Current knowledge and future challenges in camelid reproduction

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Reproductive biology research on camelids offers some interesting peculiarities and challenges to scientists and animal production specialists. The objective of this paper is to review camelid reproduction, advances in reproductive physiology and reproductive biotechnologies in camelids and discuss some areas for further research. In the female, the focus has been on understanding follicular dynamics. This has allowed development of synchronization and superovulation strategies to support embryo transfer technologies which are now commonly used in camels. Some advances have been achieved in preservation of embryos by vitrification. Fertilization, early embryo development and embryo signaling for maternal recognition of pregnancy are still not fully understood. New information on the interaction of the developing embryo and the endometrium may shed some light on this signaling as well as the mechanism of prevention of luteolysis. The presence of a seminal ovulation-inducing factor (OIF) was confirmed in llamas and alpacas. Chronology of oocytes maturation has been described. In vitro production of embryos has been achieved resulting in successful pregnancies and births in the dromedary. These techniques offer a new tool for the production and study of interspecies/cross-species embryos and their effect on pregnancy. Male reproductive function remains poorly studied. Semen preservation and artificial insemination still present many challenges and are not used in production at the moment. The involvement of climatic and nutritional conditions as well as the role of leptin in the regulation of reproductive function need to be evaluated.

Introduction

Camelidae are economically important in many countries. There are six species, two old world camelids (OWC); *Camelus dromedarius* and *Camelus bactrianus* and four new world camelids (NWC); *Lama glama, Lama pacos* (renamed *Vicugna pacos*), *Lama guanacoe* and *Vicugna vicugna*. Domestic camelids show a variety of "breeds" with specific production characteristics. Camelid reproductive biology research has seen tremendous development in recent years due to renewal of interest for these species. The application of hormone assays and ultrasonog-

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raphy have permitted great advances in this area (Anouassi *et al.* 1988; Tibary & Anouassi 1996). The objective of the present paper is to review camelid reproduction and to point out areas for future research, focusing on reproductive physiology and applied biotechnologies. Recent reviews are available for discussion of reproductive disorders in camelids (Tibary *et al.* 2005b; Tibary & Vaughan 2006; Vaughan & Tibary 2006).

Reproduction in the male camelid

Reproductive parameters of the male are critical for evaluation of the prospective herd sire and are summarized in Table 1.

Table 1: Reproductive parameters in male camelids (adapted from Tibary & Anouassi 1997; Bravo 2002; Tibary & Vaughan 2006)

Parameter	*C. dromedaries*	*C. bactrianus*	*L. pacos*	*L. glama*
Testicular descent	Present at birth	Present at birth	Present at birth	Present at birth
Puberty	3 to 5 years	3 to 5 years	15 to 24 months	15 to 24 months
Sexual maturity (years)	5 to 6	5	4	4
Seasonality	Yes	Yes	sometimes	sometimes
Testicular size at maturity	36.4 cm*	?	3.7 x 2.5**	5.4 x 3.3**
Duration of Spermatogenesis	?	?	?	?
Sperm production per gram of testis	20 to 61 x 10^6	?	?	?
Daily sperm production	0.751 x 10^9	?	?	?
Gonadal sperm reserve reserves	1.7 – 3.4 x 10^9	?	?	?
Epididymal transit	4.3. days	?	?	?
Epididymal sperm reserve	2.3-6.1 x 10^9	?	?	?
Mating duration (minutes)	7 – 20	3 – 20	5 -50	5-65
Ejaculate characteristics				
Volume (ml)	2-13	2.5-12.5	0.4-4.3	0.2-7.9
Concentration (x 10^6/ml)	140-1,300	450-5,590	0.06-170	15-640
Total spermatozoa (x 10^6)	600- 5,690	?	0.06-167	2.9 -246
Motility (%)	20-80	70-90	85	50-95
Normal Morphology (%)	40-70	50-90	40-75	40-85
Sperm cell dimensions (μm)				
Total length	51.1 ± 0.9	42 ± 1.9	?	49.5
Head length	6.6 ± 0.5	6.0 ± 0.6	?	5.3
Head width	3.8 ± 0.1	3.9 ± 0.1	?	3.8
Midpiece	6.8 ± 0.5	6.2 ± 0.7	?	5.3
Tail	37.6 ± 0.9	30.8 ± 1.9	?	36.6

* scrotal circumference, **Length by width

Sexual development and puberty

Anatomy of genital organs has been reviewed extensively (Tibary & Anouassi 1997; Tibary & Vaughan 2006). The most notable feature is the absence of seminal vesicles. The testicles are already descended at birth but may not be easily palpable until 6 months of age in OWC.

Male NWC can display sexual activity as early as 11 months of age. Preputial detachment and complete erection and intromission are possible at 15 months but may take up to 3 years. Presence of spermatozoa in the seminiferous tubules was reported at 15-18 months of age in the alpaca. Testicular growth is slow and does not plateau until 3 years of age (Tibary & Vaughan

2006). In the vicuna, spermatogenesis is present at 16 months of age. Guanaco and vicuna males live in a bachelor band until they reach maturity between 4 and 6 years of age, at which point they start forming their own harem (Tibary & Vaughan 2006).

In the OWC, rutting behavior may be seen as early as 2 years of age, but spermatogenesis and fertilization are not possible until 3 years at the earliest. Sexual maturity (5 to 6 years) is signaled by the plateau in spermatogeneic activity (Al-Qarawi *et al.* 2001). Reduction of spermatogenic activity, libido and expression of male behavior are observed after 20 years (Tibary & Anouassi 1997).

