In camels, females are usually bred first at 4 to 6 years of age and thereafter are bred only every other year (Yagil, 1985). Although little data are available on hormonal factors related to puberty in camels, with better management camels could probably be bred first at 2 to 3 years of age. Although it is stated by some authors that camels could have a calf nearly every calendar year, maximum fecundity is not given high priority among herders, with other sociologic factors being of primary importance to Bedouin camel breeders. Since the gestation period of camels is 13 months and camels are seasonal breeders, it is difficult to have one calf/female/calendar year unless the female is bred very early in the breeding season in one year and late in the season on alternate years. One author stated that a reasonable goal for camel herds would be for females to have 2 calves in 3 years (Arthur, 1992).

Llamas and alpacas are bred to have an offspring or cria each year if possible. Although the female SAC may be receptive to the male within hours of giving birth, higher conception rates occurred at breeding 20 or 30 days postpartum when compared to breeding 10 days postpartum (Bravo et al., 1994). It has been suggested that the limiting factor in re-establishing postpartum fertility in the llama is the time needed for

uterine involution although this is not usually fully achieved by the time the animal is bred (Johnson, 1989, 1994). Waiting for a period of time after parturition prior to rebreeding also allows time for postpartum infections to be identified and treated. Most llama owners prefer to rebreed animals at approximately 2 weeks postpartum. There is some anecdotal evidence among owners that waiting longer than 4 weeks post-partum before breeding results in lowered conception rates and prolonged postpartum intervals. By 4 to 6 weeks postpartum the dam is approaching peak lactation and the energetics of heavy lactation may result in lowered fertility, similar to lactational anestrous observed in other species. It has also been observed that lactation in llamas alters the interval of dominant follicle development (Adams et al., 1990a).

In cattle, which normally do not cycle for a period of time after calving, lowered ovulation rates and fewer signs of estrus are often evident until after the peak of lactation has passed (Hafez, 1993). Although SAC do not have estrous cycles, it is likely that the hormones and nutritional requirements of lactation interfere to some degree with follicular development and/or ovulation. If a SAC suffers a postpartum uterine infection, injury or illness or if there is a lapse in management that causes a delay in breeding, the birthing interval becomes prolonged beyond a year and there is a loss of production for the animal. It is thus desirable to quickly note and treat infections and utilize good management procedures to keep the postpartum breeding interval as short as possible.

While camelids can be successfully bred well into their teens or even twenties (especially in the case of camels), this usually does not happen. By the time most llamas are 12 to 15 years of age, they have commonly had a dystocia, uterine infection

or other problem which results in impaired fertility. Older animals also frequently require a higher plane of nutrition to maintain body condition and successfully carry a pregnancy to term and nurse young. In North America, such care is often given to llamas and many cases of llamas over 15 years of age giving birth are known.

II.2.3. **Reproductive behavior: season and receptivity.**

While domesticated SAC do not exhibit any inherent seasonal variation in reproductive parameters, this is not true for OWC. Female camels have alterations in hormone levels, follicular activity and behavior during the breeding season (Shalash, 1965; ElWishy, 1987; Ismail, 1987). Female camels are also described by many authors as having estrous cycles, although since they are induced ovulators this likely refers to periods of behavior in which they are more receptive to the male interspersed with periods of diminished sexual receptivity. Female SAC are generally continuously receptive to the male, except for brief periods of nonreceptivity which are often assumed to be associated with times of ovarian inactivity between successive follicular waves (England et al, 1969a). Some researchers have found, however, that little correlation exists between follicular activity and receptivity in the alpaca, with periods of lowered receptivity to the male apparently occurring at random (Bravo and Sumar, 1989). Nonreceptivity in llamas is also sometimes associated with particular male-female pairings.

The SAC were once considered to be seasonal breeders as in the OWC because in their native climate they breed during the rainy season, which is during the summer in

South America. This allows them to give birth in the next year's rainy season when adequate forage is available. Vicuñas in particular have a distinct breeding season (Franklin, 1981). Llamas and alpacas are capable of breeding year-round, but it is not clear if season plays any role on fertility. Definitive data on reproductive hormones and fertility in different seasons is lacking. A zoo survey report noted that while llama and alpaca births occurred year-round, births were concentrated in spring and summer months. Vicuña and guanaco births were even more strongly concentrated seasonally, suggesting that seasonal effects on breeding are more pronounced in the nondomesticated SAC (Schmidt, 1973).

Female dromedaries undergo estrous behavioral cycles with greater follicular activity during the rainy season in their native climates. This occurs most frequently during the late winter, spring and early summer months (Gauthier-Pilters and Dagg, 1981). In Somalia, which has a spring and shorter fall rainy season, changes in hormones corresponding with rutting and mating occur in both rainy seasons, although most breeding occurs during the spring rainy season (Bono et al., 1989). There is also evidence to suggest that in OWC, which do have hormonal changes associated with seasonal breeding, breeding is associated with rainfall and forage availability as in SAC rather than with photoperiod. It has been suggested that camels on adequate forage are capable of breeding year-round (Arthur et al., 1985). Some reports state that wild Bactrian camels in Russia breed year-round, while in captivity they are seasonal breeders (Yagil, 1982). Other investigators report a spring breeding season similar to that in the dromedary, both in the wild and domestic situations (Zhao et al., 1994). The previously mentioned zoo survey noted that 95.8% of Bactrian camel births took place

in February to May, while 72.3% of dromedary births occurred during this same time period (Schmidt, 1973).

Although camels undergo changes in reproductive hormones and behavior during the breeding season, these changes are somewhat different than those observed in photoperiod-induced seasonal breeders such as the sheep and the horse. Although changes in circulating glucocorticoid concentrations are not normally associated with seasonal breeding in other species, rises in corticoids were the most striking change in dromedaries during the rainy season which is usually associated with the breeding season. Corticoids also rose during periods of drought (Bono et al., 1989). In female dromedaries, estrogens increased during the breeding season, as well as LH (Bono et al., 1989), although LH concentrations appear to be relatively low in all camelids at all times and rise significantly only after breeding with an ovulatory surge.

In photoperiodically controlled seasonal breeders, such as sheep (short day breeders), basal LH concentrations increase during the estrous season and decrease during anestrous. Both LH and FSH are more clearly produced as pulsatile hormones in these species than in camelids (Hafez, 1993).

Estrous behavior in camels is similar to other species. The female camel produces copious amounts of vaginal mucus, urinates frequently (especially in the presence of the male), vocalizes and becomes restless. Periods of estrus may last up to 2 weeks in the absence of a male and mating but signs of estrus diminish 3 to 4 days following mating (Arthur et al., 1985). Another author reports that the dromedary has estrous cycles of 2 to 3 weeks duration with estrus lasting 3 to 4 days, while the Bactrian camel is reported to have intervals of 10 to 40 days between periods of estrus (Novoa,

1970). Receptivity of 3 to 4 days duration followed by 10 to 12 days of nonreceptivity in the dromedary has also been described (Yagil, 1985). The periods of receptivity occur only when a mature follicle is present.

In the alpaca and llama, the female shows no overt signs of estrus and is generally receptive to the male unless an active CL is present. The level of receptivity is not, however, constant and brief periods of nonreceptivity or lessened receptivity may occur (England et al., 1969a). These periods of nonreceptivity are not clearly correlated with follicular activity or lack thereof (Sumar et al., 1993). Certain females may not show immediate receptivity and some females exhibit decided preferences and dislikes for particular males (Smith et al., 1994). Receptivity takes the form of the female assuming the sitting or "cush" position at the approach of the male. While the male may initially have to chase the receptive female and mount her one or more times before she assumes the cush position, females that are clearly non-receptive will kick and spit at the male and will not submit to mating unless forced down by an unusually aggressive male (Fernandez-Baca, 1993). The female will generally be nonreceptive only if high levels of progesterone are present (Smith et al., 1994).

Mating in camelids occurs in the cush position and takes place over a prolonged period of time. Duration of coitus in the camel varies from 1 to 35 minutes (Wilson, 1984; Arthur, 1992) with an average length of 10 to 20 minutes (Merkt et al., 1987). In the llama and the alpaca, copulation varies from 3 to 65 minutes in duration (Smith et al., 1994) with an average length of 18 to 20 minutes (Bravo, 1994). During periods of copulation the female moves little except to occasionally look at the male and perhaps shift her weight. Some females will roll onto their sides which may or may not interrupt

copulation. If mating occurs in a pasture situation with other animals present, herdmates will frequently gather around the mating pair and receptive females may assume the cush position nearby (Smith et al., 1994).

II.2.4. **Ovulation**.

Ovulation is copulation-induced in camelids although rare instances of spontaneous ovulation have been reported (England et al., 1969b; Sumar, 1994). Copulation-induced ovulation in the camelid was originally established via studies of reproductive tracts of camels collected from abattoirs (Shalash and Nawito, 1964; ElWishy, 1988) and studies of alpacas in which animals were sacrificed and the reproductive tracts were examined (Fernandez-Baca et al., 1970a). In these types of studies, functional corpora lutea were found only during pregnancy or shortly after known breedings. Laparotomy studies in alpacas support the theory of induced ovulation although it was reported that spontaneous ovulation occurred occasionally with an overall rate of 3.5% (Bravo and Sumar, 1989). Other reports indicate that the incidence of spontaneous ovulation may be as high as 10% (Sumar, 1994). There is the suggestion that most of these spontaneous ovulations occur early in the breeding season with reintroduction of males into female herds or with other pheromonal stimuli. In South America, male alpacas are usually kept separated from females except during the breeding season when several males are pastured with large numbers of females (Condorena and Valasco, 1978). Spontaneous ovulation may occur soon after male introduction to herds in camels (Marie and Anouassi, 1987) or when males are

introduced briefly as "teaser" animals to check for pregnancy in alpacas and llamas (Sumar, 1994). The author of this thesis has observed spontaneous ovulations in females during the course of other studies when animals were not exposed to the male or ovulation-inducing treatment.

Follicular waves occur at approximately 11 day intervals in llamas, with ovaries alternating as to the presence of the dominant follicle 81% of the time (Bravo et al., 1990a). Llamas mated in the presence of a growing or mature follicle ovulated the dominant follicle of that follicular wave (Bravo et al., 1991a). If an animal was mated when only small (4 to 5 mm) follicles were present, no ovulation occurred. Furthermore, if follicles were already beginning to regress, ovulation did not occur although luteinization of the follicular walls did. These luteal structures had a shortened life span (5.1 days) compared to normal (12 days) (Bravo et al., 1991a). Hemorrhagic follicles have also been described in llamas. These do not, however, appear to have significant effect on fertility (Adams et al., 1991).

In the dromedary, ovulation occurs 32 to 40 hours following mating, while in the Bactrian camel it occurs 36 to 48 hours post-coitus (Merkt et al., 1987). Manual stimulation of the cervix and mounting of the female by the male is not sufficient to promote ovulation in camelids. This is in contrast to other induced ovulators such as the rabbit and cat, in which cervical stimulation is sufficient to induce ovulation (Nalbandov, 1976; Cupps, 1991). Although cervical stimulation does not result in ovulation in camelids, it is of note that the male's penis enters the uterus during coitus and uterine stimulation may be necessary for ovulation. In the author's experience

(unpublished observations), manipulation of the uterus to take biopsies or instill saline and/or antibiotic flushes very rarely results in ovulation in llamas.

In Bactrian camels, infusion of semen was noted to result in ovulation. Seminal plasma without sperm and bull semen worked as well as whole camel semen and ovulation rates and times were similar to those after natural service. It is suggested that camel semen contains an LH-like factor to help promote ovulation (Chen et al., 1985). Intramuscular injection of camel seminal plasma was also effective at inducing ovulation in Bactrian camels (Zhao et al., 1994). Studies in llamas show that insemination does result in ovulation in a proportion of animals, while mounting alone does not (Fernandez-Baca et al., 1970b; Fernandez-Baca, 1993).

Ovulation in SAC appears to occur somewhat sooner after mating than in OWC. The usual timing of ovulation as detected by transrectal ultrasonography is within 2 days after mating in llamas (Adams et al., 1989) and has been reported as 26 hours post-coitus in alpacas sacrificed at various times after mating (San-Martin et al., 1968). In alpacas undergoing laparoscopy immediately, 30 hours and 72 hours after coitus, 50% of the animals had ovulated by 30 hours post-coitus and an additional 24% by 72 hours post-coitus. The remainder of the animals in the experiment were classified as non-ovulatory (Sumar et al., 1993).

Ovulation can also be induced in camelids following injections of GnRH, LH or hCG. Timing of ovulation after such treatment is similar to that following mating although ovulation may occur 1 to 2 hours sooner with hormonal treatment than after mating. In Bactrian camels, 300 IU LH, 1000-2000 IU hCG or 250-500 μg GnRH induced ovulation with similar timing and rates as with seminal infusion and natural

mating (Chen et al., 1985). Fifty μg GnRH administered every 2 hours to female dromedaries also resulted in an end of estrus followed by development of a short-lived CL (Homeida et al., 1991). In alpacas, 750 IU hCG given intramuscularly resulted in a 100% ovulation rate (Fernandez-Baca et al., 1970). Different doses ranging from 25 to 1600 IU hCG given intravenously to alpacas resulted in ovulation as well, although rates were lower with 25 IU compared to higher doses (San-Martin et al., 1968). Llamas given a single intramuscular dose of 500 or 750 IU hCG resulted in 9 of 10 animals ovulating (Adam et al., 1989) while 1000 μg GnRH administered iv resulted in ovulation in 89% of alpacas and 92% of llamas treated in another study (Bravo et al., 1992a). England and others in 1969 gave different doses of hCG to llamas and found variable ovulation rates following different dosages. Administration of 25 IU hCG resulted in a 50% ovulation rate while 500 IU hCG resulted in a 100% ovulation rate. These researchers also found a non-significant trend towards fewer ovulations during the late April to May period than in March, the peak breeding season. Intramuscular injections of 300 IU hCG to alpacas resulted in higher peak progesterone levels and lower 20 alpha-hydroxy-4-pregnen-3-one than with natural service that resulted in pregnancy (Fernandez-Baca et al., 1970b).

The number and duration of copulations appear to be unrelated to ovulation rate in the llama. Although there is a general feeling among llama owners in North America that multiple services improve conception rate, a study by Bravo and others (1992a) found no evidence that repeated copulations increased LH levels or the likelihood of ovulation. Generally, animals ovulated with the first service with a maximal release of LH and further services did not result in further LH release. In alpacas, multiple

services had no effect on conception rate when compared to single service (Fernandez-Baca et al., 1970c). In contrast, in the cat, another induced ovulator species, multiple services produce a greater release of LH (Concannon et al., 1980) and may contribute to more follicles ovulating when compared with queens mated only once (Wildt et al., 1980).

Length of time spent copulating has also not been correlated with conception rates in camelids. Interruption of service after the first 5 minutes in the alpaca does not appear to interfere with fertility (Fernandez-Baca et al., 1970c). Time spent copulating in all camelids appears to be largely determined by male preference, although it is not always consistent with the same male.

Although fetal development in camelids occurs almost exclusively in the left uterine horn, ovulation occurs bilaterally. Whether there is a predisposition for ovulation from the left ovary or not differs according to study. While some researchers report that ovulation alternates between ovaries, other studies in camels report a significantly higher rate of ovulation in the left ovary based on regressed CL found at slaughter. In a review of camel reproduction literature , reports from 50.2 to 56.5% of corpora lutea were found on the left ovary (ElWishy, 1987). Most studies in camels suggest nearly equal rates of ovulation in the left and right ovaries with about a 1 to 2% higher rate in the left ovary. In alpacas and llamas, ovulation rates from each ovary are approximately equal. It has been reported that 85% of alpacas had succeeding dominant follicles in alternating ovaries as determined by laparoscopy (Bravo and Sumar, 1989) and that 81% of cycles alternate ovaries in llamas as determined by ultrasound (Bravo et al., 1990a).

Multiple (usually two) ovulations are relatively common in camelids, being reported as 12.5 to 14.7% (Merkt et al., 1987), 14.7 to 18.6% (ElWishy, 1987) and 17.1% (ElWishy, 1988a) in one-humped camels and 5.5 to 22.2% in alpacas (Novoa, 1970). Multiple births are extremely rare in all camelids. This suggests that most multiple ovulations result in only a single conception and(or) only one conceptus survives the early embryonic period. Twin conceptuses have been observed in alpacas on day 31 but not thereafter in South American studies (Fernandez-Baca et al., 1970a) and were diagnosed by ultrasound on day 47 in a llama in one study but only one had a heartbeat (Adams et al., 1989). Occasionally twin fetuses are spontaneously aborted after 3 to 6 months of gestation. There have been a few reported cases of live twin llama births in the United States (Fowler, 1990).

Following copulation, LH concentrations increase to a maximum 1.4 to 2.0 hours later in the llama (Bravo et al., 1990) with an initial increase 15 minutes after copulation began and a return to basal concentration by 7 hours post-mating. Basal LH concentrations in this study were low in llamas (0.7 ng/ml) and the ovulatory peak concentrations were lower (4.4 ± 1.5 ng/ml, representing a five- to seven-fold increase) than those usually observed in other species, including other induced ovulators. LH concentrations reached following GnRH administration in the llama are similar to those observed after natural mating but rise somewhat sooner (Bravo et al., 1992a).

Basal LH concentrations in the dromedary are somewhat higher (0.7 to 3.0 ng/ml) than in the llama and reach higher ovulatory peak concentrations (2.9 to 19.1 ng/ml) 180 to 240 minutes following mating, returning to basal concentrations by 10.5 hours after mating (Marie and Anouassi, 1986) and LH concentration changes following

GnRH administration are similar in magnitude but the increase occurs somewhat sooner (Marie, 1992). In the Bactrian camel, basal LH concentrations have been reported to be 2.7 ± 1.2 ng/ml (mean ± SEM) and to peak at 6.9 ± 1.0 ng/ml 4 hours after insemination (Xu et al., 1985).

The cat, another induced ovulator, undergoes an ovulatory LH surge 30 to 100 times greater than basal LH concentrations (1.8 to 3.2 ng/ml), with the surge typically lasting about 90 minutes (Wildt et al., 1981). Further increases in LH can occur with multiple matings in cats. The rabbit has an LH peak that increases 100 times over basal concentrations (19 to 30 ng/ml) by 1 to 2 hours after mating and this peak is sustained for 1 hour (Scaramuzzi et al., 1972).

In the cow, a non-induced ovulator, LH undergoes a preovulatory surge that is a 19 to 20 fold increase over basal concentrations of 1.4 ± 0.1 (mean ± SEM) ng/ml and lasts about 8 hours (Swanson and Hafs, 1971). In sheep, the LH surge is even more dramatic with increases of 40-fold or more over basal concentrations of 2.9 ± 0.9 (mean ± SEM) ng/ml and occurs over approximately 10 hours (Goding et al., 1969).

II.2.5. **Pregnancy and corpus luteum life span.**

After mating or induction of ovulation with hormones, progesterone concentrations increase within 3 to 5 days in the llama. The llama and alpaca may cease to be receptive to the male before measurable concentrations of progesterone are observed. If pregnancy does not occur, progesterone concentrations reach a maximum at day 6 to 9 post-coitus, then decrease to basal concentrations by day 10 to 13 after

mating (Adam et al., 1989; Leon, 1990). Basal progesterone concentrations can be somewhat variable and some females have concentrations of approximately 0.5 ng/ml when not pregnant. For most animals, however, progesterone concentrations in the non-pregnant animal will be less than 0.5 ng/ml and are usually undetectable by routinely used assay methods. If pregnancy occurs, progesterone levels remain elevated until shortly before parturition (Leon et al., 1990). In the dromedary that ovulates but does not conceive, progesterone concentrations reach a maximum 7.2 days following ovulatory stimulation before dropping to undetectable concentrations by day 17 post-coitus (ElWishy, 1987).

In llamas undergoing daily transrectal ultrasonography, the CL have usually formed by day 3.1 ± 0.2 (mean ± SEM) after ovulatory stimulus, reaching maximum size by day 5.9 ± 0.3, begun regression by day 9 and are not detectable by day 12 (Adams et al., 1990b). Studies in alpacas in which animals were sacrificed after treatments with hCG or vasectomized breeding reported that the CL had reached maximum size and secretion by day 8 to 9, decreased in size by day 12 and regression was completed by day 18 post-treatment (Fernandez-Baca et al., 1970b). The CL is required for the length of gestation in alpacas and llamas, since removal of ovaries or CL ablation at any time in gestation resulted in abortion or premature delivery (Sumar, 1988).

Although pseudopregnancy has been reported in the alpaca, it is unclear if the condition described would meet the generally accepted definition of pseudopregnancy (San-Martin et al., 1968). True pseudopregnancy with hormonal, behavioral and physical changes such as occurs in cats and dogs is not generally recognized to occur in

SAC. While true pseudopregnancy is not generally recognized as occurring in the camelids, a persistent or retained CL is a recognized cause of infertility. This problem is generally not identified until the normal gestation length (342 ± 10 days) has passed without ensuing parturition in situations in which only serum progesterone has been used for pregnancy diagnosis. In some cases, elevated progesterone concentrations have been reported for more than a year in the absence of pregnancy (Smith et al., 1994). Although this condition has been referred to by some as a pseudopregnancy, it lacks the behavioral and physical characteristics associated with pregnancy which are observed in true pseudopregnancy as well.

The female SAC will usually remain unreceptive to the male as long as progesterone concentrations remain elevated to concentrations associated with an active CL (usually > 1.5 ng/ml) and is frequently used to diagnose pregnancy (Smith et al., 1994). Elevated progesterone concentrations (> 1 ng/ml) at 1 to 2 weeks post-breeding are indicative of ovulation while progesterone concentrations which remain elevated past 2 weeks post-breeding in the absence of further exposure to the male are generally indicative of pregnancy. Pregnancy can also be confirmed by rectal palpation and use of rectal or transabdominal ultrasound. Transrectal ultrasonography has been used to detect embryos as early as 9 to 11 days after mating but accuracy increases at 15 days or greater (Adams et al., 1989). Accuracy of rectal palpation alone increases past 45 days of gestation (Bravo, 1994). Transabdominal ultrasonography becomes reliable past 3 to 4 months of gestation (Smith et al., 1994). External ballottement of the fetus is used in South America to diagnose pregnancy and works well at later stages of pregnancy (Smith et al., 1994). Transrectal ultrasonography can be used to predict fetal age by

using measurements of fetal head width (Haibel and Fung, 1991) or other features (Bravo and Varela, 1993). Since early embryonic mortality is relatively high in camelids, any pregnancy diagnosed prior to 30 days should be reconfirmed at 45 to 60 days.

The length of gestation for SAC is about 11.5 months and about 13 months for camels. Some reported ranges in days for alpacas are 342 to 345 (San-Martin et al., 1968), for llamas 350.0 ± 4.5 (Leon et al., 1990), for the dromedary 383 ± 9 (Agarwal et al., 1987), and 406 (Gauthier-Pilters and Dagg, 1981) for the Bactrian camel. Reports vary as to what is normal variability for all these species, since uncertainty of breeding dates may exist in some herds and animals may be mated more than once. As a result, a diagnosis of prematurity is best made by appearance and behavior of the newborn and not presumptive gestational length. In SAC, full-term crias should have erect ears, at least 2 sets of incisors erupted and be able to stand and nurse shortly after birth (Paul-Murphy, 1989). As in foals, dysmaturity, or behavior of a premature animal with an adequate gestational age, is sometimes a problem. Trans-rectal or trans-abdominal ultrasonography are recommended to confirm pregnancy and assess fetal age in the llama, since it has been shown that fetal head growth can reliably estimate stage of gestation (Haibel and Fung, 1991).

Hormonal changes associated with pregnancy, other than progesterone, have not been widely studied. Serum estrogens have been found to rise throughout pregnancy in the llama (Leon et al., 1990) and in the last few months of pregnancy in the camel, although they are lower in camels pregnant with male fetuses than with female progeny (Agarwal et al., 1987). Changes in serum estrogens are reflected in higher urinary

estrogens in late pregnancy in camels (ElWishy, 1987) and in llamas during the last 20 days of gestation (Bravo et al., 1991b). Changes in glucocorticoid concentrations throughout gestation in the llama are similar to those reported in other species, rising approximately 24 hours prior to parturition, then declining immediately after birth (Leon et al., 1990).

Although the ovaries appear to function almost equally, pregnancy in camelids occurs almost exclusively in the left horn of the uterus (> 95%). In the dromedary, left horn pregnancy rates in slaughterhouse studies were reported as 98.2 to 100% in a review by ElWishy in 1987. Most right horn pregnancies were observed only at very early stages. In llamas, all animals in a study in which ultrasonography was used to examine the reproductive tract had left horn pregnancies (Adams et al., 1989). There is evidence of embryo migration from the right to the left horn since corpora lutea have been found in right ovaries of alpacas with left horn pregnancies (Fernandez-Baca et al., 1970). In these alpacas, no right horn pregnancies were observed past 87 to 95 days of gestation. Based on a study in which various parts of the oviduct and uterus of alpacas were removed it has been postulated that the right uterine horn possesses only a local luteolytic effect while the left one possesses local and systemic effects, requiring the embryo to be in the left horn to maintain pregnancy (Fernandez-Baca et al., 1979).

Twinning is very rare in camelids despite relatively high multiple ovulation rates as previously described. The incidence of twins in camels is reported to be 0.13 to 0.40% (Shalash and Nawito, 1964; Arthur et al., 1985) and twin embryos were found only at early stages of gestation. Alpacas examined at less than 2 months of gestation revealed one set of twin embryos in the left uterine horn in one animal and one embryo

each in two other animals with more than one CL present (Fernandez-Baca et al., 1970). The placenta of camelids is epitheliochorial and diffuse in nature as in the horse. Although the fetus is found in the left horn, the placenta normally fills the entire uterus, including the surface of both horns in llamas (Fowler and Olander, 1990) and camels (Arthur et al., 1985). It is likely that, as in the horse (Ginther, 1979), the entire uterine surface is needed to provide sufficient nutrition to the developing fetus, making live twin births unlikely. Some documented instances of live twin births in llamas have been reported (Fowler, 1990).

The classification and orientation of placental membranes of camelids are as in other species, with the exception of an additional membrane of fetal epithelial origin that encompasses the fetus closely and is apposed to mucous membranes of body orifices (Merkt et al., 1988; Fowler and Olander, 1990). This translucent membrane is observed at birth and dries up and falls away shortly thereafter, since the dam does not lick her offspring as do most other species. The anatomic importance of this membrane is unresolved.

Fetal fluids in camelids are similar in origin and placement within the fetal-placental unit to other species although the amniotic fluid tends to be more watery and not as viscous as in other species. Both amniotic and allantoic fluids are of lesser quantity relative to the size of the animal than in other species (Arthur et al., 1985; Fowler and Olander, 1990). The epidermal membrane and smaller amounts of watery amniotic fluid may be an adaptation for water conservation and facilitate slipperiness of the newborn for delivery.

The signs and stages of parturition in camelids are similar to those observed in other species. First stage labor is characterized by restlessness, vocalization, separation from the herd and typically lasts 2 to 6 hours. Expulsion of the fetus is usually rapid, occurring in less than 1 hour. The placenta is usually delivered within 45 minutes to 3 hours post-partum. The offspring gets to its feet quickly, usually within 30 minutes, and starts nursing within 1 to 2 hours post-partum. The dam may deliver either standing or lying down. Normal fetal presentation is similar to other species with an anterio-dorsal presentation. The most common dystocias are caused by entanglement of the long forelegs and neck or flexion of a leg or the neck (Arthur et al., 1985; Johnson, 1989; Bravo, 1994). Camelids usually give birth during daylight hours. A behavioral study in wild vicuñas reported that 23 of 24 animals gave birth in the morning hours and stated that vicuñas in zoos also give birth in the morning. A similar phenomenon is observed in free ranging guanacoes with > 80% of deliveries occurring between 1000 and 1400 hours. Most llama births occur from 0500 to 1400 hours and it has been postulated that this is an adaptation to allow the cria to dry before the stormy afternoons and freezing nights in the Andes during the spring/summer birthing season (Franklin, 1981). Camels are also reported to give birth predominantly during the morning hours (Wilson, 1984).

While induction of parturition has inadvertently occurred after administration of dexamethasone, termination of early pregnancy is better accomplished with prostaglandin (PG)$F_{2\alpha}$ and its analogs. In cattle, $PGF_{2\alpha}$ and the analog cloprostenol have been utilized to successfully terminate pregnancy up to 5 months of gestation (Lauderdale, 1975). Beyond that time, effectiveness in terminating pregnancy decreases although parturition can be induced near term with prostaglandins in cattle. Retained

placentas, however, often becomes a problem. In horses and llamas, adverse side effects have been reported after administration of dinoprost. These problems range from sweating and signs of abdominal discomfort to death in some llamas (Fowler, 1989). Dosages used in llamas have been extrapolated from other species and in some cases were seemingly quite high, perhaps accounting for the observed side effects, particularly when death was the ultimate outcome. Cloprostenol appears to be efficacious to terminate early pregnancy without serious side effects in the llama (see Chapter IV).