Spermatogenesis, sperm production and sperm maturation

The cycle of the seminiferous tubule in the male llama and dromedary presents 8 stages (Delhon & Von Lawzewitsch 1987; Bustos-Obregon *et al.* 1997). Differences in the frequency of each stage exist between the two species. Epididymal maturation and transit has been estimated to be 4.3 days in the dromedary. Histologically and histochemically different segments were identified in the epididymis in llamas (Delhon & Von Lawzewitsch 1994) and camels (Tingari & Moniem 1979).

Testicular weight is correlated with spermatogenesis and epididymal sperm reserves. However, sperm production and gonadal sperm reserves are lower than those of ruminants. Sperm production is affected by age and season (Tibary & Anouassi 1997; Tibary & Vaughan 2006).

Seasonality of reproduction in the male OWC and vicuna has been established through endocrinological, behavioral and histological studies (Tibary & Vaughan 2006). Testicular size decreases in winter in vicunas (Urquieta *et al.* 1994) and in summer in camels (Tibary & Anouassi 1997). Sperm production and quality is lower in summer in llamas (Tibary & Vaughan 2006). The mechanism regulating seasonality in male camelids remains poorly studied.

Mating behavior and ejaculation

Mating behavior has been extensively described (Tibary & Anouassi 1997; Tibary & Vaughan 2006). In OWC, rutting season is evidenced by increase poll gland secretions, exteriorization of the soft palate and increased urine marking behavior. All camelids mate with the female in a sternal recumbency and mating lasts an average of 20 minutes. Duration of copulation in alpacas is affected by the age of females (longer for multiparous) and by the presence of other males (shorter) (Tibary & Vaughan 2006).

Semen is delivered continuously throughout mating, in non-fractioned small quantities, via a combination of pelvic thrusts and rhythmic urethral pulses throughout copulation (Lichtenwalner *et al.* 1996; Lichtenwalner *et al.* 1997; Tibary & Anouassi 1997). Copulation length affects ovulation induction rate but does not affect conception rates of ovulating females (Table 2). Sperm cells are present in the ejaculate within 2 to 5 minutes of copulation. Semen is deposited partly deep in both uterine horns and partly in the cervix and vagina (Tibary & Anouassi 1997; Tibary & Vaughan 2006).

Semen characteristics

Specially designed artificial vaginas allow reliable collection of ejaculates (Lichtenwalner *et al.* 1996; Tibary & Vaughan 2006), unfortunately a large number of ejaculates do not contain spermatozoa (Flores *et al.* 2002; Deen *et al.* 2003).

Table 2: Effect of copulation length on ovulation and embryo recovery in dromedary camels (Tibary & Anouassi 1997)

Mating duration (minutes)	Number of females	% non-ovulating females	% females with at least one embryo
Less than 1.5 minutes	45	35.6	44.9
1.5 to 3	232	13.8	53.5
4	102	13.9	56.8
4.5-5	102	16.7	65.9
5.5-6	57	14	77.6
>6	94	8.5	55.3

Camelid semen has high viscosity, low sperm concentration and poor motility (Garnica *et al.* 1993; Tibary & Anouassi 1997). Viscosity is attributed to mucopolysaccharides secreted from the bulbourethral glands or the prostate (Garnica *et al.* 1993). Liquefaction of camelid semen may take several hours and may be enhanced by exposure to hydrolytic and proteolytic enzymes (trypsin, collagenase, fibrolysin, hyalurodinase). A trypsin solution of 1:250 seems to be effective with minimal negative effects on spermatozoa (Bravo *et al.* 2000a).

The effects of the various sperm abnormalities on fertility have not yet been determined. Incidence of sperm abnormalities in the ejaculates is highly variable (Lichtenwalner *et al.* 1996; Flores *et al.* 2002; Urquieta *et al.* 2005). The incidence of cytoplasmic droplets is relatively high. The incidence of abnormalities is not affected by rank of ejaculate (Bravo *et al.* 1997). Concentration of the ejaculate remains stable if a period of at least 12 hours is allowed between successive ejaculations (Bravo *et al.* 1997; Urquieta *et al.* 2005). Male camelids may differ in their ability to induce ovulation (Tibary & Anouassi 1997).

Reproduction in the female camelid

Reproductive parameters in the female (Table 3) present striking differences compared to ruminants. There are several distinct anatomical differences in the genital organs compared to the ruminant (i.e. large ovarian bursa, absence of intercornual ligaments, a well-developed papillae of the utero-tubal junction, arrangement of the cervical rings) (Fig. 1) (Tibary & Anouassi 1997; Vaughan & Tibary 2006).

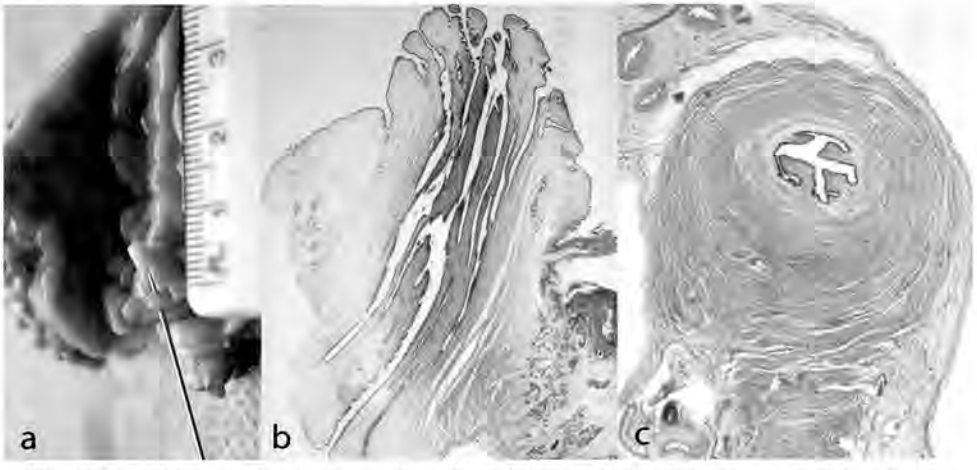

Fig. 1 Gross (a) and microanatomy (b and c) of the papillae of the utero-tubal junction in camelidae. Note the projection into the uterine lumen (2 to 5 mm) and the strong smooth muscle sphincter. The utero-tubal junction serves as a sperm reservoir and selective passage of healthy embryos into the uterus

Table 3: Reproductive parameters in female camelids (adapted from Tibary & Anouassi 1997; Bravo 2002; Vaughan & Tibary 2006,)

Parameter	*C. dromedarius*	*C. bactrianus*	*L.pacos*	*L. glama*
Puberty (months)	24-48	18-24	4-6	6-8
Age at first breeding (months)	36-48	24-36	12-18	15-18
Follicular wave phases duration (days)				
Growth (days)	10.5±0.5	10.9 ± 3	3-9	3-9
Maturation	7.6 ± 0.8	7 ± 4.2	2-8	2-8
Regression	11.9±0.8	11.9 ± 4.2	3-8	3-8
Ovulatory follicle characteristic				
Minimum size (mm)	9	9	7	8
Growth rate/day (mm)	1.8	1.8	0.43	0.5-0.9
Average size(mm)	10-18	10-18	8-10	9-12
Maximum size(mm)	25	22	12	13
Incidence of anovulatory follicles (%)	40-50	?	5	10-40
Anovulatory follicle regression (days)	8-45	?	?	4-22
Corpus luteum characteristics				
Size (mm)	15-25	15-25	11-15	11-18
Day at CL maximum size	7.2±1.7	7.3	7-8	8
Luteolysis day	10 ± 1.2	10.5	10-12	10-12
Pregnancy and Parturition				
Implantation (days)	14	14	12-14	12-14
Average and range of pregnancy length (days)	384 (330-396)	402 (374-419)	344 (330-376)	345 (333-382)
Expulsion of fetus (minutes)	10.5 ± 4.7 (5-45)	26.8 ± 12 (5-45)	10-15	10-15
Delivery of placenta (minutes)	65 ± 4.2 (30-180)	69 ± 47	88 (40-143)	77 (45-240)
Postpartum				
Uterine involution (days)	20-45	?	20	17
Postpartum ovarian activity (days)	14-300*	?	7	5

*Extreme variation in onset of postpartum ovarian follicular activity is primarily due to nutritional condition and effect of lactation anoestrus and seasonality

Puberty

Puberty is generally defined in the female as the age at which ovulation and fertilization are possible. Endocrinological and clinical studies show that recruitment, growth and ovulation of follicles are possible in female NWC as early as 4 months of age. However early breeding may result in poor fertility due to ovulation failure and increased early pregnancy loss. Females are bred when they reach 60% of the adult weight (Vaughan & Tibary 2006).

In OWC, normal follicular dynamics is present at 8 to 12 months of age in some breeds and under intensive management. However in nomadic conditions sexual activity does not start until 2 or 3 years of age. Nutritional status, season of birth, and breed of camel can affect the age at onset of puberty, age at first conception, and consequently the age at first parturition (Tibary *et al.* 2005b).

Seasonality of reproduction in the female

Seasonal pattern of reproduction in the female camelid is probably largely due to nutritional conditions (OWC and wild NWC) and management (NWC) (Tibary & Anouassi 1997; Sghiri &

Driancourt 1999; Tibary *et al.* 2005b). The breeding season coincided with warmer months when ample forage is available. However, the relationship between nutrition and reproduction has not been thoroughly studied in camelids. In the dromedary, body condition score and particularly hump fat content was shown to have a direct effect on ovarian activity (Tibary & Anouassi 1997). Also, leptinemia is positively related to hump fat content and is correlated to water and food deprivation. Recent studies show that this species has much higher hepatic lipogenic enzymes activity, an expression of leptin and leptin receptors, than in other ruminants. This suggests that leptin may be an important regulator of reproduction in this species (Bartha *et al.* 2005; Chillar *et al.* 2005a; 2005b; Sayed-Ahmed *et al.* 2005).

Ovarian follicular activity

In the absence of mating, there is a succession of overlapping follicular waves with highly variable rhythm showing 3 phases: growth, maturation and regression (Fig. 2) [(dromedary; (Skidmore *et al.* 1996a; Tibary & Anouassi 1996); llamas (Chaves *et al.* 2002), alpacas (Vaughan *et al.* 2004) and vicunas (Miragaya *et al.* 2004)]. Follicular wave patterns are orchestrated by endocrinological events involving both hypothaloamo-pituitary-gonadal axis and local (inhibin) regulation (Vaughan & Tibary 2006). Little is known about the mechanisms of recruitment of each follicular wave but these may involve FSH.