Progesterone declines within 48 hours following $PGF_{2\alpha}$ administration to luteal phase or pregnant cattle, sheep, horses and pigs. In sheep, serum progesterone concentrations had declined significantly by 4 hours after $PGF_{2\alpha}$ administration concurrent with increased phosphodiesterase activity in the CL (Agudo et al., 1984). In goats, intramuscular injection of $PGF_{2\alpha}$ at 52 to 63 days of gestation or in cycling luteal-phase animals resulted in progesterone concentrations of < 1 ng/ml by 24 hours after administration and pregnant goats aborted (Bretzlaff et al., 1988). Cloprostenol has also been used to abort gilts at 60 days of pregnancy, with progesterone declining immediately after administration and reaching very low concentrations by 30 to 40 hours post-administration (Cort et al., 1986). Cloprostenol administered to cycling beef cows caused a decline in serum progesterone concentrations by 12 hours post-administration (Harrison et al., 1985). After administration of $PGF_{2\alpha}$ to cycling mares, a transient increase in serum progesterone concentrations was observed but was followed by a decrease to 50% of preadministration concentrations by 1 hour and to 10% of preadministration concentrations by 24 hours (Noden et al., 1978).

II.2.6. **Infertility**.

Although infertility has been noted as a significant problem in camelid production, major advances in the diagnosis and treatment of reproductive problems have been slow. This slow pace has been partially due to the lack of information about specific aspects of the reproductive biology of these animals. In their native countries, some of the fertility problems of camelids may be due to breeding practices. Camels are first bred at a relatively old age (4 to 6 years) and then every other year (Yagil, 1985). Llamas and alpacas are usually bred in large groups so that identification of infertile animals (both males and females) is difficult (Condorena and Valasco, 1978). Infertility due to infection is often not diagnosed and aggressively treated.

Infertility has been noted to be as high as 50% in camels in some pastoral herds (Merkt et al., 1987) and fertility as being 43% or less (Gauthier-Pilters and Dagg, 1981). Other reports, however, state that camels under intensive management systems can achieve an 80 to 90% calving rate (Arthur, 1992) and Bedouins often report that 100% of adult females in their herds give birth in a given year. The discrepancy is often due to the way in which fertility rates are calculated and the fact that fertility in camels is higher in good years and poorer in drought years. In South American alpaca herds, fertility rates have been reported to be low as well, perhaps as low as 50% (Novoa, 1970, Raggi et al., 1994). Putative causes of infertility in camelids have been listed as male sterility, failure to conceive, high embryonic mortality and failure of the female to ovulate with mating. Little research has accurately pinpointed true causes, although

early embryonic mortality seems to account for a high degree of losses in camels (Merkt et al., 1987; Wilson, 1994) and alpacas (Fernandez-Baca et al., 1970a).

In camels, poor management is probably the leading cause of poor fertility. Many female camels are 4 to 6 years old before being bred for the first time, they are bred only every other year and inbreeding is common in some herds. Animals could also benefit substantially from nutritional supplementation, particularly in young animals just before their first breeding season (Kamoun and Wilson, 1994). Malnutrition is a problem in many areas, especially in drought years, and results in anestrous and lack of ovulation and may contribute to embryonic mortality (Arthur, 1992). Often, poor breeding performance is not used as a criteria for culling in camels. Yagil (1985) relates poor breeding performance to inbreeding in herds.

Endometritis is sometimes observed in camels at slaughter, usually when a regressing CL is present (Arthur et al., 1985). This can occur post-partum or post-abortion (Arthur and Al-Rahim, 1982). Acquired infertility due to endometritis or metritis, pyometra or injury to the reproductive tract with subsequent scarring has also been observed in the llama (Johnson, 1989).

In a study of camel carcasses at a Cairo slaughterhouse, a high rate of embryonic mortality was observed, especially in twin pregnancies, as measured by the presence of corpora lutea without a fetus or fetuses in the uterus (Shalash and Nawito, 1964). There is also high calf mortality, up to 50% in some herds, with 30% considered usual in the first months of life, further decreasing production (Merkt et al., 1987; Wilson, 1984). Numbers of calves lost are sometimes included in fertility and production records and can result in a skewing of percentages.

Various reproductive tract anatomical abnormalities have been observed in camels and llamas. These include persistent hymen, vaginal stricture, aplasia or hypoplasia of all or parts of the reproductive tract, congenital recto-vaginal fistulas and hermaphrodites or pseudohermaphrodites (Shalash, 1965; Johnson, 1989). The condition known as uterus unicornous or segmental aplasia of one uterine horn appears to be relatively common in llamas and alpacas (Johnson, 1989).

In alpacas, as in camels, early embryonic mortality is apparently high. One laparoscopic study observed a 70% pregnancy rate on day 3 post-breeding, but the rate dropped to 35% by days 28 to 31 and then remained fairly constant (Fernandez-Baca et al., 1970). Other sources have cited approximately 10% rate of embryonic death up to 45 days in llamas and 5% abortion rate thereafter (Johnson, 1989).

Failure of a female llama to ovulate is often perceived to be the main reproductive problem by owners in the United States, but this may relate to improper breeding methods or lack of supervision of coitus. The male may not always accomplish intromission. Blood samples taken at 1 week post-breeding can indicate if the animal ovulated if progesterone is > 1.0 to 1.5 ng/ml but subsequent samples need to remain high at 2 weeks and thereafter to be consistent with pregnancy (Bravo, 1994).

In the alpaca, low sperm counts in the male and pseudopregnancy in the female have been named as contributors to poor reproductive performance (San-Martin et al, 1968). It is unclear what is meant by the term pseudopregnancy in the SAC. True pseudopregnancy with physical, behavioral and hormonal signs as in cats and dogs, does not exist in SAC, although they do occasionally have persistently elevated progesterone without pregnancy, commonly known as a retained CL. In these animals, progesterone

apparently remains high indefinitely until a luteolytic compound is given. Most cases of retained CL appear to result from a uterine pathology with fluid present in the uterus which perhaps interferes with $PGF_{2\alpha}$ release from the endometrium to lyse the CL (Smith et al., 1994).

II.3. The Male Camelid

II.3.1. **Anatomy**.

The reproductive anatomy of the male camel has been described in some detail. The reproductive anatomy of the male SAC has not been so extensively covered in the literature as the camel, but as in the female the SAC appears to be similar to the camel, the primary difference being variations in size. In the male llama and alpaca there is the added difference from the camel that there is no rutting season with associated changes in libido, testicular size or sperm production (Novoa, 1970).

The testes of camelids are carried high near the body under the anus as in the cat, dog and boar rather than in a pendulous scrotum as in the bull or ram (ElWishy, 1988; Bravo and Johnson, 1994). The testes of the mature llama are approximately 2.5 to 3.5 cm wide by 5 to 7 cm long by 3.4 cm deep and weigh approximately 24 g (Smith et al., 1994). Those of the camel are approximately 5.08 cm wide by 9.07 cm long by 4.34 cm deep although breed differences exist (Yagil, 1985). In the alpaca, testes average 4 to 5 cm in length and 2.5 to 3 cm in width (Smith et al., 1994). In comparison to other domestic species, the testicular size in camelids are smaller in relation to the size of the

adult animal. Seminiferous tubules within the testes are tightly packed, and in the camel (ElWishy, 1988) and the vicuña (Urquieta et al., 1994), exhibit seasonal changes in histology, with more active spermatogenesis during the breeding season, although spermatogenesis does not entirely cease at any season. The epididymis is similar to other species, although the tail is not easily evaluated on palpation. The vas deferens of the llama is 2 to 3 mm in diameter and about 40 cm long with lack of well-defined ampulla (Bravo and Johnson, 1994).

The penis of camelids is generally considered to be of the fibroelastic type as in the bull and the ram and possesses a sigmoid flexure similar to those species. One report on penis structure in the dromedary camel considered it to be of an intermediary type between fibroelastic and hemodynamic because of increased blood cavities compared to the penis of bulls and rams (Moborak et al., 1972). The penis of the llama is 35 to 45 cm long and ends with a cartilaginous process on the glans which is oriented clockwise with the urethral opening adjacent to the base of the cartilaginous process. The process may be important in dilating the cervix of the female both for induction of ovulation and for ejaculation to occur within the uterus of the female. The penis is covered by a prepuce which is normally caudally directed until erection occurs, at which time powerful protractor prepuce muscles pull it forward. Urination occurs in a caudal direction (Bravo and Johnson, 1994).

Camelids possess no seminal vesicles, but do have small bulbourethral glands lateral to the base of the penis and a prostate gland dorsolateral to the pelvic urethra at the trigone of the bladder (Smith et al., 1994). These produce alkaline fluid added to semen during ejaculation but add little to total semen volume. Total seminal volume is

small even though ejaculation occurs in dribbling amounts throughout the 5 to 45 minute copulation period (Johnson, 1989).

II.3.2. **Puberty**.

The definition of puberty may vary according to source. Often puberty is defined as the ability to produce fertile gametes (Hafez, 1994). In male camelids, hormonal changes and ability to produce spermatozoa probably occur well before published ages of puberty. Most male camels are not used for breeding until they are 4 to 6 years of age (Arthur et al., 1985), while alpacas and llamas are first utilized between 2 or 3 years of age (Novoa, 1970; Johnson, 1989). Although little data on puberty in these species are available, the primary limiting factor in their use for breeding is the presence of adhesions of the penis to the prepuce, lasting in most cases until 21 to 26 months in llamas (Bravo et al., 1992b). While a few animals may have freedom from adhesions earlier, in others adhesions may persist until 2 to 3 years of age. In other farm animal species, these adhesions are normally gone before viable spermatozoa are present in ejaculates and are not a factor involved with defining puberty (Hafez, 1981). If one defines puberty as the ability to impregnate a female by natural service, llamas do not reach puberty until these adhesions are broken down. In cattle and sheep, these adhesions break down with a combination of rising testosterone concentrations, good nutrition and sexual experience. In ram lambs adhesions were broken down completely by 63 to 70 days of age and rate of breakdown depended highly on growth rate (Dun, 1955).

In bulls, changes initiating the event of puberty start around 10 to 12 weeks of age when LH concentrations rise with increasing pulse frequency and amplitude (Amann and Walker, 1983; Amann et al., 1986). Testosterone concentrations begin to increase around 4 to 6 months of age (McCarthy et al., 1979; Amann and Walker, 1983). After testosterone increases, LH concentrations decline somewhat, although pulse amplitude continues to gradually increase with age (Deaver and Peters, 1988). Sperm production is usually evident within the testes at 7 to 8 months of age in the bull (MacMillan and Hafs, 1968). First ejaculates usually have lower numbers of sperm with more abnormalities and less motility than later ejaculates. The pituitary of bull calves is responsive to GnRH at 2 months of age, but testosterone increases following GnRH administration were not observed until 6 months of age in bulls sampled at 2, 4 and 6 months (Mongkonpunya et al., 1975).

In rams, the sequence of events is similar to the bull, but occurs sooner in life as the animals mature at a younger age. Concentrations of LH increase around 1 to 7 weeks of age (sooner in rapidly growing breeds) from very low or undetectable levels at birth (Lee et al., 1976a). Pulse frequency of LH and FSH increased in ram lambs from 1 to 8 weeks of life with increasing basal concentrations of these hormones (Foster et al., 1978; Olster and Foster, 1986). Concentrations of LH were observed to peak at 6 weeks of age in ram lambs sampled weekly, and then declined at approximately 16 weeks of age (Wilson and Lapwood, 1979a).

Testosterone concentrations in rams increased slowly from 5 to 25 weeks of age with marked increases observed at puberty (Illius et al., 1976; Lee et al., 1976a). Testosterone pulses followed LH pulses at 14 and 22 weeks of age (Wilson and

Lapwood, 1979a). The ratio of androgens has also been observed to change with age. The ratio of androstenedione to testosterone was narrow at birth in lambs, then testosterone became predominant. Androgen content increased with testicular weight in these lambs (Skinner et al., 1968).

Response of LH to exogenous GnRH is evident at birth in ram lambs, although the sensitivity is increased at 2 to 3 months of age (Lee et al., 1976b). Response of testosterone to GnRH administration is low at 6 weeks of age, but increased up to 32 weeks of age in animals sampled and treated weekly with GnRH (Wilson and Lapwood, 1979b).

Sperm production in ram lambs is evident by 16 to 18 weeks of age (Olster and Foster, 1986). Fructose and citric acid content in the ejaculate precede spermatogenesis and the first appearance of sperm was noted to be breed and size dependent by other investigators (Skinner and Rowson, 1968). These investigators also noted an increase in sperm motility and decreased abnormalities with age.

In stallions, LH concentrations are low between 8 to 32 weeks of age, then increase from 36 to 40 weeks of age. At 60 weeks of age, LH concentrations decline and remain low to 80 weeks of age. Testosterone is undetectable until 32 weeks of age, then increases through 56 weeks of age followed by a slight decrease after 60 weeks of age and a dramatic increase at 75-80 weeks of age. Semen volume and sperm concentration is lower than in the adult stallion and the percentage of progressively motile sperm increase with age (Clay and Clay, 1992).

In camelids, little data are available on hormonal or physical status at puberty. In a group of young male llamas, it has been observed that testosterone concentrations

rose gradually from birth to 21 months of age, then increased exponentially to 30 months at which time levels reached a plateau (Bravo et al., 1992b). This was concurrent with testicular growth and body weight increases in a linear fashion. It has been observed that scrotal circumference increases in a linear fashion with body weight in stallions (Clay and Clay, 1992) and ram lambs (Mukasa-Mugerwa and Ezaz, 1992).

In both rams and bulls, attainment of puberty is highly dependent on breed and nutrition (Hafez, 1993). High planes of nutrition and larger, faster growing breeds typically attain puberty sooner. The influence of body size and type on puberty is well documented in other species but largely unknown in camelids.

II.3.3. **Season: behavior and hormone levels.**

Male camels have a distinct rutting season associated with behavioral and physiological changes, while most male SAC do not have similar seasonal changes (Novoa, 1970). In the camel, the mating season varies somewhat with geography but appears to be closely correlated with rainfall and availability of vegetation, and poorly associated with photoperiod (Wilson, 1984). In SAC and OWC populations living at near-equatorial sites and therefore without dramatic shifts in daylight length, breeding still tends to be seasonal. Reports exist from zoos around the world that suggest that births in all camelids follow a seasonal pattern and that adequate forage is not the only factor that can account for this (Schmidt, 1973). Vicuñas do have seasonal variation in testosterone levels, testes diameter and spermatogenesis (Urquieta et al., 1994). It has

been suggested that llamas have less of a seasonal pattern of reproduction than do vicuñas and guanacos because of the domesticity of the llama.

In northern Africa, the camel rutting season is from December to May. In India, camels mate from November to February, in Pakistan from December to March, in Israel from January to March, and from April to May in Somalia (ElWishy, 1988). In Russia, wild Bactrian camels have been reported to be seasonal, mating in winter and early spring, but domesticated ones were said to be capable of mating year-round (Gauthier-Pilters and Dagg, 1981). Other authors reported a breeding season in Chinese Bactrian camels from mid-December to April with most females showing estrus in January and February (Abdel-Rahim et al., 1994). Some evidence suggests that camels might be capable of mating year-round if adequate forage is provided, but this is contradicted by the fact that zoo camels often experience a short breeding season by latitude, usually in the spring (Schmidt, 1973).

Typical changes in the male camel's behavior occur with the onset of the rut. The animals are more prone to fighting each other, increased aggression toward people, vocalization and establishment of territorial boundaries. They protrude the elongated soft palate, the dulaa, while vocalizing. The poll glands increase secretion of a foul-smelling dark substance which the camel rubs on his own shoulders, surrounding fences, trees and any upright surface. He also urinates copiously, beating his tail down between his legs against the flow and spreading urine on his hindquarters and back and surrounding objects (ElWishy, 1988). No such changes are observed in male SAC.

Hormonal changes associated with the rut in camels during the rainy season in Somalia include increased corticoids and testosterone (Bono et al., 1989). The increase

in testosterone was associated with increased steroidogenic enzymes in the testes (Bedrak et al., 1983). Increased testosterone concentrations during the breeding season in camels is associated with increased testicular size, changes commonly observed in other seasonal breeding species such as the ram and the stallion. The stallion undergoes increases in testicular size with increasing day length, testosterone concentrations increase and sexual behavior becomes more pronounced (Thompson et al., 1977). Sperm count increases in long-day months in stallions and fewer abnormal spermatozoa are present (Clay et al., 1987). Mean LH concentrations also increase in stallions during the breeding season (Burns et al., 1984).

Similar findings have been reported for short day breeders such as the ram, with increased number of spermatozoa and fewer abnormalities observed during the breeding season (Amir et al., 1986). Basal testosterone concentration and pulse frequency was lower in the nonbreeding season than during the breeding season in rams (Katangole et al., 1974). This has been associated with increased LH concentrations in the ram when days begin to shorten (Schanbacher and Lunstra, 1976). Clear changes in LH concentrations have not been observed with season in male camels (Bono et al., 1989), but changes in testosterone concentrations and testicular characteristics are associated with increasing corticoid concentrations during the rainy season. How much importance photoperiodic cues may have is unknown, but corticoid concentrations increase both during periods of drought and the rainy season yet rut occurs only during the rainy season.

Unlike the llama and alpaca, the vicuña does have increases in testicular diameter and testosterone production in the mating season but with little change in

behavior (Urquieta et al., 1994). The male llama has no apparent seasonal changes in testosterone concentrations (Bravo et al., 1992b).

II.3.4. **Castration.**

Castration may or may not be routinely performed on male camelids not kept for breeding stock. Intact male camelids are generally tractable although some fighting behavior is common. Male camels in the rut can be difficult or impossible to handle. Castration does improve behavior of camelids kept together in mixed-sex groups to decrease aggression between males and sexual behavior toward females. With proper training, however, intact male llamas can be used as pack or show animals with little trouble (Gauthier-Pilters and Dagg, 1981; Fowler, 1989).

There is little or no information available in the camelid regarding alterations of hormone levels following castration. It is assumed that changes would be similar to those in other species. In dogs, testosterone concentrations decreased rapidly following castration and were undetectable by 4 hours after the procedure (Taha et al., 1982). In bulls castrated and sampled at intervals to determine the half-life of testosterone (every minute for 10 minutes, then every 5 minutes for 20 minutes, then every 10 minutes for 30 minutes), the hormone was found to decline in a 2-phase clearance pattern with half-lives of 8 and 30 minutes for each phase (Haynes et al., 1976). These data agree closely with work done in humans in which tritiated testosterone was injected and blood and urine samples used to determine volume of distribution and half-lives (Horton et al., 1965).

Another finding after castration is that of increased LH concentrations. Gonadal steroids exhibit inhibitory influences on the pituitary and removal of the gonads results in increased gonadotropin concentrations. In the bull, basal LH concentrations increased approximately 4 to 5 times compared to precastrate concentrations (Schanbacher et al., 1983). In sheep, wethers have been reported to have basal LH concentrations almost 9 times those of intact rams (D'Occhio et al., 1982). Interestingly, LH concentrations in geldings in one study were not different from concentrations observed in intact stallions (Hoffman et al., 1987). In the boar, LH concentrations have been noted in some studies to increase following castration, but only temporarily (Allrich et al., 1982). Other studies in boars showed an increase in LH post-castration similar to that in bulls and rams (Liptrap et al., 1986). Concentrations of LH in the neutered male dog are also increased approximately three-fold compared to intact male dogs (Olson et al., 1992).

Age at castration has some impact on the timing of the LH rise. If ram lambs are castrated early, such as shortly after birth before LH concentrations have become detectable, the LH rise will be delayed until the time LH concentrations would ordinarily become detectable (Olster and Foster, 1988). At this time, LH concentrations in the castrate become comparable to those in animals castrated at later ages. Similar results have been reported for bulls castrated before 1 month of age, with the exaggerated post-castration LH rise being delayed until the normal time of the prepubertal LH rise (McCarthy et al., 1979).

II.3.5. **Infertility**.

Infertility in male camelids remains an area that needs further investigation. Reports of sterile male alpacas from South America do not always state the reason for sterility. In general, only very valuable males are fully investigated for causes of infertility even in the United States. Difficulty in obtaining semen further complicates such investigation.

In llamas, some of the causes for infertility that have been described are congenital abnormalities of the penis and prepuce, low sperm counts, and acquired injuries to the penis (Johnson, 1989). Penile damage may occur when a male is mated to a long-wooled female and wool becomes wrapped around the penis. This may not be noticed until permanent damage to the glans has resulted. Other breeding difficulties may be attributed to behavior, as some males, particularly young ones, do not seem to know how to approach a female and also there may be trouble with intromission. Often owners will manually aid the male (Smith et al., 1994). This is true of camels as well and such practices may inadvertently genetically select for males with poor libido and/or reproductive performance (Wilson, 1983).

Analysis of alpaca semen has suggested that many males (20% or more) may have low sperm counts (San-Martin et al., 1967), but methods of semen collection in this study may be questionable. Due to the nature of dribble ejaculation over a prolonged period of time, semen collection is difficult in camelids. Electroejaculation often yields undesirable samples. The most successful method used has been an artificial vagina fitted inside a dummy with the male being trained to mount the dummy.

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CHAPTER III. COMPARISON OF OVULATORY STIMULI IN THE FEMALE LLAMA

III.1. Abstract.

While llamas normally ovulate only following mating, ovulation can be induced with hormonal treatment. This study was designed to determine appropriate dosages of hCG and GnRH to induce ovulation and to compare hormonal profiles of such treatment with those following mating.

Serum progesterone (P_4) concentrations were measured in 8 mature female llamas on alternate days for 16 days following treatment with saline; 1 or 5 μg gonadotropin-releasing hormone (GnRH)/kg body weight (BW); 1.25, 6.25 or 31.25 international units (IU) human chorionic gonadotropin (hCG)/kg BW; or sterile mating to a vasectomized male. Following completion of these treatments, an additional dose of 6.25 (n = 5) or 31.25 (n = 3) IU hCG/kg BW was administered to assess the repeatability of the ovarian response to hCG stimulation. The repeat dosage was based on whether the animal had ovulated with the initial 6.25 IU hCG/kg BW dose. Four animals were mated to a fertile male, resulting in pregnancy, after all other treatments were given. Treatments were administered at approximately 4 week intervals with 2 weeks of rest after the completion of sampling for the previous treatment. Ovulation rate for each treatment was as follows: saline, 0 of 8; 1 μg GnRH/kg BW, 7 of 8; 5 μg GnRH/kg BW, 8 of 8; 1.25 IU hCG/kg BW, 6 of 8; 6.25 IU hCG/kg BW, 5 of 8; 31.25 IU hCG/kg BW, 8 of 8; sterile mating, 7 of 7; repeat dose of 6.25 or 31.25 IU hCG/kg BW, 4 of 8. Concentrations of P_4 peaked 8 days following all treatments except saline

with an average peak P_4 concentration of 4.4 ± 0.4 ng/ml (mean ± SEM). With all ovulation-inducing treatments except fertile breeding, P_4 declined to basal concentrations (< 0.5 ng/ml) by day 12 post-treatment. There was no difference ($P >$ 0.05) between treatments in time course of P_4 rise and fall, magnitude of P_4 or in peak P_4 concentrations. Progesterone concentrations following fertile mating remained elevated throughout the balance of the 16 day sampling period. All treatments induced higher (P > 0.05) P_4 concentrations than observed following saline administration.

Release of LH was examined at 15 minute intervals for 1 hour prior to and 10 hours following treatment with 1 μg GnRH/kg BW (n = 8), breeding with a vasectomized male (n = 7) or breeding with a fertile male (n = 4). Release of LH was similar in magnitude, peak concentration and duration of peak for both types of breeding, peaking at 3.7 ± 0.4 (mean ± SEM) hours post-mating. While the magnitude of the LH surge and maximum peak concentration following treatment with GnRH were similar to those observed following sterile and fertile matings, the time to the peak following GnRH administration was shorter ($P < 0.01$), 1.9 ± 0.3 (mean ± SEM) hours. Overall average peak LH concentration was 4.3 ± 0.6 ng/ml (mean ± SEM) for all treatments.

III.2. Introduction.

Frequent reproductive problems in llamas in North America require more information on normal function and possible treatments for reproductive failures. The llama is an induced ovulator, requiring either mating or hormonal stimulus to release

LH and ovulate (England et al., 1969; Bravo, 1994). One fertility problem that has been described is failure of the female llama to ovulate with breeding (Johnson, 1989). Treatment with hCG or GnRH at the time of mating has been proposed to stimulate the ovaries of infertile or subfertile llamas at the time of breeding. Treatment with hCG or GnRH and the measurement of elevated P_4 at 7 to 9 days post-stimulation can also aid a clinician in determining if an apparently infertile female has functional ovaries. Although dosages of hCG and GnRH have been proposed for the llama, they have been extrapolated from other species or gathered from individual cases. Little or no comparative information is available relating hCG or GnRH dosages to their efficacy at inducing ovulation in the llama. Accordingly, the primary objective of this study was to establish an appropriate dosage of hCG or GnRH to reliably induce ovulation. A secondary objective was to compare the characteristics of the LH surge following mating and GnRH stimulation.

III.3. Materials and Methods.

III.3.1. **Animals.**

Ten adult female, one mature vasectomized male and 4 sexually intact adult male llamas were used in this study. Females were 1.5 to 12.0 yrs of age (5.8 ± 3.4 yrs, mean ± SEM) and weighed 99.2 to 171.8 kg (141.8 ± 23.8 kg, mean ± SEM). All females had normally functioning ovaries as previously determined by response to mating and(or) GnRH administration with a P_4 increase on day 7 post-treatment. Not

all females used were capable of sustaining a pregnancy to term, mostly due to acquired defects in the tubular reproductive tract (see Appendix 1). One animal had a congenital rectovaginal fistula and one animal had infertility of unknown origin.

The vasectomized male was surgically altered 2.5 months prior to study initiation and was determined by electroejaculation and semen examination not to have sperm in the ejaculate prior to initiation of the study. All intact males had been shown to be fertile by either electroejaculation and semen evaluation or previous successful use as breeding animals. Animals were kept on pasture throughout the study period with free access to water and mineral mix. Males and females were kept in separate pastures except for times of controlled breeding.

III.3.2. **Experimental design, treatment and sampling protocol.**

Eight female llamas were used in a completely randomized study with repeated treatments on animals within blocks. Animals were assigned at random to one of 7 treatments on day 1 of the experiment. Treatments were: 1) saline injection at a rate of 0.01325 ml/kg BW intramuscularly (i.m.) and 0.1 ml/kg BW intravenously (i.v.), 2) i.v. injection of GnRH[1] at 1 μg/kg BW, 3) i.v. injection of GnRH at 5 μg/kg BW, 4) i.m. injection of hCG[2] at 1.25 IU/kg BW, 5) i.m. injection of hCG at 6.25 IU/kg BW, 6) i.m. injection of hCG at 31.25 IU/kg BW, and 7) sterile mating with a vasectomized male.

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[1]Cystorelin[R], gonadorelin, Abbott Laboratories, North Chicago, IL for CEVA Laboratories, Inc., Overland Park, KS

[2]Chorulon[R], human chorionic gonadotropin, Intervet International, Boxmeer, Holland for Intervet, Inc., Millsboro, DE

Samples were collected by jugular venipuncture into evacuated tubes[3] on days 0, 2, 4, 6, 8, 10, 12, 14 and 16. All treatments were given between 0800 and 1000 on day 0. Blood samples were collected during the same time period on all subsequent sampling days. Following 2 weeks of rest after collection of the last sample from the previous treatment, treatments were rotated among the animals until all 8 animals had received all 7 treatments, with the exception of the animal (#216) whose rectovaginal fistula precluded her use for breeding. After the administration of the seventh treatment, all animals received an additional dose of 6.25 or 31.25 IU hCG/kg BW. The dosage was determined by examining the response of each animal to the first dose of 6.25 IU hCG/kg BW. If the animal failed to ovulate with that dose, a 2nd dose of 31.25 IU hCG/kg BW (n = 3) was given, otherwise a second dose of 6.25 IU hCG/kg BW (n = 5) was administered. Four females known to be fertile (2 of which were included in the other treatments in the study and 2 others added for sufficient fertile matings for comparison) were bred to 4 fertile males, as the last treatment for these animals.

All animals except #216 were palpated on day 8 of each treatment sampling period to confirm ovulation by presence of a corpus luteum (CL) on at least one ovary. In addition, pregnancies were confirmed by palpation and/or ultrasound at > 20 days.