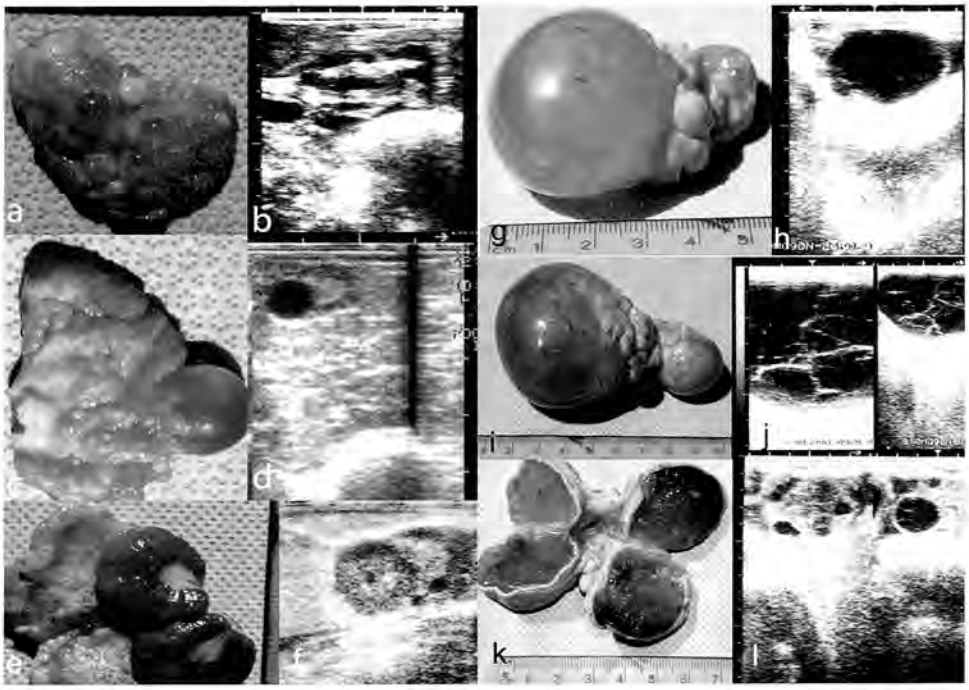

Fig. 2 Gross and ultrasonographic appearance of ovarian structures in camelids. Emergence of a new follicular wave growth (a,b), dominant follicle (c,d), mature corpora lutea (e,f), anovulatory follicle (g,h), anovulatory hemorrhagic follicle (i,j) luteinized hemorrhagic follicles (k,l)

Follicle size is correlated with plasma 17ß-oestradiol (oestradiol), oestrone sulphate and urinary oestrone sulphate in alpacas (Aba *et al.* 1995), llamas (Aba *et al.* 1995; Chaves *et al.* 2002) and

dromedaries (Skidmore *et al.* 1996a; Tibary & Anouassi 1997). Peak plasma oestradiol concentrations coincide with maximum follicle size (Chaves *et al.* 2002). Mature follicles may develop into large anovulatory follicles (Fig. 2). These regress slowly but do not seem to affect follicular growth. Some may become luteinized (Tibary & Anouassi 1996).

Ovulation

Camelids are induced ovulators. An LH surge is observed within 15 to 40 minutes after mating and ovulation occurs in most females within 24 to 48 hours after mating (Marie & Anouassi 1987; Bravo *et al.* 1992; Aba *et al.* 1995). Incidence of ovulation following mating varies from 60 to 100%, occurring equally in the left and right ovaries (Tibary & Anouassi 1996; Vaughan & Tibary 2006).

Mechanism of induction of ovulation

Ovulation is induced by intramuscular injection of seminal plasma. The presence of an ovulation-induction factor (OIF) in semen was demonstrated in the bactrian camel (Zhao *et al.* 2001) and NWC recently (Adams *et al.* 2005). Absorption of the OIF seems to be dependant on hyperemia and endometrial irritation caused by copulation (Ratto *et al.* 2005b).

Natural multiple ovulations

Multiple ovulations (double and even triple) occur in 5 to 20 % of natural matings (Tibary & Anouassi 1996; Vaughan & Tibary 2006). However, twin pregnancies and twin births are extremely rare.

Spontaneous ovulations in camelidae

Spontaneous ovulations have been reported, particularly in the postpartum female. Their incidence range from 3.5% to 40% (Nagy *et al.* 2005; Vaughan & Tibary 2006). Some of these spontaneous ovulations could simply be luteinization of anovulatory follicles.

Corpus luteum (CL) and lutyeolysis

The luteal phase is short (Marie & Anouassi 1987; Aba *et al.* 1995). The CL reaches its maximum size by 7 days post-breeding and luteolysis is initiated 9 to 10 days post-mating through prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) release (Aba *et al.* 1995; Skidmore *et al.* 1998). The role of oxytocin in luteal regression in camelids is unknown. There seems to be a difference in the luteolytic effects of the right vs. left uterine horn. Luteolytic activity of the right uterine horn is local while the left uterine horn has both a local and a systemic effect (Fernandez Baca *et al.* 1979). It is postulated that the left horn may induce luteolysis of a corpus luteum in the right ovary via a local veno-arterial pathway (Ghazi 1981; Del Campo *et al.* 1996).

Pregnancy

Fertilization and early embryo development

Fertilization rate is very high and embryo development is rapid. Embryos enter the uterine

cavity approximately 5-6 days after ovulation at the hatching or early hatched blastocyst stage (McKinnon *et al.* 1994; Tibary & Anouassi 1997; Tibary 2001b; Tibary 2001c). The embryo starts to elongate by Day 10 and the trophoblast establishes close contact with the endometrium by day 14 (Fig. 3) (Tibary 2001b).

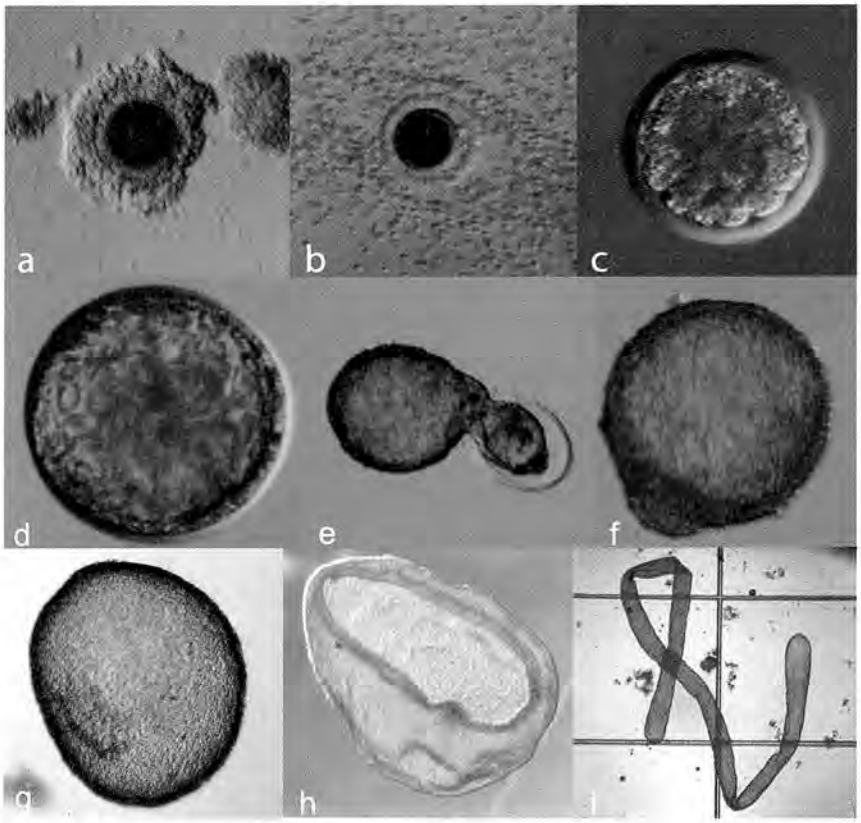

Fig. 3 Early embryo development in camelids. a) immature compact cumulus-oocyte-complex, b) expanded cumulus after maturation, c) day 5 morula (260 μm), d) day 6.5 blastocyst (380 μm), e) day 7 hatching blastocysts; f) day 7.5 hatched blastoyst, g) day 8.5 collapsing blastocyst (5 mm); h) day 9.5 embryo (9 mm), i) day 13 elongating trophoblast (5.5 cm)

Maternal recognition and embryo migration

The majority (98%) of pregnancies occur in the left uterine horn regardless of the side of ovulation. Differential luteolysis occurs in the left and right uterine horns and may explain embryo migration from the right to the left uterine horn (Tibary & Anouassi 1997; Vaughan & Tibary 2006).

Embryonic signals have not been thoroughly investigated but they must be present before Day 9 after mating in order to rescue the corpus luteum of pregnancy (Aba *et al.* 1997). In the dromedary, the early embryo (Day 10) exhibits high aromatizing activity and synthesizes large amounts of estrogens. This coincides with the time of luteolysis and may be the first signal for maternal recognition and prevention of luteolysis. No interferon-like protein has yet been identified (Skidmore *et al.* 1994). High levels of oestrone sulphate are observed between Days 21 and 27 of pregnancy in alpacas and may be involved in maternal recognition of pregnancy (Vaughan & Tibary 2006).

Placentation and fetal development

The macro and microanatomy of the uteroplacental interface was recently described. Camelid placentation is eptheliochorial, diffuse microcotyledonary with the fetus completely covered by an epidermal membrane (Skidmore *et al.* 1996b; Tibary & Anouassi 1997; Olivera *et al.* 2003a; Olivera *et al.* 2003b).

Implantation and placental development begins in the left horn around day 14 then extends to the right (Olivera *et al.* 2003a). Complete interdigitations between the membranes of uterine epithelial and trophoblastic cells are observed exclusively in the left horn in areas around the embryo. Specialized pre-contact zones, ectoplasmic pads, provide initial points of attachment and early metabolic maternal-fetal exchange (Abd-Elnaeim *et al.* 2003). Aerolae are present at the gland regions and may play a role in nutrient transfer (Abd-Elnaeim *et al.* 2003; Olivera *et al.* 2003b). The contact surfaces of trophoblast and uterine epithelial cells show a richly glycosylated layer and many classes of glycan that may play a role in attachment, materno-fetal interactions and embryo fixation at the implantation site (Jones *et al.* 2002; Olivera *et al.* 2003a).

Tropoblastic binucleated and multinucleated cells are present, but their function is not clear. There is no evidence of production of chorionic hormones (Jones *et al.* 2002; Olivera *et al.* 2003a).

Endocrinology of pregnancy

Ovulation, CL growth and progesterone production are similar in pregnant and non-pregnant camelids for the first 8 days after ovulation. A temporary decline in blood progesterone was reported from day 8 to 12 after mating during the period of maternal recognition of pregnancy (Aba *et al.* 1995). The CL is required to maintain pregnancy (Tibary & Anouassi 1997; Vaughan & Tibary 2006). Plasma progesterone concentration remains above 2 ng/mL but fluctuates throughout gestation then declines in the last 2 weeks of gestation (Tibary & Anouassi 1997; Raggi *et al.* 1999).

Plasma oestradiol levels in pregnant camelids are generally low in early gestation, rise in mid-gestation, are very high in the last month of pregnancy then decline rapidly when the feto-placental unit is disrupted at term (Aba *et al.* 1995; Tibary & Anouassi 1997).

Parturition

Gestation length is highly variable and is affected by season (Davis *et al.* 1997; Tibary & Anouassi 1997; Vaughan & Tibary 2006). Parity of the dam and sex of the cria do not influence gestation length in NWC (Davis *et al.* 1997). However, these factors, as well as nutrition, are important in OWC (Tibary & Anouassi 1997). Stages of parturition have been thoroughly described and are relatively short compared to ruminants (Table 3).