In addition to alternate day sampling, blood samples were collected via indwelling jugular catheters[4] at 15 minute intervals for 1 hour prior to and 10 hours following treatment with 1 μg GnRH/kg BW, sterile or fertile mating. The catheters

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[3]SST[R] Vacutainer, Becton Dickinson and Co., Rutherford, NJ

[4]Angiocath[R], Becton Dickinson and Co., Sandy, UT

were placed the day prior to treatment and sampling and sample procedure was as outlined in Appendix II with heparinized saline (5 IU/ml) instilled in catheters between samples to retain patency. Immediately following collection, samples taken via catheter were transferred into tubes containing approximately 4.5 mg disodium EDTA, mixed gently by inversion, then placed on ice until centrifugation at 1500 x *g*, at 45 to 60 minute intervals. Plasma was removed and stored in duplicate aliquots at -20° C until assayed.

III.3.3. **Hormone analysis.**

Alternate day samples (days 0 to 16) for each treatment were analyzed for P_4 using coated tube radioimmunoassay techniques (DPC Coat-A-Count[5]). Inter- and intra-assay coefficients of variation for assays (obtained from measurement of hormone in pools of llama plasma with high and low P_4 concentrations in each assay) used in this study were 10.1% and 6.8%, respectively. The assay had been previously validated for use in the llama by exhibiting parallelism of llama plasma with the P_4 standard curve and recovery of standard from llama plasma (Leon et al., 1990). The lower limit of detection for this assay was 0.2 ng/ml. Crossreactivities of various steroids with the antibody as reported by DPC were: androstenediol, cortisol, danazol, estradiol, medroxyprogesterone, pregnane, pregnenolone and testosterone undetectable; 5β-pregnan-3α-ol-20-one 0.2%; 17α-hydroxyprogesterone 0.3%; corticosterone 0.4%; 5α-

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[5]Coat-A-Count[R] Progesterone, Diagnostic Products Corp., Los Angeles, CA

pregnan-3,20-dione 0.8%; 5β-pregnan-3,20-dione 1.3%; 11-deoxycorticosterone 1.7%; 20α-dihydroprogesterone 2.0%; and 11-deoxycortisol 2.4%.

Concentrations of LH were measured using the method described by Matteri and others in 1987 and modified for use in the llama by Bravo and others in 1990. The first antibody was a mouse monoclonal anti-bLHβ at a concentration of 1:1,000,000. Characterization and crossreactivity of this antibody was done by the previously mentioned authors, showing crossreactivity of less than 4% with follicle stimulating hormone, thyroid stimulating hormone, prolactin and growth hormone. In addition, potency of LH from various species compared to the eLH standard used by those authors ranged from 15.4% for hLH to 200% for pLH and all hormones which showed measurable activity exhibited displacement curves parallel to the eLH standard. Iodination and cold standard hormone used in our laboratory was bLH (USDA-I-1 AFP6000). This assay was validated for use in the llama in this laboratory, exhibiting parallelism of llama plasma added to assay tubes in triplicate in 25, 50, 100 and 200 μl amounts with the standard curve when plotted on a log-logit scale and recoveries of 0.1, 0.2 and 0.5 ng/tube standard in 50 μl llama plasma averaging 96.3 ± 10.2% (mean ± SEM, n = 6). Inter- and intraassay coefficients of variation were 16.4% and 8.9%, respectively. The lower limit of detection was 0.4 ng/ml. Complete assay protocol used in our laboratory is described in Appendix III.

III. 3.4. **Statistical analysis.**

Progesterone concentrations on individual sampling days were compared among treatments for all animals by multifactor ANOVA using the Statgraphics[R] program for the IBM PC[6] and choosing the LSD procedure to differentiate between significant differences. The day to maximal P_4 concentrations and the maximal P_4 concentrations were compared for all animals among all treatments in a similar fashion, as was the total area under the curve of P_4 release for all animals among all treatments. The total area of P_4 release was determined using trapezoidal integrals calculated with the RSTRIP program (for the IBM PC[7]) designed for analyzing pharmacological clearance data. Within each treatment, mean P_4 concentrations among all animals for each day were compared using the same Statgraphics procedure as described previously.

The following parameters for LH were analyzed in a similar fashion as P_4: hour to maximal LH release, area under the curve of total LH release (determined by RSTRIP) and maximal LH concentrations. These parameters were compared among all animals for relevant treatments: 1μg GnRH/kg BW, sterile mating and fertile mating by the same Statgraphics procedures used for comparing similar parameters for P_4.

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[6]Statgraphics[R], Version 5, STSC, Inc., Rockville, MD

[7]RSTRIP Polyexponential Curve Stripping/Least Squares Parameter Estimation, MicroMath Scientific Software, Salt Lake City, UT

III. 4. Results.

All treatments except saline resulted in ovulation in at least some animals, as evidenced by P_4 release in the days following treatment (Figure 1) and confirmed by rectal palpation of a CL on day 8. Multiple CL were palpated in 4 different animals, 2 animals after treatment with the highest dose of hCG, and 1 animal each after 1 or 5 μg/kg dosages of GnRH. Saline treatment resulted in no significant change in P_4 concentrations ($P > 0.05$) over the course of sampling in any of the animals. Some animals did not ovulate with all doses of hCG, although all animals responded to hCG by ovulating with at least one dose, forming a palpable CL and P_4 concentrations of > 1.5 ng/ml. Ovulation rates and palpation data for each treatment are summarized in Table 1.

Both doses of GnRH (1 and 5 μg/kg BW) were effective at causing ovulation, although one animal failed to ovulate with the 1 μg GnRH/kg BW dose. She did, however, exhibit an LH rise comparable to that in the other animals given this dose of GnRH.

Increases of P_4 were first detectable at day 4 post-treatment as being significantly higher ($P < 0.05$) in animals treated with 1 μg GnRH/kg BW, 1.25, 6.25 or 31.25 IU hCG/kg BW, sterile mating, repeated 6.25 or 31.25 IU hCG/kg BW and fertile mating (0.5 ± 0.1, 0.6 ± 0.1, 0.6 ± 0.1, 0.7 ± 0.1, 0.8 ± 0.1, 0.7 ± 0.1 and 0.9 ± 0.1 ng/ml; mean ± SEM, respectively) compared to saline (0.2 ± 0.1 ng/ml). After treatment with the 5 μg GnRH/kg BW dose, P_4 had risen on day 4 but did not become significantly different from saline until day 6, at which time it had risen appreciably

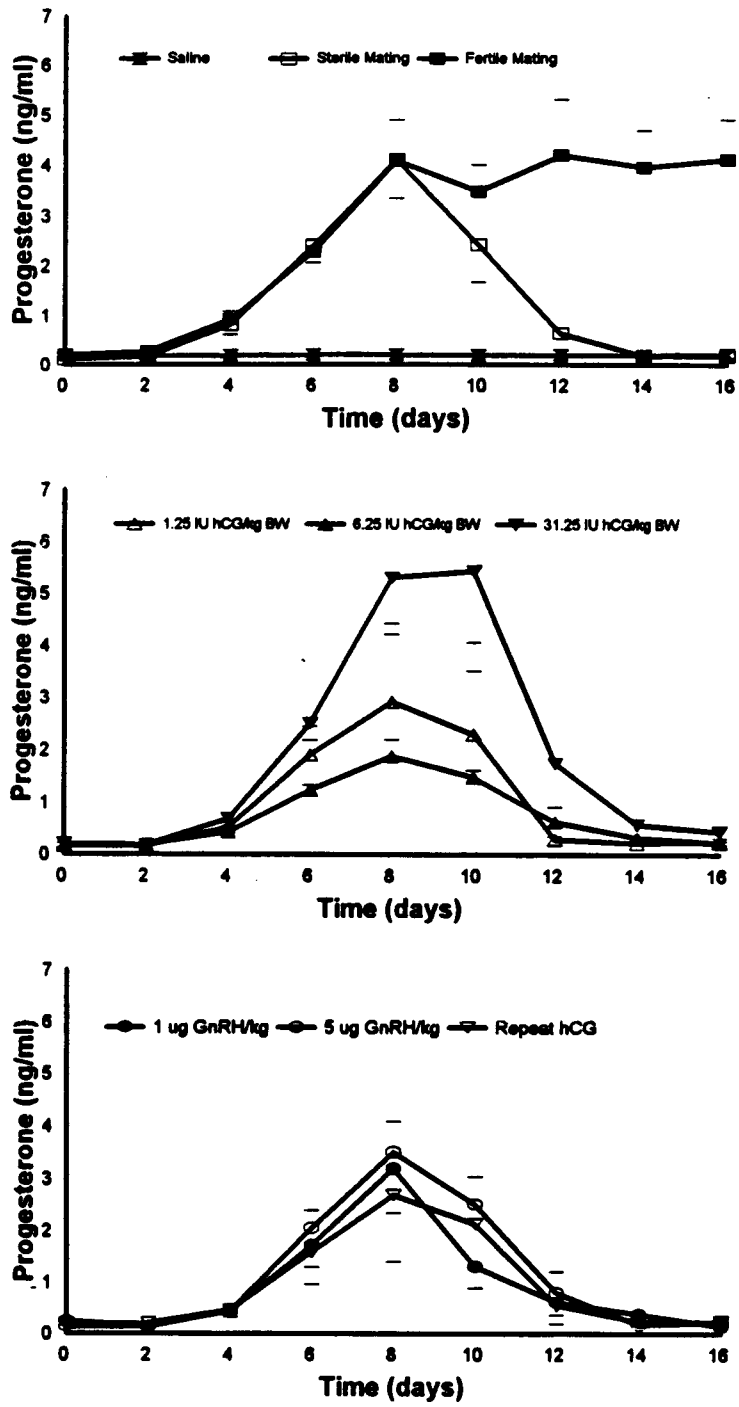

Figure 1. Mean progesterone concentrations (± SEM) following saline, sterile or fertile mating (upper panel), administration of 1.25, 6.25, or 31.25 IU hCG/kg BW (middle panel); and 1 or 5 μg GnRH/kg BW or repeat hCG administration (lower panel).

Table 1. Ovulation and palpation data following each treatment. Presented as number of animals ovulating (as determined by P_4 increase)/number of animals treated and number of CL palpated/number of animals palpated.

	Treatment (per kg BW)							
	Saline	1.0 μg GnRH	5.0 μg GnRH	1.25 IU hCG	6.25 IU hCG	31.25 IU hCG	Sterile mating	Repeat hCG
Ovulations	0/8	7/8	8/8	6/8	5/8	8/8	7/7	4/8
# CL	0/7[a,b]	7/7[a,c]	7/7[a,d]	6/7[a,e]	5/6[a,f]	9/7[a,g]	6/6[a,h]	3/7[a,e]

[a]#216 not palpated for any treatment due to rectovaginal fistula.

[b]#235 missing palpation data.

[c]#217 not palpated due to behavioral problems (jumping and agitated in chute); #230 had 2 palpable CL.

[d]#236 missing palpation data; #176 had 2 palpable CL.

[e]#198 missing palpation data; #217 recorded as having CL but P_4 indicated no ovulation had occurred.

[f]#230 not palpated due to behavioral problems (lying down and rolling in chute); #236 recorded as having CL but P_4 indicated ovulation had not occurred.

[g]#198, #162 each had 2 palpable CL.

[h]#230 missing palpation data; #216 not mated due to rectovaginal fistula.

(2.1 ± 0.3 ng/ml). Progesterone concentrations for most treatments peaked by day 8, then remained high or declined to undetectable from saline on day 10 (1 μg GnRH/kg BW, 1.5 ± 0.8 ng/ml and 6.25 IU hCG/kg BW, 2.2 ± 0.9 ng/ml compared to 0.2 ± 0.7 ng/ml for saline) or 12 (5 μg GnRH/kg BW, 0.8 ± 0.4 ng/ml; 1.25 IU hCG/kg BW, 0.3 ± 0.5 ng/ml; sterile mating, 0.6 ± 0.4 ng/ml; repeated dose of 6.25 or 31.25 IU hCG/kg BW, 0.8 ± 0.6 ng/ml). The highest dose of hCG had a tendency to cause more total P_4 to be released. Higher concentrations of P_4 on day 10 (5.4 ± 0.8 ng/ml) were observed for this treatment when compared with all other treatments except the repeat dose of 6.25 or 31.25 IU hCG/kg BW and fertile mating, and concentrations of P_4 remained higher longer, first being undetectable from saline on day 14. Fertile mating produced a P_4 profile similar to that of other ovulation-inducing treatments, with peak concentrations reached on day 8 (4.1 ± 1.0 ng/ml). Progesterone then remained elevated on days 10, 12, 14 and 16 (3.5 ± 1.0, 4.2 ± 0.6, 4.0 ± 0.2 and 4.1 ± 0.2 ng/ml, respectively) with no significant differences between concentrations on those days. Mean P_4 concentrations for each treatment group are shown in Figure 1.

Total area under the graphical curve of P_4 release averaged 22.9 ± 1.7 ng/ml over the 16 day period for all treatments which resulted in ovulation (excluding fertile matings). There were no significant differences ($P > 0.05$) observed between treatments. Maximum P_4 concentrations averaged 4.5 ± 0.7 ng/ml over all ovulation-inducing treatments, again with no differences observed between treatments. When time to maximum P_4 concentration was averaged over all animals within each treatment and differences among treatments analyzed by ANOVA, there were no differences due to treatment, averaging 8.4 ± 0.2 days for all ovulating animals. Time back to basal P_4

(< 0.5 ng/ml) was also not significantly different among treatments, averaging 12.7 ± 0.2 days from the day of treatment. The highest dosage of hCG had a tendency to cause greater P_4 release with higher maximum concentrations, greater area under the curve and somewhat delayed return to basal P_4, although none of these differences were significant ($P > 0.05$). Table 2 summarizes individual treatment data for maximum P_4 concentrations, time to peak and time to basal P_4 and area under the P_4 curve.

Table 2. Progesterone curve analysis: means ± SEM for various parameters. All differences were non-significant ($P > 0.05$).

Treatment	Area under the Curve (ng/ml over 16 days)	Time to peak (days)	Maximum P_4 concentrations (ng/ml)	Days to basal (< 0.5 ng/ml)
1 μg GnRH/kg BW	17.9 ± 2.6	8.8 ± 0.4	3.7 ± 0.9	12.0 ± 0.6
5 μg GnRH/kg BW	19.6 ± 5.5	8.5 ± 0.4	3.8 ± 0.8	12.2 ± 0.6
1.25 IU hCG/kg BW	21.0 ± 16.6	8.0 ± 0.4	4.4 ± 0.9	12.0 ± 0.6
6.25 IU hCG/kg BW	18.2 ± 8.2	8.0 ± 0.5	3.1 ± 1.0	14.0 ± 0.7
31.25 IU hCG/kg BW	33.6 ± 15.7	9.5 ± 0.4	6.2 ± 0.9	13.4 ± 0.6
Sterile mating	21.8 ± 8.7	8.0 ± 0.4	4.2 ± 0.9	11.8 ± 0.6
Repeat hCG	28.6 ± 12.0	8.0 ± 0.6	5.5 ± 1.1	13.5 ± 0.8
Fertile mating	---	8.0 ± 0.6	4.6 ± 1.0	---

Similar parameters were compared for LH for the following treatments only: 1 μg GnRH/kg BW (n = 8), fertile mating (n = 4) and sterile mating with a vasectomized male (n = 7). Maximum LH peak concentrations averaged 4.3 ± 0.6 ng/ml (Figure 2) with no significant differences ($P > 0.05$) observed between treatments. Time to maximum LH concentration was similar for sterile and fertile matings (3.7 ± 0.4 h) but was significantly $P < 0.05$) longer than when animals were treated with GnRH (1.9 ± 0.3 h). Area of total LH release averaged 20.6 ± 2.6 units and was not different among treatments. A summary of LH surge dynamics is presented in Table 3.

Table 3. LH curve analysis: means ± SEM for various parameters. Means within columns with different superscripts differ significantly ($P < 0.05$).

Treatment	Area under curve (ng/ml over 10 hours)	Time to peak (hours)	Maximum LH level (ng/ml)
1 μg GnRH/kg BW	15.9 ± 3.9^{a}	1.9 ± 0.3^{b}	3.1 ± 1.0^{d}
Sterile mating	18.4 ± 4.2^{a}	4.1 ± 0.3^{c}	3.9 ± 1.0^{d}
Fertile mating	27.5 ± 5.5^{a}	3.3 ± 0.4^{c}	5.9 ± 1.4^{d}

III. 5. Discussion.

The time course and amount of LH and P_4 released by the animals in this study are comparable to those reported by Bravo and others in 1990 for fertile matings and observed by Leon (unpublished observations) for P_4 release following hCG administration, sterile and fertile matings. The study reported herein was useful in

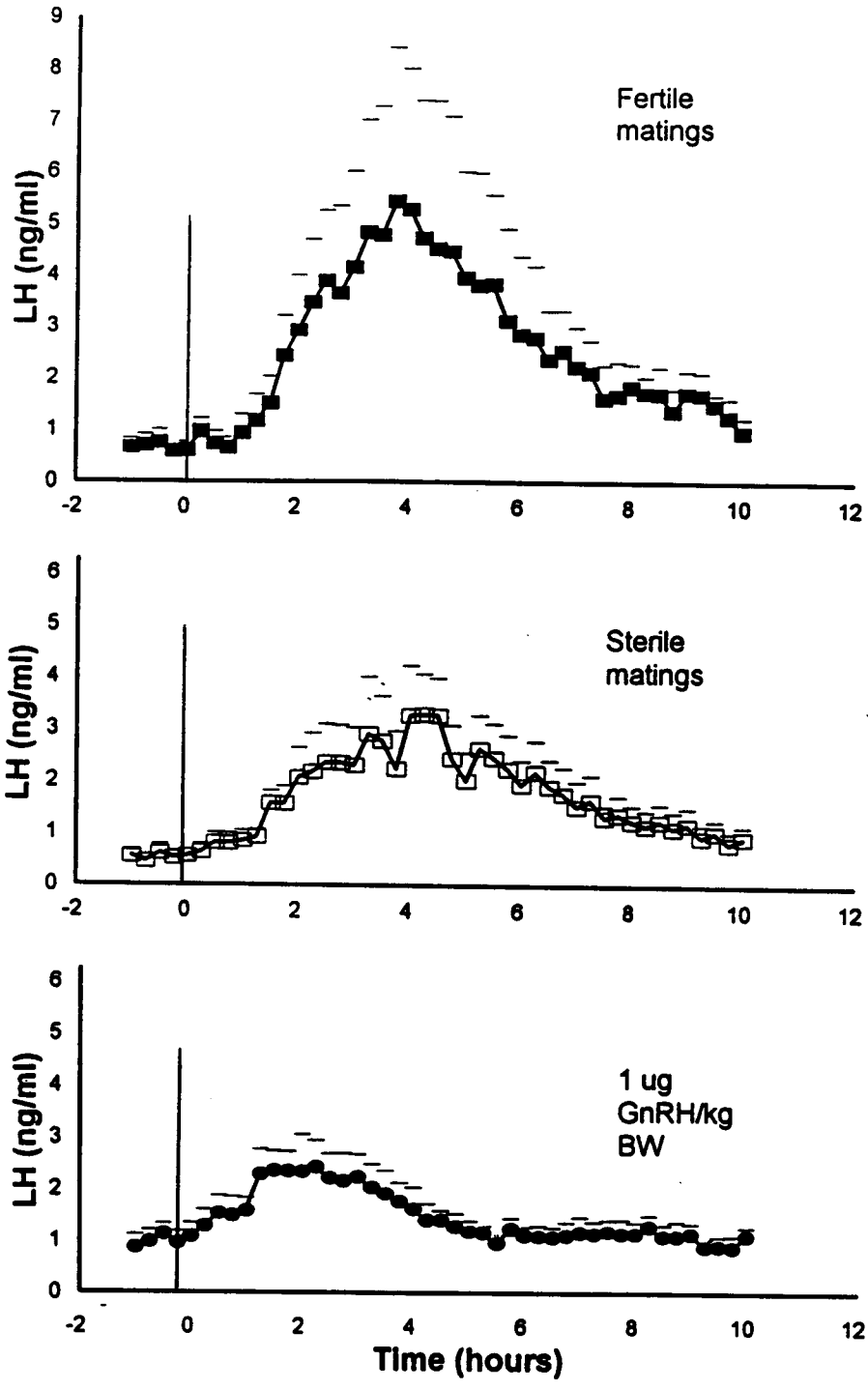

Figure 2. Change in mean LH concentrations (+ SEM) following fertile (upper panel) and sterile (middle panel) matings, and intravenous administration of 1 μg GnRH/kg BW. All treatments occured at time = 0.

comparing different doses of hCG and GnRH which have not previously been reported in a single study.

While not all animals used in this study ovulated with all treatments, all were shown capable of ovulating with at least one dose level of all hormonal treatments given and all animals ovulated with a sterile mating (with the exception of the one animal which was not mated). Some animals utilized in the study were not fertile in the sense of being able to produce viable offspring. In all cases, however, these animals had been previously shown to have functional ovaries and appeared to be infertile because of problems with the tubular reproductive tract and unrelated to ovarian function.

The lack of ovulation following treatment in some animals may have been due to a lack of ovarian follicular readiness at the time of treatment. Studies (Bravo and Sumar, 1989; Bravo et al., 1990, 1991; Adams et al., 1990) have shown that ovarian follicular development in the llama occurs in waves of approximately 14 days total length for each dominant follicle, with an interwave interval of approximately 11.1 days, since the next follicle begins development while the previous one is regressing. This results in periods of approximately 2 to 3 days duration between succeeding waves in which there are no follicles of sufficient size to respond to an ovulatory stimulus.

In addition, Bravo and others in 1991 demonstrated that some regressing follicles developed luteinized walls rather than rupturing and forming a mature CL when the animal was treated with an ovulatory stimulus prior to the next follicle attaining ovulatory size ($\geq$ 7 mm in diameter). These luteinized follicles produced lower P_4 concentrations than a normal CL. In the study reported herein, this did not appear to happen, as P_4 concentrations for individual animals for treatments which did

not result in ovulation were similar to those for saline treatment in those animals rather than being elevated slightly as in Bravo's study when animals had luteinized follicles. Furthermore, in most animals in which a P_4 rise was documented, a CL was palpated on day 8 post-treatment and a luteinized follicle would not have been identified as a CL on rectal palpation. Since high resolution ultrasound equipment was not available at the time of this study, we were unable to evaluate follicular size or CL morphology. In addition, some of the animals were difficult to palpate due to anatomical and(or) behavioral problems.

There did appear to be some lack of repeated response to hCG. One animal (#216) responded only to the highest dose and only with the first injection of that dosage. The lack of response to repeated doses of hCG appears to be real and was not necessarily due only to follicular size, since half of the animals did not respond to the final dosage of hCG administered following all other hormonal treatments. All animals initially responded to at least one dosage of hCG with ovulation and the response of ovulation in only 4 of 8 animals with the final dosage of hCG represents a decrease from initial response to individual dosages. One possibility for lack of repeated response to hCG is the formation of antibodies that may inhibit response after multiple exposure. Such antibodies have been shown to occur following repeated doses of hCG in the horse, although they have not been definitively shown to inhibit response (Wilson et al, 1990).

The highest dosage of hCG used was comparable on a dosage per kg body weight basis to that used by Leon (unpublished observations) in which P_4 concentrations following this dose were higher than those following fertile matings. Sterile matings

also resulted in higher maximum P_4 concentrations compared to fertile matings in that study. In the study reported herein, there was not a significant difference in maximum P_4 concentrations for any treatment, but many animals exhibited higher P_4 concentrations after the highest dose of hCG compared to any other treatment. The high dose of hCG treatment also tended to elicit a P_4 response that lasted longer than other treatments. One animal (#217) exhibited an extremely high P_4 concentration (peaking at > 12 ng/ml) after the highest dose of hCG that remained elevated for over 20 days, returning to baseline only by 32 days. Because this concentration of P_4 was so high and prolonged, the statistical analysis was skewed and this animal was excluded from the overall analysis for this treatment. It was felt by the authors that such a response was atypical based on results of this study and previous observations after the use of hCG.

An explanation for the trend for an increase in P_4 production after the highest dose of hCG is unknown. One possibility might be multiple ovulations caused by this high level of hCG. In two animals in this group, 2 CL were clearly palpated on day 8 following treatment but not all animals responded in a similar manner. In addition, 2 CL were also palpated in 2 other animals after each of the doses of GnRH. Multiple ovulations are relatively common in camelids, being documented in alpacas and dromedaries (Novoa, 1970). While the incidence of multiple ovulations in alpacas has been reported to be as high as 5 to 22%, the incidence of twinning is much lower (0 to 0.4%) (Fernandez-Baca et al., 1970a). Live twin births have been reported in llamas (Fowler, 1990). Multiple ovulations in llamas that have received no hormone treatments at the time of mating have been identified by the author (unpublished observations).

The high dose of hCG may have been capable of luteinizing follicles in addition to causing one follicle to rupture, resulting in higher P_4 concentrations. A further possibility is one similar to a phenomenon observed in a study by Fernandez-Baca and others (1970) in the alpaca, in which 20 alpha-hydroxy-4-pregnen-3-one was higher in pregnant animals on day 8 after mating than animals mated to a sterile male or given hCG. A similar increase in this progestin has been reported in the pseudopregnant rabbit, another induced ovulator (Orstead et al., 1988). This might explain higher P_4 concentrations measured in vasectomized and hCG-treated animals compared to pregnant animals (Leon, unpublished observations), although it does not seem to fully explain the results reported here, since only the highest dose of hCG exhibited any difference and this was not statistically significant.

In this study, GnRH appeared to be more reliable than hCG at repeatedly inducing ovulation. A single intravenous dose was sufficient to promote ovulation, although multiple injections have been suggested (Johnson, 1989). The one animal (#235) that failed to ovulate with the lower dose of GnRH did exhibit an LH rise after this treatment which was not different from that in other animals. This would seem to indicate that this animal may have lacked a follicle of sufficient size to ovulate. This animal did ovulate in response to the higher dose of GnRH in the study and has been known to ovulate with the lower dose of GnRH at other times (unpublished observations).

A single intravenous dose of 1 μg GnRH/kg BW following palpation and(or) ultrasound to determine follicular size at the time of treatment and subsequent P_4 determination at 7 days post-treatment would appear to be an appropriate dosage to

reliably evaluate the ovulatory ability of an infertile female llama. In addition, blood samples taken at hourly intervals for 4 hours following this dosage of GnRH could be used for LH quantification to determine if an infertile female is capable of responding with an LH surge. If such a response were absent, a pitiutary deficiency would be a likely cause of infertility. Due to the small size of the GnRH molecule, the chances of development of inhibiting antibody are less with repeated use than with repeated use of hCG. GnRH is widely available to the veterinary practitioner and is relatively inexpensive in the llama due to the small doses required. Although GnRH was used intravenously in this study, it has been administered by the intramuscular route to llamas by other researchers with resulting ovulation (Adam et al., 1992).

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CHAPTER IV. CLOPROSTENOL-INDUCED ABORTION IN THE LLAMA

IV.1. Abstract.

In order to determine the efficacy of injection of the $PGF_{2\alpha}$ analog cloprostenol (Estrumate^R) at causing abortion in llamas and to examine the decline in serum P_4 following such an injection, 1.7 μg cloprostenol/kg BW was administered to five mature female llamas (day 28 to 70 of pregnancy). One animal was used twice in separate pregnancies 2 years apart. All animals aborted subsequent to treatment with no adverse effects. Progesterone declined following two phase clearance dynamics with an initial rapid decline to approximately one half of pretreatment concentrations occurring within one hour of treatment (0.25 ± 0.06 hour, mean half-life ± SEM) and a slower secondary decline to nonpregnant levels (< 0.5 ng/ml) over the subsequent 48 hours (21.46 ± 6.35 hours, mean half-life ± SEM).

IV.2. Introduction.

It is occasionally necessary for the llama owner to abort an animal due to mismating and a safe, effective treatment is desirable. A persistent CL without pregnancy is also a problem in the llama and has been estimated to occur in 1 to 3% of all female llamas (Smith et al., 1994). Administration of $PGF_{2\alpha}$ or its analogues is usually the treatment of choice for both abortion and persistent CL. In the llama, $PGF_{2\alpha}$ (Lutalyse^R) administration has been associated with serious side effects and ultimately death in a few animals (Johnson, 1989). While effective in removing the CL, the side

effects have precluded its widespread use in the llama. In contrast, use of other $PGF_{2\alpha}$ analogues have been reported to sometimes require multiple dosages, which can be an inefficient use of time and money. For these reasons, the following study was designed to examine the effect of cloprostenol, a $PGF_{2\alpha}$ analogue, on the CL of early pregnancy in the llama.

IV.3. Materials and Methods.

IV.3.1. **Animals.**

Five adult female llamas from the Oregon State University (OSU) research herd were used, with one animal used during 2 separate pregnancies. Animals ranged in age from 4.5 to 9.0 yrs (6.9 ± 0.8, mean ± SEM) and in weight from 126.8 to 188 kg (155.6 ± 8.9, mean ± SEM). All animals had undergone normal previous pregnancies. Animals were maintained on pasture from the time of breeding until initiation of sampling. They were kept in stalls in the research facility during the period of sampling with *ad lib* access to food and water.

Five of six of the pregnancies had known breeding dates, while the sixth had a presumptive breeding date based on ultrasound examination of the fetus and size of the fetal vesicle. All pregnancies were confirmed by high P_4 concentrations and ultrasound or palpation prior to the initiation of sampling. Two animals were treated on day 28 of pregnancy, one on day 35 (approximately), two on day 64 and one on day 70.

IV.3.2. **Sampling and treatment protocol.**

On the day prior to initiation of sampling, animals were catheterized as described in Appendix I. Beginning 3 hours prior to cloprostenol[1] treatment, samples were taken at 15 minute intervals for determination of basal P_4 concentrations. At time 0, cloprostenol was administered intramuscularly at a dosage of 1.7 μg/kg body weight, based on the comparable per body weight dosage given to the bovine. Samples were taken at 15 minute intervals for 3 hours following treatment, then at hourly intervals for an additional 15 hours, followed by samples at 24, 30, 36 and 48 hours post-treatment. Immediately following collection, all samples were transferred into tubes containing disodium EDTA and centrifugation aid beads[2], mixed gently and placed on ice until centrifugation at 1500 x *g*, which occurred within one hour. Plasma was harvested and frozen at -20° C until assayed.

IV.3.3. **Hormone analysis.**

Samples were analyzed for P_4 by RIA (DPC Coat-A-Count), as previously described in Chapter III. Inter- and intra-assay coefficients of variation for the assays in this study were 10.1% and 6.8%, respectively.

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[1]Estrumate[R], cloprostenol sodium, Haver, Mobay Corp., Shawnee, KS

[2]Centrifuge aid beads, Sarsedt Corp., Numbrecht, W. Germany

IV.3.4. **Statistical analysis.**

Each animal's P_4 data were analyzed using the RSTRIP program for IBM compatible personal computers, which determined the best-fit exponential curve, then fitted and stripped the data to give graphical output and statistical parameters such as goodness of fit and half-lives for each phase of the curves. In general, the best fit was a 2-exponential curve for each data set. Half-lives for the initial and final phases of the progesterone decline curve were determined using the program, then averaged among animals for the overall mean.

IV.4. Results.

All animals aborted subsequent to treatment with 1.7 μg cloprostenol/kg body weight delivered in a single intramuscular dose. Progesterone concentrations declined to < 0.5 ng/ml by 48 hours post-treatment (Figure 3). No side effects or passage of fluid or fetal tissues were observed in any of the animals. All animals had subsequent normal pregnancies and deliveries after rebreeding following the study, although one animal (#242) had a uterine infection after abortion in this study and being rebred within one week of the end of sampling.

Four of six P_4 curves were adequately described with a 2-phase exponential model, resulting in 2 half-lives being calculated by the RSTRIP program. One of the curves (llama #236-'91), although sharing the same general shape as the others, had a slower 2nd phase decline in relation to the time 0 value when compared with the other curves. This result tended to flatten the curve and made the program disregard the time

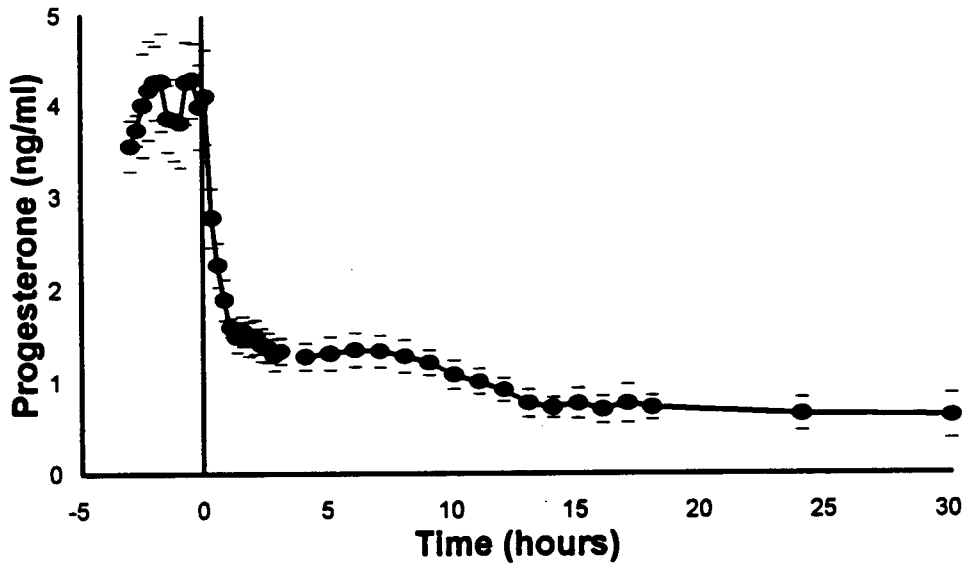

Figure 3. Mean (± SEM) P_4 concentration as a function of time preceding and following cloprostenol administration at time = 0 (n = 6).

0 value in calculations for the best fit curve and required that the initial value be given a weighting factor in the analysis to make the program begin calculations at that level and not count it as an aberrent point. Another animal (llama #242) had a transient increase in P_4 5 to 10 hours after treatment which necessitated an overall weighting factor (suggested and calculated by the RSTRIP program) in the model to more closely fit the data to the 2-exponential curve. The fitted curves for all animals, including pre- and post-weighted curves for #236-'91 and #242 are shown in Figure 4.

The half-lives for both phases of the disappearance curve of P_4 for each animal are shown in Table 4. Overall mean for the initial phase of P_4 decline was 0.25 ± 0.06 hour (mean ± SEM) which was less variable than the 2nd phase which averaged 21.46 ± 6.35 hours (mean ± SEM).

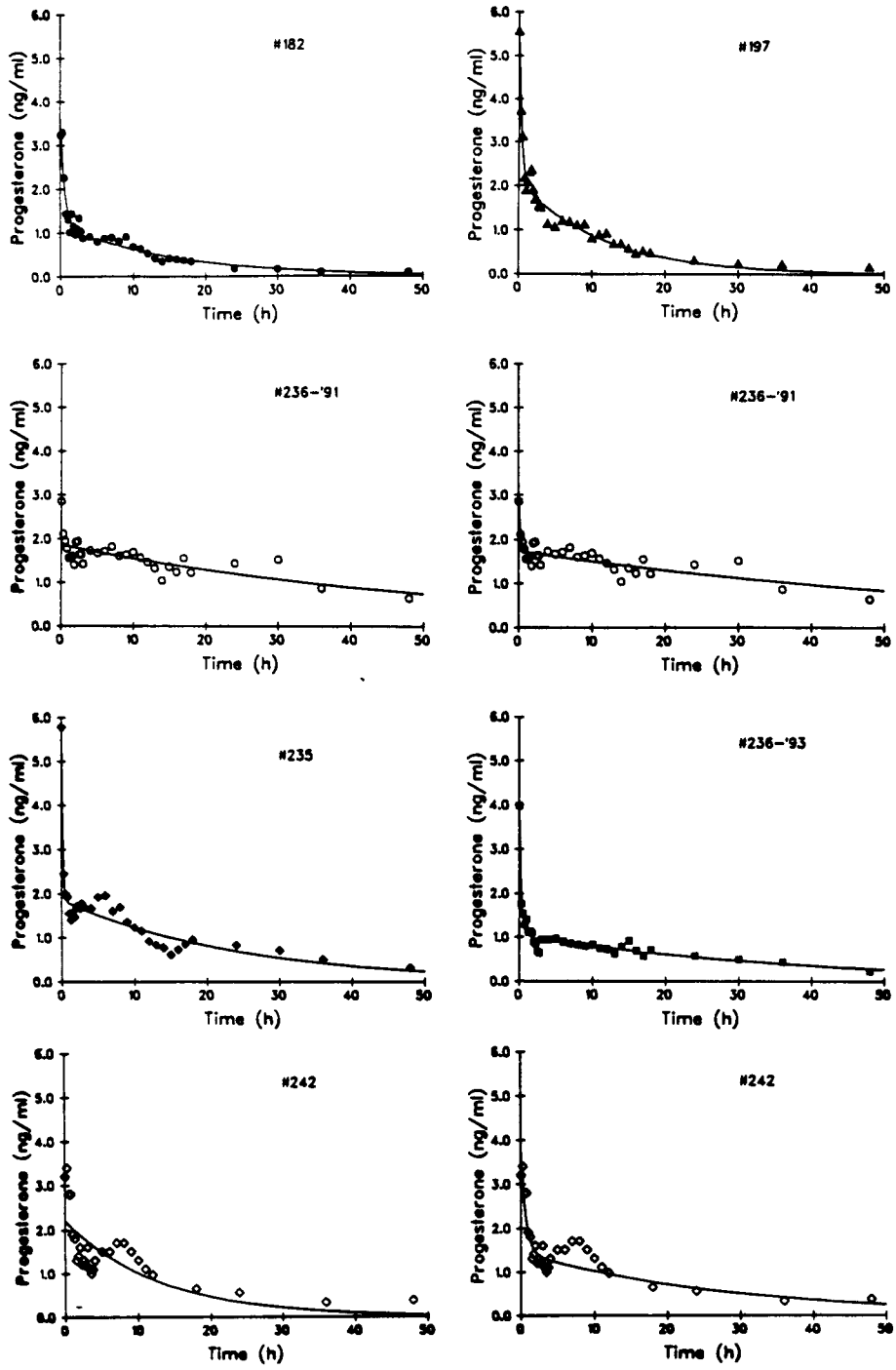

Figure 4. Fitted clearance curves for P_4 (RSTRIP) following cloprostenol administration. Curves for animals #236-'91 and #242 are shown before (left panel) and after (right panel) weighting factors applied to individual points (#236-'91) or the overall curve (#242).

Table 4. 1st and 2nd phase P_4 decline half-lives in hours for individual animals with mean, SD and SEM.

Animal #	1st Phase	2nd Phase
182	0.39	10.82
197	0.25	8.08
235	0.09	16.86
236-'91	0.16	47.48
236-'93	0.16	25.52
242	0.45	19.98
Mean	0.25	21.46
SD	0.14	14.21
SEM	0.06	6.35

IV.5. Discussion.

A single intramuscular injection of cloprostenol appears to be safe and effective as an abortifacient in the llama during the early stages of pregnancy. No significant side effects were noted with this treatment. The efficacy of cloprostenol at this dosage for the treatment of persistent CL has not been assessed.

Although data in this study was examined for disappearance rates of P_4 in a similar fashion to pharmacological clearance data, this is not a true clearance rate because the lysing of the corpus luteum by $PGF_{2\alpha}$ is not a sudden event with subsequent disappearance of P_4 from the circulation. The lysing of the CL, once initiated by $PGF_{2\alpha}$ presumptively takes place over a period of hours to days, with continuing production of

P_4 by the CL for some period of time. Our goal in fitting the P_4 data to clearance curves and determining half-lives was to provide a basis of describing the cessation of P_4 production by the CL and subsequent disappearance from circulation. Such data are actually a mixture of clearance and slowing of production of P_4.

The change in P_4 concentrations following $PGF_{2\alpha}$ analog administration are similar to data obtained from other species such as cycling cattle (Louis et al., 1973, 1974; Mutiga et al., 1993), sheep (Acritopoulou et al., 1977) and horses (Noden et al., 1978). The P_4 profiles following cloprostenol injection in pregnant swine (Diehl and Day, 1974) and cattle (Lulai et al., 1992), were similar to those observed in this study, although the sampling frequency was longer in these studies compared to this study.

It is of interest that most of the llamas exhibited a transient rise in P_4 3 to 9 hours after an initial dramatic fall. This phenomenon has been observed in the mare (Noden et al., 1974, 1978) and heifer (Fogwell et al., 1978) following $PGF_{2\alpha}$ injection. Such a rise may have multiple explanations. It may indicate that the CL tends to increase production of P_4 briefly during its regression after an initial cessation of production and the start of clearance from the body. Alternatively, the regressing CL may continue production of P_4 until the CL reaches a size or functional state that can no longer support further production and there may then be a transient increase in P_4 in the serum of some animals as stored P_4 is released in a burst when a certain number of cells autolyze.

While there was considerable variability in the second phase of P_4 decline, the first phase was remarkably similar among animals. This may support the previously mentioned possibility that the first phase of clearance approximates P_4 clearance as the

CL is initially affected by $PGF_{2\alpha}$ and begins the lytic process while the second phase represents some degree of continuing production as cellular death slows. There may also be some repartitioning of P_4 from peripheral compartments in a true 2-phase physiological clearance pattern.

In summary, cloprostenol in this study was a safe, effective method of terminating early pregnancy in the llama. Progesterone concentrations declined to basal concentrations (< 0.5 ng/ml) by 48 hours post-treatment. Additional time should be allowed before rebreeding, to allow uterine involution and return to normal state.

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CHAPTER V. STUDIES ON REPRODUCTION IN THE MALE LLAMA: PARAMETERS ASSOCIATED WITH PUBERTY AND THE EFFECT OF SEASON ON TESTOSTERONE CONCENTRATIONS IN THE ADULT LLAMA.

V.1. Abstract.

V.1.1. **Study A.**

Five male llamas were studied at 2 mo intervals from 8 to 20 mo of age. Blood samples were collected via intravenous catheter at 15 min intervals for 30 h, with GnRH (1 μg/kg BW, i.v.) administered at 24 h. All samples were analyzed for LH and 30 min interval samples were analyzed for testosterone (T) by RIA. On the day following the 30 h blood sampling period, animals were sedated with xylazine (0.66 mg/kg BW, i.v.) and testicular measurements recorded. Semen was then collected by electroejaculation from each animal and the penis was examined for presence of adhesions to the prepuce. When obtained, semen was examined microscopically for presence of normal spermatozoa.

Mean 24 h T concentrations increased from 8 (1.0 ± 0.3 ng/ml, mean ± SEM) to 20 mo (1.6 ± 0.2 ng/ml) of age with a tendency toward lower T at 14 mo in all animals (0.4 ± 0.2 ng/ml). Concentrations of T at 18 and 20 mo were significantly ($P < 0.05$) higher than at other mo. Basal LH concentrations averaged 0.8 ± 0.1 ng/ml over all sampling periods with no significant ($P > 0.05$) differences over age. Likewise, response of LH to GnRH stimulation did not change significantly as a function of time. Response of T to GnRH tended to increase with age and was significantly greater at 20

mo compared to other ages studied. Testicular dimensions increased in a linear fashion, as did body weight. All animals had normal spermatozoa in ejaculates by 20 mo (3 of 5 at 14 mo, 4 of 5 at 16 months, 5 of 5 at 20 mo). At 20 mo, 1 of 5 animals still had complete adhesion of the glans penis to the prepuce and 2 of 5 had the only the tip of the glans free. One each of the 2 remaining llamas had complete extension of the penis at 18 and 20 mo. Thus, it would appear that the limiting factor in the use of young male llamas for breeding is the rate of breakdown of preputial adhesions.

V.1.2. **Study B.**

Six adult male llamas (age 2.5 to 4.5 yr) were utilized in an experiment to evaluate seasonal changes in T secretion. Animals were sampled as in Study A in March, June, September and December and were maintained under natural lighting conditions throughout the year. Samples were analyzed at 30 min intervals for T. Testicular dimensions were measured on standing animals at the end of each sampling period. There was no significant effect ($P > 0.05$) of season on 24 h mean T concentrations, difference in mean T in the 6 h prior to and after GnRH administration or on testicular dimensions.

V.2. Introduction.

Inefficient reproduction and high rates of infertility have been reported in all camelids (Novoa, 1970) and in South American camelids both in South America (San-Martin et al., 1968; Foote et al., 1968) and in North America (Johnson, 1989). Various

causes of poor reproductive performance in these species have been suggested, including sterility or poor fertility of the male (Foote et al., 1968; San-Martin et al., 1968). Breeding practices in South America, usually consisting of maintaining relatively large groups of females with multiple males (Condorena and Valasco, 1978), frequently do not allow for individual male evaluation. Llama males in North America are usually not utilized to their full capacity, frequently breeding < 10 females/year, making evaluation of fertility by pregnancy rate somewhat difficult. In addition, the fact that male llamas copulate over a relatively long period of time (often 15 to more than 40 min) with dribble ejaculation occurring throughout this period, makes collection of high-quality semen samples for evaluation problematical.

One problem in the evaluation of llama fertility in North America has been lack of information on normal reproduction, including age at puberty. Research data on puberty in South American camelids is scarce and word-of-mouth opinions of owners can lead to erroneous assumptions. The male llama is generally considered to be sexually mature by 2 to 3 yr of age, although some owners start breeding them successfully at 18 mo of age, while some individual animals appear unsuccessful at causing pregnancy until 3 yr of age. Study A was performed to further our understanding of puberty in the male llama.

Camelids are usually considered to be seasonal breeders in their native climates. In the dromedary, seasonal variations in testicular steroidogenesis (Bedrak et al., 1983) and in concentrations of LH, corticoids and sex steroids (Bono et al., 1989) have been observed along with seasonal changes in sexual behavior (Yagil and Etzion, 1980). Such variations, however, correspond with rainy seasons and may reflect adequate

forage conditions for breeding and birth of the young rather than influences of photoperiod as in true seasonal breeders. Additional work has shown that with adequate forage the dromedary can breed year-round (Gauthier-Pilters and Dagg, 1981) as does the Bactrian camel in Russia (Novoa, 1970). In the SAC, no seasonal variations in reproductive hormones have been reported, although vicuñas and guanacos breed seasonally in the wild (Franklin, 1981) and llamas and alpacas are managed to be bred during the rainy season (Foote et al., 1968). Due to an overall decrease in T observed during the winter months in animals on Study A and because reports on the effect of season on hormones in male llamas could not be identified in the literature, Study B was designed to examine the effects of season on T secretion in the male llama.

V.3. Materials and Methods.

V.3.1. **Animals.**

V.3.1.1. ***Study A.***

Five animals of similar age and genetic background from a commercial llama herd in the Willamette Valley of Oregon were utilized for this study. Animals had birthdates within two weeks of one another and were progeny of two sires and similar type females in the herd. At the beginning of the study, animals were 8 months of age and averaged 62.8 ± 1.9 (mean ± SEM) kg body weight. At the end of the study,

animals were 20 mo of age and averaged 100.2 ± 8.4 (mean ± SEM) kg bodyweight. Animals were provided with food and water *ad lib* throughout the study period.

V.3.1.2. ***Study B.***

Six adult male llamas from the OSU llama herd and ranging in age from 2.5 to 4.5 years (3.5 ± 0.8 yrs, mean ± SEM) and an average weight of 131.0 ± 11.2 (mean ± SEM) kg were utilized in Study B. Animals were maintained on pasture between sampling periods. During sampling periods, animals were confined in an outdoor enclosure under natural conditions without artificial lighting beginning at least one week prior to and extending throughout the sampling period. Animals had free choice access to food, water and trace mineral mix throughout the study.

V.3.2. **Blood sampling.**

V.3.2.1. ***Study A.***

Animals were sampled at 2 mo intervals from 8 to 20 mo of age. Animals were brought in to the OSU College of Veterinary Medicine research facility at least one day prior to each sampling period and weighed, then placed in stalls (2 to 3 animals/stall) and allowed to acclimate to their surroundings. On the morning of initiation of each sampling period, animals were fitted with indwelling jugular venous catheters[1] as

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[1]B-D I.V. CATH, Becton, Dickinson and Co., Rutherford, NJ

described in Appendix I except that catheters were 16 ga x 4". In most cases catheterization of all 5 animals was accomplished by 1 to 2 h prior to scheduled initiation of sampling and that time was used for sham sampling to accustom the animals to the sampling procedure. Sample procedure and handling was as previously described.

Sampling was initiated at 1200 h for each sampling period and blood samples were taken at 15 min intervals for 30 h. At 24 h after the beginning of each sampling period, 1 μg GnRH/kg BW was administered i.v. via the catheter immediately after the 24 h sample was taken.

V.3.2.2. ***Study B.***

Animals were sampled 4 times during one calendar year, during March, June, September and December. These times were within one week of the summer and winter solstices and the spring and fall equinoxes. Catheterization and blood sampling procedures were as previously described.

V.3.3. **Testicular dimensions and semen analysis.**

In Study A, on the day following the conclusion of blood sampling, animals were taken to the OSU Veterinary teaching hospital where each animal was given 0.66 mg xylazine hydrochloride[2]/kg BW via the i.v. catheter. Animals were placed in left

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[2]Rompun[R], xylazine hydrochloride, Miles Laboratories, Inc., Shawnee, KS

lateral recumbency and width and length of each testicle + scrotum were measured with calipers and recorded. The animals were then electroejaculated using a portable battery powered electroejaculation unit with a ram probe. Pulses of approximately 1 second duration were administered at 5 second intervals for 30 seconds, followed by a rest period of 30 seconds and repeating this sequence until semen was collected or approximately 5 minutes had passed. Presence or absence of preputial adhesions was visually evaluated as ability to manually extrude the penis under sedation and with electroejaculation. Semen was examined for presence of spermatozoa. In Study B, testicular dimensions were measured on standing animals immediately at the conclusion of blood sampling and semen samples were not collected.

V.3.4. **Hormone analysis.**

In Study A all samples were analyzed for LH by RIA as previously described in Chapter III. Intra- and interassay coefficients of variation for assays in this study were 6.7 and 17.6%, respectively. Odd numbered samples (collected at 30 min intervals) for both studies were analyzed for T by a commercial coated tube radioimmunoassay (DPC Coat-A-Count Total Testosterone[3]). This RIA was validated for the llama in this laboratory, exhibiting parallelism of 12.5, 25, 50 and 100 µl of llama plasma with the standard curve when graphed on a log-logit scale and recoveries of standard diluted in equal volumes of llama plasma and added to assay tubes at a rate of 0.5, 2.0 and 4.0 ng/tube standard averaging 101.0 ± 5.3% (mean ± SD, n=9). Intra- and interassay

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[3]Coat-A-Count[R] Total Testosterone, Diagnostic Products Corp., Los Angeles, CA

coefficients of variation for the testosterone assays were 7.9 and 10.5%, respectively for Study A and 5.1 and 9.3% for Study B. Crossreactivity of the antibody to testosterone were reported by DPC to be < 1% for: aldosterone, corticosterone, cortisol, 11-deoxycortisol, estradiol, estrone, progesterone, androsterone, DHEA, DHEA sulfate, 5β-androstan-3α,17β-diol, 5α-androstan-3β,17β-diol, 5-androsten-3β,17β-diol, 11β-hydroxytestosterone and 5α-androstan-3,17-dione and 1.5% for 19-hydroxytestosterone, 3% for androstenedione, 3.2% for 11-ketotestosterone and 7.9% for 5α-dihydrotestosterone. Samples below the detection limits of the assays were assigned a value of the lower detection limit (0.3 ng/ml for T, 0.6 ng/ml for LH) for calculation purposes.

V.3.5. **Statistical analysis.**

Twenty-four h means for LH and T were compared among sampling periods for both studies by one-way ANOVA using Statgraphics for the IBM PC using the LSD procedure to determine differences. Likewise, the means for the 6 h following GnRH for both LH and T were compared to the means for the 6 h preceding GnRH using the same Statgraphics procedure to determine responsiveness to GnRH. The differences between the means for the 6 h prior to GnRH and the 6 h following GnRH were compared among ages for Study A and among seasons for Study B to determine age and season effect on response to GnRH. All differences reported as significant were at the $P < 0.05$ level.

V.4. Results.

V.4.1. **Study A.**

There were no significant ($P > 0.05$) differences in 24 hour mean LH between sampling periods. Basal LH averaged 0.8 ± 0.1 (mean ± SEM) ng/ml throughout the study. Response of LH to GnRH was minimal at all ages, with an average increase across all sampling periods of 0.1 ± 0.1 (mean ± SEM) ng/ml in the 6 h following GnRH administration compared to the 6 h immediately preceding GnRH administration (Figures 5 and 6). There were no differences in LH response to GnRH between sampling periods. All mean LH data for Study A are summarized in Table 5.

Table 5. Mean concentrations of LH (ng/ml) at various ages in the peripubertal male llama during the 24 h basal period and the 6 h intervals preceding and following GnRH administration. None of the between or within column comparisons were significant ($P > 0.05$).

Age (months)	24 h (mean ± SEM)	6 h pre-GnRH (mean ± SEM)	6 h post-GnRH (mean ± SEM)
8	0.7 ± 0.2	0.6 ± 0.1	0.7 ± 0.1
10	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
12	0.7 ± 0.2	0.8 ± 0.1	0.9 ± 0.1
14	0.8 ± 0.1	0.9 ± 0.1	1.2 ± 0.1
16	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
18	0.9 ± 0.1	1.0 ± 0.1	1.1 ± 0.1
20	0.9 ± 0.1	1.0 ± 0.1	1.3 ± 0.1

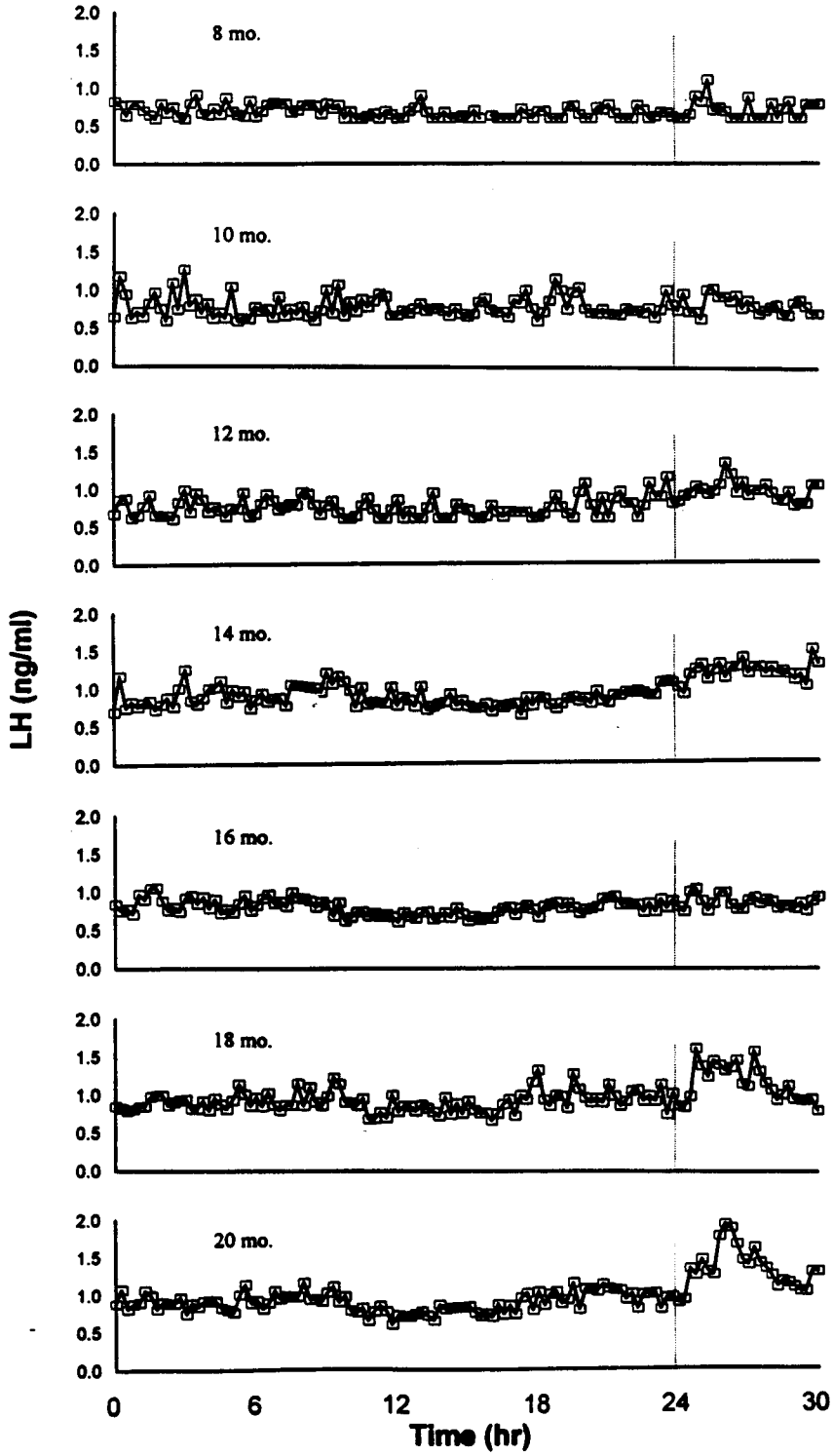

Figure 5. Mean LH concentrations between 8 and 20 mo of age in the male llama. GnRH (1 μg/kg BW) was administered i.v. at 24 h (dashed vertical line).