Postpartum

Uterine involution is rapid (Tibary & Anouassi 1997; Bravo 2002). Gross anatomical involution is completed by 21 days postpartum. The interval from parturition to resumption of ovarian activity is 5 to 7 days in NWC. In NWC, mating and ovulation are possible by 10 days postpartum (Bravo *et al.* 1994). Conception rate returns to normal by 2 to 3 weeks postpartum, allowing production of one cria per year (Vaughan & Tibary 2006). Lactational anoestrus is a problem in lactating camels particularly under desert conditions. Early weaning and nutritional supplementation allow early return to cyclicity and fertility (Tibary *et al.* 2005b). In the dromedary, early weaning (3 to 7 days of life) resulted in earlier onset postpartum ovarian activity (9.07 ±

7.77 days) and an average interval from calving to mating of 17.92 ± 9.11 days with a 76.8% pregnancy rate at the first postpartum mating (Khorchani *et al.* 2005). In addition, lactating females with good body condition at the time of calving were shown to have an earlier onset of postpartum ovarian activity and a shorter intercalving interval (i.e. 13 to 14 months instead of 22 to 24 months) (Sghiri 1988).

Reproductive biotechnologies

Semen preservation and artificial insemination

The use of artificial insemination (AI) has been reported in camelidae since the 60's (Tibary & Anouassi 1997; Tibary 2001a). Most of the reported work is on *Camelus bactrianus* (Zhao *et al.* 1994).

Semen preservation technology in camelids

Short-term preservation of camelid semen has been attempted at different temperatures (25°C, 30°C or 4°C) using various extenders (Tibary 2001a). Complete liquefaction of semen before adding the extender poses some technical problems (Bravo *et al.* 2000b). Normal liquefaction of camelid semen may take several hours and may be enhanced by exposure to hydrolytic and proteolytic enzymes (trypsin, collagenase, fibrolysin, hyalurodinase). A trypsin solution of 1:250 seems to be effective with minimal negative effects on spermatozoa (Bravo *et al.* 2000a).

Semen has been frozen successfully for over 3 decades (Bravo *et al.* 2000b; Tibary 2001a). However, insemination trials were carried out mainly in the bactrian camel (Zhao *et al.* 1994). Llama and alpaca semen has been frozen using a variety of common ruminant techniques after liquefaction (Bravo *et al.* 2000b). Skim milk-egg-yolk ethylene glycol seems to result in higher motility and acrosome integrity (Santiani *et al.* 2005). Freezing and thawing procedures depend on the packaging method used (Bravo *et al.* 2000b; Tibary 2001a; Deen *et al.* 2003; Miragaya *et al.* 2006).

Artificial insemination

Insemination trials using cooled or frozen-thawed semen are scarce. Pregnancy rates following AI with fresh un-extended or extended semen are variable. AI is usually carried out 24 hours after induction of ovulation with hCG or GnRH. However, the overall pregnancy rate after AI is lower compared to natural service in most species except the bactrian camel (Tibary 2001a).

The method of insemination used in large camelids is similar to that used in the bovine (i.e. rectal-vaginal manipulation and use of either a cassou gun or an infusion pipette). In alpacas, insemination is performed by guiding an insemination pipette through a vaginoscope. A dose of 1 ml of semen containing $400x10^6$ spermatozoa seem to be adequate to guarantee ovulation and fertilization in the bactrian camel (Zhao *et al.* 1994). This dose can be reduced if ovulation is induced by hormonal treatment and the timing of insemination is determined with precision. In alpacas, conception was achieved following AI of $8x10^6$ sperm cells (Bravo *et al.* 2000b). In our laboratory, deposition of $15x10^6$ motile sperm cells, deep into the uterine horn ipsilateral to the side of ovulation 24 hours after administration of buserelin (25 ìg), resulted in a 77.8% conception rate in ovulating females (n = 18) (A. Anouassi unpublished data).

Conception rates following artificial insemination with frozen semen vary between 50 and 80% in bactrian camels. On the other hand, conception rate achieved with frozen-thawed

dromedary semen exhibiting very good post-thaw motility has been disappointing (10 to 15%). There are few reports on AI with frozen-thawed NWC semen (Bravo *et al.* 2000b; Tibary 2001a; Miragaya *et al.* 2006)

Interspecies artificial insemination trials

Cross species insemination has long been performed in South America (Tibary 2001a). Studies using vicuna semen and paco-vicuna semen with female alpacas and llamas have yielded variable results. Trials to cross the guanaco with the dromedary camel have been reported resulting in the birth of three calves, however these pregnancies register a high rate of early loss (Skidmore *et al.* 2000). Cross-breeding between species of the same genus (i.e. *C. bactrianus* x *C. dromedaries*, *L. pacos* x *L. glama*, *L. guanacoe* x *L. glama*) are fertile. Offspring resulting from *C. dromedaries* and *L. guanacoe* do not seem to be fertile.

Multiple Ovulation and Embryo Transfer (MOET)

MOET procedures for camelids are similar to those used for ruminants (McKinnon *et al.* 1994; Tibary & Anouassi 1997; Tibary 2001c; Skidmore *et al.* 2002; Miragaya *et al.* 2006).

Donor management

Donors are superovulated using ovine (oFSH), porcine (pFSH), camel (cFSH), eCG or a combination of FSH and eCG. Treatment is initiated after synchronization of a follicular wave with progesterone, elimination of the dominant follicle by GnRH or hCG treatment, or at the early stage of the follicular wave (no follicle > 2mm). Optimal dose and frequencies of gonadotropin treatments vary from species to species (McKinnon *et al.* 1994; Tibary & Anouassi 1997; Tibary 2001c; Miragaya *et al.* 2006). Development of mature follicles takes 6 to 8 days after initiation of gonadotropin treatment. Dromedary females seem to become refractory to eCG and oFSH or pFSH following multiple treatments. Superovulation problems are of two types: lack of response (20%) or overstimulation (15 to 20%) (Tibary & Anouassi 1997). Embryo collection can also be performed without superovulation resulting in up to 29 pregnancies per female per season (A. Anouassi & A. Tibary unpublished data).