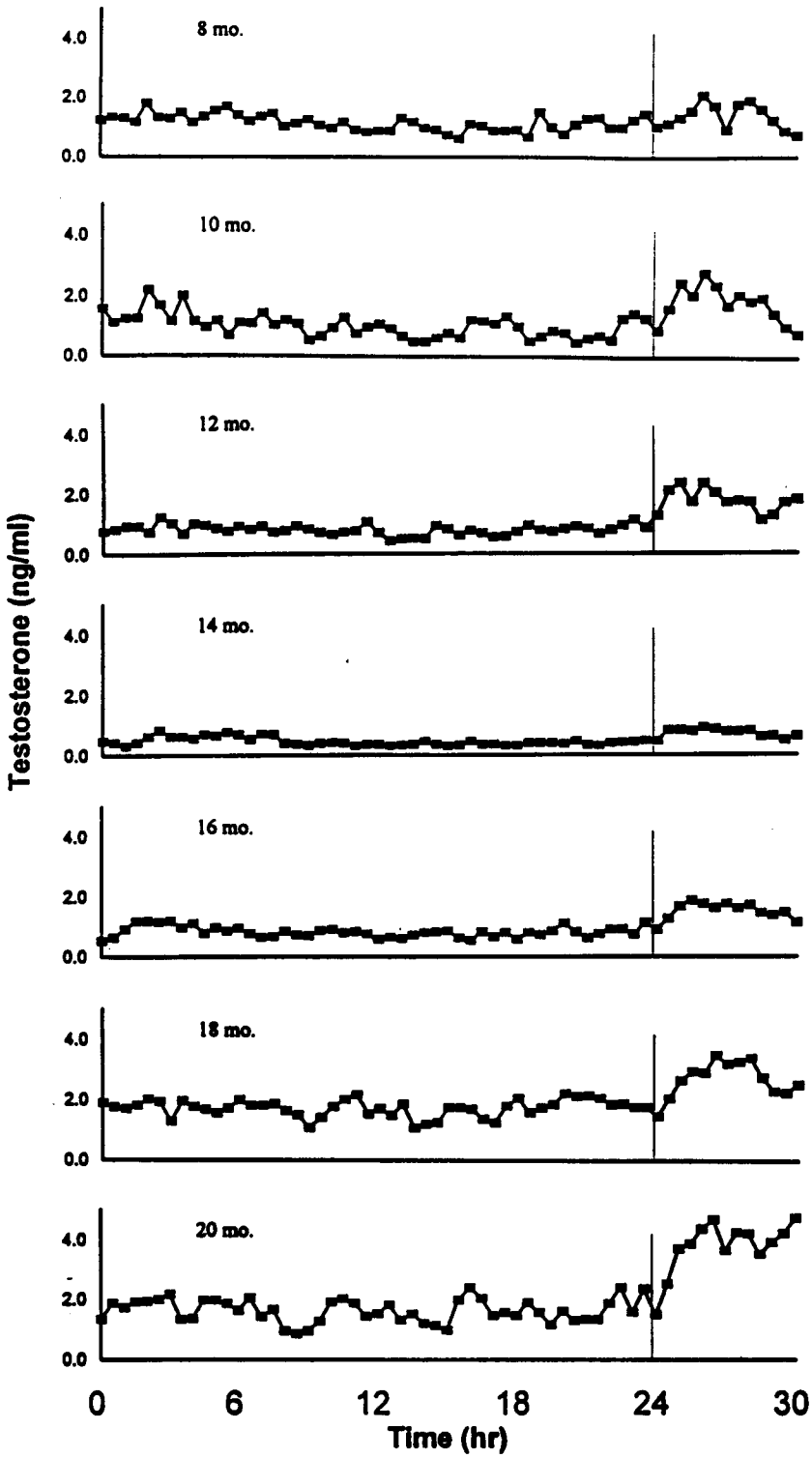

Figure 6. Mean T concentrations between 8 and 20 mo of age in the male llama. GnRH (1 μg/kg BW) was administered i.v. at 24 h (dashed vertical line).

Testosterone in the 24 h basal period increased ($P < 0.05$) from a mean at 8 mo of age of 1.0 ± 0.3 (mean ± SEM) ng/ml to 1.7 ± 0.2 (mean ± SEM) ng/ml at 18 mo of age with no further increase at 20 mo of age (1.6 ± 0.2 ng/ml, mean ± SEM). Concentrations at 8, 10, 12 and 16 mo of age were not significantly different from one another ($P > 0.05$) nor were concentrations at 18 or 20 mo of age different from one another. Response of T to GnRH administration was greater at 20 mo of age than at all other ages, with an increase of 2.5 ± 0.3 (mean ± SEM) ng/ml in the 6 h period following GnRH compared to the 6 h period preceding GnRH at 20 mo of age. This difference averaged only 0.7 ± 0.3 (mean ± SEM) ng/ml for all other sampling periods. There was a trend for increasing response to GnRH with increasing age, but the difference between the 6 h post-GnRH mean and the 6 h pre-GnRH mean was only significant ($P < 0.05$) at 20 mo of age. Testosterone data for Study A are summarized in Table 6. Average LH and T for each sample in each 30 h sampling period are shown in Figures 7 (LH) and 8 (T). Mean LH and T concentrations for the samples in the 6 h immediately preceding and following the administration of GnRH 24 hours after the beginning of sampling at each age are shown in Figures 5 (8, 10, 12 and 14 mo of age) and 6 (16, 18 and 20 mo of age). Overall mean LH and T for the 24 h periods prior to administration of GnRH are shown in Figure 9.

Mean T at 14 mo of age was lower (0.4 ± 0.2, mean ± SEM, ng/ml, Figure 9) than at other time periods, although the difference between age periods was not statistically significant. The decrease in T at 14 mo of age compared to other age periods occurred in all animals. The actual mean may have been even lower due to the assignment of minimum assay detection limit values for calculation purposes to all

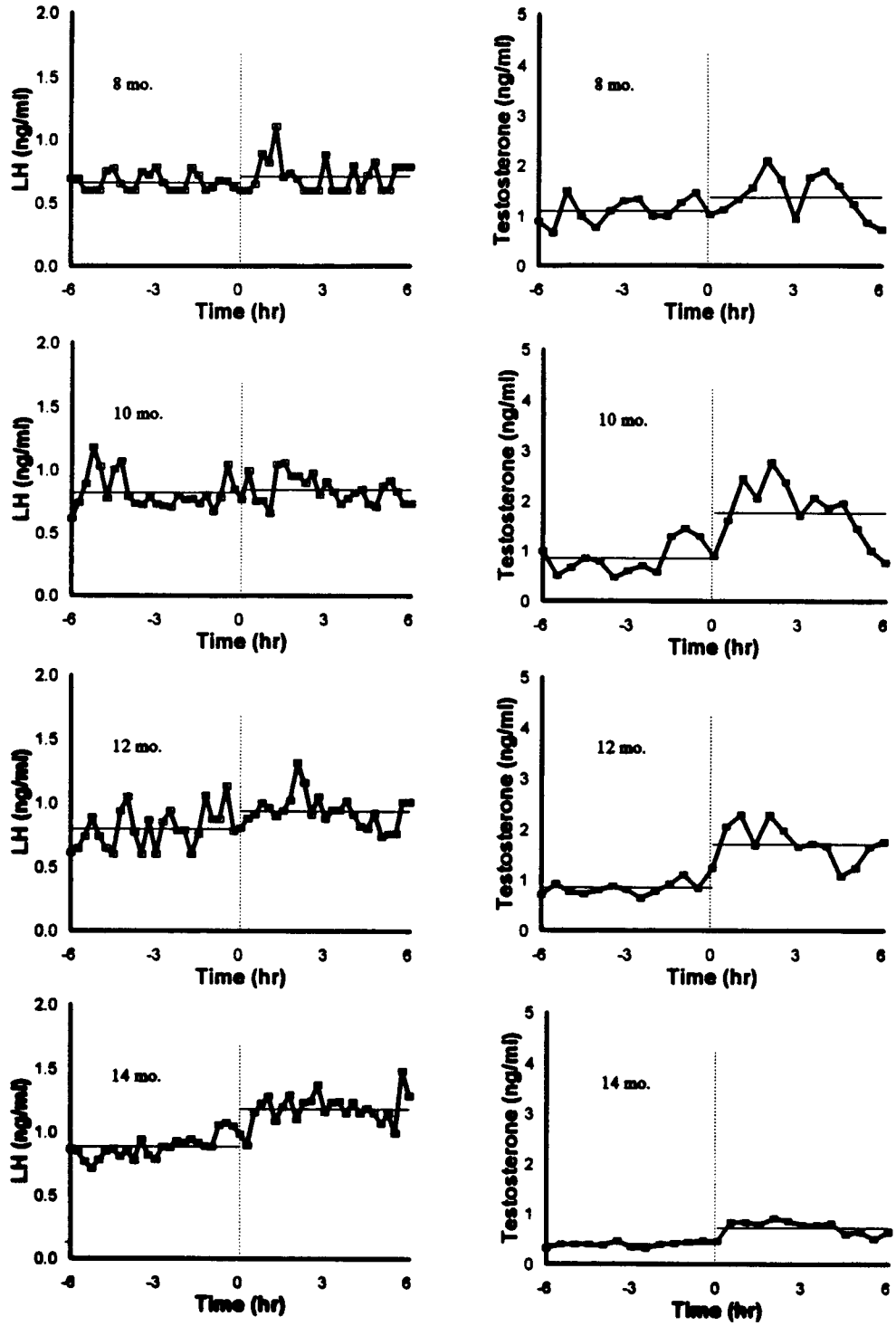

Figure 7. Mean LH and T concentrations in 8 to 14 mo old male llamas for 6 h preceding and following i.v. GnRH (1μg/kg BW) administration at 0 h (dashed vertical line). The mean LH or T concentration for each time period is indicated with a solid horizontal line.

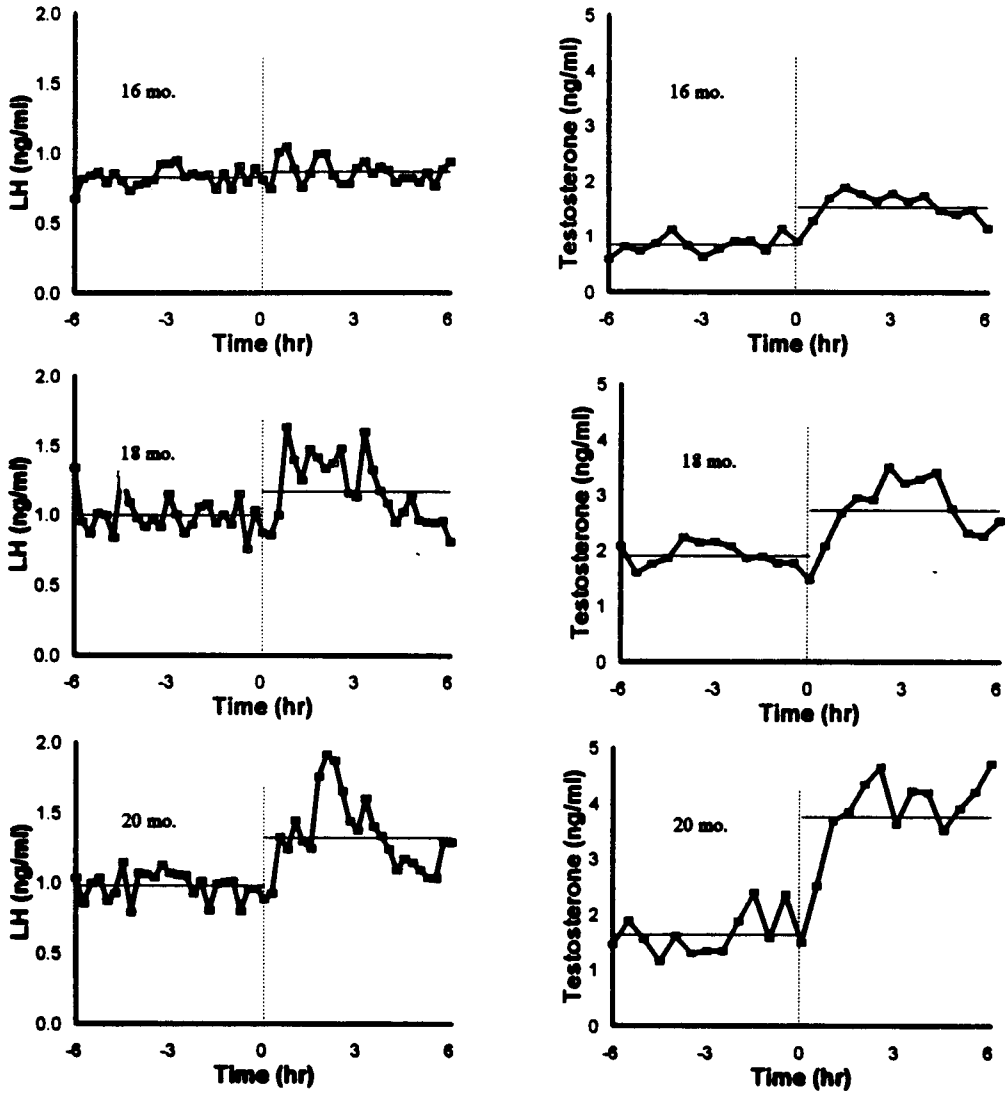

Figure 8. Mean LH and T concentrations in 16 to 20 mo old male llamas for 6 hours preceding and following i.v. GnRH (1 μg/kg BW) administration at 0 h (dashed vertical line). The mean LH or T concentration for each time period is indicated with a solid horizontal line.

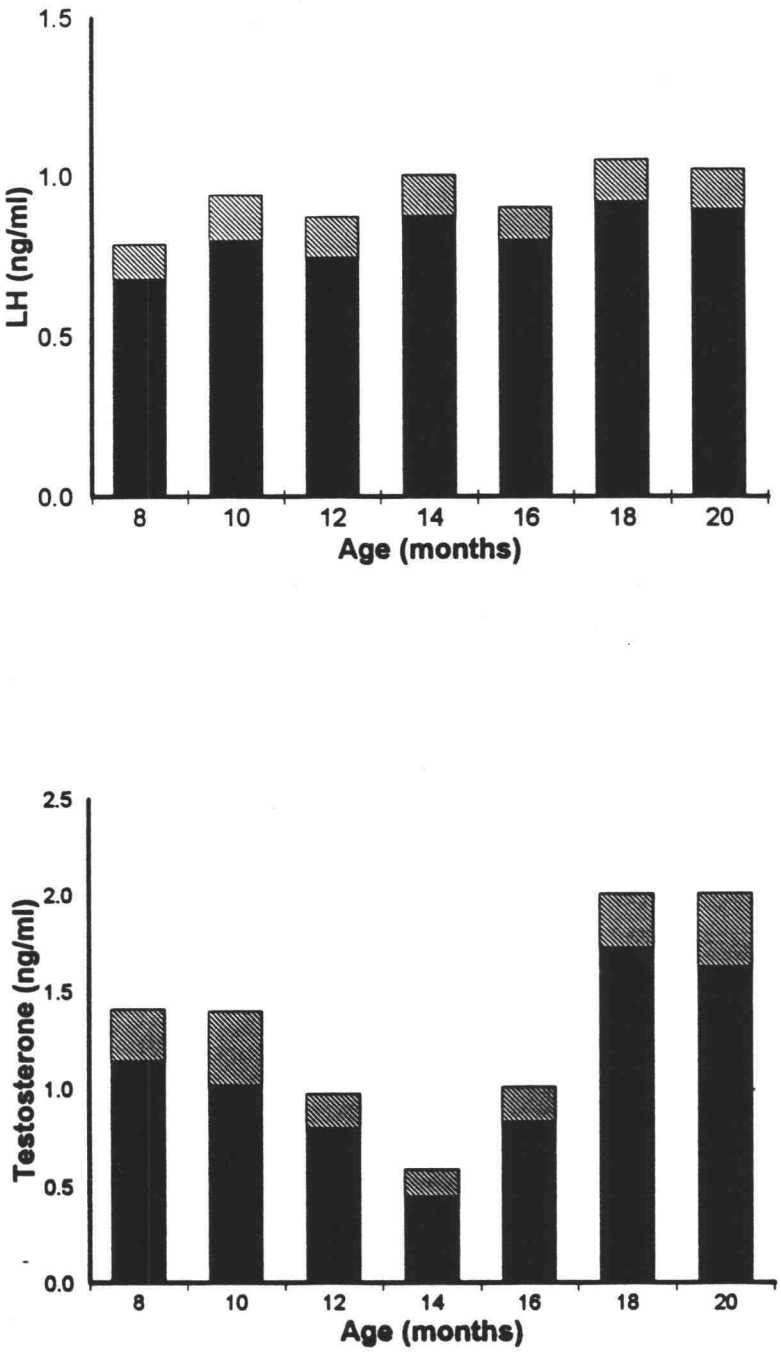

Figure 9. Change in 24 h mean (+ SEM) LH (upper) and T (lower) concentrations between 8 and 20 mo of age in the male llama.

Table 6. Mean testosterone concentrations (ng/ml) as a function of age in the peripubertal llama during the 24 h basal period and the 6 h intervals preceding and following GnRH administration. Different letter superscripts within the second column indicate means which differ significantly ($P < 0.05$). Asterisk (*) in the fourth column indicates that the difference in 6 h pre- and post-GnRH means is significantly different from this difference at other ages.

Age (months)	24 h (mean ± SEM)	6 h pre-GnRH (mean ± SEM)	6 h post-GnRH (mean ± SEM)
8	1.0 ± 0.3^{ab}	0.9 ± 0.1	1.3 ± 0.2
10	0.9 ± 0.2^{a}	0.8 ± 0.1	1.6 ± 1.0
12	0.8 ± 0.2^{a}	0.9 ± 0.1	1.6 ± 0.1
14	0.4 ± 0.2^{a}	0.4 ± 0.1	0.8 ± 0.1
16	0.8 ± 0.2^{a}	0.8 ± 0.1	1.6 ± 0.1
18	1.7 ± 0.2^{c}	1.9 ± 0.1	2.8 ± 0.1
20	1.6 ± 0.2^{bc}	1.6 ± 0.1	4.0 ± 0.2*

samples in which T was undetectable. There was no corresponding change in 24 h basal LH concentrations in these animals. In addition, there was a tendency for decreased response of T to GnRH at 14 mo of age compared to other months although this difference was also not significant.

Body weight increased as a function of increasing age, as did testicular dimensions (Figure 10). There was no significant difference between right and left testicular length or width.

Adhesion of the penis to the prepuce was complete at the beginning of the study in all animals. With successive sampling periods, most animals had progressive breakdown of adhesions and at the end of the study, 2 animals were capable of full penile extension. Both of these had the tip of the glans penis free at 14 mo of age,

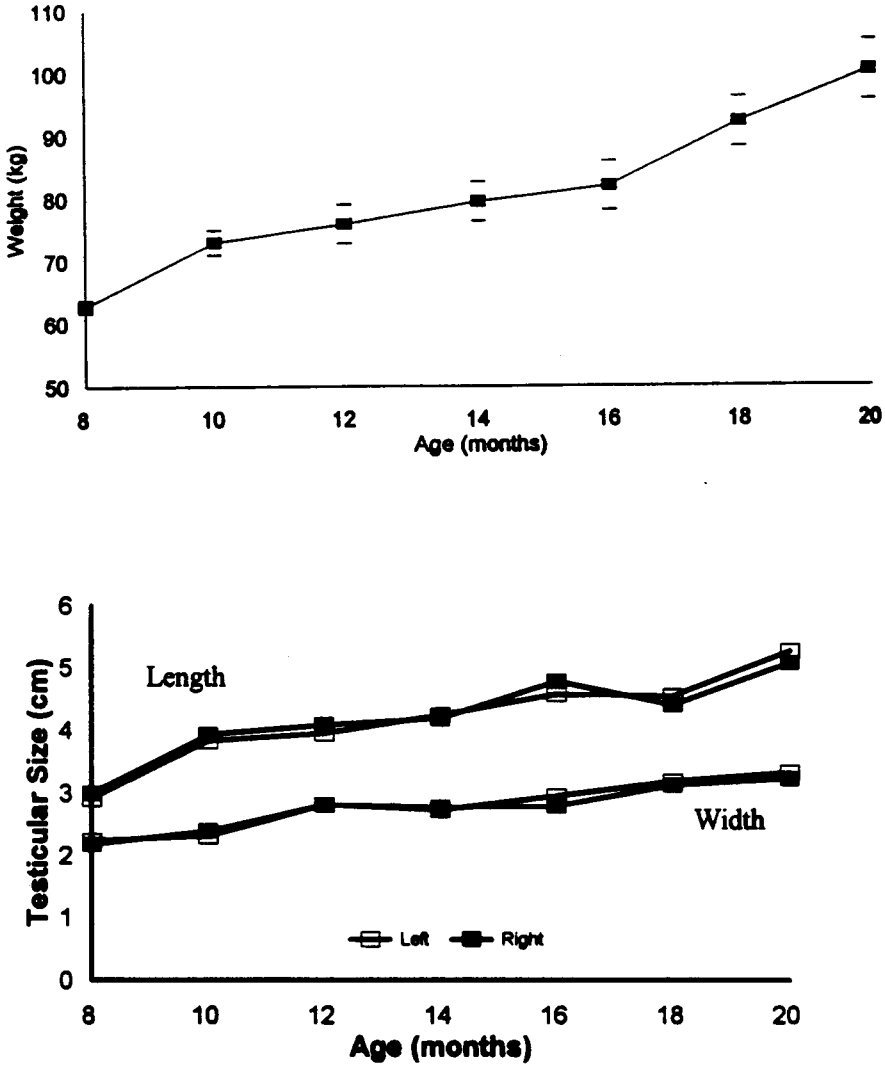

Figure 10. Change in mean BW (± SD) (upper) and mean testicular length and width (lower) between 8 and 20 mo of age.

which coincided with the first appearance of spermatozoa in the ejaculates of these animals. A third animal did not have even the tip of the glans free until 20 mo of age, well after the first collection of spermatozoa in this animal at 14 mo of age. Another animal had the tip of the glans free at 16 mo of age along with first collection of spermatozoa, but still had extensive adhesions at 20 mo of age and the remaining animal had complete penile-to-preputial adhesions at 20 mo of age, which is when spermatozoa were first collected in that animal.

Low volumes of fluid (often unmeasurable, as only droplets were expressed and these adhered to the prepuce and sides of collection tubes or were only enough to smear on a slide for evaluation) were collected upon electroejaculation, therefore, final evaluation of semen was for presence or absence of viable spermatozoa without quantification of total numbers, abnormalities or volume of collection. All 5 animals had viable, motile spermatozoa with normal morphology in the ejaculate by 20 mo of age (3 of 5 at 14 mo of age, 4 of 5 at 16 mo of age and 5 of 5 at 20 mo of age). Subjectively, the first ejaculate in which spermatozoa were observed contained larger numbers of abnormal and dead spermatozoa than found in subsequent collections. Quantity of semen collected did not appear to increase with age and was more dependent on each individual collection.

V.4.2. **Study B.**

There was no significant ($P > 0.05$) effect of season on 24 h mean T concentrations, which averaged 3.6 ± 0.4 (mean ± SEM) ng/ml for all seasons over all

animals. Figure 11 shows the mean T concentration for the 24 h basal secretion period for each season. Figure 12 shows the 24 h T secretion of a typical animal (llama #222). There was also no effect of season on differences between 6 h pre-GnRH means and 6 h post-GnRH means with the average difference being 2.1 ± 0.3 (mean ± SEM) ng/ml for all seasons. Figure 13 shows mean T concentrations for the 6 h preceding and following GnRH stimulation as a function of season and Figure 14 shows this time period for llama #222. Table 7 shows averages of 24 h T and response to GnRH for each season. Testicular dimensions did not change over the course of the study.

Table 7. Changes in testosterone concentration (ng/ml) in the male llama as a function of season. None of the between or within column comparisons were significant ($P > 0.05$).

Season	24 h (mean ± SEM)	6 h pre-GNRH (mean ± SEM)	6 h post-GnRH (mean ± SEM)
Spring	3.4 ± 0.1	3.7 ± 0.3	6.1 ± 0.4
Summer	4.4 ± 0.2	4.9 ± 0.4	6.9 ± 0.4
Fall	3.0 ± 0.1	3.6 ± 0.2	6.1 ± 0.3
Winter	3.3 ± 0.1	3.4 ± 0.3	4.7 ± 0.3

V.5. Discussion.

In relationship to its size, the male llama appears to be relatively slow in attaining sexual maturity. Puberty has been reported to take place at 2.5 to 3.0 yr in the male llama (Johnson, 1989). Male llamas are most commonly used extensively for breeding in both South and North America at approximately 3 yr of age (Jessup and

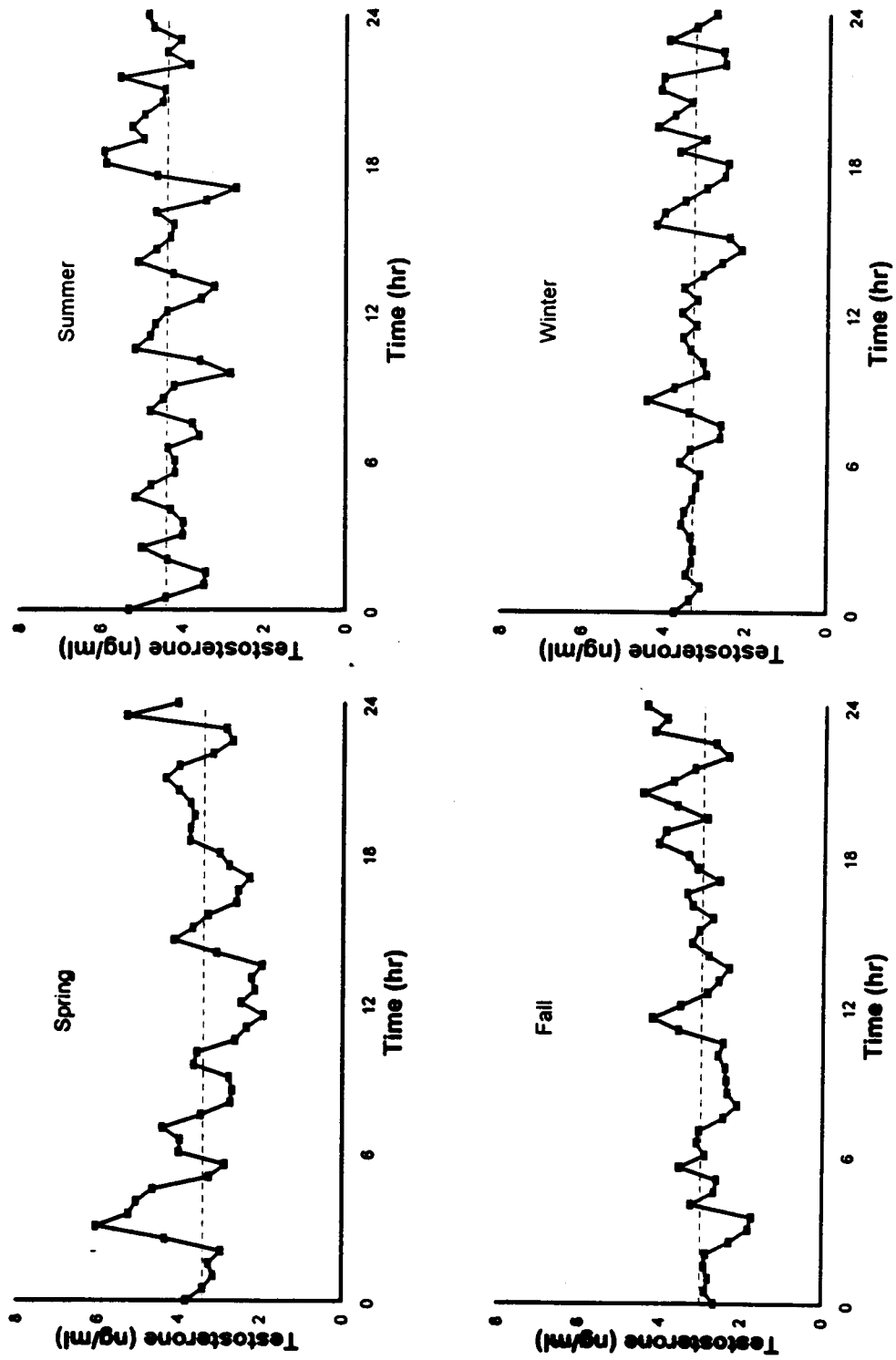

Figure 11. Seasonal changes in mean T concentrations in the male llama (n = 6). The overall mean T concentration for the 24 h period is indicated by the dashed horizontal line.

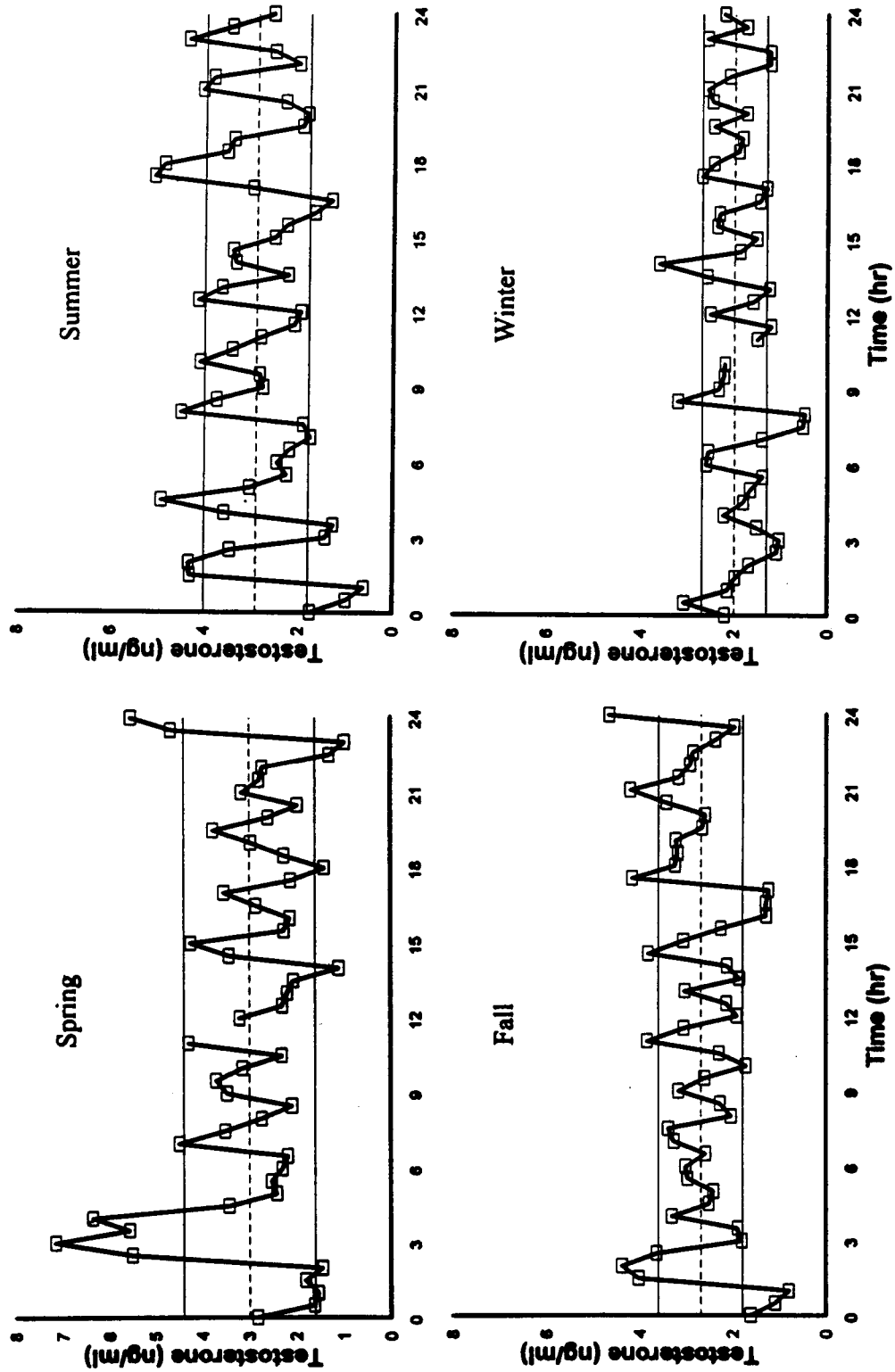

Figure 12. Changes in T concentrations during a 24 h period in a representative animal (#222). Mean (dashed line) and ± SEM (solid lines) T concentrations are shown.

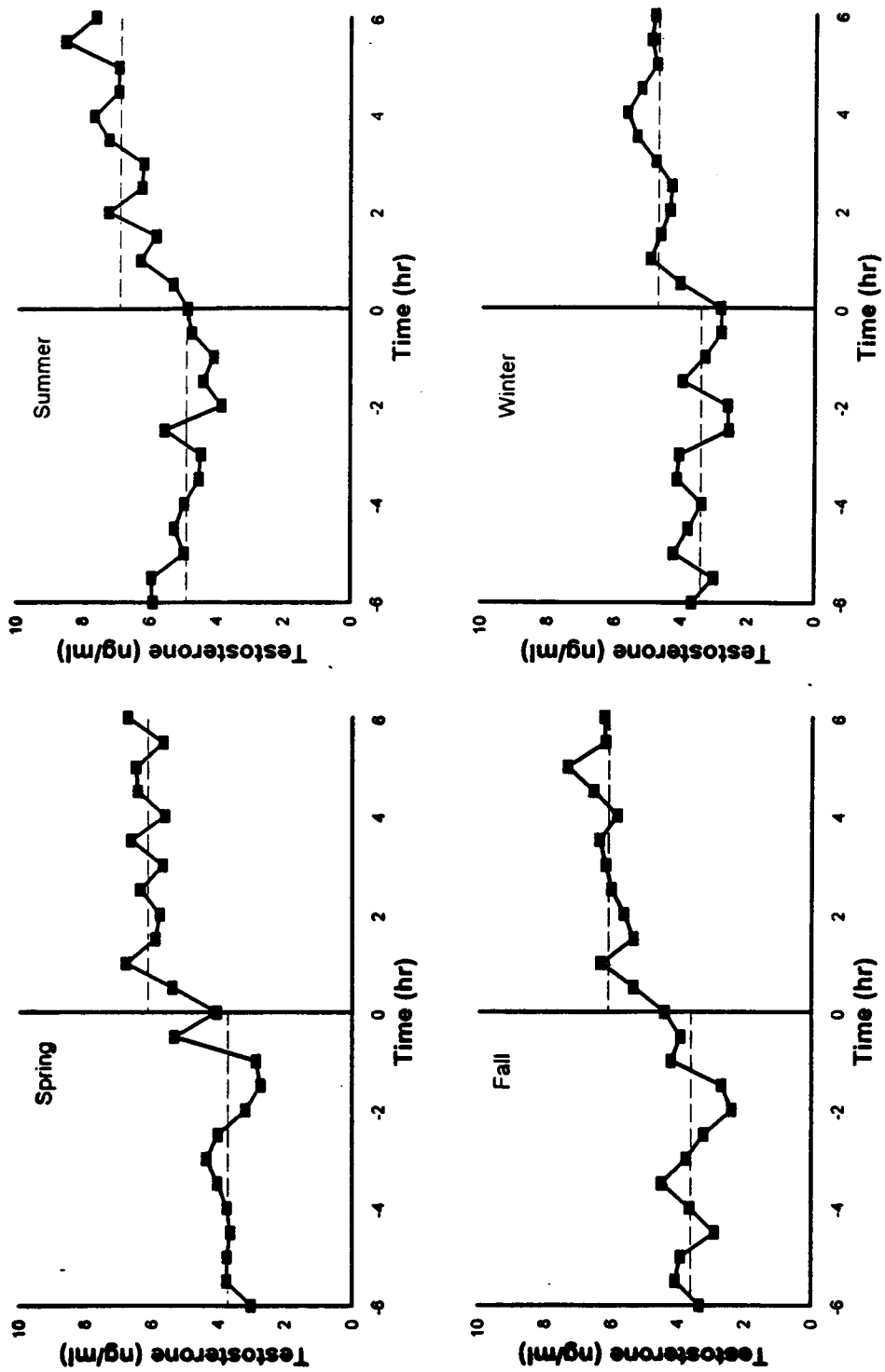

Figure 13. Changes in mean T concentrations for 6 h preceding and following GnRH administration (1 μg/kg BW i.v.) as a function of season in the male llama (n = 6). The dashed horizontal lines indicate the mean T concentration for each 6 h interval.

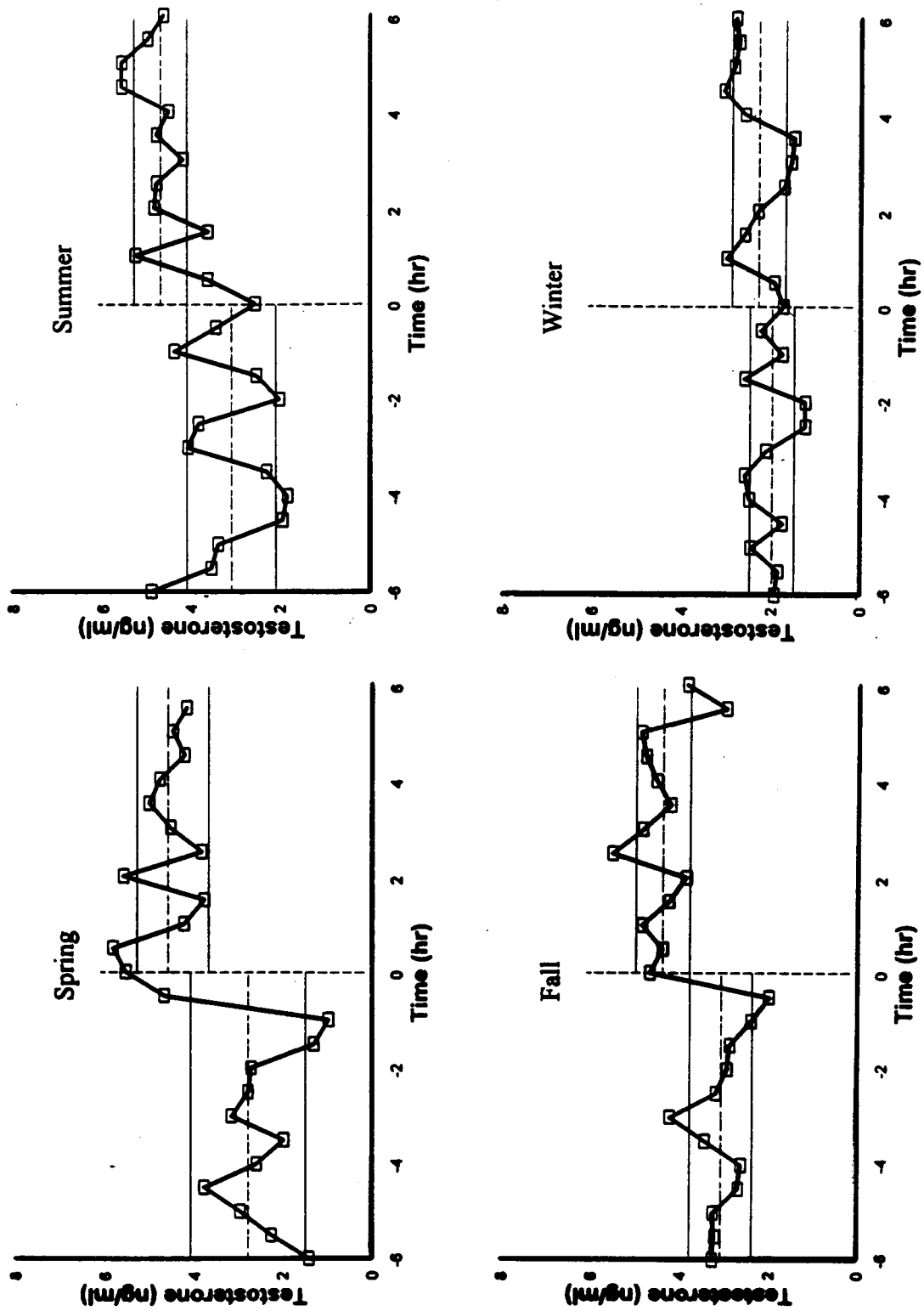

Figure 14. Changes in mean T concentrations for 6 h preceding and following GnRH administration (1 μg/kg BW i.v.) as a function of season in a representative animal (#222). Mean (dashed line) and ± SEM (solid lines) T concentrations are shown.

Lance, 1982) although some animals are used starting at about 2 yr of age. The occasional owner reports young males causing pregnancy as early as 6 mo of age, while some animals are unable to cause conception until well past 3 yr of age. In camels, males are considered to attain puberty between 3 to 5 years of age and reach full sexual activity at 6 to 7 years, but much of this is due more to common breeding and herding practices than to physiology (Yagil, 1982; Wilson, 1984). Similarly, in South America, camelids probably physically attain puberty earlier than they are actually utilized for breeding (Novoa, 1970). Often, the limiting factor in use of young male camelids for breeding is the prolonged presence of penile-to-preputial adhesions (Bravo et al., 1992).

In Study A, average basal LH concentrations did not change over the time period of the study. In other species, an early increase in LH secretion has been reported before increased T and onset of puberty. In ram lambs, for example, basal LH concentrations have been observed to increase by 5 wk of age (Lee et al., 1976a) with a further increase at 41 wk. This second increase was associated with rapidly increasing T concentrations. In another study , LH concentrations in sheep appeared to peak early (6 wk), then declined while T had a slow steady increase to peak at 28 to 32 wk (Wilson and Lapwood, 1979a), which is about the time other investigators first found sperm in the ejaculate of rams (Skinner and Rowson, 1968). In bulls, basal LH concentrations have been observed to rise with increasing pulse frequency beginning at 3 to 4 mo of age (Macmillan and Hafs, 1968; McCarthy et al., 1979; Amann and Walker, 1983; Amann et al., 1986) while T increases were observed at 4 to 6 mo of age (McCarthy et al., 1979; Amann and Walker, 1983). Young stallions also show an increase in LH (at 36 to 40 wk) prior to increased T which becomes detectable at 32 wk and increases

gradually through 56 wk before falling somewhat then increasing dramatically at 75 to 80 wk of age, near the average age of puberty (Clay and Clay, 1992). In boars sampled prior to castration at 40, 70, 100, 130, 160, 190, 220 or 250 d of age, however, there were no differences in average LH as a function of age while T increased linearly with age (Allrich et al., 1982) an observation similar to what was observed in the llamas in Study A.

The increase in basal LH concentrations in rams is associated with more frequent LH pulses and T pulses begin to follow LH pulses with a clear association between the two hormones as the animal approaches puberty (Foster et al, 1978; Wilson and Lapwood, 1979a). In the llamas in Study A, T pulses were not clearly associated with LH pulses. There was likewise no increase in basal LH observed, but it is likely that any increase had occurred prior to initiation of the study at 8 mo of age. Testosterone, however, did show an overall increase with age. In a study by Bravo and others in 1992, male llamas were shown to have slowly increasing T to about 21 mo of age, at which time concentrations increased dramatically to plateau at about 30 mo. Unpublished results from our laboratory indicate that T concentrations tend to be quite variable among male llamas and older animals tend to have greater average T.

Response to i.v. administration of GnRH at 1 μg/kg produced little release of either LH or T in the prepubertal llamas although it did produce an increase in T in the adult llamas in Study B. In other work in our laboratory (some of which is presented in Chapter VI), it appears that response of LH or T to GnRH in male llamas is quite variable between animals and tends to be minimal at any age, although GnRH does produce an ovulatory release of LH with ovulation in the female llama (Chapter III).

There was some trend for the llamas in Study A to release more T at later sampling periods and the difference in T in the 6 h before and after GnRH was significantly greater at 20 mo of age ($P < 0.05$) compared to other months. In ram lambs, highest response of LH to GnRH was achieved at 6 wk and response decreased in magnitude and was delayed at later ages (Wilson and Lapwood, 1979b). In contrast, T response to GnRH increased with age in these lambs. Lee and others in 1976(b) found that ram lambs were responsive to GnRH at birth but sensitivity was heightened at 2 to 3 mo of age. In 2, 4 and 6 mo old bulls, all responded to GnRH with increased LH by 45 min following GnRH administration with a return to basal concentrations by 6 h but T response was apparent only in 6 mo old bulls with peak increases at about 2 h and return to basal levels by 6 h (Mongkonpunya et al., 1975).

The decrease in T at 14 mo of age is difficult to explain. Although the decrease was not statistically significant when compared to other time periods it consistently occurred in all 5 animals. While a seasonal effect was considered because the low T concentrations occurred during December, in a subsequent study (Study B) we found no effect of season on T or on testicular dimensions in adult male llamas. The possibility exists that there is an effect of season in peripubertal llamas. It has been noted that goat kids and ram lambs born in autumn experience a delay in puberty compared to spring-born kids (Skinner and Rowson, 1968; Deveson et al., 1992). Both of these species also exhibit seasonal changes in reproductive parameters as adults, such as increased testicular size and increased sperm production, changes which have not been consistently observed in domesticated SAC. In cattle, a non-seasonal breeding species, increased light has been shown to stimulate a transient increase in T but not LH

(Stanisiewski et al., 1987). It is also possible that this change is a normal part of sexual maturation in llamas, as similar transitory decreases in T after initial slow increases have been reported in red deer stags (Suttie et al., 1991), stallions (Clay and Clay, 1992) and bulls (Amann, 1983). In the studies in stags and stallions, the period of T decrease were followed by rapid and more dramatic increases. The data mentioned earlier from a study by Bravo and others in 1992, wherein young male llamas experienced a dramatic increase in T from 21 to 30 mo of age, suggests the possibility that the llamas in our study may have been beginning this type of increase when the study ended at 20 mo of age.

Whether any camelid is a true photoperiod-induced seasonal breeder is debatable. Although both dromedaries and Bactrian camels have been considered to be true seasonal breeders, evidence is somewhat lacking. Some investigators report seasonal variations in reproductive hormones for the dromedary (Bono et al., 1989) with increasing androgens in the rainy season. This change seems more closely correlated with rainfall itself than with photoperiodic cues since androgen concentrations rose with both spring and fall rainy seasons in Somalia, as did corticoids. Male camels do, however, undergo a physiological and behavioral rut with a corresponding period of cyclic receptivity in the female, whereas SAC do not (Novoa, 1970; Yagil, 1985). It has also been noted that dromedaries on adequate planes of nutrition are capable of breeding year round (Merkt et al., 1987). In contrast, camels in European zoos retained a seasonal reproductive pattern, including Bactrian camels, which breed year-round in Russia (Gauthier-Pilters and Dagg, 1981). Many places in which camels live are near-

equatorial, with minimal changes in photoperiod, reducing the possibility that photoperiodic cues are responsible for seasonal breeding.

The llama has widely been held to be a non-seasonal breeder in North America, although it was once considered to be purely seasonal in South America and one reference states that the seasonal breeding was reversed in the northern hemisphere (Jessup and Lance, 1982). It is widely known that mating and births can occur throughout the year in the United States. Study B supports the assumption that llamas are non-seasonal breeders in the United States. Season may, however, play some role on fertility of llamas, particularly in certain individuals, as there was a tendency towards increased basal T secretion in summer and decreased secretion and response to GnRH in winter. Although the overall differences we observed were not significant, results of the study indicate that individual animals may exhibit greater tendencies towards lowered T (Figure 12) and response to GnRH (Figure 14) in the winter, which may be associated with decreased fertility and(or) libido. Most SAC in North America are bred for spring or fall progeny to avoid heat stress on breeding animals and cria in the summer and the stress of cold, damp weather on the cria in the winter. These practices would fit well with the trends observed for decreased T in the winter.

Semen was not evaluated in Study B due to difficulties in reproducibly electroejaculating llamas. Testicular size (an indirect indicator of sperm production) did not, however, change over the course of the study. In true seasonal breeders, such as rams (Sanford et al., 1983; Lincoln, 1984) and stallions (Clay et al., 1987) testicular size decreases during the non-breeding season. This has also been observed in OWC (Merkt et al., 1987).

In the peripubertal llamas in Study A, testicular size increased as a linear function of increasing age. Similar observations have been made in the bull (Amann, 1983), ram (Mukasa-Mugerwa and Ezaz, 1992) and stallion (Clay and Clay, 1992). In species other than the llama, growth has been shown to be an important factor in attainment of puberty. Puberty in both males and females of many species usually occurs when a certain percentage of adult body weight is attained. Thus, breed types in domestic animals and nutrition play important roles in attainment of body size necessary for the events of puberty to take place (Mukasa-Mugerwa and Ezaz, 1992). In Study A, all llamas were of a similar genetic type, minimizing any "breed" effect and nutrition was constant for these animals.

Low volumes of ejaculate were obtained from these young llamas, due in part to presence of glans penis to preputial adhesions which made collections difficult and quality of samples poor. Electroejaculation rarely produces quality samples even in adult llamas, perhaps because of the nature of "dribble" ejaculation and low volume of concentrated semen under natural mating conditions. Seminal characteristics did appear to improve with age in the young llamas, as has been previously noted in rams (Alexopoulos et al., 1991), stallions (Clay and Clay, 1992) and bulls (Almquist and Amann, 1976). Although spermatozoa characteristics were not evaluated in the llamas, there appeared to be more live cells with greater motility and fewer abnormalities with successive ejaculations.

One feature llamas share with other species with a fibroelastic type of penis is the presence of adhesions of the glans penis to the prepuce. In contrast to the bull and ram, however, these adhesions persist for a relatively long period of postnatal life

(Bravo et al., 1992). In this study, 3 of 5 llamas still had extensive preputial adhesions at 20 mo of age, even though all animals had spermatozoa in their ejaculate by this age. In contrast, detachment of the penis from the prepuce occurs at least 1 to 2 weeks prior to appearance of spermatozoa in the ejaculate in the bull (Almquist and Amann, 1976). Breakdown of these adhesions has been associated with spermatogenesis (Dun, 1955) and growth rate (Skinner and Rowson, 1968) in rams and occurs with or shortly after the time of rising testosterone in rams and bulls. There is probably a physical component of breakdown of these adhesions associated with increased sexual experience, which may come in the way of attempted mounts and(or) masturbation. In Study A, the electroejaculation procedure itself may have contributed to breakdown of adhesions. Since these adhesions often persist beyond 20 mo of age in llamas, this would appear to be the limiting factor to the use of young male llamas for breeding.

V.6. References

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CHAPTER VI. REPRODUCTIVE HORMONES IN THE ADULT MALE LLAMA: LUTEINIZING HORMONE AND TESTOSTERONE PRECEDING AND FOLLOWING CASTRATION.

VI.1. Abstract.

Three studies were performed in adult male llamas examining luteinizing hormone (LH) and testosterone (T) dynamics preceding and following castration. Prior to castration, LH and T were examined for a 24 h basal period to determine the relationship between secretion of these two hormones, then the response of both hormones to GnRH was determined. Secretion of LH and response to GnRH were compared prior to and following castration and T decline immediately following castration was examined.

VI.1.1. **Study A.**

Four intact adult male llamas were bled via jugular catheter at 15 min intervals for 30 h with gonadotropin releasing hormone (GnRH, 1 μg/kg BW, i.v.) administered 24 h after initiation of sample collection. Approximately 1 mo following initial sample collection, animals were castrated under xylazine sedation (0.66 mg/kg BW, i.v.) and sampling was repeated 1 mo post-castration with the same protocol as in intact animals. Concentrations of LH were analyzed in all samples while T was analyzed at 30 min intervals in pre-castration samples only. Both hormones were secreted in a pulsatile manner pre-castration although T pulses did not always clearly follow LH pulses. Mean 24 h LH increased significantly ($P < 0.05$) from 0.7 ± 0.1 (mean ± SEM) ng/ml pre-

castration to 1.2 ± 0.1 (mean ± SEM) ng/ml 1 mo post-castration. Response of LH or T to GnRH administration was minimal and there was no significant difference ($P > 0.05$) in response of LH to GnRH stimulation before or after castration.

VI.1.2. **Study B.**

Blood samples were taken via catheter at hourly intervals for T from 4 adult male llamas for 12 h after each of 4 i.v. doses of GnRH: 0.0, 0.1, 1.0 and 5.0 μg/kg BW. Although mean T concentrations for the 12 h following 0.1, 1.0 and 5.0 μg GnRH/kg BW doses increased significantly over the 0.0 μg GnRH/kg BW control dose (8.9 ± 1.1 ng/ml, 8.8 ± 1.2 ng/ml and 8.2 ± 0.9 ng/ml compared to 6.6 ± 0.9 ng/ml, mean ± SEM), there were no significant differences between doses and individual animal response was highly variable.

VI.1.3. **Study C.**

Four adult male llamas were bled via jugular catheter at 15 min intervals for 3 h prior to castration under xylazine sedation (0.44 mg/kg BW, i.v.), then at 5 min intervals for 1 h post-castration, then at 15 min intervals for an additional 2 h. Testosterone in samples decreased rapidly following castration, with a 2-phase clearance pattern. Half-lives for the first and second phases were 0.23 ± 0.02 (mean ± SEM) h and 1.65 ± 0.29 (mean ± SEM) h respectively. Testosterone was virtually undetectable in all animals by 3 h post-castration.

VI.2. Introduction.

Although llamas, native to South America, have been popular animals in North America for the last 10 to 15 years, it is only recently that many aspects of their anatomy and physiology are becoming understood. Reproduction in the male llama is still poorly understood; partly because males are usually available in greater numbers than females and less attention has focused on male reproduction. Occasionally an owner desires an infertility workup for an outstanding male and normal concentrtations of LH and T need to be established for purposes of comparison. Normal concentrations of LH and T and the response of these hormones to GnRH have not been reported in adult male llamas, although some information is available for the phylogenetically related Old World camels. A series of studies was designed to evaluate secretion patterns of LH and T over a 24 h period and response of LH and T to GnRH stimulation in male llamas, secretion of LH 1 mo after castration and clearance of T immediately following castration in llamas.

VI.3. Materials and Methods.

VI.3.1. **Animals.**

VI.3.1.1. ***Study A.***

Four adult male llamas from the OSU research herd ranging in age from 4 to 15 yr (average age about 10 yr, exact birthdates unknown for some animals) and averaging

125.9 ± 5.1 kg (mean ± SEM) BW were used. Animals were housed in stalls in the OSU research facility for 1 d prior to and throughout each of the two sampling periods. Food pellets and water were available *ad lib* throughout each sampling period. The llamas were maintained on pasture with free-choice access to water and mineral mix for approximately one mo after the first sampling period, then castrated after xylazine (0.44 mg/kg BW, i.v.) sedation and kept on pasture for an additional mo following castration before the second sampling period occurred.

VI.3.1.2. ***Study B.***

Four adult males (three of which were utilized in Study A) ranging in age from 4 to 15 yr (average 10 yr) and averaging 129.5 ± 8.9 (mean ± SEM) kg BW were used. Animals were housed indoors at the research facility for the study period. Food and water were provided *ad lib*.

VI.3.1.3. ***Study C.***

Four different adult male llamas, averaging 4.5 yr in age (range 3.9 to 4.8 years) and 125.5 ± 21.0 (mean ± SEM) kg BW were used for Study C. Animals were brought in from pasture the day prior to initiation of sampling and tethered in an outdoor enclosure throughout the sampling period. No food or water was available throughout this period. Animals were castrated using xylazine sedation (0.44 mg/kg i.v.) during the sampling period. Xylazine was given via the i.v. catheter and animals were recumbent within 1 to 4 minutes. Castration was accomplished using the method described by

Dargatz and Johnson (1987) and required a total of 5 to 8 minutes for each animal. Animals remained recumbent for 30 to 75 minutes following xylazine administration.

VI.3.2. **Sampling.**

On the morning of initiation of each blood sampling period in Study A and on the day prior to the blood sampling periods in Studies B and C, animals were fitted with indwelling jugular venous catheters as described in Appendix I. Sampling was initiated at 1200 h for each sampling period in Study A and samples were taken at 15 min intervals for 30 h. At 24 h after the beginning of each sampling period, 1 μg GnRH/kg BW was administered via the catheter immediately after the 24 h sample was taken. Samples were collected and handled using the same procedures as described in previous chapters. In Study B, samples were taken at hourly intervals for 12 h after each of the following sequential i.v. doses of GnRH were given: 0.0 μg/kg BW, 0.1 μg/kg BW, 1.0 μg/kg BW, and 5.0 μg/kg BW. For Study C, sampling procedures for individual animals were started at 30 min intervals to allow time for castration and close interval sampling immediately following castration. Samples were taken at 15 min intervals for 3 h, then 0.44 mg/kg xylazine was administered to each animal via the catheter immediately after the time 0 sample was taken and the catheter was then flushed with heparinized saline. As soon as each animal was recumbent, the castration procedure was performed. Samples were taken at 5 min intervals for 1 h following xylazine administration, then at 15 m intervals for 2 additional h.