Preliminary experiments on immunization of dromedaries and alpacas against a synthetic peptide fragment of a-inhibin are very encouraging. An increased number of ovulations (4 to 10) was observed in 60% of the immunized females (Tibary & Anouassi 1997).

A single mating or insemination of donors is sufficient once follicles reach mature size. Donors are usually treated with hCG or GnRH agonist after mating in order to maximize ovulation rate.

Embryo collection

Non-surgical collection of embryos is done in the same manner described for cattle. Low epidural anesthesia is recommended in llamas and alpacas and in young dromedaries because of the smallness of the pelvis (Tibary 2001c).

Camelid embryos enter the uterus 6 to 6.5 days after ovulation. For maximum embryo recovery, flushing should be performed 7 to 8 days after ovulation. Embryo recovery rates (embryos recovered/ovulations) are highly variable and depends on many factors including superovulation treatment, fertility, management, collection date and technician experience (McKinnon *et al.* 1994; Tibary & Anouassi 1997; Tibary 2001c). Reported recovery rates in non-superovulated llamas range from 52 to 61%. Our recovery rate from the dromedary is 85% in single ovulators and 165% in double ovulators (Tibary & Anouassi 1996). Only hatched blastocysts are transferable (Tibary 2001c).

Management of recipients

Our results on over 6000 transfers show that recipients should ovulate one or two days after the donor. Synchronization of follicular development in donors and recipients has been attempted using progestagen with variable degrees of success (Tibary 2001c; Miragaya *et al.* 2006). We select females on the basis of follicular size from a large pool of recipients and induce ovulation with GnRH or hCG (Tibary & Anouassi 1997). Some recipients lose their primary CL, however, induction of new accessory corpora lutea by treating recipients with eCG/hCG during the first 2 months of pregnancy may be an approach for such recipients (Tibary 2001c).

Transfer of embryos into oestradiol/progesterone-treated, bilaterally ovariectomized recipient resulted in a 30% pregnancy rate. However, daily progesterone treatment is thereafter required throughout pregnancy. Spontaneous parturition has been reported in dromedary females receiving exogenous progesterone but this is not always the case. Moreover, progesterone treatment seems to increase the risk of dystocia, incomplete dilation of the cervix, premature placental separation and inadequate milk production.

Pregnancy results with fresh embryos

Embryos are generally transferred non-surgically in OWC, llamas and large alpacas. Whilst surgical transfer may be indicated in alpacas and vicunas, laparoscopy has been used in our laboratory for transfer of embryos in alpacas (Tibary 2001c).

Factors affecting pregnancy rates have been extensively studied in the dromedary (McKinnon *et al.* 1994; Tibary & Anouassi 1997; Skidmore *et al.* 2002). Pregnancy rates are affected by season, quality of recipients and synchronization between donors and recipients. However, factors such as side of transfer, method of transfer, and age of the embryo do not have any effect on pregnancy rates. In our laboratory, MOET has been practiced on a commercial basis since 1992. During the period between 1992 and 1998, a total of 2653 fresh embryos were transferred, resulting in an overall pregnancy rate of 62%. Pregnancy rates improved steadily from 30% to 70% over that period. It is not uncommon to achieve a pregnancy rate of 100% with some batches of embryos. Low pregnancy rates and high early pregnancy loss rates are observed when transfers are performed during the hottest months of the year (McKinnon *et al.* 1994; Tibary & Anouassi 1997).

Alpaca embryos have been successfully transferred into llama recipients, resulting in normal births. Transfer of vicuna embryos into llamas or alpacas could be a good technique for the multiplication of this wild species (Miragaya *et al.* 2006).

Cryopreservation of camelid embryos

Freezing camelid embryos according to the protocols that are in widespread use for cryopreservation of ruminant embryos results in poor (0 to 15%) pregnancy rates (McKinnon *et al.* 1994; Tibary & Anouassi 1997), probably due to the stage of development at which they are collected (i.e hatched blastocyst) and to their size (i.e. 400 to 2500 microns). More recently the use of vitrification techniques resulted in higher pregnancy rates in llamas and dromedaries (Aller *et al.* 2002; Nowshari *et al.* 2005; Skidmore *et al.* 2005).

In vitro production of embryo

Oocyte collection

Ovarian slicing or follicular aspiration have both been used as sources of oocytes in llamas (Del Campo *et al.* 1995) and camels (Khatir *et al.* 2004; Nili *et al.* 2004; Kafi *et al.* 2005; Nowshari

2005). In llamas, follicular aspiration results in a 62% recovery rate (i.e. number of oocytes complexes per aspirated follicle) with an average of 6 oocytes per female. Mincing produces higher yields of oocytes (27 per female) but a lower maturation rate (Del Campo *et al.* 1995). In dromedaries, recovery rates range from 31 to 49% following aspiration (Khatir *et al.* 2004) and 94% after follicle dissection (94%) (Nowshari 2005). Cumulus-oocyte-complexes (COCs) are embedded in a large sheet of granulosa cells and are difficult to retrieve without direct visualization (Nowshari 2005). In dromedaries, more oocytes are recovered during the breeding season and in the absence of a corpus luteum (Tibary *et al.* 2005a).

Oocyte recovery rates of up to 80% have been reported in alpacas and vicunas using laparotomy. Laparoscopic ovum pickup results in slightly lower recovery rate but is safer. Recovery rates range from 75 to 85% after FSH/eCG stimulation (Tibary *et al.* 2005a; Miragaya *et al.* 2006).