VI.3.3. **Hormone analysis.**

All samples for Study A were analyzed for LH by RIA as described in Chapter III. Intra- and inter-assay coefficients of variation for a llama plasma pool were 6.4 and 17.9%, respectively. Half hourly samples in the Study A pre-castration sampling period and all samples for Studies B and C were analyzed for T by RIA as described in chapter V. Intra- and inter-assay coefficients of variation for a llama plasma pool were 7.9 and 10.5%, respectively for Studies A and B and 5.1 and 9.3% for Study C.

VI.3.4. **Statistical analysis.**

Pre- and post-castration 24 h mean LH for Study A were compared by one-way ANOVA using the LSD procedure on Statgraphics for the IBM PC. Similarly, 12 h mean T concentrations following each dose of GnRH in Study B were compared. In Study A, the differences between mean LH in the 6 h preceding GnRH and the 6 h following GnRH administration were compared between pre- and post-castration periods by the same Statgraphics procedure to determine if there was a difference in response to GnRH before and after castration. In the precastration samples for LH and T, and the postcastration samples for LH, the 6 h means before GnRH were compared to the 6 h means following GnRH in a similar fashion to determine response to GnRH. In Study C, T profiles were fitted and stripped by the using the RSTRIP program for pharmacological data on the IBM PC as described in Chapter IV. Decline of T after castration followed a 2-exponential curve, which was selected for statistical output parameters such as goodness of fit and half lives of phases.

VI.4. Results.

VI.4.1. **Study A.**

Individual temporal changes in LH and T concentrations in intact adult male llamas over a 30 h period are shown in Figure 15. At 24 h, GnRH (1 μg/kg BW) was administered as an i.v. bolus. Although secretion of both hormones was pulsatile in the 24 h basal period, the LH and T pulses were not clearly related to one another. Mean T concentrations over the 24 h period before GnRH were more variable between animals than was mean LH. Overall 24 h means and SEM were 7.2 ± 0.4 ng/ml for T and 0.7 ± 0.1 ng/ml for LH while individual animals' mean 24 h T were 8.5 ± 0.7, 2.3 ± 0.4, 12.6 ± 0.6 and 5.2 ± 0.3 ng/ml and 24 h mean LH were 0.7 ± 0.1, 0.7 ± 0.1, 0.8 ± 0.1 and 0.8 ± 0.1 ng/ml for llamas #164, #175, #177 and #184, respectively. Animals #164 and #177 were the oldest animals on the study and were both > 10 years of age (exact birthdates unknown). Animal #175 had what appeared to be very low T with little pulsatile release.

The change in LH and T concentrations following GnRH administration was minimal when evaluated either as an absolute change or a relative increase when the mean concentration for each animal during the basal period (0 to 24 h) was set equal to 100% (Figure 16). While both LH and T increased, LH from an average of 0.8 ± 0.1 (mean ± SEM) ng/ml in the 6 h immediately preceding GnRH administration to 1.3 ± 0.1 (mean ± SEM) ng/ml in the 6 h following GnRH and T from 7.4 ± 0.7 (mean ±

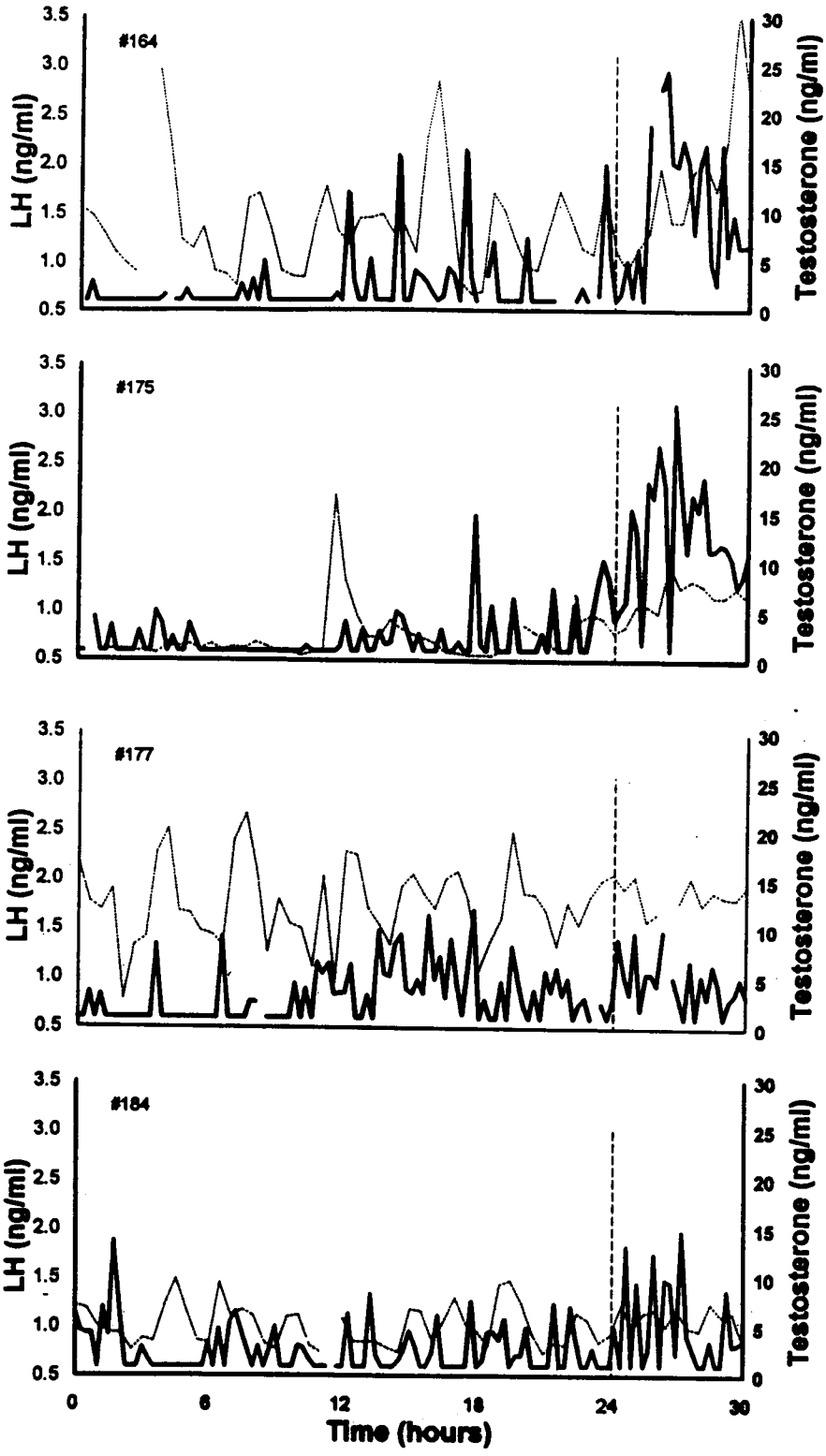

Figure 15. Changes in individual T (dashed line) and LH (solid line) concentrations as a function of time for four llamas 1 mo prior to castration. GnRH (1μg/kg BW) was administered as an i.v. bolus at 24 h (vertical lines).

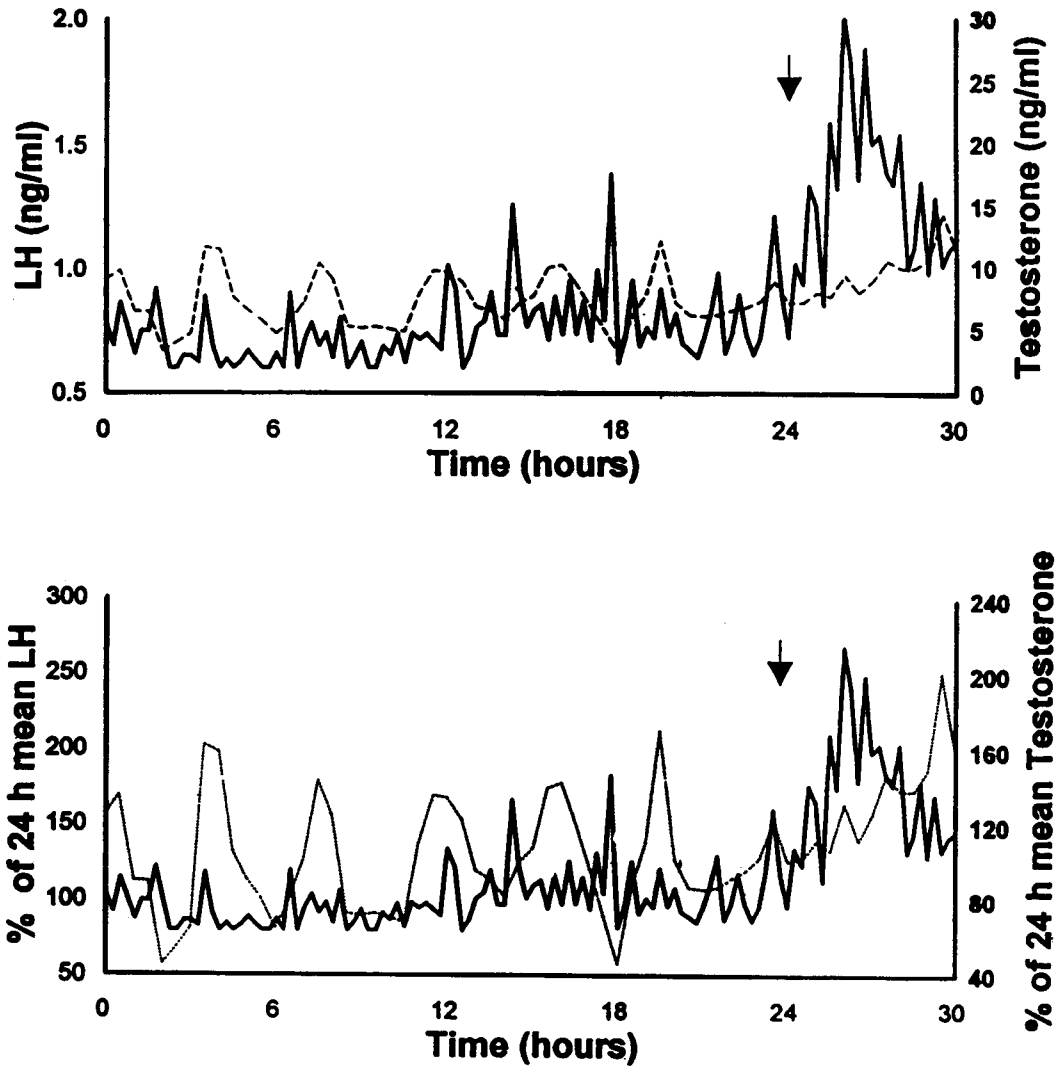

Figure 16. Mean T (dashed line) and LH (solid line) concentrations as a function of time 1 mo prior to castration (n = 4) (upper panel). Mean T (dashed line) and LH (solid line) concentrations presented as a % of the individual 24 h mean T and LH concentrations (lower panel). GnRH (1 μg/kg BW, i.v.) was administered at 24 h (vertical line).

SEM) ng/ml to 9.8 ± 0.8 (mean ± SEM) ng/ml in the same time periods as Figure 15 indicates, the response was quite variable between animals.

One month following castration, 24 h mean LH was increased significantly ($P < 0.05$) compared to pre-castration levels (overall mean and SEM of 1.2 ± 0.1 ng/ml post-castration vs. 0.7 ± 0.1 ng/ml precastration, Figure 17). Difference of mean LH concentration in the 6 h following GnRH administration compared to mean LH in the 6 h preceding GnRH administration was significant at the $P < 0.05$ level precastration (0.5 ± 0.2 ng/ml, mean ± SEM), although there was no detectable response to GnRH administration post-castration. When responses to GnRH stimulation pre- and post-castration were compared, the difference was not significant at the 0.05 level. Figure 17 depicts individual animal response to GnRH, while Figure 18 shows mean LH and % change for LH in all 4 animals.

VI.4.2. **Study B.**

In comparison to the 12 h pretreatment period, mean T concentrations increased during the 12 h following each treatment (Figure 19). The largest increase occurred in the first 6 h following each dose (Figure 20). Average T for the first 12 h of sampling (pretreatment control period, 0.0 μg GnRH/kg BW) was 6.6 ± 0.9 ng/ml (mean ± SEM) and increased to 8.9 ± 1.1 (mean ± SEM) ng/ml in the 12 h after 0.1 μg GnRH/kg BW. For the 12 h periods following 1.0 and 5.0 μg/kg BW doses of GnRH, average T concentrations were 8.8 ± 1.2 (mean ± SEM) ng/ml and 8.2 ± 0.9 (mean ± SEM) ng/ml, respectively. Although there appeared to be more T released over each time period

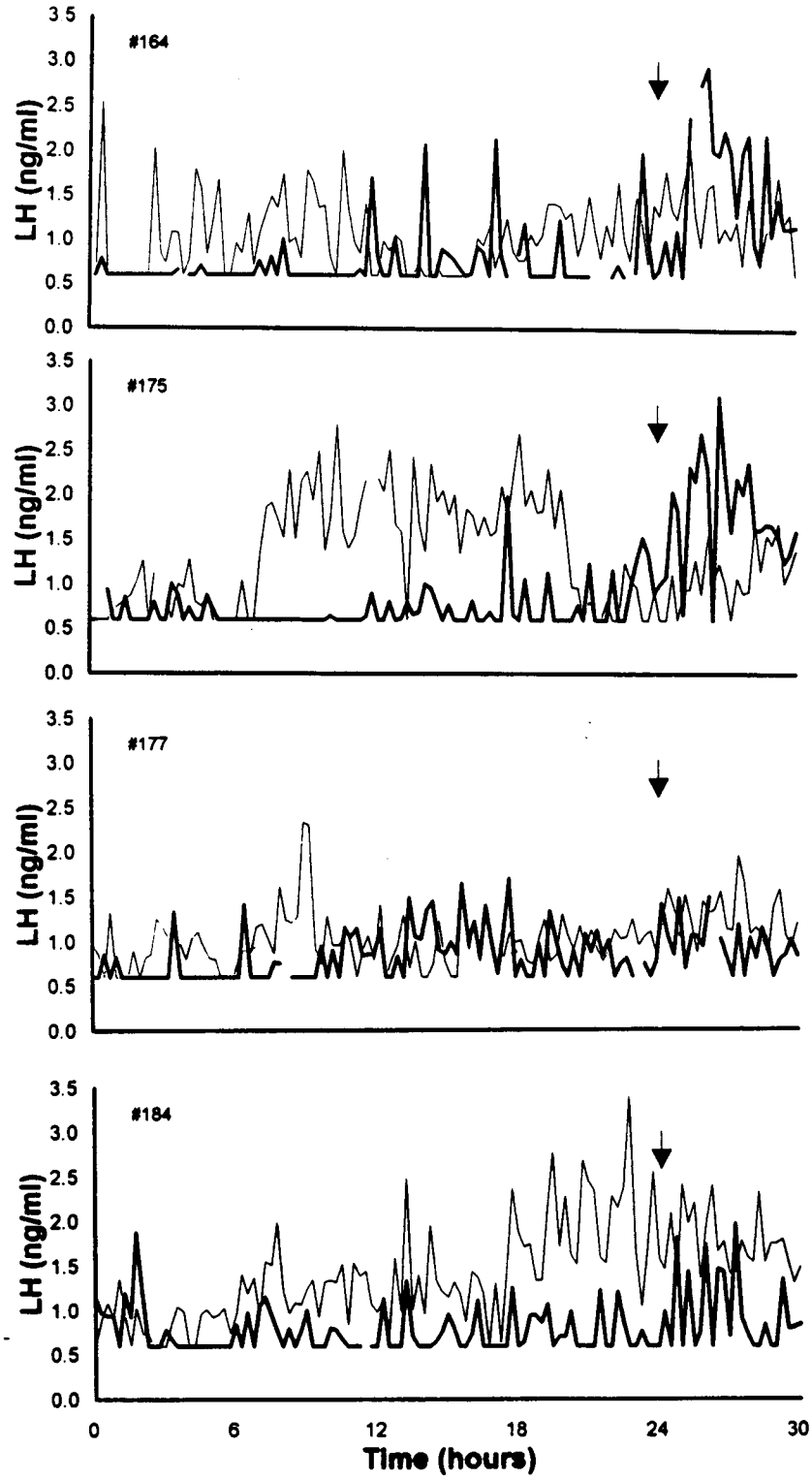

Figure 17. Comparison of individual LH concentrations in four animals 1 mo prior to (heavy line) and 1 mo following (light line) castration in Study A. GnRH (1 μg/kg BW, iv) was administered at 24 h (vertical line).

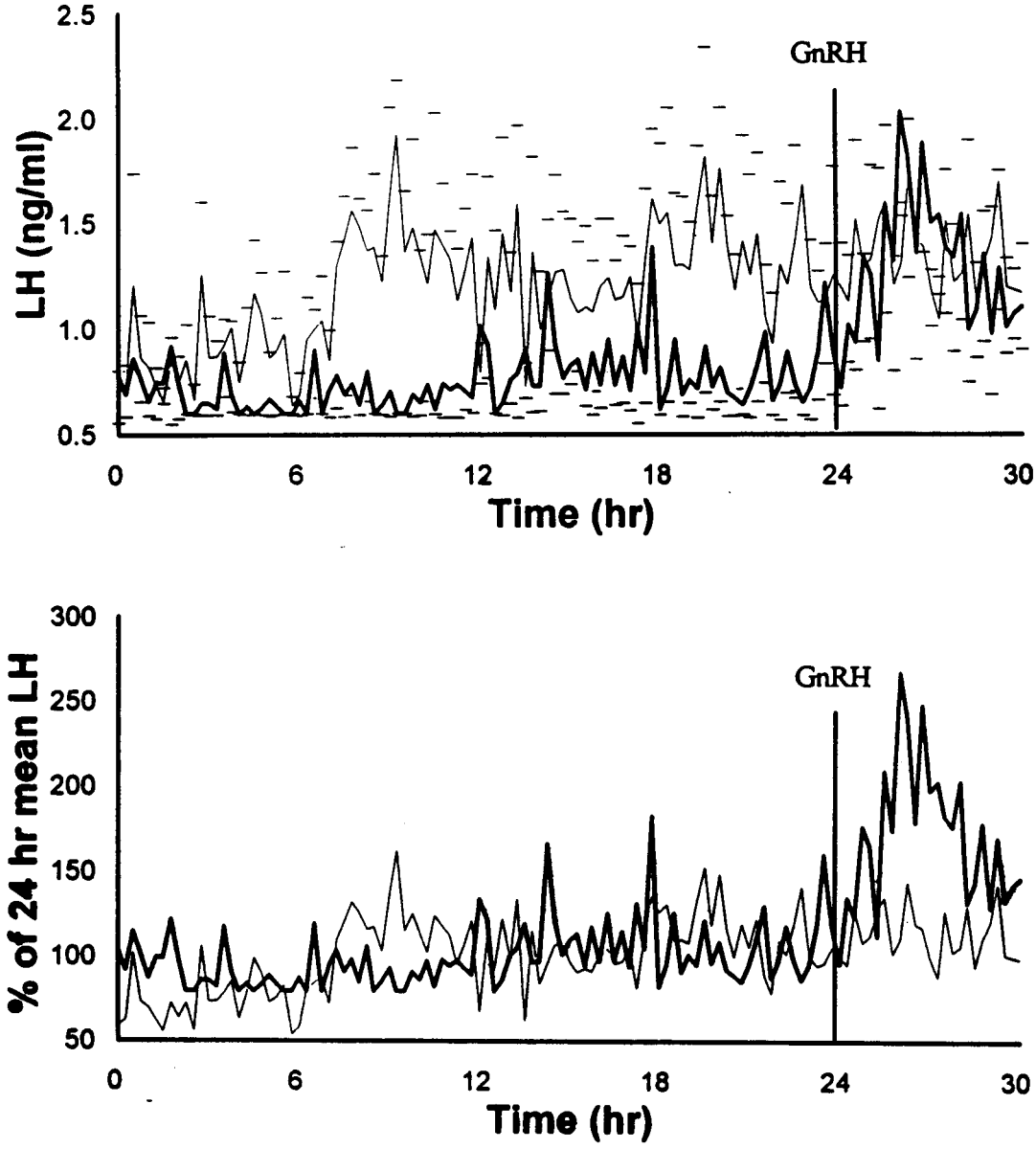

Figure 18. Mean LH concentrations for llamas (n = 4) in Study A for the 30 h sampling periods 1 mo prior to (heavy lines) and 1 mo following (light lines) castration, expressed as raw data (top panel) and as a % of 24 h mean values (bottom panel). GnRH (1 μg/kg BW, i.v.) was administered at 24 h (vertical lines).

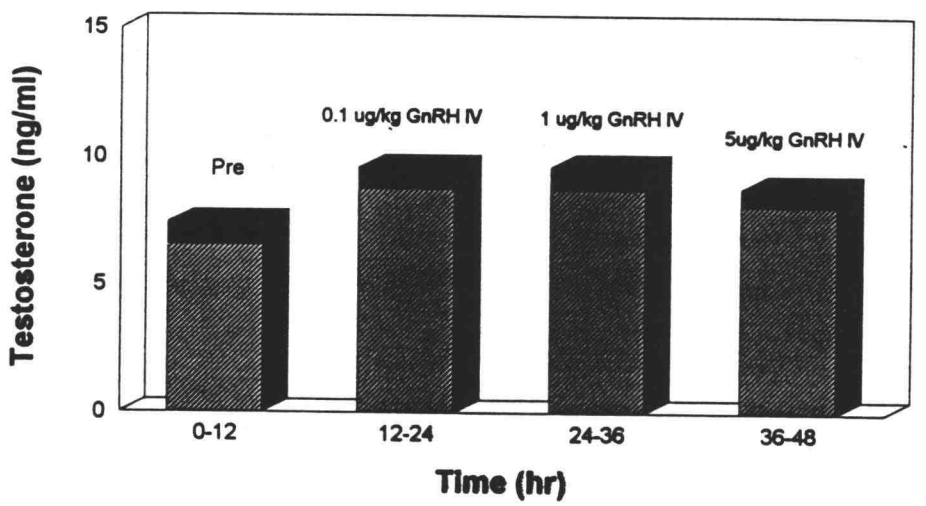

Figure 19. Mean T (+ SEM) concentrations for male llamas (n = 4) for the 12 h intervals following each dosage of GnRH.

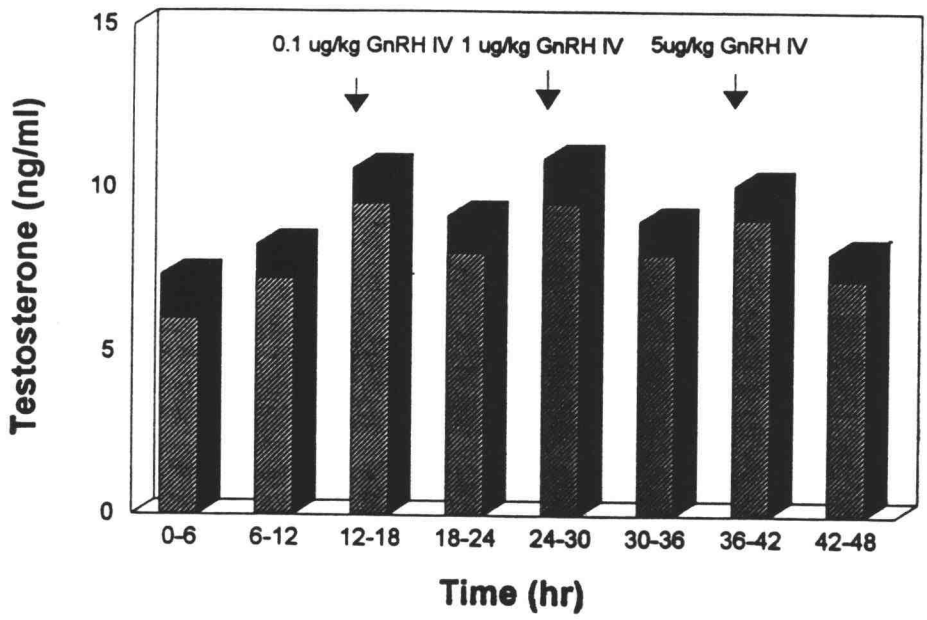

Figure 20. Mean T (+ SEM) concentrations for male llamas (n = 4) for 6 h periods in Study B with arrows denoting administration of each dosage of GnRH.

following the 3 doses of GnRH compared to the control time period, there was no increased release of T with increased dose of GnRH. Individual animal T patterns were highly variable (Figure 21).

VI.4.3. **Study C.**

Following castration, detectable T quickly disappeared from circulation (Figure 22). As determined by the RSTRIP program, the disappearance most closely approximated a 2-phase clearance curve. When each set of data was fitted to a 2-phase clearance curve, half-lives of each phase were found to average 0.23 ± 0.02 h (mean ± SEM) and 1.65 ± 0.29 (mean ± SEM) h. Figure 22 shows the averages for each sample throughout the sampling period, while Figure 23 shows a representative fitted clearance curve (llama #225).

VI.5. Discussion.

In the study discussed in Chapter V, season had no effect on T or testicular measurements in adult male llamas. The studies in Chapter VI were performed on all animals in the same calendar month for each study and all studies occurred within 4 calendar months. It was therefore expected that season would play little or no role in results noted.

Although the secretion of LH and T in the adult male llama were pulsatile in nature, the pulse patterns were not consistently synchronized with some LH pulses not followed by T pulses, nor were all T pulses preceded by an LH pulse (Figure 15).

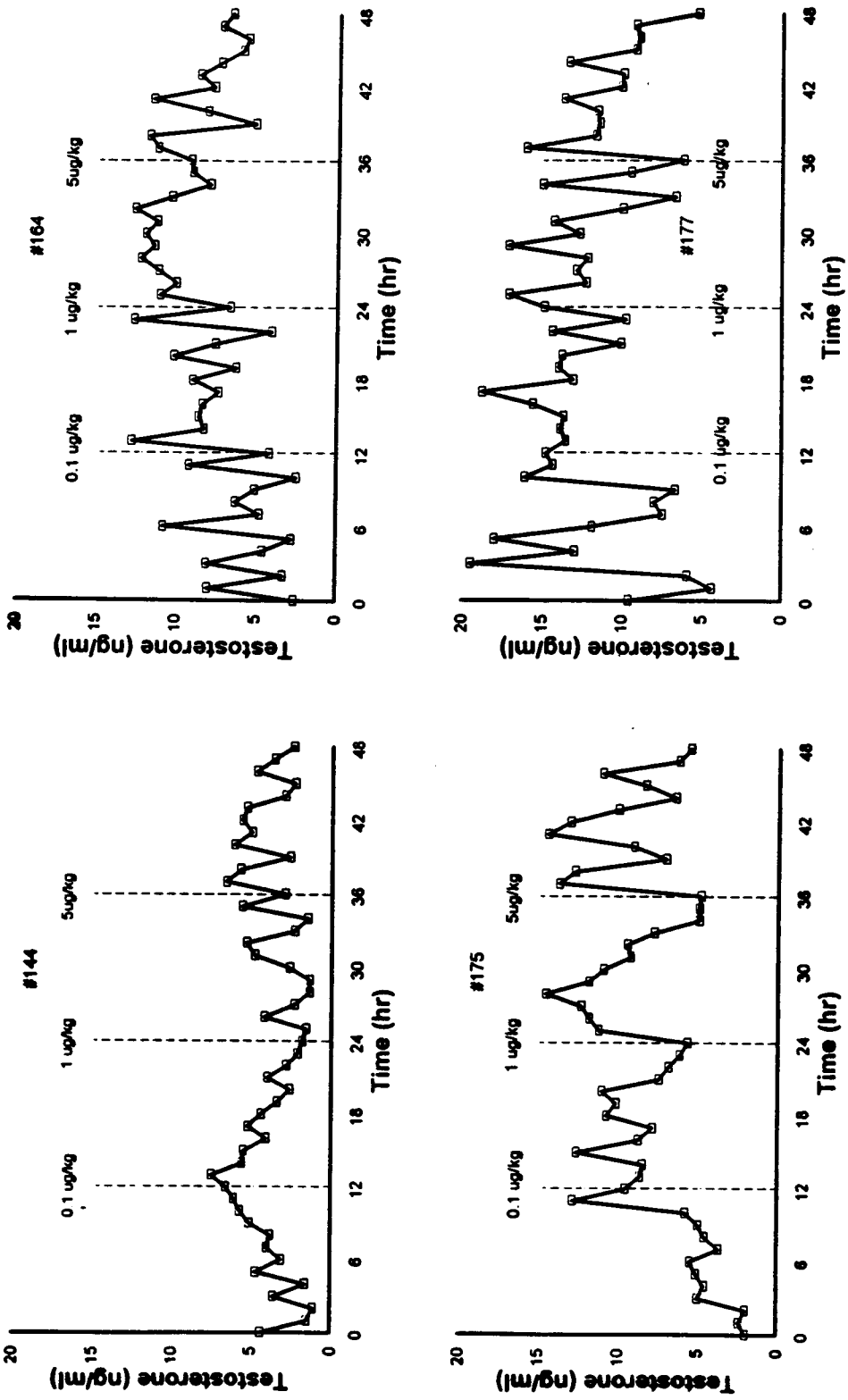

Figure 21. Change in individual T concentrations for each dosage of GnRH in Study B.