Transvaginal ultrasound-guided aspiration has been used in NWC and camels with collection rates ranging from 30 to 75% (Ratto *et al.* 2005a; Tibary *et al.* 2005a). Gonadotropin stimulation does not improve recovery rates but increases the number of oocytes obtained per female (Ratto *et al.* 2005a; Tibary *et al.* 2005a). Ovarian stimulation with eCG or FSH provides a uniform population of oocytes and offers a means to collect in vivo (cumulus expanded) matured COCs.

In vitro oocyte maturation (IVM)

Camelid oocytes can be matured *in vitro* in conditions similar to those described for ruminants (Tibary *et al.* 2005a). Camelid oocytes display a dark cytoplasm, due to presence of lipid particles, which makes evaluation very difficult. The perivitelline space (PVS) increases as the maturation process progresses until 24 h to a size which is maintained until 36 h of culture. A large number of microvilli are observed in the PVS in Metaphase II oocytes. Cortical granules migrate towards the peripheral areas of the ooplasm and form a lining in the suboolemmal area (Kafi *et al.* 2005).

In llamas, the optimal maturation rate (62%) of oocytes is obtained after incubation for 32 to 36 h (Del Campo *et al.* 1995). Higher maturation rate (80.6%) is obtained after 30 h incubation of oocytes aspirated after ovarian stimulation but oocyte quality and developmental ability may suffer (Ratto *et al.* 2005a). A 62% maturation rate in a medium without added hormones was reported for oocytes collected 22 h after induction of an LH surge (Miragaya *et al.* 2006). In alpacas, the oocyte maturation rates are 40 to 46% for compact COCs collected 18 to 24 h after hCG administration to stimulated females and incubation of 26 h. Similar results were obtained with vicuna oocytes (Miragaya *et al.* 2006).

In dromedaries, oocytes maturation rates vary from 50 to 83% after 30 to 36 hours of culture depending on conditions (Tibary *et al.* 2005a). Maturation rate of oocytes from non-stimulated animals are lower than those from stimulated animals (63% vs. 83%). The addition of EGF and cysteamine to the maturation medium has a beneficial effect on nuclear and cytoplasmic maturation (Khatir *et al.* 2004; 2005). In the bactrian camel, 46.7% of oocytes achieved meiotic maturation after 24 to 26 h of culture. As with ruminant oocytes, maturation rate and development ability post-fertilization are dependant on follicular size (Tibary *et al.* 2005a).

In vitro fertilization

The first successful production of embryos by IVM/IVF was reported in llamas using epididymal sperm enriched by percoll gradient in presence of heparin (2 or 5μg/ml) (Del Campo *et al.* 1995). The oocyte penetration and development to the pronuleus stage rates were 29.2% and 57.1%, respectively. Llama epididymal sperm was used to produce interspecies (*L. pacos* x *L. glama*) embryos. In vitro production of camel embryos was reported using fresh ejaculated

(Khatir *et al.* 2004; Tibary *et al.* 2005a) and epididymal sperm (Nowshari 2005). Penetration (68%) and cleavage rates (40%) are promising with fresh ejaculated semen (Khatir *et al.* 2004; Khatir *et al.* 2005). The first dromedary offspring from transfer of IVP embryos using IVM/IVF was recently reported (Khatir & Anouassi 2006).

Intracytoplasmic sperm injection (ICSI)

ICSI may be a valuable tool for production of interspecies embryos within the camelidae family. The only report of ICSI produced morulas was undertaken with llamas (Miragaya *et al.* 2006).

Cloning

Production of embryos by nuclear transfer using adult cells was reported in llamas. Fusion of the couplets was successful in 62.5% of attempts (n = 80) followed by cleavage rates of 32% to 40%. Oviductal transfer of 8- to 32-cell embryos and uterine transfer of a morula did not result in any pregnancy (Sansinena *et al.* 2003). Eight cells embryos were recently obtained by nuclear transfer in the dromedary after activation of oocytes with ionomycine followed by incubation in cyclohexidine (A. Anouassi unpublished data).

Culture of IVP embryos

In camels, we recently showed that embryos obtained by IVM/IVF and cultured to the hatched stage in semi-defined medium (mKSOMaa) have better *in vivo* development ability after transfer than those cultured with oviductal cells (Khatir *et al.* 2005). Factors affecting developmental ability of IVP embryos are currently being investigated.

Conclusion

Camelid reproductive biology offers interesting challenges to the scientist. Camelids are reputed to have poor reproductive performance, but in fact, these highly adapted species can maintain excellent reproductive performances even under harsh condition. Although our understanding of some of the reproductive phenomena has improved, there is still a need for research in areas that are critical for the management of individuals and herds. Breed differences within each species should be investigated. In males, spermatogenesis and sperm production studies are needed to elucidate the effect of season, ability to induce ovulation (OIF concentration) and interaction with some production characteristics (such as fiber, draught, etc.).

In the female, the mechanisms of luteolysis and maternal recognition of pregnancy remain unclear. This area is critical to understanding the causes of early pregnancy loss that typify IVP embryos and heat stressed animals. Studies using interspecific embryo transfer, interspecific embryo production by ICSI, or cross-species nuclear transfer would allow more genomic studies on reproductive phenomena.

Semen physiology and artificial insemination studies are limited by the lack of an easy and reliable method for semen collection, as well as the gelatinous nature of the semen. Data on use of frozen-thawed semen remains limited to the bactrian camel. Further studies on factors affecting oocyte maturation and activation, and developmental ability of IVP embryos are needed. Embryo transfer technology is well established and could be an excellent tool to study interspecies placentation. Interspecific pregnancies (alpaca in llama, bactrian in dromedary) have been obtained and resulted in births but no trials have been conducted between OWC and NWC (e.g. bactrian in camel, alpaca in camel, ect.).

Finally, to improve herd performance it is necessary to study the interaction among season, nutrition and lactation with special attention to the role of leptin in regulation of reproductive activity and effect of metabolism on hormone levels.

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