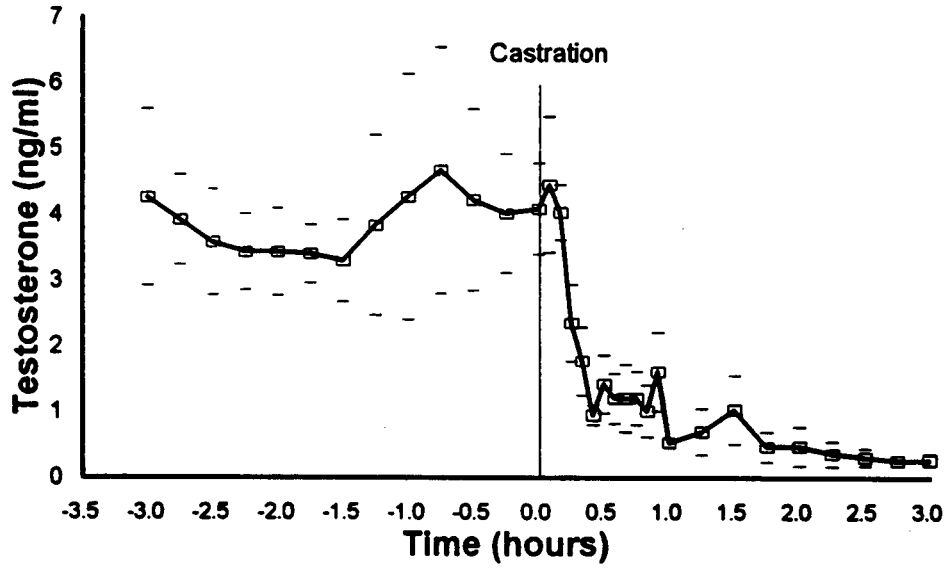

Figure 22. Mean serum T concentrations preceding and following castration (n = 4).

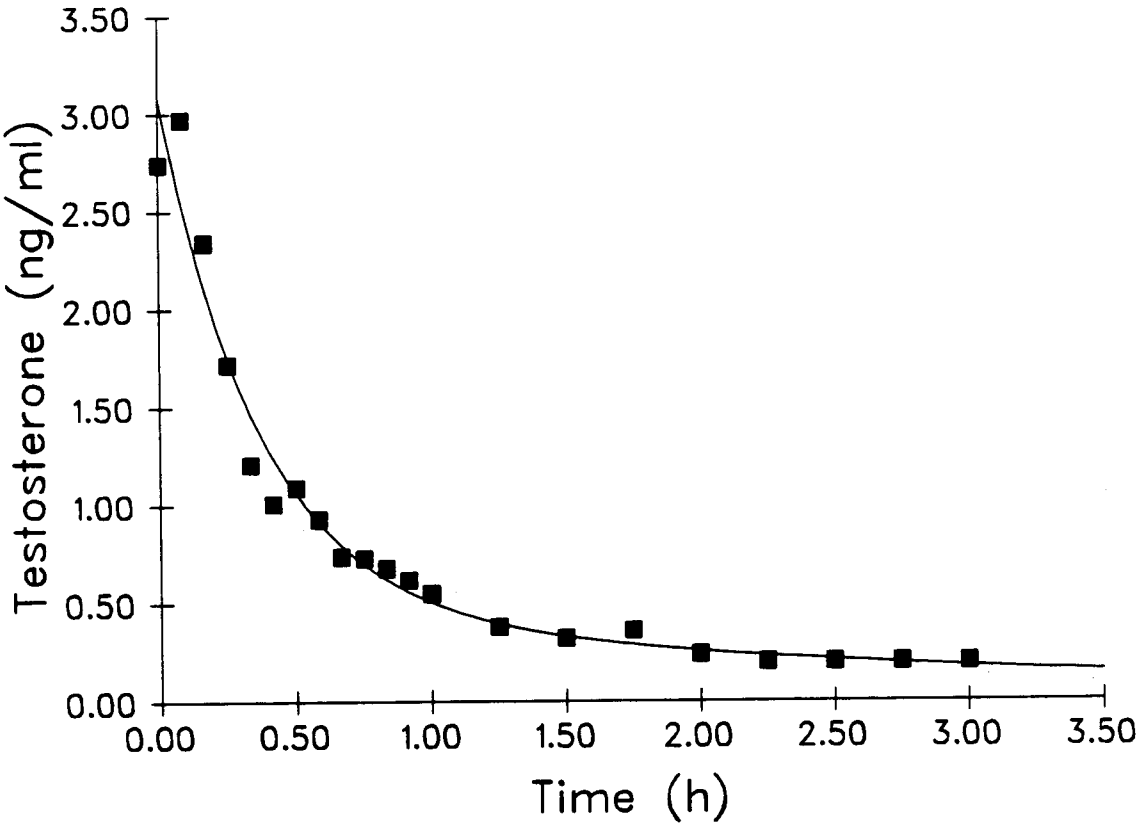

Figure 23. Fitted T clearance curve (RSTRIP) for a representative llama (#225) following castration.

While there is a clear association between LH and T pulse release in the ram (Schanbacher and Ford, 1976) and bull (Katangole et al., 1971), a lack of clear association between pulses of these two hormones similar to that observed in the llamas has been demonstrated in the in the boar (Liptrap et al., 1986) and stallion (Clay and Clay, 1992).

Average T concentrations varied widely between animals. This and other unpublished work from our laboratory suggests that older llamas have greater average T compared to young and middle-aged llamas. Although one previous study in our laboratory indicated that puberty (as defined by presence of normal spermatazoa in the ejaculate) occurs by 20 mo of age in the male llama and other sources have reported puberty at 2 to 3 years in llamas (Johnson, 1989), basal T appears to continue to increase at least to mature body weight at 4 to 5 years of age and perhaps even longer. Similar changes have been described in bulls (Amann, 1983).

Basal LH concentrations in the llama are relatively low compared to other species. The overall 24 h mean LH concentration in Study A of 0.7 ± 0.1 ng/ml is much lower than mean LH concentrations reported in species such as stallions (varying from approximately 20.7 ng/ml in November to approximately 96.2 ng/ml in June, Thompson et al., 1977), dogs (6.0 ± 5.2 ng/ml, Olson et al., 1992) and bulls (3.3 ± 1.8 ng/ml, McCarthy et al., 1979) and about half that observed in rams (varying from 1.81 ± 0.18 ng/ml in the nonbreeding season to 2.46 ± 0.36 ng/ml in the breeding season, Schanbacher and Ford, 1976). LH concentrations observed in the llamas were, however, only slightly lower than what has been reported for boars (0.83 to 1.25 ng/ml, Allrich et al., 1982). Camels also appear to have low LH concentrations, ranging from

0.5 ± 0.3 ng/ml in the nonbreeding season to 1.1 ng/ml during the rut (Bono et al., 1989).

The post-castration rise in LH due to removal of testicular steroid inhibition on the pituitary has been well documented in other species and was observed in llamas as well. In the ram, LH concentrations increased approximately threefold during the first week following castration with an increase in pulse frequency and amplitude compared to intact rams (Schanbacher et al., 1983). In bull calves, LH has been reported to double within hours of castration (McCarthy and Swanson, 1976) and pubertally castrated bulls had LH concentrations of 9.2 ± 3.1 ng/ml compared to concentrations of 3.3 ± 1.8 ng/ml in intact bulls (McCarthy et al., 1979).

While LH concentrations did rise significantly following castration in the llama, the increase was relatively small, averaging less than a 2-fold increase. The post-castration increase in LH concentrations appears to vary widely between species and sometimes within species. For example, neutered male dogs showed an approximate doubling of LH compared to intact males (Olson et al., 1992) while male rats gonadectomized as adults had an increase in LH concentrations within 8 h of castration, eventually reaching as much as 50 times that of precastration concentrations (Gay and Midgley, 1969). A study in boars, in which they were castrated at different ages, showed a slight initial increase in LH concentrations which then declined and remained at precastration concentrations through d 16 post-castration (Allrich et al., 1982). Another study in boars castrated postpubertally showed a doubling of the area under the curve of total LH production for the period sampled along with an increased number of LH pulses when compared to intact boars (Liptrap et al., 1986). Hoffman and others

(1987) reported that long-term geldings had mean LH concentrations within the range of those reported for intact stallions.

Response patterns of LH and T to GnRH stimulation remain unclear in the llama. While llamas in Study A did appear to respond as a whole, the response was less than that observed for other species. For example, in sheep increases in LH concentrations after comparable GnRH dosages are tenfold or greater than basal concentrations (D'Occhio et al., 1982) while in bulls, 24 to 30 fold increases in LH and three- to seven-fold increases in T have been reported following injection of comparable or lower dosages of GnRH (Mongkonpunya et al., 1975; Schanbacher and Echternkamp, 1978). In the boar, mean LH concentrations had approximately doubled by 40 min following GnRH administration (Kittock et al., 1989). In the stallion, the response of both LH and T to GnRH administration was influenced by season as well as dosage. Mean LH and T concentrations doubled following GnRH administration at the height of the breeding season but showed little or no change following the same dosage of GnRH administered during the nonbreeding season (Clay et al., 1989).

Removal of gonadal steroid inhibition on the pituitary by castration usually leads to increased response of LH to GnRH in castrate animals compared to the response in intact animals. This has been reported in rams (D'Occhio et al., 1982), boars (Kittock et al., 1989) and bulls (McCarthy and Swanson, 1976). This was not apparent in the castrated llama, with no significant change in average LH concentrations in the 6 h following GnRH administration compared to the 6 h immediately preceding GnRH administration. These results suggest that the steroid inhibition of LH secretion in the llama may not be as great as that in other species, resulting in smaller increases in basal

LH and response to GnRH in castrate llamas compared to other species. Another possibilty is that the relatively small but significant increase in basal LH concentrations that does occur in llamas following castration obscures the LH response to GnRH, since response to GnRH in llamas is inherently low. The time course of sampling following GnRH administration in Study A was based on observations in the bull (Mongkonpunya et al., 1975; Schanbacher and Echternkamp, 1978) in which T rose by 2 to 4 h after GnRH administration and decreased to basal levels by 5 to 7 h. The time course of the LH rise and decline after GnRH is usually one to two h more rapid than that for T. Changes in Study B in which samples were taken for longer periods of time following GnRH administration compared to Study A and higher and lower doses of GnRH in Study B failed to show differences in response of T to GnRH from the dosage and sampling period used in Study A. It appears, therefore, that llamas do not have a delayed response to GnRH administration compared to that observed in other species. Furthermore, the dosage of GnRH used in Study A was apparently not so great it overwhelmed any physiological response or so little that response was not complete. The dosage of GnRH used in Study A was the same used in the study in Chapter III, in which female llamas ovulated in response to this dosage. This dosage is somewhat higher than what has been suggested for the llama (50 μg, Johnson, 1989).

Another possible explanation for the lack of response to GnRH in the male llama is that endogenous GnRH is somehow different in biochemical form or action in the llama or in the male compared to the female. This explanation is unlikely, however, since essentially all mammalian species have identical circulating GnRH and sexual dimorphism of GnRH is unknown to occur (Beattie, 1982). While it is theoretically

possible that camelids have a different type of releasing hormone or that regulation of the hypothalamo-pituitary-testicular axis is fundamentally different than in other species, it seems more likely that camelids require little LH for basal T secretion and the increases observed following injection of GnRH are physiological for this species. In another study by this laboratory (Chapter III) as well as unpublished work, female llamas did ovulate after a similar dose of GnRH (1 μg/kg i.v.) and had a release of LH similar (except for a shorter time to peak) to that accompanying ovulation induced by either vasectomized or fertile mating, although all LH releases were smaller than ovulatory surges observed in other species.

The decline of T following castration most closely approximated a 2-phase pharmacological clearance curve, in which the first phase typically represents disappearance of circulating hormone (central compartment) while the second phase represents repartitioning into peripheral tissue compartments in which hormone may be stored and/or bound and is more slowly released. In the case of T, most circulating hormone in the llama is probably bound to serum proteins, primarily albumin and testosterone-binding globulin and the second phase of clearance may partially represent dissociation from these proteins and elimination. Testosterone decline following castration showed a somewhat different time course than has been noted in bulls (Haynes et al, 1976) and man (Horton et al, 1965) wherein first and second phase half-lives of approximately 8 minutes and 38 minutes were reported. In study C, samples were taken less frequently in the initial hour than in either of the other studies, so some differences in fitting of the clearance curves may have occurred. The use of xylazine sedation for the castration procedure may also have affected the clearance rate. The

bulls in the study cited above were castrated under local anesthesia only while the human study was a classical metabolic clearance study in which exogenous tritiated T was administered and samples of blood and urine were taken and analyzed to calculate volume of distribution and clearance characteristics. Work in the sheep has shown that xylazine alters progesterone clearance in the ovariectomized ewe progesterone-implanted ewe (Reed et al., 1984) and it is a reasonable assumption that clearance of all steroids may be altered by sedatives and/or anesthetics.

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CHAPTER VII. SUMMARY

When compared to other domestic species, there are several unique features of reproduction in the llama. The results from the studies reported in this thesis add to the knowledge of basic camelid reproduction, including some aspects that have not been previously reported.

Camelids ovulate only following mating rather than having estrous cycles. While previous studies have demonstrated that ovulation can be induced in camelids by treatment with hCG or GnRH, few comparative dose response studies have been conducted to evaluate ovulation rates and subsequent progesterone concentrations. In the first series of studies, it was demonstrated that GnRH and hCG can be used to induce ovulation in the llama, with P_4 concentrations in the days following treatment similar to those following nonfertile or fertile mating in a similar time course to maximum P_4 concentrations. It is also of interest that while the temporal aspects of the ovulatory LH surge in the female llama were similar to those observed in other species, the peak LH concentrations in the llama were markedly lower than those observed during the ovulatory surge of other species. Based on these studies a dosage of 1 μg GnRH/kg BW i.v. appears to be the most appropriate treatment to reliably induce ovulation in the llama.

In comparison to other domestic livestock species, there are several aspects of the hypothalamic-pituitary-gonadal axis of the llama that are unusual. Basal LH concentrations were low in both male and female llamas. While GnRH was capable of stimulating an ovulatory surge of LH and ovulation in the female llama even though

concentrations were lower than in other species, comparable dosages of GnRH in male llamas did not consistently stimulate release of either LH or T. Furthermore, although LH concentrations did increase following removal of gonadal steroid inhibition by castration in the male llama, the magnitude of the increase at 30 d post-castration was less than two-fold, markedly less than that observed in other species.

These results suggest that gonadotropins have a lower threshold for gonadal activity in the llama, as evidenced by induction of ovulation with GnRH in the female llama. Maximum P_4 concentrations reached following ovulation in the llama were similar to those observed in some domestic species (such as sheep). The low maximal concentrations of LH released in the ovulatory surge of llamas apprear to be sufficient to allow formation of a functional CL capable of supporting pregnancy. The corpus luteum of early pregnancy in the llama was responsive to administration of a $PGF_{2\alpha}$ analog, with decreases in P_4 concentrations and induced abortion consistent with lysis of the CL. The time course of the decline in P_4 was similar to that observed in other domestic species.

The testes of the llama apparently do exert an inhibitory influence on the hypothalamo-hypophyseal axis, as demonstrated by a significant increase in circulating LH concentrations following castration in the male. Again, although some characteristics of LH secretion in the male llama following castration are similar to those of other species, the magnitude of the increase in LH secretion is less than that observed in other species. Testosterone itself is secreted by the testes in concentrations and pulsatile patterns of release comparable to other species, although T release seems to be less correlated with LH pulses than in cattle and sheep and T is not predictably

responsive to exogenous GnRH administration. The two-phase clearance pattern of T following castration does not appear to be appreciably different from reports in other species.

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APPENDICES

APPENDIX I

Reproductive histories of animals utilized for the study described in Chapter III.

#162 -- Infertile, shown to ovulate normally prior to study, questionable previous fertility. Has since died and had normal ovaries on necropsy, uterus was thickened with increased glands.

#176 -- Normal fertility with 1 pregnancy prior to study and 1 resulting from fertile breeding in study. Has since died.

#182 -- Normal fertility with 5 pregnancies prior to study and 1 since. Used only for fertile matings in study. Has since become infertile with scarring of the endometrium, presumably due to uterine infection.

#197 -- Normal fertility with 2 pregnancies prior to study and 4 since. Used only for fertile matings in study.

#198 -- 1 pregnancy prior to study and 3 since. Difficult to get pregnant. Mild abnormality of vulva.

#216 -- Congenital recto-vaginal fistula with chronic vaginitis. Shown to ovulate normally prior to study. Has since died and had normal ovaries and uterus on necropsy.

#230 -- Normal fertility at younger age, with 3-4 pregnancies, then a C-section caused chronic uterine infection and scarring and subsequent infertility. Shown to ovulate normally prior to study and uterine infection was treated and cleared. Has since died and ovaries were normal on necropsy, although cervix had strictured closed with subsequent mucometra.

#235 -- Normal fertility, tendency to twin with 2 twin pregnancies prior to study and 1 singleton since.

#236 -- Normal fertility with 2 pregnancies prior to study, 1 resulting from fertile mating in study and 1 since study.

APPENDIX II

Catheterization and sampling procedure

On each animal a 2" square area was clipped on the neck over the jugular groove with #40 clipper blades, the area was scrubbed with surgical grade iodine soap[1], rinsed with water, and then sprayed with 70% ethanol and 1/2 cc of lidocaine hydrochloride[2] (2%) was instilled in the skin directly over the area of the jugular vein. A #15 scalpel blade was inserted through the lidocaine-prepared skin at a 45° angle to the skin and directly over the jugular vein and a commercial iv catheter was guided through the incision and into the jugular vein. The catheter was flushed with 1-2 ml of heparinized (5 IU/ml) normal saline solution, fitted with an extension and valve assembly and sutured in place. The catheter and extension was then wrapped to the neck with brown roll gauze and Elastikon*[3] tape. Sample procedure via catheter was as follows: a) withdrawal of 4-5 ml of saline + blood from the catheter + extension and discard of this volume b) withdrawal of a 3 ml blood sample c) flush with 4-5 ml of heparinized saline.

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[1] Prepodyne[R], Amsco Medical Products Division, American Sterilizer Co., Erie, PA

[2] Lidocaine Hcl, Phoenix Pharmaceutical Co., St. Joseph, MO

[3] Elastikon*, Johnson and Johnson Medical, Inc., Arlington, TX

APPENDIX III

Llama LH Assay Protocol

REAGENTS:

0.01 M Phosphate Buffered Saline (PBS) -- used to make assay buffers

1. Weigh:

   8.182g NaCl
   0.101g monobasic sodium phosphate
   1.318g dibasic sodium phosphate (anhydrous)
   0.1g thimerosol

2. Place all in 1 l volumetric flask. QS to 1 l with dd H_2O.

3. Adjust pH to 7.4. Store at 4° C.

0.5% BSA-PBS -- used as assay buffer, for iodination fraction collection and to dilute trace.

1. Weigh 5 g bovine serum albumin fraction V.

2. Place in 500 ml beaker with ~ 400 ml PBS.

3. Stir and heat gently until dissolved.

4. Let cool to room temperature. Adjust pH to 7.4 with NaOH.

5. Place in 500 ml volumetric flask. QS to 500 ml with PBS. Store at -20° C. Thaw day prior to use.

0.05 M EDTA-PBS 1.5% normal mouse serum (NMS)-- 1st Antibody diluent

1. Weigh 18.6125 g disodium EDTA.

2. Place in 1 l beaker with ~ 800 ml PBS.

3. Heat and stir until dissolved.

4. Let cool to room temperature. Adjust pH to 7.4 with NaOH.

5. Place in 1 l volumetric flask. QS to 1 l. Remove 15 ml. Add 15 ml

normal mouse serum. Store at -20° C. Thaw day prior to use.

0.05 M phosphate buffer -- used to elute iodination column, to dilute chloramine-t and metabisulfite for iodination reaction, and as 2nd antibody diluent.

1. Weigh:

   1.095 g monobasic sodium phosphate.
   5.96 g dibasic sodium phosphate.

2. Place in 1 l volumetric flask and QS to 1 l with dd H_2O.

3. Adjust pH to 7.5. Store at 4° C or -20° C.

Chloramine-T solution -- starts iodination reaction.

1. Weigh 10 mg chloramine-T (kept at -20° C in individual vials- remove and warm to room temperature before weighing).

2. Place in 10 ml volumetric flask. QS to 10 ml with 0.05 M phosphate buffer.

Note: make fresh within 1 hour of use.

Sodium metabisulfite solution -- ends iodination reaction.

1. Weigh 10 mg sodium metabisulfite.

2. Place in 10 ml volumetric flask. QS to 10 ml with 0.05 M phosphate buffer.

0.5 M phosphate buffer -- used in iodination

1. Weigh:

   0.579 g monobasic sodium phosphate.
   2.953 g dibasic sodium phosphate.

2. Place in 50 ml volumetric flask. QS to 50 ml with dd H_2O.

3. pH to 7.4. Aliquot in 200 ul aliquots and store at -20° C.

1st antibody: mouse monoclonal anti-bLH Beta 518B7. Our current stock is used at a titer of 1:1,000,000. Retitrate if necessary. Stock is stored at - 70° C

at a dilution of 1:400 in 0.05 M EDTA-PBS.

1. Fill a 1 l volumetric flask to the neck with 0.05 M EDTA-PBS 1.5% NMS.

2. Add 400 µl 518B7 at 1:400.

3. QS to 1 l. Use immediately or store leftover at -20° C. Thaw day prior to use.

Note: Dilute antibody bears refreezing and thawing well without appreciable loss of binding.

Trace: Calculate amount needed by multiplying # of tubes in assay by 0.05. Add 5-10 ml extra for waste.

1. Place calculated amount of 0.5% BSA-PBS in disposable container with screwtop lid.

2. Add enough concentrated tracer from iodination tube determined by binding tests to be suitable for use to make a final concentration of 10,000-12,000 cpm/5 µl.

2nd antibody: sheep antimouse IgG. Current stock is at -70° C in 40 ml bottles. Use at 1:50 in 5% PEG-0.05 M phosphate buffer. Retitrate if necessary.

1. Weigh 50 g polyethylene glycol (PEG-MW approx. 8000) and place in 1000 ml beaker.

2. Add 20 ml sheep antimouse IgG serum.

3. Bring to 1000 ml with 0.05 M phosphate buffer.

4. Stir without heat until dissolved. Store at 4° C or -20° C. (Best if used within 24 hours and stored at 4° C)

IODINATION PROCEDURE:

1. Prepare hormone for iodination.

   a. Weigh 15-40 µg bLH USDA-I-1 AFP6000.

   b. Dissolve in equal amount of dd H2O (1µg/µl)

   c. Label conical microvials with date, hormone ID and volume.

d. Place 5 µl at tip of conical vial.

e. Store at -70° C until use. (or use fresh). Remove to room temperature immediately before iodination.

2. Prepare column.

a. Break tip off of disposable glass 10 ml pipet. Leave as much reservoir space as possible at top.

b. Place glass bead at tip. Put tygon tubing (~3inches) on tip (can use 1/2 inch or so of silastic tubing to attach).

c. Place on ring stand and pour in or add with pasteur pipet the Sephadex slurry.

To make Sephadex slurry: Add some Sephadex G-75 to 0.05 M phosphate buffer and swirl until a slurry is formed. Needs to be liquid enough to pour or pipet. Let swell overnight at room temperature before use. On standing, should end up with a layer of buffer (~1/4 inch) on top. Swirl to mix as adding to column. Store at 4° C. Remove to room temperature about 1 hour before pouring column.

d. Let column settle and pack to about 1 cm from the top of the pipet.

e. Run buffer (0.05 M phosphate buffer) through for 10-15 minutes to thoroughly pack.

Above steps can be done day prior to iodination and column left set at room temperature. Alternatively, the column can be stored at 4° C for 1 to 2 weeks. It will need to be warmed to room temperature before use. Air bubbles may form but these do not usually affect the purification of this iodinated hormone.

f. An hour or 2 before iodinating, add 1 ml 5% BSA-PBS to the column after draining buffer to level of gel-bed. When this has entered column, add 0.05 M phosphate buffer and let column run for 10-15 minutes. This lets the column equilibrate with the protein. I like to move the column to the fraction collector to complete this step to ensure that all is working properly.

g. Drain column to level of gel bed and shut off.

3. Iodination: Wear 2 pairs latex gloves and have hood running throughout. Minimize unnecessary personnel in the lab during the reaction if possible.

a. Make chloramine-T and metabisulfite solutions.

b. Clean chloramine-T and iodine LH Hamilton syringes by filling and discarding 10 times in each of the following solutions:

   i. 1:10 radiacwash

   ii. 70% ethanol
   iii. dd H_2O

c. Remove hormone from freezer if necessary.

d. Warm 0.5 M phosphate buffer to room temperature.

e. Add 25 µl 0.5 M phosphate buffer to hormone vial. Cover vial with parafilm.

f. Measure 0.05 ml metabisulfite solution in TB syringe.

g. Measure 0.1 ml 0.5% BSA-PBS into each of 2 TB syringes.

h. Measure 10 µl Chloramine-T solution into appropriate Hamilton syringe.

i. Add 5 µl Na I^{125} to the reaction vial through the parafilm.

j. Cover vial with more parafilm. Set aside, close iodine vial and fill Hamilton syringe with radiacwash.

k. Add 10 µl Chloramine-T solution. Gently rotate and tap to mix for 30 seconds.

l. Add 50 µl metabisulfite solution. Gently rotate and tap to mix.

m. Add 100 µl transfer solution to vial, remove contents and gently add to column.

n. Add 2nd 100 µl transfer solution to vial, remove contents and gently add to column.

o. Open column. When liquid has entered, fill to top with 0.05 M phosphate buffer. Collect 10 drops/tube into tubes containing 200 µl 0.5% BSA- PBS. Keep column full of buffer and collect 30-40 tubes.

p. Count 5 µl aliquots of each tube for 0.1 min on gamma counter. Cap tubes! Plot counts vs tube # to see peak iodinated hormone and peak free iodine.

q. Clean Hamilton syringes in same procedure as before and store.

r. Choose tubes to test--usually I test the peak and 1-2 tubes after.

IODINATION TEST:

Tube	ID	Buffer	Std or Sample	1st Ab/NMS	trace	2nd Ab
1 2	TC	0	0	0	50 µl to all tubes	0
3 4	NSB-buffer	100 µl	0	150 µl NMS-0.05 M EDTA-PBS		1 ml to all tubes
5 6	NSB-plasma	0	100 µl llama plasma	150 µl NMS-0.05 M EDTA-PBS		
7 8 9	0 Std.	100 µl	0	150 µl 1st Ab to all tubes		
10 11 12	0.06 Std.	97 µl	3 µl 2 ng/ml std			
13 14 15	0.2 Std.	90 µl	10 µl 2 ng/ml std			
16 17 18	1.0 Std.	95 µl	5 µl 20 ng/ml std			
19 20 21	4.0 Std.	80 µl	20 µl 20 ng/ml std			
22 23 24	Serum Pool	0	100 µl high llama serum			

Assay procedure:

Day 1: Add std, buffer and sample to tubes as per worksheet.
Add 1st antibody and trace with Micromedic.
Incubate at 25° C for 24 hrs.

Day 2:

Add 2nd antibody. Incubate at room temperature for 1 hour. Centrifuge at full speed (1500 X *g*) for 45 minutes. Decant liquid from tubes. Let tubes sit upside down for at least 15 minutes. Turn over and let set to dry tops of tubes 1 more hour. Count on gamma counter 1 min/tube.

A regular assay is done as above with the addition of the following standard points: 0.1, 0.5, 1.0, 8.0 ng. Unknown samples follow the serum pool in the assay.

PARALLELISM:

Parallelism was established by measuring llama serum pools in 4 separate assays in triplicate in each assay in the following amounts: 25, 50, 100 and 200 µl. Percent binding was graphed on a log-logit scale versus the standard curve and slopes of regression lines compared and found to be no different ($P > 0.05$).

RECOVERIES:

Recovery of various amounts of standard in plasma was established by adding the appropriate amount of standard for 0.1, 0.2 and 0.5 ng standard points to tubes containing 50 µl of llama serum pool. Assay buffer was added to tubes to bring volume to 100 µl. Each point was assayed in triplicate in 4 separate assays. The recovery percentage was calculated using the following formula:

$$\frac{\text{ng/ml total in tube - ng/ml for 50 µl llama serum}}{\text{ng/ml of standard point}} * 100$$